Chemical and Toxicological Characterization of Dissolved Organics from Oil Sands Waters

by

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Author's Declaration

This thesis consists of material all of which I authored or co-authored: see Statement of Contributions included in the thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

I understand that my thesis may be made electronically available to the public.

Statement of Contributions

The organization of this thesis is such that it is composed of seven chapters, five of which have been structured in a manuscript format for publication in peer-review journals. Chapter 1 is a general introductory chapter outlining information contained in the subsequent chapters. The following five chapters are in the process of submission (Chapters 2, 3, 4, 5, and 6). Chapter 2 is a method development for isolation and fractionation of soluble organics in aged OSPW. Chapter 3 is an effects-directed analysis of generated fractions from aged OSPW soluble organics. Chapter 4 involved isolation and fractionation of soluble organics in bitumen-influenced groundwaters. Chapter 5 is an effects-directed analysis of generated fractions from soluble organics in bitumen-influenced groundwaters. Chapter 6 is a review and risk assessment of toxicity of acid-extractable organics and represents an overview and summary of concepts throughout this thesis. Chapter 7 is a concluding chapter briefly summarizing the major findings in the present thesis. Due to the thesis structure, the contents of discussion present some repetition herein. The titles and authorship of each manuscript are listed below, accompanied by the contributions from each co-author. All chapters, excluding Chapter 5 were exclusively written by Anthony E. Bauer as indicated by primary authorship. Chapter 4 was written primarily by Richard A. Frank based on the experimentation organized and lead by Anthony E. Bauer. Drs. Dixon, Frank, Hewitt, Farwell, and Hall contributed editorial comments and points of discussion on all manuscripts.

Chapter 2.	Anthony E. Bauer, R. A. Frank, J. V. Headley, C. B. Milestone, S. Batchelor, K. M. Peru, M. D. Rudy, S. Barrett, R. Vanderveen, D. G. Dixon, L. M. Hewitt. 2018. A preparative method for the isolation and fractionation of dissolved organics from bitumen-influenced waters. Contribution of co-authors: -R. A. Frank and L. M. Hewitt (Environment and Climate Change Canada, Burlington, ON) provided oil sands extracts, equipment, supplies, and aided in experimental design and method development for extraction and fractionation of dissolved organics. Editorial comments and points of discussion were also provided. -J. V. Headley and K. M. Peru (Environment and Climate Change Canada, Saskatoon, SK) provided ESI-HRMS for analysis of dissolved organics.

-C. B. Milestone (Sheridan College, Toronto, ON) performed LC-QToF analysis of dissolved organics.
-S. Batchelor (Environment and Climate Change Canada, Burlington, ON) provided GC-MS/MS analysis of dissolved organics.
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-R. A. Frank provided organisms, equipment and expertise for bioassay exposures for *V. fischeri* and *D. magna*, as well as editorial comments and points of discussion.
-J. L. Parrott (Environment and Climate Change Canada, Burlington, ON) provided equipment and personnel responsible for exposures of *P. promelas.*
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-P. Gillis (Environment and Climate Change Canada, Burlington, ON) provided equipment and personnel responsible for exposures of *L. cardium.*
-L. E. Deeth (University of Guelph, ON) aided in statistical analysis of bioassay data.
-M. D. Rudy, R. Vanderveen, and L. Brown (Environment and Climate Change Canada, Burlington, ON) aided in bioassay exposures of *V. fischeri, D. magna,* and *H. azteca.*
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-R. A. Frank and L. M. Hewitt wrote the bulk of the chapter using data generated in experiments lead by A. E. Bauer, provided insight on method development, equipment, supplies, and aided in experimental design for extraction and fractionation of dissolved organics.
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-S. Batchelor provided GC-MS/MS and LC-QToF analysis of dissolved organics.
-C. B. Milestone performed LC-QToF analysis of dissolved organics.
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-A. Farwell provided pertinent data and editorial comments.
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ABSTRACT

CHEMICAL AND TOXICOLOGICAL CHARACTERIZATION OF DISSOLVED ORGANICS FROM OIL SANDS WATERS

The surface mining of oil sands from the Athabasca deposit north of Fort McMurray, Alberta produces considerable tailings waste which is stored in large tailings ponds on industry lease sites. With the advent of oil sands end-pit lakes and decommissioned tailings ponds, viable strategies for the detoxification of oil sands process affected water (OSPW) are under investigation. One such strategy relies on the biodegradation of toxic organic compounds by indigenous microbes, resulting in aged tailings waters with potentially reduced toxicity. Determining drivers of toxicity within OSPW poses a great challenge because differences in ore quality and bitumen extraction methods influence organic and inorganic chemistry, and therefore, toxicity.

In order to assess the toxic potential of the suite of dissolved organics in OSPW, a method for extraction and fractionation was developed. This was achieved with a liquid chromatography approach using reversed-phase solid phase extraction coupled with soxhlet extraction. The method successfully separated organic compounds from 180 L of an aged OSPW source into three fractions (F1-F3) with increasing polarities. Chemical characterization of the generated fractions included electrospray ionization high-resolution mass spectrometry, liquid chromatography quadrupole time-of-flight mass spectrometry, gas chromatography triple quadrupole time-of-flight mass spectrometry, and synchronous fluorescence spectroscopy. Method validation included fractionations with surrogate reference standards and labelled

standards, which also confirmed separation according to polarity and verified high recovery of dissolved organics. This method was designed to generate bulk quantities of extract which provide enough material for a suite of toxicity bioassays.

Using this novel method, aged OSPW and four bitumen-influenced groundwater sites (2 influenced by natural bitumen; 2 influenced by a mixture of natural bitumen and OSPW sources) were fractionated. The whole water and isolated fractions were then exposed to seven different aquatic species; *Pimephales promelas* (embryo)*, Oryzias latipes* (embryo)*, Vibrio fischeri, Daphnia magna* (neonates)*, Lampsilis cardium* (glochidia)*, Lampsilis siliquiodea* (glochidia) and *Hyalella azteca* (juveniles). Chemically, bitumen-influenced groundwater sites were predominantly composed of O_2 and O_4 species while aged OSPW was dominated by O_4 species. Analysis also revealed a high variability in composition and abundance of organic and inorganic constituents across groundwater sites. Of the organic fractions assessed, F1 (least polar) and F3 (most polar) appeared most toxic overall while F2 displayed little toxicity to all species evaluated. Organisms were identified as differentially more sensitive to whole waters, likely as a result of inorganics (*D. magna* and *L. siliquiodea*), or dissolved organics (*P. promelas* and *H. azteca*).The present study indicates that although an aged tailings source (≥18 years) displayed low toxicity overall, inorganic and polyoxygenated organic components may pose a persistent concern to aquatic organisms. A general comparison of groundwater sites containing OSPW-derived constituents vs. natural bitumen-derived constituents revealed that whole water toxicities were quite similar. It is therefore likely that toxicity associated with tailings seepage into groundwater is mitigated by chemical changes as a result of soil composition and groundwater mixing.

Finally, an ecotoxicological risk assessment of OS acid-extractable organics (AEO) produced a joint probability curve which predicted that the probability of producing an effect in 10% of fish and invertebrates species was 100% and 97.7%, respectively. In general, at AEO exposures in the range of 17 – 104 mg/L, an acute species sensitivity distribution revealed vertebrates (embryonic) to be more sensitive than invertebrate organisms. The risk assessment recommends a monitoring program that accounts for current anthropogenic dissolved organic input from tailings seepage, and its effect on particularly sensitive fish species. Additionally, future efforts regarding the wet landscape strategy should account for changes in dissolved organic concentrations and reduction in toxicity over time.

In summation, for those organisms that display sensitivity to dissolved organics in oil sands waters, aging by natural biodegradation appears to be a viable strategy. Moreover, industrially-influenced groundwaters do not appear to pose a greater risk to aquatic organisms than groundwaters influenced by naturally-derived bitumen. Nonetheless, due to possible invertebrate sensitivities to inorganic components within whole waters, a strategy to deal with these bio-persistent compounds warrants investigation. In order to identify and characterize OSPW-derived dissolved organics that pose the greatest environmental risk, the bioactive fractions (F1 and F3) will be further fractionated and assessed toxicologically. Finally, it is recommended that organisms identified as being most acutely sensitive to OSPW-derived organic and inorganic constituents be the focus of future effects-directed analysis of OSPW toxicity, as well as impact assessment monitoring and future remediation/reclamation of industrial lease sites.

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List of Abbreviations

AEO	Acid-extractable organics
ANOVA	Analysis of variance
DBE	Double-bond equivalents
DP	Drive-point
ECCC	Environment and Climate Change Canada
ESI-HRMS	Electrospray ionization high-resolution mass spectrometry
EtOAc	Ethyl Acetate
F1	Fraction 1
F2	Fraction 2
F3	Fraction 3
FTIR	Fourier-transform infrared spectroscopy
GC/MS	Gas chromatography mass spectrometry
GC-MS/MS	Gas chromatography tandem mass spectrometry
HCl	Hydrochloric acid
LC_{50}	Median lethal concentration
LC-QToF	Liquid chromatography quadrupole time-of-flight mass spectrometry
MeOH	Methanol
MOA	Mode of action
NA	Naphthenic acids
NaOH	Sodium hydroxide
OSPW	Oil sands process-affected waters
PAC	Polycyclic aromatic hydrocarbons
SFS	Synchronous fluorescence spectroscopy
SPE	Solid-phase extraction

Chapter 1. General Introduction

1.1. Introduction

The oil sands deposits in northern Alberta encompasse 142,000 km^2 and contain the third largest reserve of oil worldwide next to Venezuela and Saudi Arabia (Alberta Energy Regulator, 2014; Canadian Association of Petroleum Producers, 2016). The vast deposits are comprised of three regions, the largest being the Athabasca deposit, followed by the Cold Lake and Peace River deposits. Oil sands are heavy crude oil deposits composed of 68% sand, 23% bitumen and 9% water (Fair, 2010). Bitumen, the sought after resource, is a viscous hydrocarbon mixture. The extraction of bitumen can be achieved in two ways; *in-situ* methods for deposits deeper than 75 metres and *surface mining* methods for deposits closer to the surface (Alberta Energy and Utilities Board, 2009-2010). In-situ methods, including steam assisted gravity drainage, require high pressure steam to be injected into oil sand deposits which allows the bitumen to flow to a lower well where it is then pumped to the surface (Royal Society of Canada Expert Panel, 2010). Bitumen deposits that are close to the surface are trapped in the underlying sand, and therefore, are surface mined and then separated from the sand. It is then transported to an extraction facility where a process known as Clark hot water extraction liberates bitumen from sand in the oil sand material (Alberta Energy, 2009; The Royal Society of Canada, 2010). The Clark hot water extraction is a separation method in which the oil sand is mixed with hot water, sodium hydroxide and steam, thus separating it into three distinct layers: sand, water and bitumen. This surface-mined bitumen represents 20 percent of Alberta's recoverable oil sands (Alberta Energy, 2008b). The bitumen is removed and then upgraded to a synthetic crude oil to be sold and used in the production of other petroleum products (Allen, 2008; Royal Society of Canada Expert Panel, 2010).

With increasing bitumen production by surface mining methods, there is a concurrent increase in mine tailings. Oil sands tailings are largely a product of waste material produced during the separation of bitumen and is composed of 70-80% process-affected and/or fresh water, 20-30% solids, and 1-3% unrecovered bitumen (Allen, 2008). These large amounts of fluid tailings, termed oil sands process-affected water (OSPW), contain contaminants of environmental concern. To mitigate this, the Alberta government has established a zero discharge policy which restricts the release of wastes associated with the oil sands industry and requires that oil sands lease sites be returned to their original state or better (Government of Alberta, 2017, FTFC, 1995a). The oil sands industry has responded to the policy by developing aquatic reclamation approaches, one of which involves filling mined pits with fluid tailings and capping them with water to create *end-pit lakes*. To be successful, there is a need to determine the magnitude of oil sands constituents that an ecosystem can safely assimilate for the establishment of a viable habitat.

1.2. Oil Sands Process-affected Water Toxicants

1.2.1. Inorganic components

Within the OSPW fraction of mine tailings are a plethora of contaminants including trace metals, major ions and dissolved organic compounds (Allen, 2008). Where whole tailings include the water soluble and insoluble waste components (sands, clays, etc.), process-affected waters represent the wastewater fraction containing only water soluble components. Some of the major inorganic contaminants within oil sands tailings water are trace metals. The source of trace metals in oil sands tailings is largely due to the concentration of naturally occurring metals in bitumen. For example, some metals dissolved in tailings pond water and process affected water

which exceed Canadian Water Quality Guidelines are As, Cr, Mo, Fe, Pb, Ni, Zn and Cu (Allen, 2008; Mahdavi et al., 2013). Because the types and amount of metals present in tailings is dependent on industrial processes, exceedances differ between tailings ponds. Metals such as Cd, Ba, Mn, Sr, Al, Fe, B, Si, As, Dc, Co Cr, Ni, Pb, Se, V, and Zn, have been shown to leach into porewater/groundwater from tailings reservoirs (Oiffer, 2006; Holden et al., 2013). Tailings leachate represents a potential hazard to natural surface waters, as well as groundwaters that act as a transport medium. One study showed Athabasca River sediments downstream of tailings ponds contained a 3-fold increase in Al, Sb, As, Cu, Pb, Sr, U, and Zn compared to upstream, suggesting direct input from the tailings pond (Timoney and Lee, 2009). It is important to note that the source of downstream metals in this study was a result of industry and major bitumen deposits, and it is unknown what proportion of the observed increase was solely due to industrial inputs. Although metals are concentrated in tailings, they can precipitate out of solution in the tailings environment reducing their overall bioavailability to aquatic organisms.

