COMPARISON OF THE REPRODUCTIVE AND EMBRYO-LARVAL EFFECTS OF COMMERCIAL AND EXTRACTED NAPHTHENIC ACID MIXTURES IN FATHEAD MINNOW (*Pimephales promelas*)

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ABSTRACT

To improve monitoring efforts and establish a guideline that is relevant to oil sands naphthenic acids, more information is needed to understand the composition of naphthenic acid components in raw OSPW and their role in determining toxicity. Commercial naphthenic acids have been used as a toxicological surrogate for naphthenic acids in raw OSPW. The primary objective of this research was to conduct a thorough source, pathway to receptor analysis for reproducing fish exposed to a commercial (Fluka) and an oil sands extracted naphthenic acid mixture. To improve the environmental relevance, reverse osmosis water was used to match water quality conditions in the Athabasca River. Steady-state naphthenic acid concentrations were achieved in the flow-through system design for both Fluka treatments. Naphthenic acid concentrations measured in the water were roughly half the nominal concentrations. The difference observed demonstrates the importance of measuring in-water concentrations in future toxicological assessments with oil sands naphthenic acids. This research is the first to apply high resolution mass spectrometry to detect and estimate the uptake of naphthenic acids in fish muscle tissue. Although the tissue estimates are semi-quantitative, the results are consistent with the current GC-MS method to analyse naphthenic acids in biological tissues. The reproductive and embryo-larval effects were more pronounced in fathead minnows exposed to the same nominal concentration (5 mg/L) of the commercial mixture relative to the oil sands extracted mixture. A significant decrease in cumulative egg production and larval survival and an increased deformity rate was observed in the commercial naphthenic acid exposure. No significant differences were observed in reproduction or embryo-larval development in the extracted naphthenic acid exposure. The results of the present study clearly demonstrate significant toxicological differences in population level endpoints between commercial and oil sands naphthenic acids.

Toxicological data from commercial mixtures should not be used in the development of a water quality guideline for naphthenic acids extracted from raw OSPW. However, toxicological assessments with oil sands extracted mixtures are limited. Similar to the conclusion reported in the 1998 CEATAG review, currently there is insufficient information to recommend a water quality guideline for the protection of aquatic life for oil sands naphthenic acids.

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OPEN HEARTS

They teach.
They guide.
They counsel.
They console.
They befriend.
They minister.
They lead.
They inspire.

Again and again
Each reprises
Just one lesson:
That we are valued,
That we are capable.

That is all.
That is everything.

That is you,
To me.

Forever grateful,
Ashley

§ DIANNE M. DEL GIORNO

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ABBREVIATIONS AND ACRONYMS

11-KT	11-Ketotestosterone
AEP	Alberta Environmental Protection
AESRD	Alberta Environment & Sustainable Resource Development
ANOVA	Analysis of Variance
AR	Androgen Receptor
BTEX	Benzene, Toluene, Ethylbenzene, and Xylene
CAPP	Canadian Association of Petroleum Producers
CCME	Canadian Council of Ministers of the Environment
CEATAG	Conrad Environmental Aquatics Technical Advisory Group
CERI	Canadian Energy Research Institute
CF	Condition Factor
CNA	Commercial Naphthenic Acid Mixture
DEAE	Diethylaminoethyl
EC_{50}	Effect Concentration to 50% of Organisms
EE	Exposed mother, Exposed embryos
EEM	Environmental Effects Monitoring
ELS	Early Life Stages
ERCB	Energy Research Conservation Board
EROD	Hepatic 7-ethoxyresorufin-O-deethylase activity
ESI-FT-ICR MS	Electrospray Ionization Fourier Transform Ion Cyclotron Resonance Mass Spectrometry
ESI-MS	Electrospray Ionization Mass Spectrometry
FTFC	Fine Tailings Fundamentals Consortium
FTIR	Fourier Transform Infrared Spectroscopy

GC-MS	Gas Chromatography Mass Spectrometry
GCxGC-MS	Two-dimension Gas Chromatography Mass Spectrometry
GSI	Gonadal Somatic Index
HPLC-ESI-HRMS	High Pressure Liquid Chromatography Electrospray Ionization High Resolution Mass Spectrometry
HPLC-HRMS	High Performance Liquid Chromatography High Resolution Mass Spectrometry
HPLC-QTOF-MS	High Performance Liquid Chromatography Quadrupole Time of Flight Mass Spectrometry
KS	Kolmogorov Smirnov
LC/MS	Liquid Chromatography Mass Spectrometry
LC_{50}	Concentration Lethal to 50% of Organisms
LC-HRMS	Liquid Chromatography High Resolution Mass Spectrometry
LC-TOF-MS	Liquid Chromatography Time of Flight Mass Spectrometry
LOEC	Lowest Observable Effect Concentration
LSI	Liver Somatic Index
MFT	Mature Fine Tailings
MLSB	Mildred Lake Settling Basin
MS	Mass Spectrometry
n	Sample size
NA(s)	Naphthenic Acid (s)
NOAEL	Not adversely affecting life processes
NOEC	No Observable Effect Concentration
NSD	No Significant Difference
OAF	Organic Acid Fraction
OECD	Organization for Economic Co-operation and Development

OSPW	Oil Sands Process-Affected Water
OSWRTWG	Oil Sands Water Release Technical Working Group
PAC(s)	Polycyclic Aromatic Compound (s)
PAH(s)	Polycyclic Aromatic Hydrocarbon (s)
PTFE	Polytetrafluoroethylene
PVC	Poly Vinyl Chloride
QA/QC	Quality Assurance Quality Control
RAMP	Regional Aquatics Monitoring Program
RE	Reference mother, Exposed embryos
ROS	Reactive Oxygen Species
RR	Reference mother, Reference embryos
SE	Standard Error
T	Testosterone
TIE	Toxicity Identification Evaluation
UPLC-HRMS	Ultra Performance Liquid Chromatography High Resolution Mass Spectrometry
US EPA	United States Environmental Protection Agency
WIP	West In Pit

CHAPTER 1: INTRODUCTION

1.1 Problem Statement

The economic importance of the Canadian oil sands industry cannot be overstated. According to recent projections by the Canadian Energy Research Institute (CERI), over the next 25 years new oil sands development is expected to contribute $2.1 trillion and over 800,000 jobs to the Canadian economy (CAPP, 2011). However, rapid development of the oil sands industry has raised concerns regarding potential environmental impacts affecting ecosystem health (Natural Resources Canada – CanmetENERGY, 2010; Royal Society of Canada, 2010).

Of the estimated 170 billion barrels of bitumen located in northern Alberta, only 20% are shallow enough to be recovered through surface mining (Alberta Government, 2012). In surface mining operations, the bitumen is separated from the sand using various modifications of the Clark Water Extraction process (Masliyah et al. 2011). The resulting froth, which contains the extracted bitumen, then undergoes further treatment before it is upgraded to synthetic crude oil (Masliyah et al. 2004; Romanova et al. 2004). Oil sands process-affected water (OSPW) is a by-product of these processes.

Provincial legislation (Alberta Environmental Protection and Enhancement Act, Section 23, 1993) prohibits the release of potentially toxic waste streams to the environment. As a result, more than 1 billion cubic meters of OSPW is currently maintained on lease sites within active settling basins, commonly known as tailings ponds (Li et al. 2014). These immense volumes are expected to increase with the ongoing expansion of oil sands mining operations (ERCB, 2009).

Oil sands process-affected water poses significant environmental challenges for both industry and regulators. OSPW has been defined as any water that has come into contact with

bitumen in the extraction process or within the mine (Natural Resources Canada – CanmetENERGY, 2010). Although this broad definition encompasses a variety water types or sources within surface mining operations, OSPW is generally divided into two categories: (i) raw (or fresh) OSPW that is stored in active settling basins, and (ii) aged or treated OSPW that is stored within various experimental reclamation sites.

In 2011, concerns about the environmental impact of oil sands operations resulted in the development of the Joint Canada-Alberta Implementation Plan for Oil Sands Monitoring (Environment Canada, 2011a-c; 2012a). This plan outlines the steps required for the implementation of a world-class monitoring system to ensure the economic benefits of this valuable resource are achieved in an environmentally sustainable fashion (Environment Canada, 2011a-c; 2012a). The success of any monitoring program requires a thorough understanding of the source of the contaminants, pathways of potential exposure, and sensitivity of ecosystem receptors to environmentally relevant concentrations (Munkittrick and McCarty, 1995).

This source, pathway and receptor concept applies to both individual contaminants and complex mixtures (e.g., pulp and paper effluents, metal mining effluents, municipal sewage and OSPW). In surface mining operations, active settling basins (or tailings ponds) are the primary source of potential migration and off-site transport of raw OSPW. Although seepage protection measures are currently implemented on all lease sites, the potential risk associated with the design of tailings ponds is still a concern for regulators (Royal Society of Canada, 2010).

OSPW contains a variety of toxic substances, including naphthenic acids (NAs), phenols, polycyclic aromatic compounds, benzene, toluene, ethylbenzene and xylene (BTEX), metals and salts (Allen, 2008; Natural Resources Canada – CanmetENERGY, 2010). Raw OSPW is acutely

and chronically toxic to a variety of aquatic organisms (Peters et al. 2007; Zubot et al. 2012; Anderson et al. 2012a; Wiseman et al. 2013b; Mahaffey and Dubé, In prep). Early studies to examine various treatment options found the acute toxicity of raw OSPW was removed through naturally occurring physical, chemical and biological processes when isolated in experimental test pits for 1 to 2 years (Boerger and Aleksiuk, 1984; MacKinnon and Boerger, 1986). The results of these studies led to the development of various wet landscape reclamation strategies to investigate the natural detoxification of OSPW (Boerger et al. 1992; FTFC, 1995).

While both raw and aged OSPW contain a complex mixture of inorganic and organic constituents, the major source of toxicity has been traced to naphthenic acids (FTFC, 1995; CEATAG, 1998; Canada Natural Resources – CanmetENERGY, 2010). Naphthenic acids are a large and complex mixture of carboxylic acid surfactants that are native to most sources of petroleum, including bitumen, and are solubilised into OSPW during the extraction process (Seifert and Teeter, 1970; Brient et al. 1995; Headley and McMartin, 2004). This family includes acyclic and alicyclic carboxylic acids fitting the classical naphthenic acid formula, $C_nH_{2n+z}O_2$, as well as hydroxy, dibasic, heteroatom, aromatic, and diamondoid adamantane acids (Bataineh et al. 2006; Frank et al. 2009; Headley et al. 2011a; Rowland et al. 2011a-e; Lengger et al. 2013).

Due to the variety of structural classes that do not adhere to the classical naphthenic acid formula, some researchers believe using the term "naphthenic acids" to describe the acid extractable fraction of OSPW is misleading (Grewer et al. 2010; Headley et al. 2013). However, while it is understood that other organic acid components are present, "oil sands naphthenic acids" or "naphthenic acid extract" are the most common terms used to describe the complex suite of organic acids in OSPW (Kavanagh et al. 2012; Woodworth et al. 2012; Headley et al. 2013; Leclair et al. 2013; MacDonald et al. 2013).

Although organic compounds that do not conform to the classical naphthenic acid formula are also found in commercially available naphthenic acid preparations (Brient et al. 1995; Grewer et al. 2010; West et al. 2011; Rowland et al. 2011a; Swigert et al. 2015), several studies have reported significant differences in the composition and biodegradation of commercial and oil sands naphthenic acid mixtures (Clemente et al. 2003; Barrow et al. 2004; Headley and McMartin, 2004; Scott et al. 2005; Grewer et al. 2010). Biodegradation studies indicate that the least cyclic naphthenic acids containing a lower degree of alkyl branching are most easily degraded. The more cyclic, highly branched naphthenic acids are recalcitrant to degradation (Bataineh et al. 2006; Han et al. 2008).

The decrease in acute toxicity of aged OSPW is generally attributed to the microbial degradation of the more labile (easily degraded) naphthenic acids in raw OSPW (Holowenko et al. 2002; Clemente et al. 2004; Bataineh et al. 2006; Han et al. 2008; 2009). While previous studies with Microtox indicated oil sands naphthenic acid toxicity was mainly influenced by lower molecular weight compounds (Holowenko et al. 2002; Clemente et al. 2004; Frank et al. 2008), the same response was not observed in fish (Bauer, 2013). Recent modelling and fractionation studies with oil sands extracted naphthenic acid mixtures indicate toxicity is likely driven by the structural properties of the various compound classes present within the organic acid fraction of raw OSPW rather than broad range molecular weight differences (Scarlett et al. 2012; 2013; Bauer, 2013).

The chronic toxicity of aged OSPW has been attributed to the remaining recalcitrant fraction of naphthenic acids (Han et al. 2008; 2009; Li et al. 2014). However, the presence of other inorganic and organic contaminants in aged OSPW confounds these conclusions (McNeill et al. 2012; van den Heuvel et al. 2012; Leclair et al. 2013). Due to the limited understanding of

factors that modify oil sands naphthenic acid toxicity, it is possible that other environmental factors, such as dissolved oxygen, temperature, salinity and hardness may increase or decrease the sensitivity of biota to naphthenic acids within reclamation environments (CEATAG, 1998; Kavanagh et al. 2012; Li et al. 2014).

Although numbers are increasing, toxicological assessments of the acid extractable fraction of OSPW are limited. Reported effects of oil sands extracted naphthenic acid mixtures include alterations in water uptake and reduced growth in aquatic plants and algae (Armstrong et al. 2008; 2009; Woodworth et al. 2012; Goff et al. 2013), decreased survival in fish (Nero et al. 2006b) and invertebrates (Armstrong et al. 2009), increased embryo-larval deformities (Farwell et al. 2006), alterations in gill and immune function (Nero et al. 2006b; Leclair et al. 2013), increased bile metabolites and liver somatic indices (Kavanagh et al. 2012; Leclair et al. 2013), and decreased fecundity, spawning, plasma sex steroids and secondary sex characteristics in fish (Kavanagh et al. 2012).

Commercial naphthenic acid mixtures have been used as a surrogate to assess the toxicity of the organic acids in OSPW. While similar effects have been reported for commercial surrogates (Dokholyan and Magomedov, 1983; Hagen et al. 2012), direct comparison studies have shown significant differences in the toxicological response of fish, plants and mice exposed to commercial and oil sands extracted naphthenic acid mixtures (Nero et al. 2006b; Armstrong et al. 2008; Garcia-Garcia et al. 2011a; 2011b). These results have led researchers to question the use of commercial mixtures as surrogates for assessing the toxicity of the organic acid fraction of OSPW (Peters et al. 2007; West et al. 2011; Tollefsen et al. 2012; MacDonald et al. 2013; Swigert et al. 2015).

There are currently no water quality guidelines for the protection of aquatic life for naphthenic acids in Canada or the United States (AESRD, 2014; Golder, 2014). In the 1998 CEATAG review, none of the toxicological assessments of naphthenic acids using both commercial surrogates and OSPW whole effluent toxicity tests met the Alberta Environmental Protection primary data standards required for the development of a water quality guideline for naphthenic acids. This included the Toxicity Identification Evaluation conducted by Verbeek et al. (1993; 1994) that identified naphthenic acids as the primary toxic component of OSPW (CEATAG, 1998). In a more recent review, Golder Associates (2014) also reported there was insufficient data in the published literature to develop a chronic effects benchmark specific for oil sands naphthenic acids using the species sensitivity distribution approach.

In order to improve monitoring efforts and establish a guideline that is relevant to oil sands naphthenic acids, more information is needed to understand the composition of naphthenic acid components in raw OSPW and their role in determining OSPW toxicity. Understanding population-level responses to organic contaminants like naphthenic acid mixtures is critical to the ecological risk assessment process (Miller and Ankley, 2004). Impacts during early development and reproduction are the ultimate determinants of population viability and status (Ankley and Villeneuve, 2006). While several studies have reported significant differences in the composition and/or toxicity of commercial and extracted naphthenic acid mixtures, a direct comparison study to assess population-level responses in fish has not been done.

1.2 Purpose of Study

The purpose of this study was to compare the reproductive and embryo-larval effects in fathead minnow exposed to a commercial and an oil sands extracted naphthenic acid mixture.

Kavanagh et al. (2011; 2012; 2013) recently conducted a series of studies to examine the potential reproductive effects of aged OSPW in various wet landscape reclamation strategies. As such, the naphthenic acid extract used in their study was artificially aged to resemble the organic acids in aged OSPW (Kavanagh et al. 2012). The work described in this thesis compares the reproductive effects of a commercial and a freshly extracted naphthenic acid mixture in fathead minnows. The naphthenic acids were extracted from raw OSPW collected from an active tailings pond and the mixture was not artificially aged or simulated.

Reproductive and embryo-larval endpoints were included in this study to increase the toxicological database of the organic acid fraction of OSPW (OSWRTWG, 1996; CEATAG, 1998). This study used a flow-through system for the exposure experiments to achieve steady-state naphthenic acid concentrations. To improve the environmental relevance, reverse osmosis water was used to match water quality conditions in the Athabasca River. A tissue extraction method that is applicable for the analysis of naphthenic acids in fish using high resolution mass spectrometry was developed as a fundamental tool to assess naphthenic acid uptake and exposure.

Results of this study can be used to help inform regulators in the development of an oil sands naphthenic acid guideline by further clarifying the differences between commercial and extracted naphthenic acid mixtures, and improving our understanding of the sublethal effects of a "fresh" naphthenic acid extract to fish exposed to environmentally relevant concentrations and reference water conditions. Development of a new tissue extraction method that is suitable for analyses using high resolution mass spectrometry would help advance the progress of alternative environmental monitoring options (Headley et al. 2009a; 2011c; 2013; van den Heuvel et al. 2014).

1.3 Research Objectives

The primary objective of this research was to conduct a thorough source (naphthenic acid mixtures), pathway (waterborne exposure) to receptor (uptake and response) analysis for reproducing fish exposed to a commercial and an extracted naphthenic acid mixture. The first set of objectives focus on the assessment and comparison of response, while the second set of objectives focus on uptake and measurement of naphthenic acids in fish muscle tissue. Specifically my objectives were to:

Objective 1a: Assess the effects of waterborne exposure to a commercial and an extracted naphthenic acid mixture on fathead minnow reproduction and embryo-larval development.

Null Hypothesis: Exposure of adult fathead minnow to environmentally relevant concentrations of naphthenic acids will have no effect on the reproduction and survival of their offspring.

Objective 1b: Compare the reproductive and embryo-larval response of fathead minnow exposed to a commercial and an extracted naphthenic acid mixture.

Null Hypothesis: The reproductive and embryo-larval response of fathead minnow will not be different between mixtures.

Objective 1c: Compare the embryo-larval response of commercial and extracted naphthenic acid mixtures in the presence and absence of parental exposure.

Null Hypothesis: Parental exposure will not affect the embryo-larval response of fathead minnow exposed to commercial and extracted naphthenic acid mixtures.

Objective 2a: Develop a tissue extraction method applicable for the analysis of naphthenic acids using high resolution mass spectrometry to measure uptake and confirm exposure of naphthenic acids to fathead minnow.

Null Hypothesis: Commercial and extracted NA mixtures will not be taken up in fathead minnow tissue.

Objective 2b: Compare uptake in male and female fathead minnow exposed to a commercial and an extracted NA mixture.

Null Hypothesis: There will be no differences in the uptake between males and females.

Objective 2c: Assess and compare differences in the uptake of a commercial vs. extracted NA mixture.

Null Hypothesis: There will be no differences in the uptake of commercial and extracted NA mixtures.

CHAPTER 2: LITERATURE REVIEW

2.1 Background

The objective of this chapter is to provide a review of the composition and toxicity of commercial and extracted naphthenic acid mixtures, and the approaches that have been used to investigate their role in the toxicity of both raw and aged OSPW. The oil sands region in northern Alberta, Canada, contains an estimated 1.7 trillion barrels of oil and represents the third largest deposit of crude oil in the world (Alberta Government, 2011). Canada's oil sands account for 56% of total world reserves open for private sector investment (CERI, 2011). Increasing energy demands, coupled with improvements in extraction technologies, have led to a rapid expansion of the oil sands industry. The development of Alberta's oil sands currently accounts for more than 50% of Canada's oil production (CAPP, 2013). Over the next 25 years, new development is expected to contribute $2.1 trillion and over 800,000 jobs to the Canadian economy (CERI, 2011).

Alberta's oil sands include three main deposits: Athabasca, Cold Lake and Peace River, covering a surface area of over 140,000 km^2 (Figure 2-1) (Alberta Government, 2012). Oil sands deposits contain a mixture of bitumen (a heavy, highly-biodegraded form of crude oil), clay, sand and formation water, which are located at varying depths beneath an overburden of muskeg, glacial tills, sandstone and shale (Schramm et al. 2000). Roughly 80% of the oil sands are only accessible by in situ methods (Alberta Government, 2012). The remaining shallower deposits (up to 75 m below the surface) are accessed via surface mines, most of which are located in the Athabasca deposit (Figure 2-1) (Allen, 2008).

In surface mining operations, the bitumen is separated from the oil sands using various modifications of the Clark Water Extraction process (Masliyah et al. 2011). Three main tailings streams are generated with the extraction of bitumen from the oil sand ore: (i) coarse tailings from the primary bitumen separation step, (ii) fine fluid tailings from the secondary and/or tertiary bitumen recovery step, and (iii) froth treatment tailings (Mikula et al. 2008). All three are aqueous slurries that contain varying portions of minerals, metals, water, dissolved inorganic salts and residual organics (Kasperski and Mikula, 2011).

Figure 2-1. Map of oil sands deposits in northeastern, Alberta, Canada. *Source*: http://www.oilsands.alberta.ca/reclamation.html

The coarse tailings fraction is mainly composed of sand and unrecovered bitumen, which quickly settles out within the settling basin and is subsequently used to build the containment dykes surrounding the tailings ponds (List and Lord, 1997). Although the sand itself is relatively

inert, the unrecovered bitumen within the sand dykes is a potential source of contaminants to the environment as a result of dissolution into groundwater (FTFC, 1995).

The fine fluid tailings stream is mainly composed of silt and clay minerals (predominantly kaolinite and illite), which gradually settle over three to five years until they reach 30 to 40 wt% (Mikula et al. 1996). At this density, the slurry is classified as mature fine tailings (MFT). Further consolidation of MFT is extremely slow and has led to the development of several tailings remediation technologies to help reduce the growing volumes of MFT currently stored on lease sites (Kasperski and Mikula, 2011).

The froth-treatment tailings stream is generated in the final step of the recovery process. Naphtha or paraffinic solvents are added to the extracted bitumen froth to decrease the viscosity and allow for more efficient separation of the bitumen from the residual minerals and water (Romanova et al. 2004; Shelfantook, 2004). The resulting froth-treatment tailings are particularly important because of the environmental impact of the residual solvent or diluents that are used. However, their contribution to the total tailings volume is relatively small (Mikula et al. 2008).

Tailings and wastewater management practices are unique to each oil sands mine and have evolved over time (List and Lord, 1997). Some operations keep the upgrader wastewater and/or froth-treatment tailings separate from the other tailings streams, while others combine all three (Kasperski and Mikula, 2011). Regardless of the individual strategies employed, tailings generated in the recovery of bitumen are pumped to active settling basins or tailings ponds. Here, the solids settle out and the overlying clear water is collected and recycled back into the extraction process (Masliyah et al. 2004; 2011).

2.2 Oil Sands Process-Affected Waters

OSPW is generally composed of 70-80 wt% water, 20-30 wt% solids (sand, silt and clay minerals) and 1-3 wt% residual bitumen (Allen, 2008). Organic compounds are the primary contaminants within OSPW; bitumen is the major source of these organic compounds (Schramm et al. 2000). Connate waters and clay minerals within the ore are the primary sources for the inorganic compounds (Mikula et al. 1996). Ore quality is the primary factor influencing raw OSPW composition because it affects residual bitumen and naphthenic acid concentrations, total dissolved and suspended solids, clay and mineral content (and associated metals), inorganic ion (or salt) concentrations, as well as the amount of additional process aids that are required to maintain extraction efficiency (FTFC, 1995; Schramm et al. 2000; Romanova et al. 2004).

OSPW contains several general classes of contaminants including: total suspended and dissolved solids, residual bitumen and other hydrocarbons such as benzene, toluene, ethylbenzene, xylene (BTEX) and polycyclic aromatic compounds (PACs), naphthenic acids, phenolics, ammonia, sulfides, trace and heavy metals, as well as other inorganic ions such as Na^{+}, Cl^{-}, $(SO_4)^{2-}$ and $(HCO_3)^{-}$ (Allen, 2008; Natural Resources Canada – CanmetENERGY, 2010). Relative to natural surface waters in the lower Athabasca River Basin, raw OSPW can be characterized by high concentrations of salts, residual hydrocarbons, and a highly complex mixture of neutral and polar organic compounds, commonly known as naphthenic acids (MacKinnon et al. 2005; Ross et al. 2012).

In Alberta, provincial legislation (Alberta Environmental Protection and Enhancement Act, Section 23, 1993) prohibits the release of potentially toxic waste streams to the environment (Headley and McMartin, 2004). As such, OSPW is not currently approved for release to the environment. However, treatment and release of process-affected waters is considered as part of

longer term tailings management strategies in combination with mine closure landscapes, which include constructed wetlands and end pit lakes (Martin et al. 2010; Anderson et al. 2012a).

Oil sands process-affected water has been defined as any water that has come into contact with bitumen in the extraction process or within the mine (Natural Resources Canada – CanmetENERGY, 2010). In surface mining operations, this broad definition encompasses a variety of water types or sources, including: connate water (water trapped in the pores of the ore), water used in the bitumen extraction and upgrading process, water released from fine fluid tailings, dyke drainage and seepage collection waters, transport water for sand storage facilities, mine run-off from overburden and coke storage areas, as well as aquifer water from the dewatering of ore deposits prior to surface mining. Although not all of these water sources are directly generated from the extraction and upgrading of bitumen, depending on the water management practices employed within individual mining operations, they are all collected and maintained in active settling basins or tailings ponds (Table 2-1). In addition to the waters described above, this definition also encompasses a variety of "process-affected" waters that are currently being investigated for tailings remediation options and wet landscape reclamation strategies (Table 2-1).

Table 2-1. Definitions of oil sands process-affected water types identified in the literature with references to selected articles in which toxicity was examined.

Major Types of OSPW in Literature	Definition
Raw OSPW	Process water collected from active settling basins (tailings ponds or external tailings facilities) that receive active or fresh inputs of wastewaters from the extraction and upgrading of bitumen (Peters et al. 2007; Zubot et al. 2012)
Consolidated tailings release water (CT)	Water released through the consolidation of a mixture of mature fine tailings and course tailings (sand) treated with gypsum or other coagulation aids (Mikula et al. 1998; MacKinnon et al. 2001)
Constructed wetlands receiving CT discharge	Series of wetlands located within the CT Demonstration Study Site to assess various approaches for reclaiming a CT landscape (Neville et al. 2011). Created in 1999 by flooding a 52-ha area with consolidated mine tailings. Since then, the site has received tailings from an active settling basin through a discharge pipe (Gentes et al. 2007b)
Dyke drainage, mine runoff & seepage collection waters	Water collected from dyke seepage control systems (seepage ponds & ditches, dyke filter drainage & finger drains) (MacKinnon et al. 2005; Toor et al. 2013a) and mine runoff and leachates from overburden and coke storage areas (Holowenko et al. 2002; Puttaswamy et al. 2010)
Wetlands composed of seepage and CT discharge	Natural Wetland: opportunistic wetland formed from dyke seepage that has received historical CT discharges (Smits et al. 2000; Wayland et al. 2008; Toor et al. 2013a)
Wetlands created from saline overburden & lean oil sands material	South Bison: most studied reclamation wetland within this category (van den Heuvel et al. 1999a; 1999b; 2000; 2012)
Reclamation ponds with fine tailings and fresh water	Demonstration Pond: most studied reclamation pond within this category (McNeill et al. 2012; Leclair et al. 2013)
Reclamation ponds with fine tailings and OSPW	Pond 5 or FE5: most studied reclamation pond within this category (Siwik et al. 2000; Nero et al. 2006a; Wiseman et al. 2013b)
Reclamation ponds with OSPW only (aged OSPW)	Pond 9: most studied reclamation pond within this category (Kavanagh et al. 2011; Anderson et al. 2012a)
Reclamation ponds with aged CT release water	Mike's Pond: most studied reclamation pond within this category (Leonhardt, 2003; Hersikorn et al. 2011; Kennedy, 2012)

2.3 Oil Sands Process-Affected Water Toxicity

Despite the diversity of sources listed above, OSPW is generally divided into two categories: (i) raw (or fresh) OSPW that is stored in active settling basins and (ii) aged (or treated) OSPW that is stored within various experimental reclamation sites. Raw OSPW is acutely and chronically toxic to a variety of aquatic organisms (MacKinnon and Retallack, 1982; Wiseman et al. 2013b; Mahaffey and Dubé, In prep) (Tables 2-2 and 2-3). Reported effects in fish exposed to raw OSPW include acute lethality in rainbow trout (Nix and Martin, 1992), decreased embryo-larval survival and increased deformity rates in Japanese medaka, yellow perch (Peters et al. 2007) and fathead minnow (He et al. 2012a), as well as alterations in gene transcripts related to oxidative stress, endocrine signalling and immune response in fathead minnow embryos (He et al. 2012a) and adults (He et al. 2012b; Wiseman et al. 2013a) (Table 2-2). Reported effects in invertebrates exposed to raw OSPW include decreased survival and reproduction in *Ceriodaphnia dubia* (Puttaswamy et al. 2010; Zubot et al. 2012), decreased growth, pupation and emergence in field and lab-reared Chironomid spp. (Whelly, 1999; Pourrezaei et al. 2011; Anderson et al. 2012a), as well as alterations in gene transcripts related oxidative stress, apoptosis and endocrine signalling in *Chironomus dilutus* (Wiseman et al. 2013b) (Table 2-3).

Early studies to examine various treatment options found that the acute toxicity of raw OSPW was removed through naturally occurring physical, chemical and biological processes in isolated experimental test pits after 1 to 2 years (Boerger and Aleksiuk, 1984; MacKinnon and Boerger, 1986). The results of these studies led to the development of various wet landscape reclamation strategies to investigate the natural detoxification of OSPW (Boerger et al. 1992; FTFC, 1995). As indicated by Table 2-1, a significant amount of research has been conducted to

Table 2-2. Study summaries of raw OSPW effects on fish. The results are summarized according to the following notations: (NSD) no significant difference relative to control, (−) significant decrease relative to control, (+) significant increase relative to control. If data were provided, the magnitude of change was calculated: (exposure - control) / control*100%

Reference	Raw OSPW	Species	Duration	Endpoint & Result
MacKinnon, 1981	MLSB[a]	Rainbow trout	Acute: 96h	Survival (LC50 < 4%)
MacKinnon & Retallack, 1982	MLSB[a]	Rainbow trout	Acute: 96h	Survival (LC50: 4-6%)
Boerger & Aleksiuk, 1984	MLSB[a]	Rainbow trout	Acute: 96h	Survival (0%)
Boerger et al. 1986	MLSB[a]	Rainbow trout	Acute: 96h	Survival (LC50: 8%)
MacKinnon, 1986	MLSB[a]	Rainbow trout	Acute: 96h	Survival (LC50: 7%)
MacKinnon & Boerger, 1986	MLSB[a]	Rainbow trout	Acute: 96h	Survival (LC50: 7%)
Rogers et al. 2007	MLSB[a]	Rainbow trout	Acute: 96h (Serial Dilution)	Survival: 5% OSPW (73%), 10% OSPW (87%), 20% OSPW (33%), 50% OSPW (0%)
Boerger et al. 1986	Recycle Pond	Rainbow trout	Acute: 96h	Survival (LC50: 15%)
Nix & Martin, 1992	Suncor Pond 1	Rainbow trout	Acute: 96h	Survival: 1981 (LC50: 17%), 1982 (LC50: 7.5-10.2%), 1984 (LC50: 4.5%), 1988 (LC50: 28%), 1989 (LC50: 3.2%)
Nix & Martin, 1992	Suncor Pond 1A	Rainbow trout	Acute: 96h	Survival: 1981 (LC50: 27%), 1982 (LC50: 24%), 1984 (LC50: 5.8%), 1988 (LC50: 92%), 1989 (LC50: 3.2%)
Nix & Martin, 1992	Suncor Pond 2	Rainbow trout	Acute: 96h	Survival: 1981 (LC50: 16%), 1982 (LC50: 4.2-5.1%), 1984 (LC50: 4.2%), 1988 (LC50: 26%), 1989 (LC50: 3.2%)
Nix & Martin, 1992	Suncor Pond 3	Rainbow trout	Acute: 96h	Survival: 1988 (LC50: 5.8%), 1989 (LC50: 3.2%)
Zubot et al. 2012	West In Pit (WIP)	Rainbow trout	Acute: 96h	Survival (LC25 >25%), (LC50: 35%), (0%)
MacKinnon & Retallack, 1982	MLSB[a]	Fathead minnow	Acute: 96h	Survival (LC50: 6-8.5%)
Peters et al. 2007	MLSB[a]	Yellow perch	Chronic: ELS[b] (Serial Dilution)	Fertilization success: 0.16% OSPW (NSD), 0.8% OSPW (NSD), 4% OSPW (NSD), 20% OSPW (NSD), 100% OSPW (0%); Incidence of deformities (+ ; optic-cephalic & spinal deformities, calculated threshold: 7.25 mg/L OSPW-NAs); Larval hatch length (− ; calculated threshold: 1.98 mg/L OSPW-NAs)

Table 2-2 (continued)

Reference	Raw OSPW	Species	Duration	Endpoint & Result
Peters et al. 2007	MLSB[a]	Japanese medaka	Chronic: ELS[b] (Serial Dilution)	Incidence of deformities (+ ; circulatory (pericardial edema & tube heart) and head region (anisophthalmia, microphthalmia & mandible) deformities, calculated threshold: 30 mg/L OSPW-NAs); Larval hatch length (− ; calculated threshold: 6.18 mg/L OSPW-NAs)
He et al. 2012 a	West In Pit (WIP)	Fathead minnow	Chronic: 7d ELS[b] 100% OSPW	Larval survival (−55.3 %); Cumulative hatch rate (+ ; at 48h, 72h, 96h, 120h & 144h post-fertilization); Spontaneous embryo movement (+ 92.1 %); Incidence of deformities: Hemorrhage (+ 50 %); Pericardial edema (+ 56.2 %); Spinal malformations (+ 37.5 %)
He et al. 2012 a	West In Pit (WIP)	Fathead minnow	Chronic: 7d ELS[b] 100% OSPW	Gene transcripts: Biotransformation / Detoxification: *cyp1a* (NSD); *cyp3a* (+ 2.35-fold); Oxidative Stress: *gst* (+ 2.15-fold); *sod* (+ 3.08-fold); Apoptosis: *casp9* (+ 3.26-fold); *apopen* (+2.38-fold); Generation of Reactive Oxygen Species (+ 1.68-fold)
He et al. 2012 a	West In Pit (WIP)	Fathead minnow	Chronic: 7d ELS[b] 100% OSPW	Summary: results suggest that caspase-activated apoptotic cell death, induced by oxidative stress resulting from metabolism of substrates by P450 enzymes that are not induced by activation of the AhR, was the primary mechanism of effects on fathead minnow embryos
He et al. 2012 b	West In Pit (WIP)	Fathead minnow	Chronic: 7d 100% OSPW	Survival (100%)
He et al. 2012 b	West In Pit (WIP)	Fathead minnow	Chronic: 7d 100% OSPW	Endocrine gene transcripts (Females) Brain: *lhβ* (+ 5.3-fold); *gnrhr* (−); Gonads: *fshr* (− 0.02-fold) and *lhr* (− 0.33-fold); Liver: *ar* (− 0.18-fold); *erα* (− 0.14-fold); *erβ* (− 0.08-fold); *vtg* (− 0.002-fold); *chg-l* (− 0.022-fold) and *chg-h* (− 0.036-fold)
He et al. 2012 b	West In Pit (WIP)	Fathead minnow	Chronic: 7d 100% OSPW	Endocrine gene transcripts (Males) Brain: *erα* (+ 5.14-fold); *kiss1r* (+ 6.11-fold); *fshβ* (+ 3.96-fold); *lhβ* (+ 3.04-fold); *cyp19b* (+ 3.44-fold) and *gnrhr* (− 0.13-fold); Gonads: *fshr* (+ 3.7 ± 0.43); *lhr* (+ 2.5 ± 0.59); *cyp11a* (+) and *3βhsd* (+); Liver: *erα* (+ 4.1-fold); *vtg* (+ 4.9-fold); *chg-l* (+ 5.4-fold) and *chg-h* (+ 3.4-fold)
He et al. 2012 b	West In Pit (WIP)	Fathead minnow	Chronic: 7d 100% OSPW	Summary: OSPW increased transcripts important for the synthesis of gonadotropins in both male & female brains, increased transcripts for gonadotropin receptors & steroidogenesis in male gonads, and increased estrogen-responsive transcripts in male livers. In contrast, OSPW decreased gonadotropin receptor and estrogen-responsive transcripts in female gonads & livers. Results suggest OSPW may impair hypothalamic-pituitary-gonadal (HPG) signaling & 17β-estradiol synthesis; decreased *fshr*, *lhr*, *cyp11a* & *cyp17* in female gonads support this mechanism of action

Table 2-2 (continued)

Reference	Raw OSPW	Species	Duration	Endpoint & Result
Wiseman et al. 2013 a	West In Pit (WIP)	Fathead minnow	Chronic: 7d 100% OSPW	Survival (100%); Observations of distress (No changes in feeding behavior or ability to maintain body position in water column)
Wiseman et al. 2013 a	West In Pit (WIP)	Fathead minnow	Chronic: 7d 100% OSPW	Male liver gene transcripts: Phase I Biotransformation/Detoxification: *cyp1a* (+ 2.1-fold); *cyp2j28* (+ 2.2-fold); *cyp2ad2* (+ 2.7-fold); *cyp2k6* (+ 10.1-fold); *cyp2k19* (+ 11.7-fold); *ao1* (+ 3.1-fold1); *aldh2* (+ 3.6-fold); *moa* (+ 3.2-fold) and *eh* (+ 2.0-fold)
Wiseman et al. 2013 a	West In Pit (WIP)	Fathead minnow	Chronic: 7d 100% OSPW	Male liver gene transcripts: Phase II & III Biotransformation/Detoxification: *gst-m* (+ 4.5-fold); *gst-c* (+ >23.3-fold); *ugt2a3* (+ 6.3-fold); *sult1,3* (+ 1.8-fold); and *ugt5f1* (− 4.3-fold)
Wiseman et al. 2013 a	West In Pit (WIP)	Fathead minnow	Chronic: 7d 100% OSPW	Male liver gene transcripts: Oxidative Stress / Antioxidant Defense: *gs* (+ 3.1-fold); *gr* (+ 3.2-fold); *gpx* (+ 1.7-fold); *tk* (+ 2.4-fold); *6-pgdh* (+ 10.1-fold) and *g6pdh* (+ 2.7-fold)
Wiseman et al. 2013 a	West In Pit (WIP)	Fathead minnow	Chronic: 7d 100% OSPW	Male liver gene transcripts: Immune Response / Immune Complement Proteins: *c8β* (− 2.1-fold); *c1gyc* (− 19.7-fold); *c3* (− 7.6-fold); *c3-h1* (− 2.1-fold) and *c4-2* (− 2.0-fold)
Wiseman et al. 2013 a	West In Pit (WIP)	Fathead minnow	Chronic: 7d 100% OSPW	Summary: OSPW decreased transcripts related to immune response and increased transcripts related to xenobiotic metabolism (biotransformation / detoxification), oxidative stress response & apoptosis in male livers. Results suggest sublethal toxicity of OSPW is due to increased generation of reactive oxygen species (ROS), via P450-mediated metabolism, which induces caspase-independent apoptosis & autophagy. OSPW may also exert chronic toxicity due to effects on the immune system

[a] MLSB: Mildred Lake Settling Basin

[b] ELS: Early Life Stages

Table 2-3. Study summaries of raw OSPW effects on invertebrates. The results are summarized according to the following notations: (NSD) no significant difference relative to control, (−) significant decrease relative to control, (+) significant increase relative to control. If data were provided, the magnitude of change was calculated: (exposure - control) / control*100%

Reference	Raw OSPW	Species	Duration	Endpoint & Result
MacKinnon & Retallack, 1982	MLSB[a]	Daphnia magna	Acute: 96h	Survival (LC50: 16-27%)
Boerger & Aleksiuk, 1984	MLSB [a]	Daphnia	Acute: 96h	Survival (0%)
MacKinnon, 1986	MLSB [a]	Daphnia pulex	Acute: 96h	Survival (LC50: 2%)
MacKinnon & Boerger, 1986	MLSB [a]	Daphnia pulex	Acute: 96h	Survival (LC50: 2%)
Zubot et al. 2012	West In Pit (WIP)	Daphnia magna	Acute: 48h	Survival (LC25 > 100%), (LC50 > 100%), (IC25 > 100%), (IC50 > 100%)
Zubot et al. 2012	West In Pit (WIP)	Ceriodaphnia dubia	Chronic: 6d	Survival (LC25: 52%), (LC50: 65%); Fecundity (IC25: 8%), (IC50: 39%)
Puttaswamy et al. 2010	Recycle Water Pond	Ceriodaphnia dubia	Chronic: 7-8d	Survival (LC50: 70.7%); Reproduction (IC50: 49.4%)
Whelly 1999 MSc	Suncor OSPW[c]	Chironomus riparius	Chronic: 14d (Serial Dilution)	Survival (NSD); Length (NSD); Emergence (EC50: 64%), (NOEC: 25%), (LOEC: 50%); Mentum deformities (NSD)
Whelly 1999 MSc	Suncor OSPW[c]	Chironomus dilutus (lab population)	Chronic: 14d (Serial Dilution)	Survival (LC50: 71%), (NOEC: 25%), (LOEC: 50%); Length (EC50: 75%), (NOEC: 25%), (LOEC: 50%); Mentum deformities (NSD)
Whelly 1999 MSc	Suncor OSPW[c]	Chironomus dilutus (field population[d])	Chronic: 14d (Serial Dilution)	Survival (LC50: 71%), (NOEC: 25%), (LOEC: 50%); Length (EC50: 200%), (NOEC: 50%), (LOEC: 100%); Mentum deformities (NSD)
Pourrezaei et al. 2011	West In Pit (WIP)	Chironomus dilutus	Acute: 10d 100% OSPW	Survival (100%); Growth (−)
Anderson et al. 2012 a	West In Pit (WIP)	Chironomus dilutus	Acute: 10d 100% OSPW	Survival WIP-2009 (−35.9 %); WIP-2010 (NSD); Growth WIP-2009 (− ; 64% less); WIP-2010 (− ; 79% less); Observations of larval case size & structure: WIP-2009 & WIP-2010 (decreased); Activity score (Days 1 & 3): WIP-2009 (NSD); WIP-2010 (+); Activity Score (Days 7 to 9): WIP-2009 & WIP-2010 (−)
Anderson et al. 2012 b	West In Pit (WIP)	Chironomus dilutus	Acute: 10d 100% OSPW	Survival WIP-2009 (NSD, p=0.06); WIP-2010 (NSD); Growth WIP-2009 (− ;64% less); WIP-2010 (− ;79% less); Observations of larval case size & structure: WIP-2009 & WIP-2010 (decreased); Activity score (Days 3): WIP-2009 (NSD); WIP-2010 (+); Activity score (Days 7 to 9): WIP-2009 (− ; days 7 & 8); WIP-2010 (− ; days 7 & 9)

Table 2-3 (continued)

Reference	Raw OSPW	Species	Duration	Endpoint & Result
Anderson et al. 2012 a	West In Pit (WIP)	Chironomus dilutus	Chronic: 60d 100% OSPW	Pupation success: WIP-2009 (−32.9 %); WIP-2010 (−73.9 %); Emergence success: WIP-2009 (−83.9 %); WIP-2010 (−90.1 %); Time to Emerge (Females): WIP-2009 & WIP-2010 (NSD); Males: WIP-2009 (+ 11.4 days); WIP-2010 (+ 9.7 days); Cumulative Emergence: WIP-2009 & WIP-2010 (−)
Anderson et al. 2012 b	West In Pit (WIP)	Chironomus dilutus	Chronic: 60d 100% OSPW	Pupation success: WIP-2009 (−32.9 %); WIP-2010 (−69.7 %); Emergence success: WIP-2009 (−83.9 %); WIP-2010 (−90.1 %); Time to Emerge (Males & Females): WIP-2009 & WIP-2010 (NSD); Cumulative Emergence: WIP-2009 & WIP-2010 (−)
Wiseman et al. 2013 b	West In Pit (WIP)	Chironomus dilutus	Acute: 4d & 7d 100% OSPW	4d & 7d Survival (NSD); Growth 4d (− ; 49% less); 7d (− ; 62% less)
Wiseman et al. 2013 b	West In Pit (WIP)	Chironomus dilutus	Acute: 4d & 7d 100% OSPW	Gene transcripts: Oxidative Stress (4d exposure): *cat* (+ 1.9-fold); *gpx* (+ 2.7-fold); 7d exposure: *gst* (− 2.4-fold) and *aif* (+ 3.9-fold); Tissue concentration of lipid peroxidase (+ 2.9-fold)
Wiseman et al. 2013 b	West In Pit (WIP)	Chironomus dilutus	Acute: 4d & 7d 100% OSPW	Gene transcripts: Endocrine Signaling (4d exposure): *eer* (− 1.8-fold); 7d exposure: *err* (+ 4.2-fold); *esr* (+ 4.8-fold) and *usp* (+ 8.9-fold)
Wiseman et al. 2013 b	West In Pit (WIP)	Chironomus dilutus	Acute: 4d & 7d 100% OSPW	Summary: OSPW increased peroxidation of lipids and transcripts related to oxidative stress, apoptosis and endocrine signaling in Chironomus dilutus. Results are consistent with the transcription response of fathead minnow exposed to OSPW

[a] MLSB: Mildred Lake Settling Basin

[c] Suncor OSPW: active settling basin not specified

[d] Chironomus dilutus (formerly C. tentans) collected from South Bison in July 1998. South Bison: On-site wetland created from saline overburden & lean oil sands material

assess the toxicity of aged or treated OSPW within various wet landscape reclamation environments (Mahaffey and Dubé, In prep).