Of potentially greater concern is the high salinity associated with inorganic ions present in tailings. Salinity in OSPW is largely a result of the caustic hot water solutions employed for the separation of bitumen which can become concentrated due to water recycling (FTFC, 1995a). The transport of inorganic ions into the environment through groundwater poses a possible threat to freshwater organisms which typically exhibit low tolerance to high salinity. However, groundwater in the oil sands region displays a wide range in salinity with Cl^- concentrations in some shallow groundwater discharges of up to 50 mg/L (Jasechko et al., 2012), suggesting some level of tolerance in the system. The persistence, transport, and bioavailability of metal ions in groundwater is largely determined by groundwater flows. Specifically, the persistence of Na^+ and Cl^- in OSPW has been proposed to be dependent upon the underlying substrate, and where

Na^+ persists, the precipitation of existing ions (SO_4^{2-}, Ca^{2+}, Mg^{2+}) will likely occur (Holden et al., 2011). Due to evidence that high Cl^- concentrations can preferentially precipitate certain organic contaminant classes within OSPW (Headley et al., 2012), the toxicological focus on salinity is typically in regard to its interaction with other contaminants (Nero et al., 2006; Celsie et al., 2016).

1.2.2. Organic components

Organic contaminants within tailings OSPW have been largely separated into neutral organic and polar organic components. Neutral components consist mainly of polycyclic aromatic compounds (PAC), while polar components were referred to as naphthenic acids (NAs) and more recently acid-extractable organics (AEO).

The relatively neutral PACs can result from natural and anthropogenic sources and are of great concern due to their mutagenic and carcinogenic potential (WHO, 2010). They are produced as a result of incomplete combustion of carbon containing fuels, where pyrogenic PACs are typically produced as a result of forest fires and petrogenic PACs are anthropogenically derived (ATSDR, 1996). PACs are lipophilic and, therefore, more commonly encountered in sediment than water, but due to the highly turbid nature of tailings and length of time it takes for fluid fine tailings to settle out (decades estimated; Royal Society of Canada Expert Panel, 2010), their persistence in tailings water is likely quite profound. The major source of PACs in oil sands tailings ponds is unrecovered bitumen remaining after the extraction process. They are concentrated in tailings as a result of the extensive re-use of water in bitumen extraction and upgrading processes. Alkylated PAC congeners are more common in reclaimed tailings ponds due to their relatively high resistance to degradation than their parent compounds

(Headley and Akre, 2001) and can be used to identify anthropogenic impact and transport in environmental samples (Akre et al., 2004; Hall et al., 2012).

A group of polar organic compounds within the acid-extractible fraction of OSPW, widely referred to as naphthenic acids (NAs), was considered to be the compounds of greatest concern in OSPW due in part to their high concentrations within tailings and their water solubility (Clemente and Fedorak, 2005; Allen, 2008). The "classical" definition of NAs included alkyl-substituted acyclic, monocyclic and polycyclic carboxylic acids classified using the general formula $C_nH_{2n+z}O_2$, where *n* is the carbon number and *z* refers to the hydrogen deficiency due to a ring formation (Clemente and Fedorak, 2005). Typically, research was conducted on samples acquired through acid-extraction methods (Frank et al., 2006), which allowed for chemical evaluation of precipitated acid compounds. More recent characterization of these components has revealed structures much more complex than the "classical" NA definition, and is more inclusive of compounds present in the acid-extractable fraction of OSPW. This more broadly defined group of organics, which include NAs, will be henceforth referred to as acid-extractable organics (AEO).

Recent research using advanced chromatographic separation techniques, such as 2-dimensional gas chromatography, and advanced spectrometry techniques, such as high resolution mass spectrometry, are beginning to uncover new AEO compound structures. Unlike classical oil sands NAs, AEO are known to be heteroatomic, containing oxygen, sulphur, and nitrogen ions within their molecular structure (Stanford et al., 2007; Bauer et al., 2015). There is also evidence that oil sands AEO contain di-carboxyl, hydroxyl, ethanoic, methyl, di-methyl, benzenoid, and ketonic moieties (Rowland et al., 2011a; Rowland et al., 2011b). Recently, AEO ring structures have been revealed to have adamantane, and diamantane configurations (Rowland et al., 2011a).

Identification of this diverse suite of AEO coupled with the lack of commercial analytical standards for oil sands AEO has presented challenges with quantitation, qualitative analysis, and toxicological interpretation.

1.2.3. Aquatic Toxicity of Bitumen-Derived Toxicants

Toxicity of OSPW is a result of both inorganic and organic constituents present in bitumen which are concentrated in a tailings environment. As described above, inorganic components typically associated with particulates tend to precipitate out of suspension in tailings, reducing their bioavailability to many aquatic species. As a result, research regarding tailings toxicity is generally concerned with dissolved organics, specifically AEO. The concern with AEO is their high concentration in tailings and persistence in aquatic environments (Brown and Ulrich, 2015). Many studies have demonstrated the AEO of OSPW to display acute and sub-acute toxicity to a variety of aquatic organisms including phytoplankton (Leung et al., 2003), benthic invertebrates (Bartlett et al., 2017), and fish (Siwik et al., 2000; Farrell et al., 2004; Nero et al., 2006; Peters et al., 2007; Lister et al., 2008; Kavanagh et al., 2011; Bauer et al., 2017; Marentette et al., 2015a). Specifically, AEO have been shown to reduce phytoplankton community diversity (Leung et al., 2003), as well as reduce plasma sex steroids (Lister et al., 2008), reduce spawning (Kavanagh et al., 2011), promote gill degeneration (Farrell et al., 2004; Nero et al., 2006), alter leukocyte levels (Farrell et al., 2004), reduce hatch success (Peters et al., 2007; Bauer et al., 2017), reduce growth parameters (Siwik et al., 2000; Peters et al., 2007; Bauer et al., 2017), and increase embryo-larval mortalities in fish (Peters et al., 2007; Bauer et al., 2017).

To date, studies have proposed a number of different physico-chemical properties related to AEO acute toxicity. These properties include AEO molecular weight (Frank et al., 2008;

Clemente et al., 2004), solubility (Stanford et al., 2007; Jones et al., 2011), carboxylic acid content (Frank et al., 2009), aromaticity (Jones et al., 2012), and carbon number (Lai et al., 1996; Clemente et al., 2004; Jones et al., 2011). Due to the surfactant nature of AEO, narcosis (non-specific membrane disruption) has been regarded as the likely mode of toxic action (MOA) (Clemente and Fedorak, 2005). Thus, an increase in a compound's lipophilicity enables greater penetration into cell membranes and greater potential for membrane disruption, resulting in greater toxic potency. More recent research has focussed on linking compound functional groups to acute toxicity, and no clear evidence suggests non-specific narcosis as the sole driver of toxicity. For example, studies investigating the degradation of OSPW by microbes suggest that AEO composition shifts to larger, more recalcitrant compounds as they are degraded over time (Lai et al., 1996). Because this compositional shift corresponds with a reduction in toxicity, the concept that larger AEO are less toxic was widely accepted (Holowenko et al., 2002; Lo et al., 2006; Frank et al., 2009). A definitive assessment revealed that the lower toxicity of higher molecular weight compounds was likely due to an increase in hydrophilic carboxylic acid groups with resulting reduction in lipophilicity (Frank et al., 2009). However, more recent research has shown that there is no definitive trend between AEO molecular weight and toxicity when sensitivities of different fish species was compared (Bauer et al., 2017). This has led to observations that AEO may exhibit additional MOA such as oxidative stress (He et al., 2012; Wiseman et al., 2013). Additionally, structural similarities between AEO and compounds like Ibuprofen and estrogen have suggested MOA resulting from competitive binding (Tollefson et al., 2012). AEO contains >1000 chemical constituents with a broad diversity in structural and chemical characteristics, therefore, specific compounds responsible for toxicity are still largely unknown (Frank et al., 2008). Furthermore, recent observations that various taxa display a range

in sensitivities to AEO (Marentette et al., 2015b; Bartlett et al., 2017) has further challenged identification of toxic drivers.

1.3. Challenges Associated with AEO Research

1.3.1. AEO quantitation

The study of the chemistry and toxicology associated with AEO is hampered by technical challenges. These challenges are rooted in the difficulty in obtaining representative oil sands AEO extracts and the fact that there are no AEO chemical standards commercially available. This poses difficulties with AEO chemical analysis because instrument calibration relies on commercial NA mixtures which aren't wholly inclusive of AEO classes and are not representative of bitumen-derived AEO.

The most common extraction method involves the acidic extraction of AEO and their reconstitution in an alkaline solution or polar solvent (Frank et al., 2006), and has been used by the majority of toxicological assessments currently in literature (Farwell et al., 2006; Nero et al., 2006; Kavanagh et al., 2012; Woodworth et al., 2012; Leclair et al., 2013; Scarlett et al., 2013; Marentette et al., 2015a; Marentette et al., 2015b; Bartlett et al., 2017; Bauer et al., 2017). Unfortunately, the volume of acids, bases and solvents required in the extraction procedure make it a major undertaking. Because the AEO extraction process is very labour intensive, many studies have used whole OSPW, commercial NA mixtures, and/or AEO surrogates for testing. Although whole OSPW contains bitumen-derived NAs, it also contains a plethora of other contaminants including metals, humic acids, and polycyclic aromatic compounds. This makes interpretation of toxicity from whole OSPW exposure difficult and not specifically attributable to AEO. Unfortunately, commercial NAs are not very representative of oil sands AEO and

consequently present challenges resulting in misrepresentation in chemical and toxicological characterizations. Five commercial preparations are typically used as analytical standards; Acros, Merichem, Aldrich, Kodak, and Fluka. Studies have shown that these commercial mixtures are of dissimilar composition to each other (Hindle et al., 2013; Lu et al., 2013). Furthermore commercial mixtures are chemically and toxicologically different from OSPW AEO (Grewer et al., 2010; Marentette et al., 2015a). For example, when compared to a Kodak or Merichem commercial NA mixture, oil sands-derived AEO display a greater distribution of acids in the mid-mass range (150-350 amu) and a higher percentage of acids with >22 carbons (Clemente et al., 2003). Commercial NAs and surrogates are representative of "classical" NA structures and lack non-classical structures such a dicarboxylic acids, and heteroatoms. Due to drastic differences between AEO and commercial NAs, surrogates, and whole OSPW, obtaining and studying OSPW-derived AEO presents great value to the interpretation of oil sands-related research.

Other challenges inherent in the analysis of AEO are due to the diversity of analytical instrumentation used for derivation of AEO concentration. These instruments include electrospray ionization mass spectrometry (ESI-MS), electrospray ionization high-resolution mass spectrometry (ESI-HRMS), gas chromatography (GC/MS), gas chromatography tandem mass spectrometry (GC-MS/MS), liquid chromatography quadrupole time-of-flight mass spectrometry (LC/MS-QToF), and fourier transform infrared spectroscopy (FTIR), which require different sample preparation methods and produce varying results. In general, high resolution analytical methods generate lower, more accurate AEO concentrations compared to low resolution methods (Brown and Ulrich, 2015). For example, FTIR and GC low res-MS have been shown to produce false high concentrations of AEO compared to other high resolution

analyses (Yen et al., 2004; Martin et al., 2008; Han et al., 2009; Headley et al., 2009b). Detection of AEO classes also differs depending on whether an ESI-MS instrument is operated in negative- or positive-ion mode (Headley et al., 2013b). For example, positive-ion mode has been shown to better detect species containing sulfur-containing AEO heteroatoms (Barrow et al., 2010). Therefore, AEO ion composition derived from analysis is largely dependent on standards used, which inevitably produce unique distributions due to their inherent variability.

1.3.2. Elucidating AEO toxicity

Similar to challenges associated with AEO quantitation, the lack of representative commercial standards has presented challenges with interpretation of AEO toxicity. A major detractor is the fact that commercial NAs display greater toxicities than oil sands AEO (Marentette et al., 2015a; Bartlett et al., 2017). More comprehensive analyses of AEO-associated toxicity have therefore utilized oils sands extracted AEO, which presents its own challenges. Specifically, the diversity of physico-chemical components results in differing toxicities, dependent upon source material, test organism,and assessed toxicological endpoint.

In order to elucidate some of the toxicity associated with AEO physico-chemical properties, chemical fractionations have been useful. For example, a fractionation method employed argentation solid phase extraction with polar solvents, and was able to separate organics based on aromaticity (Jones et al., 2012). Another fractionation method involving cyano column chromatography was able to separate AEO into four fractions based on polarity using different solvent mixtures (Borgund et al., 2007). Both of these methods are useful for characterization of organics analytically but neither is suitable for the preparative bulk isolations required for toxicity testing. Previous attempts to fractionate OSPW based on solubility have generated

fractions with a substantial degree of overlap with regard to AEO species present (Lo et al., 2006; Grbovic et al., 2012; Huang et al., 2015). Unfortunately, fractionations based on solubility have not led to the assignment of structure-toxicity relationships for specific compound classes. Therefore, the challenge remains to develop methods to isolate distinct physico-chemical fractions of this complex mixture of oil sands AEO in quantities sufficient to conduct toxicity tests.

1.3.3. Tailings contaminant fate

Industry operators have invested substantial effort to prevent tailings from leaching into underlying groundwaters and contaminating surface waters. As such, tailings pond construction methods include perimeter dykes, low-permeability clay-till dyke material, internal dyke drainage, and tailings interceptor ditches (Ferguson et al., 2009; Yasuda et al., 2010; Holden et al., 2011). Despite these efforts, recent evidence suggests OSPW infiltration into underlying groundwaters is occurring (Ferguson et al., 2009; Oiffer et al., 2009; Yasuda et al., 2010; Ross et al., 2012; Frank et al., 2014; Roy et al., 2016). Although seepage has been identified, determining the degree to which it affects the natural environment remains difficult. Studies have identified that OSPW can change chemically due to interaction with underlying substrate. For example, total polar organic concentrations decrease in seepage plume samples further from the source (Ahad et al., 2013), likely due to sorption by soils (Janfada et al., 2006). Overall, tailings pond seepage components appear to become less distinguishable from constituents present in surface waters the further they are sampled from the plume source, due to transport through substrate and mixing with natural groundwaters (Janfada et al., 2006; Holden et al., 2011; Headley et al., 2012; Ahad et al., 2013). In addressing this concern, recent research has focussed

on chemically profiling OSPW sources and developing methods to differentiate OSPW from natural surface and groundwaters. Chemical fingerprinting of anthropogenically-derived constituents has been achieved using parameters such as molecular charge/weight ratio (Barrow et al., 2015; Holowenko et al., 2002), AEO fluorescence spectra (Frank et al., 2014; Kavanagh et al., 2009), heteroatom proportions (Barrow et al., 2015; Frank et al., 2014; Headley et al., 2011b), and double-bond equivalents (Barrow et al., 2015; Headley et al., 2011b). Regardless, quantifying the toxicological risk present is a challenge due to the difficulty in determining the level of environmental contamination that is directly a result of industrial activity.

Research in the area of oil sands toxicology is inundated with analytical and toxicological challenges. These challenges include a lack of chemical standards, the variability in analytical methods, variability in AEO composition spatially and temporally, and the presence of natural background contamination. The development of representative oil sands standards could greatly improve the current knowledge gaps inherent in oil sands research. Additionally, a comprehensive analysis of AEO constituents from naturally and industrially-derived bitumen in conjunction with toxicological comparisons is necessary for the advancement of this field of research.

1.4. Research Objectives and Experiments

The overall scope of this study involved contributing to the understanding of chemical composition and toxicity of the oil sands dissolved organic components. Broadly, the present study intended to identify components driving toxicity of bitumen-influenced waters. Specifically, the focus was to compare AEO composition and toxicity between industrially-derived and naturally-derived sources. Furthermore, a toxicological approach proposed to

evaluate and compare multiple taxa for identification of relatively sensitive species. To accomplish this, three objectives were identified. These were a comparison between: isolated AEO fractions, natural and industry-influenced AEO, and relative sensitivities of aquatic organisms. The following four experiments were designed to meet the objectives.

1.4.1. Experiment 1

Because of difficulties in extracting oil-sands dissolved organics, related literature is lacking chemical and toxicological experimentation utilizing AEO derived from industry and natural sources. A novel bulk extraction method would serve to increase AEO yield and allow for the greater cross-laboratory comparability. Subsequent fractionation at a preparative scale would allow more comprehensive toxicological analyses to a variety of taxa.

Objective: To develop a method for bulk extraction and fractionation of AEO. The method was developed using an aged OSPW source, followed by chemical characterization of generated fractions.

Hypothesis: Utilizing adsorbent resins in the extraction of AEO will improve comprehensive yield and be applicable to the fractionation of AEO from a variety of water sources in the oil sands region. The isolated fractions will display distinct AEO compound distributions and toxicities.

Methodology: The extraction and fractionation relies on the range in individual compound chemical properties present in the suite of dissolved organics. The method involves the use of synthetic adsorbent resins which selectively trap polar organic compounds. A preliminary pH adjustment of OSPW facilitates the precipitation of select dissolved organics rendering them amenable to extraction on a resin. This is followed by a reversed-phase solid phase extraction

(SPE) technique to trap precipitated organics. A soxhlet extraction of the resin using solvents varying in polarity enables the isolation of fractions containing organics with differing polarities. Chemical characterization of isolated fractions was conducted using a suite of analytical techniques including; ESI-HRMS, GC-MS/MS, LC-QToF, and SFS.

1.4.2. Experiment 2

Assessing the chemical characteristics of dissolved organics in aged tailings has been studied extensively with regard to compound cyclicity, molecular weight, and carbon number and their relation to biodegradation and bioavailability. Information regarding the relative polarity of dissolved organics and their relation to toxicity has not been fully explored. Furthermore, research regarding the toxicity of aged tailings OSPW has typically evaluated the marine bacterium *Vibrio fischeri* (Microtox® assay) while relative sensitivities of more relevant taxa is largely lacking. An understanding of dissolved organic fraction toxicities from aged industrial sources to multiple organisms could aid in the development of a reclamation strategy of decommissioned tailings ponds.

Objective: To toxicologically characterize previously isolated aged OSPW fractions using a suite of bioassays including representative aquatic species indigenous to the oil sands region.

Hypothesis: The range in polarities of the three fractions will display a concurrent range in associated toxicities to all bioassays, but display similar internal trends within each assay.

Methodology: Short-term acute and sub-lethal data were generated for *V. fischeri, H. azteca, D. magna, L. cardium, P. promelas,* and *O. latipes*. All organisms were exposed to whole water, three isolated dissolved organic fractions, and a Recombined treatment representing all three fractions recombined and absent of inorganics.

1.4.3. Experiment 3

The natural background level of bitumen contamination in aquatic systems in the oil sands region makes determining the degree of anthropogenic contamination difficult. Therefore, there is a need for a comprehensive chemical analysis of naturally-derived AEO. Tailings pond OSPW has recently been documented to leach into groundwater, which acts as a transportation vector for surface water contamination. Identifying compositional differences between naturally-influenced and OSPW-influenced groundwaters is important for understanding contaminant fate, transport, and potential environmental impact of dissolved organics in the oil sands region. Fractionation-based investigations allow for the isolation of chemically distinct properties. Subsequent comparison of fractions within and between sites enables identification of relative AEO composition variability.

Objective: To chemically characterize dissolved organics in natural bitumen-influenced and OSPW-influenced groundwaters.

Hypothesis: Naturally-derived AEO will display chemically distinct AEO composition compared to industrially-derived AEO.

Methodology: Four bulk samples (~200L) of bitumen-influenced groundwaters were collected for extraction and fractionation using the previously developed method (Experiment 1). Natural groundwater sites included two natural bitumen-influenced groundwater sites which were within the Athabasca oil sands deposit but outside of industrial operations. Two industry-influenced sites were selected by their close proximity to oil sands operations and their previously documented contamination by tailings seepage. The three generated fractions for each site were chemically analyzed using ESI-HRMS, GC-MS/MS, LC-QToF, and SFS.

1.4.4. Experiment 4

There is a natural background level of bitumen contamination in aquatic systems in the oil sands region. There is a need for a comprehensive toxicological analysis of naturally-derived vs anthropogenically-derived AEO. Also, little is known about the relative sensitivities of aquatic organisms exposed to bitumen-influenced groundwater AEO. Monitoring would benefit from a comprehensive chemical and toxicological comparison between naturally-derived and industry-derived AEO.

Objective: To compare overall toxicity between industry-derived and naturally derived bitumen-influenced groundwater sources. To compare drivers of toxicity across groundwater sites and identify species sensitivities relating to toxic components therein.

Hypothesis: There will be an observable difference between the toxic potencies of natural and bitumen- + natural-influenced groundwaters. Species sensitivities will correlate to identified drivers of toxicity and remain consistent across sites.

Methodology: Previously generated groundwater fractions (Experiment 3) were prepared for toxicity evaluation. Toxicological assessments involved the comparison of *V. fischeri, H. azteca, D. magna, L. cardium, P. promelas,* and *O. latipes.* All organisms were exposed to whole water, three isolated dissolved organic fractions, and a Recombined treatment representing the re-assemblage of all three fractions.

Chapter 2. A Preparative Method for the Isolation and Fractionation of Dissolved Organics from Bitumen-Influenced Waters

2.1. Overview

The surface mining of oil sands from the Athabasca deposit north of Fort McMurray, Alberta produces considerable tailings waste which is stored in large tailings ponds on industrial lease sites. With the advent of oil sands end-pit lakes and decommissioned tailings ponds, viable strategies for the detoxification of oil sands process affected water (OSPW) are under investigation. One of the greatest challenges in this regard is determining drivers of toxicity within OSPW, recognizing that differences in ore quality, and bitumen extraction and separation methods influence chemistry and toxicity. Dissolved organic compounds concentrated in tailings are recognized as having a significant influence on toxicity, while high levels of inorganic contaminants are also of concern. In order to assess the toxic potential of the suite of dissolved organics in OSPW, a method for their extraction and fractionation was developed. The objectives of the method required high dissolved organic recovery, adequate separation of fractions, and ability to process a variety of water sources at a preparative scale. This was achieved with a solid phase extraction approach. The method successfully separated organic compounds from 180 L of an aged OSPW source into three fractions (F1-F3) with increasing polarities. Chemical characterization of the generated fractions including electrospray ionization high-resolution mass spectrometry (ESI-HRMS), liquid chromatography quadrupole time-of-flight mass spectrometry, gas chromatography triple quadrupole time-of-flight mass spectrometry, and synchronous fluorescence spectroscopy (SFS), verified fractionation based on polarity. Additionally, ESI-HRMS class distribution data and SFS identified increased degree of oxygenation and degree of aromaticity, respectively, as associated with increased polarity. Method validation, which included method and matrix spikes with surrogate and labelled standards, confirmed separation according to polarity and verified high recoveries (75 - 96.3%). Because this novel method is

capable of extracting large volumes of source water types it is amenable to thorough chemical characterization and toxicological assessments with a suite of bioassays. As such, this protocol will facilitate the identification of toxic components within bitumen influenced waters from a variety of sources.

2.2. Introduction

The Canadian oil sands region, located in northern Alberta, contains one of the largest petroleum deposits worldwide, and extraction of the mineable bitumen has increased nearly 1000% in the last 4 decades (Royal Society of Canada Expert Panel, 2010). Bitumen is a viscous hydrocarbon mixture which is extracted via in-situ or surface mining methods and is ultimately upgraded to a synthetic crude oil. Extraction of bitumen results in the generation of oil sands process-affected water (OSPW) and tailings waste. According to the Alberta Government, in 2013 the surface area of associated oil sands tailings in containments reached 77 square kilometers (Alberta Energy, 2013). In accordance with the Alberta Environment Protection and Enhancement Act, the release of substances that may cause adverse effects to the environment is prohibited and Crown-leased land must be reclaimed (Government of Alberta, 2017, FTFC, 1995a). To address the growing reserve of OSPW on industrial leases, the development and testing of large-scale landscape reclamation strategies has begun.

Much of the difficulty associated with oil sands research is due to the complexity of OSPW. There are a plethora of inorganic and organic constituents present as a complex mixture, many of which are not fully characterized and remain undiscovered. Among the most toxic constituents of OSPW is the polar organic fraction (Allen, 2008; Royal Society of Canada Expert Panel, 2010), which includes a subclass of O_2 compounds which is referred to as naphthenic

acids (NA) (Headley and McMartin, 2004; Clemente and Fedorak, 2005). Industrially-derived NA have been shown to be acutely toxic to a wide variety of aquatic organisms (Headley and McMartin, 2004; Allen, 2008; Royal Society of Canada Expert Panel, 2010) and also to elicit reproductive impairment in fish (Kavanagh et al., 2012). As a result, research during the past decade has focussed on NA characterization and toxicity. However, recent research has identified that oil sands polar organics contain a plethora of diverse constituents, collectively termed acid-extractable organics (AEO) (Brown and Ulrich, 2015). Specifically, while AEO include the classical NAs, they have also been shown to contain heteroatoms and acids that contain dicarboxyl, hydroxyl, dihydroxy, and aromatic moieties (Bataineh et al., 2006; Barrow et al., 2009; Grewer et al., 2010; Headley et al., 2011b; Rowland et al., 2011b; Bauer et al., 2015). Recent toxicological assessments have begun to identify the toxic chemical classes, and O_2-containing substances (which includes NA) have emerged as the principal toxicants within OSPW (Morandi et al., 2015; Hughes et al., 2017). Accordingly, the Alberta government has recently advocated for Canadian Council of Ministers of the Environment (CCME) water quality guidelines for NA (Energy Resources Conservation Board and Canadian Environmental Assessment Agency, 2011; Minister of Environment, 2013). To create these guidelines, a more comprehensive understanding of the chemical composition and toxicity of not only AEO but other dissolved organics is required. This is critical in developing methods to reduce tailings toxicity and demonstrate end pit lakes as a viable reclamation strategy.

Effects-directed analysis (EDA) approaches are well suited to investigate the classes of compounds associated with toxicity to aquatic species which are present in complex mixtures (Brack et al., 2016). However, when applied to the oil sands, these investigations have been hampered by obstacles of scale with regard to sample volumes required for bioassays. A typical

7-day, static-renewal embryo-larval fish bioassay with a 5-concentration dilution series and three replicates can require up to 1 litre of sample volume. Bioassays involving flow-through systems, juvenile/adult fish, chronic test durations, etc., often require even greater sample volumes (USEPA, 2002). As a result, varying degrees of success in characterizing bioactive substances have been achieved, and it is worth noting that the advances that have been made were often with single bioassays (Armstrong et al, 2009; Frank et al., 2006; Johnston et al., 2017; Kavanagh et al, 2012; Lo et al., 2006; Nero et al., 2006; Scarlett et al., 2013), often in an in vitro scale that requires extrapolation to effects at the individual or population levels.