Chronic effects of aged or treated OSPW have been reported in several species, including: plants (Crowe et al. 2002), algae (Leung et al. 2001; 2003), amphibians (Pollet and Bendell-Young, 2000), mallard ducklings (Gurney et al. 2005), tree swallows (Gentes et al. 2006; 2007a; 2007b) and bacteria (Holowenko et al. 2002); however, the majority of studies have focused on fish and invertebrates. A detailed summary of the studies conducted with fish and invertebrates for each of the reclamation water types described in Table 2-1 is provided in Appendix A. The exposures detailed in Appendix A have been broken down by species, endpoint and magnitude of change relative to the control if the data were provided.

A comprehensive review and synthesis of consistent responses reported in fish and invertebrates following exposure to the various reclamation water types described in Table 2-1 are in preparation (Mahaffey and Dubé, In prep). In contrast to fish, limited effects have been observed in invertebrates exposed to oil sands reclamation waters (Appendix A). These include decreased growth, pupation and emergence in *Chironomus dilutus* following acute and chronic exposure to aged OSPW collected from two reclamation ponds: Demonstration Pond and Pond 9 (Anderson et al. 2012b).

A wide range of responses have been described in fish exposed to aged or treated OSPW in reclamation environments (Appendix A). Reported effects include decreased egg size and larval hatch length in yellow perch (Peters, 1999); decreased reproduction, plasma sex steroids and secondary sex characteristics in fathead minnow (Kavanagh et al. 2011); increased EROD activity and bile metabolites in yellow perch and rainbow trout (van den Heuvel et al.1999b;

McNeill et al. 2012), altered blood hematology and immune response in rainbow trout (Leclair et al. 2013); increased gill cavity deformities in fathead minnow (Kavanagh et al. 2013); significant gill and liver tissue alterations (Nero et al. 2006a) and increased disease observations in yellow perch (van den Heuvel et al. 2000) (Appendix A).

While both raw and aged OSPW contain a complex mixture of inorganic and organic constituents, the major source of toxicity has been traced to naphthenic acids (CEATAG, 1998). Naphthenic acids were first described and tentatively identified as the main toxic component of raw OSPW by MacKinnon and Boerger (1986). To further investigate these findings, a modified Toxicity Identification Evaluation (TIE) was conducted by Verbeek et al. (1993; 1994) using Microtox as the first screening step to assess the acute toxicity of the different compound classes within raw OSPW. In other words, if a significant reduction in Microtox acute toxicity was observed following the removal of a specific class of compounds, further tests were then selectively carried out using *Daphnia magna* and rainbow trout (Verbeek et al. 1993; 1994).

Initial screening results with Microtox indicated that organic compounds that have a non-polar component, as removed by solid phase extraction with C_{18} sorbent, and organic acids, as removed by precipitation at pH 2.5, accounted for 100% of the acute toxicity of raw OSPW collected from Syncrude's Mildred Lake Settling Basin (MLSB) and Suncor's Pond 2 (Verbeek et al. 1993). As the C_{18} column extraction removes simple non-polar organic compounds, as well as surfactants, the overlap in the removal of acute toxicity between these two treatments indicated that the organic acids that precipitate at pH 2.5 must also have a non-polar component (Verbeek et al. 1993). This was consistent with previous mass spectral analyses that found the organic acid fraction of raw OSPW was composed of approximately 95% carboxylic acids with characteristics similar to naphthenic acids (Zenon, 1986; Morales et al. 1993).

The removal of acute toxicity with the precipitation of organic acids at pH 2.5 was also observed in the follow-up bioassays with both Daphnia and rainbow trout (Verbeek et al. 1993). Similar to the overlap response observed with Microtox, acute toxicity was eliminated following the C_{18} removal of organic compounds with a non-polar component in Daphnia. The response of rainbow trout following the C_{18} removal of organic compounds was not reported (Verbeek et al. 1993; 1994). While previous mass spectral analyses were referenced to draw conclusions in the TIE conducted by Verbeek et al. (1993), a method to quantify naphthenic acids was not developed at the time of their study (Jivraj et al. 1995). As such, concentrations of naphthenic acids were not measured or assessed in relation to the toxicity observed or LC_{50} values reported (Verbeek et al. 1993; 1994; CEATAG, 1998).

A dramatic increase in Microtox acute toxicity was reported at lower pH levels (Verbeek et al. 1994). While compounds affected by the manipulation of pH include: cyanide, hydrogen sulfide, pentachlorophenol, as well as other polar and non-polar organics (Traas and van Leeuwen, 2007), the Microtox results, in combination with previous mass spectral analyses (Zenon, 1986; Morales et al. 1993), suggested the increase in acute toxicity was due to the relative degree of unionized naphthenic acids (Verbeek et al. 1993). An increase in acute toxicity with lower pH levels was also observed in the follow-up bioassays with rainbow trout (Verbeek et al. 1993). According to Verbeek et al. (1994), this response was consistent with a previous study that reported increased acute toxicity of two organic acids (oleic and linoleic acid) with lower pH levels in rainbow trout (Hrudey and Tookwinas, 1982). The response of Daphnia following this manipulation was not reported (Verbeek et al. 1993; 1994).

As can be seen from the discussion above, the conclusions generated from the Phase I TIE conducted by Verbeek et al. (1993; 1994) were largely inferential and were not supported by

independent lines of evidence (i.e., confirmation manipulations in Phase II and III TIE assessments). For example, while removal of toxicity with the C18 manipulation indicated toxicity was due to organics, confirmation and fraction isolation of the organic toxicity in methanol elutions of the C18 column was not conducted. Similarly, the conclusion that organic toxicity was largely due to naphthenic acids was based on the precipitation of organic acids at pH 3. However, other non-polar organics would also be expected to partition into the colloidal phase due to differential affinities relative to water. Thus, complete loss of toxicity does not necessarily mean that all of the toxicity was due to naphthenic acids (Dr. Howard Bailey, Nautilus Environmental, personal communication, March 1, 2015).

Differences in species sensitivity to raw OSPW were reported in the bioassays conducted by Verbeek et al. (1993). Rainbow trout were significantly more sensitive than both Microtox and Daphnia. Based on EC_{50} and LC_{50} values, rainbow trout were approximately 3 times more sensitive than Microtox, and 7 times more sensitive than Daphnia. Daphnia were the least sensitive species to raw OSPW (Verbeek et al. 1993). According to Verbeek et al. (1994), the observed differences in species sensitivity demonstrated that the acute toxicity of oil sands wastewaters should not be tested with Microtox alone. The authors recommended, at the very minimum, Microtox results indicating no acute toxicity should be validated with bioassays using other test organisms such as Daphnia and rainbow trout (Verbeek et al. 1994). However, a validation of these results (i.e., another TIE to assess raw OSPW) has not been conducted or reported in the published literature (Mahaffey and Dubé, In prep).

As noted previously, because a decrease in acute toxicity using the Microtox bioassay was not observed in any of the other TIE manipulations, further tests with Daphnia and/or rainbow trout were not conducted (Verbeek et al. 1993; 1994). This is an important modification

of the standard protocol for TIE Phase I assessments (USEPA, 1991). In standardized laboratory assessments, acute toxicity tests are conducted using a suite of aquatic organisms (bacteria, algae, invertebrates and fish) to identify which species is most sensitive to the whole effluent. Once identified, the most sensitive species is then used to assess the toxicity of the individual compound classes that are isolated following each of the TIE manipulations (Maxxam Analytics, 2014).

In some cases, the USEPA (1991) protocol is modified to specifically select ecologically-relevant aquatic-test species that are representative of major trophic levels, such as Daphnia and fathead minnow. However, even with modified protocols, the most sensitive species is still identified and used to assess the toxicity of the different compound classes within the effluent or wastewater (Headley et al. 2001). While *Vibrio fischeri* (Microtox) can be identified as the most sensitive species (Headley et al. 2001), extrapolation of the results to higher level organisms is difficult (Frank et al. 2009; Scarlett et al. 2012). As such, Microtox would not be used as the initial screening step in an exploratory TIE assessment in standardized laboratories, especially following the identification of a more sensitive species to the effluent (Curtis Eickhoff, Maxxam Analytics, personal communication, October 1, 2014).

As discussed in Section 2.2, a considerable amount of research has been conducted to investigate potential wet landscape reclamation strategies with aged or treated OSPW (Table 2-1) (Mahaffey and Dubé, In prep). Recalcitrant (less amenable to natural biodegradation processes) naphthenic acids are the most widely reported compounds implicated for the chronic effects observed in reclamation environments (Li et al. 2014). However, the presence of other contaminants in aged or treated OSPW makes it difficult to link chemical causes with the effects observed (Nero et al. 2006a; van den Heuvel et al. 2012; Leclair et al. 2013) (Appendix A). In a

TIE assessment conducted by HydroQual Laboratories, three potential classes of toxic materials were identified in dyke seepage water: ammonia, naphthenic acids and phenolic substances. Of these three, ammonia was largely responsible for the toxicity associated with fathead minnows in constructed wetlands receiving dyke seepage water (Goudey, 1994; FTFC, 1995) (Table 2-1).

In a recent synthesis of the toxic components within OSPW, Li et al. (2014) reported that compared to NAs, there is limited data available on the concentrations of other organics (e.g., PAHs, BTEX, phenols) that may contribute to the observed organic toxicity of OSPW. Dissolved ions and trace metals were also identified as possible constituents that may contribute to and/or affect the overall toxicity of OSPW (Li et al. 2014). They concluded that OSPW is an extremely complex mixture and more research is needed to evaluate the potential additive, synergistic and/or antagonistic effects caused by its many different constituents (Li et al. 2014).

A similar conclusion was reported in the present literature review on the composition and toxicity of OSPW. Mahaffey and Dubé (In prep) found that only ten of the 342 articles (2.9%) reviewed over the period from 1975 to 2013 included chemical analysis of raw OSPW concurrent with standard acute and chronic bioassay assessments of fish and invertebrate toxicity. Recommendations for future studies conducted to examine the composition and toxicity of OSPW included: (i) clear differentiation and reporting of different OSPW types and sources; (ii) use of consistent terminology for process waters; (iii) consideration and inclusion of detail on mine type and processing that can affect raw OSPW composition (e.g., source, froth treatment type, consolidated tailings additives, tailings management practices, etc.); (iv) use of consistent and standardized chemical and toxicological methods; (v) concurrent chemical and toxicological analysis; and (vi) toxicological assessments at environmentally relevant exposure concentrations (Mahaffey and Dubé, In prep).

2.4 Naphthenic Acids

Despite the uncertainties discussed above, naphthenic acids (NAs) are considered to be the major toxic component in both raw and aged OSPW (Natural Resources Canada – CanmetENERGY, 2010). Naphthenic acids are conventionally described as a complex mixture of acyclic, monocyclic and polycyclic carboxylic acids that are naturally found in petroleum, oil sands bitumen and crude oil (Seifert and Teeter, 1970; Brient et al. 1995; Frank et al. 2008). This mixture includes hundreds of individual NA compounds that contain different functional groups, numerous isomers, and cover a wide range of molecular weights (Martin et al. 2008; Headley et al. 2009a). Classical NAs are represented by the general formula $C_nH_{2n+z}O_2$, where n is the number of carbon atoms, and z is either zero or an even, negative integer that represents the number of hydrogen atoms that are lost as the structure becomes more compact (i.e., through the formation of a ring or double bond) (Qian et al. 2008; Grewer et al. 2010; Reinardy et al. 2013) (Figure 2-2). A z value of zero is used to describe acyclic acids, a z value of -2 is used to describe monocyclic acids; -4 represents bicyclic, and so on (Zhao et al. 2012).

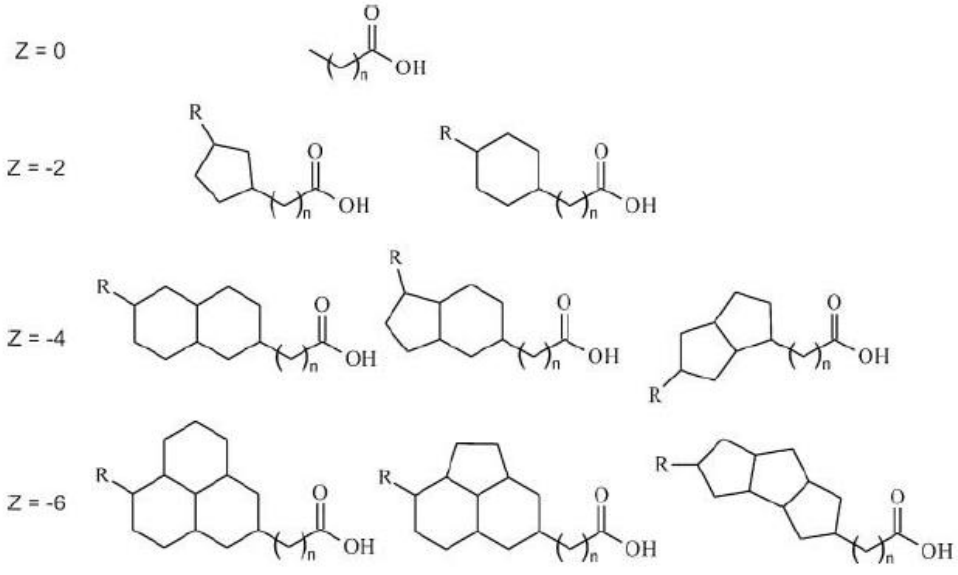


Figure 2-2. Examples of representative classical naphthenic acid structures grouped according to z family (z = 0 to z = -6) (Headley et al. 2011a).

Naphthenic acid mixtures include a variety of organic acids that do not conform to the classical NA formula (Figure 2-3). For example, oxy-NAs or the O_x species ($C_nH_{2n+z}O_x$, where x = 3 to 7), which include hydroxyl acids (O_3 species), dicarboxylic acids (O_4), and possibly humic, fulvic or other weathered acids (O_{5-7}); as well as various heteroatomic species that contain nitrogen and/or sulfur atoms (e.g., NO_x, SO_x), have been identified in both commercial and oil sands extracted naphthenic acid mixtures (Brient et al. 1995; Bataineh et al. 2006; Barrow et al. 2009; Frank et al. 2009; Han et al. 2009; Headley et al. 2009b; Grewer et al. 2010; Lengger et al. 2013; Swigert et al. 2015). Other structures that have either been firmly, or tentatively, identified include: aromatic acids (Rudzinski et al. 2002; Mohamed et al. 2008; Frank et al. 2009; Kavanagh et al. 2009; Barrow et al. 2010; Rowland et al. 2011d; Jones et al. 2012; MacDonald et al. 2013), estrogen-like steroidal acids (Rowland et al. 2011e), and diamondoid adamantane acids (Rowland et al. 2011a-c). In addition to classical and non-classical NA structures, phenolic and polycyclic aromatic hydrocarbon (PAH) impurities have also been identified in both commercial (Brient et al. 1995; West et al. 2011; Rowland et al. 2011a; 2011c) and oil sands extracted NA mixtures (Peters, 1999; Rogers et al. 2002a; Kavanagh et al. 2012).

On top of the structural differences imparted by various functional groups (Figure 2-3), the polarity and non-volatility of individual naphthenic acids increase with molecular weight (Frank et al. 2009). As a result, individual compounds within the mixture will contain various physical, chemical and toxicological properties (Jones et al. 2011; Scarlett et al. 2012; 2013). However, as a group, naphthenic acids contain general physical and chemical characteristics that can be used to describe the overall mixture (Headley and McMartin, 2004).

Naphthenic acids are characterized by their surfactant properties, owing to the presence of both a hydrophilic carboxyl group and hydrophobic alkyl group within their molecular

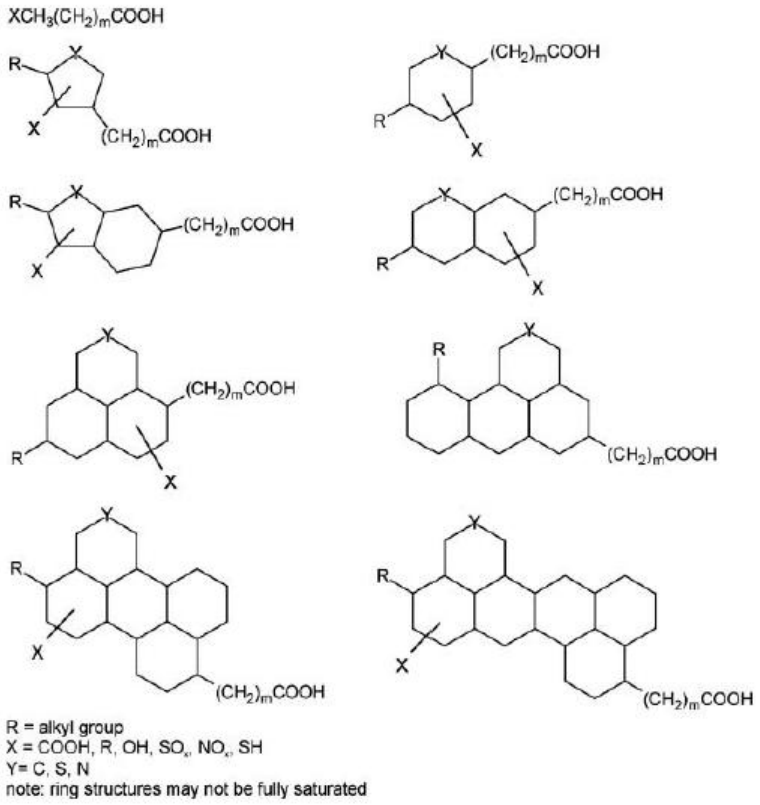


Figure 2-3. Example structures of classical and non-classical naphthenic acids within the acid extractable fraction of OSPW (Headley et al. 2011a).

structure (Frank et al. 2008). Chemically, naphthenic acids behave like typical carboxylic acids (weak organic acids), with pKa values ranging between 5 and 6 (Headley and McMartin, 2004). As such, their solubility in water is pH-dependent (Headley et al. 2002). Naphthenic acids are more soluble at neutral and alkaline pH levels. They are readily deprotonated by sodium hydroxide (NaOH) and sodium bicarbonate ($NaHCO_3$) to form sodium naphthenate salts, which are more water soluble than the corresponding naphthenic acid (Headley et al. 2011a).

2.5 Analytical Challenges

The variety and sheer number of individual NA compounds present within the organic acid fraction of OSPW poses significant analytical and environmental monitoring challenges for industry and regulators (Headley et al. 2013). There are two aspects to consider in the analysis of

naphthenic acid mixtures: quantification (measurement of total NA concentrations) and characterization (identification of individual NA components and their relative composition within the mixture). To help describe the composition of NA mixtures, NAs have been divided into sub-groups based on carbon number and z series (Holowenko et al. 2002; Clemente et al. 2003), oxidation (or O_x species) (Han et al. 2009; Grewer et al. 2010; Headley et al. 2011c) and aromaticity (Jones et al. 2012; Reinardy et al. 2013; Scarlett et al. 2013). Advances in the compositional assessment of NA mixtures continue to support the conclusion that the toxic effects of NA mixtures appear to be more a function of composition than concentration alone (Nero et al. 2006b; Garcia-Garcia et al. 2011a; 2011b; Tollefsen et al. 2012). However, the development of a more reliable method to assess total NA concentrations in surface and process water samples, combined with meaningful toxicological data, is still needed (CEATAG, 1998; Headley et al. 2009a; Grewer et al. 2010; RAMP, 2013).

A number of analytical methods have been used to estimate total NA concentrations in environmental samples (Headley et al. 2009a; Zhao et al. 2012; Brown and Ulrich, 2015). The Fourier transform infrared (FTIR) spectroscopy method (Jivraj et al. 1995) has become the industry standard for the quantification of NAs and has been extensively used by researchers (Zhao et al. 2012). However, because this method is based on the absorption intensity of the carbonyl group, it is prone to interferences from naturally occurring carboxylic acids and has been shown to overestimate NA concentrations, especially in surface water samples (Yen et al. 2004; Scott et al. 2008; Grewer et al. 2010). Similar problems were identified with early chromatographic methods using unit resolution mass spectrometry (MS) (Clemente et al. 2004; Zhao et al. 2012). For example, it is now known that the double derivatization of oxy-NAs (O_3 and O_4 species) resulted in the misclassification and conclusion that various "process-affected"

water sources contained a higher relative proportion of NAs with more than 22 carbon atoms (Holowenko et al. 2002). However, when coupled with high or ultra-high resolution MS, issues pertaining to false positives and misidentification of transformation products are corrected (Bataineh et al. 2006; Headley et al. 2009a; Brown and Ulrich, 2015).

Despite the improved specificity of high resolution MS techniques, the absolute quantification of any complex environmental NA mixture should be considered, at best, semi-quantitative, regardless of the analytical method employed (Martin et al. 2008; Headley et al. 2009a; Headley et al. 2013). Total naphthenic acid concentrations reported by FTIR and MS methods are influenced by several factors. These include: (i) differences in extraction methods and clean up techniques, (ii) differences in the calibration standard that is used, and (iii) inclusion or exclusion specific NA congeners when summing the peak area ratios used to determine total NA concentrations (Scott et al. 2005; Ross et al. 2012; Zhao et al. 2012; MacDonald et al. 2013; Leclair et al. 2013; van den Heuvel et al. 2014).

The primary limitation of quantitative MS methods is they are based on the assumptions that (i) the response factors of the various components within NA mixtures are the same, and (ii) the calibration curve obtained from either a commercial or extracted naphthenic acid mixture can be applied for the quantification of naphthenic acids in any given sample (Headley et al. 2009a; Headley et al. 2013). While differences in the total NA concentrations reported using FTIR and MS methods have been noted (Scott et al. 2008; Grewer et al. 2010), recent comparison studies have also found significant differences between total NA concentrations reported with higher resolution MS methods currently used for environmental monitoring programs (RAMP, 2011; RAMP, 2012; Ross et al. 2012).

2.6 Naphthenic Acids in the Environment

Naphthenic acids are a contaminant of concern within the Athabasca Oil Sands region due to their high concentrations in OSPW produced from the bitumen extraction process, the storage of increasing volumes of OSPW within active settling basins, and the proximity of these settling basins to the Athabasca River and its tributaries (Royal Society of Canada, 2010). Depending on the analytical method used, a wide range of NA concentrations have been reported in raw OSPW collected from active settling basins (Table 2-4). Additional concern regarding the fate and transport of naphthenic acids within the environment is due to their increased water solubility (as naphthenates) in neutral and alkaline waters, which makes them highly mobile in petroleum-contaminated waters (Clemente and Fedorak, 2005). NAs are also a toxicological concern in the aquatic environment as they are believed to be the major source of toxicity in raw OSPW (Canada Natural Resources – CanmetENERGY, 2010) (Tables 2-2 and 2-3).

As the scale of oil sands development increases in the lower Athabasca River basin, so do concerns regarding potential effects of development on the natural environment, particularly on surface water quality (Ross et al. 2012). Naphthenic acid concentrations are routinely monitored in surface waters within the lower Athabasca River basin (Environment Canada, 2011b). However, as discussed in Section 2.5, environmental monitoring and NA source determination studies have been limited by the complexity of the mixture and naturally occurring interferences that affect their measurement (Headley et al. 2009a; Zhao et al. 2012; Brown and Ulrich, 2015). The bulk of previous monitoring data for naphthenic acids in the Athabasca region have been collected using low (or unit) resolution MS or infrared spectroscopy (RAMP, 2010; 2011; 2012; Ross et al. 2012).

Table 2-4. Raw OSPW naphthenic acid concentrations reported in the literature

Publication	Active Settling Basin (Source)	Collection Date	FTIR NAs (mg/L)	UPLC-HRMS [a] NAs (mg/L)	GC-MS [b] NAs (mg/L)
FTFC, 1995	MLSB [c]	Not Reported	112		
FTFC, 1995	Suncor Pond 1/1A	Not Reported	82		
Renault et al. 1998	Suncor Pond 5 (CT [d] Pond)	1996	73		
Holowenko 2000 MSc	MLSB [c]	1997	84		
Holowenko 2000 MSc	MLSB [c]	1998	86		
Holowenko 2000 MSc	West In Pit	1998	61		
Leung et al. 2001	MLSB [c]	1997	52.7		
Leung et al. 2001	MLSB [c]	1997	57		
Holowenko et al. 2002	MLSB [c]	Not Reported	49		
Holowenko et al. 2002	Suncor Pond 5 (CT [d] Pond)	Not Reported	38		
Del Rio et al. 2006	Suncor Pond 5 (CT [d] Pond)	2000	77		
Del Rio et al. 2006	Suncor Pond 5 (CT [d] Pond)	2001	74		
Han et al. 2009	MLSB [c]	Not Reported	50		
Han et al. 2009	West In Pit	Not Reported	77		
Han et al. 2009	Aurora Tailings Pond	Not Reported	60		
Han et al. 2009	South East Pond	Not Reported	77		
Tompkins 2009 MSc	Suncor South Tailings Pond	2008	42.8 ± 1.9 [e]		
Grewer et al. 2010	MLSB [c]	Not Reported	44		28
Grewer et al. 2010	West In Pit	Not Reported	60		36
Grewer et al. 2010	Suncor Pond 2/3	Not Reported	63		47
Grewer et al. 2010	Suncor Pond 5 (CT [d] Pond)	Not Reported	38		26
Grewer et al. 2010	Albian Tailings Pond	Not Reported	35		18
Zubot 2010 MSc	West In Pit	1997	68		
Zubot 2010 MSc	West In Pit	1998	66		
Zubot 2010 MSc	West In Pit	1999	73		
Zubot 2010 MSc	West In Pit	2000	70		
Zubot 2010 MSc	West In Pit	2001	83		
Zubot 2010 MSc	West In Pit	2002	50.2		
Zubot 2010 MSc	West In Pit	2003	51.4		
Zubot 2010 MSc	West In Pit	2004	80.1		
Zubot 2010 MSc	West In Pit	2005	67.3		
Zubot 2010 MSc	West In Pit	2006	78		
Zubot 2010 MSc	West In Pit	2007	75.1		
Zubot 2010 MSc	West In Pit	2008	68		
Gamal El-Din et al. 2011	West In Pit	Not Reported	75		
He et al. 2011; 2012a; b	West In Pit	Feb 2010		19.7	
Pourrezaei et al. 2011	West In Pit	2009		23.6	
Wang 2011 MSc	West In Pit	Oct 2009		23.6	
Wang 2011 MSc	West In Pit	Jan 2010		19.7	

Table 2-4 (continued)

Publication	Active Settling Basin (Source)	Collection Date	FTIR NAs (mg/L)	UPLC-HRMS [a] NAs (mg/L)	GC-MS [b] NAs (mg/L)
Wang 2011 MSc	West In Pit	May 2010		21.8	
Anderson et al. 2012a	West In Pit	2009 (Summer)		23.6	
Anderson et al. 2012b	West In Pit	2009 (Summer)	72 ± 5.0		
Anderson et al. 2012a	West In Pit	2010 (Winter)		19.7	
Anderson et al. 2012b	West In Pit	2010 (Winter)	70 ± 0.5		
Small et al. 2012	Suncor South Tailings Pond	2009	56 ± 0.2		
Small et al. 2012	Suncor South Tailings Pond	2010	64.7 ± 0.5		
Wiseman et al. 2013a	West In Pit	2010		19.7	
Wiseman et al. 2013b	West In Pit	2009	71.7		
		Mean ± SE	64 ± 2.5	21 ± 0.7	31 ± 4.9

[a] Ultra performance liquid chromatography high resolution mass spectrometry (UPLC-HRMS)

[b] Unit-resolution gas chromatography mass spectrometry (GC-MS)

[c] Mildred Lake Settling Basin (MLSB)

[d] Consolidated tailings (CT)

[e] Calculated mean and standard deviation of OSPW groundwater injection samples (n = 4) reported in thesis

Naphthenic acid concentrations (based on FTIR analysis) within the Athabasca River basin are typically < 1 mg/L, with concentrations up to 20 mg/L (Athabasca River, September 1998) (RAMP, 2010). Concentrations of organic acids (or Oil Sands Acid Extractables) based on the unit resolution gas chromatography mass spectrometry ion-trapping method (GC/MS ion-trapping) reported since 2009, are also typically < 1 mg/L, with concentrations up to 11.9 mg/L (McLean Creek, September 2011) (RAMP, 2013). In a recent study using reverse phase liquid chromatography time of flight MS (LC-TOF-MS), Ross et al. (2012) reported NA concentrations in the Athabasca River basin were 100-fold lower (< 2 to 80.8 µg/L) than previously estimated with both FTIR and the Alberta Innovates Technology Futures GC/MS ion-trapping method (RAMP, 2012). The study conducted by Ross et al. (2012) further highlights the inherent differences between currently available high resolution MS methods, and the challenges faced by industry and regulators in assessing the risk of NAs in aquatic environments. As noted by Brown

and Ulrich (2015), significant progress in all areas of naphthenic acid research has been hampered by the abundance of analytical methods and the lack of a uniformly accepted quantitative methodology.

While the potential exists for seepage of raw OSPW from active settling basins into surface and groundwaters (Gibson et al. 2011; Frank et al. 2014), natural erosion of exposed bitumen deposits and groundwater mixing may also contribute NA loadings to surface waters in the lower Athabasca River basin (Headley and McMartin, 2004). Naphthenic acid concentrations (based on FTIR analysis) in near surface aquifers that are drained prior to mining have been reported to range from 2 to 5 mg/L in Suncor mine drainage waters and 5 to 20 mg/L in Syncrude mine drainage waters (CEATAG, 1998). FTIR-NA concentrations in surficial aquifers collected from groundwater wells on Suncor's Steepbank Mine have been reported to range from < 4 to 7 mg/L (Golder and Klohn-Crippen, 1996; CEATAG, 1998).

More recently, NA concentrations (based on low-resolution MS analysis calibrated with an OSPW-NA standard) in groundwater samples collected within lower Athabasca River basin were reported by Frank et al. (2014). The majority of the far-field samples (> 1 km upstream or downstream from active settling basins) were < 10 mg/L, with concentrations up to 27 mg/L (Ells River, 2010) (Frank et al. 2014). Groundwater samples collected along the Athabasca River where the McMurray deposit outcrops at the river edge ranged from 20 to 48 mg/L (Frank et al. 2014). For comparison purposes, NA concentrations from two active settling basins were reported to range from 54 to 60 mg/L (Frank et al. 2014). Thus, a key challenge facing aquatic monitoring programs today is the development of a more selective and accurate/consistent quantitative method that can differentiate between anthropogenic and natural sources of NAs within Athabasca River basin (Ross et al. 2012; Headley et al. 2013; Frank et al. 2014).

2.7 Naphthenic Acid Composition

Oil sands crude oils typically contain naphthenic acids in quantities of up to 4% by weight (Headley et al. 2009a). Oil sands are mined from different depths and locations within the Athabasca oil sands deposit (Allen, 2008). As no two oil sand formations are exactly the same, the composition and concentration of naphthenic acids in the bitumen are also not exactly the same. For example, in addition to compositional differences, Clemente et al. (2003) reported that the naphthenic acid content in a bitumen sample collected from one of Syncrude's lease sites was 150 mg/kg, while a second sample, collected from a Suncor lease site about 30 km away, contained 370 mg/kg. The composition of NAs in the ore also differs from the composition of NAs in raw OSPW (Han et al. 2009). This is because raw OSPW is recycled back into the extraction process. Therefore the composition of naphthenic acids in raw OSPW represents a steady-state of "new" NAs from the ore and "old" NAs from the recycle water (Han et al. 2009).

Due to the complexity of naphthenic acid mixtures, it is not surprising that numerous studies have reported significant differences in the concentration and composition, both among and between, various naphthenic acid sources. These sources include oil sands ore, commercial mixtures and OSPW (Clemente et al. 2003; Barrow et al. 2004; Scott et al. 2005; Nero et al. 2006b; Armstrong et al. 2008; Han et al. 2009; Grewer et al. 2010; Headley et al. 2011c). In general, commercial mixtures have a lower molecular mass range and are much less complex relative to oil sands extracted NA mixtures (Headley and McMartin, 2004) (Figure 2-4).

Grewer et al. (2010) recently conducted a comparison study to investigate the concentration and composition of various naphthenic acid sources using ultrahigh resolution electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry (ESI-FT-ICR MS) and additional analyses. Relative to commercial preparations, NA mixtures extracted

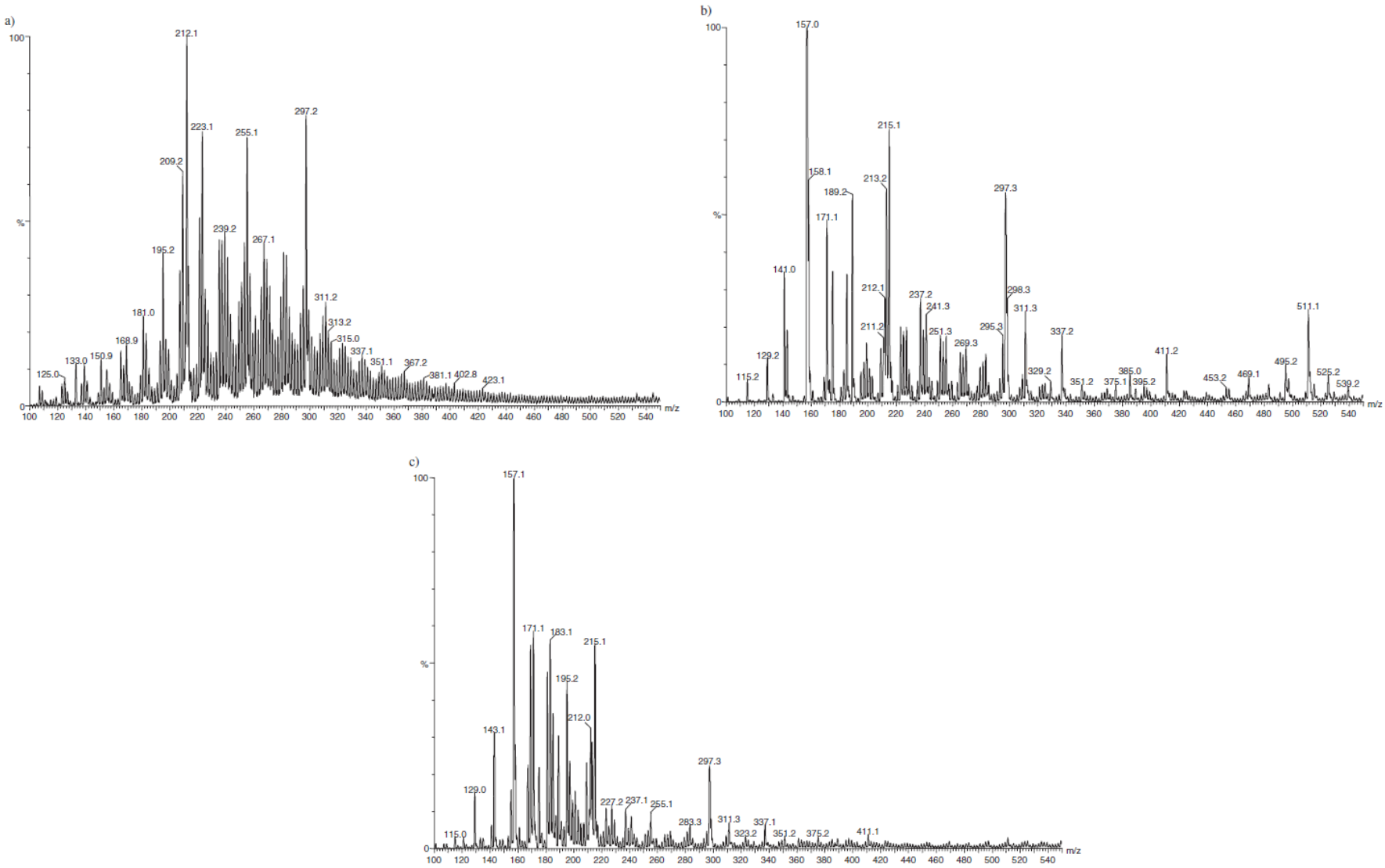


Figure 2-4. Mass spectra of naphthenic acids in an (a) oil sands extracted mixture, (b) Acros commercial mixture, and (c) Fluka commercial mixture analyzed by negative ion electrospray mass spectrometry (ESI-MS). Each peak represents a mass, which may contain several or hundreds of individual naphthenic acid compounds or isomers (Headley and McMartin, 2004)

Table 2-5. Comparison of naphthenic acid mixtures by Ox species [a], as reported by Grewer et al. (2010) using ESI-FT-ICR [b] analysis

Oxy-NAs (O_x species)	x = 2	x = 3	x = 4	x = 5	Sum of classical & oxy-NAs relative to total peak abundance (%)
Commercial NA mixtures					
Merichem	43.5	0.1	0.2	0.0	43.9
Acros	33.2	0.0	0.9	0.0	34.1
Kodak	41.8	0.3	0.9	0.1	43.1
Raw OSPW-NAs					
Syncrude Mildred Lake Settling Basin (MLSB)	28.6	7.5	3.8	0.4	40.3
Syncrude West In Pit (WIP)	24.4	7.4	3.8	0.5	36.1
Suncor Pond 2/3	35.6	6.9	3.1	0.3	45.9
Shell Albian Tailings Pond	19.5	10.3	5.8	0.6	36.2
Aged OSPW-NAs					
Reclamation Pond 9 [c] (OSPW only)	10.7	16.3	9.1	1.7	37.7
Demonstration Pond [d] (MFT + fresh water)	17	17.2	11.2	2	47.4

[a] Values are based on the relative abundance (%) of total peaks corresponding to the oxy-NA formula $C_nH_{2n+z}O_x$

[b] Ultrahigh resolution electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry

[c] Reclamation pond composed of OSPW only (aged OSPW)

[d] Reclamation pond composed of mature fine tailings (MFT) capped with fresh water (aged OSPW)

Table 2-6. Comparison of naphthenic acid mixtures by z series [a], as reported by Grewer et al. (2010) using ESI-FT-ICR [b] analysis

Hydrogen deficiency (z series)	0	-2	-4	-6	-8	-10	-12
Commercial NA mixtures							
Merichem	31	37	28	3	1	0	0
Acros	80	8	8	3	1	0	0
Kodak	15	46	33	4	2	0	0
Raw OSPW-NAs							
Syncrude Mildred Lake Setting Basin (MLSB)	17	10	35	26	6	3	3
Syncrude West In Pit (WIP)	18	8	31	28	9	4	3
Suncor Pond 2/3	12	9	40	27	6	4	3
Shell Albian Tailings Pond	28	6	24	25	8	4	4

[a] Values are based on the relative abundance (%) of peaks corresponding to classical NA formula $C_nH_{2n+z}O_2$, where n = 8 to 30

[b] Ultrahigh resolution electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry

from raw OSPW contained a higher proportion of heteroatomic species (nitrogen and sulfur content), oxidized species (oxy-NAs) and higher molecular weight NAs (based on z series) (Grewer et al. 2010) (Tables 2-5 and 2-6). Similar to differences reported in NA concentrations, the percent contributions of the different NA classes shown in Tables 2-5 and 2-6 vary depending on the analytical and extraction methods used (Zhao et al. 2012; Brown and Ulrich, 2015).

For example, several studies using high performance liquid chromatography high resolution MS (HPLC-HRMS) (Bataineh et al. 2006; Martin et al. 2008; Han et al. 2009) have reported much lower contributions of the z = 0 and z = -2 series in raw OSPW than those reported by Grewer et al. (2010) (Tables 2-7 and 2-8). Using HPLC-HRMS, Han et al. (2009) found that compared to raw OSPW, aged OSPW contains a significantly higher proportion of less cyclic NAs (based on lower mean z values) and a higher proportion of oxidized NAs relative to classical NAs (based on the sum of O_3 + O_4 species / O_2 species). Dramatic differences in the composition of oxidized NAs in aged OSPW and oil sands extracted NA mixtures have also been reported using LC-HRMS (Leclair et al. 2013; MacDonald et al. 2013) (Table 2-9). Recent attempts to extract naturally degraded NA mixtures from aged OSPW have shown that oxidized NAs are excluded in the bulk extraction process (Leclair et al. 2013; MacDonald et al. 2013). Leclair et al. (2013) found that less than 1% of the original oxidized NA content in aged OSPW was present in the extracted NA mixture following a modified version of the bulk extraction procedure developed by Frank et al. (2006) (Table 2-9).

In addition to source-specific differences, Grewer et al. (2010) reported that less than 50% of the total peak abundance within both the commercial and OSPW (raw and aged) naphthenic acid mixtures could be attributed to classical and oxidized NAs (Table 2-5). In a

Table 2-7. Comparison of ESI-FT-ICR [a] and HPLC-QTOF-MS [b] analyses of commercial and raw OSPW naphthenic acid mixtures by z-series

Hydrogen deficiency (z series)	0	-2	-4	-6	-8	-10	-12
ESI-FT-ICR[a]							
Commercial NA (Merichem)	31	37	28	3	1	0	0
Raw OSPW (West In Pit)	18	8	31	28	9	4	3
HPLC-QTOF-MS[b]							
Commercial NA (Merichem)	20	41	32	5.8	1.7	0.3	Not reported
Raw OSPW (West In Pit)	0.4	6.7	36	36	5.6	7.8	7.8

[a] Ultrahigh resolution electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry (ESI-FT-ICR), as reported by Grewer et al. (2010)

[b] High resolution reverse-phase capillary high performance liquid chromatography quadrupole time of flight mass spectrometry (HPLC-QTOF-MS), as reported by Bataineh et al. (2006)

Table 2-8. Comparison of ESI-MS [c] and HPLC-ESI-HRMS [d] analyses of the same raw OSPW naphthenic acid mixture (unnamed source) by z-series, as reported by Martin et al. (2008)

Hydrogen deficiency (z series)	0	-2	-4	-6	-8	-10	-12
Raw OSPW (ESI-MS)	15	19	22	17	9	9	9
Raw OSPW (HPLC-ESI-HRMS)	2	4	33	34	10	9	8

[c] Direct injection unit resolution electrospray ionization (ESI-MS)

[d] High pressure liquid chromatography electrospray ionization high resolution mass spectrometry (HPLC-ESI-HRMS)

Table 2-9. Comparison of the composition of classical and oxidized NAs in aged OSPW between reclamation ponds, as well as before and after bulk extraction procedure using LC-HRMS [e], as reported by Leclair et al. (2013)

Oxy-NAs (O_x species)	x = 2	x = 3	x = 4	x = 5
Demonstration Pond [f] (MFT + fresh water)	88.7	9.3	2.0	Not reported
Reclamation Pond 10 [g] (MFT + OSPW)	60	22.9	17.2	Not reported
NA Extract from Pond 10 [h]	99.8	0.15	0.03	Not reported

[e] Liquid chromatography high resolution mass spectrometry (LC-HRMS)

[f] Reclamation pond composed of mature fine tailings (MFT) capped with fresh water (aged OSPW)

[g] Reclamation pond composed of mature fine tailings (MFT) capped with OSPW (aged OSPW)

[h] NAs were extracted following a modified version of the diethylaminoethyl (DEAE)-cellulose bulk extraction method developed by Frank et al. (2006). See Frank et al. (2006), Leclair et al. (2013) and MacDonald et al. (2013) for discussion on compositional changes resulting from bulk extraction methods.

separate study using ultra pressure liquid chromatography/high resolution mass spectrometry (UPLC-HRMS) and a modified extraction method to isolate both the neutral and acid extractable organics within raw OSPW, Garcia-Garcia et al. (2011b) found that only 74.6% of the total mass of the extract could be attributed to classical and mono-oxidized NAs. Therefore 25.4% of the extracted NA mixture used in their assessment remained uncharacterized (Garcia-Garcia et al.

2011b). Based on their results, both studies concluded that the presence of these yet to be identified organic components confounds the conclusion that classical naphthenic acids are responsible for the organic toxicity of OSPW (Grewer et al. 2010; Garcia-Garcia et al. 2011b).

Although it is generally understood that hydrogen deficiency (or z-series) in the classical NA formula is a function of the number of alicyclic rings present, hydrogen deficiency may also be due to aromaticity (i.e., the formation of double bonds within the ring structure) (Reinardy et al. 2013). Thus, aromatic NAs are included in the formulaic definition of classical naphthenic acids (Qian et al. 2008; Reinardy et al. 2013). As discussed in Section 2.4, aromatic NAs have been identified in both commercial and oil sands extracted NA mixtures. Early studies using synchronous fluorescence spectroscopy reported that despite having very different congener distributions, naphthenic acids extracted from raw OPSW contained a similar fluorescence profile to the commercial NA mixture, Fluka (Kavanagh et al. 2009). Although Fluka naphthenic acids had previously been reported to contain 7.1% aromatic carbons (based on ^{13}C-Nuclear magnetic resonance spectroscopy) (Rudzinski et al. 2002), the concentration and identification of the aromatic compounds responsible for the fluorescent signature in the oil sands extracted NAs could not be determined with synchronous fluorescence spectroscopy (Kavanagh et al. 2009).