Previous attempts to fractionate OSPW based on solubility have generated fractions with a substantial degree of overlap with regard to NA species present (Lo et al., 2006; Grbovic et al., 2012; Huang et al., 2015). One solubility-based study incorporated anion-exchange chromatography, eluting with buffers encompassing a range of pH values (Lo et al., 2006). While this study was still limited in its capacity to generate distinct fractions, it was able to observe a pKa-dependent trend where higher pKa NAs exhibited lower potencies using Microtox assays (Lo et al., 2006). Unfortunately, fractionation attempts based on solubility have not led to the assignment of structure-toxicity relationships for specific compound classes. Argentation solid phase extraction (SPE) achieved separation of dissolved acids based on aromaticity through the use of different mobile phases (Jones et al., 2012). This study was able to generate two main fractions, one containing alicyclic acids and the other containing aromatic carboxylic acids. A separate study by Borgund et al. (2007) used cyano HPLC column chromatography to separate NAs into four fractions based on polarity using different solvent mixtures (hexane, dichloromethane, and methanol): non-polar compounds, carboxylic acids, phenols, and polyfunctional compounds. Both of these methods were suitable for the analytical

characterization of acids, however neither generated sufficient quantities required for toxicity testing using ecologically relevant species. Lack of sufficient fraction volume in these studies is likely due to the absence of commercially available high-volume chromatography columns, and methodology for high-volume throughput of samples. Finally, a study utilizing preparative fractional distillation was able to produce 5 fractions of AEOs isolated from fresh OSPW based on boiling point (Frank et al., 2008). Chemical characterization revealed that the distillation method was able to isolate fractions with increases in boiling point associated with increasing mean molecular weight, aromaticity, and heteroatom content (Frank et al., 2008; Bauer et al., 2015). Although this method produced sufficient material to conduct toxicity assays with several test organisms, the extraction method was biased toward O_2 species and each fraction contained a high degree of overlap in molecular weight.

In order to determine principal toxic components of OSPW and other bitumen sources, EDA studies need to utilize large volumes of source waters for use in bioassays of relevant vertebrate and invertebrate species. Therefore, the challenge remains to develop a method capable of isolating all soluble organics and producing chemically distinct fractions in the large quantities required. Worthy of note, one successful fractionation approach involved OSPW collected from the oil sands industry's first end pit lake and incorporated an initial extraction at two different pH's, followed by preparative HPLC chromatography fractionation (Morandi et al., 2015). Using an in vivo bioassay to an ecologically relevant test species (96 h fathead minnow embryos), the authors determined that the toxicity of OSPW was largely attributable to the O_2 NA species, but non-acid species also contributed to the overall toxicity. While these findings highlighted the contributions of non-acid species to the toxicity of OSPW, there remains a

paucity of information regarding the identity of bioactive substances in surface and groundwaters influenced by natural bitumen-derived dissolved organics.

To add further complexity, the majority of fractionation and toxicity analyses on OSPW have used fresh tailings as a source material. Unfortunately, this has led to major knowledge gaps regarding constituents present in aged tailings and the relative toxicities of components therein. There is ample research identifying both chemical and toxicological differences between fresh and aged tailings (Siwik et al., 2000; Bataineh et al., 2006; Han et al., 2009; Marentette et al., 2015a; Bartlett et al., 2017), specifically with regard to natural degradation of dissolved organics by algae and bacteria (MacKinnon and Boerger, 1986; Herman et al., 1993; Lai et al., 1996; Clemente et al., 2004). With the advent of decommissioned tailings ponds and end-pit lakes, risk assessments of aged mixtures and comparisons to natural mixtures are needed. Previous attempts by the present authors to recover dissolved organics from aged OSPW using a method developed for fresh tailings (Frank et al. 2006) yielded low recoveries (<10%). This exemplified the differences in chemistry and consequential difficulty in working with aged mixtures and underscored the need for a revised method that could be used for all bitumen-influenced waters.

The objective of the present study was to develop a robust preparative-scale extraction and fractionation method that could be applied to the range of relevant bitumen-influenced water sources. Moreover, this objective required that the method be capable of producing large quantities of each fraction to enable chemical characterization and toxicity evaluations using a suite of ecologically relevant test organisms and endpoints.

2.3. Methods and Materials

2.3.1. Chemicals and Reagents

Chemicals used for the preparative scale fractionation and chemical analysis (methanol (MeOH), ethyl acetate (EtOAc), toluene, and hydrochloric acid (HCl)) were purchased from Fisher Scientific (Mississauga, ON). Sodium hydroxide (NaOH) was purchased from Sigma-Aldrich® (Oakville, ON). Chemicals used for method development (hexane, ethanol, acetonitrile (ACN), and dichloromethane (DCM)) were purchased from Fisher Scientific (Mississauga, ON). Chromatography resin Isolute® ENV+ was purchased from Biotage® (Charlotte, NC), Diaion® HP20 and Sepabeads® SP825 were supplied by Itochu Chemicals America (Farmington Hills, MI), Oasis® HLB, and Oasis® MAX resins were supplied by Waters Ltd (Mississauga, ON).

2.3.2. OSPW Sample Source

An aged OSPW sample was chosen for method development to provide an example of conditions relevant to end pit lakes as well as a proxy for surface water bitumen-derived organic sources influenced by natural degradation processes. Approximately 2000L were pumped from a test pond (Pond 9) on Syncrude Canada Ltd.'s lease in 2011, into two 1000-L polypropylene totes. This test pond was originally filled in 1993 with OSPW from an active tailings pond (Mildred Lake Settling Basin), with no subsequent amendment other than natural precipitation and evaporative processes (Siwik et al, 2000). Following method development experiments and scale-up, the preparative scale fractionation was conducted on 180 L.

2.3.3. Bench Scale: Extraction Method Development

Three bench scale extraction techniques were evaluated. Throughout, "bench scale" is defined as small-scale experiments and involved sample volumes in the range of 30 - 3000 mL. The main objective of the bench-scale experiments was to determine which method and resin type provided the greatest AEO yield. The results of these evaluations are presented in Figure 2.1, where the y-axes indicate recovery of dissolved organics (mg/L). The three evaluated methods included a previously defined acid precipitation method (Frank et al., 2006), a resin settling/flocculent approach, and an SPE approach (Figure 2.1B). The settling/flocculent approach involved mixing methanol pre-conditioned SPE resins (described below) with water samples and allowing them to settle or floc for 24 h or 48 h. The method followed SPE theory (International Sorbent Technologies, 2001; Argonaught Technologies, 2002) such that acidic compounds present in solution were expected to adsorb to the resin where they were extracted via acid precipitation methods (Frank et al., 2006). The SPE approach followed standard SPE protocols (International Sorbent Technologies, 2001; Argonaught Technologies, 2002) adapted for the concentration and clean-up of AEO as described previously (Frank et al., 2008; Gagné et al., 2011). Briefly, the current SPE method involved the use of an organic solvent to pre-condition a porous resin (stationary phase) that was packed in a column, in order to adsorb organic compounds. Resins with different chemical and structural properties (hydrophobic, ionic, surface area, etc.) can be selected with the intention of best capturing an analyte of interest. In this case, the sample water was acidified to precipitate AEO in order that they preferably adsorbed to the resin. Following loading and passage of the water sample through the column, sorbed AEO were then removed and isolated by elution with an organic solvent (mobile phase) through the same column. The latter two methods (settling/flocculent and SPE) were themselves

evaluated with five different solid phase resin types: hydroxylated polystyrene divinylbenzene (ENV+, Biotage®, NC USA), unsubstituted polystyrene divinylbenzene copolymers (HP20 and SP825, Diaion®, MI USA), divinylbenzene copolymer and divinylbenzene copolymer modified with dimethylbutylamine (HLB and MAX, respectively, Oasis®, ON Canada), representing a range of adsorptive properties. Although the settling/flocculent approach generally provided better results than the acid precipitation method (Figure 2.1A), the SPE method provided the best recovery overall regardless of resin type (Figure 2.1B). Based on these results, the SPE method was selected for further experimentation to determine the best resin. Throughout the SPE extraction tests, the ENV+ stationary phase outperformed all other resins tested, displaying the most consistently high AEO recovery (Figure 2.1C) and diminishing loss associated with breakthrough (Figure 2.1D), measured using high-resolution electrospray ionization mass spectrometry methods described in Appendix A (A1). In applying an SPE approach, separation of inorganics from organics was initially achieved. The result was an extraction method which utilized ENV+ resin as a stationary phase to recover AEO from acidified aged OSPW, which were then eluted with methanol.

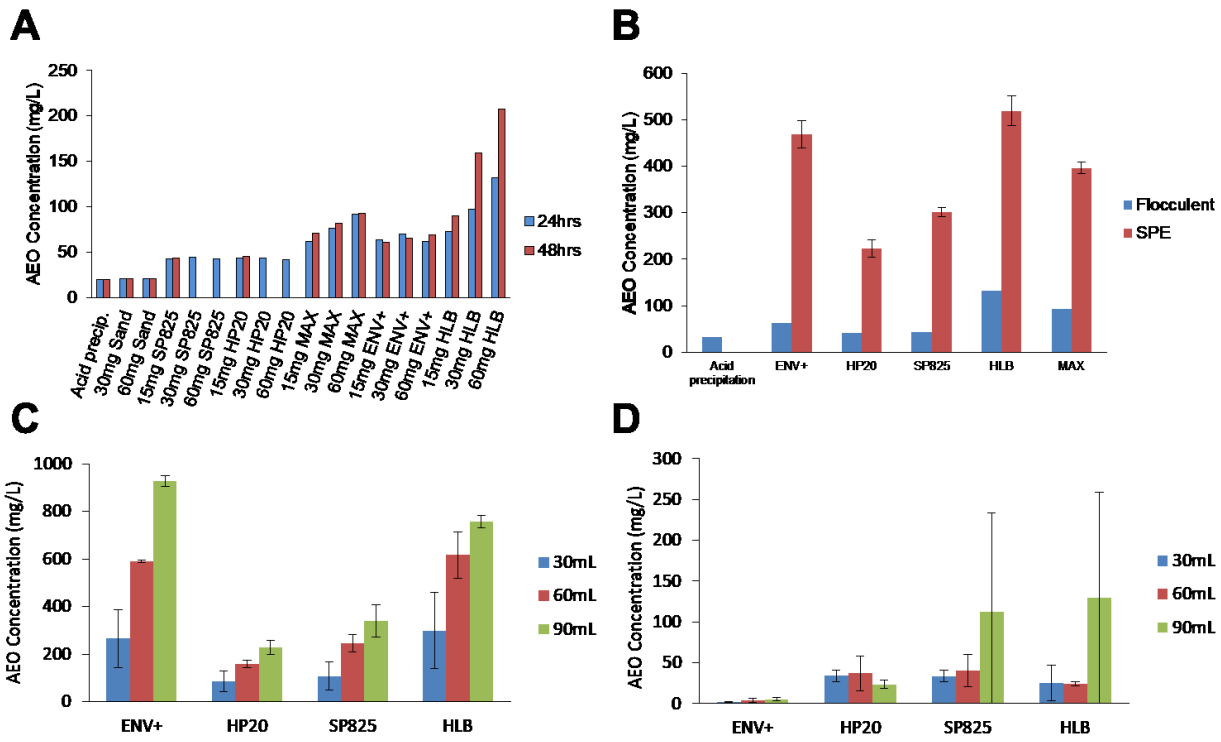


Figure 2.1. Bench-scale method development to select best application of a stationary phase resin. AEO concentrations represent equivalently concentrated sample volumes. Extraction of AEO from 30mL OSPW with different resin types was assessed using a settling/flocculent method at 24 h and 48 h settling times (A). The SPE method was assessed and compared to the settling/flocculent method using 30 mL of OSPW and 60 mg of different resins (B). SPE method AEO yield (C), and breakthrough (D) using 60 mg resin with increasing OSPW volumes were compared across resin types.

2.3.4. Scale-up to Preparative Extraction

Scale-up experiments of 10x and 100x relative to the previous 30 mL OSPW loading volume (300 mL and 3 L OSPW, respectively) were conducted using custom packed ENV+ columns for which recovery of AEO was optimized (Figure 2.2). The procedure was run in duplicate with the addition of a method blank, which involved conducting the extraction using de-ionized water as the source material. Importantly, in order to maintain high yields with increasing cartridge diameter, resin bed heights (resin mass) and loading/elution flow rates were altered according to equations, outlined previously (Rathore and Velayudhan, 2002), accordingly at each step (Figure 2.2). Experimentation to evaluate resin capacity was conducted using 60 mg of ENV+ resin with increasing OSPW volume (Figure 2.2A). Subsequently, flow rates were assessed at each OSPW loading volume scale (1x, 10x, 100x) in order to optimize method efficiency (Figure 2.2B, C, D).

Following optimization of an extraction method, established parameters were employed for development of a fractionation procedure. During fractionation method development, the separation of the least polar from the most polar compounds was ultimately achieved by conducting the extraction in two stages, initially at pH 11, and then at pH 2. For each stage, the previously optimized SPE conditions (flow rates, resin ratio) were applied and elutions with various solvents of different polarities were evaluated to achieve maximal solubilities of recovered organics.

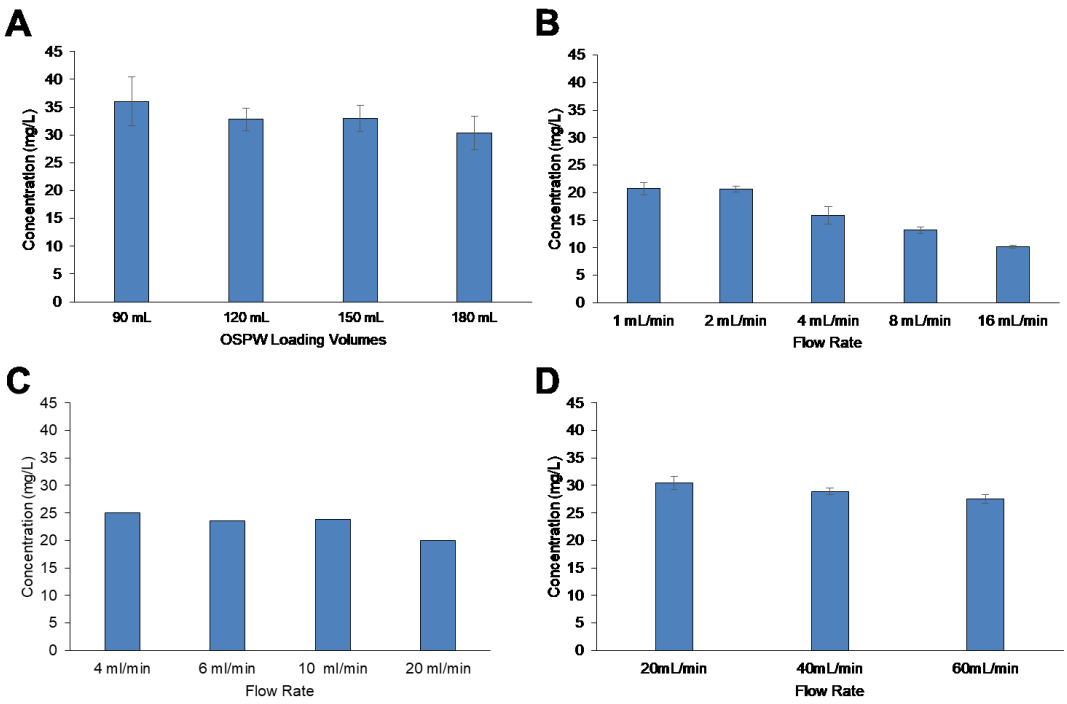


Figure 2.2. Scale up of ENV+ SPE method showing AEO yield (using 60 mg resin) with increasing OSPW volume (A). Scale up experiments were conducted at 1x (B), 10x (C), and 100x (D) the original 30-mL OSPW volume and 20 mg resin mass. Scale up experiments (A - D) were normalized to 1-L for comparison of AEO concentration (y-axes).

2.3.5. Preparative Fractionation

Previous bench-scale and scale-up experimentation ultimately led to the development of a preparative fractionation method with the ability to process 200 L of OSPW. The preparative fractionation procedure was conducted in two stages; addition of base to the OSPW (Stage 1; pH 11) and the subsequent acidification stage (Stage 2; pH 2) (Figure 2.3). The preparative fractionation apparatus consisted of a glass column with plunger (10 cm ID x 30 cm height, Spectrum Chromatography, Houston, TX), two 200-L HDPE barrels, and a controller and motor (Cole-Palmer) with a rotary vane pump head (Procon Pumps). The column was operated as an SPE cartridge where feedstock flow was directed onto the resin bed using an adjustable plunger. In place of a vacuum pump typical of SPE, a water pump was used to pull the initial sample from the sample barrel through the resin in the first column (SPE-1, Figure 2.3) with negative pressure and transfer of the filtrate to a second barrel.

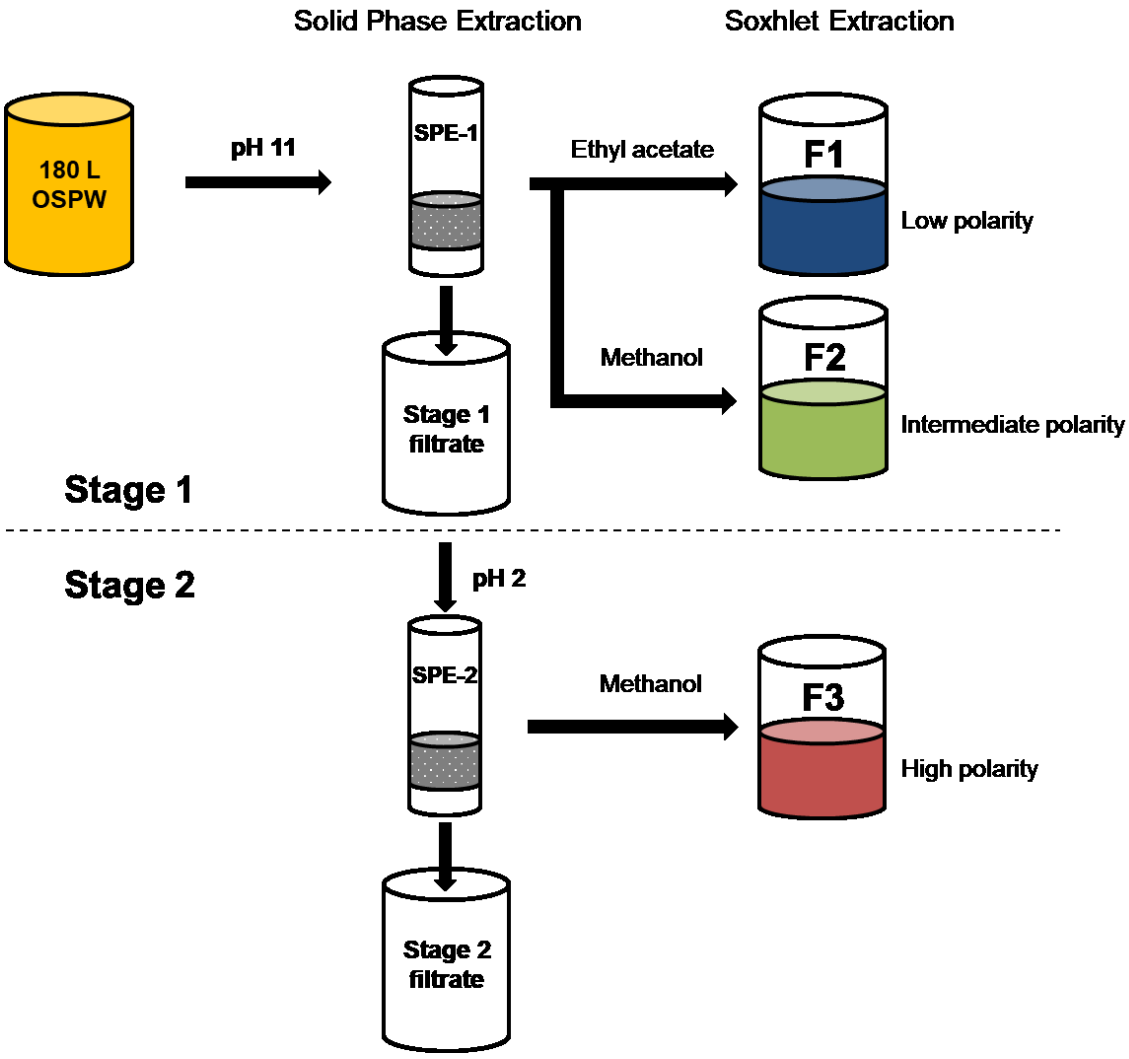


Figure 2.3. Fractionation method schematic displaying Stage 1 and Stage 2 SPE loading followed by soxhlet extraction using solvents indicated. The fractionation resulted in the generation of fractions containing dissolved organic constituents of relative lower polarity (F1), intermediate polarity (F2), and higher polarity (F3).

2.3.6. Stage 1: Preparative Neutral Extraction

Using a water pump, a loading volume of 180 L of OSPW was transferred from 1000-L containers to 200-L barrels and the pH was raised to 11.0 ± 0.5 with 10 M NaOH. The OSPW was then mixed for approximately 1 hour with a hand drill fitted with a teflon mixing rod and allowed to stand for 12 hours. Following the equilibration period, the pH was adjusted, re-mixed, and left to stand for at least another 6 hours until the pH was stable. The ENV+ resin bed was added to the column as a slurry (120 g in 600 mL EtOAc) and was first conditioned with 1.5 L of EtOAc, a second solvent wash of 1.5 L of MeOH, and a final step with 6 L of pH 11 de-ionized (DI) water.

The column (SPE-1) was then plumbed to two barrels (one containing OSPW, one empty) in order for the OSPW to be extracted through the conditioned column and the filtrate to flow into an empty barrel. Throughout all conditioning, equilibration, and OSPW-loading steps described herein, the solvent/water in the column was maintained at a height of 10 cm above the resin bed and the plunger at a height of 1 cm above the solvent/water to avoid disturbance of the resin. Likewise, all conditioning, equilibration, and loading herein were pumped through the column at a rate of 100 ± 10 mL/min. Following filtration of 180 L of OSPW, the column was disassembled and the resin carefully transferred into a 4-L glass beaker. The beaker containing the resin was covered with a large Kimwipe® left in a fume hood for 12-24 hrs to dry.

Analytes were extracted from SPE-1 using a large soxhlet apparatus. The dried resin was split between two glass thimbles, sandwiched in each between 500 g sodium sulphate ($NaSO_4$). Analytes were extracted with 1.5 L of EtOAc in each soxhlet apparatus (3 L total) for 12 h. The soxhlet extraction method differs from standard SPE methods that involve solvent elution directly through the SPE cartridge, but still incorporates the dissolution of analytes into the

solvent (discussed below). The EtOAc was pooled and filtered 4 times through 400 g $NaSO_4$ and 8 µm pore filter paper (Whatman grade 40 ashless, Sigma-Aldrich®, Oakville, ON) to remove any water, hereafter referred to as Fraction 1 (F1). The resin was then removed from the thimbles, allowed to dry, and placed in new thimbles with fresh $NaSO_4$. A second soxhlet extraction was performed as described above using 3 L of MeOH, hereafter referred to as Fraction 2 (F2).

2.3.7. Stage 2: Preparative Polar Extraction

For extraction of more polar analytes from OSPW, the Stage 1 filtrate was acidified to pH 2 using HCl (12 M) in the second barrel, in a manner similar to the initial pH adjustment in Stage 1. For preparation of the SPE-2 stationary phase, 120 g fresh ENV+ resin was placed into the cleaned column, conditioned, and equilibrated as described previously for Stage 1, with the exceptions that only MeOH was used and the final conditioning was with pH 2 DI water. Following SPE-2 conditioning, the acidified stage 1 filtrate was pumped from the second barrel, through the conditioned column, and back into barrel 1. The soxhlet extraction of SPE-2 was the same as Stage 1, but only required MeOH, hereafter referred to as Fraction 3 (F3). The extraction of 180 L of OSPW with 3 L of solvent for each fraction represented a 60-fold concentration of dissolved organics. These large volume, highly concentrated batches of each fraction were stored at 4°C until further use.

2.3.8. Chemical Characterization

In addition to the 3 fractions, samples of OSPW were collected for chemical analysis at several points throughout the extraction/fractionation procedure. Samples were collected as

follows: pre-Stage 1 pH 11 OSPW, Stage 1 pH 11 filtrate (after ~10 L, ~100 L, ~160 L), pre-Stage 2 pH 2 filtrate, Stage 2 pH 2 filtrate (after ~10 L, ~100 L, ~160 L). It is important to note that unlike water samples, fraction aliquots did not represent equivalent concentrations of organics, only equivalent fraction volumes. Moreover, although F1-F3 were aliquots from equivalent volumes of solvent and represented concentrated samples, all other samples were not concentrated and only comparable to F1-F3 qualitatively. The chemical characterization conducted on each sample employed the use of several analytical techniques including: negative-ion electrospray ionization high-resolution mass spectrometry with Orbitrap (ESI-HRMS), liquid chromatography quadrupole time-of-flight mass spectrometry (LC-QToF), gas chromatography triple quadrupole mass spectrometry (GC-MS/MS), synchronous fluorescence spectroscopy (SFS), and analysis of total dissolved metals and major ions, all of which are described in Appendix A (A1). All chemical analyses were run in duplicate with respective solvent blanks and internal standards.

For quantitative analyses, including derivation of soluble organic concentrations, double-bond equivalents (DBE) of O_2, and ion class distributions, ESI-HRMS with Orbitrap was conducted at Environment and Climate Change Canada's (ECCC) National Hydrology Research Centre in Saskatoon, SK according to methods outlined in Bauer et al. (2015) and briefly described therein. Qualitative analyses of soluble organics fractions for comparison of relative polarity and abundance from chromatograms were conducted at ECCC's Canadian Centre for Inland Waters in Burlington, ON using LC-QToF and GC-MS/MS. Additional qualitative analyses of soluble organic aromaticity using SFS was conducted at ECCC's National Water Research Institute in Burlington, ON as outlined previously (Peuravuori et al., 2002), with minor modifications which are provided in greater detail in Appendix A (A1).