Using a more selective, higher resolution technique (two-dimension gas chromatography-mass spectrometry (GCxGC-MS)), numerous naphtheno-monoaromatic acids have either been firmly, or tentatively, identified in both commercial and oil sands extracted naphthenic acid mixtures (Rowland et al. 2011d; 2011e). However, until recently, it was not known how much these aromatic compounds contributed to the overall composition of oil sands extracted NA mixtures. Based on the GCxGC-MS analyses conducted by Jones et al. (2012), two major fractions were identified within an esterified NA mixture extracted from raw OSPW. Alicyclic

acids (or non-aromatic NAs) were estimated to comprise 60 to 70% of the mixture, while the second major fraction, containing several aromatic NAs (or naphtheno-monoaromatic acids), was estimated to comprise 30 to 40% (relative to the alicyclic fraction) (Jones et al. 2012).

As can be seen from the discussion above, there is a significant amount of source- and measurement-dependent variability in the composition of naphthenic acid mixtures. Despite advancements in the identification, synthesis and modelling of individual NA compounds, the primary toxic component(s) have not been clearly established (Headley et al. 2011c; Rowland et al. 2011a-e; Scarlett et al. 2012; 2013; West et al. 2013). The complexity and compositional variability of naphthenic acid mixtures poses significant analytical and experimental challenges in the evaluation and understanding of NA toxicity. The presence of yet to be identified naphthenic acid components, potential additive, synergistic and/or antagonistic effects between the various NA compound classes which have been identified, the abundance of analytical methods, and the lack of a uniformly accepted methodology to allow both quantitative and qualitative comparisons between studies, all add to the uncertainty surrounding the reported effects of naphthenic acids (Grewer et al. 2010; Garcia-Garcia et al. 2011b; Tollefsen et al. 2012; Reinardy et al. 2013; Brown and Ulrich, 2015).

2.8 Naphthenic Acid Toxicity

As discussed previously, early field investigations found the acute toxicity of raw OSPW was removed through naturally occurring physical, chemical and biological processes when isolated in experimental test pits for 1 to 2 years (Boerger and Aleksiuk, 1984; MacKinnon and Boerger, 1986; MacKinnon, 1986). While this is generally attributed to the microbial degradation of the more labile (easily degraded) naphthenic acids within raw OSPW (Bataineh et

al. 2006; Han et al. 2009), at the time these early field investigations were conducted, a method to quantify and/or examine the composition of naphthenic acids had not yet been developed (Jivraj et al. 1995). Therefore, it is important to note that in addition to the expected mineralization of the acidic fraction and other toxic organics, dramatic decreases in the concentration of phenols (0.15 to 0.01 mg/L), cyanide (1 mg/L to 1 µg/L), ammonia (4 to 0.02 mg/L), chemical oxygen demand (400 to 150 mg/L), and suspended solids (1000 to 15 mg/L) were measured in raw OSPW after isolation in experimental test pits for 1 to 2 years (MacKinnon and Boerger, 1986). All of which could have contributed to the decrease in acute toxicity of raw OSPW isolated in experimental test pits for 1 to 2 years.

Besides their presence in OSPW, naphthenic acids are known contaminants in produced waters from offshore oil production platforms (Thomas et al. 2009; West et al. 2011; Tollefsen et al. 2012) and oil refinery effluents (Dorn, 1992; Wong et al. 1996; Misiti et al. 2013a; 2013b). As such, commercial naphthenic acid preparations, which are easier to obtain in larger quantities than extracted samples, have been used as surrogates in a range of toxicity studies and assumed to be somewhat representative of oil sands or petroleum-derived naphthenic acid mixtures (Kannel and Gan, 2012). Effects of commercial and extracted naphthenic acids have been examined in a variety of organisms, including rats (Rogers et al. 2002b), mice (Garcia-Garcia et al. 2011a; 2011b), birds (Gentes et al. 2007c), amphibians (Melvin and Trudeau, 2012a; 2012b; Melvin et al. 2013), aquatic plants and invertebrates (Armstrong et al. 2008; 2009; Swigert et al. 2015). Several studies have reported acute and chronic effects of NA mixtures in fish. These studies are presented below by source, species, life stage, exposure duration, endpoint and reported effect concentrations (nominal and measured) (Tables 2-10 and 2-11).

Due to their surfactant-like characteristics, narcosis has been suggested as the probable mode of action for the acute toxicity of naphthenic acids (Frank et al. 2008; 2009; 2010; Swigert et al. 2015). Narcosis is a non-specific mode of action that disrupts cell membrane function due to the presence of a hydrophobic compound in the lipid bilayer, the accumulation of which can ultimately lead to cell death (Frank et al. 2009). While previous studies with Microtox indicated the acute toxicity of oil sands naphthenic acids was mainly influenced by lower molecular weight compounds (Holowenko et al. 2002; Clemente et al. 2004; Frank et al. 2008), the same response was not observed in fish (Bauer, 2013) (Table 2-10).

Using the same fractional distillation procedure developed by Frank et al. (2008) to assess the toxicity of individual molecular weight fractions of an oil sands extracted NA mixture, Bauer (2013) found the lowest molecular weight fraction (Fraction 1) was the least toxic of all the fractions tested in the embryo-larval bioassays with Japanese medaka and fathead minnow. Comparison of the individual fraction toxicities, relative to the whole extracted NA mixture, indicated that constituents in the mid-molecular mass range (Fractions 2 and 3) had the greatest effect on survival of Japanese medaka embryos. The estimated LC_{50} of the whole extracted NA mixture was 37.6 mg/L (Bauer, 2013) (Table 2-10). Embryo survival in fathead minnow exposed to the whole extracted NA mixture was not assessed by Bauer (2013), however Kavanagh et al. (2012) reported an LC_{50} range of 32.6 to 32.8 mg/L in fathead minnow embryos exposed to an artificially aged naphthenic acid extract (Table 2-10).

In a similar study to assess the relative toxicity of the aliphatic and aromatic fractions of an esterified oil sands extracted naphthenic acid mixture, Scarlett et al. (2013) found the aromatic fraction had a greater effect on the 96 h survival of zebrafish larvae (LC_{50} 8.1 mg/L), relative to the alicyclic fraction (LC_{50} 13.1 mg/L) (Table 2-10). The estimated LC_{50} value of the acidified

NA extract (i.e., whole extracted NA mixture before esterification) was ~8 mg/L (Scarlett et al. 2013) (Table 2-10). The 96 h LC_{50} value for fathead minnow larvae exposed to an artificially aged naphthenic acid extract was 51.8 mg/L (Kavanagh et al. 2012) (Table 2-10). Based on the three species examined, zebrafish larvae appear to be the most sensitive to oil sands extracted naphthenic acid mixtures. However, whether this is due to differences in the extraction method, analytical measurement and/or manipulation of the naphthenic acid extracts is not known (Table 2-10).

Acute lethality in juvenile fish exposed to an oil sands naphthenic acid extract was reported in one study (Table 2-10). In the comparison exposure conducted by Nero et al. (2006b), 100% mortality was observed in young of the year yellow perch exposed for 96 h to naphthenic acids extracted from raw OSPW at a nominal concentration of 6.8 mg/L. No significant differences in survival were reported in adult fathead minnow exposed for 14 to 21 days to an artificially aged NA extract at a nominal concentration of 10 mg/L (Kavanagh et al. 2012) (Table 2-10). Limited mortality was observed in rainbow trout (age not specified) exposed for 7 days to naphthenic acids extracted from aged OSPW at a nominal concentration of 8 mg/L (Table 2-10). Based on the three species examined, young of the year yellow perch appear to be the most sensitive to oil sands extracted naphthenic acid mixtures. However, similar to the embryo-larval comparisons, whether this is due to differences in the source of the OSPW, extraction method, analytical measurement, and/or manipulation of the naphthenic acid extracts is not known (Table 2-10).

A wide range of acute (96 h) LC_{50} concentrations have been observed in fish exposed to commercial NA mixtures (Table 2-11). These concentrations have been shown to vary depending on species, age, water quality characteristics and method of measurement (i.e.,

Table 2-10. Study summaries of oil sands extracted naphthenic acid mixture effects in fish and invertebrates. Results are summarized according to the following notations: significant decrease relative to control (−), significant increase relative to control (+). Concentrations reported are nominal unless otherwise noted.

Study List	NA Extract (Source of acid extractable fraction of OSPW)	Species	Life stage	Test Endpoint	Test Duration	Effect Endpoint	Reported Effect Concentration	Response / Notes
Farwell et al. 2006	NA Extract (MLSB[a])	Japanese medaka (*Oryzias latipes*)	Embryo-larval	Survival & hatch length	18 days		16 mg/L	No significant difference relative to control (NSD)
Farwell et al. 2006	NA Extract (MLSB[a])	Japanese medaka (*Oryzias latipes*)	Embryo-larval	Heart & cranio-skeletal deformities	18 days	LOEC[c]	16 mg/L	
Farwell et al. 2006	NA Extract (MLSB[a])	Japanese medaka (*Oryzias latipes*)	Embryo-larval	Percent normal larvae & yolk sac-pericardial deformities	18 days		16 mg/L	No significant difference relative to control (NSD)
Nero et al. 2006b	NA Extract (WIP[b])	Yellow perch (*Perca flavescens*) from Mildred Lake	Young of the year	Survival	96 h		6.8 mg/L	0% Survival
Nero et al. 2006b	NA Extract (WIP[b])	Yellow perch (*Perca flavescens*) from Mildred Lake	Young of the year	Gill & Liver histopathology	21 days	LOEC[c]	1.7 mg/L	100% Survival; Significant gill alterations relative to control; Liver histopathology (NSD)
Lister et al. 2008	NA Extract (MLSB[a])	Goldfish (*Carassius auratus*)	Sexually immature	Gonadosomatic Index (GSI)	7 days		6.1 mg/L	No significant difference relative to control (NSD); males & females
Lister et al. 2008	NA Extract (MLSB[a])	Goldfish (*Carassius auratus*)	Sexually immature	Male plasma sex steroids	7 days		1.5 mg/L	17β-estradiol (+); Testosterone (NSD)
Lister et al. 2008	NA Extract (MLSB[a])	Goldfish (*Carassius auratus*)	Sexually immature	Male plasma sex steroids	7 days		3 mg/L	17β-estradiol (NSD); Testosterone (NSD)
Lister et al. 2008	NA Extract (MLSB[a])	Goldfish (*Carassius auratus*)	Sexually immature	Male plasma sex steroids	7 days		6.1 mg/L	17β-estradiol (+); Testosterone (NSD)
Lister et al. 2008	NA Extract (MLSB[a])	Goldfish (*Carassius auratus*)	Sexually immature	Female plasma sex steroids	7 days		6.1 mg/L	17β-estradiol (NSD); Testosterone (NSD)
Armstrong et al. 2009	NA Extract (unknown source) spiked in hydroponic medium	*Daphnia magna*	Adult	Survival	48 h	LC50	45.2 % (v/v)	Nominal exposure concentration of 60 mg/L at pH 5.0 (NAs in non-ionized form)

Table 2-10 (continued)

Study List	NA Extract (Source of acid extractable fraction of OSPW)	Species	Life stage	Test Endpoint	Test Duration	Effect Endpoint	Reported Effect Concentration	Response / Notes
Armstrong et al. 2009	NA Extract (unknown source) spiked in hydroponic medium	*Daphnia magna*	Adult	Survival	48 h	LC50	62.5 % (v/v)	Nominal exposure concentration of 60 mg/L at pH 7.8 (NAs in ionized form)
Kavanagh et al. 2012 (Bioassay 1)	Aged NA Extract (WIP[b])	Fathead minnow (*Pimephales promelas*)	9 months	Liver somatic Index (LSI)	21 days		5 mg/L	Significant increase (+) relative to control
Kavanagh et al. 2012 (Bioassay 1)	Aged NA Extract (WIP[b])	Fathead minnow (*Pimephales promelas*)	9 months	Male plasma sex steroids (11-KT)	21 days		5 mg/L	Significant decrease (-) relative to control
Kavanagh et al. 2012 (Bioassay 1)	Aged NA Extract (WIP[b])	Fathead minnow (*Pimephales promelas*)	9 months	Liver somatic Index (LSI)	21 days		10 mg/L	Significant decrease (-) relative to control
Kavanagh et al. 2012 (Bioassay 1)	Aged NA Extract (WIP[b])	Fathead minnow (*Pimephales promelas*)	9 months	Fecundity rate (eggs/female/day)	21 days		10 mg/L	Significant decrease (-) relative to control
Kavanagh et al. 2012 (Bioassay 1)	Aged NA Extract (WIP[b])	Fathead minnow (*Pimephales promelas*)	9 months	Spawning rate (mean # spawns)	21 days		10 mg/L	Significant decrease (-) relative to control
Kavanagh et al. 2012 (Bioassay 1)	Aged NA Extract (WIP[b])	Fathead minnow (*Pimephales promelas*)	9 months	Male secondary sex characteristics (mean # tubercles)	21 days		10 mg/L	Significant decrease (-) relative to control
Kavanagh et al. 2012 (Bioassay 1)	Aged NA Extract (WIP[b])	Fathead minnow (*Pimephales promelas*)	9 months	Male plasma sex steroids (11-KT & T)	21 days		10 mg/L	Significant decrease (-) relative to control
Kavanagh et al. 2012 (Bioassay 1)	Aged NA Extract (WIP[b])	Fathead minnow (*Pimephales promelas*)	Embryo-larval	Survival	9 days	LC50	32.6 mg/L	Artificially aged (aerated at room temperature for ~1.5 mos)
Kavanagh et al. 2012 (Bioassay 1)	Aged NA Extract (WIP[b])	Fathead minnow (*Pimephales promelas*)	Embryo-larval	Fertilization, hatch success & larval survival	9 days		10 mg/L	No significant difference relative to control (NSD)
Kavanagh et al. 2012 (Bioassay 2)	Aged NA Extract (WIP[b])	Fathead minnow (*Pimephales promelas*)	12 months	Fecundity rate (eggs/female/day)	14 days		10 mg/L	Significant decrease (-) relative to control

Table 2-10 (continued)

Study List	NA Extract (Source of acid extractable fraction of OSPW)	Species	Life stage	Test Endpoint	Test Duration	Effect Endpoint	Reported Effect Concentration	Response / Notes
Kavanagh et al. 2012 (Bioassay 2)	Aged NA Extract (WIP[b])	Fathead minnow (*Pimephales promelas*)	12 months	Spawning rate (mean # spawns)	14 days		10 mg/L	Significant decrease (-) relative to control
Kavanagh et al. 2012 (Bioassay 2)	Aged NA Extract (WIP[b])	Fathead minnow (*Pimephales promelas*)	12 months	Male secondary sex characteristics (mean # tubercles)	14 days		10 mg/L	Significant decrease (-) relative to control
Kavanagh et al. 2012 (Bioassay 2)	Aged NA Extract (WIP[b])	Fathead minnow (*Pimephales promelas*)	12 months	Male plasma sex steroids (11-KT & T)	14 days		10 mg/L	Significant decrease (-) relative to control
Kavanagh et al. 2012 (Bioassay 2)	Aged NA Extract (WIP[b])	Fathead minnow (*Pimephales promelas*)	Larvae (5 days)	Survival	96 h	LC50	51.8 mg/L	Artificially aged (aerated at room temperature for ~1.5 mos)
Bauer 2013 MSc	NA Extract (WIP[b]-2005)	Japanese medaka (*Oryzias latipes*)	Embryo-larval	Survival	~12 days	LC50 (2d post-hatch)	37.6 mg/L	Not sure if Whole Extract was Acidified Extract or Esterified Extract
Leclair et al. 2013	NA Extract (Pond 10: MFT+OSPW rec pond aged 17yrs)	Rainbow trout (*Oncorhynchus mykiss*)	Not specified	LSI, leukocyte count, bile fluorescence & hepatic activity	7 days		0.76 mg/L	LSI (+); Phenanthrene bile metabolites (+); Head kidney T-thrombocytes (+); EROD[d] (NSD). In-water exposure concentrations measured at 0.76 mg/L; Nominal concentration was 1 mg/L.
Leclair et al. 2013	NA Extract (Pond 10: MFT+OSPW rec pond aged 17yrs)	Rainbow trout (*Oncorhynchus mykiss*)	Not specified	Immune response & antibody production	21 days		0.76 mg/L	Differential leukocyte count (NSD); Antibody production (NSD)
Leclair et al. 2013	NA Extract (Pond 10: MFT+OSPW rec pond aged 17yrs)	Rainbow trout (*Oncorhynchus mykiss*)	Not specified	Survival & blood erythrocyte count	7 days		6.62 mg/L	No significant difference relative to control (NSD). In-water exposure concentrations measured at 6.62 mg/L; Nominal concentration was 8 mg/L.
Leclair et al. 2013	NA Extract (Pond 10: MFT+OSPW rec pond aged 17yrs)	Rainbow trout (*Oncorhynchus mykiss*)	Not specified	LSI, leukocyte count, bile fluorescence & hepatic activity	7 days		6.62 mg/L	LSI (+); Phenanthrene bile metabolites (+); Spleen thrombocytes (-); EROD[d] (NSD)

Table 2-10 (continued)

Study List	NA Extract (Source of acid extractable fraction of OSPW)	Species	Life stage	Test Endpoint	Test Duration	Effect Endpoint	Reported Effect Concentration	Response / Notes
Leclair et al. 2013	NA Extract (Pond 10: MFT+OSPW rec pond aged 17yrs)	Rainbow trout (*Oncorhynchus mykiss*)	Not specified	Immune response & antibody production	21 days		6.62 mg/L	Differential leukocyte count (NSD); Antibody production (NSD)
MacDonald et al. 2013	NA Extract (Pond 10: MFT+OSPW rec pond aged 17yrs)	Rainbow trout (*Oncorhynchus mykiss*)	1+ year old females	Intraperitoneal injection & immune response	5 days		100 mg/kg	Total blood leukocyte count (-); T-lymphocytes (-); Spleen thrombocytes (-); Bile metabolites (NSD); EROD[d] (NSD)
MacDonald et al. 2013	NA Extract (Pond 10: MFT+OSPW rec pond aged 17yrs)	Rainbow trout (*Oncorhynchus mykiss*)	1+ year old females	Intraperitoneal injection & immune response	21 days		100 mg/kg	Total blood leukocyte count (NSD); Bile metabolites (NSD); EROD[d] (NSD); Fin erosion (NSD)
Reinardy et al. 2013	Acidified OSPW Extract (Standard NA Extract before Esterification) (WIP[b]-2009)	Zebrafish (*Danio rerio*)	Larvae (Hatched 72 h post fertilization)	Expression of vitellogenin genes (*vtg*)	96 h		2000 ug/L	Did not induce expression of vitellogenin genes (NSD). Consistent with He et al. (2011) found 100% raw OSPW with ~20 mg/L NA (based on HPLC-HRMS analysis) needed to elicit an *in vitro* estrogenic response
Reinardy et al. 2013	Whole Esterifiable NA Extract (i.e. All Esterifiable acids within NA Extract) (WIP[b]-2009)	Zebrafish (*Danio rerio*)	Larvae (Hatched 72 h post fertilization)	Expression of vitellogenin genes (*vtg*)	96 h		200 ug/L	Expression vtg (+); at highest exposure concentration (2000 ug/L) expression vtg (+; 8-fold)
Reinardy et al. 2013	Alicyclic (non-aromatic NA) fraction of Esterifiable acids within NA Extract (WIP[b]-2009)	Zebrafish (*Danio rerio*)	Larvae (Hatched 72 h post fertilization)	Expression of vitellogenin genes (*vtg*)	96 h		1000 ug/L	No significant difference relative to control (NSD). Consistent with model predictions of individual NA structures (Scarlett et al. 2012)
Reinardy et al. 2013	Aromatic fraction of Esterifiable acids within NA Extract (WIP[b]-2009)	Zebrafish (*Danio rerio*)	Larvae (Hatched 72 h post fertilization)	Expression of vitellogenin genes (*vtg*)	96 h		100 ug/L	Expression vtg (+); at highest exposure concentration (1000 ug/L) expression vtg (+; 12-fold)

Table 2-10 (continued)

Study List	NA Extract (Source of acid extractable fraction of OSPW)	Species	Life stage	Test Endpoint	Test Duration	Effect Endpoint	Reported Effect Concentration	Response / Notes
Scarlett et al. 2013	Acidified OSPW Extract (Standard NA Extract before Esterification) (WIP[b]-2009)	Zebrafish (*Danio rerio*)	Larvae (Hatched 72 h post fertilization)	Survival	96 h	LC50	~ 8 mg/L	Approximate value; concentration of initial extract determined using ESI-MS. LC50 based on nominal serial dilutions of stock solution
Scarlett et al. 2013	Whole Esterifiable NA Extract (i.e. All Esterifiable acids within NA Extract) (WIP[b]-2009)	Zebrafish (*Danio rerio*)	Larvae (Hatched 72 h post fertilization)	Survival	96 h	LC50	5.4 mg/L	Measured value; concentration determined by weighing on a 7 figure microbalance
Scarlett et al. 2013	Alicyclic (non-aromatic NA) fraction of Esterifiable acids within NA Extract (WIP[b]-2009)	Zebrafish (*Danio rerio*)	Larvae (Hatched 72 h post fertilization)	Survival	96 h	LC50	13.1 mg/L	Measured value; concentration determined by weighing on a 7 figure microbalance
Scarlett et al. 2013	Aromatic fraction of Esterifiable acids within NA Extract (WIP[b]-2009)	Zebrafish (*Danio rerio*)	Larvae (Hatched 72 h post fertilization)	Survival	96 h	LC50	8.1 mg/L	Measured value; concentration determined by weighing on a 7 figure microbalance

[a] Mildred Lake Settling Basin (Active Settling Basin)
[b] West In Pit (Active Settling Basin)
[c] Lowest Observable Effect Concentration
[d] Hepatic 7-ethoxyresorufin-O-deethylase activity (EROD) is used as a measure of liver cytochrome P4501A (CYP1A) enzyme activity

Table 2-11. Study summaries of commercial naphthenic acid mixture effects in fish. Results are summarized according to the following notations: significant decrease relative to control (−), significant increase relative to control (+). Concentrations reported are nominal unless otherwise noted

Study List	Commercial NA mixture (CNA)	Species	Life stage	Test Endpoint	Test Duration	Effect Endpoint	Reported Effect Concentration	Response / Notes
Cairns, 1957 (*In*: CEATAG, 1998)	CNA (Kodak)	Bluegill (*Lepomis macrochirus*)	Not specified	Survival	96 h	LC50	5.6 mg/L	Soft water conditions (CaCO3: 0.01 g/L hardness)
Cairns, 1957 (*In*: CEATAG, 1998)	CNA (Kodak)	Bluegill (*Lepomis macrochirus*)	Not specified	Survival	96 h	LC50	7.1 mg/L	Hard water conditions (CaCO3: 21.78 g/L hardness)
Cairns, 1957 (*In*: CEATAG, 1998)	CNA (Kodak)	Bluegill (*Lepomis macrochirus*)	Not specified	Survival	96 h	LC50	2 mg/L	Periodic low dissolved oxygen conditions
Dokholyan and Magomedov, 1983	CNA (Sodium naphthenate [a])	Kutum (*Rutilus frisii kutum*)	2 months	Survival	96 h	LC50	50 mg/L	Source of CNA not specified
Dokholyan and Magomedov, 1983	CNA (Sodium naphthenate [a])	Kutum (*Rutilus frisii kutum*)	2 months	Survival	60 days	LC50	2 mg/L	Source of CNA not specified
Dokholyan and Magomedov, 1983	CNA (Sodium naphthenate [a])	Kutum (*Rutilus frisii kutum*)	2 months	Not adversely affecting life processes	60 days	NOAEL	0.57 mg/L	Source of CNA not specified
Dokholyan and Magomedov, 1983	CNA (Sodium naphthenate [a])	Chum salmon (*Oncorhynchus keta*)	Juveniles	Survival	96 h	LC50	25 mg/L	Source of CNA not specified
Dokholyan and Magomedov, 1983	CNA (Sodium naphthenate [a])	Chum salmon (*Oncorhynchus keta*)	Juveniles	Survival	60 days	LC50	1.4 mg/L	Source of CNA not specified
Dokholyan and Magomedov, 1983	CNA (Sodium naphthenate [a])	Chum salmon (*Oncorhynchus keta*)	Juveniles	Not adversely affecting life processes	60 days	NOAEL	0.32 mg/L	Source of CNA not specified
Dokholyan and Magomedov, 1983	CNA (Sodium naphthenate [a])	Roach (*Rutilus rutilus caspius*)	Young	Survival	96 h	LC50	50 mg/L	Source of CNA not specified
Dokholyan and Magomedov, 1983	CNA (Sodium naphthenate [a])	Roach (*Rutilus rutilus caspius*)	Young	Survival	45 days	LC50	10 mg/L	Source of CNA not specified
Dokholyan and Magomedov, 1983	CNA (Sodium naphthenate [a])	Roach (*Rutilus rutilus caspius*)	Young	Not adversely affecting life processes	45 days	NOAEL	4.8 mg/L	Source of CNA not specified
Dokholyan and Magomedov, 1983	CNA (Sodium naphthenate [a])	Roach (*Rutilus rutilus caspius*)	Adult (2 years)	Survival	96 h	LC50	75 mg/L	Source of CNA not specified

Table 2-11 (continued)

Study List	Commercial NA mixture (CNA)	Species	Life stage	Test Endpoint	Test Duration	Effect Endpoint	Reported Effect Concentration	Response / Notes
Dokholyan and Magomedov, 1983	CNA (Sodium naphthenate [a])	Roach (*Rutilus rutilus caspius*)	Adult (2 years)	Survival	96 h	LC50	75 mg/L	Source of CNA not specified
Dokholyan and Magomedov, 1983	CNA (Sodium naphthenate [a])	Roach (*Rutilus rutilus caspius*)	Adult (2 years)	Survival	60 days	LC50	14 mg/L	Source of CNA not specified
Dokholyan and Magomedov, 1983	CNA (Sodium naphthenate [a])	Roach (*Rutilus rutilus caspius*)	Adult (2 years)	Not adversely affecting life processes	60 days	NOAEL	8.9 mg/L	Source of CNA not specified
Dokholyan and Magomedov, 1983	CNA (Sodium naphthenate [a])	Roach (*Rutilus rutilus caspius*)	Adult (2 years)	Not affecting hematological parameters	45 days	NOEC	0.5 mg/L	Source of CNA not specified
Dokholyan and Magomedov, 1983	CNA (Sodium naphthenate [a])	Roach (*Rutilus rutilus caspius*)	Adult	Not affecting biochemical parameters	45 days	NOEC	1 mg/L	Source of CNA not specified
Dokholyan and Magomedov, 1983	CNA (Sodium naphthenate [a])	Caspian round goby (*Neogobius melanostomus affinis*)	Fingerlings	Survival	96 h	LC50	75 mg/L	Source of CNA not specified
Dokholyan and Magomedov, 1983	CNA (Sodium naphthenate [a])	Caspian round goby (*Neogobius melanostomus affinis*)	Fingerlings	Survival	60 days	LC50	13.5 mg/L	Source of CNA not specified
Dokholyan and Magomedov, 1983	CNA (Sodium naphthenate [a])	Caspian round goby (*Neogobius melanostomus affinis*)	Fingerlings	Not adversely affecting life processes	60 days	NOAEL	9 mg/L	Source of CNA not specified
Dokholyan and Magomedov, 1983	CNA (Sodium naphthenate [a])	Sturgeon (*Acipenser gueldenstaedti*)	2 years	Survival	96 h	LC50	50 mg/L	Source of CNA not specified
Dokholyan and Magomedov, 1983	CNA (Sodium naphthenate [a])	Sturgeon (*Acipenser gueldenstaedti*)	2 years	Survival	60 days	LC50	11 mg/L	Source of CNA not specified
Dokholyan and Magomedov, 1983	CNA (Sodium naphthenate [a])	Sturgeon (*Acipenser gueldenstaedti*)	2 years	Not adversely affecting life processes	60 days	NOAEL	6.3 mg/L	Source of CNA not specified

Table 2-11 (continued)

Study List	Commercial NA mixture (CNA)	Species	Life stage	Test Endpoint	Test Duration	Effect Endpoint	Reported Effect Concentration	Response / Notes
Dokholyan and Magomedov, 1983	CNA (Sodium naphthenate [a])	Sturgeon (*Acipenser gueldenstaedti*)	2 years	Not affecting hematological parameters	45 days	NOEC	0.5 mg/L	Source of CNA not specified
Dokholyan and Magomedov, 1983	CNA (Sodium naphthenate [a])	Sturgeon (*Acipenser gueldenstaedti*)	2 years	Not affecting biochemical parameters	45 days	NOEC	1 mg/L	Source of CNA not specified
Dorn, 1992	CNA (Kodak NA spiked in non-toxic refinery effluent)	Three-spine stickleback (*Gasterosteus aculeatus*)	Juveniles	Survival	96 h	LC50	5 mg/L	
Dorn, 1992	CNA (Kodak NA spiked in non-toxic refinery effluent)	Three-spine stickleback (*Gasterosteus aculeatus*)	Juveniles	Survival	96 h		2.5 mg/L	60% Survival
Dorn, 1992	CNA (Kodak NA spiked in non-toxic refinery effluent)	Three-spine stickleback (*Gasterosteus aculeatus*)	Juveniles	Survival	96 h		5 mg/L	10% Survival
Dorn, 1992	CNA (Kodak NA spiked in non-toxic refinery effluent)	Three-spine stickleback (*Gasterosteus aculeatus*)	Juveniles	Survival	96 h		10 mg/L	0% Survival
Lai et al. 1996	CNA (Kodak NA spiked in non-toxic aged OSPW from a reclamation pond)	Fathead minnow (*Pimephales promelas*)	Larvae (9 days)	Survival	96 h		45 mg/L	0% Survival
Nero et al. 2006b	CNA (Acros NA sodium salts)	Yellow perch (*Perca flavescens*)	Young of the year	Survival	96 h		3.6 mg/L	0% Survival
Nero et al. 2006b	CNA (Acros NA sodium salts)	Yellow perch (*Perca flavescens*)	Young of the year	Survival	96 h		1.8 mg/L	20% Survival
Nero et al. 2006b	CNA (Acros NA sodium salts)	Yellow perch (*Perca flavescens*)	Young of the year	Gill & Liver histopathology	21 days		0.9 mg/L	100% Survival; Significant gill alterations relative to control; Liver histopathology (NSD)

Table 2-11 (continued)

Study List	Commercial NA mixture (CNA)	Species	Life stage	Test Endpoint	Test Duration	Effect Endpoint	Reported Effect Concentration	Response / Notes
Peters et al. 2007	CNA (50% aqueous sodium salt soln; Pfaltz & Bauer)	Yellow perch (*Perca flavescens*)	Embryo-larval	Optic-cephalic & spinal deformities	Not specified	Threshold	1.67 mg/L	Threshold effect concentrations were estimated (from measured exposure concentrations) by calculating the geometric mean of the lowest observable effect concentration (LOEC) and the no observable effect concentration (NOEC)
Peters et al. 2007	CNA (50% aqueous sodium salt soln; Pfaltz & Bauer)	Yellow perch (*Perca flavescens*)	Embryo-larval	Hatch length	Not specified	Threshold	0.88 mg/L	
Peters et al. 2007	CNA (50% aqueous sodium salt soln; Pfaltz & Bauer)	Japanese medaka (*Oryzias latipes*)	Embryo-larval	Optic-cephalic, tube heart & edema deformities	Not specified	Threshold	1.51 mg/L	
Peters et al. 2007	CNA (50% aqueous sodium salt soln; Pfaltz & Bauer)	Japanese medaka (*Oryzias latipes*)	Embryo-larval	Hatch length	Not specified	Threshold	1.44 mg/L	
Young et al. 2007	CNA (Merichem NAs)	Rainbow trout (*Oncorhynchus mykiss*)	Fingerlings	Survival	96 h		3 mg/L	100% Survival
Young et al. 2011	CNA (Merichem NAs)	Rainbow trout (*Oncorhynchus mykiss*)	4 to 5 years	Survival	10 days		2.8 mg/L	100% Survival; measured exposure concentrations, (calculated mean)
Hagen et al. 2012	CNA (Merichem NAs)	Goldfish (*Carassius auratus*)	Not specified	Gene transcripts & immune responses	1 week		20 mg/L	Pro-inflammatory genes (+); Resistance to infection (+); Post-infection mortality (NSD)
Hagen et al. 2012	CNA (Merichem NAs)	Goldfish (*Carassius auratus*)	Not specified	Gene transcripts & immune responses	8 weeks		5 mg/L	Pro-inflammatory genes (+); Resistance to infection (NSD); Post-infection mortality (NSD)
Hagen et al. 2012	CNA (Merichem NAs)	Goldfish (*Carassius auratus*)	Not specified	Gene transcripts & immune responses	8 weeks		20 mg/L	Pro-inflammatory genes (-); Decreased resistance to infection; Post-infection mortality (+)

Table 2-11 (continued)

Study List	Commercial NA mixture (CNA)	Species	Life stage	Test Endpoint	Test Duration	Effect Endpoint	Reported Effect Concentration	Response / Notes
Swigert et al. 2015	CNA (Merichem NAs)	*Daphnia magna*	Larvae (≤ 24 h)	Survival	48 h	LL50 [b]	24 mg/L	Nominal (WAF[c] - Loading Rate) concentration
Swigert et al. 2015	CNA (Merichem NAs)	*Daphnia magna*	Larvae (≤ 24 h)	Survival	48 h	LC50	20 mg/L	Measured (WAF[c] - Dissolved) concentration
Swigert et al. 2015	CNA (Merichem NAs)	Fathead minnow (*Pimephales promelas*)	Larvae (≤ 24 h)	Survival	96 h	LL50 [b]	9 mg/L	Nominal (WAF[c] - Loading Rate) concentration
Swigert et al. 2015	CNA (Merichem NAs)	Fathead minnow (*Pimephales promelas*)	Larvae (≤ 24 h)	Survival	96 h	LC50	5.6 mg/L	Measured (WAF[c] - Dissolved) concentration

[a] Determination of nominal vs. in-water exposure concentrations not specified in article

[b] LL50: Concentration lethal to 50% of organisms; Lethal Loading (LL) concentrations were based on nominal concentrations of the commercial NA mixture

[c] WAF: Water Accommodated Fraction

nominal vs. measured exposure concentrations) (Table 2-11). For example, the LC_{50} values for the freshwater species, Bluegill (*Lepomis macrochirus*), ranged from 2 mg/L under periodic low dissolved oxygen conditions to 7.1 mg/L under hard water conditions (Cairns et al. 1957) (Table 2-11). The acute (96 h) LC_{50} concentrations reported for five euryhaline fish species, ranged from 25 to 75 mg/L depending on species and age (juveniles were more sensitive than adults) (Dokholyan and Magomedov, 1983) (Table 2-11). In a more recent assessment, Swigert et al. (2015) reported a 96 h LL_{50} value of 9 mg/L for fathead minnow larvae (based on nominal concentrations of a Merichem commercial NA mixture). Using a water accommodated fraction method to measure the dissolved (in-water exposure) concentrations of the Merichem mixture, the 96 h LC_{50} value dropped to 5.4 mg/L (Swigert et al. 2015) (Table 2-11).

In the chronic (45 to 60 days) exposures conducted by Dokholyan and Magomedov (1983), significant sub-lethal changes in blood leukocyte counts and carbohydrate metabolism were observed at concentrations ranging from 1 to 5 mg/L depending on the age and species examined. The no observable adverse effect level (NOAEL) concentrations ranged from 0.32 to 9.0 mg/L (Dokholyan and Magomedov, 1983) (Table 2-11). The source of the commercial mixture and determination of nominal vs. measured concentrations were not specified by Dokholyan and Magomedov (1983). Chronic exposures conducted with commercial and extracted NA mixtures are described in more detail according the endpoints that were examined in the sections below.

While the exact mechanism(s) of sub-lethal naphthenic acid toxicity remains unknown, it is likely this diverse mixture of compounds exhibits multiple modes of toxic action (Scarlett et al. 2012; Tollefsen et al. 2012). For example, in a recent modelling study to assess the toxicity of 54 individual NA compounds (8 NA structural classes), polycyclic monoaromatic acids were

predicted to be the most toxic to fathead minnows and possess human estrogenic, androgenic and reproductive disrupting properties (Scarlett et al. 2012). Aliphatic pentacyclic (diamantane) acids were also predicted exhibit androgenic activity and act as substrates for the cytochrome P450 enzyme CYP3A4 (Scarlett et al. 2012). CYP3A4 represents the largest family of xenobiotic biotransformation enzymes involved in the hydroxylation of many polycyclic aliphatic hydrocarbons and the catabolism of testosterone (Gagne et al. 2012).

Several studies have reported significant differences in the toxicological and biodegradation properties between various commercial NA mixtures (e.g., Kodak, Merichem, Fluka, Acros, etc.) (Clemente et al. 2004; Thomas et al. 2009; Tollefsen et al. 2012). Similar differences have also been reported between commercial and oil sands extracted naphthenic acids (Scott et al. 2005; Armstrong et al. 2008; Garcia-Garcia et al. 2011a; 2011b). For example, in an *in vitro* study to assess the endocrine disrupting properties of the naphthenic acid fraction of off-shore produced waters, Thomas et al. (2009) reported the androgen receptor (AR) antagonist activity of an Acros commercial mixture was almost 4 times higher than the Fluka mixture. However, both the commercial preparations had higher AR potencies relative to the naphthenic acid fractions extracted from the off-shore produced waters (Thomas et al. 2009). In a comparison study to examine immune response in mice, Garcia-Garcia et al. (2011b) found that while the ability of mouse macrophages to phagocytose zymosan (measure of innate host defense) was decreased in animals exposed to a commercial NA mixture, it was significantly enhanced in animals exposed to the neutral and acidic fraction of raw OSPW.

2.8.1 Gill and Liver Histopathology

To date, only one study has been conducted to compare the sub-lethal response in fish exposed to a commercial and an oil sands extracted naphthenic acid mixture (Nero et al. 2006b)

(Tables 2-10 and 2-11). 100% mortality was observed at the highest exposure concentrations for both NA mixtures: commercial (3.6 mg/L; Acros sodium naphthenate) and extracted (6.8 mg/L; raw OSPW). At comparable (nominal) concentrations of a commercial and an extracted NA mixture, dramatic differences in the survival of young-of-the-year yellow perch were reported (Nero et al. 2006b). At 1.8 mg/L commercial NAs, 80% mortality was observed. In contrast, 0% morality was observed at 1.7 mg/L of oil sands extracted NAs.

Although both naphthenic acid mixtures caused significant proliferative alterations and total gill pathologies in yellow perch, only the extracted NAs caused significant histological gill alterations in the combined salt treatments (NA + salt) (Nero et al. 2006b) (Tables 2-10 and 2-11). Interestingly, despite the increased proliferative, inflammatory and structural gill alterations in the combined salt treatment with the oil sands NAs, the addition of salt decreased mortality by 50% in the extracted NA (3.4 mg/L) and 40% in the commercial NA (1.8 mg/L) exposures (compared to the NA only treatments) (Nero et al. 2006b). No significant histological alterations were reported in the livers (Tables 2-10 and 2-11).

Nero et al. (2006a) conducted the same assessment of gill and liver histopathology in adult yellow perch exposed in the field to aged OSPW in two reclamation ponds (Pond 3 and Pond 5) (Table 2-1) (Appendix A). Based on the mortality and histopathology reported in these two studies, young-of-the-year yellow perch exposed in the lab to a combination of extracted NAs (1.7 mg/L) and salinity (0.9 ppt) were much more sensitive than adults exposed in the field to aged OSPW containing 3.6 mg/L NAs and 0.2 ppt salinity (i.e., no mortality, gill or liver alterations were observed in yellow perch captured from Pond 3). Significant gill alterations were reported in yellow perch exposed to aged OSPW containing 24 mg/L NAs and 1.4 ppt salinity (Pond 5), however significant liver alterations were also observed (Nero et al. 2006a).

Although no mortalities were reported in either of the aged OSPW exposures, Nero et al. (2006a) noted that the observations were limited by the size and depth of the ponds (i.e., stock and capture study). The authors attributed the increased sensitivity of yellow perch in the lab exposures (relative to the field exposures with aged OSPW as a mixture) to compositional differences between the naturally degraded NAs in aged OSPW and NAs extracted from raw OSPW (Nero et al. 2006b).

2.8.2 Immune Function

Two studies have examined immune response endpoints in fish exposed to commercial naphthenic acids (Table 2-11). Dokholyan and Magomedov (1983) examined the effects of a sodium naphthenate mixture (unknown source) on the leukocyte count in adult roach and sturgeon exposed to 0.5, 1.0 and 5.0 mg/L concentrations. They reported an initial stimulation after 6 days, followed by a significant depression after 15 days. Some signs of recovery were observed after 30 to 45 days in both species. Based on the responses observed, the threshold or no observable effect concentration (NOEC) was 0.5 mg/L (Dokholyan and Magomedov, 1983) (Table 2-11).

Hagen et al. (2012) examined immune response in goldfish exposed to a Merichem NA mixture at nominal concentrations ranging from 1 to 20 mg/L. They reported an initial stimulation of pro-inflammatory gene expression and increased resistance to infection following a 1 week exposure to 20 mg/L. However, after 8 weeks of exposure, immune genes began to down-regulate, fish showed decreased resistance to infection and increased post-infection mortality (Table 2-11). Goldfish exposed to 5 mg/L showed no significant alterations in gene expression after 1 week. After 8 weeks of exposure, the goldfish displayed increased pro-inflammatory gene expression and increased resistance to infection (Table 2-11). No alterations

of gene expression were observed in goldfish exposed to 1 mg/L Merichem NAs after 8 and 12 weeks exposure (Hagen et al. 2012).

While the two exposures conducted with commercial NA mixtures clearly indicate an immune response in fish, due to the lack of comparison studies, it is uncertain whether these results can be extrapolated to exposure to oil sands-derived NAs (MacDonald et al. 2013). To date, only one study has examined immune response endpoints in fish following a waterborne exposure to an oil sands extracted NA mixture (Leclair et al. 2013) (Table 2-10). In order to better understand the potential immunotoxicity of naturally degraded naphthenic acids in reclamation environments, the NA mixture used in the experiments conducted by Leclair et al. (2013) was extracted from aged OSPW (Pond 10; Table 2-9).

Leclair et al. (2013) reported initial (7 d) alterations in differential leukocyte counts in adult rainbow trout at both exposure concentrations (0.76 mg/L and 6.62 mg/L; in-water concentrations based on direct injection LC-HRMS analysis). However, no significant differences in leukocyte counts or antibody production against *A. salmonicida* were observed after 21 days (Table 2-10). While increased liver somatic indices and elevated bile fluorescence at phenanthrene wavelengths were reported at both exposure concentrations after 7 days, no significant differences were observed in hepatic CYP1A enzymes, as measured by hepatic 7-ethoxyresorufin-O-deethylase (EROD) activity (Leclair et al. 2013) (Table 2-10).

Leclair et al. (2013) conducted the same assessment of immune response endpoints in rainbow trout exposed to aged OSPW containing 6.25 mg/L NAs (based on direct injection LC-HRMS analysis). Despite comparable in-water exposure concentrations of oil sands NAs, significant differences were reported. Although differential leukocyte counts were initially (7 d)

affected in both the aged OSPW and extracted NA exposures, the response was more pronounced in rainbow trout exposed to aged OSPW (Leclair et al. 2013) (Demonstration Pond; Appendix A). In addition, while exposure to both aged OSPW and extracted NAs increased bile fluorescence at the phenanthrene wavelength, elevated fluorescence was only detected at the benzo[a]pyrene wavelength in rainbow trout exposed to aged OSPW.

In contrast to the extracted naphthenic acid exposure, a significant decrease in antibody production and spleen size was observed following exposure to aged OSPW from Demonstration Pond (Leclair et al. 2013) (Appendix A). However, elevated bile fluorescence at the phenanthrene wavelength and decreased antibody production were also observed in rainbow trout exposed to natural waters collected from a near-by compensation lake (Horizon Lake) (Leclair et al. 2013). CYP1A induction, as measured by EROD, was not observed following exposure to waters collected from Demonstration Pond (aged OSPW) or Horizon Lake.

According to Leclair et al. (2013), a key difference between the exposures with aged OSPW and extracted NAs was the detection of increased bile fluorescence at the benzo[a]pyrene wavelength in trout exposed to aged OSPW. Although the extracted NA mixture tested in their study did not possess significant immunotoxic potential (relative to aged OSPW), the extracted mixture only contains a refined subset of the complex suite of organics present within aged OSPW (Leclair et al. 2013; MacDonald et al. 2013). The bulk extraction procedures used to isolate the organic acid fraction from OSPW are selective for compounds that precipitate with acidification and are designed to exclude the neutral and more hydrophobic organics, like PAHs, from the extract (Rogers et al. 2002a; Frank et al. 2006).