2.3.9. Surrogate and Matrix Spike Validations

In order to validate the method and investigate what type of organic compounds may be eluting in each fraction, two different stock solutions containing commercial standards as surrogates were fractionated at a bench scale according to the described method. In the first stock solution, surrogate organic compounds with a range in structural and chemical properties (Table 2.1) were spiked into deionized water (referred to as "surrogate standards"). The second fractionated stock solution contained isotopically labelled compounds (Table 2.1) spiked into aged OSPW ("matrix standards"). Treatments were prepared by adding 1 mL of each spiking solution into 99 mL of deionized water or aged OSPW (100 mL total loading volume), then mixing for 10 min at 30°C. Fractionation parameters such as resin conditioning, elution solvents and solvent orders were conducted as described previously for the preparative fractionation above. Specifically, SPE cartridges were packed with 67 mg of ENV+ resin. All resin was conditioned and standards eluted with 10 mL of appropriate solvents at a rate of 1 mL/min. For further quality assurance, a method blank was included and all treatments were run in triplicate. For chemical analysis each 10 mL fraction generated was evaporated under N_2 in a 30°C water bath to dryness and reconstituted in 1 mL of MeOH. Samples were then analyzed using a LC-QToF according to parameters described in Appendix A (A1). Recoveries were determined by comparison of sample results against the original spiking solutions.

2.4. Results and Discussion

2.4.1. Extraction Method Development and Scale-up

The objective of this study was to develop a robust extraction method suitable for the range of bitumen influenced sources in the Athabasca oil sands region that would generate

sufficient quantities of isolated organics for toxicological evaluations using a suite of aquatic test organisms. Bench-scale experimentation evaluated an acid/base precipitation method, solid phase settling/flocculent method, and a SPE method, and identified SPE using ENV+ resin as the method which provided optimal AEO yield. This method was then subjected to scale-up experimentation in order to proceed from a bench-scale to a preparative scale extraction, using loading volumes of 30 mL and 180 L of OSPW, respectively. Reverse-phase SPE was explored for the isolation of soluble organics within bitumen-influenced waters. This method allowed for simple isolation of organics from the initial water matrix and also provided the opportunity for fractionation. In so doing, it also avoided large solvent volumes associated with liquid/liquid extractions on a preparative scale, was amenable to scale-up, and a small-scale variation of this approach was already routinely used for sample clean-up prior, and directly during, NA analysis of OSPW samples (Verbeek et al., 1993; de Campos et al., 2006; Gagné et al., 2011; Headley et al., 2013a). Furthermore, a study that compared different solvents and SPE systems for the extraction of AEO from OSPW reported that the ENV+ resin provided the highest recovery and was able to capture the greatest range in different oxygenated AEO species (Headley et al., 2013a).

Experimental data from AEO extraction methods are presented in Figure 2.1 which are presented with dilution factors incorporated for within-graph comparison. Initial applications of the original acid precipitation method to aged OSPW produced very low AEO recoveries (20 mg/L) as determined by a mass balance approach and hi-res electrospray analysis (Figure 2.1A, B). As the original method (Frank et al., 2006) was developed for AEOs, namely O_2s, for fresh OSPW, we hypothesize that the lower recovery for the aged source was due to compositional differences that occurred during aging. The first set of bench-scale experiments determined that

the use of a resin dispersed in aged OSPW achieved more than 10-fold greater recovery (208 mg/L vs 19.8 mg/L) than the original method (Figure 2.1A). Utilizing the adsorbent resins as a stationary phase for SPE achieved up to 16-fold greater recovery (519 mg/L) than the acid precipitation method (32 mg/L) (Figure 2.1B). In order to determine which stationary phase resin possessed the highest capacity, 60 mg of the four resins were subjected to increasing OSPW volumes. The results for the ENV+ phase were superior, as it maintained the highest recoveries with increasing OSPW volume, exhibited low breakthrough and with little variability (Figure 2.1C, D).

Experimental data assessing ENV+ capacity with increasing OSPW loading volume and flow rates are presented in Figure 2.2 and are standardized to whole OSPW volumes to enable comparisons. In order to determine the resin capacity of ENV+ for dissolved organics, 60 mg was used to extract AEO from increasing loading volumes of OSPW (90 mL, 120 mL, 150 mL, 180 mL) (Figure 2.2A). From 120 mL to 150 mL, and 90 mL to 120 mL OSPW loading, AEO recoveries dropped 2.6 mg/L and 3.2 mg/L, respectively (Figure 2.2A). The 90 mL loading volume displayed the highest AEO recovery overall (36 mg/L). Therefore, a conservative ratio of OSPW (mL) to resin (mg) of 3:2 was adopted. Finally, loading and elution flow rates were optimized. These were conducted as part of the scale-up work, and were initiated using the 3:2 OSPW:resin ratio. As scale-up increased to 10x and 100x, the optimal flow rate chosen was 10 mL/min and 20 mL/min, respectively (Figure 2.2B,C,D). Optimal flow rates did not increase linearly with OSPW volume and resin weight, likely due to changes in resin bed dimensions changing OSPW linear velocity, which required slower relative flow rates to prevent breakthrough (International Sorbent Technologies, 2001). These assessments determined that at

the bench scale a conservative flow rate of 1mL for every 30mL of OSPW could be sufficiently processed every minute, provided the previously established resin mass ratio was used.

2.4.2. Fractionation Method Development

Although we are aware that dissolved organics other than organic acids may be present in the OSPW mixture, instrumentation required the use of organic acid standards. Therefore, the method was developed based on predicted properties of organic acids and described as such herein. Our fractionation method was designed to separate organic compounds by utilising two properties; ionization states, and polarity. Stage 1 involved deprotonating (ionizing) a higher percentage of organic acids by raising the pH to 11 prior to SPE-1 (Figure 2.3). This allowed for only the non-acidic organic compounds in the aged OSPW to be un-ionized, allowing them to be adsorbed to the resin. These compounds could then be extracted from the resin by elution or soxhlet extraction with an organic solvent. The remaining more acidic (ionized) compounds, still in an ionized state, would remain in solution to be later extracted in the second stage. Stage 2 involved protonating the remaining more acidic compounds by lowering the pH of the solution to pH 2 (Figure 2.3). This allowed acidic compounds to precipitate out of solution and prevented loss/breakthrough of those highly polar compounds bound to sample water. In effect, mechanical filtration and molecular adsorption ensured greater capture of polar organic compounds. These compounds were then differentially extracted from the resin using organic solvents with different polarities in the same manner as Stage 1.

The following briefly explains the experimentation leading to the final fractionation method which is described in detail in the Methods and Materials section above. With the development of an extraction procedure complete, subsequent experiments were conducted to

develop a preparative scale fractionation method using SPE. Fractionation parameters such as conditioning/extraction solvents, extraction solvent order, flow rates, and number of stages (columns) were modified. Specifically, extraction solvents hexane, EtOAc, and MeOH, representing a range in polarities, were assessed for their ability to recover dissolved organics and generate fractions with distinct polarities. These were applied to a Stage 1 (pH 11) and Stage 2 (pH 2) OSPW treatment (Figure 2.3) in order of increasing polarity (Hexane < EtOAc < MeOH). Samples from each solvent application were analysed using GC-MS/MS and LC-QToF to determine overlap in fraction chemical composition, while ESI-HRMS determined areas of potential loss in yield. This experimentation identified hexane as a poor extraction solvent with low recovery, resulting in its exclusion. Stage 1 extraction with EtOAc and MeOH generated distinct fractions containing an appreciable amount of organics. Conversely, extraction with EtOAc followed by MeOH in Stage 2 resulted in low dissolved organic recovery in both elutions. This experimentation identified that MeOH alone was able to capture the remaining dissolved organics in Stage 2.

Extraction of organic compounds from the solid phase resin was performed using a soxhlet apparatus. The soxhlet extraction allowed for continual extraction of organics from the resin over a longer duration (12 h) compared to elution, as described in the final method above (Figure 2.3). Quantitative analysis of resulting fractions confirmed scale up experiments (Figure 2.2) that identified a 3:2 ratio of OSPW volume : resin weight capable of optimal recovery. At very conservative resin weights (60 mg resin for 30 mL OSPW), previous experimentation determined that re-use of resin up to three times resulted in loss of recovered organics and increased variability in yield (Appendix A, A2). Due to the modification to a soxhlet extraction step, Stage 1 and Stage 2 were therefore conducted using two separate batches of ENV+ resin.

This ensured that the resin capacity was maintained throughout the procedure by reducing potential loss of organics associated with re-use of resin.

In order for organic compounds to be adsorbed, the resin required a conditioning step. Conditioning of the resin was performed using 1.5 L of the same solvents in the same order as applied for elution as described by solid phase resin manufacturers (International Sorbent Technologies, 2001; Argonaught Technologies, 2002). The conditioning solvent volume was determined by adherence to International Sorbent Technologies (IST) guidelines of 1-2 mL for every 100 mg of resin. Thus, the use of 120 g of resin in the final method allowed for 1.2 – 2.4 L of conditioning solvent. As further described by the IST guidelines (2001), during elution the resin should be saturated by the solvent for at least 1-4 minutes regardless of flow rate. This recommendation, in addition to flow rates established in the scale-up, guided conditioning and sample loading flow rates for the preparative-scale fractionation. Thus, for conditioning the resin, a flow rate for 1.5 L of solvent to saturate the resin for 4 minutes during conditioning allowed a flow rate of 375 – 1500 mL/min. Consequently, a conservative flow rate of 100 mL/min was adopted for solvent conditioning of resin and within apparatus allowances. Resin manufacturer sample loading rates are typically provided for much lower sample volumes and resin weights, and are most dependent on resin bed height and width, which determine sample linear velocities. For this reason, IST (2001) and Argonaught (2002) guidelines suggest increasing flow rates until breakthrough of analytes is observed. Although previous scale-up experiments identified that loading of 300 L of OSPW could be processed at a flow rate of 10 L/min, this was not feasible with the apparatus set-up. Instead, IST (2001) guidelines which suggested 10-120 mL/min flow rates for a 6 mL sample were incorporated. For a conservative approach to the methodology and to maintain consistency with conditioning rates, a loading rate

of 100 mL/min was applied. This new flow rate demonstrated the ability to capture organics at concentrations exceeding those observed in scale-up experiments as confirmed by ESI-HRMS.

The resulting pH adjustments, solvents used, and solvent order were incorporated into the final method, as described in Methods and Materials (Figure 2.3). Briefly, in Stage 1, ENV+ resin was conditioned using 1.5 L EtOAc and MeOH at a flow rate of 100 mL/min, followed by a pH 11 water wash. OSPW at pH 11 was then loaded onto the column and pumped through at a flow rate of 100 mL/min. In Stage 2, a fresh batch of ENV+ resin was conditioned using MeOH, followed by a pH 2 water wash at parameters identical to Stage 1. Stage 1 filtrate was then acidified (pH 2) and passed through the preconditioned ENV+. The ENV+ resin from Stage 1 and Stage 2 were soxhlet extracted for 12 hours for each solvent, using 3 L of EtOAc/MeOH (separately) and MeOH, respectively. These extractions generated three fractions described herein as F1, F2, and F3. Methodology for separation of the dissolved organic analytes resulted in F1, F2, and F3 containing organics ranging from least polar, intermediate polarity, and most polar constituents, respectively (Figure 2.4).

2.4.3. Method Validation

For quality assurance at the preparative scale, a complete fractionation method blank was run which substituted 180 L of deionized water for OSPW. Analysis of samples from the method blank using LC-QToF and GC-MS/MS displayed no appreciable peaks of organic analytes above those of representative solvent blanks (Appendix A, A3). For further quality assurance and assessment of repeatability, the preparative scale OSPW fractionation was run in duplicate. There were no considerable differences between the first and second fractionation of Pond 9 OSPW as observed by LC-QToF (Appendix A, A4), GC-MS/MS, and ESI-HRMS. Although

presented singly herein, all chemical analyses were run in duplicate which were not different in all cases.

Table 2.1. Method recoveries of surrogate standards spiked into deionized water and isotopically labelled standards spiked into an aged OSPW matrix. Mean recoveries ± standard error were derived from LC-QToF analysis against the spiking solutions for each fraction which were then summed for a total method recovery.