As described in Section 2.7, in addition to the exclusion of neutral organic compounds, oxidized naphthenic acids were also removed in the bulk extraction process (Leclair et al. 2013; MacDonald et al. 2013). Leclair et al. (2013) found that less than 1% of the original oxidized NA content in the aged OSPW was present in the extracted NA mixture following a modified version of the bulk extraction procedure developed by Frank et al. (2006) (Table 2-9). The authors concluded that due to compositional differences between the naphthenic acids in aged OSPW (i.e., acidic organic component) and the extracted NA mixture, in addition to the presence of neutral organics within aged OSPW, the exact compound(s) responsible for the immunotoxic effects seen in Demonstration Pond remain to be determined (McNeill et al. 2012; Leclair et al. 2013) (Appendix A). However, the results do suggest that naphthenic acids (extracted from aged OSPW and conforming to the classical NA formula) are most likely not the cause of the immunotoxicity found in aged OSPW (Leclair et al. 2013) (Tables 2-9 and 2-10).

2.8.3 Reproduction

Although previous *in vitro* experiments have demonstrated the endocrine disrupting properties of commercial NA mixtures, an *in vivo* study to examine the reproductive effects of a commercial NA mixture has not been done. As discussed in Section 2.8, *in vitro* comparisons have shown commercial NA mixtures exhibit much higher androgen receptor antagonist activity relative to naphthenic acid fractions extracted from off-shore produced waters (Thomas et al. 2009). In a similar comparison to investigate the estrogen and androgen receptor-mediated effects of raw OSPW and a Merichem NA mixture, He et al. (2011) found that the commercial NA mixture exhibited a weaker estrogenic and androgenic response relative to raw OSPW. However, as the purpose of the study was to assess the effectiveness of ozone treatment on raw OSPW, an *in vitro* comparison of the endocrine disrupting potential of commercial NA mixture

relative to the acid extractable fraction of raw OSPW was not conducted (He et al. 2011). Similarly, an *in vivo* comparison of the reproductive effects of a commercial and an oil sands extracted NA mixture has not been done.

In a follow up study to investigate the mechanistic basis of the endocrine disrupting effects observed *in vitro* (He et al. 2011), He et al. (2012b) examined the gene transcript response of adult male and female fathead minnows following a 7 day exposure to 100% raw OSPW containing 19.7 mg/L of naphthenic acids (based on UPLC-HRMS analysis) (Tables 2-2 and 2-4). The results indicated that undiluted raw OSPW had endocrine disrupting effects at all levels of the Brain-Gonad-Liver axis in both male and female fathead minnows. Although raw OSPW increased gene transcripts involved in the synthesis of gonadotropins in both male and female brains, the transcriptional responses observed in the gonads and livers of male and female fathead minnows were not the same (He et al. 2012b) (Table 2-2).

Exposure to 100% raw OSPW increased gene transcripts for gonadotropin receptors and steroidogenesis in male gonads, and increased the abundance of estrogen-responsive genes in male livers (He et al. 2012b). In contrast, undiluted raw OSPW decreased gene transcripts for gonadotropin receptors and steroidogenesis in female gonads, and decreased the abundance of estrogen-responsive genes in female livers (Table 2-2). According to He et al. (2012b), the presence of steroidal-aromatic or "estrogen-like" NAs in raw OSPW might explain the up-regulation of estrogen receptor signaling in males and the down-regulation steroidogenic activity and estrogen-responsive genes in females.

However, as raw OSPW contains both neutral and acidic organic components, in addition to other contaminants, it is difficult to determine if the effects observed were solely due to oil

sands naphthenic acids. A study to examine the gene transcript response of fathead minnows exposed to an oil sands extracted NA mixture (i.e., acidic fraction of raw OSPW) has not been done. Similarly, an experiment to assess the reproductive effects of an oil sands extracted NA mixture that was not artificially aged to simulate the composition of naturally degraded NAs in aged OSPW has not been done.

To investigate the tentative identification of steroidal aromatic or "estrogen-like" NAs and their predicted endocrine disrupting effects (Rowland et al. 2011e; Scarlett et al. 2012), Reinardy et al. (2013) examined the estrogenic effects of the alicyclic and aromatic fractions of an esterified oil sands extracted NA mixture in zebra fish larvae. Based on the gene production of vitellogenin, the aromatic fraction was more estrogenic than the alicyclic fraction (Table 2-10). However, relative to other anthropogenic steroids, the aromatic NAs in raw OSPW were only weakly estrogenic (Reinardy et al. 2013).

Interestingly, although vitellogenin gene production was significantly increased following exposure to the aromatic fraction, no significant differences were observed in zebra fish larvae exposed to the whole extracted NA mixture (Reinardy et al. 2013) (Table 2-10). According to Reinardy et al. (2013), the lack of response observed following exposure to 2000 µg/L of the acidified NA extract (whole NA extract before esterification) was consistent with the study conducted by He et al. (2011), who found that 100% raw OSPW containing 19.7 mg/L of NAs (based on UPLC-HRMS analysis) was needed to produce an estrogenic response *in vitro*. For comparison purposes, this equates to 70 ± 0.5 mg/L of naphthenic acids based on FTIR analysis (Anderson et al. 2012b) (Table 2-4; raw OSPW from West In Pit, winter 2010).

To date, only one study has been conducted to assess the reproductive effects of an oil sands extracted NA mixture in sexually mature fish (Table 2-10). As noted previously, in order to simulate the composition of naturally degraded naphthenic acids in aged OSPW, the NA mixture used in the experiments conducted by Kavanagh et al. (2012) was extracted from raw OSPW and artificially aged (aerated at room temperature for ~1.5 months). Kavanagh et al. (2012) examined the reproductive response of fathead minnows exposed to nominal concentrations (5 mg/L and 10 mg/L) of an extracted NA mixture for 14-21 days under static renewal conditions. Water renewals occurred every 2 days.

Kavanagh et al. (2012) reported increased liver somatic indices and decreased plasma levels of 11-ketotestosterone in male fathead minnows exposed to 5 mg/L of an extracted NA mixture (Table 2-10). Male fathead minnows exposed to 10 mg/L had increased liver somatic indices, lower plasma concentrations of both 11-ketotestosterone and testosterone, and decreased tubercles (secondary sex characteristic) (Table 2-10). No significant differences in physiological indices or plasma sex steroids were reported in female fathead minnows at both exposure concentrations (Kavanagh et al. 2012). No significant differences in reproduction rates were observed at 5 mg/L, however at 10 mg/L, fecundity and spawning rates were significantly decreased (Table 2-10). Fathead minnow survival was not significantly affected at either exposure concentration (Kavanagh et al. 2012).

Kavanagh et al. (2011) conducted the same assessment of reproduction endpoints in fathead minnows exposed to aged OSPW collected from various reclamation ponds (Appendix A) (Table 2-1). The reproductive response of male and female fathead minnows was not significantly affected following exposure to aged OSPW containing 10.7 to 19.2 mg/L NAs (based on FTIR analysis) (Kavanagh et al. 2011). Although a similar response (i.e., decreased

fecundity, spawning, male tubercles and male plasma hormone levels) was observed following exposure to aged OSPW containing 28.6 mg/L NAs, significant decreases in female hormone levels (17β-estradiol) and gonadal somatic indices (GSI) were also reported (Kavanagh et al. 2011) (Appendix A). Survival was not affected in any of the aged OSPW exposures.

As noted by Kavanagh et al. (2012), the impaired reproduction observed in both the aged NA extract and aged OSPW exposures could be the result of naphthenic acids acting directly or indirectly on the endocrine system of fathead minnows. Previous studies have shown that both stress and hypoxia can indirectly affect the reproductive physiology of fish and reduce plasma sex steroids (Wu et al. 2003; Pollock et al. 2007; Schreck, 2010). Significant histopathological gill alterations have been reported in yellow perch exposed to both commercial and extracted NA mixtures, as well as aged OSPW (Nero et al. 2006a; 2006b) (Tables 2-10 and 2-11; Appendix A). Therefore it is possible that respiratory stress, due to impaired gill function and reduced gas exchange, could have affected the reproductive physiology in fathead minnows exposed to the aged naphthenic acid extract and aged OSPW (Kavanagh et al. 2011; 2012). However, based on the high mortality rates reported by Nero et al. (2006b) using a "fresh" extracted NA mixture, Kavanagh et al. (2012) speculated that in addition to reducing the acute toxicity, the artificial aging of the extracted NA mixture may have helped to reduce or eliminate the effects of respiratory stress in the fathead minnow reproduction assays (Table 2-10).

Despite the loss of the lower molecular weight NAs during the bulk extraction procedure (Frank et al. 2006), and the aeration of the extracted NA mixture afterwards, the reproductive response of fathead minnow was more pronounced at lower nominal concentrations of the aged NA extract relative to aged OSPW containing similar or higher NA concentrations (based on FTIR analysis) (Kavanagh et al. 2012). Although the authors recognized there may have been

differences in the composition of the NAs in the aged extract relative to what has been observed in aged OSPW, a compositional comparison was not conducted (Kavanagh et al. 2012). Reduced bioavailability of naphthenic acids due to binding interactions with colloidal clays and/or humic acids was also suggested as possible environmental factors that may have contributed to the reduced response observed in the aged OSPW exposures (Kavanagh et al. 2012).

2.8.4 Embryo-larval Development

Only one study has examined the embryo-larval effects of a commercial NA mixture in fish (Table 2-11). Peters et al. (2007) compared the embryo-larval response of yellow perch and Japanese medaka exposed to serial dilutions of raw OSPW and a commercial sodium naphthenate solution (Tables 2-2 and 2-11). While a similar response (i.e., increased deformities and decreased hatch length) was observed following exposure to both raw OSPW and the commercial NA mixture, the effects were more pronounced at lower concentrations of the commercial naphthenic acids (Peters et al. 2007) (Tables 2-2 and 2-11).

Peters et al. (2007) attributed the increased toxicity of the commercial preparation to differences in the composition of naphthenic acids in the commercial mixture and raw OSPW. However, as raw OSPW contains both neutral and acidic organic components, in addition to other contaminants, it is difficult to determine if the embryo-larval response to the raw OSPW dilutions was solely due to naphthenic acids (i.e., acid extractable fraction of raw OSPW). A study to compare the embryo-toxicity of a commercial and an oil sands extracted NA mixture has not been done.

It is important to note that the naphthenic acid concentrations reported by Peters et al. (2007) were measured in-water exposure concentrations (i.e., not nominal concentrations). As

the commercial NA mixture they were working with was a 50% (w/v) sodium naphthenate solution, the nominal concentrations were much higher than the actual in-water exposure concentrations. For example, the 20 mg/L nominal concentration ranged from 4.49 to 4.89 mg/L when measured in the water using FTIR analysis (Peters et al. 2007).

Yellow perch were much more sensitive to raw OSPW than Japanese medaka (Peters et al. 2007) (Table 2-2). The authors speculated this may have been due to experimental differences between the bioassays. The Japanese medaka embryos were not fertilized or water hardened in the treatment waters. According to Peters et al. (2007) the increased sensitivity of the perch may have been due to increased toxicant entry into the eggs during the water hardening stage. Exposure during fertilization would have also allowed more time for the surfactant properties of the NAs to interact or disrupt the regulatory function of the egg chorion, again allowing for increased toxicant entry into the perch eggs relative to the medaka eggs (Peters et al. 2007).

Interestingly, the differences in the sensitivity of yellow perch and Japanese medaka were much less apparent in the commercial NA exposures (Peters et al. 2007) (Table 2-11). This may suggest the presence of other contaminants in the raw OSPW dilutions played a role in the species sensitivity differences observed (Table 2-2). However, a comparison study to investigate the embryo-larval effects of a commercial and an oil sands-extracted NA mixture (i.e., organic acid fraction in isolation) on eggs spawned and hardened in clean waters prior to exposure has not been done.

Larval length at hatch was the most sensitive endpoint for both species and treatments in the exposures conducted by Peters et al. (2007). The calculated threshold concentrations for decreased hatch length in yellow perch were 0.88 mg/L commercial NAs and 1.98 mg/L raw

OSPW-NAs (based on FTIR analysis). In Japanese medaka, the threshold concentrations for decreased hatch length were 1.44 mg/L commercial NAs and 6.18 mg/L raw OSPW-NAs (Tables 2-2 and 2-11). The threshold concentrations for increased deformities in yellow perch were 1.67 mg/L commercial NAs and 7.25 mg/L raw OSPW-NAs. In Japanese medaka, the deformity threshold concentrations were 1.51 mg/L commercial NAs and 30 mg/L raw OSPW-NAs (Peters et al. 2007) (Tables 2-2 and 2-11).

The predominant deformities in yellow perch exposed to both raw OSPW and commercial NAs were optic-cephalic abnormalities and spinal malformations (Tables 2-2 and 2-11). As discussed by Peters et al. (2007), possible explanations for the spinal malformations included antioxidant depletion or neuromuscular spasms in the caudal region of the embryos due to ionic imbalance. The predominant deformities in Japanese medaka included optic-cephalic abnormalities and deformities associated with the circulatory system and osmoregulation (Tables 2-2 and 2-11). The more severe circulatory abnormalities included pericardial edema and tube heart. Decreased yolk utilization and incomplete hatching were also observed, suggesting possible metabolic complications (Peters et al. 2007).

Similar deformities were observed in fathead minnow embryos exposed to 100% raw OSPW containing 19.7 mg/L NAs (based on UPLC-HRMS analysis) (He et al. 2012a). Relative to the control, fathead minnow embryos had greater incidences of premature hatch, spontaneous movement and larval deformities (Table 2-2). Embryo survival was 43.8 ± 7.12% (He et al. 2012a). The predominant deformities were hemorrhage, pericardial edema and spinal malformations (Table 2-2). To identify the mechanism of toxicity, He et al. (2012a) measured the production of reactive oxygen species (ROS) and the gene transcript response in fathead minnow embryos. Based on the increased production of ROS and abundance of gene transcripts

that play a key role in the clearance of ROS (Table 2-2), He et al. (2012a) concluded the likely mechanism of embryo-toxicity in fathead minnows exposed to 100% raw OSPW was oxidative stress.

Oxidative stress results when antioxidant defense mechanisms become saturated and concentrations of ROS exceed normal levels produced in functioning cells (He et al. 2012a). When the capacity of the cell to reduce ROS is exceeded, increased ROS concentrations in the cell can damage proteins, lipids, DNA, and ultimately induce apoptosis (programmed cell death) (Bauder et al. 2005; He et al. 2012a; Wiseman et al. 2013a). In addition to the greater abundance of gene transcripts related to oxidative stress response and apoptosis, *cyp3a* gene transcripts were also significantly increased in fathead minnow embryos exposed to raw OSPW (He et al. 2012a) (Table 2-2). Metabolism of substrates by P450 enzymes (such as CYP1A and CYP3A) have been linked to greater generation of ROS (He et al. 2012a; Wiseman et al. 2013a).

He et al. (2012a) also compared the embryo-larval response in fathead minnow exposed to ozone-treated and activated charcoal-treated OSPW. Removal of the organics through ozone and activated charcoal significantly attenuated the embryo-toxicity in fathead minnows (He et al. 2012a). However, as raw OSPW contains both neutral and acidic organic components, in addition to other contaminants, it is difficult to determine if the embryo-larval response observed was solely due to the acidic fraction of raw OSPW. A study to examine the gene transcript response of fathead minnow embryos exposed to an oil sands extracted NA mixture (i.e., acidic fraction of raw OSPW) has not been done. Similarly, a study to examine the embryo-larval development (i.e., time to hatch, hatch length, incidence and type of deformities) in fathead minnow exposed to a whole extracted NA mixture has also not been done.

In the fractional distillation experiments conducted by Bauer (2013), decreased hatch length was reported in both Japanese medaka and fathead minnow embryos exposed to various molecular weight fractions of an oil sands extracted NA mixture. Delayed time to hatch was observed in Japanese medaka, while pre-mature hatch was reported in fathead minnows (Bauer, 2013). In contrast to the increased deformity rates reported in previous raw OSPW exposures (Peters et al. 2007; He et al. 2012a) (Table 2-2), relatively few deformities were observed in Japanese medaka and fathead minnow embryos exposed to separated molecular weight fractions of an oil sands extracted NA mixture (Bauer, 2013). However, as the purpose of the study by Bauer (2013) was to assess the composition and embryo-toxicity of the individual molecular weight fractions, embryo-larval development was not examined following exposure to the whole extracted NA mixture.

Farwell et al. (2006) examined the embryo-larval development of Japanese medaka following exposure to a whole extracted NA mixture (Table 2-10). In contrast to the responses observed in the fractional distillation experiments (Bauer, 2013) and the raw OSPW dilution exposures (Peters et al. 2007), Farwell et al. (2006) found that incidence of deformity was the most sensitive endpoint in Japanese medaka embryos exposed to nominal concentrations of a "fresh" oil sands extracted NA mixture (Table 2-10). The lowest observable effect concentration (LOEC) for heart and cranial-skeletal deformities was 16 mg/L (Table 2-10). Relative to the control, survival and hatch length were not significantly affected in medaka embryos exposed to 16 mg/L oil sands extracted NAs (highest concentration tested) (Farwell et al. 2006) (Table 2-10).

Comparison of the raw OSPW and extracted NA exposures suggests the presence of other contaminants in raw OSPW may have influenced the embryo-larval development of

Japanese medaka (Farwell et al. 2006; Peters et al. 2007) (Tables 2-2 and 2-10). Based on the response observed with the extracted NA mixture, it would appear that the organic acid fraction in isolation is a much more potent inducer of deformities (Farwell et al. 2006), and other contaminants in raw OSPW contributed to the decreased hatch length in Japanese medaka embryos (Peters et al. 2007). As discussed in Section 2.8, Bauer (2013) examined the survival of Japanese medaka embryos exposed to the whole extracted NA mixture. The estimated LC_{50} value of 37.6 mg/L reported by Bauer (2013) is consistent with the lack of effects on embryo survival in Japanese medaka exposed to 16 mg/L of an extracted NA mixture (Farwell et al. 2006) (Table 2-10).

As can be seen from the discussion above, a variety of approaches have been used to investigate the role of naphthenic acids in the toxicity of raw and aged OSPW. While naphthenic acids have been implicated as the primary toxic component, a considerable amount of uncertainty still surrounds this conclusion (Li et al. 2014). Several studies have reported attenuation of effects following the reduction and/or removal of the organic component in raw OSPW (He et al. 2011; 2012a; 2012b; Anderson et al. 2012a; Wiseman et al. 2013a). However, it is not known whether the effects were due to the acidic component, the neutral component, or a combination of both. Although attenuation was attributed to the removal of organics, it is also possible that the interaction of salts and/or metals with the organic component contributed to the effects observed in the exposures with undiluted raw OSPW (He et al. 2012a; 2012b; Anderson et al. 2012a; Wiseman et al. 2013a) (Table 2-2).

Similar uncertainties have been reported in toxicity assessments of aged OSPW in reclamation environments (Nero et al. 2006a; McNeill et al. 2012; van den Heuvel et al. 2012). Despite attempts to extract and/or simulate the composition of naturally degraded NA mixtures,

the presence of neutral organics and other contaminants, as well as additional environmental factors, have been cited to explain differences in the toxicological response in comparison exposures with extracted NA mixtures and aged OSPW (Kavanagh et al. 2012; Leclair et al. 2013). Mixture composition is often cited to explain differences in the toxicity of various naphthenic acid sources (Nero et al. 2006b; Peters et al. 2007; Armstrong et al. 2008). However, the primary toxic component(s) within NA mixtures is not known (Headley et al. 2011c; Tollefsen et al. 2012). While previous studies have reported differences in the toxicity of commercial and extracted NA mixtures (Thomas et al. 2009; Garcia-Garcia et al. 2011a; 2011b), a comparison exposure to examine the reproductive and embryo-larval effects of a commercial and an oil sands extracted NA mixture in fish has not been done.

2.9 Naphthenic Acids in Tissue

Previous work has shown that naphthenic acids can be taken up by fish and measured in the tissue (Young et al. 2007; 2008; 2011; Kavanagh et al. 2011; 2012; van den Heuvel et al. 2014). Initial experiments conducted by Young et al. (2007) found that naphthenic acids could be detected in the muscle tissue of rainbow trout using a modified method developed by Merlin et al. (2007) to detect NAs in water samples. This method is specific for naphthenic acids with 13 carbon atoms and 2 rings (n = 13, z = -4) (Section 2.4, Figure 2-2). The ions that are detected with this method correspond to the major fragments of the *t*-butyldimethylsilyl esters (or derivatives) of naphthenic acid isomers with the formula $C_{13}H_{22}O_2$. These NA isomers elute as an unresolved hump at nominal mass m/z = 267 in the single ion monitoring chromatograms obtained from GC-MS analyses at low (Young et al. 2008) and high resolution peak width (Young et al. 2011).

In the initial experiments conducted by Young et al. (2007) naphthenic acids (or $C_{13}H_{22}O_2$ isomers) were detected in the muscle tissue of rainbow trout exposed for 96 h to both waterborne and diet borne commercial NAs (Merichem), as well as aged OSPW collected from a reclamation pond (Pond 9) (Table 2-1). Young et al. (2008; 2011) modified the detection method used in their 2007 study to estimate naphthenic acid concentrations in the tissue. This was done by spiking the muscle tissues with various concentrations of a commercial NA mixture (Merichem) and creating a calibration plot based on the ratio of the integrated area of the naphthenic acids hump ($C_{13}H_{22}O_2$ isomers at m/z = 267) to the peak area of a surrogate standard, 9-fluorenecarboxylic acid (9-FCA) (Young et al. 2008; 2011). The tissues extracted from the exposed fish were then compared to the calibration plot to estimate the amount of naphthenic acids (or $C_{13}H_{22}O_2$ isomers) in the tissue. The minimum detectable concentration reported was 0.1 mg/kg of fish flesh (Young et al. 2008; 2011).

2.9.1 Uptake and Depuration

Young et al. (2008) used this method to estimate uptake and depuration of naphthenic acids in rainbow trout exposed for 96h to a nominal concentration of 3 mg/L Merichem NAs. Within 1 day of transferring the fish to clean water, about 95% of the NAs were depurated. The estimated bioconcentration factor in rainbow trout exposed for 9 days to 3 mg/L Merichem NAs was ~2 at pH 8.2 (Young et al. 2008). In a subsequent study using in-water exposure concentrations, the estimated bioconcentration factor in rainbow trout exposed for 10 days to a target concentration of 3 mg/L Merichem NAs was ~4 at pH 7.9 (Young et al. 2011). The mean in-water concentration throughout the 10 d exposure was 2.8 mg/L Merichem NAs (based on GC-MS analysis).

Young et al. (2011) found that one day after adding 3 mg/L of a Merichem stock solution, the measured in-water naphthenic acid concentration was 1.1 mg/L. To achieve their target concentration of 3 mg/L Merichem NAs, stock solutions were added daily throughout the remainder of the exposure (Young et al. 2011). The authors attributed the decrease in the measured NA concentrations to sorption of NAs to the surface of the exposure tanks (Headley et al. 2010), aeration (Han et al. 2009), and uptake into the fish (Young et al. 2011). This study illustrates the importance of measuring in-water exposure concentrations, and why it is a requirement to meet the primary study designation for the development of water quality guidelines for the protection of aquatic life in Alberta (AEP, 1996; CEATAG, 1998).

In addition to the assessment of uptake and depuration in lab-exposed fish, Young et al. (2008) also examined naphthenic acid concentrations in the muscle tissue of four wild fish species collected from Athabasca river basin. These species included northern pike, lake whitefish, white sucker and walleye. Naphthenic acids (or $C_{13}H_{22}O_2$ isomers) were detected in 4 (one of each species) of the 23 fish analysed. The NA concentrations in the muscle tissue of the wild fish ranged from 0.2 to 2.8 mg/kg (Young et al. 2008).

Some reproducibility problems were noted in the tissue analyses of the wild fish samples (Young et al. 2008). The NA concentrations in the duplicate tissue samples were generally half the concentrations measured in the initial sample. For example, the estimated NA concentration in walleye muscle tissue collected from the Athabasca river basin was 2.8 mg/kg. However, when a second portion of the walleye muscle tissue (duplicate sample from the same fish) was analyzed, the concentration was 1.8 mg/kg (Young et al. 2008). Similar decreases were observed in all of the duplicate wild fish tissue samples. Considerable variability in the NA tissue concentrations was also observed when the analyses were conducted months apart. However, as

there were no consistent trends in the repeated analyses, the cause for the variability in the estimated NA tissue concentrations could not be found (Young et al. 2008).

As discussed by Young et al. (2008), the application of the calibration plot to estimate the uptake and depuration rate of naphthenic acids in rainbow trout, assumes that all the NAs within the commercial mixture are taken up and depurated at the same rate, and to the same extent, as the $C_{13}H_{22}O_2$ isomers. Verifying this assumption would be difficult because the high fatty acid content in the fish tissues would mask many of the NAs in the commercial mixture (Young et al. 2008). Similarly, when applying the calibration plots to the analyses of wild fish that would be exposed to NAs in the Athabasca river basin (rather than commercial NAs), it was assumed that quantifying the $C_{13}H_{22}O_2$ isomers would be representative of all the structural classes found within oil sands-derived naphthenic acids (Young et al. 2008). Based on previous mass spectral analyses (Bataineh et al. 2006; Han et al. 2008) and calculations conducted by Young et al. (2008), the $C_{13}H_{22}O_2$ isomers comprise ~8% of the total NA mixture in both the Merichem preparation and raw OSPW collected from West In Pit.

2.9.2 Distribution in Target Organs

Young et al. (2011) assessed the distribution of naphthenic acids (or $C_{13}H_{22}O_2$ isomers) in the tissues and target organs of adult rainbow trout exposed for 10 days to 2.8 mg/L Merichem NAs (mean in-water exposure concentration based on GC-MS analysis). Naphthenic acids were detected in all the exposed tissues (µg/g wet weight) at mean concentrations that were significantly greater than the control tissues (Young et al. 2011). The mean NA concentration in the livers was 88 ± 54 µg/g, 56 ± 41 µg/g in the gills, 45 ± 30 µg/g in the kidneys, 19 ± 11 µg/g in the hearts, 17 ± 6 µg/g in the eggs, and 11 ± 2 µg/g in the muscle. According to Young et al. (2011), despite the high variabilities in the estimated NA concentrations within the individual

tissues examined, non-parametric pairwise comparisons showed that the NA concentrations in the gills and livers were significantly higher than the muscle tissue. The authors speculated that the detection of naphthenic acids (or $C_{13}H_{22}O_2$ isomers) in the eggs of the lab-exposed rainbow trout suggests that exposure to these acids begins *in vivo* (Young et al. 2011).

It is important to note that the presence of small distinct peaks (or "native peaks") at nominal mass m/z = 267 were detected in the control tissues of adult rainbow trout (Young et al. 2011). These native peaks were not found in the muscle tissues, but were present in the gills, heart, liver, kidney and eggs. Because the native peaks (or "apparent NAs") were not always detected in the control tissues, the peak areas contributed by the apparent NAs were not subtracted from the control tissues in the statistical comparisons. If the native peaks were present in the exposed tissues, they could not be detected due to the dominance of the unresolved hump from the $C_{13}H_{22}O_2$ isomers (Young et al. 2011).

Naphthenic acid concentrations in the muscle and livers of 8 wild fish (4 fish species) collected during the 2009 RAMP fish survey were also analysed by Young et al. (2011). Naphthenic acids (or $C_{13}H_{22}O_2$ isomers) were not detected in the muscle or liver tissues of the wild fish (Young et al. 2011). However, "apparent NAs" were detected in the livers of all 8 fish collected from Athabasca River basin. According to Young et al. (2011), although naphthenic acids were previously detected in the muscle tissues of wild fish in their 2008 study, the high mobility of fishes and their ability to quickly depurate NAs (Young et al. 2008) would reduce the likelihood of finding NAs in the tissues of wild fish.

2.9.3 Biliary Excretion

Resin acids are a mixture of organic carboxylic acids found in untreated pulp and paper effluents (Hewitt et al. 2008). Resin acids contain similar structures, molecular weights and pKa values to some naphthenic acids (Young et al. 2008; 2011; van den Heuvel et al. 2014). As described by Young et al. (2008; 2011), previous studies have measured resin acids in fish tissue. Similar to the method developed by Young et al. (2008; 2011), resin acids are extracted from the tissue, derivatized and analysed by GC or GC-MS. However, resin acids are quantified based on selected model compounds that elute as individual peaks in the chromatogram (Young et al. 2011). As noted by Young et al. (2011), the method developed to measure naphthenic acids in fish tissue does not have the ability or resolution to focus on a few well defined peaks in the chromatograms obtained from the GC-MS analysis. Thus, the presence of an unresolved hump that is indicative of naphthenic acids (or $C_{13}H_{22}O_2$ isomers) is used to detect and estimate the concentration of naphthenic acids in fish tissues (Young et al. 2008; 2011).

Resin acids have been demonstrated to have relatively low levels of accumulation and substantial biliary excretion (van den Heuvel et al. 2014). Due to the rapid excretion of naphthenic acids observed in the commercial NA exposure by Young et al. (2008), and the structural similarity of resin acids to some naphthenic acids, van den Heuvel et al. (2014) recently conducted a study to determine if naphthenic acids accumulated in the muscle tissue and/or were excreted by a biliary route in yellow perch exposed to aged OSPW. As noted previously, because the GC-MS method developed by Young et al. (2008; 2011) does not have the resolution to differentiate individual NA compounds as well-defined peaks in the GC chromatogram, van den Heuvel et al. (2014) developed a high resolution method (based on LC-HRMS analysis) to examine specific NA ions in the bile of the exposed fish.

In the study conducted by van den Heuvel et al. (2014), muscle tissue and bile samples were collected from yellow perch exposed in the field to aged OSPW within two reclamation environments (Demonstration Pond and South Bison) (Table 2-1). The exposed fish were compared to yellow perch collected from two off-lease reference lakes and Mildred Lake reservoir. Mildred Lake serves as an on-lease reservoir for extraction plant water that is pumped into the lake from the Athabasca River. The muscle tissue and bile samples from the exposed and reference sites were analysed for naphthenic acids (or $C_{13}H_{22}O_2$ isomers) using the GC-MS method developed by Young et al. (2008; 2011).

To identify and examine specific NA ions using high resolution mass spectrometry, the pooled bile samples from each location were initially screened by direct injection into a Thermo Velos Orbitrap mass spectrometer (van den Heuvel et al. 2014). Target ions (or NA isomers) were then selected on the basis of their relative abundance in the pooled bile sample from fish exposed to aged OSPW (Demonstration Pond), with some further refinement to eliminate ions that showed high background concentrations in the pooled bile sample collected from one of the reference lakes. The seven target NA ions that were selected corresponded to following NA isomers: C13, z = -4 (m/z 209.1536); C13, z = -2 (m/z 211.1693); C15, z = -8 (m/z 233.1536); C15, z = -6 (m/z = 235.1693); C15, z = -4 (m/z 237.1849); C15, z = -2 (m/z 239.2006); and C17, z = -8 (m/z 261.1849) (van den Heuvel et al. 2014). An average of the results of all seven ions were used to quantify total NAs against an oil sands NA mixture extracted from aged OSPW (MacDonald et al. 2013) (Table 2-9).

Based on the GC-MS tissue analyses, naphthenic acids did not accumulate in the muscle tissue of yellow perch exposed to aged OSPW collected from two different reclamation environments (van den Heuvel et al. 2014) (Table 2-1). Naphthenic acids (or $C_{13}H_{22}O_2$ isomers)

were not detected in any of the muscle tissues in yellow perch collected from Demonstration Pond (n = 4) and South Bison (n = 4). However, low concentrations of NAs were detected in 3 of the 8 samples collected from the off-lease reference lakes and in all 4 of the samples collected from Mildred Lake reservoir (i.e., 7 of the 12 reference perch collected) (van den Heuvel et al. 2014).

The reason for the absence of naphthenic acids (or $C_{13}H_{22}O_2$ isomers) in the muscle tissues of yellow perch exposed to aged OSPW from Demonstration Pond and South Bison is not known (van den Heuvel et al. 2014). Previous studies have detected NAs in the muscle tissues of rainbow trout and fathead minnows exposed to aged OSPW collected from other reclamation environments (i.e., Pond 5 and Pond 9) (Young et al. 2007; Kavanagh et al. 2011) (Table 2-1). Similarly, the reason for the presence of low NA concentrations in the muscle tissue of yellow perch collected from the reference sites is not known (van den Heuvel et al. 2014). As discussed in Section 2.9.2, although "native peaks" or "apparent NAs" were identified in the target organs of adult rainbow trout (control tissues), they were not present in any of the muscle tissues analysed (Young et al. 2011).

In contrast to the muscle tissues, naphthenic acids were detected in the bile of yellow perch exposed to aged OSPW using both the GC-MS and LC-HRMS methods (van den Heuvel et al. 2014). However, the GC-MS method was not able to distinguish between the reference and exposed bile samples. According to van den Heuvel et al. (2014), the LC-HRMS method for naphthenic acids was superior at differentiating exposed vs. reference perch compared to the GC-MS method. The authors speculated this was likely due to the high fatty acid content in the bile that may have interfered with the low resolution GC-MS method. Based on the results observed, naphthenic acids are excreted by a biliary route in fish and the LC-HRMS method developed was

shown to be a highly sensitive, selective and promising technique as an indicator of exposure of biota to oil sands-derived naphthenic acids (van den Heuvel et al. 2014). A method to analyse naphthenic acids in the muscle tissue of fish using high resolution mass spectrometry (HRMS) has not been developed (Headley et al. 2013).

2.9.4 Uptake and Response

While tissue concentrations in fish exposed to commercial NA mixtures were examined by Young et al. (2008; 2011), only one study has estimated NA concentrations in the muscle tissue of fish exposed to an oil sands extracted NA mixture. Using the GC-MS method developed by Young et al. (2008; 2011), Kavanagh et al. (2012) reported a mean NA concentration of 8.9 ± 3.3 µg/g wet weight in the muscle tissue of fathead minnows exposed for 14 days to 10 mg/L of an aged extracted NA mixture. As noted in the introduction, the purpose of the studies conducted by Kavanagh et al. (2011; 2012; 2013) was to examine the reproductive effects of aged OSPW in wet landscape reclamation environments (Appendix A) (Table 2-1). Therefore, in addition to simulating the composition of naturally degraded NAs in aged OSPW, Kavanagh et al. (2012) also examined the reproductive response of fathead minnows exposed to oil sands extracted NAs and 700 mg/L $NaHCO_3$. This concentration was selected because it is comparable to the concentrations of HCO_3^- in aged OSPW collected from various reclamation ponds (Kavanagh et al. 2012) (Table 2-1).

Interestingly, in addition to improved reproductive response, Kavanagh et al. (2012) reported reduced uptake (5.4 ± 2.1 µg/g wet weight) in the muscle tissue of fathead minnows with the addition of 700 mg/L $NaHCO_3$ to the nominal 10 mg/L aged NA extract treatment (14 d exposure). According to the authors, the reason(s) for this is not known, however the reduced uptake suggests that HCO_3^- interferes with naphthenic acids (or $C_{13}H_{22}O_2$ isomers) and their

movement through the cell membrane (Kavanagh et al. 2012). Uptake in relation to sub-lethal response based on increasing exposure concentrations of the aged NA extract was not examined. However the addition of 700 mg/L of $NaHCO_3$ was shown to reduce the acute toxicity of the aged extracted NA mixture in fathead minnow embryos and larvae (Kavanagh et al. 2012). In the embryo exposure, the addition of 700 mg/L of $NaHCO_3$ increased the LC_{50} from 32.6 mg/L to 59.9 mg/L. In the larval exposure, the addition of 700 mg/L of $NaHCO_3$ increased the LC_{50} from 51.8 mg/L to 121 mg/L (Kavanagh et al. 2012).

Smits et al. (2012) recently modified the GC-MS method developed by Young et al. (2008; 2011) to estimate naphthenic acid (or $C_{13}H_{22}O_2$ isomers) concentrations in the muscle tissue of northern leopard frogs exposed to increasing nominal concentrations of Merichem NAs. The frogs were exposed for 28 days under saline conditions comparable to reclaimed wetlands in the Athabasca oil sands region (Table 2-1). Concurrent with uptake, Smits et al. (2012) examined a suite of sub-lethal endpoints including immune function, thyroid hormone levels and hepatic detoxification. The authors reported a mean NA concentration of 4.9 ± 0.4 µg/g wet weight in frog muscle tissue in the 20 mg/L Merichem NA exposure, and 9.2 ± 0.8 µg/g wet weight in the 40 mg/L Merichem NA exposure.

Interestingly, although NA tissue burdens increased with increasing nominal exposure concentrations, the commercial NA mixture produced little to no evidence of sub-lethal toxicity in northern leopard frogs after a month of continuous exposure (under static renewal conditions, with water renewals every 2 days) (Smits et al. 2012). A study to examine uptake in relation to response in fish exposed to increasing concentrations of a commercial NA mixture has not been done. Similarly, a study to compare the uptake of naphthenic acids in the muscle tissue of fish exposed to a commercial and an oil sands extracted NA mixture has also not been done.

The quantification of naphthenic acids in biological tissues poses significant analytical challenges (Headley et al. 2013). Despite the noted reproducibility problems and high background interferences from the presence of natural fatty acids in biological tissues, the GC-MS method developed by Young et al. (2008; 2011) has, until recently, been the only method available to estimate NA concentrations in tissue. While the application of high resolution mass spectrometry improved the specificity and accuracy of the analysis of naphthenic acids in the bile of yellow perch exposed to aged OSPW, considerable variability in the relative abundance of the individual NA isomers was observed depending on the source of the naphthenic acids (van den Heuvel et al. 2014). In other words, the relative abundance of the individual NA isomers (and resulting concentration) in the bile of the yellow perch varied depending on the source of the aged OSPW they were exposed to (i.e., Demonstration Pond vs. South Bison) (Table 2-1).

As discussed in Section 2.7, the relative abundance and composition of individual NA structural classes vary depending on the source examined (i.e., aged OSPW, raw OSPW, commercial NA mixtures and extracted NA mixtures) (Tables 2-5 to 2-9). Thus, similar to the analyses of naphthenic acids in water samples, the development of a more selective and accurate/consistent quantitative method to measure NAs in biological tissues is needed (Headley et al. 2013). The application of high resolution mass spectrometry to assess naphthenic acid concentrations in the muscle tissue of fish exposed to isolated naphthenic acid mixtures has not been done.

CHAPTER 3: METHODOLOGY

3.1 Extraction of Naphthenic Acids from Oil Sands Process-Affected Water

For this study, two naphthenic acid mixtures were examined: a commercial mixture from Fluka Chemicals (Fluka, Sigma-Aldrich Canada, Inc., Oakville, ON, Canada), and a freshly extracted oil sands mixture from raw OSPW. The raw OSPW was collected from an unspecified oil sands operation in Alberta, Canada, and shipped in 20 L carboys to Environment Canada, Water Science and Technology Directorate, in Saskatoon, Saskatchewan, during the winter of 2010. The suspended solids (and absorbed hydrophobic compounds) were removed through gravity settling in the Environment Canada chemistry lab in Saskatoon.

The organic acid fraction (OAF) (i.e., oil sands extracted NA mixture) used in this study was extracted following a modified version of the method developed by Rogers et al. (2002a). The overlying water layer from each of the 20 L carboys was decanted and acidified to pH < 2 with H_2SO_4. The organic acid fraction was solvent extracted three times using a ~1:2 dichloromethane to water ratio. The organic acid layers from each solvent cycle were collected and combined.

The dichloromethane was separated from the organic acids by evaporation in a rotary evaporator at ~85 ˚C and recycled back into the extraction process to reduce solvent costs. This process was repeated for all 25 carboys (~500 L). The combined organic acid extract was then re-dissolved in 0.1N NaOH, adjusted to pH 10 using H_2SO_4, and filtered through a 0.45 µm glass microfilter. The filtrate was then subjected to a 1000 MW cut off ultra-filtration, adjusted to pH 8 using H_2SO_4 and stored at 4 ˚C in an amber glass bottle with a Teflon cap for ~2 weeks prior to beginning the fish exposure trials.

3.2 Reference Water

A key component in the study design of this experiment was the use of reverse osmosis water to match the average water quality conditions present in the Athabasca River (Squires et al. 2010). This "synthetic river water" approach has been used by other researchers to improve the environmental relevance of their exposures (Rozon-Ramilo et al. 2011a; 2011b; Ouellet et al. 2013a-c). The Athabasca River is considered to be the receiving environment for potential seepage of raw OSPW, and hence organic acids, from active settling basins (Environment Canada, 2011a-c), and potential discharges of treated OSPW in future treatment and release scenarios (Golder, 2014).

The control or reference water was composed of 60% reverse osmosis and 40% de-chlorinated Saskatoon city water. As previous studies have reported the toxicity of commercial and extracted naphthenic acid mixtures are influenced by pH and hardness (Cairns, 1957; Armstrong et al. 2009), this approach enabled matching the reference water to the average pH (8.2), alkalinity (75 mg/L $CaCO_3$) and hardness (127 mg/L $CaCO_3$) levels recorded for the Athabasca River near the mouth between 1996-2006 (Squires et al. 2010). The 60:40 dilution of reverse osmosis and city water was held within a separate tank in the lab and pumped via March pumps (Model LC-3CP-MD, March Manufacturing, Glenview, IL, USA) into each of the head tanks daily.

The "synthetic river water" was used for both the controls and treatment dilutions. Similar to previous studies, a solvent control (NaOH) was included to account for the NaOH solvent used to dissolve the commercial and extracted naphthenic acid mixtures (Nero et al. 2006b; Lister et al. 2008; Kavanagh et al. 2012). The NaOH control had a NaOH concentration

(3.2 mg/L) equivalent to the highest Fluka treatment. All of the treatment waters, including the NaOH control, were adjusted to pH ~8.2 using a 10% (v/v) dilution of HCl.

3.3 Experimental Design

The flow-through system and table design were based on previous mesocosm studies that examined the reproductive and embryo-larval effects of pulp and paper and metal mining effluent mixtures in fathead minnow (Rickwood, 2006; Rozon-Ramilo, 2011; Ouellet, 2013). Artificial stream systems (or mesocosms) are the recommended monitoring alternative in Environmental Effects Monitoring (EEM) programs for pulp and paper and metal mining when standard EEM field surveys cannot be conducted (Environment Canada, 2010; 2012b).