Compound	Molecular Mass (g/mol)	Solubility ($LogK_{ow}$)[a]	Retention Time (min)	Recoveries (%)[b]			
				F1	F2	F3	Total
Surrogate Standards							
3,4-dihydroxybenzoic acid	154.12	0.86 - 1.16	3.05	-	-	26.8 ± 0.5	26.8
adipic acid	146.14	0.08 - 0.23	4.26	-	-	44.4 ± 5.4	44.4
3-thiopheneacetic acid	142.17	1.18 - 1.25	7.15	-	-	80.1 ± 5.8	80.1
1,4-cyclohexanedicarboxylic acid	172.18	0.5 - 0.95	7.62	-	-	82.7 ± 1.4	82.7
3-Methyl-2-thiophenecarboxylic acid	142.17	2.03 - 2.24	9.74	-	-	114.2 ± 7.9	114.2
cyclohexane carboxylic acid	128.17	1.77 - 2.36	11.15	-	-	16.4 ± 0.5	16.4
diphenic acid	242.22	2.02 - 2.83	11.43	0.3 ± 0.0	1.2 ± 0.0	109.7 ± 1.2	111.2
2-naphthylacetic acid	186.21	2.74 - 2.81	12.82	1.7 ± 0.2	7.2 ± 0.2	66.1 ± 2.0	75.0
5-(2-thienyl) pentanoic acid	184.26	2.38 - 3.09	13.03	3.4 ± 0.3	3.7 ± 0.2	64.2 ± 3.4	71.3
3-cyclopentylpropionic acid	142.20	2.27 - 2.85	13.29	-	-	36.0 ± 3.3	36.0
decanoic acid	172.26	3.96 - 4.09	16.91	74.1 ± 2.2	17.9 ± 1.3	9.1 ± 0.8	101.1
cyclohexanepentanoic acid	184.28	3.90 - 4.32	16.93	75.5 ± 3.6	15.4 ± 2.2	8.5 ± 1.0	99.4
dehydroabietic acid	300.44	6.35 - 6.52	19.07	95.1 ± 1.9	21.1 ± 0.8	2.7 ± 1.1	118.9
Labelled Standards							
Benzoic-d_5 acid	122.12	1.87 - 1.89	8.72	-	-	93.6 ± 2.4	93.6
9-anthracene-d_9-carboxylic acid	222.24	4.36	13.76	6.8 ± 2.7	30.0 ± 2.2	75.3 ± 0.9	112.1
Decanoic-d_{19} acid	172.27	3.96 - 4.1	16.81	21.3 ± 3.0	8.0 ± 1.4	2.4 ± 1.0	31.7

[a] Predicted values from online resources: https://pubchem.ncbi.nlm.nih.gov and http://www.chemspider.com/
[b] Fraction percentage recoveries are means of three replicates

To validate the method and investigate the type of organic compounds recovered in each fraction, authentic standards of various organic acids were fractionated at a bench scale (100 mL sample volume). In two separate experiments, a method spike containing 13 standards was added

to deionized water while a matrix spike containing 3 deuterated standards in MeOH was added to the aged OSPW. Table 2.1 displays the relative mean recoveries in each fraction as well as the total recoveries across fractions. Surrogates are listed in ascending order of LC-QToF retention time, where longer retention on the instrument reverse phase column indicates lower polarities. The decrease in relative compound polarities coincides with relative abundance in each fraction, as more polar compounds are more abundant in F3, while F1 contains lower polarity compounds. This observation verifies the fractionation methodology in which subsequent fractions were designed to capture dissolved organics with increasing polarity. The majority of the compounds in the standard spike experiment displayed >70% recovery with the exception of 3,4-dihydroxybenzoic acid (26.8%), adipic acid (44.4%), cyclohexane carboxylic acid (16.4%), and 3-cyclopentylpropionic acid (36%). According to the water solubilities of these compounds, they are among the least soluble compounds tested (Table 2.1) and may not have completely dissolved during the spiking preparation. Overall, recoveries were very good (average 76%) and separation by compound polarity was demonstrated. In the matrix spiking experiment, two of the 3 labelled compounds displayed recoveries >90% (Benzoic-d5 acid and 9-anthracene-d9-carboxylic acid), while only 31% of the Decanoic-d19 acid standard was recovered. This low recovery for Decanoic-d19 acid may be due to its low water solubility and potential adsorption onto matrix components as the method recovery for native decanoic acid was optimal. Overall, both standards fractionation experiments validated that the method does show separation of organic compounds based on polarity with adequate recoveries.

Interestingly, results from the method validation experiments indicated that in addition to F3, acidic compounds were captured in F1 and F2, which incorporated a pH 11 pre-loading adjustment to sample water. With this adjustment, it would be expected that only neutral-basic

compounds would be un-ionized, allowing adsorption to the resin. However, although Stage 1 involved a basic extraction, the resin was pre-conditioned to adsorb organic compounds, which was observed herein. For example, class distribution data revealed that the majority of O_2 compounds (including NA) were present in the high pH fraction F1. A similar fractionation study which utlilized pH adjustments and SPE extractions, also observed an abundance of dissolved organics consistent with NA isolated in a high pH fraction (Morandi et al., 2015). This suggests that factors influencing the polarity of OSPW-derived dissolved organics are not solely driven by protonation and deprotonation of carboxylic acids. It is more likely that polarity is governed by factors such as molecular size, functional groups, water solubility, and molecular structure. Therefore, although separation of dissolved organics was not governed by compound ionization, in combination with differential solvent extractions, pH adjustments contributed to successful isolations based on polarity.

The analysis of major ions and metals was performed on water samples taken directly from pre- and post- Stage 1 and 2 steps in the procedure, providing a means to track inorganics in the method (Appendix A, A5). The fractionation design provided for the removal of soluble organics, with the final filtrate theoretically containing all inorganic species. The relatively constant concentration of all major ions and metals from unaltered OSPW to final Stage 2 filtrate after Stage 2 (Appendix A, A5), supports this assertion. The exceptions to this were sodium and chloride. Sodium increased slightly in the pre-Stage 1 sample and chloride increased substantially in the pre-Stage 2 sample, because NaOH was used to raise the pH prior to Stage 1 and and HCl was used to acidify prior to Stage 2. The fact that all other ion/metal concentrations were relatively unaltered, and those that were could be accounted for in the final filtrate following SPE Stage 2, indicate the method was specific in its recovery of dissolved organics.

Table 2.2. Concentration of dissolved organics in fractions and filtrate of aged OSPW determined by ESI-HRMS. Values represent concentrations based on original 180 L water sample.

	Concentration (mg/L)	Contribution (%)
Fraction 1	5.4	15.6
Fraction 2	0.4	1.1
Fraction 3	27.7	79.5
Post-Stage 2 filtrate	1.3	3.8
Total	34.8[a]	100.0

[a] represents a derived theoretical recovery based on all other measured values

2.4.4. Fraction Characterization

Chemical characterization of aged OSPW fractions consisted of a suite of instrumental applications including ESI-HRMS which provided mass-charge distribution, ion class distribution, and double-bond equivalents (of O_2 ions), such as classical naphthenic acids. Additionally, GC-MS/MS and LC-QToF profiling was conducted along with SFS, which provided an analysis of aromaticity.

Dissolved organic concentrations for each fraction and the final filtrate were measured by ESI-HRMS analysis (Table 2.2). Data indicate that the fractionation procedure was able to capture 96.2 % of dissolved organics detectable by ESI-HRMS with 3.8 % loss via breakthrough. Breakthrough represents a loss in recovery due to constituents which did not adsorb to the resin and, therefore, passed through at both stages. This breakthrough/loss was confirmed and quantified by a separate extraction of Stage 2 filtrate using SPE and extraction with MeOH. The distribution of dissolved organics could not be assigned with acceptable error using ESI-HRMS

analysis and is, therefore, not presented herein. In the present study, breakthrough was likely due to a combination of the resin reaching capacity in the area of the resin bed with highest linear velocity or the presence of very polar compounds. The bulk of dissolved organic compounds were captured in F3 (most polar) which comprised 79.5 % of all organics observed, while only 1.1 % were captured in F2 (intermediate polarity).

For determination of ion class distribution and DBE, ESI-HRMS analysis was performed on the three generated fractions. This high-resolution analysis has been successfully used for analysis of AEO in previous research (Scarlett et al., 2013; Marentette et al., 2015a; Morandi et al., 2015; Bauer et al., 2017). Generally, high-resolution analysis has been identified as producing lower, more accurate concentration determinations compared to low-resolution analyses such as FTIR and GC-MS (Brown and Ulrich, 2015). For this reason, and for comparison with some of our previous work (Marentette et al., 2015a; Bartlett et al., 2017; Bauer et al., 2017), ESI-HRMS was conducted. Infusion experiments with ESI-HRMS showed qualitative differences between fraction spectra (Appendix A, A6). A comparison of sample mass spectra revealed very similar distributions between fractions with the majority of the compounds ranging from 200 – 400 m/z, analogous to observations made in previous work (Bauer et al., 2015). The notable exception was a minor "hump" appearing between 0 – 200 m/z in F3. The relative electronegativity of oxygen means that, in similar compounds, those containing more oxygen atoms are relatively more polar. For class distribution data, F1 was comprised of predominantly O_3 and O_4 ions, F2 was dominated by O_4, O_5, and O_5S, and F3 displayed a major contribution from O_4, O_5, and O_6 ions (Figure 2.4A). All fractions displayed minor contributions (<10% each) from O_2 (classical NA), and sulfur-containing ions (O_3S, O_4S, and O_5S). Degree of oxygenated ions increased with fraction number with F1, F2 and F3

displaying no oxygen class ions greater than O_6, O_7, and O_9, respectively (Figure 2.4A). The relative increase in degree of oxygenation with fraction number validates the methods ability to separate dissolved organics based on polarity.

Recent research (Ajaero et al., 2017) has suggested that WAX (weak anion-exchange) resin may provide a slightly improved recovery of O_2 classes relative to ENV+, however these differences appear to be minimal. Taken collectively and considering the margin of error, ENV+ was essentially shown to be equivalent to WAX in recovering the broad range of species within the AEO mixture that was measured and may represent an alternate solid phase for extractions. In addition, our previous work identified a predominance of O_2 classes in fresh tailings using an ENV+ SPE cleanup (Bauer et al., 2015), therefore, the low relative contribution of O_2 ions observed for the aged source is likely due to compositional changes that occur through aging.

DBE analysis represents the double-bond formed in a compound due to the absence of a hydrogen atom, but can also signify a ring formation and degree of aromaticity. In the present analyses, only the O_2 class DBE were examined and the DBE are a percentage abundance relative to the total abundance of O_2 species (the total percent DBE equals the percent O_2 for class distribution) (Figure 2.4B). DBE data for F1-F3 ranged from 1-9 for F1 and F2, and 1-7 for F3 (Figure 2.4B). Given that F1 contained a greater abundance of O_2 species compared to F2 and F3, it is no surprise that F1 contains a greater overall abundance of O_2 DBE. All three fractions display the greatest percent abundance at DBE 4 and second highest at DBE 5.

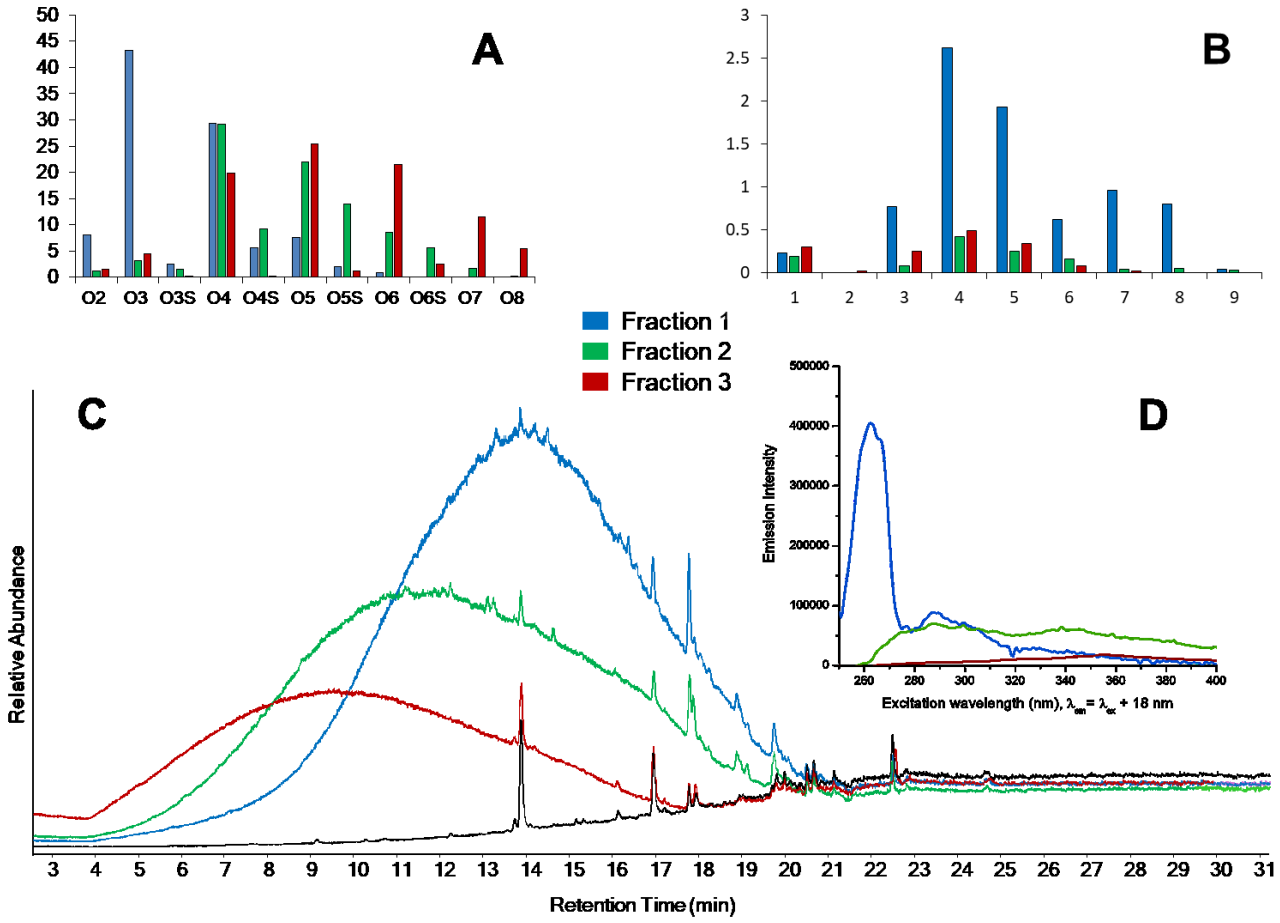


Figure 2.4. Fraction characterization of F1-3 isolated from aged OSPW. Chemical properties analysed for F1-3 include double-bond equivalents of O_2 ions (A), ion class distribution for those species with >2% contribution (B), LC-QToF total ion chromatograms (C), and SFS fluorescence spectra indicating degree of aromaticity (D).