The experimental design consisted of three treatments and two controls for a total of five tables each containing six 10L flow-through glass tanks (Figure 3-1). Each table contained five replicate (fish or breeding) tanks and one egg tank (Figure 3-2). Each replicate fish tank contained one fathead minnow breeding pair and a spawning tile (~15 cm long PVC pipe cut in half) (Figure 3-3). The egg tanks contained the eggs and larvae which were held in egg cups that

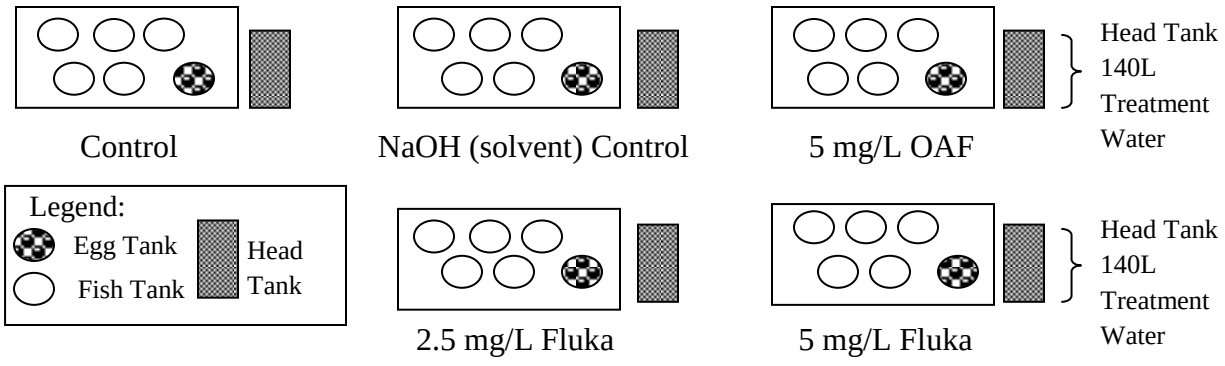


Figure 3-1. Experimental design included five tables with three treatments and two controls. Each table contained six 10L glass flow-through tanks: five replicate breeding tanks and one egg tank. Water was delivered from the glass head tank (140 L) to each replicate tank via peristaltic pumps and Teflon tubing.

were suspended in the water by an egg plate (Figure 3-3). The egg cups were made of glass tubes with a 250 µm screen mesh bottom which was held in place by a Teflon ring (Figure 3-3). All of the tanks (fish and egg tanks) were continuously aerated with air stones via standard airline tubing. Glass tanks and Teflon® PTFE (Polytetrafluoroethylene) tubing were specifically used to reduce sorption losses of the naphthenic acid mixtures.

a) b)

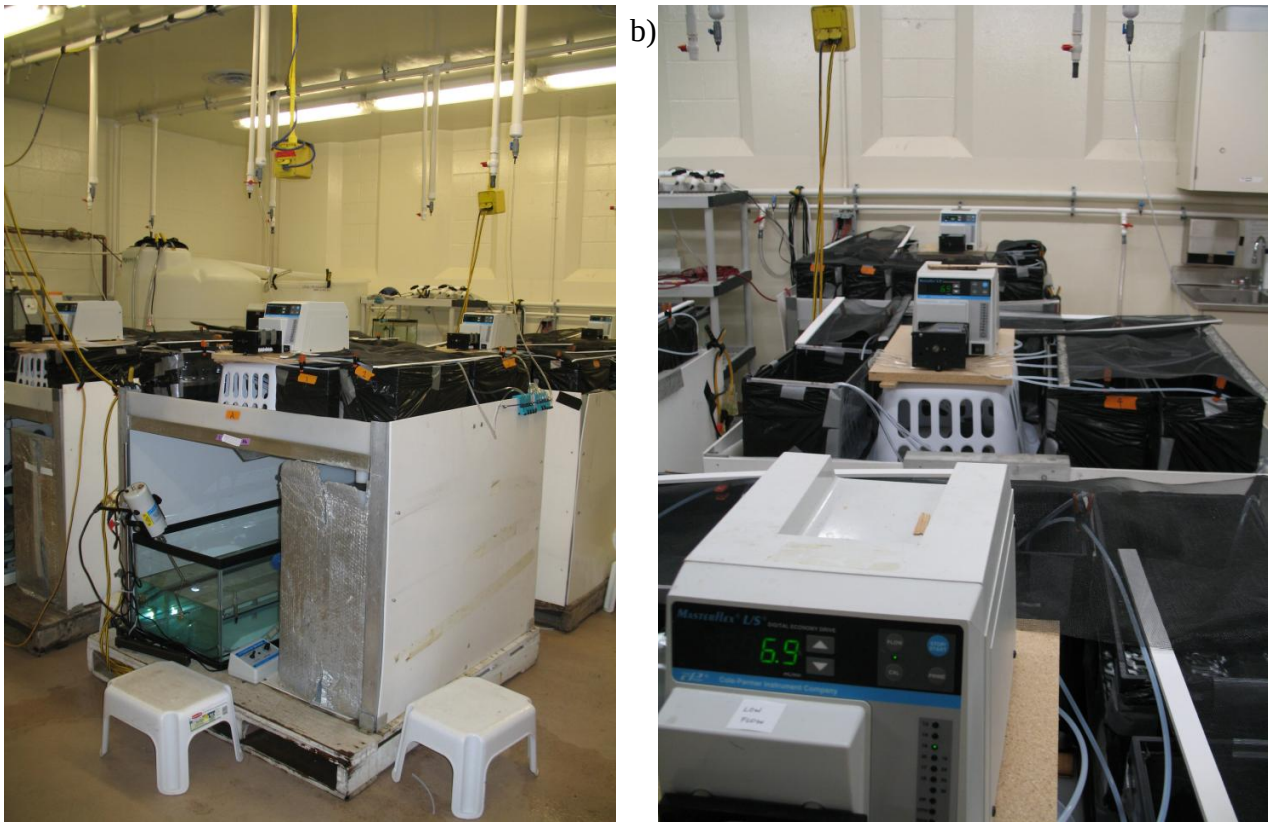
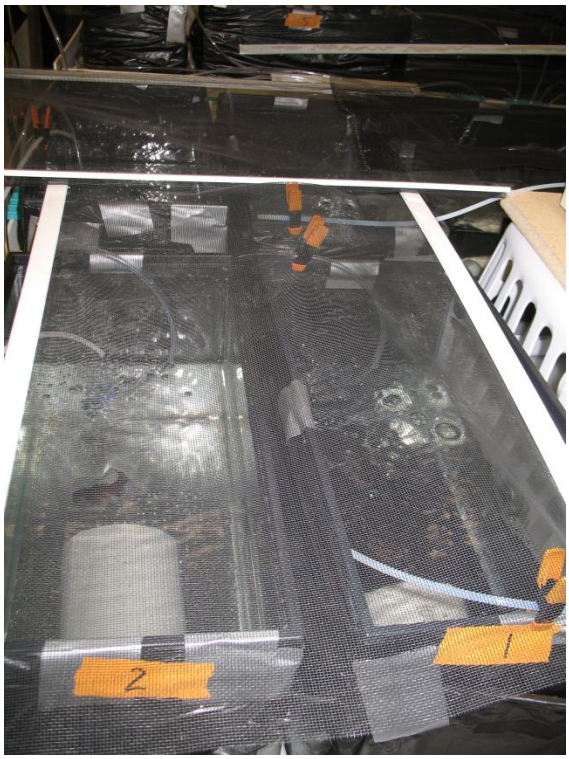


Figure 3-2. Artificial stream systems used for the 21d fathead minnow reproduction assay: (a) Photo shows top and bottom of the modified modular mesocosm units developed by Rickwood (2006). Glass head tank with Stir-Pak mixer and collection reservoir were housed below the wet table; (b) Photo shows top of wet table with replicate fish and egg tanks. Water was delivered from the head tank via peristaltic pumps and Teflon tubing. Flow-through water from each tank was collected through a drain in the center of the wet table and directed to the collection reservoir below for disposal.

a)

b)



c)

Figure 3-3. Fish and egg tanks used in the 21d fathead minnow reproduction assay: (a & b) Photos show replicate flow-through glass aquaria with fathead minnow breeding pairs and spawning tile. The sides of the glass tanks were covered with dark plastic and screens were placed on top. (c) Photo shows glass egg cups suspended in the treatment water by the egg plate inside the glass flow-through egg tank.

The treatments included two nominal Fluka concentrations (2.5 mg/L and 5 mg/L) and one nominal OAF concentration (5 mg/L). The treatment concentrations chosen were based on environmental relevance and the available quantity of organic acid extract (OAF). As discussed in Section 2.6, naphthenic acid concentrations (based on FTIR analysis) within the Athabasca River basin are typically < 1 mg/L, with concentrations up to 20 mg/L (Athabasca River, September 1998) (RAMP, 2010). Concentrations of organic acids or Oil Sands Acid Extractables (based on GC/MS ion-trapping analysis) reported since 2009 are also typically < 1 mg/L, with concentrations up to 11.9 mg/L (McLean Creek, September 2011) (RAMP, 2013). Fresh dilutions of both the Fluka and OAF stock solutions in 0.1N NaOH were mixed daily and added to the 40 gallon glass head tanks that contained ~140 L of synthetic river water (Figure 3-2).

The head tanks were continuously mixed (Stir-Pak® Heavy Duty Mixer, Model 50007-30, Cole Parmer, Vernon Hills, IL, USA) and heated to 25ºC±1 ºC using 400 W submersible aquatic heaters (Figure 3-2). The head tanks were continuously mixed to prevent the naphthenic acids from plating out of solution. The control and treatment waters were continuously delivered to each of the six streams from the head tanks via peristaltic pumps (Masterflex® L/S, Model 7524-50, Vernon Hills, IL, USA) and Teflon tubing at a rate of one turn-over per day (0.042 L/min). Flow-through waters overflowed from each replicate tank into the wet table and then drained from the top of the table into an 85 L collection reservoir that was connected to a separate drainage hose for disposal (Figure 3-2). The peristaltic pumps and tubing were checked every day to ensure consistent flow rates and proper maintenance of tubing.

3.4 Study Species

The fathead minnow (*Pimephales promelas*) was an ideal test species for this experiment for several reasons. In addition to being one of the most-commonly used fish species for acute and chronic toxicity testing, fathead minnows are a native forage fish species within the Athabasca River basin (Siwik et al. 2000; van den Heuvel et al. 1999a; Kavanagh et al. 2013). The fathead minnow reproduction assay was specifically designed to identify endocrine disrupting chemicals in the environment through the inclusion of several reproductive endpoints known to be affected by alterations in endocrine systems controlled by androgens and estrogens (Ankley et al., 2001). As a result of its extensive use in government and industry for regulatory purposes, considerable information exists regarding its life history, reproduction and spawning behaviour (Rand, 1995; Ankley et al. 2001; OECD, 2004; Rickwood et al. 2006a-c; USEPA, 2007).

Fathead minnows are tolerant of a wide range of water quality parameters and are easily cultured in the laboratory. They have been shown to be very responsive to a variety of inorganic and organic contaminants (Benoit and Holcombe, 1978; Ankley and Villeneuve, 2006; Squires et al. 2013). The partial lifecycle reproductive assay with fathead minnows has also been shown to be a useful tool in assessing the effects in both adult fish and their offspring following exposure to pulp and paper and metal mining effluents (Rickwood et al. 2006a-c; 2008; Rozon-Ramilo et al. 2011a; 2011b; Ouellet et al. 2013a-c).

Fathead minnows have a relatively short life cycle reaching reproductive maturity within four or five months (under optimal conditions). The timing of their reproduction cycle can be easily manipulated in the lab through temperature and photoperiod (25 ± 1ºC, 16:8 h light:dark

photoperiod) (Ankley et al. 2001). Adult fathead minnows are sexually dimorphic, allowing males and females to be easily distinguished from each other (Parrot and Wood, 2002).

Male fathead minnows are larger, generally weighing between 4 to 5 g while females usually weigh between 2 to 3 g (Ankley et al. 2001). Males are typically darker in color (dark grey to black) and have two distinct light-coloured vertical banding patterns on their sides. Sexually mature males develop large nuptial tubercles on their snout, a dark coloured dorsal fin dot on their tails, and an elongated fleshy pad (fat pad) that extends from the nape to the dorsal fin that they use to clean the eggs after they are spawned. Sexually mature females develop a large fleshy ovipositor that is easily recognized during reproduction (Ankley et al. 2001; Parrot and Wood, 2002).

Fathead minnows are fractional substrate spawners, releasing only a portion of their eggs at each spawning. Individual females typically spawn every 3 to 5 days, and under optimal lab conditions, generally releasing between 50 to 150 eggs per spawn (Ankley et al. 2001). Fertilization success is determined by the ratio of infertile to fertile eggs which are examined the day after they are spawned. Infertile eggs are opaque or clear with a white dot where the yolk has precipitated. Fertile eggs remain clear for 36-48 h until they reached the eyed stage. Embryos typically hatch within 4 to 5 days (Ankley et al. 2001).

3.5 Fathead Minnow 21 Day Reproductive Assay

The reproduction assay was conducted in the Aquatic Toxicology Laboratory Facility in the Western College of Veterinary Medicine at the University of Saskatchewan. The methodology has been outlined by Ankley et al. (2001) and modified by Rickwood (2006). Ten

to eleven month old, naive fathead minnows from stock cultures purchased from Osage Catfisheries Inc. (Osage Beach, MO, USA) were used for this study.

The assay consisted of two phases: pre-exposure and exposure. The pre-exposure phase typically ranges from 7 to 21 days, depending on the reproductive output of the breeding groups or pairs. Assays can include the use of breeding groups (four females and two males or two females and one male) or breeding pairs (one female and one male). Pair breeding has been used to investigate the link between specific effects and individual fish (Rickwood et al. 2006a-c; Rozon-Ramilo et al. 2011a; 2011b; Ouellet et al. 2013a-c). For the pre-exposure phase in the present experiment, breeding pairs were randomly selected from the culture stock. Fork length, weight and secondary sex characteristics were recorded and 60 breeding pairs were randomly assigned to aquaria in the laboratory. Secondary sex characteristics were assessed according to the methodology developed by Parrot and Wood (2002). In this system, each characteristic is assigned a value which is then summed and expressed as a score or index (Table 3-1).

Table 3-1. Point system for grading fathead minnow secondary sex characteristics [ab]

Male Index				Female Index
Nuptial Tubercles	Dorsal pad	Dorsal fin dot	Banding	Ovipositor Index
Absent: 0	Absent: 0	Absent: 0	Absent: 0	Absent: 0
Present: 1	Small: 1	Present: 1	Present: 1	Medium: 1
	Medium: 2			Large: 2
	Large: 3			Very Large: 3

[a] Developed from Parrot and Wood, (2002) and Martel et al. (2003)
[b] Table modified from Rickwood, (2006)

After 10 days of pre-exposure, 25 exposure breeding pairs were selected according to the following criteria: 100% survival of adults, production of at least one spawn within seven days, and greater than 80% fertilization of the eggs produced (Ankley et al., 2001; OECD, 2006).

Statistical analyses were conducted on the breeding pairs that fit the above criteria to confirm there was no statistical difference between them before exposure (i.e., all stream replicates were equal). After the breeding pairs were selected and analysed, they were randomly assigned to the replicate treatment tanks to begin the 21 day exposure to the treatments described in Section 3-3.

During both the pre-exposure and exposure phase, fathead minnow breeding pairs were fed half a gram of frozen blood worms (Sally's bloodworms™, San Francisco Bay Brand, Inc., Newark, CA, USA) twice daily. Spawning tiles were checked daily for eggs. As fathead minnows typically spawn at dawn, eggs were checked mid-morning to give them time to water harden. Eggs were gently rolled off the spawning tile into a petri-dish, where they were photographed using a digital camera (Powershot Model A620, Canon, Mississauga, ON, Canada) and examined using a tri-nocular microscope (Vista Vision™ Model 48402-00, VWR International, Mississauga, ON, Canada). Egg size measurements (n = 20 per brood) were later taken from these photographs using image analysis software (Image-Pro Plus 6.1, Media Cybernetics, MD, USA). The images were calibrated in the software program using a micrometer that was placed under the petri dish at the time the photograph was taken.

After the eggs were photographed and transferred into the egg cups, the spawning tile was returned to its original tank. Once the eggs reached the eyed stage (day 3), they were photographed again, re-counted to determine fertilization success, and returned to their egg cups. The egg cups were checked daily to monitor time-to-hatch. Larvae were collected 5 days post-hatch and examined (alive) using a tri-nocular microscope (Vista Vision™ Model 48402-00, VWR International, Mississauga, ON, Canada) for spinal deformities (scoliosis, lordosis, kyphosis), yolk sac and craniofacial deformities, edema (yolk sac and pericardial), and hemorrhaging of the body, yolk sac, ocular, and pericardial regions. Non-deformed larvae (n = 5

to 10 larvae per brood) were photographed and measured for length using image analysis software (Image Pro-Plus 6.1, Media Cybernetics, MD, USA). The images were calibrated in the software program using a micrometer that was placed under the petri dish at the time the photograph was taken.

At the end of the exposure period, fathead minnows were anaesthetized with methane tricainesulfonate (MS-222) and euthanized by spinal severance. Male and female fish were assessed again for secondary sex characteristics and measured for total length and body weight. The livers, gonads, gills and carcasses were dissected and weighed (to 0.001 g). The carcasses (filet and skin) were placed in pre-labelled amber glass tubes with Teflon® PTFE caps and frozen (approximately -20˚C) for naphthenic acid analyses.

Standard individual biological endpoints were assessed. These included: survival, gonadal somatic indices (GSI), liver somatic indices (LSI), condition factor (CF), body weight, fork length, as well as male and female secondary sex characteristic indices. Reproductive endpoints included: mean total egg production (total mean number of eggs produced / breeding pair (or replicate) over the 21d exposure period), mean egg production (average number of eggs produced / breeding pair (or replicate) over the 21d exposure period), cumulative egg production (cumulative number of eggs produced / female / day; factoring in mortality), cumulative spawning events (cumulative number of spawning events / female / day; factoring in mortality), egg size (diameter (mm)), and fertilization success (number of eyed eggs / total number of eggs). Embryo-larval development endpoints included: time-to-hatch (days), hatching success (number of eggs hatched / number of eggs fertilized), larval length (mm), larval survival (number larvae survived 5 days post-hatch / total number of larvae) and mean total deformities (# of larvae

deformed / total # larvae hatched from each breeding pair (or replicate) throughout the 21d exposure period).

3.6 In-situ Water Quality Analysis

Daily in-situ water quality measurements were collected and recorded for all treatments during both the pre-exposure and exposure phases of the experiments. Conductivity, dissolved oxygen and temperature were measured using a handheld YSI portable meter (Yellow Springs Instrument, Yellow Springs, OH, USA). Ammonia levels (Low Range Ammonia Meter HI93700, Hanna Instruments, Woonsocket, RI, USA), pH (pHTestr30, Oakton Instruments, Vernon, IL, USA), alkalinity and hardness (Hatch Test Kit, Model 5-EP MG-L, Loveland, CO, USA) were also measured.

Quality Assurance/Quality Control (QA/QC) samples were collected each week and sent to ALS Laboratories in Saskatoon, Saskatchewan, Canada, for ammonia and routine/general water analysis. With the exception of chloride, major ions were analysed by ALS using inductively coupled plasma optical emission spectrometry (ICP-OES). Chloride concentrations were determined colorimetrically. Water samples for naphthenic acid analysis were collected on days 0, 7, 15 and 21 from the head, breeding and egg tanks for all three treatment groups in 120 mL amber glass jars with Teflon® PTFE caps, and stored in the dark at ~4ºC for later analysis. Water samples from the control and NaOH (solvent) control were also collected weekly for QA/QC analyses of naphthenic acids (6 samples).

3.6.1 Naphthenic Acid Analysis in Water

In-water exposure concentrations of naphthenic acids in the Fluka treatments and both controls (QA/QC) were analysed by direct injection using a linear ion trap-orbitrap mass

spectrometer (LTQ Orbitrap Velos, Thermo Fisher Scientific, San Jose, CA, USA) equipped with an electrospray ionization (ESI) interface operated in the negative ion mode. Data were acquired in the full-scan mode from m/z 80 to 600. All observed ions were singly charged and the average mass resolving power was 100,000. Naphthenic acid concentrations were calculated using a 25-component Fluka calibration curve. This was done by summing the area of the top 25 peaks in the mass spectra for each of the water samples collected and plotting them against a 25-component Fluka standard concentration-area calibration curve (i.e., the summed area of the top 25 components observed with known concentration injections with the Fluka standard). Due to time and budget constraints, in-water exposure concentrations of naphthenic acids in the water samples collected from the OAF treatment could not be analysed.

3.7 Method Development for Analyses of Naphthenic Acids in Fish Muscle Tissue

One of the objectives of this thesis was to develop a tissue extraction method for the analysis of naphthenic acids using ultra-high resolution mass spectrometry. The extraction method was modified from previous work that distinguished oil sands organic acids from co-extracted plant tissue components using the Orbitrap (LTQ Orbitrap Velos, Thermo Fisher Scientific, San Jose, CA, USA) (Headley et al. 2011b). The exposed fish muscle tissues (filet and skin with head removed) were analysed prior to the spike recovery trials (described in Section 3.7.3 below).

The fathead minnow muscle tissues were pooled per treatment group to ensure there was enough tissue for instrument detection. These included both the Fluka treatments (2.5 and 5.0 mg/L), the OAF treatment (5 mg/L), and both controls (synthetic river water and NaOH). Male and female tissues were analysed separately. All equipment was cleaned and rinsed with liquid

chromatography mass spectrometry (LC/MS) grade methanol prior to use. A blank was included in both the spike recovery trials and exposed tissue extractions.

3.7.1 Exposed Tissue Homogenization and Extraction

Frozen muscle tissue was ground-up using a ceramic mortar and pestle. The homogenized tissues were distributed evenly for a total of six replicates (3 male and 3 female replicates) for each treatment group. Approximately 3.2 g of male muscle tissue and ~1.2 g of female muscle tissue were added to pre-weighed conical centrifuge tubes (30 tubes total). 15 mL of 1 mM NaOH was added to each tube and mixed using a vortex mixer. The samples were placed in a sonic bath for 1 hour and then centrifuged for 30 min at 15,000 rpm (Beckman Coulter, Model TJ-6, Mississauga, ON, Canada).

The supernatant was removed using a glass pipette and transferred into a second set of pre-labelled centrifuge tubes. The supernatant was acidified to pH < 2 using drop-wise additions of formic acid and extracted three times with 20 mL of dichloromethane. After each 20 mL addition of dichloromethane, the samples were centrifuged for 10 min at 15,000 rpm (Beckman Coulter, Model TJ-6, Mississauga, ON, Canada). The combined organic fractions were removed using a glass pipette, transferred into pre-labelled glass test tubes, and evaporated to dryness under a nitrogen stream.

3.7.2 Solid Phase Extraction Clean-up

To reduce background ion interferences, the dried tissue extracts were cleaned up using ENV+ solid phase extraction cartridges (Biotage, Charlottesville, VA, USA) (Headley et al. 2002). The extracts were re-dissolved in 10 mL Milli-Q water and acidified to pH < 2 using drop- wise additions of formic acid. The cartridges were washed with 6 mL of methanol

followed by 6 mL of 0.1% formic acid. The acidified extracts were then loaded into the cartridges and washed with 10 mL of 0.1% formic acid. The analytes were then eluted with 8 mL of methanol and evaporated to dryness under a nitrogen stream. The extracted analytes were re-dissolved in a 50:50 solution of acetonitrile:Milli-Q water and 0.1% ammonium hydroxide prior to analysis with the Orbitrap.

3.7.3 Spike Recovery Trials with Commercial and Extracted Naphthenic Acid Mixtures

Spike recovery trials were performed using lab-reared fathead minnow (culture stock aged 9 to 12 months) obtained from the Aquatic Toxicology Laboratory Facility in the Western College of Veterinary Medicine at the University of Saskatchewan. The same homogenization and extraction procedure was used for both the exposed tissues and the spike recovery trials. The muscle tissues were spiked with the same pre-made stock solutions of the Fluka naphthenic acid mixture (1232 mg/L) and the raw OSPW-extracted naphthenic acid mixture (8700 mg/L) used in the reproduction assay.

For the Fluka mixture, the homogenized male (~3.2 g) and female (~1.2 g) muscle tissues were divided into six replicates (3 male and 3 female), and spiked to a concentration of 24 µg/g of tissue. For the extracted (OAF) mixture, homogenized male (~3.2 g; n = 3) and female (~1.2 g; n = 3) muscle tissue were spiked to a concentration of 48 µg/g of tissue. The spiked tissues were covered and left to sit (refrigerated) for 1 hour prior to beginning the rest of the extraction procedure described in Sections 3.7.1 and 3.7.2.

3.8 Statistical Analyses

Statistical analyses were performed using SPSS® 19 (SPSS Inc., Chicago, IL, USA). Males and females were assessed separately for individual biological and reproductive endpoints.

Student t-tests were conducted for every endpoint to test for differences between the control and the NaOH (solvent) control to ensure no statistically significant differences were present prior to pooling the control data sets. One-way analysis of variance (ANOVA) tests were used to analyze most of the endpoints, provided parametric assumptions of normality (Shapiro-Wilks) and homogeneity of variance (Levene's) were met. Data that did not meet these assumptions (after log-transformation) were analysed using the non-parametric equivalent test (Kruskal-Wallis).

Differences among treatment groups were assessed using Tukey's *post hoc* or the non-parametric Mann-Whitney-U test applying the appropriate *Bonferronni* correction (σ (0.05) / number of comparisons made) to reduce the Type I error rate. Kolmogorov Smirnov (KS) tests were used to analyze cumulative egg production and cumulative spawning events. Chi-square tests were used to analyze adult survival and secondary sex characteristic indices. Differences were considered significant at $p \leq 0.05$.

3.9 Cross-Over Egg Experiment

To investigate the embryo-larval response in the presence and absence of parental exposure to the commercial and extracted naphthenic acid mixtures, a cross-over egg experiment was conducted where eggs that were fertilized and water hardened in clean (or reference) water were transferred into each of the reproduction assay treatment waters (10d exposure) (Figure 3-4). A single spawn was collected from three different breeding pairs (lab culture stock). The eggs from each single spawn were then divided equally into 15 egg cups (n = 3 per treatment group, with each replicate containing 20 to 40 eggs depending on size of brood) and transferred into the treatments waters in the reproduction assay: Control, NaOH solvent control, Fluka (2.5 and 5 mg/L) and OAF (5 mg/L). Similar to the reproduction assay, one-way analysis of variance

(ANOVA) or the non-parametric equivalent test (Kruskal-Wallis) were used to examine hatching success, time to hatch, larval survival, larval length, and mean total larval deformities.

The embryo-larval responses in the cross-over egg experiment (embryos exposed after water hardening) were then compared to the responses observed in the reproduction assay (embryos exposed in the mother) (Figure 3-4). As this was not a full factorial experimental design (i.e., a treatment to examine eggs exposed in the mother and then transferred to clean water was not included/conducted), Student t-tests were used to compare the responses between the following treatment groups: (i) RR: Reference mother, Reference embryos; (ii) RE: Reference mother, Exposed embryos; (iii) EE: Exposed mother, Exposed embryos (Figure 3-4). The endpoints compared included: hatching success, time to hatch, larval survival, larval length, and mean total deformities.

	Reference Water (Reference Mother)	Treatment Water (Exposed Mother)
Reference Water (Reference Embryos)	Complete Control (**RR**)	Not conducted
Treatment Water (Exposed Embryos)	Cross-Over Egg Experiment (**RE**)	Reproduction Assay (**EE**)

Figure 3-4. Treatment groups used to compare the embryo-larval response of fathead minnows in the presence and absence of parental exposure to commercial and extracted naphthenic acid mixtures. Where RR = Reference mother, Reference embryos, RE = Reference mother, Exposed embryos, EE = Exposed mother, Exposed embryos.

CHAPTER 4: RESULTS

4.1 Water Quality and Naphthenic Acid Analysis

No significant differences were observed among treatments for any of the water quality parameters collected daily in the lab compared to the controls (ANOVA and Kruskal-Wallis; $p > 0.05$) (Table 4-1). Likewise, no significant differences were observed for any of the routine water chemistry parameters (major ions, TDS, etc.) analysed weekly by ALS Environmental (ANOVA and Kruskal-Wallis; $p > 0.05$) (Table 4-1).

Table 4-1. Summary of water chemistry measured in the lab and by ALS Environmental. [a]

	Parameter	Units	Control	NaOH Control	Fluka 2	Fluka 5	OAF
Water Quality (Lab)	pH		8.30 ± 0.01	8.29 ± 0.02	8.33 ± 0.02	8.33 ± 0.02	8.32 ± 0.02
	Temperature	°C	25.4 ± 0.30	25.3 ± 0.25	25.2 ± 0.41	25.3 ± 0.40	25.5 ± 0.45
	Dissolved Oxygen	mg/L	7.52 ± 0.04	7.57 ± 0.05	7.59 ± 0.10	7.56 ± 0.07	7.54 ± 0.10
	Conductivity	µS/cm	328 ± 16	372 ± 17	375 ± 22	359 ± 25	422 ± 9
	Salinity	ppt	0.2 ± 0.01	0.2 ± 0.01	0.2 ± 0.01	0.2 ± 0.01	0.2 ± 0.00
Water Chemistry (ALS)	HCO3	mg/L	88.9 ± 2.32	85.8 ± 4.93	89.3 ± 2.36	91.7 ± 2.45	91.0 ± 2.08
	Cl	mg/L	12.0 ± 0.49	37.3 ± 12.76	23.9 ± 0.63	23.7 ± 0.29	30.1 ± 4.78
	Ca	mg/L	26.6 ± 0.72	26.3 ± 0.65	26.4 ± 0.66	26.2 ± 0.60	26.8 ± 0.74
	K	mg/L	4.4 ± 0.32	4.3 ± 0.32	4.3 ± 0.32	4.3 ± 0.29	4.5 ± 0.35
	Mg	mg/L	14.2 ± 0.52	14.0 ± 0.47	14.0 ± 0.49	14.0 ± 0.47	14.3 ± 0.57
	Na	mg/L	27.9 ± 1.52	44.6 ± 10.27	36.1 ± 1.55	36.3 ± 1.74	41.3 ± 4.79
	S (as SO4)	mg/L	86.8 ± 3.20	87.7 ± 2.31	87.5 ± 2.90	87.5 ± 2.19	90.1 ± 2.06
	Ammonia	mg/L	0.238 ± 0.13	0.199 ± 0.10	0.230 ± 0.05	0.226 ± 0.06	0.195 ± 0.07
	TDS (Calculated)	mg/L	219 ± 2.65	260 ± 22.03	239 ± 3.18	240 ± 3.28	254 ± 10.65
	Total Alkalinity	mg/L	72.8 ± 1.90	70.3 ± 4.04	73.2 ± 1.92	75.1 ± 2.01	74.6 ± 1.70
	Hardness (as CaCO3)	mg/L	125 ± 4.04	123 ± 3.51	124 ± 3.61	123 ± 3.38	126 ± 4.16

[a] Values are means ± SE (n = 21 for parameters measured daily in the lab, and n = 3 for parameters analyzed weekly by ALS Environmental).

Water samples were collected throughout the 21 d exposure period to monitor naphthenic acid concentrations within the flow-through system (i.e., NA concentrations from the head tank to the exposure tanks). As expected with a 24 hr turnover rate, the NA concentrations measured in the exposure tanks on day 0 were lower than (roughly half) the concentrations measured in the head tanks for both Fluka treatments (Table 4-2). However by day 7, NA concentrations in the exposure tanks were comparable to the head tanks (Table 4-2). Comparison of the in-water NA concentrations measured throughout the exposure indicated that the concentrations remained relatively stable (steady state conditions) in both the head and exposure tanks from days 7 to 21 for both Fluka treatments (Table 4-2). Due to time and budget constraints, NA concentrations in the water samples collected from the OAF treatment could not be determined.

Table 4-2. Naphthenic acid (NA) concentrations in water samples collected throughout the 21 day Fluka exposure. Samples were collected from both the head (n=1) and exposure tanks (mean ± SE, n=3) to monitor NA concentrations within the flow-through exposure system.

Treatment (Nominal NA Concentration)	Flow-through system tank	Measured NA Concentrations (mg/L)			
		Day 0	Day 7	Day 15	Day 21
Fluka 2 (2.5 mg/L)	Exposure Tank	0.64 ± 0.02	1.26 ± 0.02	1.21 ± 0.02	1.22 ± 0.08
	Head Tank	1.92	1.45	1.48	1.57
Fluka 5 (5 mg/L)	Exposure Tank	1.61 ± 0.07	2.40 ± 0.15	2.39 ± 0.14	2.90 ± 0.16
	Head Tank	4.33	2.97	3.05	2.68

In the Fluka treatments, the NA concentrations measured in the water were roughly half the nominal concentrations based on a 25-component Fluka calibration curve (Table 4-2) (see Section 3.6.1). The NA concentrations measured in the exposure tanks for the nominal 2.5 mg/L Fluka treatment ranged from 1.21 mg/L to 1.26 mg/L from days 7 to 21 (Table 4-2). The mean concentration measured over the 21 d exposure period was 1.2 ± 0.29 mg/L (mean ± SD; n = 12). Similarly, the NA concentrations measured in the exposure tanks for the nominal 5 mg/L Fluka

treatment ranged from 2.39 mg/L to 2.90 mg/L from days 7 to 21 (Table 4-2). The mean concentration measured over the 21 d exposure period was 2.3 ± 0.52 mg/L (mean ± SD; n = 12).

4.2 Naphthenic Acid Analysis in Fish Muscle Tissue

Analyses of the exposed muscle tissues identified the presence (or uptake) of one naphthenic acid congener detected as a peak in the Orbitrap mass spectra corresponding to m/z = 237.1857 (Appendix B). Based on the m/z ratio, this peak corresponds to NA isomers containing 15 carbons and 2 rings (n = 15, z = -4), fitting the classical naphthenic acid formula $C_{15}H_{26}O_2$. The NA congener (or $C_{15}H_{26}O_2$ isomers) was detected in both the male and female muscle tissues exposed to all three NA treatments (Fluka 2, Fluka 5 and OAF) (Figure 4-1). The NA congener (n = 15, z = -4) was detected in all the exposed tissues analysed, with the exception of one female replicate in the pooled homogenized tissue exposed in the 5 mg/L OAF treatment (i.e., the peak was detected in 17 of the 18 exposed tissue samples analysed). The peak corresponding to m/z = 237.1857 was not detected in any of the control tissues (n = 12) (Figure 4-1).

Although the $C_{15}H_{26}O_2$ isomers were detected in the exposed tissues, they were not detected in any of the spike recovery trials conducted with both the commercial (Fluka) and extracted (OAF) naphthenic acid mixtures. This prevented the generation of a spiked-tissue calibration plot (with known NA concentrations) to estimate naphthenic acid concentrations in the exposed muscle tissue. However, a semi-quantitative comparison based on the peak area count of the NA congener identified (m/z = 237.1857) per gram of wet muscle tissue was conducted to examine gender, treatment and exposure concentration differences in uptake using the equation below:

Uptake estimate = $\frac{\text{mass spectra area count of peak corresponding to m/z = 237.1857}}{\text{grams of wet muscle tissue in each of the replicates analyzed}}$

In the Fluka treatments, uptake estimates in both male and female fathead minnows increased with increasing exposure concentration (Figure 4-1). Based on peak area counts per gram of tissue, a 2.8-fold increase was observed in the male muscle tissues exposed to the 5 mg/L Fluka treatment (higher concentration) relative to the 2.5 mg/L Fluka treatment (lower concentration). A 1.2-fold increase was observed in the female muscle tissues exposed to the 5 mg/L Fluka treatment relative to the 2.5 mg/L Fluka treatment (Figure 4-1).

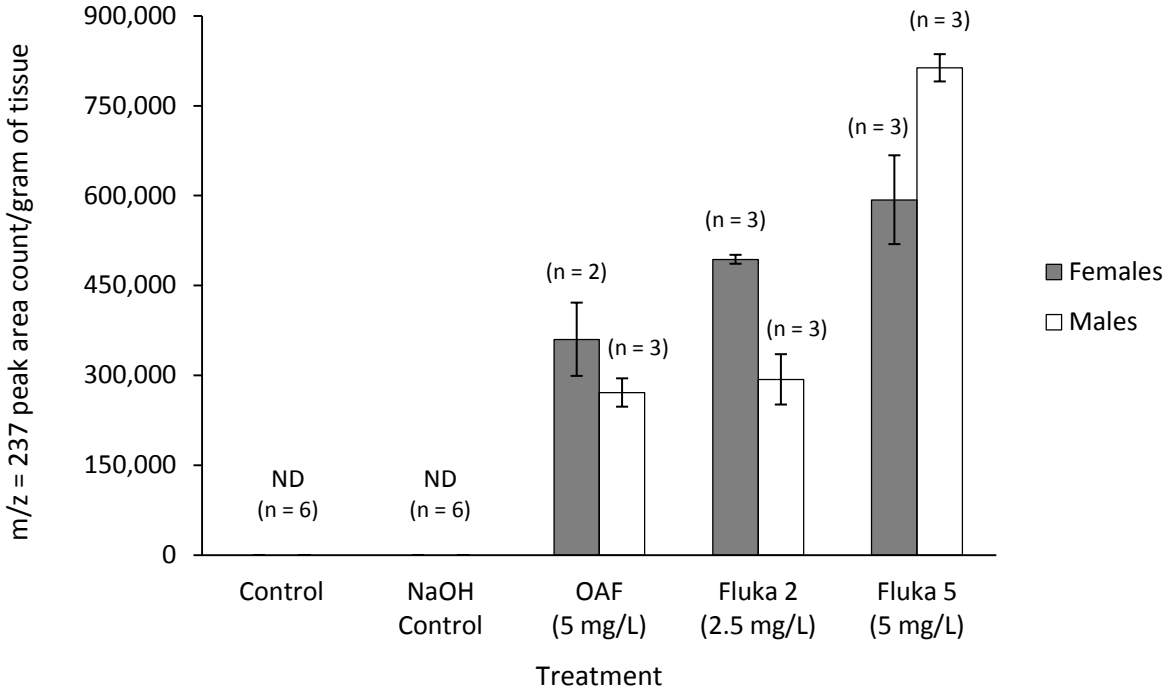


Figure 4-1. Semi-quantitative uptake comparisons in male and female fathead minnow muscle tissue following a 21d exposure to commercial (Fluka) and extracted (OAF) naphthenic acid mixtures. Values represent the mean ± SE of pooled homogenized tissues separated into 3 individual replicates (with the exception of the female OAF tissue where n=2). The identified NA congener was not detected (ND) in any of the male or female control tissues (Control and NaOH Control).

No consistent trends were observed with respect to gender-specific differences in uptake (i.e., uptake was not consistently higher or lower in males than in females). In the 2.5 mg/L Fluka treatment, uptake estimates were higher (~68%) in females than in males. In the 5 mg/L Fluka treatment, uptake estimates were higher (~37%) in males than in females (Figure 4-1). In the 5 mg/L OAF treatment, uptake estimates were ~33% higher in the female muscle tissues compared to the male tissues (Figure 4-1).

Comparison of the semi-quantitative uptake estimates between the commercial (Fluka) and extracted (OAF) naphthenic acid treatments indicated uptake was higher in both the male (3-fold) and female (1.6-fold) muscle tissues exposed to the 5 mg/L Fluka treatment compared to the 5 mg/L OAF treatment (Figure 4-1). Uptake estimates were also higher (~37%) in the female muscle tissue exposed to the 2.5 mg/L Fluka treatment compared to the 5 mg/L OAF treatment. However, in the male muscle tissues, uptake estimates were similar in both the 2.5 mg/L Fluka and the 5 mg/L OAF treatments (Figure 4-1).

4.3 Individual Biological Endpoints

As discussed in Section 3.8, Student t-tests were conducted for every endpoint to test for differences between the control and the NaOH (solvent) control. No significant differences were observed between the two controls, so the data were pooled and all three treatments (Fluka 2, Fluka 5 and OAF) were analysed relative to the pooled control data sets. No significant differences were observed among treatments for male and female survival (Chi-square; $p > 0.05$) compared to the control. No significant differences were observed (ANOVA or Kruskal-Wallis; $p > 0.05$) among treatments for mass, fork length, condition factor and liver somatic indices in male and female fathead minnows compared to the pooled control (Table 4-3).

4.4 Individual Reproductive Endpoints

No significant differences were observed among treatments for male GSI (ANOVA; p = 0.095) and female GSI (ANOVA; p = 0.514) compared to the pooled control (Table 4-3). No significant differences were observed among treatments for male secondary sex characteristics or female ranked ovipositor size (Chi-square; $p > 0.05$) compared to the control (data not shown).

Table 4-3. Fork length, mass, condition factor (CF), gonadal somatic indices (GSI), and liver somatic indices (LSI) for male and female fathead minnows exposed to commercial (Fluka) and extracted (OAF) naphthenic acid mixtures. [a]

	Treatment	Fork Length (cm)	Mass (g)	CF (%)	LSI (%)	GSI (%)
Male	Control	6.83 ± 0.09	3.95 ± 0.22	1.23 ± 0.06	1.63 ± 0.07	0.93 ± 0.10
	Fluka 2	6.80 ± 0.21	4.24 ± 0.35	1.35 ± 0.05	2.00 ± 0.45	1.12 ± 0.14
	Fluka 5	6.68 ± 0.06	3.77 ± 0.11	1.27 ± 0.02	1.76 ± 0.18	0.84 ± 0.19
	OAF	6.97 ± 0.03	4.28 ± 0.20	1.27 ± 0.07	1.86 ± 0.30	1.28 ± 0.09
Female	Control	5.38 ± 0.10	1.82 ± 0.12	1.16 ± 0.04	1.69 ± 0.19	8.68 ± 0.76
	Fluka 2	5.65 ± 0.10	2.05 ± 0.15	1.13 ± 0.04	1.81 ± 0.15	9.15 ± 1.32
	Fluka 5	5.36 ± 0.12	1.95 ± 0.16	1.25 ± 0.02	1.83 ± 0.07	10.27 ± 1.18
	OAF	5.28 ± 0.14	1.90 ± 0.08	1.30 ± 0.08	1.83 ± 0.13	8.13 ± 0.77

[a] Values are means ± SE (n = 3 to 9 depending on gender and endpoint)

4.5 Egg Production

During the 21 d exposure there was a significant decrease (36%) in cumulative egg production (cumulative # of eggs /female/day) in the 5 mg/L Fluka treatment compared to the pooled control (Kolmogorov Smirnov; p = 0.009) (Figure 4-2). No significant differences were observed in the 2.5 mg/L Fluka treatment or the 5 mg/L OAF treatment (Kolmogorov Smirnov; $p > 0.05$) compared to the pooled control (Figure 4-2).

No significant differences were observed among treatments for cumulative spawning events (Kolmogorov Smirnov; $p > 0.05$) and mean total spawning events (Chi-square; $p > 0.05$) over the 21 d exposure period compared to the control. Similarly, no significant differences were observed among treatments for mean total egg production (total # of eggs produced by each

female in a breeding pair (or replicate) over the 21d exposure period), mean egg production (average # of eggs produced by each female in a breeding pair (or replicate) over the 21d exposure period), fertilization success (# of eggs fertilized/# of eggs spawned), and egg size (ANOVA or non-parametric equivalent, Kruskal-Wallis; $p > 0.05$) compared to the pooled control.

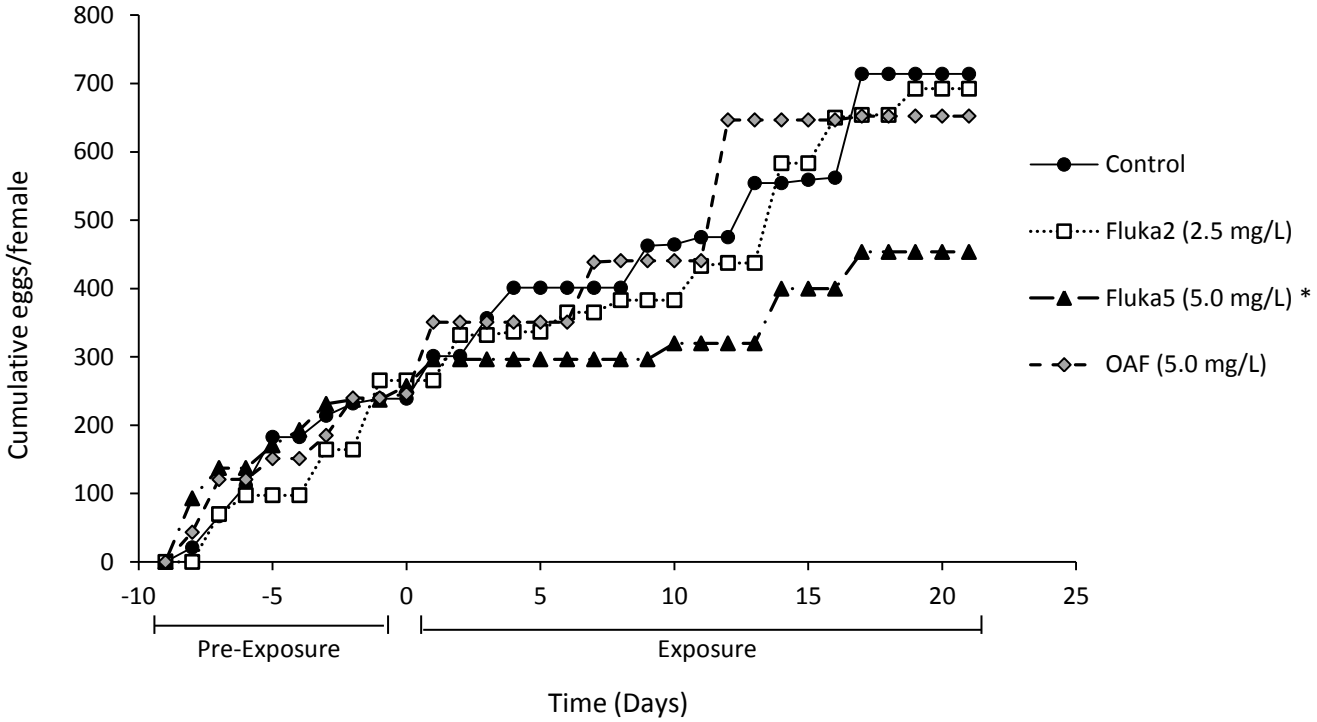


Figure 4-2. Cumulative egg production in fathead minnow breeding pairs during the 10 d pre-exposure and 21 d exposure to commercial (Fluka) and extracted (OAF) naphthenic acid mixtures. Nominal exposure concentrations reported in brackets. Asterisks (*) denotes significant decrease in cumulative egg production in the 5 mg/L Fluka treatment (Kolmogorov Smirnov; $p = 0.009$) compared to the pooled control.

4.6 Embryo-Larval Endpoints

Larval survival was significantly decreased (46%) in the 5 mg/L Fluka treatment (ANOVA; $p = 0.025$) compared to the 5 mg/L OAF treatment (Figure 4-3). Larval survival was decreased by 36% in the 5 mg/L Fluka treatment compared to the pooled control, however, this result was not significantly different in the post-hoc comparisons (Tukey HSD; $p = 0.106$)

(Figure 4-3). No significant differences were observed in the post-hoc comparisons between the pooled control and the OAF treatment (Tukey HSD; $p > 0.05$). There were not enough replicates (n = 2) in the 2.5 mg/L Fluka treatment to assess larval endpoints in the ANOVA comparison.

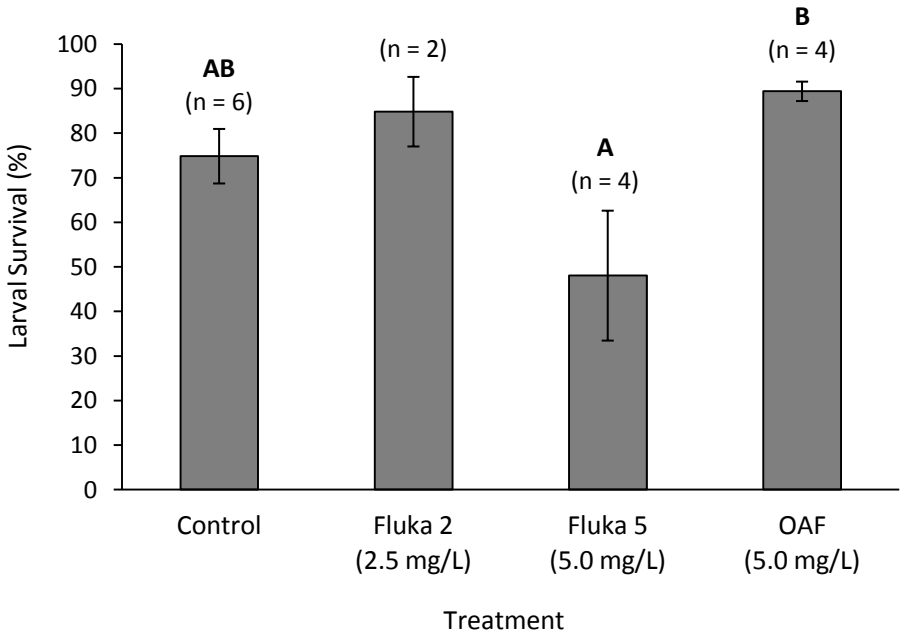


Figure 4-3. Mean larval survival (5 days post-hatch) in fathead minnow exposed to commercial (Fluka) and extracted (OAF) naphthenic acid mixtures. Values represent the mean ± SE for all broods collected from each breeding pair (n) per treatment over the 21d reproduction assay. Different letters designate statistically significant differences in larval survival as determined by ANOVA and Tukey's test ($p < 0.05$).

A significant increase in mean total larval deformities (# of larvae deformed / total # larvae hatched from each breeding pair (or replicate) throughout the 21d exposure period) was also observed in the 5 mg/L Fluka treatment (ANOVA; $p = 0.015$) compared to the pooled control and the 5 mg/L OAF treatment (Figure 4-4). No significant differences were observed in the post-hoc comparisons between the pooled control and the OAF treatment (Tukey HSD; $p > 0.05$). In the Fluka treatments, the rate and severity of the observed deformities increased with increasing exposure concentrations (Figure 4-4). The rate of larval deformities increased by ~2.5-fold in the 2.5 mg/L Fluka treatment, and ~7.5-fold in the 5 mg/L Fluka treatment relative

to the pooled control (Figure 4-4). In contrast to the Fluka treatments, the mean total deformity rate in the 5 mg/L OAF treatment (3.21 ± 1.26%) was comparable to the rate observed in the pooled controls (5.97 ± 3.01%) (Figures 4-4 and 4-5).

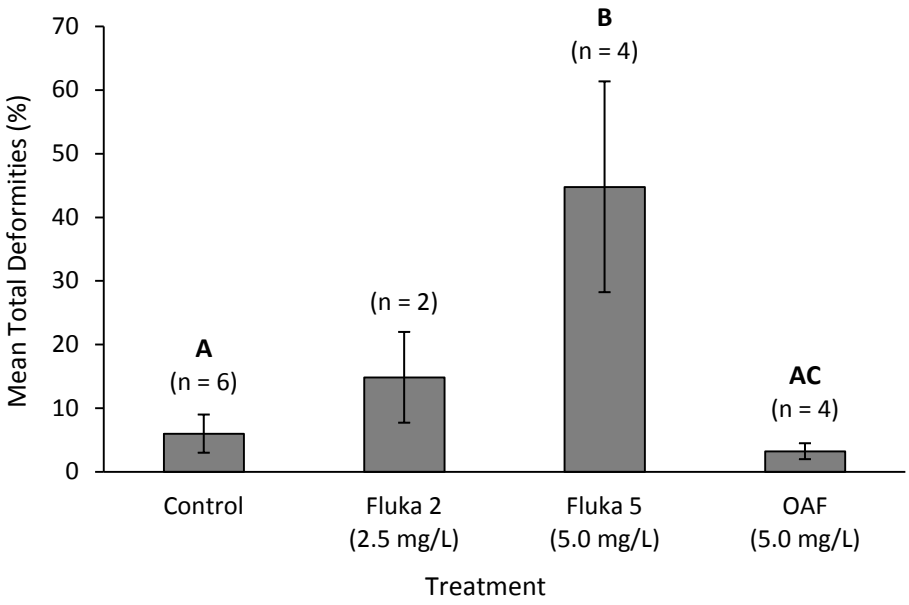


Figure 4-4. Mean total larval deformities in fathead minnow exposed to commercial (Fluka) and extracted (OAF) naphthenic acid mixtures. Values represent the mean ± SE for all broods collected from each breeding pair (n) per treatment over the 21d reproduction assay. Different letters designate statistically significant differences in larval deformities as determined by ANOVA and Tukey's test ($p < 0.05$).

The dominant malformations included pericardial and yolk sac edema, yolk-sac deformities, as well as craniofacial and skeletal malformations (kyphosis, lordosis, scoliosis) (Figure 4-6). The most consistent deformity was a combination that included pericardial and yolk-sac edema, yolk-sac deformity and craniofacial malformations (PE+YE+YSD+CF) (Figure 4-6 a). In the Fluka treatments, the rate and severity of this deformity in combination with various spinal malformations (e.g. PE+YE+YSD+CF + kyphosis) was higher in the 5 mg/L Fluka treatment (Figure 4-6 c) compared to the 2.5 mg/L Fluka treatment (Figure 4-6 b).

No significant differences were observed among treatments for mean days to hatch, mean hatching success (# eggs hatched / # of eggs fertilized per breeding pair (or replicate) over the 21d exposure period), and larval length (5 days post-hatch) (ANOVA or non-parametric equivalent, Kruskal-Wallis; $p > 0.05$) compared to the pooled control.

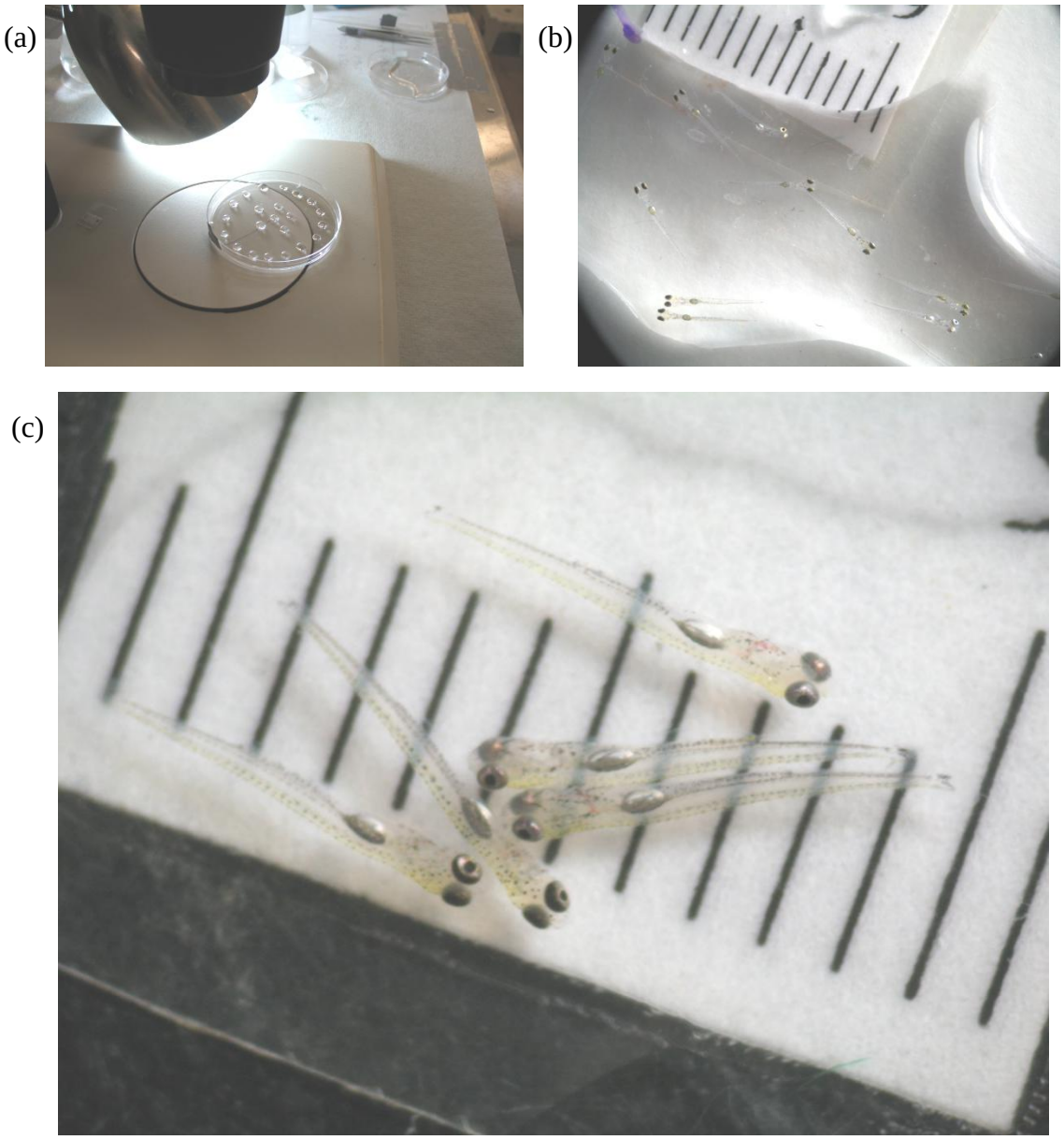


Figure 4-5. Normal fathead minnow larvae collected from the pooled controls throughout the 21d reproductive assay: (a) 5d post-hatch larvae were examined (alive) using the VistaVision™ tri-nocular microscope; (b) Normal larvae viewed at 4x magnification; (c) Normal larvae viewed at 40x magnification. Photos by A. Mahaffey (2011)

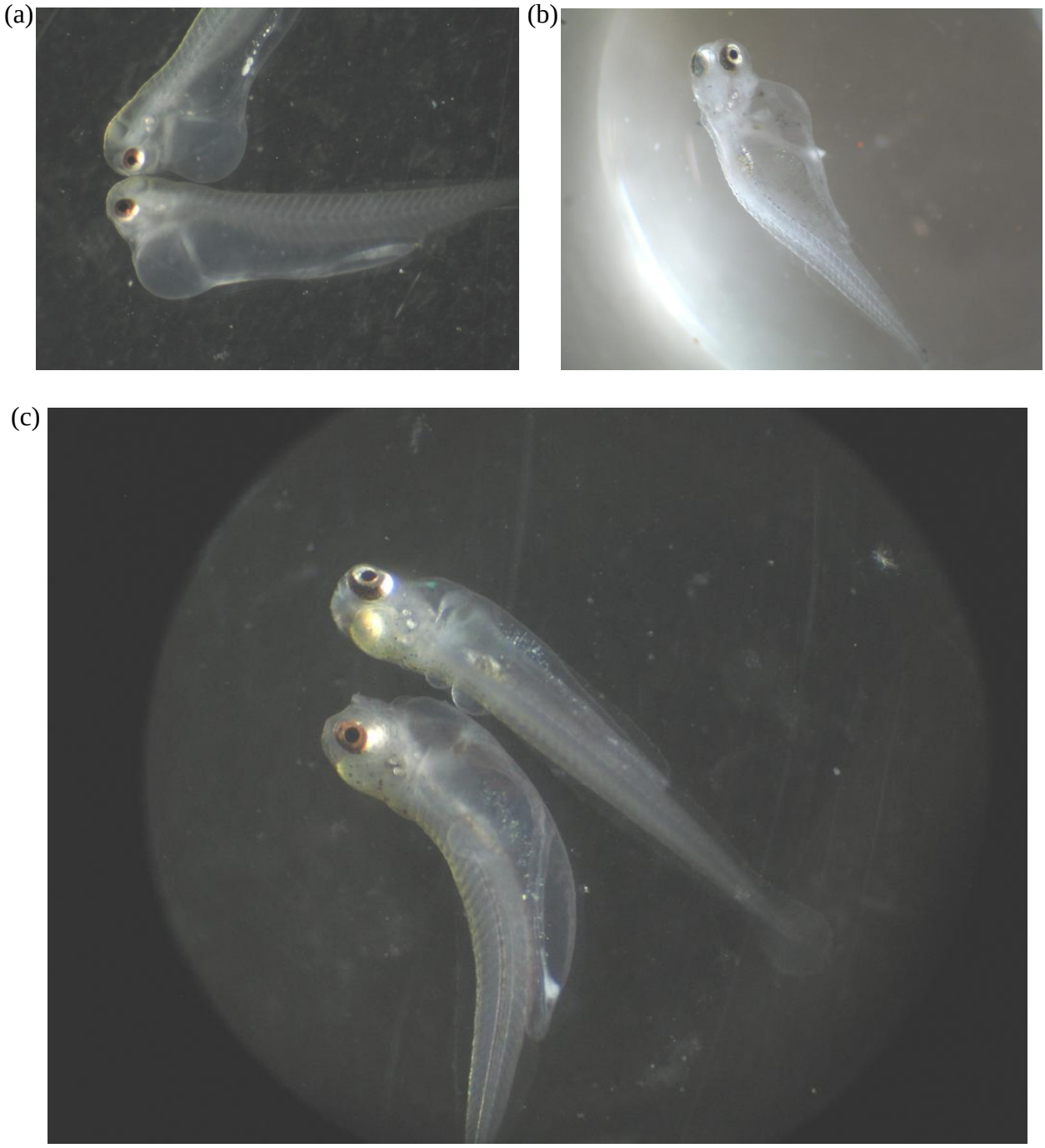


Figure 4-6. Deformities in fathead minnow larvae (5d post-hatch) exposed to the commercial (Fluka) NA mixture: (a & b) Deformities observed in fathead minnow larvae collected from 2.5 mg/L Fluka treatment: (a) Photo shows craniofacial deformity, pericardial and yolk-sac edema, yolk-sac deformity and slight kyphosis (convex rounding of the spine); (c) Larval deformities observed in 5 mg/L Fluka treatment: photo shows craniofacial deformity, pericardial and yolk-sac edema, yolk sac deformity and more pronounced kyphosis. Photos by A. Mahaffey (2011)

4.7 Cross-Over Egg Experiment

In the cross-over egg experiment, fertilization success was tested to ensure no significant differences were present prior to placing the reference eggs (spawned from non-exposed parents) into the treatment waters. No significant differences were observed among treatments (ANOVA; $p = 0.709$) compared to the pooled control. A significant increase in mean total larval deformities was observed in the 5 mg/L Fluka treatment (ANOVA; $p = 0.039$) compared to both the pooled control (~5.7 fold) and the 5 mg/L OAF treatment (~5.8-fold) (Figure 4-7). No deformities were observed in any of the larvae examined in the 2.5 mg/L Fluka treatment (n = 3 broods; 58 individual larvae) (Figure 4-7). No significant difference in mean total deformities was observed in the post-hoc comparisons between the 5 mg/L OAF treatment and the pooled control (Tukey HSD; $p > 0.05$). The rate of larval deformities observed in the cross-over egg experiment in the 5 mg/L OAF treatment (4.78 ± 2.63%) was comparable to the rates observed in the pooled control (4.85 ± 3.13%) (Figure 4-7).

No significant differences were observed among treatments for larval survival (ANOVA; $p > 0.05$) in the cross-over egg experiment. Similarly, no significant differences were observed among treatments in the cross-over egg experiment for mean days to hatch, mean hatching success (# eggs hatched / # of eggs fertilized) and larval length (5 days post-hatch) (ANOVA or Kruskal-Wallis; $p > 0.05$) compared to the pooled control.

As discussed in Section 3.9, Student t-tests were conducted to compare the response of fathead minnow embryos in the presence (EE: exposed mother, exposed embryos) and absence (RE: reference mother, exposed embryos) of parental exposure for each of the treatment waters examined in the reproduction assay (Figure 3-4). Student t-test comparisons could not be

conducted for the 2.5 mg/L Fluka treatment because there were not enough replicates (n = 2) in the 21d reproduction assay.

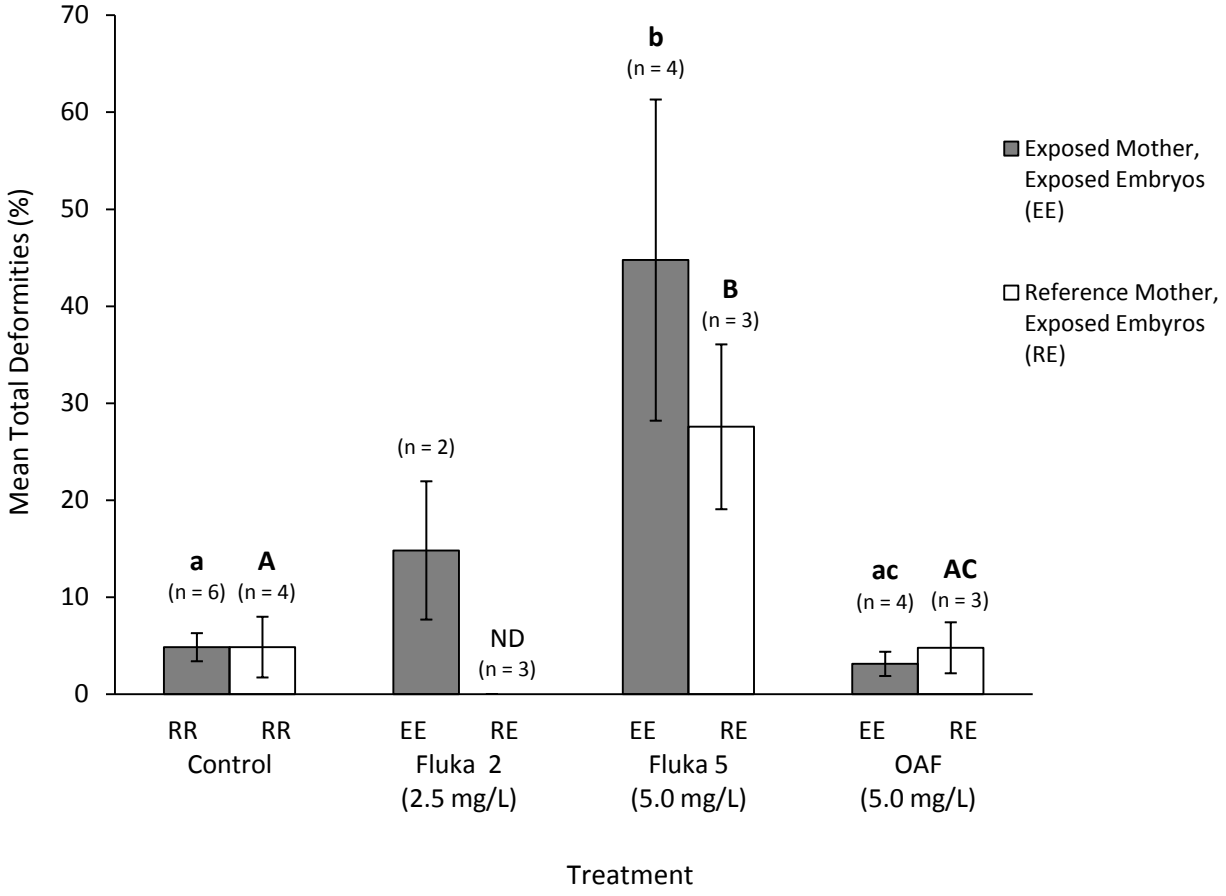


Figure 4-7. Comparison of the mean total deformities observed in fathead minnow larvae in the presence (EE) and absence (RE) of parental exposure to commercial and extracted NA mixtures. Different letters designate statistically significant differences in larval deformities as determined by ANOVA and Tukey's test ($p < 0.05$). Lower case letters designate results observed in the presence of parental exposure (EE). Upper case letters designate results observed in the absence of parental exposure (RE). No deformities (ND) were observed in the 2.5 mg/L Fluka treatment in the cross-over egg experiment.

No significant differences were observed in any of the Student t-test comparisons between the treatment groups for larval survival, mean days to hatch, mean hatching success (# eggs hatched / # of eggs fertilized), larval length (5 days post-hatch), and mean total deformities (Student t-test; $p > 0.05$) (Figure 4-7). It is interesting to note, however, that while the frequency

of deformities in the pooled control and the 5 mg/L OAF treatment were similar in the cross-over egg experiment and the reproduction assay, the frequency of deformities in both the Fluka treatments were considerably reduced (Figure 4-7). In the 5 mg/L Fluka treatment, the deformity rate decreased from 44.76 ± 16.56% in presence of parental exposure (EE: exposed mother, exposed embryos) to 27.57 ± 8.5% in the absence of parental exposure (RE: reference mother, exposed embryos) (Figure 4-7). In the 2.5 mg/L Fluka treatment, the deformity rate in the presence of parental exposure (EE) was 14.82 ± 7.13%. In the absence of parental exposure (RE), no deformities were observed (Figure 4-7).

CHAPTER 5: DISCUSSION

5.1 Water Quality

Overall, water quality was similar among treatments (Table 4-1). Non-significant increases were observed in conductivity, sodium and chloride relative to the control (Table 4-1). These were expected and can be attributed to the NaOH solvent and diluted HCl used to adjust the pH. One of the objectives for this experiment was to assess the effects of commercial and oil sands extracted NA mixtures at environmentally relevant exposure concentrations in water conditions comparable to the Athabasca River. The Athabasca River was chosen because it is considered to be the receiving environment for potential seepage of raw OSPW (and naphthenic acids) from active settling basins (Environment Canada, 2011a-c), and potential discharges of treated OSPW in future treatment and release scenarios (Golder, 2014).

Although the current understanding of factors that modify naphthenic acid toxicity are limited (Li et al. 2014), previous work has shown the toxicity of commercial and extracted NA mixtures are influenced by pH and water hardness (Cairns, 1957; Armstrong et al. 2009) (Tables 2-10 and 2-11). Squires et al. (2010) recently examined water quantity and quality changes in the Athabasca River basin across historical (1966-1976) and current day (1996-2006) time periods. The average pH, alkalinity and hardness levels recorded for the mouth reach of the Athabasca River between 1996 and 2006 were 8.2, 75 mg/L $CaCO_3$, and 127 mg/L $CaCO_3$, respectively (Squires et al. 2010). Comparison of the pH, alkalinity and hardness levels measured throughout the 21 day exposure indicated they were within the range of the average levels recorded in the mouth reach of the Athabasca River for all treatments including the "synthetic river water" control (Table 4-1).

As discussed in Section 1.1, there are no water quality guidelines for the protection of aquatic life for naphthenic acids in Canada or the United States (AESRD, 2014; Golder, 2014). In Alberta, guidelines for the protection of aquatic life are based on high quality, scientific information for many different organisms (AEP, 1996). As such, the data used to develop water quality guidelines are evaluated and classified as primary, secondary or unacceptable according to CCME (1991) criteria. To develop a full guideline according to the Alberta protocol, the minimum data set must meet the primary data criteria (AEP, 1996).

One of the key requirements to meet the primary data classification is the measurement of in-water concentrations at the beginning and end of the exposure period (AEP, 1996). Ideally, primary data are generated from toxicological studies that use flow-through conditions and confirm, by direct measurement, the chemical concentrations and conditions in each experiment chamber. Calculated and/or nominal concentrations based on measurements taken in stock solutions are not acceptable (AEP, 1996). Generally, static tests are not acceptable unless it can be shown that the chemical concentrations did not change (i.e., less than 10%) during the test, and that adequate environmental conditions for the test species were maintained. Measurement of abiotic variables such as temperature, pH, dissolved oxygen and water hardness should be reported so that any factors that may affect toxicity can be included in the evaluation process (AEP, 1996).

The use of secondary data from flow-through and static tests in which concentrations were measured and did not change significantly throughout the exposure, are allowed for the derivation of interim Alberta guidelines (AEP, 1996). However, the results from static toxicological tests with nominal concentrations cannot be used. As discussed in the Alberta protocol, during static tests the concentration of a test chemical may be reduced by adsorption

onto particulates or test vessels (AEP, 1996). In these situations, an effect would be observed at concentrations that are actually lower than the nominal concentrations initially added to the vessel. Therefore, static tests with nominal concentrations are not used because they could result in guidelines that do not provide sufficient protection (AEP, 1996).

Significant differences between nominal and in-water exposure concentrations have been reported in toxicological assessments of commercial NA mixtures (Peters et al. 2007; Young et al. 2011). For example, Young et al. (2011) reported that one day after adding 3 mg/L of the Merichem NA stock solution, the measured in-water naphthenic acid concentration was 1.1 mg/L. To achieve the target concentration of 3 mg/L Merichem NAs, stock solutions were added daily throughout the remainder of the exposure (Young et al. 2011). However, despite daily additions of the Merichem NA stock solution, the measured in-water concentrations did not reach 3 mg/L until the 4^{th} day of the 10 d exposure, and ranged from 3 mg/L to 4 mg/L from days 6 to 10 (Young et al. 2011). The authors attributed the decrease in the measured NA concentrations to sorption of NAs to the surface of the exposure tanks (Headley et al. 2010), aeration (Han et al. 2009), and uptake into the fish (Young et al. 2011).

In the present study, the naphthenic acid concentrations measured in the treatment waters were roughly half the nominal concentrations (Table 4-2). In the nominal 2.5 mg/L Fluka treatment, the measured in-water NA concentrations ranged from 1.21 mg/L to 1.26 mg/L from days 7 to 21 (Table 4-2). The mean NA concentration measured over the 21 d exposure period was 1.2 mg/L. In the nominal 5 mg/L Fluka treatment, the in-water NA concentrations ranged from 2.39 mg/L to 2.90 mg/L from days 7 to 21 (Table 4-2). The mean NA concentration measured over the 21 d exposure period was 2.3 mg/L. Sorption losses were expected and are consistent with previous reports in the literature (Headley et al. 2010; Young et al. 2011). In a

comparison study to examine the dissipation of commercial (Fluka) and extracted (OAF) naphthenic acid mixtures by lake biofilms, Headley et al. (2010) reported that the initial dissolved NA concentration was generally less than half of the original spike. This observation was reproducible and observed in the control bioreactors (i.e., without biofilms), suggesting sorption of NAs to the bioreactor contributed to the decrease in the measured NA concentrations (Headley et al. 2010).

Comparison of the in-water NA concentrations measured on days 7, 15 and 21 indicated that steady-state concentrations were achieved in the flow-through system design for both the Fluka treatments (Table 4-2). Unfortunately, time and budget constraints prevented analysis of the in-water exposure concentrations in the OAF treatment. Due to the compositional differences between the commercial and extracted mixtures, it is difficult to determine whether or not the sorption losses would have been similar in the OAF treatment. However, it is reasonable to assume that steady-state concentrations were achieved in the nominal 5 mg/L OAF treatment.

The significant difference between the nominal and in-water exposure concentrations of naphthenic acids observed in this study and others (Peters et al. 2007; Headley et al. 2010; Young et al. 2011), demonstrates the importance of measuring in-water concentrations in future toxicological assessments of oil sands extracted NA mixtures and why it is required to meet the primary study designation for the development of water quality guidelines in Alberta (AEP, 1996). Although this has been recommended in previous reviews (OSWRTWG, 1996; CEATAG, 1998), the number of studies that have measured in-water exposure concentrations in toxicological assessments with oil sands extracted NA mixtures are extremely limited (Table 2-10). To date, only one study has reported in-water concentrations of a whole extracted NA mixture in a toxicological assessment with fish (Leclair et al. 2013). However, as reviewed in

Section 2.6, there is a considerable amount of variability in the measurement of total NA concentrations depending on the analytical method that is used. Therefore, the standardization or use of the same quantitative method would be required to allow cross-study comparisons of in-water NA concentrations and toxicological response, especially in the development of water quality guideline specific to oil sands naphthenic acids (CEATAG, 1998).

5.2 Semi-Quantitative Naphthenic Acid Uptake Estimates in Muscle Tissue

The present study is the first to apply high resolution mass spectrometry (HRMS) to detect and estimate the uptake of naphthenic acids in fish muscle tissue. The absence of the n = 15, z = -4 NA congener (or $C_{15}H_{26}O_2$ isomers) in the spiked tissue trials prevented the generation of a calibration plot to estimate NA concentrations similar to method employed by Young et al. (2008; 2011). The reason(s) for the detection of the n = 15, z = -4 NA congener in only the exposed muscle tissues is not known, however, it is possible that the concentrations used in the spiked tissue trials were too low.

In the Fluka treatments, uptake estimates of NAs (or $C_{15}H_{26}O_2$ isomers) in the muscle tissue of fathead minnows increased with increasing exposure concentration (Figure 4-1). Although the NA tissue estimates are semi-quantitative, this study is the first to report increased uptake with increasing exposure concentrations of a commercial NA mixture in fish. As discussed in Section 2.9.4, Smits et al. (2012) reported increased uptake of NAs (or $C_{13}H_{22}O_2$ isomers) with increasing nominal exposure concentrations of Merichem NAs in the muscle tissues of northern leopard frogs. Therefore, the increased uptake of $C_{15}H_{26}O_2$ isomers detected in the muscle tissues of fathead minnows exposed to increasing concentrations of Fluka NAs using the high resolution method developed in the present study is consistent with the results

reported by Smits et al. (2012) using the GC-MS method developed by Young et al. (2008; 2011).

Kavanagh et al. (2012) used the GC-MS method developed by Young et al. (2011) to estimate NA concentrations (or $C_{13}H_{22}O_2$ isomers) in the muscle tissue of fathead minnows exposed to nominal concentrations (10 mg/L) of an aged extracted NA mixture. They reported no significant difference in the estimated NA tissue concentrations between male and female fathead minnows (i.e., no gender specific differences in uptake were reported) (Kavanagh et al. 2012). Likewise, no consistent trends were observed with respect to gender in the uptake estimates of $C_{15}H_{26}O_2$ isomers in fathead minnow muscle tissues (i.e., uptake was not consistently higher or lower in males than in females) exposed to the commercial (Fluka) and extracted (OAF) naphthenic acid mixtures using the high resolution method developed in the present study (Figure 4-1).

While gender-specific differences were not observed, uptake estimates of $C_{15}H_{26}O_2$ isomers were higher in the muscle tissue of fathead minnows exposed to the same nominal concentration of a commercial NA mixture relative to an oil sands extracted NA mixture (Figure 4-1). In other words, the semi-quantitative uptake estimates of naphthenic acids (or $C_{15}H_{26}O_2$ isomers) in the muscle tissue of both male and female fathead minnows were higher in the nominal 5mg/L Fluka treatment compared to the nominal 5 mg/L OAF treatment (Figure 4-1). Whether this is due to a higher relative proportion of $C_{15}H_{26}O_2$ isomers in the Fluka mixture (compared to the oil sands extracted mixture), or if this is due to compositional differences between the commercial and extracted mixtures as a whole, is not known.

As described in Section 2.9.3, van den Heuvel et al. (2014) recently developed a high resolution mass spectrometry method to identify and examine specific NA isomers in the bile of yellow perch exposed to aged OSPW. Although the high resolution method was superior (relative to the GC-MS method) at differentiating between the exposed vs. reference perch, the relative abundance of the individual NA isomers in the bile of the yellow perch varied depending on the source of the aged OSPW they were exposed to (van den Heuvel et al. 2012). As reviewed in Section 2.7, several studies have shown the relative abundance and composition of individual NA structural classes vary depending on the source examined (i.e., aged OSPW, raw OSPW, commercial and extracted NA mixtures) (Tables 2-5 to 2-9). Therefore, it is possible that the differences in the semi-quantitative uptake of $C_{15}H_{26}O_2$ isomers in the muscle tissue of fathead minnows exposed to the commercial and extracted NA mixtures were due to differences in the relative abundance of the n = 15, z = -4 NA congener between the mixtures (Figure 4-1). A detailed compositional analysis to examine the relative proportion of individual NA congeners within the commercial (Fluka) and extracted (OAF) naphthenic acid mixtures was not conducted.

Methods to quantify naphthenic acids in biological tissues are still in the early stages of development (Headley et al. 2013). Thus, little is known about the relationship between naphthenic acid uptake (i.e., tissue burdens) and toxicological response. As discussed in Section 2.9.4, Kavanagh et al. (2012) reported reduced uptake and improved reproductive response in fathead minnows exposed to an aged extracted NA mixture with the addition of 700 mg/L $NaHCO_3$. The reason(s) for this are not known (Kavanagh et al. 2012). To date, a study to examine uptake of naphthenic acids (i.e., tissue burdens) in relation to sub-lethal response in fish exposed to increasing concentrations of an oil sands extracted NA mixture has not been done.

Only one previous study has examined uptake (i.e., estimated NA tissue concentrations) in relation to sub-lethal response following exposure to a commercial NA mixture (Smits et al. 2012). Although tissue burdens of naphthenic acids (or $C_{13}H_{22}O_2$ isomers) increased with increasing nominal exposure concentrations to Merichem NAs (20 mg/L and 40 mg/L), little to no evidence of sub-lethal toxicity was observed in northern leopard frogs following a 28 day exposure under saline conditions comparable to reclamation environments (Smits et al. 2012). In contrast to the results reported by Smits et al. (2012), increased uptake estimates of $C_{15}H_{26}O_2$ isomers in fathead minnows exposed to increasing concentrations of Fluka NAs corresponded to a significant decrease in cumulative egg production (Figure 4-2) (discussed in the following section). Differences in the species and endpoints examined, composition of the commercial mixtures used, test conditions, tissue extraction and analytical methodology, as well as the addition of salts in the exposures conducted by Smits et al. (2012) could all account for the difference between naphthenic acid uptake and sub-lethal response observed in the present study.

5.3 Naphthenic Acid Effects on Fathead Minnow Reproduction Endpoints

As reviewed in Section 2.8.3, previous *in vitro* experiments have shown that commercial NA mixtures (Fluka and Acros) exhibit much higher androgen receptor antagonist activity relative to naphthenic acid fractions extracted from off-shore produced waters (Thomas et al. 2009). Similar *in vitro* experiments indicated that Merichem NAs exhibited weaker estrogen and androgen receptor-mediated responses relative to raw OSPW (He et al. 2011). However, an *in vitro* comparison of the endocrine disrupting potential of a commercial and an oil sands-extracted NA mixture (i.e., acidic fraction of raw OSPW in isolation) was not conducted. The present study is the first to compare the reproductive response in fish exposed to a commercial

and an extracted NA mixture. As noted in the introduction, the extracted NA mixture used in this thesis was isolated from raw OSPW and was not artificially aged or simulated.

The fathead minnow reproduction assay has been used in numerous studies to examine the reproductive and embryo-larval effects of complex mixtures (Rickwood et al. 2006a-c; 2008; Rozon-Ramilo et al. 2011a; 2011b; Ouellet et al. 2013a-c). These studies have consistently identified cumulative egg production as one of the most sensitive reproductive endpoints in fathead minnows. While a significant decrease in cumulative egg production was observed in the nominal 5 mg/L Fluka treatment, no significant differences were observed at the same nominal concentration of an extracted (OAF) naphthenic acid mixture (Figure 4-2).

Based on the difference in cumulative egg production in fathead minnows exposed to the same nominal concentration (5 mg/L) of a commercial and an extracted naphthenic acid mixture, the reproductive effects of the commercial NA mixture were more pronounced than the oil sands extracted NA mixture (Figure 4-2). Although aromatic NAs have been identified in commercial mixtures (Rudzinski et al. 2002; Kavanagh et al. 2009; Rowland et al. 2011d), the steroidal aromatic acids in raw OSPW were not found in the commercial mixture analysed by Rowland et al. (2011e). Steroidal or "estrogen-like" NAs have been implicated in the reproductive effects observed in fish exposed raw and aged OSPW (Kavanagh et al. 2011; He et al. 2012b), as well as artificially aged oil sands extracted NA mixtures (Kavanagh et al. 2012). However, recent work has shown that the aromatic NA fraction, isolated from raw OSPW, is only weakly estrogenic relative to other anthropogenic steroids (Reinardy et al. 2013) (Table 2-10).

As discussed in Section 2.8.3, the impaired reproduction observed in this study and others (Kavanagh et al. 2011; 2012) could be the result of naphthenic acids acting directly or indirectly

on the endocrine system of fathead minnows. Previous studies have shown that both stress and hypoxia can indirectly affect the reproductive physiology of fish and reduce plasma sex steroids (Wu et al. 2003; Pollock et al. 2007; Schreck, 2010). Significant histopathological gill alterations have been reported in yellow perch exposed to both commercial (0.9 mg/L) and oil sands extracted (1.7 mg/L) NA mixtures, as well as aged OSPW (Nero et al. 2006a; 2006b) (Tables 2-10 and 2-11; Appendix A). Therefore, it is possible that respiratory stress, due to impaired gill function and reduced gas exchange, affected the reproductive physiology in fathead minnows exposed to the commercial (Fluka) naphthenic acid mixture in this study (Figure 4-2).

Although cumulative egg production was not assessed in the reproduction assay conducted by Kavanagh et al. (2012), there do not appear to be any considerable differences in the egg production rates of fathead minnow exposed to the same nominal (5 mg/L) concentration of a "fresh" vs. an "artificially aged" oil sands extracted NA mixture. The mean fecundity rate in fathead minnows exposed to 5 mg/L of the "fresh" extracted NA mixture used in this study was 6.5 ± 2.1 eggs /female/day. The mean fecundity rate in fathead minnows exposed to 5 mg/L of an "artificially aged" extracted NA mixture was 6.9 ± 1.7 eggs /female/day (Kavanagh et al. 2012).

Similar to the results observed with the "fresh" extracted NA mixture used in this thesis, Kavanagh et al. (2012) reported no significant differences in fertilization success, spawning rates, and male secondary sex characteristics (assessed as number of male tubercles) at a nominal 5 mg/L concentration of an "artificially aged" extracted NA mixture. However, due to differences in the extraction methods and experimental design between the present study (i.e., flow-through system) and the exposures conducted by Kavanagh et al. (2012) (i.e., static test with water renewals every 2 days), the comparison of the reproductive effects of a "fresh" vs. an "artificially aged" extracted NA mixture should be interpreted with caution.

Egg size has been identified as a sensitive reproductive endpoint in fathead minnow reproduction assays with complex mixtures (Weber et al. 2008; Driessnack et al. 2011; Rozon-Ramilo et al. 2011a; 2011b), and is a required endpoint in the Canadian Environmental Effects Monitoring (EEM) programs for metal mining and pulp and paper (Environment Canada, 2010; 2012b). Previous studies have shown that reduced egg size can affect the fitness of the resulting larvae (Einum and Fleming, 1999; 2000). Peters (1999) reported an inverse relationship between conductivity and naphthenic acid concentrations in aged OSPW and egg size in yellow perch (Appendix A). However, the present study is the first to examine egg size in fish exposed to naphthenic acid mixtures in isolation. No significant differences were observed in the egg size of fathead minnows in either of the commercial (Fluka) or oil sands extracted (OAF) naphthenic acid exposures.

5.4 Naphthenic Acid Effects on Fathead Minnow Embryo-Larval Endpoints

As reviewed in Section 2.8.4, only one previous study has examined the embryo-larval effects of a commercial NA mixture in fish (Table 2-11). Peters et al. (2007) compared the embryo-larval response of yellow perch and Japanese medaka exposed to serial dilutions of raw OSPW and a commercial sodium naphthenate solution (Tables 2-2 and 2-11). While a similar response (i.e., increased deformities and decreased hatch length) was observed following exposure to both the raw OSPW dilutions and the commercial NA mixture, the effects were more pronounced at lower in-water concentrations of the commercial naphthenic acids (Peters et al. 2007) (Tables 2-2 and 2-11).

Peters et al. (2007) attributed the increased toxicity of the commercial preparation to differences in the composition of naphthenic acids within the commercial mixture and raw

OSPW. However, as raw OSPW contains both neutral and acidic organic components, in addition to other contaminants, it is difficult to determine if the embryo-larval response to raw OSPW was solely due to naphthenic acids (i.e., acid extractable fraction of raw OSPW). The present study is the first to examine the embryo-larval development of fathead minnows exposed to a whole extracted NA mixture and to compare the embryo-toxicity of a commercial NA mixture and the organic acid fraction of raw OSPW in isolation. Similar to the results reported by Peters et al. (2007), the commercial mixture was more embryo-toxic than the oil sands extracted NA mixture.

Significantly decreased survival (Figure 4-3) and increased deformity rates (Figure 4-4) were observed in fathead minnow larvae exposed to the commercial (Fluka) mixture relative to the same nominal 5 mg/L concentration of the extracted (OAF) naphthenic acid mixture. These results are consistent with previous studies that have demonstrated significant toxicological differences between commercial and extracted NA mixtures (Nero et al. 2006b; Armstrong et al. 2008; Garcia-Garcia et al. 2011a; 2011b), and support the conclusion that commercial NA mixtures should not be used as a surrogate to estimate sub-lethal effects of oil sands naphthenic acids (Peters et al. 2007; West et al. 2011; Tollefsen et al. 2012). In contrast to the commercial NA mixture, no significant differences were observed relative to the control for any of the endpoints examined to assess the embryo-larval development of fathead minnows exposed to a nominal 5 mg/L concentration of an oil sands extracted NA mixture. These endpoints included: hatching success, time to hatch, larval survival, length, and deformities.

In the bioassays conducted by Peters et al. (2007), the threshold effect concentrations for increased deformity rates in yellow perch and Japanese medaka larvae exposed to a commercial NA mixture were 1.67 mg/L and 1.51 mg/L, respectively. The naphthenic acid concentrations

reported by Peters et al. (2007) were measured in the water (i.e., actual exposure concentrations) based on FTIR analysis. In the present study, the mean in-water naphthenic acid concentration measured over the 21 d exposure period in the nominal 5 mg/L Fluka treatment was 2.3 mg/L (based on HRMS analysis) (Table 4-2). As reviewed in Section 2.6, several studies have reported considerable variability in the measurement of total NA concentrations depending on the analytical method that is used. However, the significant increase in the deformity rate of fathead minnow larvae exposed to 2.3 mg/L of a commercial NA mixture, measured in the water using the HRMS analysis in the present study, is consistent with the calculated deformity threshold effect concentrations reported by Peters et al. (2007) (Figure 4-3).

In contrast to the decreased hatch length observed in both yellow perch and Japanese medaka in the commercial NA exposures conducted by Peters et al. (2007), no significant differences were observed in larval length (5 d post-hatch) in fathead minnows in either of the commercial (Fluka) or extracted (OAF) naphthenic acid exposures. As noted in Section 2.8.4, only one previous study has examined embryo-larval development in fish exposed to a whole extracted NA mixture (Farwell et al. 2006) (Table 2-10). Similar to the results observed in the present study, survival and hatch length were not significantly affected in Japanese medaka embryos exposed to a nominal 16 mg/L concentration of a “fresh” oil sands extracted NA mixture (Farwell et al. 2006). The lowest observable effect concentration (LOEC) for heart and cranial-skeletal deformities in Japanese medaka was 16 mg/L (Farwell et al. 2006) (Table 2-10).

In the cross-over egg experiment to examine embryo-larval response of fathead minnows in the presence and absence of parental exposure to commercial and extracted naphthenic acids, no significant differences were observed in the Student t-test comparisons between the treatment groups (Figures 3-4 and 4-7). In other words, the same response was observed regardless of

parental exposure. However, it is interesting to note that the deformity rates in fathead minnow larvae were considerably reduced in the absence of parental exposure in both Fluka treatments (Figure 4-7). As discussed in Section 2.9.2, naphthenic acids (or $C_{13}H_{22}O_2$ isomers) were detected in the eggs collected from adult rainbow trout exposed for 10 days to a nominal 3 mg/L concentration of a commercial NA mixture (Young et al. 2011). This may suggest that maternal transfer and/or increased naphthenic acid uptake during the water hardening stage contributed to the increased larval deformities observed in presence of parental exposure in fathead minnows exposed to the nominal 5 mg/L Fluka treatment in the present study (Figure 4-7).

While the results observed in this study clearly demonstrate significant toxicological differences between the commercial (Fluka) and the oil sands extracted (OAF) naphthenic acid mixtures, the reason or causative agent(s) for these differences is not known. Composition is often cited to explain to explain toxicity differences between various naphthenic acid sources (Nero et al. 2006b; Peters et al. 2007; Armstrong et al. 2008). As reviewed in Section 2.7, commercial NA mixtures have a lower molecular mass range and are much less complex relative to oil sands extracted naphthenic acids (Headley and McMartin, 2004) (Figure 2-4).

Relative to commercial preparations, naphthenic acids in raw OSPW contain a higher proportion of heteroatomic species, oxidized species, and higher molecular weight NAs (based on z series) (Grewer et al. 2010) (Tables 2-5 and 2-6). While aromatic NAs have been identified in both commercial and extracted mixtures (Rudzinski et al. 2002; Kavanagh et al. 2009), the steroidal aromatic or “estrogen-like” NAs have not been observed in commercial preparations (Rowland et al. 2011d; 2011e). Alkylphenol and hydrocarbon impurities have been identified in, and are suspected to contribute to the toxicity of commercial NA mixtures (West et al. 2011; Tollefsen et al. 2012). Therefore, it is possible that overall mixture composition (i.e., relative

proportions of different naphthenic acid structural classes or congeners), unidentified organic acids and/or impurities, as well as possible additive, synergistic and/or antagonistic effects between the various compound classes within these complex mixtures, contributed to the differences in the reproductive and embryo-larval responses observed in fathead minnows exposed to the commercial (Fluka) and oil sands extracted (OAF) mixtures used in this study.

CHAPTER 6: CONCLUSIONS

A variety of approaches have been used to investigate the role of naphthenic acids in the toxicity of raw OSPW within active settling basins on lease sites and aged or treated OSPW within various reclamation environments. While naphthenic acids have been implicated as the primary toxic component, a considerable amount of uncertainty still surrounds this conclusion. The science on how to accurately measure naphthenic acids is still emerging. Thus, we currently have a limited understanding of the link between naphthenic acid concentration and toxicity (i.e., at what concentration we can expect to see a response), as well as the potential factors that may modify or contribute to the toxicity of naphthenic acids in raw OSPW as a mixture (Li et al. 2014) and in aged or treated OSPW within different reclamation environments (e.g., increased salinity, alkalinity and pH) (van den Heuvel et al. 2012; Mahaffey and Dubé, In prep). Despite attempts to extract and/or simulate the composition of naturally degraded NA mixtures, toxicological differences have been reported in the comparison exposures with oil sands extracted NA mixtures and aged OSPW (Kavanagh et al. 2011; 2012; Leclair et al. 2013).

Mixture composition is often cited to explain differences in the toxicity of various naphthenic acid sources. However, there is also a significant amount of variability in the composition of naphthenic acid mixtures depending on the source, extraction procedure and analytical method used to measure them (Grewer et al. 2010; Zhao et al. 2012). The presence of yet to be identified naphthenic acid components, potential additive, synergistic and/or antagonistic effects between the various NA compound classes which have been identified, and the lack of a uniformly accepted methodology to allow both quantitative and qualitative comparisons between studies, further add to the uncertainty surrounding the reported effects of naphthenic acids (Garcia-Garcia et al. 2011b; Scarlett et al. 2012; Brown and Ulrich, 2015).

The cost and environmental risk associated with the storage and maintenance of large volumes of OSPW on lease sites presents significant challenges to both industry and regulators, and considerable concern for stakeholders. OSPW must eventually be returned to the environment and incorporated into mine closure landscapes. To improve monitoring efforts and establish a guideline that is relevant to oil sands naphthenic acids in preparation for future return scenarios, more information is needed to understand the composition of naphthenic acid components in raw OSPW and their role in determining OSPW toxicity. Understanding population-level responses to organic contaminants like naphthenic acid mixtures is critical to the ecological risk assessment process (Miller and Ankley, 2004). Impacts during early development and reproduction are the ultimate determinants of population viability and status (Ankley and Villeneuve, 2006). While several studies have reported significant differences in the composition and/or toxicity of commercial and extracted naphthenic acid mixtures, a direct comparison study to assess population-level responses in fish had not been done.

One of the goals of this research was to assess the effects of commercial (Fluka) and oil sands extracted (OAF) naphthenic acid mixtures at environmentally relevant exposure concentrations in water conditions comparable to the Athabasca River. Comparison of the pH, alkalinity and hardness levels measured throughout the 21 day exposure indicated they were within the range of the average levels recorded in the mouth reach of the Athabasca River from 1996 to 2006 (Squires et al. 2010) for all treatments including the "synthetic river water" control. Steady-state naphthenic acid concentrations were achieved in the flow-through system design for both Fluka treatments. In the Fluka treatments, the NA concentrations measured in the water were roughly half the nominal concentrations. Time and budget constraints prevented the

analysis of the in-water exposure concentrations in the OAF treatment. However, it is reasonable to assume that steady-state concentrations were achieved in the nominal 5 mg/L OAF treatment.

This research is the first to apply high resolution mass spectrometry to detect and estimate the uptake of naphthenic acids in fish muscle tissue. Analyses of the exposed fathead minnow muscle tissues identified the presence (or uptake) of one naphthenic acid congener detected as a peak in the Orbitrap mass spectra corresponding to m/z = 237.1857. Based on the m/z ratio, this peak corresponds to NA isomers containing 15 carbon atoms and 2 rings (n = 15, z = -4), fitting the classical naphthenic acid formula $C_{15}H_{26}O_2$. Although the NA tissue estimates are semi-quantitative, this is the first report of increased uptake with increasing exposure concentrations of a commercial NA mixture in fish. Gender-specific differences in uptake were not observed. Uptake estimates of naphthenic acids were higher in fathead minnows exposed to the commercial mixture relative to the extracted mixture. Whether this is due to a higher relative proportion of $C_{15}H_{26}O_2$ isomers in the Fluka mixture (compared to the oil sands extracted mixture), or if this is due to compositional differences between the commercial and extracted NA mixtures as a whole, is not known. The results obtained with the high resolution method developed for this thesis are consistent with previous studies that used the current GC-MS method to analyse naphthenic acids (or $C_{13}H_{22}O_2$ isomers) in biological tissues.

The reproductive and embryo-larval effects were more pronounced in fathead minnows exposed to the same nominal concentration (5 mg/L) of the commercial mixture relative to the oil sands extracted mixture. While a significant decrease in cumulative egg production was observed in the nominal 5 mg/L Fluka treatment, no significant differences were observed at the same nominal concentration of an oil sands extracted NA mixture. Significantly decreased larval survival and increased larval deformity rates were also observed in the 5 mg/L Fluka treatment.

No significant differences in embryo-larval development were observed in fathead minnows exposed to the same nominal concentration of an oil sands extracted NA mixture. This research is the first to examine uptake in relation to sublethal response in fish exposed to increasing concentrations of a commercial NA mixture. Increased uptake estimates of $C_{15}H_{26}O_2$ isomers in the muscle tissues of fathead minnows corresponded to a significant decrease in cumulative egg production.

The findings of this research are consistent with previous studies that have demonstrated significant toxicological differences between commercial and extracted NA mixtures and support the conclusion that commercial naphthenic acids should not be used as a surrogate to estimate sublethal effects of oil sands naphthenic acids. This study and others have shown that commercial naphthenic acids are more toxic than oil sands naphthenic acids (Nero et al. 2006b; Armstrong et al. 2008). As such, any recommendations for water quality guidelines and/or treatment targets for OSPW in future water return scenarios should not be based on commercial NA mixtures. The reason or causative agent(s) for the toxicological differences between commercial and extracted naphthenic acid mixtures are not known. It is possible that overall mixture composition (i.e., relative proportions of different naphthenic acid structural classes or congeners), unidentified organic acids and/or impurities, as well as possible additive, synergistic and/or antagonistic effects between the various compound classes within these complex mixtures, could have contributed to the differences in the uptake, reproductive and embryo-larval responses observed in fathead minnows exposed to the commercial (Fluka) and oil sands extracted (OAF) mixtures used in the present study.

6.1 Recommendations

The research performed in this thesis has highlighted several important issues that are relevant for improving monitoring efforts to assess the potential environmental impacts of oil sands mining operations, namely the development of a high resolution method to detect and estimate the uptake of naphthenic acids in fish muscle tissue, and providing further clarification on the toxicological differences between commercial and oil sands extracted NA mixtures. However, several challenges still exist with respect to the development of an oil sands specific naphthenic acid guideline and improving our understanding of the link between naphthenic acids and OSPW toxicity. These challenges and recommendations are provided below.

6.1.1 Guideline Development for Oil Sands Naphthenic Acids

As discussed previously, there are no water quality guidelines for the protection of aquatic life for naphthenic acids in Canada or the United States (AESRD, 2014; Golder, 2014). The significant difference between the nominal and in-water exposure concentrations of naphthenic acids observed in the present study and by others (Peters et al. 2007; Headley et al. 2010; Young et al. 2011), demonstrates the importance of measuring in-water concentrations in future toxicological assessments of oil sands naphthenic acids, and why it is a requirement for guideline development in Alberta (AEP, 1996). While this has been recommended in previous reviews (OSWRTWG, 1996; CEATAG, 1998), there is a considerable amount of variability in the measurement of total NA concentrations depending on the analytical method that is used (Grewer et al. 2010; Brown and Ulrich, 2015). Therefore, the application of a uniformly accepted quantitative methodology would be needed to allow cross-study comparisons of in-water NA concentrations and toxicological response in a variety of aquatic species, especially for the development of a water quality guideline specific to oil sands naphthenic acids.

The significant toxicological differences between commercial and extracted NA mixtures observed in the present study and by others (Nero et al. 2006b; Armstrong et al. 2008; Garcia-Garcia et al. 2011a; 2011b) further support the conclusion that commercial naphthenic acids should not be used as a toxicological surrogate for the assessment and development of oil sands specific naphthenic acid guideline. However, while the separation of commercial and oil sands naphthenic acids is clear, the conclusion reported in the 1998 CEATAG review has not changed. Although the number of toxicological assessments with oil sands extracted NA mixtures have increased since the 1998 review, due to the limited number of studies that meet Alberta's primary data standards, there is still insufficient information to recommend a water quality guideline for the protection of aquatic life for oil sands naphthenic acids (CEATAG, 1998).

In a more recent assessment, Golder (2014) reported there was insufficient data in the published literature to develop a chronic effects benchmark specific for oil sands naphthenic acids using the species sensitivity distribution approach. Therefore, the naphthenic acid benchmarks they proposed were based on the most sensitive individual endpoints documented in the toxicological assessments they reviewed (Tables 2-10 and 2-11). As previous biodegradation studies have shown, commercial NA mixtures contain a higher proportion of labile (easily degraded) naphthenic acids relative to the organic acids in raw OSPW (Scott et al. 2005; Han et al. 2008). Golder (2014) proposed separate benchmarks for the labile and refractory fractions of the total naphthenic acid mixture. However, it is important to note that neither the labile or refractory fractions of oil sands extracted NAs have been separated or assessed for toxicity in the peer-reviewed literature (Table 2-10).

To derive a chronic effects benchmark for the labile fraction of the total naphthenic acid mixture, Golder (2014) conservatively assigned the gill anomalies reported by Nero et al.

(2006b) to be ecologically relevant developmental effects, and used the nominal 0.9 mg/L concentration as the lowest threshold concentration reported in the literature for a total naphthenic acid mixture that contained a high proportion of labile NAs (Table 2-11). However, because this concentration was based on a commercial mixture, rather than the extracted NA mixture which had a higher nominal threshold of 1.7 mg/L (Nero et al. 2006b), it could be considered overly conservative. Therefore, to account for this uncertainty, Golder (2014) rounded the nominal 0.9 mg/L concentration reported for the commercial NA mixture to 1 mg/L to provide a conservative threshold for the labile (more toxic) fraction of the naphthenic acid mixture in raw OSPW.

To derive a chronic effects benchmark for refractory naphthenic acids, Golder (2014) proposed an approximate threshold of 19 mg/L (based on FTIR analysis of NAs in aged OSPW). This value was chosen to be representative of refractory naphthenic acids because it was below the no-effect level of 25 mg/L reported in the fathead minnow reproduction assays with aged OSPW (Kavanagh et al. 2011), and below the effects level of 24 mg/L reported in the histopathological assessments of gill and liver tissues in yellow perch exposed to aged OSPW (Nero et al. 2006a) (Appendix A). However, in situations where the relative proportions of labile and refractory naphthenic acids are not known, the lower chronic effects benchmark of 1 mg/L for the labile naphthenic acids was recommended for total naphthenic acid concentrations (Golder, 2014).

While the approach used by Golder (2014) was due to the limited number of toxicological assessments with oil sands extracted NA mixtures at the time of their review (Table 2-10), it does illustrate an important consideration for the development of a water quality guideline specific for oil sands naphthenic acids. That is, whether the guideline should be based

on the composition of naphthenic acids in raw OSPW (i.e., “fresh” extracted NA mixtures), or aged OSPW (i.e., “aged” extracted NA mixtures). Some researchers may argue that a future guideline should be based on naturally degraded naphthenic acid mixtures found in aged OSPW which have been shown to contain a larger proportion of oxidized NAs (Han et al. 2009). However, recent studies have demonstrated the limitations of bulk extraction methods to efficiently extract oxidized NAs from aged OSPW (MacDonald et al. 2013; Leclair et al. 2013) (Table 2-9). Recent studies have also shown there is a considerable amount of variability in composition of naturally degraded naphthenic acid mixtures depending on the reclamation water source and the analytical method used to measure them (Leclair et al. 2013; van den Heuvel et al. 2014) (Table 2-9). How, or if, these compositional differences influence the sublethal toxicity reported in different wet landscape reclamation strategies, is not known (Table 2-1) (Appendix A).

It could also be argued that establishing a guideline based on the composition of naphthenic acids extracted from raw OSPW (i.e., “fresh” extracted NA mixtures) would be more desirable as it would likely be more conservative. However, based on the comparison of the exposures conducted by Kavanagh et al. (2012) and the present study, there do not appear to be any considerable differences in the reproductive and embryo-larval response of fathead minnow exposed to the same nominal (5 mg/L) concentration of a “fresh” vs. an “artificially aged” oil sands extracted NA mixture. Conversely, there were significant differences in the acute lethality observed in young-of-the-year yellow perch exposed to a “fresh” extracted NA mixture in the study conducted by Nero et al. (2006b). While no significant differences in mortality were observed in adult fathead minnows in the present study, Nero et al. (2006b) reported 100% mortality within 96 hours of exposure to a nominal concentration of 6.8 mg/L. Similar to the

challenges faced with most cross-study comparisons of oil sands extracted naphthenic acids (Table 2-10), whether these differences are due to differences in species, age, source, extraction procedure or analytical measurement is not known.

As discussed throughout this thesis, the link between OSPW toxicity and naphthenic acids (i.e., the acidic fraction of OSPW) has not been clearly established (OSWRTWG, 1996; CEATAG, 1998; Garcia-Garcia et al. 2011b; Li et al. 2014). Despite significant advancements in the compositional analysis of naphthenic acid mixtures, the primary toxic component(s) within the acidic fraction of raw OSPW has yet to be determined (Headley et al. 2011c; Rowland et al. 2011a-e; Scarlett et al. 2012; 2013). The uncertainties associated with the measurement and compositional variability of these complex mixtures, add weight to a third argument. That is, whether we should consider further pursuit in the development of a guideline specific to oil sands naphthenic acids. The whole effluent toxicity technique allows for evaluation and management of discharge waters where residual toxicity in the discharge cannot be clearly correlated with the presence of specific chemicals. Thus the management of naphthenic acids, like other substances for which in-stream ambient water quality guidelines do not exist, could be achieved through the whole effluent toxicity approach (CEATAG, 1998).

This was also recommended by the Oil Sands Water Release Technical Working Group (OSWRTWG) in 1996. The OSWRTWG carefully considered the background information on naphthenic acids and evidence for the linkage of this group to toxicity. They concluded that although NAs appear to be the major source of potential toxicity, the data suggest a weak correlation between the level of naphthenic acids and the degree of toxicity (OSWRTWG, 1996). As such, the whole effluent toxicity approach was recommended as it eliminates the need to

explicitly define substances and their potential effects, and addresses the issue of potential interactive and synergistic effects of multiple chemicals in an effluent (OSWRTWG, 1996).

However, as discussed in the 1996 review, understanding water quality from both a chemical and toxicological perspective would be required for all potential reclamation and operational oil sands water releases (OSWRTWG, 1996). In a recent synthesis of the toxic components within OSPW, Li et al. (2014) reported that compared to NAs, there is limited data available on the concentrations of other organics (e.g., PAHs, BTEX, phenols) that may contribute to the observed organic toxicity of OSPW. Dissolved ions and trace metals were also identified as possible constituents that may contribute to and/or affect the overall toxicity of OSPW (Li et al. 2014).

Li et al. (2014) concluded that OSPW is an extremely complex mixture and more research is needed to evaluate the potential additive, synergistic and/or antagonistic effects caused by its many different constituents. A similar conclusion was determined in the literature review conducted for this thesis on the composition and toxicity of OSPW. Mahaffey and Dubé (In prep) found that only ten of the 342 articles (2.9%) reviewed over the period from 1975 to 2013 included chemical analysis of raw OSPW concurrent with standard acute and chronic bioassay assessments of fish and invertebrate toxicity. Therefore, while the whole effluent toxicity approach could be used for the management of naphthenic acids in the absence of a guideline, more information is needed to improve our understanding of the whole effluent (i.e., the composition and toxicity of raw OSPW) (OSWRTWG, 1996; CEATAG, 1998; Li et al. 2014; Mahaffey and Dubé, In prep).

6.1.2 Future Research

Given the discussion provided above, recommendations for future research include:

i. Whole effluent toxicity approach for naphthenic acids in future water return scenarios.
ii. Toxicity Identification Evaluation on raw OSPW (conducted according to accredited laboratory standards that included a concentration and compositional analyses of naphthenic acids using more than one analytical method).
iii. Ensure Alberta Environmental Protection protocol is followed in future toxicological assessments with raw OSPW and oil sands extracted NA mixtures.
iv. Development and application of a standardized method to measure naphthenic acid concentrations (both quantitatively and compositionally).
v. Development and application of a standardized bulk extraction method to isolate naphthenic acids (i.e., the acidic organic fraction) from raw OSPW.
vi. Dose response studies with raw OSPW and extracted NA mixtures to determine LOEC.
vii. The use of Athabasca River water or synthetic river water in future exposures with raw OSPW and extracted NA mixtures.
viii. Inclusion of neutral organic component to examine potential role in raw OSPW toxicity.

6.1.3 Improvements to Experimental Design and Methodology

Recommendations for improvements to the experimental design and methods used in this thesis include:

i. Increased sample size (i.e., additional replicates or breeding pairs of fathead minnows) to help reduce the variability around the mean and increase the power to detect a significant difference among treatments.

Previous research has recommended a sample size between 8 and 16 for fathead minnow reproduction assays with complex mixtures (Rozon-Ramilo, 2011). The level of replication used in this research was limited due to the available quantity of the naphthenic acid extract. Thus, future experiments should ensure adequate quantities of available extract.

ii. Measurement of the in-water naphthenic acid concentrations in the OAF treatment.

iii. Inclusion of additional reproductive endpoints such as plasma steroid hormones, vitellogenin, and number of tubercles in males.

iv. Inclusion of a detailed compositional analysis to examine the relative proportion of individual NA congeners within the commercial (Fluka) and extracted (OAF) naphthenic acid mixtures.

v. Higher spiking concentrations in the spiked tissue trials may allow for the high resolution detection of the n = 15, z = -4 NA congener (or $C_{15}H_{26}O_2$ isomers) in the non-exposed muscle tissue.

vi. Inclusion of Liquid Chromatography may also improve the sensitivity and ability to detect the $C_{15}H_{26}O_2$ isomers rather than using loop injection with no chromatographic separation.

vii. Increased replication (i.e., more fish) would increase the amount of tissue and possibly allow for the analysis of uptake and distribution in target organs (e.g., livers, gonads, gills, etc.).

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APPPENDIX A

Table A1. Study summaries of Consolidated tailings (CT) release water effects on fish. The results are summarized according to the following notations: (NSD) no significant difference relative to control, (−) significant decrease relative to control, (+) significant increase relative to control. If data were provided, the magnitude of change was calculated: (exposure - control / control) *100%

Reference	Consolidated tailings (CT) release water	Species	Duration	Endpoint & Result
Marr et al. 1996	CT release water	Rainbow trout	Acute: 96h	Survival (LC50: 60%)
MacKinnon et al. 2001	CT release water (aged 1 month)	Rainbow trout	Acute: 96h	Survival (LC50: 71%; estimated from graph presented in article)

Table A2. Study summaries of wet landscape reclamation pond effects on fish

Reference	Aged CT release water	Species	Duration	Endpoint & Result
MacKinnon et al. 2001	CT release water (aged 1 year)	Rainbow trout	Acute: 96h	Survival (100%); (LC50: 100%)
MacKinnon et al. 2001	CT release water (aged 1 year)	Fathead minnow	Acute: 96h	Survival (100%); (LC50: 100%)

Table A3. Study summaries of Consolidated tailings (CT) wetlands effects on fish

Reference	Consolidated tailings (CT) wetlands	Species	Duration	Endpoint & Result
Farrell et al. 2004	Constructed wetland channel receiving CT	Fathead minnow	Acute: 96h (Field; cage; 1996)	Survival (100%); Blood hematology: Hematocrit (+ 36.5 %); Leucocrit (−50.6 %); Lymphocytes (−59.7 %); Thrombocytes (+ 79.8 %); Neutrophils (+ 929 %); Gill histological index (NSD)
Farrell et al. 2004	Constructed wetland channel receiving CT	Fathead minnow	Chronic: 28d (Field; cage; 1996)	Survival (0%); Reduced survival in control channel (67%). Authors concluded fish could be caged up to 14 d without untoward effects in control fish; further studies needed to determine cause

Table A4. Study summaries of Seepage collection water effects on fish

Reference	Dyke drainage & seepage collection	Species	Duration	Endpoint & Result
Lake, 1976	Dyke filter drainage (DD)	Rainbow trout	Acute: 96h (Serial Dilution)	Survival (LC50: 3.45 - 9.4%); Time to 100% mortality: 20% DD (24 - 36 hrs); 40% DD (12 - 24 hrs); 60% DD (5 - 8 hrs); 80% DD (2.3 - 4 hrs); 100% DD (1.5 - 2 hrs)

Table A4 (continued)

Reference	Dyke drainage & seepage collection	Species	Duration	Endpoint & Result
Lake, 1976	Dyke filter drainage (DD)	Brook stickleback	Acute: 96h 100% DD	Survival (0%); Time to 100% mortality (1.95 - 2.9 hrs)
Nix & Bishay, 1996	Dyke seepage (wetland channel)	Rainbow trout	Acute: 96h	Survival: In-flow (1994 LT50: 2 hrs); Out-flow ("wetland treated" dyke seepage) (1994 LT50: 16-39 hrs); In-flow (1995 LC50: 42%); Out-flow (1995 LC50 > 100%). Note: 1995 study incorporated a pond component into the wetland channel treatment system
Nix & Bishay, 1996	Dyke drainage pond	Rainbow trout	Acute: 96h	Survival (LC50: 45 - 71%)
Nix & Bishay, 1996	Dyke seepage (wetland channel)	Fathead minnow	Chronic: ELS[a] (Field; in situ; 1994)	Larval survival: In-flow (0%); Out-flow ("wetland treated" dyke seepage) (6%). Note: low survival in control wetland channels (43%)
Farrell et al. 2004	Dyke seepage pond	Fathead minnow	Acute: 96h (Field; cage; 1996)	Survival (100%); Blood hematology: Hematocrit (+ 38.8%); Leucocrit (−50.6%); Lymphocytes (−51.1%); Thrombocytes (+ 109%)Neutrophils (NSD); Gill histological index (epithelial lifting & thickening): Overall score (−51.8 %); 1997 Critical swim speed (−8.2%)
Farrell et al. 2004	Dyke seepage pond	Fathead minnow	Chronic: 12d (Field; cage; 1996)	Survival (60%); 1997 Critical swim speed (NSD)
Farrell et al. 2004	Dyke seepage pond	Fathead minnow	Chronic: 28d (Field; cage; 1996)	Survival (0%). Reduced survival in control channel (67%). Authors concluded fish could be caged up to 14 d without untoward effects in control fish; further studies needed to determine cause
Toor et al. 2013 a	Sand dyke drainage	Rainbow trout	Acute: 96h	Survival (LC50: 67%)

Table A5. Study summaries of Natural wetland effects on fish

Reference	Natural wetland	Species	Duration	Endpoint & Result
Toor et al. 2013 a	Natural wetland	Rainbow trout	Acute: 96h	Survival (LC50 > 100%)

Table A6. Study summaries of wetlands created from saline overburden and lean oil sands material effects on fish

Reference	Saline overburden & lean oil sands	Species	Duration	Endpoint & Result
Peters, 1999 MSc	South Bison	Yellow perch	Chronic: ELS[a] (Lab)	Fertilization success (NSD); Embryo mortality (NSD); Egg size (− 7.1%); Larval hatch length (−)
Peters, 1999 MSc	South Bison	Yellow perch	Chronic: ELS[a] (Field; in situ)	Fertilization success (NSD); Embryo mortality (NSD); Egg size (− 4.7%); No larvae were found (netted) in South Bison for field comparison
van den Heuvel et al. 1999 a	South Bison	Yellow perch	Chronic: 5 months (Field Capture; 1995)	Female CF[b] (+ 6.7%); Female LSI[c] (+ 44.6%); Female GSI[d] (NSD); Fecundity index (NSD); Male CF[b] (NSD); Male LSI[c] (+ 48.1%); Male GSI[d] (NSD)
van den Heuvel et al. 1999 a	South Bison	Yellow perch	Chronic: 5 months (Field Capture; 1996)	Female CF[b] (+ 9.4 %); Female LSI[c] (+ 28.2%); Female GSI[d] (+); Fecundity index (+); Male CF[b] (NSD); Male LSI[c] (+ 9.6%); Male GSI[d] (+)
van den Heuvel et al. 1999 a	South Bison	Yellow perch	Chronic: 11 months (Field Capture; 1997)	Female CF[b] (NSD); Female LSI[c] (+ 77.2%); Female GSI[d] (+); Fecundity index (+); Male CF[b] (NSD); Male LSI[c] (+ 54.1%); Male GSI[d] (+)
van den Heuvel et al. 1999 a	South Bison	Yellow perch	Chronic: 5-11 months (Field; 1995-1997)	Summary: Catch per unit effort (CPUE): (NSD) 2-3 mos post-stocking; (-) 11 mos post-stocking *after* collection for sampling (NSD; 2 of 3 sample efforts). CF[b], LSI[c] & GSI[d] for both sexes fell within or exceeded the natural range of variability found in the off-site reference lakes
van den Heuvel et al. 1999 b	South Bison	Yellow perch	Chronic: 5-11 months (Field; 1995-1997)	Hepatic MFO/EROD (+) in males & females compared to Mildred Lake & off-site reference lakes; PAH metabolites (+) compared to both Mildred Lake & off-site reference lakes; Male & female plasma hormones (−) compared to both Mildred Lake & off-site reference lakes after 11 months; Female hepatic GST & UDPGT enzyme activity (NSD)
van den Heuvel et al. 1999 b	South Bison	Yellow perch	Chronic: 5-11 months (Field; 1995-1997)	Summary: Hepatic MFO/EROD & PAH metabolites followed gradient of oil sands influence: highest in experimental ponds, lowest in off-site reference lakes. No relationship between exposure indicators & physiological indices. No relationship between steroid hormones & exposure to oil sands related compounds or gonad size indices over 2 year study period

Table A6 (continued)

Reference	Saline overburden & lean oil sands	Species	Duration	Endpoint & Result
van den Heuvel et al. 2000	South Bison	Yellow perch	Chronic: 3-10 months (Field; 1995-1997)	Increase in percent occurrence of viral-induced tumors, caudal fin erosion & gill histopathology indices (aneurysms, chloride & epithelial cell proliferation) compared to Mildred Lake control; Female plasma ion concentrations (NSD)
Siwik et al. 2000	South Bison	Fathead minnow	Chronic: 7d ELS[a] (Lab)	Larval survival (NSD); Larval mass (NSD)
McNeill et al. 2012	South Bison	Rainbow trout (female strain)	Chronic: 21d (Field; cage; 2010)	Survival (97%); CF[b] (NSD); LSI[c] (NSD); SSI[j] (NSD); Fin erosion index (NSD); Bile Phenanthrene metabolites (NSD); Hepatic MFO/EROD (+ 2-fold); Mean total leukocytes (NSD); Differential blood leukocyte count: Thrombocytes (+); Lymphocytes, Granulocytes & Monocytes (NSD); *A. salmonicida* antibody production (NSD)
van den Heuvel et al. 2012	South Bison	Yellow perch	Chronic: 5 months (Field Capture; 2009)	Year comparison (1996 vs 2009): Female CF[b] (+ 8.8%); Male CF[b] (NSD); Female LSI[c] (NSD); Male LSI[c] (−11.2%); Female GSI[d] (−); Male GSI[d] (NSD, exceeded estimated regional norms); Fecundity index (+ 30.1%)
van den Heuvel et al. 2012	South Bison	Yellow perch	Chronic: 5 months (Field Capture; 2009)	Site comparison: Female plasma estradiol 1996: (−) 1 of 2 reference lakes, NSD Mildred Lake; 2009: (NSD) 2 of 2 reference lakes; Female plasma testosterone 1996: (NSD) all sites; 2009: (+) 2 of 2 reference lakes; Male plasma testosterone & 11-ketotestosterone 1996: (NSD) all sites; 2009: (NSD) all sites

Table A7. Study summaries of wet landscape reclamation pond effects on fish

Reference	Mature fine tailings & fresh water	Species	Duration	Endpoint & Result
Peters, 1999 MSc	Demonstration Pond & Pond 3	Yellow perch	Chronic: ELS[a] (Lab)	Fertilization success (NSD); Embryo mortality (NSD); Egg size (− 3.5%); Larval hatch length (−)
Peters, 1999 MSc	Demonstration Pond	Yellow perch	Chronic: ELS[a] (Field; in situ)	Fertilization success (NSD); Embryo mortality (NSD); Egg size (+ 6.6%); Larval mass (− 32.3%); Larval length (− 8.7%)

Table A7 (continued)

Reference	Mature fine tailings & fresh water	Species	Duration	Endpoint & Result
van den Heuvel et al. 1999 a	Demonstration Pond	Yellow perch	Chronic: 5 months (Field Capture; 1995)	Female CF[b] (+ 9.2%); Female LSI[c] (+ 60%); Female GSI[d] (NSD); Fecundity index (NSD); Male CF[b] (NSD); Male LSI[c] (+ 30.9%); Male GSI[d] (NSD)
van den Heuvel et al. 1999 a	Demonstration Pond	Yellow perch	Chronic: 5 months (Field Capture; 1996)	Female CF[b] (NSD); Female LSI[c] (+ 34.3%); Female GSI[d] (+); Fecundity index (NSD); Male CF[b] (NSD); Male LSI[c] (+ 50%); Male GSI[d] (+)
van den Heuvel et al. 1999 a	Demonstration Pond	Yellow perch	Chronic: 11 months (Field Capture; 1997)	Female CF[b] (NSD); Female LSI[c] (+ 82%); Female GSI[d] (+); Fecundity index (+); Male CF[b] (NSD); Male LSI[c] (+ 75.6%); Male GSI[d] (+)
van den Heuvel et al. 1999 a	Demonstration Pond	Yellow perch	Chronic: 5-11 months (Field; 1995-1997)	Summary: Catch per unit effort (CUPE): (−) 2-3 mos post-stocking; (−) 11 mos post-stocking *after* collection for sampling (−; 3 of 3 sample efforts). CF[b], LSI[c] & GSI[d] for both sexes fell within or exceeded the natural range of variability found in the off-site reference lakes
van den Heuvel et al. 1999 b	Demonstration Pond	Yellow perch	Chronic: 5-11 months (Field; 1995-1997)	Hepatic MFO/EROD: (+) in males & females compared to Mildred Lake & off-site reference lakes); PAH metabolites: (+) compared to off-site reference lakes only); Male & female plasma hormones: (−) compared to both Mildred Lake & off-site reference lakes after 11 months); Female hepatic GST & UDPGT enzyme activity (NSD)
van den Heuvel et al. 1999 b	Demonstration Pond	Yellow perch	Chronic: 5-11 months (Field; 1995-1997)	Summary: Hepatic MFO/EROD & PAH metabolites followed gradient of oil sands influence: highest in experimental ponds, lowest in off-site reference lakes. No relationship between exposure indicators & physiological indices. No relationship between steroid hormones & exposure to oil sands related compounds or gonad size indices over 2 year study period
van den Heuvel et al. 2000	Demonstration Pond	Yellow perch	Chronic: 3-10 months (Field; 1995-1997)	Increase in percent occurrence of viral-induced tumors, caudal fin erosion & gill histopathology indices (aneurysms, chloride & epithelial cell proliferation) compared to Mildred Lake control; Female plasma ion concentrations (NSD)
Siwik et al. 2000	Demonstration Pond & Pond 3	Fathead minnow	Chronic: 7d & 56d ELS[a] (Lab)	Larval survival (NSD); Larval mass (NSD); Larval length (NSD). Control survival in 56d bioassay varied; results treated with caution

Table A7 (continued)

Reference	Mature fine tailings & fresh water	Species	Duration	Endpoint & Result
Siwik et al. 2000	Demonstration Pond	Fathead minnow	Chronic: 21d ELS[a] (Field; cage; 1997)	Larval survival (NSD); Larval mass (−)
Nero et al. 2006 a	Pond 3	Yellow perch	Chronic: 22d (Field Capture; 2001)	Survival (NSD); Male & Female CF[b] (NSD); Male & Female LSI[c] (NSD); Liver histopathology indices (NSD); Gill histopathology indices (NSD); Gill morphometric indices: SLL[e] (−22.3%); SLW[f] (+ 23%); ID[g] (NSD); BET[h] (NSD); PAGE[i] (NSD)
Nero et al. 2006 a	Pond 3	Goldfish	Chronic: 19d (Field; cage; 2001)	Survival (NSD); Male & Female CF[b] (NSD); Male & Female LSI[c] (NSD); Liver histopathology indices (NSD); Gill histopathology indices (NSD); Gill morphometric indices: SLL[e] (NSD); SLW[f] (NSD); ID[g] (−41.1%); BET[h] (NSD); PAGE[i] (NSD)
Lister et al. 2008	Pond 3	Goldfish	Chronic: 19d (Field; cage; 2001)	Survival (NSD); Male & Female LSI[c] (NSD); Male & Female GSI[d] (NSD); Male & Female Plasma estradiol & testosterone (−); Male & Female *In vitro* basal & stimulated testosterone (NSD); Male Plasma cortisol (+ 49%)
Lister et al. 2008	Pond 3	Goldfish	Chronic: 19d (Field; cage; 2003)	Survival (NSD); Male & Female LSI[c] (NSD); Male & Female SSI[j] (NSD); Male & Female GSI[d] (NSD); Male & Female Plasma estradiol & testosterone (NSD); Male Plasma 11-ketotestosterone (NSD)
Kavanagh et al. 2011	Demonstration Pond	Fathead minnow	Chronic: 21d reproduction (Lab)	Survival (NSD); Male & Female CF[b] (NSD); Male & Female LSI[c] (NSD); Male & Female GSI[d] (NSD); Number of tubercles (NSD); Mean fecundity rate (NSD); Mean number of spawns (NSD)
Kavanagh et al. 2011	Demonstration Pond	Fathead minnow	Chronic: ELS[a] (Lab)	Fertilization success (NSD); Hatch success (NSD); Larval survival (NSD)
McNeill et al. 2012	Demonstration Pond	Rainbow trout (female strain)	Chronic: 21d (Field; cage; 2010)	Survival (86%); CF[b] (NSD); LSI[c] (NSD); SSI[j] (−18.3%); Fin erosion index (+ 150%); Bile Phenanthrene metabolites (+ 6-fold); Hepatic MFO/EROD (+ 3-fold); Mean total leukocytes (−); Differential blood leukocyte count: Lymphocytes (−); Granulocytes (+); Thrombocytes & Monocytes (NSD); *A. salmonicida* antibody production (−60.7%)

Table A7 (continued)

Reference	Mature fine tailings & fresh water	Species	Duration	Endpoint & Result
van den Heuvel et al. 2012	Demonstration Pond	Yellow Perch	Chronic: 5 months (Field Capture; 2009)	Year comparison (1996 vs 2009): Male & Female CF[b] (NSD); Female LSI[c] (NSD); Male LSI[c] (−44.2%); Male GSI[d] (−) below estimated regional norm; Female GSI[d] (−); Fecundity index (NSD)
van den Heuvel et al. 2012	Demonstration Pond	Yellow Perch	Chronic: 5 months (Field Capture; 2009)	Site comparison: Female plasma estradiol 1996: (−) 1 of 2 reference lakes, NSD Mildred Lake; 2009: (NSD) 2 of 2 reference lakes; Female plasma testosterone 1996: (NSD) all sites; 2009 (NSD) 2 of 2 reference lakes; Male plasma testosterone & 11-ketotestosterone 1996: (NSD) all sites; 2009 (−) all sites
Kavanagh et al. 2013	Demonstration Pond	Fathead minnow	Chronic: Field Survey (2006-2008)	Female CF[b] (+ ; 4 of 5 sample periods); Male CF[b] (+ ; 3 of 5 sample periods); Female LSI[c] (+ ; 4 of 5 sample periods); Male LSI[c] (+ ; 2 of 5 sample periods); Male & Female SSI[j] (− ; all sample periods: 10 observations); Note: reference fish had enlarged spleens with white nodules throughout
Kavanagh et al. 2013	Demonstration Pond	Fathead minnow	Chronic: Field Survey (2006-2008)	Female GSI[d] (+ ; 3 of 5 sample periods); Male GSI[d] (+ ; 4 of 5 sample periods); Number of tubercles: start of spawning season (−); end of spawning season (+); Female plasma estradiol & testosterone (NSD); Male plasma testosterone (NSD); Male plasma 11-ketotestosterone (−); same response 2 sampling periods, 2 reference sites
Kavanagh et al. 2013	Demonstration Pond	Fathead minnow	Chronic: Field Survey (2006-2008)	Opercula (gill cavity) deformities (all fish collected); Gill histopathology (increased proliferative & degenerative alterations); Presence of protozoan parasites in gill filaments (*Trichodina spp*. observed all sites including reference; Black spot (trematodes) & tapeworms observed reference sites only)
Leclair et al. 2013	Demonstration Pond	Rainbow trout	Chronic: 7d (Lab)	Survival (95%); CF[b] (NSD); LSI[c] (NSD); SSI[j] (− 30.7%); Hepatic MFO/EROD (NSD); Phenanthrene bile metabolites: (+); Benzo[a]pyrene bile metabolites (+); *A.salmonicida* antibody production (−49%)

Table A7 (continued)

Reference	Mature fine tailings & fresh water	Species	Duration	Endpoint & Result
Leclair et al. 2013	Demonstration Pond	Rainbow trout	Chronic: 7d (Lab)	Total blood erythrocyte count (NSD); Differential leukocyte count (Blood): B-lymphocytes (−48%); T-lymphocytes (−55%); Thrombocytes & Myeloid cells (NSD); Differential leukocyte count (Spleen): B-lymphocytes (−83%); Myeloid cells (−46%); Thrombocytes & T-lymphocytes (NSD); Differential leukocyte count (Head kidney): B-lymphocytes (+ 57%); T-lymphocytes, Myeloid cells & Thrombocytes (NSD); Differential leukocyte count (Gills): (NSD)

Table A8. Study summaries of wet landscape reclamation pond effects on fish

Reference	Mature fine tailings & OSPW	Species	Duration	Endpoint & Result
Peters, 1999 MSc	Pond 5	Yellow perch	Chronic: ELS[a] (Lab)	Fertilization success (NSD); Embryo mortality (NSD); Egg size (− 7.1%); Larval hatch length (NSD)
Peters, 1999 MSc	Pond 10	Yellow perch	Chronic: ELS[a] (Lab)	Fertilization success (NSD); Embryo mortality (NSD); Egg size (− 8.9%); Larval hatch length (−)
Siwik et al. 2000	Pond 5	Fathead minnow	Chronic: 7d & 56d ELS[a] (Lab)	Larval survival (NSD); Larval mass (NSD); Larval length (+ at 7d; NSD at 28d & 56d). Note: control survival in 56d bioassay varied; results treated with caution
Siwik et al. 2000	Pond 10	Fathead minnow	Chronic: 7d ELS[a] (Lab)	Larval survival (−); Larval mass (NSD)
Siwik et al. 2000	Pond 10	Fathead minnow	Chronic: 21d ELS[a] (Field; cage; 1997)	Larval survival (NSD); Larval mass (NSD)
Nero et al. 2006 a	Pond 5	Yellow Perch	Chronic: 22d (Field Capture; 2001)	Survival (NSD); Male & Female CF[b] (NSD); Male & Female LSI[c] (NSD); Liver histopathology indices: Total index, degenerative & inflammatory alterations (+); Gill histopathology indices: Total index, proliferative alterations (+); Gill morphometric indices: SLL[e] (−33%); SLW[f] (+ 53.8%); ID[g] (−37.9%); BET[h] (NSD); PAGE[i] (−12.9%)

Table A8 (continued)

Reference	Mature fine tailings & OSPW	Species	Duration	Endpoint & Result
Nero et al. 2006 a	Pond 5	Goldfish	Chronic: 19d (Field; cage; 2001)	Survival (NSD); Male & Female CF[b] (NSD); Male & Female LSI[c] (NSD); Liver histopathology indices: Total index, degenerative & cytoplasmic alterations (+); Gill histopathology indices: Total index, degenerative & inflammatory alterations (+); Gill morphometric indices: SLL[e] (NSD); SLW[f] (NSD); ID[g] (−41.1%); BET[h] (NSD); PAGE[i] (−32.6%)
Lister et al. 2008	Pond 5	Goldfish	Chronic: 19d (Field; cage; 2001)	Survival (NSD); Male & Female LSI[c] (NSD); Male & Female GSI[d] (NSD); Male & Female Plasma estradiol & testosterone (−); Male & Female *In vitro* basal testosterone (−); Male & Female *In vitro* stimulated testosterone (NSD); Male Plasma cortisol (+ 49%)
Lister et al. 2008	Pond 5	Goldfish	Chronic: 19d (Field; cage; 2003)	Survival (NSD); Male & Female LSI[c] (NSD); Female SSI[j] (NSD); Male SSI[j] (+); Female GSI[d] (−53.3%); Male GSI[d] (NSD); Female Plasma estradiol (−); Male Plasma estradiol (NSD); Male & Female Plasma testosterone (NSD); Male Plasma 11-ketotestosterone (NSD)
Kavanagh et al. 2011	Pond 5	Fathead minnow	Chronic: 21d reproduction (Lab)	Survival (NSD); Female mass (NSD); Male mass (−27.7%); Male & Female CF[b] (NSD); Male & Female LSI[c] (NSD); Male & Female SSI[j] (NSD); Male & Female GSI[d] (NSD); Number of tubercles (NSD); Mean fecundity rate (NSD); Mean number of spawns (NSD); Female plasma estradiol & testosterone (NSD); Male plasma testosterone & 11-ketotestosterone (NSD)
Kavanagh et al. 2011	Pond 5	Fathead minnow	Chronic: ELS[a] (Lab)	Fertilization success (NSD); Hatch success (NSD); Larval survival (NSD)
Kavanagh et al. 2011 (1 mos. salt-acclimation)	Mature Fine Tailings North (MFTN)	Fathead minnow	Chronic: 21d reproduction (Lab)	Survival (NSD); Male & Female CF[b] (NSD); Male & Female LSI[c] (NSD); Male & Female GSI[d] (NSD); Number of tubercles (NSD); Mean fecundity rate (−77.5%); Mean number of spawns (−68.4%); Female plasma estradiol & testosterone (NSD); Male plasma testosterone & 11-ketotestosterone (−)
Kavanagh et al. 2011 (1 mos. salt-acclimation)	Mature Fine Tailings North (MFTN)	Fathead minnow	Chronic: ELS[a] (Lab)	Fertilization success (NSD); Hatch success (NSD); Larval survival (NSD)

Table A8 (continued)

Reference	Mature fine tailings & OSPW	Species	Duration	Endpoint & Result
Kavanagh et al. 2011 (1 mos. salt-acclimation)	Mature Fine Tailings South (MFTS)	Fathead minnow	Chronic: 21d reproduction (Lab)	Survival (NSD); Male & Female CF[b] (NSD); Male & Female LSI[c] (NSD); Male & Female GSI[d] (NSD); Number of tubercles (NSD); Mean fecundity rate (−57.3%); Mean number of spawns (−50%); Female plasma estradiol & testosterone (NSD); Male plasma testosterone (NSD); Male plasma 11-ketotestosterone (−)
Kavanagh et al. 2011 (1 mos. salt-acclimation)	Mature Fine Tailings South (MFTS)	Fathead minnow	Chronic: ELS[a] (Lab)	Fertilization success (NSD); Hatch success (NSD); Larval survival (NSD)
Kavanagh et al. 2011 (1 mos. salt-acclimation)	50% MFTS (50% Gregorie Lake)	Fathead minnow	Chronic: 21d reproduction (Lab)	Survival (NSD); Male & Female CF[b] (NSD); Male & Female LSI[c] (NSD); Male & Female GSI[d] (NSD); Number of tubercles (NSD); Mean fecundity rate (NSD); Mean number of spawns (NSD); Female plasma estradiol & testosterone (NSD); Male plasma testosterone & 11-ketotestosterone (NSD)
Kavanagh et al. 2011 (1 mos. salt-acclimation)	50% MFTS (50% Gregorie Lake)	Fathead minnow	Chronic: ELS[a] (Lab)	Fertilization success (NSD); Hatch success (NSD); Larval survival (NSD)

Table A9. Study summaries of wet landscape reclamation pond effects on fish

Reference	Aged OSPW	Species	Duration	Endpoint & Result
Boerger & Aleksiuk, 1984	Pit 1 & 2 (aged 10 months)	Trout	Acute: 96h	Survival (60 - 100%)
Boerger et al. 1986	Pit 3 & 4 (aged 5 months)	Rainbow trout	Acute: 96h	Survival (LC50: 60%)
Boerger et al. 1986	Pit 1 (aged 10-14 months)	Rainbow trout	Acute: 96h	Survival (LC50 > 100%)
Boerger et al. 1986	Pit 2 (aged 14 months)	Rainbow trout	Acute: 96h	Survival (LC50: 90%)
MacKinnon & Boerger, 1986	Pit 1 & 2 (aged 1 & 2 years)	Rainbow trout	Acute: 96h	Survival (LC50 > 100%)

Table A9 (continued)

Reference	Aged OSPW	Species	Duration	Endpoint & Result
Nix & Martin, 1992	Suncor Test Pit 2 (aged 2 years)	Trout	Acute: 96h	Survival (LC50 > 100%)
Nix & Martin, 1992	Suncor Test Pit 2 (aged 2 years)	Trout	Chronic: 19d ELS[a] (Lab)	Hatch success (NSD); Larval survival (100%)
Peters, 1999 MSc	Pond 9 (aged 4 years)	Yellow perch	Chronic: ELS[a] (Lab)	Fertilization success (NSD); Embryo mortality (NSD); Egg size (− 8.9%); Larval hatch length (−)
Siwik et al. 2000	Pond 9 (aged 4 years)	Fathead minnow	Chronic: 56d ELS[a] (Lab)	Larval survival (NSD); Larval mass (NSD); Larval length (NSD). Control survival in 56d bioassay varied; results treated with caution
Kavanagh et al. 2011	Pond 9 (aged >15 years)	Fathead minnow	Chronic: 21d reproduction (Lab)	Survival (NSD); Male & Female CF[b] (NSD); Male & Female LSI[c] (NSD); Female GSI[d] (−34.2 %); Male GSI[d] (NSD); Number of tubercles (−57.4%); Mean fecundity rate (0%; complete cessation of spawning); Mean number of spawns (0; complete cessation of spawning)
Kavanagh et al. 2011	Pond 9 (aged >15 years)	Fathead minnow	Chronic: ELS[a] (Lab)	Larval endpoints could not be assessed due to lack of spawning
Kavanagh et al. 2011	Pond 9 (aged >15 years)	Fathead minnow	Chronic: 21d reproduction (Lab)	Survival (NSD); Male & Female CF[b] (NSD); Male & Female LSI[c] (NSD); Male & Female SSI[j] (NSD); Male & Female GSI[d] (NSD); Number of tubercles (−54.6%); Mean fecundity rate (−78.1%); Mean number of spawns (−71.6%); Female plasma estradiol (−); Female plasma testosterone (NSD); Male plasma testosterone & 11-ketotestosterone (−)
Kavanagh et al. 2011	Pond 9 (aged >15 years)	Fathead minnow	Chronic: ELS[a] (Lab)	Larval endpoints could not be assessed due to limited spawning

a) ELS: Early life stages
b) CF: Condition factor
c) LSI: Liver somatic index
d) GSI: Gonadal somatic index
e) SLL: Secondary lamellar length
f) SLW: Secondary lamellar width
g) ID: Interlamellar distance
h) BET: Basal epithelial thickness
i) PAGE: Proportion of the secondary lamellae available for gas exchange
j) SSI: Spleen somatic index

Table B1. Study summaries of Consolidated tailings (CT) release water effects on invertebrates. The results are summarized according to the following notations: (NSD) no significant difference relative to control, (−) significant decrease relative to control, (+) significant increase relative to control. If data were provided, the magnitude of change was calculated: (exposure - control / control) *100%

Reference	Consolidated tailings (CT) release water	Species	Duration	Endpoint & Result
MacKinnon et al. 2001	CT release water (aged 1 month)	Daphnia magna	Acute: 96h	Survival (100%); (LC50: 100%)

Table B2. Study summaries of wet landscape reclamation pond effects on invertebrates

Reference	Aged CT release water	Species	Duration	Endpoint & Result
MacKinnon et al. 2001	CT release water (aged 1 year)	Daphnia magna	Acute: 96h	Survival (LC50: 100%); (100%)
Leonhardt, 2003 MSc	CT Pond (aged 4 years)	Benthic invertebrates	Chronic: Field Survey (2000-2001)	Statistics based on calculated F-ratios between various OSPM-affected vs high & low conductivity reference wetlands. Reference sites included constructed wetlands from saline overburden & lean bitumen, interceptor mine runoff ditches & groundwater diversions. Sediment Core Richness & Abundance: (NSD); Artificial Substrate Richness (NSD); Abundance (-); Sweep Richness (-); Abundance (NSD).
Kennedy, 2012 MSc	Mike's Pond (aged 18 years)	Emergent Chironomidae	Chronic: Field Survey (2009)	Exuviae abundance (NSD); Length (NSD); Richness of genera (NSD); Community assemblages (NSD)

Table B3. Study summaries of Consolidated Tailings (CT) wetland effects on invertebrates

Reference	Consolidated tailings (CT) wetlands	Species	Duration	Endpoint & Result
Whelly, 1999 MSc	Hummock wetland	Benthic invertebrates	Chronic: Field Survey (1997)	Standardized abundance (NSD); Taxa richness: total, chironomid & other (NSD); Relative chironomid abundance (NSD); Chironomid generic richness (NSD); Mentum deformities (NSD)
Leonhardt, 2003 MSc	CT Wetland	Benthic invertebrates	Chronic: Field Survey (2000-2001)	Statistics based on calculated F-ratios between various OSPM-affected vs high & low conductivity reference wetlands. Reference sites included constructed wetlands from saline overburden & lean bitumen, interceptor mine runoff ditches & groundwater diversions. Sediment Core Richness & Abundance: (NSD); Artificial Substrate Richness (NSD); Abundance (-); Sweep Richness (-); Abundance (NSD).

Table B3. Study summaries of Consolidated Tailings (CT) wetland effects on invertebrates

Reference	Consolidated tailings (CT) wetlands	Species	Duration	Endpoint & Result
Kennedy, 2012 MSc	4m-CT	Emergent Chironomidae	Chronic: Field Survey (2009)	Exuviae abundance (NSD); Length (NSD); Richness of genera (NSD); Community assemblages (NSD)

Table B4. Study summaries of Seepage collection water effects on invertebrates

Reference	Dyke drainage & seepage collection	Species	Duration	Endpoint & Result
Nix & Bishay, 1996	Dyke seepage (wetland channel)	Daphnia magna	Chronic: 21d (Lab)	Number of progeny (NSD); Authors noted that results were sporadic. In general, marginal difference between in-flow (dyke seepage) and out-flow ("wetland treated" dyke seepage) waters
Nix & Bishay, 1996	Dyke drainage pond	Ceriodaphnia dubia	Chronic: 7d (Lab)	Survival (60%); Reproduction (LC50: 29%); Mean number of progeny per female (Control wetland outflow: 35.3 ± 4.4; Dyke drainage pond: 0.5 ± 1.6)
Nix & Bishay, 1996	Dyke seepage (wetland channel)	Ceriodaphnia dubia	Chronic: 7d (Lab)	In-flow waters not tested; Out-flow ("wetland treated" dyke seepage): Survival (70%); Reproduction (LC50: 43%); Mean number of progeny per female (Control wetland outflow: 35.3 ± 4.4; Dyke seepage outflow: 2.9 ± 2.6)
Nix & Bishay, 1996	Dyke seepage (wetland channel)	Ceriodaphnia dubia	Chronic: 7-10d (Field; in situ; 1994)	Mean survival rate (as a % of control wetland channels): In-flow (2%); Out-flow (73%); Mean number of progeny (as a % of control wetland channels): Out-flow (22%). Note: acute & sublethal toxicity was observed in control wetland channels
Leonhardt, 2003 MSc	Seepage Control Pond	Benthic invertebrates	Chronic: Field Survey (2000-2001)	Statistics based on calculated F-ratios between various OSPM-affected vs high & low conductivity reference wetlands. Reference sites included constructed wetlands from saline overburden & lean bitumen, interceptor mine runoff ditches & groundwater diversions. Sediment Core Richness & Abundance: (NSD); Artificial Substrate Richness (NSD); Abundance (-); Sweep Richness (-); Abundance (NSD).

Table B5. Study summaries of Natural wetland effects on invertebrates

Reference	Natural wetland	Species	Duration	Endpoint & Result
Whelly, 1999 MSc	Natural wetland	Benthic invertebrates	Chronic: Field Survey (1997)	Standardized abundance (NSD); Taxa richness: total, chironomid & other (NSD); Relative chironomid abundance (NSD); Chironomid generic richness (NSD); Mentum deformities (NSD)
Ganshorn, 2002 MSc	Natural wetland	Benthic & pelagic invertebrates	Chronic: Field Survey (2001)	Results based on observed estimates rather than statistical analyses. Annual production of predatory benthic & pelagic dipterans: Dipteran component of the benthic food web was more productive than the pelagic in both Natural Wetland & reference sites; Chironomid generic richness: Tanypodinae density was 3-4 times greater compared to reference sites. 2 reference sites were High Sulfate Pond (equivalent to South Bison: saline overburden & lean bitumen) and Shallow Wetland South Ditch. Note: Natural Wetland received fresh inputs of CT discharge during this study.
Leonhardt, 2003 MSc	Natural wetland	Benthic invertebrates	Chronic: Field Survey (2000-2001)	Statistics based on calculated F-ratios between various OSPM-affected vs high & low conductivity reference wetlands. Reference sites included constructed wetlands from saline overburden & lean bitumen, interceptor mine runoff ditches & groundwater diversions. Sediment Core Richness & Abundance: (NSD); Artificial Substrate Richness (NSD); Abundance (-); Sweep Richness (-); Abundance (NSD). Note: Natural Wetland received fresh inputs of CT discharge during this study.
Kennedy, 2012 MSc	Natural wetland	Emergent Chironomidae	Chronic: Field Survey (2009)	Exuviae abundance (NSD); Length (NSD); Richness of genera (NSD); Community assemblages (NSD)

Table B6. Study summaries of wetlands created from saline overburden and lean oil sands material effects on invertebrates

Reference	Saline overburden & lean oil sands	Species	Duration	Endpoint & Result
Whelly, 1999 MSc	South Bison	Benthic invertebrates	Chronic: Field Survey (1997)	Standardized abundance (NSD); Taxa richness: total, chironomid & other (NSD); Relative chironomid abundance (NSD); Chironomid generic richness (NSD); Mentum deformities (NSD)

Table B7. Study summaries of wet landscape reclamation pond effects on invertebrates

Reference	Mature fine tailings & fresh water	Species	Duration	Endpoint & Result
Leonhardt, 2003 MSc	Demonstration Pond	Benthic invertebrates	Chronic: Field Survey (2000-2001)	Statistics based on calculated F-ratios between various OSPM-affected vs high & low conductivity reference wetlands. Reference sites included constructed wetlands from saline overburden & lean bitumen, interceptor mine runoff ditches & groundwater diversions. Sediment Core Richness & Abundance: (NSD); Artificial Substrate Richness (NSD); Abundance (-); Sweep Richness (-); Abundance (NSD).
Anderson et al. 2012 b	Demonstration Pond	Chironomus dilutus	Acute: 10d (Lab)	Survival (NSD); Growth (−19% less); Observations of larval case size & structure (slightly smaller but relatively intact); Activity score (NSD)
Anderson et al. 2012 b	Demonstration Pond	Chironomus dilutus	Chronic: 60d (Lab)	Pupation success (NSD); Emergence success (NSD); Time to Emerge (NSD); Cumulative emergence (NSD)

Table B8. Study summaries of wet landscape reclamation pond effects on invertebrates

Reference	Mature fine tailings & OSPW	Species	Duration	Endpoint & Result
Leonhardt, 2003 MSc	Pond 5 / Mature Fine Tailings North (MFTN)	Benthic invertebrates	Chronic: Field Survey (2000-2001)	Statistics based on calculated F-ratios between various OSPM-affected vs high & low conductivity reference wetlands. Reference sites included constructed wetlands from saline overburden & lean bitumen, interceptor mine runoff ditches & groundwater diversions. Sediment Core Richness & Abundance: (NSD); Artificial Substrate Richness (NSD); Abundance (-); Sweep Richness (-); Abundance (NSD).
Anderson et al. 2012 b	Pond 5 (FE5)	Chironomus dilutus	Acute: 10d (Lab)	Survival (NSD); Growth (NSD); Observations of larval case size & structure (smaller & more fragile); Activity score (NSD)
Anderson et al. 2012 b	Pond 5 (FE5)	Chironomus dilutus	Chronic: 60d (Lab)	Pupation success (NSD); Emergence success (NSD); Time to Emerge (NSD); Cumulative emergence (NSD)
Wiseman et al. 2013 b	Pond 5 (FE5)	Chironomus dilutus	Acute: 4 & 7d (Lab)	4d & 7d Survival (NSD); 4d & 7d Growth (NSD)
Wiseman et al. 2013 b	Pond 5 (FE5)	Chironomus dilutus	Acute: 4 & 7d (Lab)	Gene transcripts: Oxidative Stress (4d exposure): (NSD); 7d exposure: *gst* (− 2-fold); Gene transcripts: Endocrine Signaling: (NSD); Tissue concentration of lipid peroxidase (NSD)

Table B9. Study summaries of wet landscape reclamation pond effects on invertebrates

Reference	Aged OSPW	Species	Duration	Endpoint & Result
Boerger & Aleksiuk, 1984	Pit 1 & 2 (aged 10 months)	Daphnia	Acute: 96h	Survival (80%)
MacKinnon & Boerger, 1986	Pit 1 & 2 (aged 1 & 2 years)	Daphnia	Acute: 96h	Survival (LC50 > 100%)
Leonhardt, 2003 MSc	Pond 9 (aged 8 years)	Benthic invertebrates	Chronic: Field Survey (2000-2001)	Statistics based on calculated F-ratios between various OSPM-affected vs high & low conductivity reference wetlands. Reference sites included constructed wetlands from saline overburden & lean bitumen, interceptor mine runoff ditches & groundwater diversions. Sediment Core Richness & Abundance: (NSD); Artificial Substrate Richness (NSD); Abundance (-); Sweep Richness (-); Abundance (NSD).
Anderson et al. 2012 b	Pond 9 (TPW) (aged 16 years)	Chironomus dilutus	Acute: 10d (Lab)	Survival (NSD); Growth (−23% less); Observations of larval case size & structure (slightly smaller but relatively intact); Activity score (NSD)
Anderson et al. 2012 b	Pond 9 (TPW) (aged 16 years)	Chironomus dilutus	Chronic: 60d (Lab)	Pupation success (−42.7%); Emergence success (−71.6%); Time to Emerge: Males (+ 9 d); Females (+ 8.6 d); Cumulative emergence (−)
Kennedy, 2012 MSc	Pond 9 (aged 17 years)	Emergent Chironomidae	Chronic: Field Survey (2009)	Exuviae abundance (NSD); Length (NSD); Richness of genera (NSD); Community assemblages (NSD)

APPENDIX B

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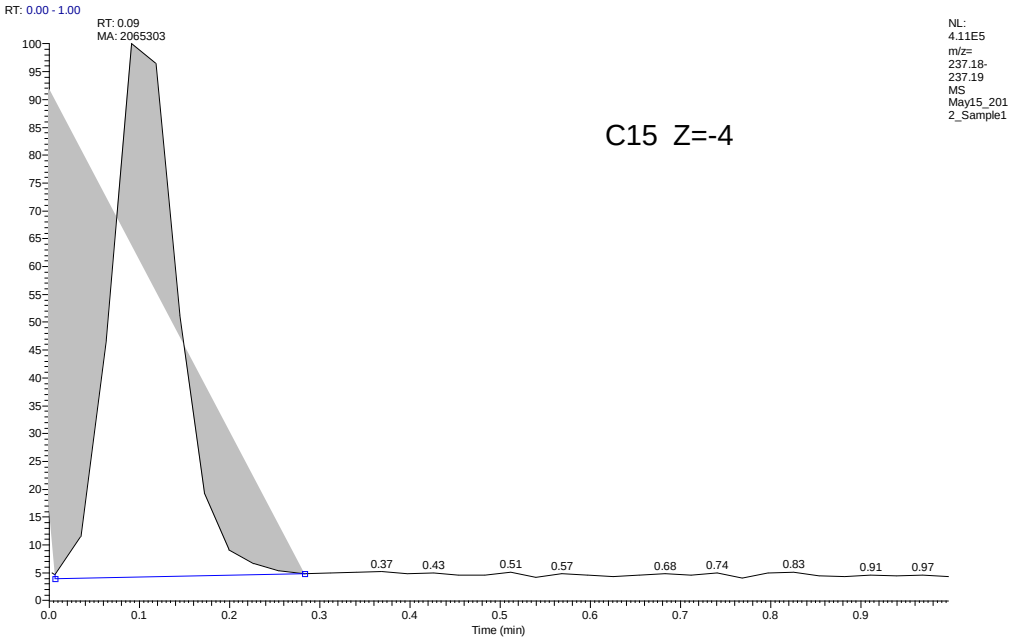

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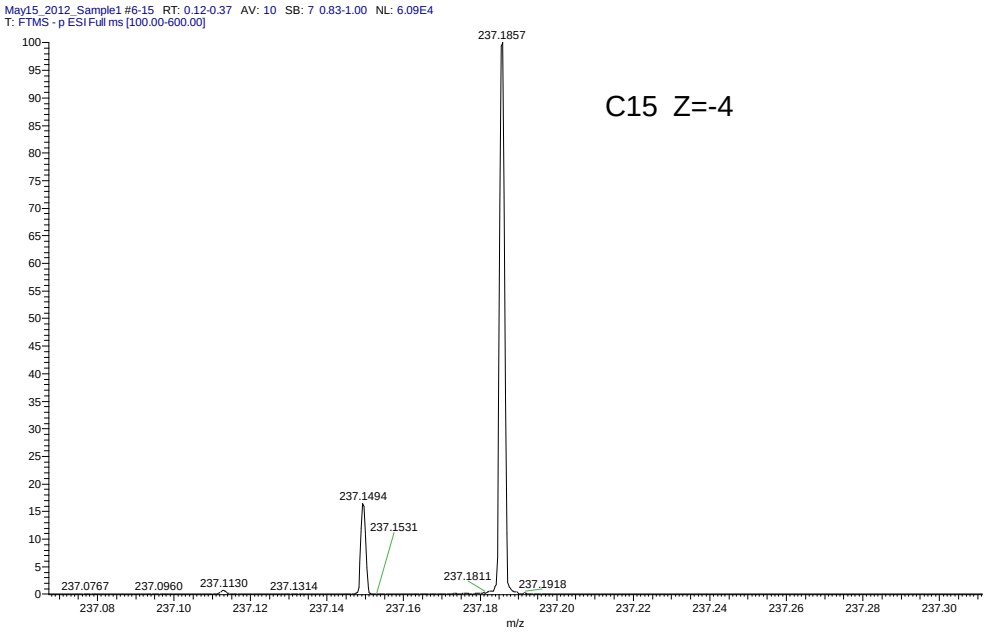

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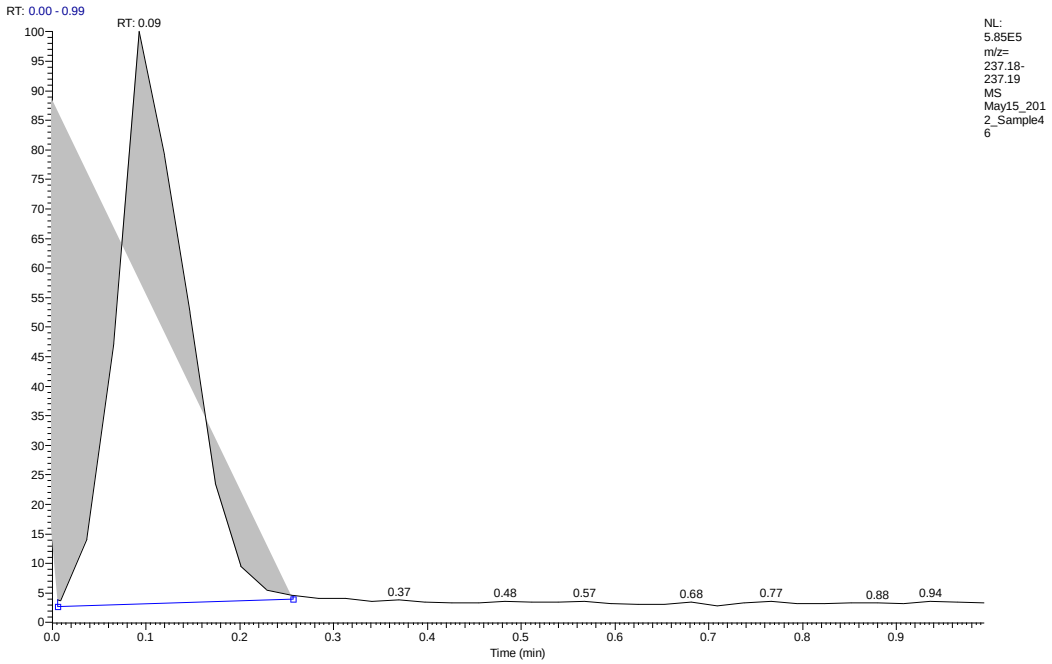

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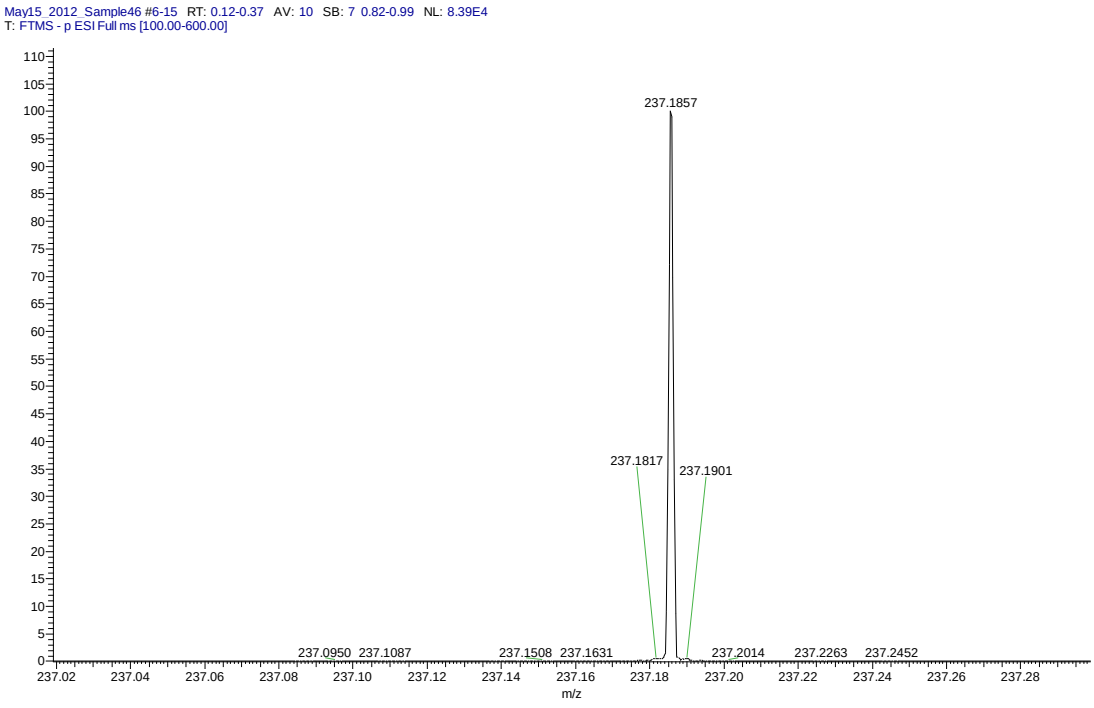

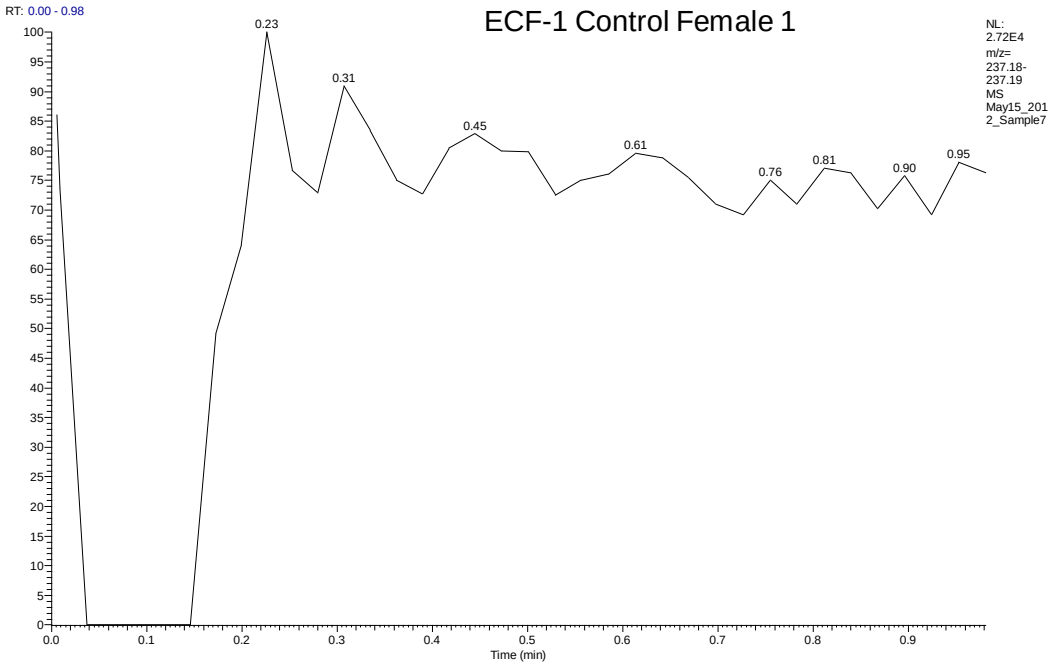
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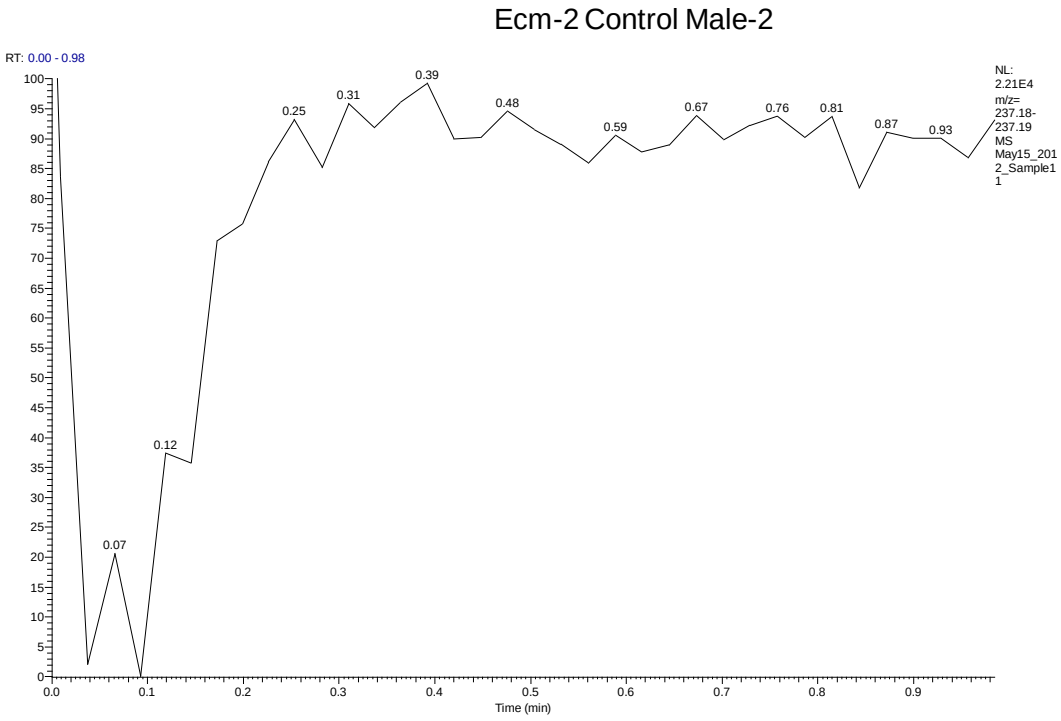
Time (min)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Time (min)

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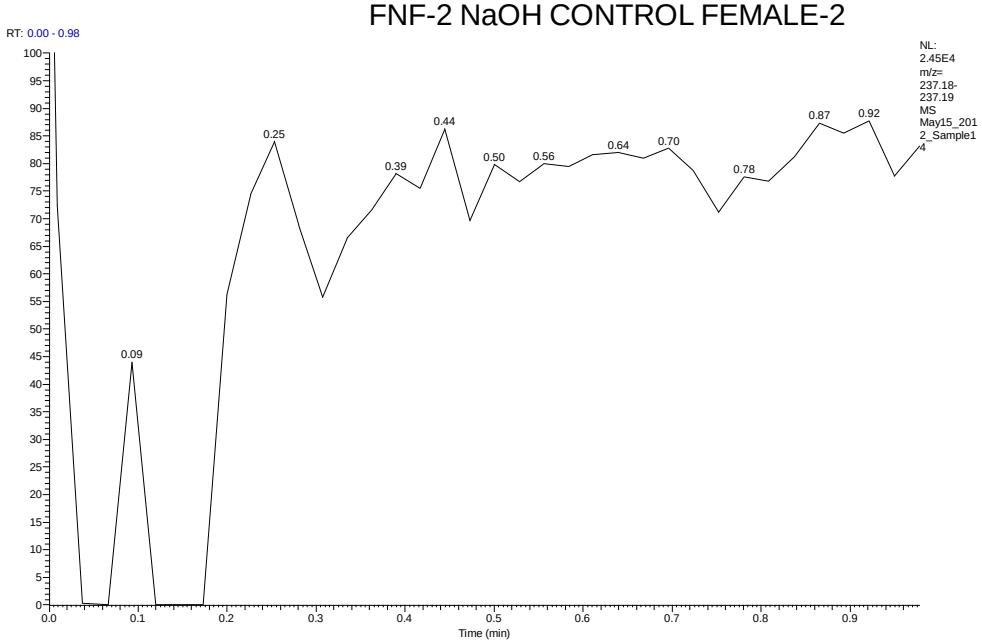

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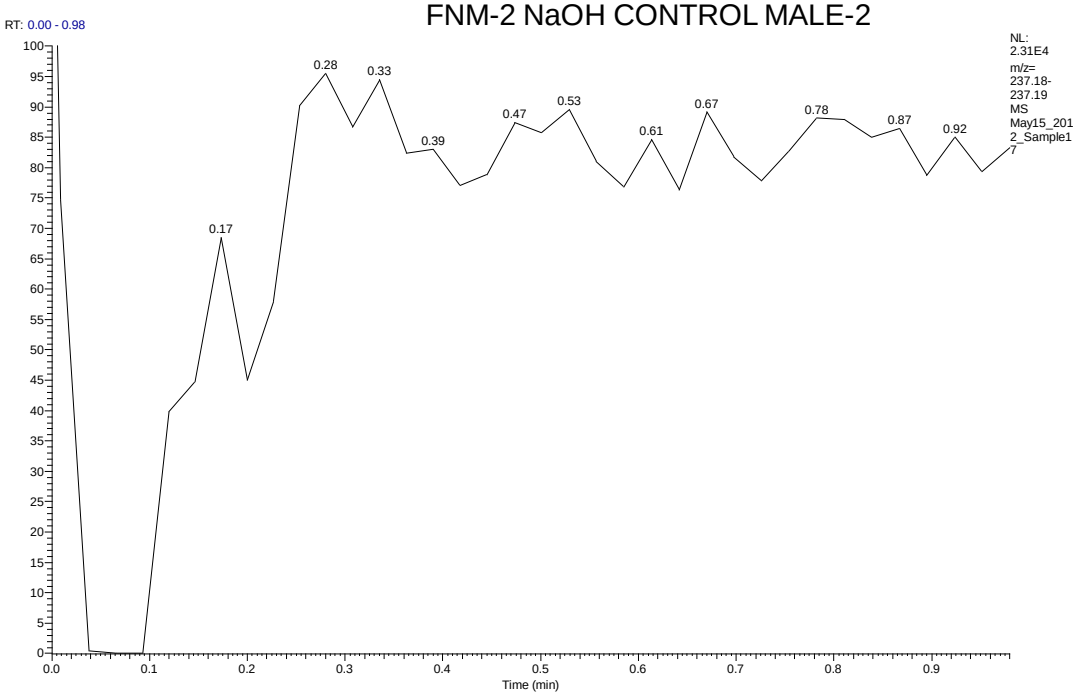

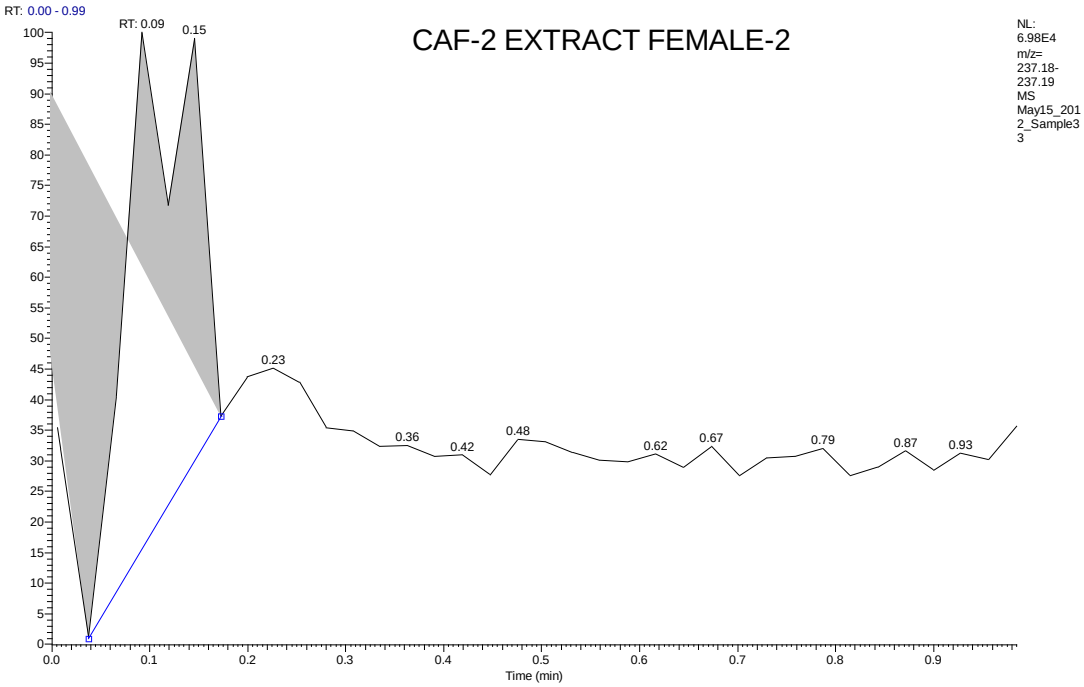
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237.19
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May15_201
2_Sample3
3
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0.67
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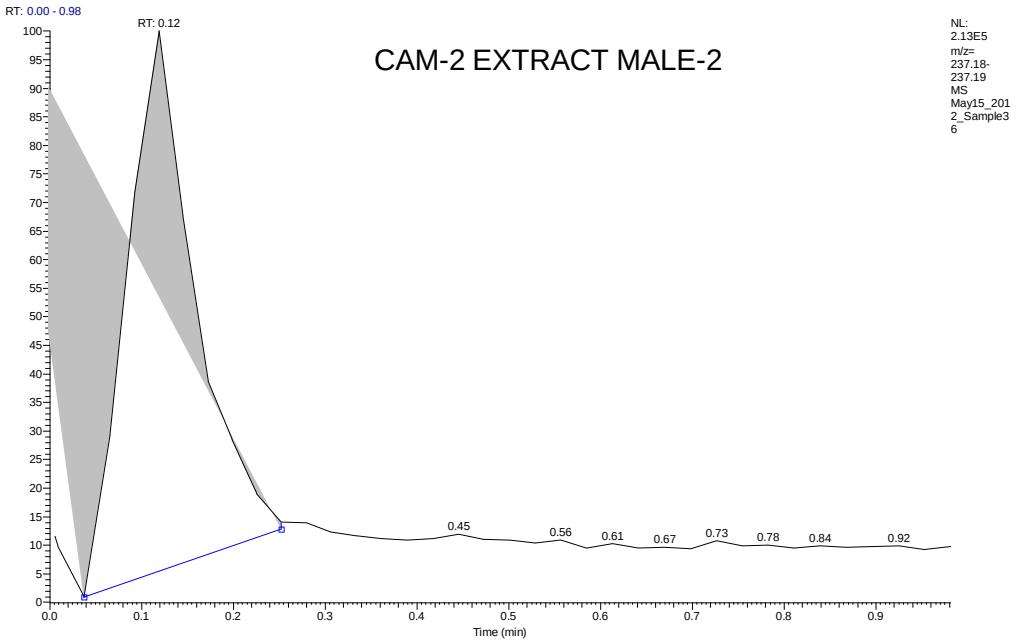
0.87
0.93
Time (min)


RT: 0.00 - 0.98
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m/z=
237.18-
237.19
MS
May15_201
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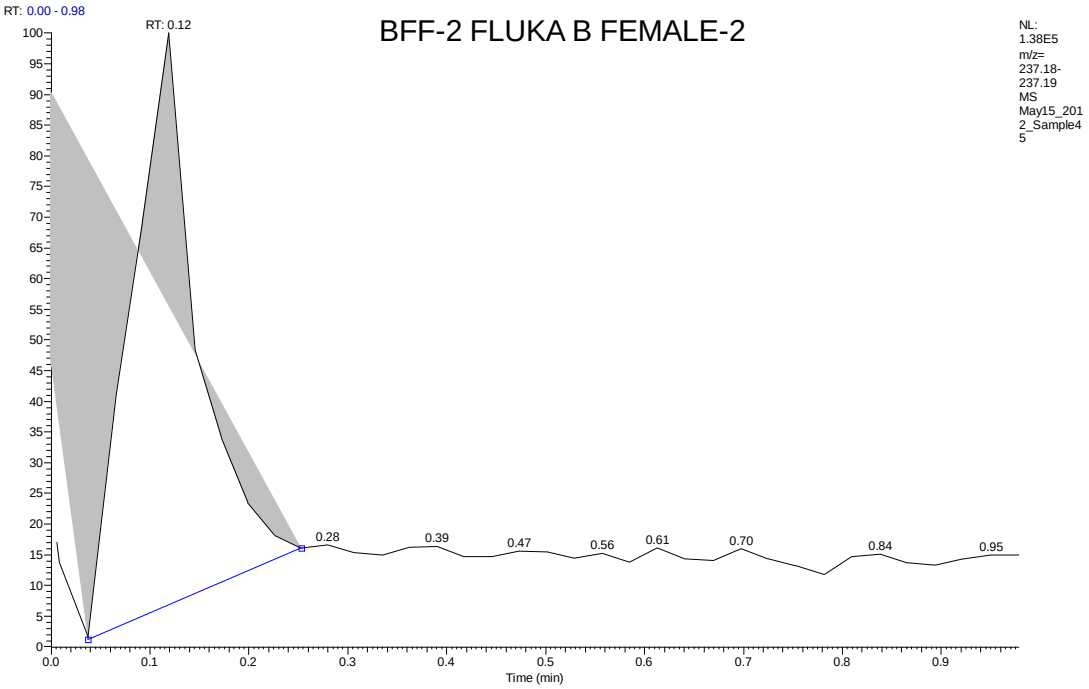
0.92
Time (min)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237.19
MS
May15_201
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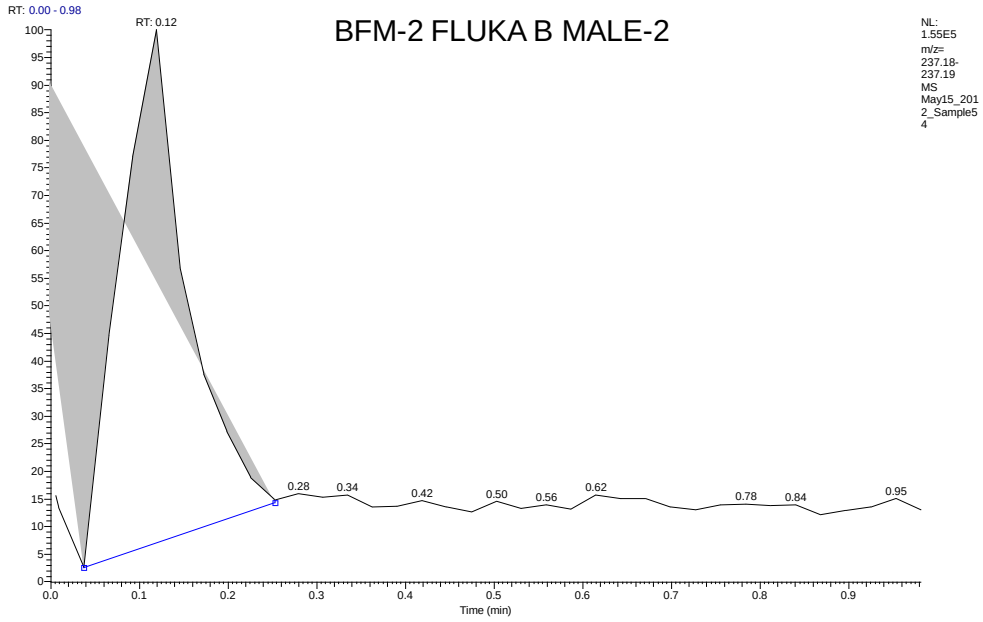
Time (min)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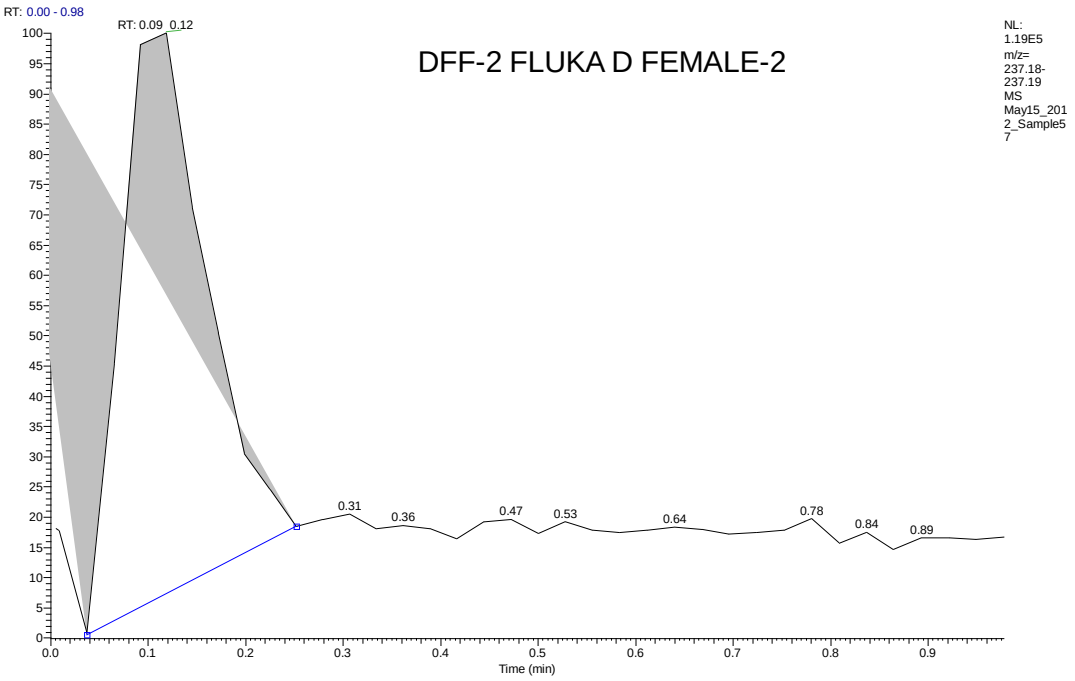
Time (min)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237.19
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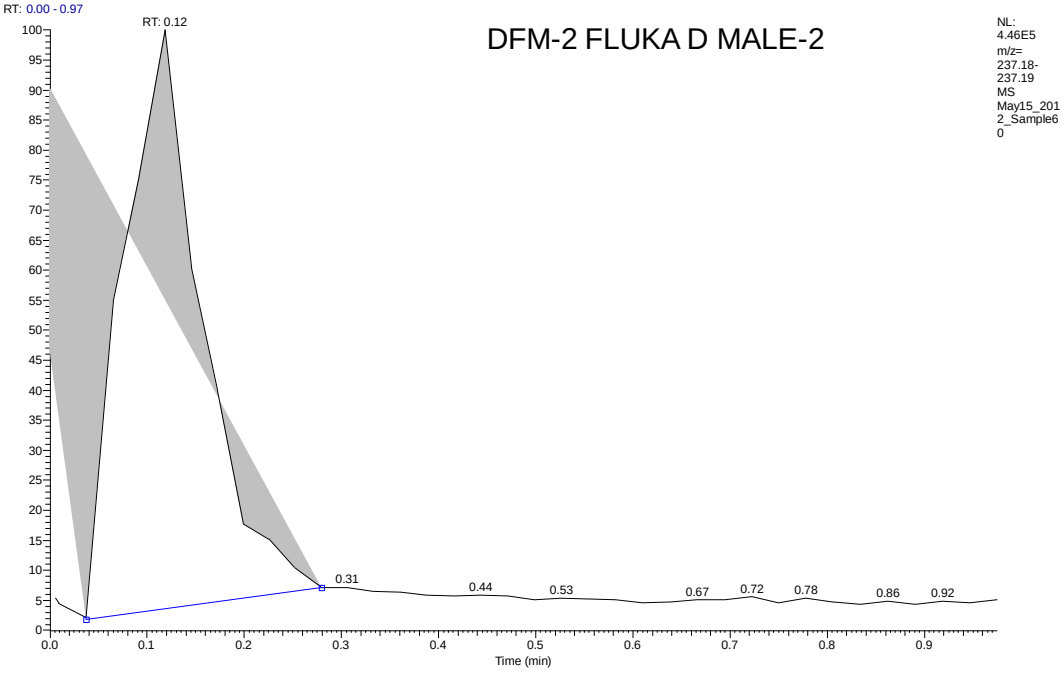
Time (min)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