LC-QToF, GC-MS/MS, and ESI-HRMS analyses verified the method's ability to isolate fractions with varying polarities. Negative ion electrospray LC-QToF profiles of fractions F1-3 are shown above in Figure 2.4C. For the reverse phase LC conditions employed, the total ion chromatograms reveal the polarity differences exhibited by the fractions. All fractions present as

individual complex mixtures that are chromatographically unresolved. The maxima of each fraction nevertheless are indicative of the differences in polarities of the components, with F3 maxima eluting first (most polar), F2 maxima intermediate (intermediate polarity) and F1 maxima eluting last (least polar). While it is possible for individual compounds to be present in one or more of the fractions, the differences apparent in these profiles suggest some degree of separation was achieved. Thus, we interpret the data as showing that from F1-F3, compound polarity increased. The LC-QToF results are supported by those obtained by GC-MS/MS (Appendix A, A7). Fraction F1 exhibited the greatest signal intensities of the three fractions, consistent with its content of neutral dissolved organics obtained from pH 11 Stage 1 extraction and solubility in EtOAc (Appendix A, A7a). Although not present in the final procedure, a fourth EtOAc soxhlet extraction (final filtrate) step in the first Pond 9 fractionation was included (shown as orange peaks in Appendix A, A7b). This extract clearly displayed several peaks between 6.6 – 7.5 minutes which were identified as phthalates and likely resulted from handling. The presence of phthalates in the final Stage 2 EtOAc extraction verified that they were likely not present in other fractions and resulted in the exclusion of that extraction step. Figure 2.4D inset displays the SFS profiles of the three fractions. The excitation wavelength of F1 exhibits a narrow peak at ~265 nm, F2 displays a slightly bimodal plateau ranging from 260 – 400+ nm, and F3 displays a broad peak at ~360 nm. The SFS data suggests that from F1 – F3 there is an increase in the degree of aromaticity, and a reduction in abundance of aromatic compounds. According to previous SFS analyses on parent PAHs and other bitumen-influenced waters (Kavanagh et al., 2009; Rowland et al., 2011b), F1 fluorescence appears to be composed primarily of monoaromatic compounds similar to toluene, while F2 and F3 contain mono- and polyaromatic compounds similar to naphthalene, fluorine, and anthracene. Finally, increases in

fraction polarity were substantiated by ESI-HRMS class distribution data (Figure 2.4A) which displayed increases in degree of oxygenation, described in detail above. Because both aromatic content and oxygen content increases the polarity of a compound, both class distribution (ESI-HRMS) and SFS data appeared to verify observations from LC-QToF and GC-MS/MS.

As high AEO recovery was of primary objective for this method development, the reduction in yield due to breakthrough was assessed. ESI-HRMS analysis of bench scale extraction alluded to possible breakthrough (Figure 2.1D). It is, therefore, likely that the final filtrate (Stage 2) at the preparative scale contained some degree of breakthrough as well. We accounted for this possibility by characterizing samples from Stage 2 filtrate. GC-MS/MS (Appendix A, A7b) and LC-QToF (Appendix A, A8) analyses of post-Stage 2 samples indicate very low levels of organic content compared to method blanks. The quantitative (ESI-HRMS) analysis showed that the organics detected in the Stage 2 filtrate comprised <4% of the total organic compounds accounted for (Table 2.2). This low level of breakthrough is consistent with ENV+ scale-up data (average 6.6 ± 7.2%).

2.5. Conclusion

According to theoretical design and analysis of data following fractionation, the procedure successfully separated organic compounds based on pH and polarity. The procedure performed well upon scale up, and was able to process 180 L of aged OSPW in 10 days. The LC-QToF data identified the three fractions as having a range in polarity from the least polar F1 fraction to the most polar F3 (Figure 2.4C). Correspondingly, the GC-MS/MS data show that F1 contains considerably more neutral organic compounds than F2 and F3 (Appendix A, A7). The relative polarity of the fractions is also illustrated by ESI-HRMS analysis, with respect to degree

of oxygenated compounds, which increases from F1 – F3 (Figure 2.4A). The success of polarity based fractionation is further substantiated by SFS data (Figure 2.4D) which indicate that the greatest abundance of aromatic compounds appear in the least polar F1, as aromatics are generally nonpolar compounds.

The success of the developed fractionation method allows for future work related to oil sands OSPW characterization. To further demonstrate the utility of this procedure, our future research will include processing large quantities of bitumen-influenced waters from a variety of sources for the creation of reference materials for all stakeholders. With this method in place, we can now begin to identify the potential compounds responsible for toxicity and if these are consistent between industrial and natural sources. To that end, our current research includes determining the relative toxicities of each fraction to a variety of aquatic species. An advantage of this method lies in the potential for each primary fraction to be sub-fractionated further using methods that require large volumes in an effects-directed analysis approach.

Chapter 3. Toxicity of Aged Oil Sands Process-Affected Water Fractions to Aquatic Species

3.1. Overview

The process of surface mining and extracting bitumen from oil sand produces large quantities of tailings and oil sands process-affected water (OSPW). Industry is currently storing OSPW on-site while investigating strategies for their detoxification. One such strategy relies on the biodegradation of organic compounds by indigenous microbes, resulting in aged tailings waters with reduced toxicity. This study evaluated the viability of this strategy by assessing the toxicity of OSPW collected from a test pond that had aged statically for approximately 18 years. Dissolved organics in aged OSPW were fractionated using a preparative solid-phase extraction method which generated three organic fractions (F1-F3) of increasing polarity. To assess toxicity, six aquatic species; *Pimephales promelas, Oryzias latipes, Vibrio fischeri, Daphnia magna, Lampsilis cardium,* and *Hyalella azteca* were exposed to whole OSPW and the derived OSPW organic fractions. Broad comparisons revealed that *P. promelas* and *H. azteca* were most sensitive to dissolved organics within aged OSPW, while whole aged OSPW was most toxic to *L. cardium* and *H. azteca*. Three cases of possible contaminant interactions within whole OSPW treatments suggested additive toxicity by organic fractions (*H. azteca*), toxicity resulting from inorganic contaminants (*L. cardium*), and amelioration of organic toxicity by whole OSPW (*P. promelas*). As such, drivers of toxicity appeared to be dependent on the species exposed. Of the organic fractions assessed, F3 (most polar) appeared most toxic overall while F2 (intermediate polarity) displayed little toxicity to all species evaluated. This presents strong evidence that classical O_2 naphthenic acids, mostly present in F1 (least polar), are not primarily responsible for the toxicity observed in an aged tailings source. The current study indicates that although the aged tailings source (≥18 years) did not display acute toxicity to the majority of organisms

assessed, inorganic components and polyoxygenated organics may pose a persistent concern to some aquatic organisms.

3.2. Introduction

The oil sands region in northern Alberta is the third largest reserve of crude oil worldwide (Canadian Association of Petroleum Producers, 2016). Although more than 80% of remaining crude oil reserves in the region can be extracted using in situ methods (Canadian Association of Petroleum Producers, 2016), surface mining has historically accounted for a considerable portion of the operations. Surface mined bitumen extraction methods produce large quantities of oil sands process-affected water (OSPW) and tailings waste material, which are very saline and contain inorganic and organic compounds that are acutely toxic to a variety of aquatic biota (Clemente and Fedorak, 2005; Brown and Ulrich, 2015). Therefore, as a precautionary measure, the release of OSPW into the natural environment is not permitted (FTFC, 1995a). With the requirement that disturbed land be reclaimed to an environmentally productive status (FTFC, 1995a), the long-term strategy by industry involves detoxifying stored OSPW. One approach to accomplishing this is to convert tailings storage ponds into viable wetland areas called end-pit lakes as part of a larger wet landscape reclamation strategy. To evaluate the viability of a wet landscape reclamation program, Syncrude Canada Ltd. constructed a number of large scale test ponds between 1989 and 1993. These ponds were established to assess different detoxification techniques including varying combinations of mature fine tailings, coarse tailings, tailings pond surface water, and tailings capped with fresh water (Siwik et al., 2000).

The most toxic component within OSPW has been attributed to the water-soluble acid-extractable organic component (Brown and Ulrich, 2015), which contains a well-studied O_2 subgroup commonly referred to as naphthenic acids (NAs). The wet landscape reclamation strategy is, therefore, currently focussed on the natural reduction of the organic acid component within OSPW. This wet landscape strategy initially showed promise with research revealing a relative reduction in toxicity associated with biodegradation of organic components within aged tailings (MacKinnon and Boerger, 1986; Herman et al., 1993; Lai et al., 1996). Research has shown that biodegradation of commercially-available NA mixtures and oil sands-derived NAs with microbes indigenous to oil sands tailings ponds resulted in preferential degradation of compounds with low carbon number and cyclicity (Clemente et al., 2004; Scott et al., 2005). Further characterization of biodegraded NAs revealed that a higher degree of alkyl branching and oxygenation contributed to their bio-persistence (Bataineh et al., 2006; Smith et al., 2008; Han et al., 2009). To confound matters, more recent research has revealed that NAs represent only a portion of the bioavailable organics present in OSPW. The broader group of water soluble organic substances include additional classes which contain heteroatomic moieties, di-carboxyl and dihydroxy groups, and aromatic rings (Headley et al., 2011b; Headley et al., 2013b; Bauer et al., 2015), and are hereafter referred to as acid-extractable organics (AEOs). It is widely recognized that overall detoxification of tailings requires the combined reduction of total acids and specific organics responsible for toxicity. Nevertheless, the long period of time required (>10 yrs) to reduce a significant amount of AEOs in tailings ponds and the persistence of particularly recalcitrant AEOs, has called into question the viability of the natural degradation of OSPW proposed in the wet landscape strategy (Quagraine et al., 2005).

Presently, there is a lack of information regarding the overall toxicity of aged OSPW and the specific components that are responsible for the toxicity. Some of the variability associated with AEO toxicity can be attributed to interspecies sensitivity differences in test organisms. Effects in a variety of aquatic species exposed to OSPW have been observed at between 8 to 65 mg/L for various endpoints (Kinley et al., 2016). However, comparisons between different studies are challenged by the variability in chemical profiles between OSPW sources (Frank et al., 2014; Frank et al., 2016). These challenges have led to research involving fractionation of whole OSPW in order to elucidate whether the toxicity can be associated with broad chemical parameters such as molecular weight, aromaticity, and solubility (Lo et al., 2006; Frank et al., 2008; Grbovic et al., 2012; Jones et al., 2012; Bauer et al., 2015; Huang et al., 2015; Bauer et al., 2017). Previous attempts to isolate toxic organics in OSPW were restricted by scale such that final fraction quantities were insufficient to allow for full chemical characterizations and toxicity testing. In an effort to permit a complete chemical and toxicological characterization of bioavailable organic components in OSPW contributing to toxicity, a method was developed (Chapter 2) for the fractionation of OSPW which was capable of isolating soluble organic compounds present. This protocol utilized differences in polarity to produce three fractions and recovered 96% of AEOs. Characterization of these fractions revealed that the increasing solvent polarities used in fraction generation corresponded to increases in the abundance of oxygenated groups as well as increased aromaticity of the constituents within fractions (Chapter 2).

As part of a broad effects-directed analysis of aged OSPW, the objectives of the present study were two-fold. The first was to assess the toxicity of the fractions of bioavailable organics isolated from aged OSPW in the aforementioned companion study (Chapter 2), and evaluate whether differences in chemical profiles for each fraction could be related to toxicological

differences. The second objective was to assess the toxicity of the aged OSPW and the isolated fractions to a suite of aquatic organisms and compare species sensitivities. Previous investigations have shown that species sensitivity can vary depending on AEO source material; therefore, a complement of organisms from different taxa should be considered (Marentette et al., 2015b; Bartlett et al., 2017). The species tested included the marine bacterium *Vibrio fischeri* (Microtox® assay), three freshwater invertebrates (*Hyalella azteca* (amphipod crustacean), *Daphnia magna* (water flea), *Lampsilis cardium* (freshwater mussel)), and two freshwater fishes (*Pimephales promelas* (fathead minnow), and *Oryzias latipes* (Japanese medaka)). The intention of this study was to identify the most toxic organic constituents within aged OSPW and to gain a better understanding of which organisms and endpoints are most sensitive; information that is critical for evaluating the viability of the wet landscape reclamation strategy.

3.3. Methods and Materials

3.3.1. Aged OSPW Sampling

An aged OSPW sample was acquired from Test Pond 9 located on the Syncrude Canada Ltd. lease site in the Athabasca region, north of Fort McMurray, Alberta. Test Pond 9 was constructed in 1993 and filled with 50,000 m^3 of tailings pond surface water from Mildred Lake Settling Basin, an active tailings discharge retention pond (Siwik et al., 2000). In 2011, approximately 2000 L of OSPW was pumped directly from Pond 9, transferred to two 1000-L polyethylene containers and shipped to Environment and Climate Change Canada (ECCC), Burlington, Ontario.

3.3.2. Aged OSPW Fractionation

The fractionation method was developed to isolate organic compounds using solid-phase extraction and was completed in two stages; one at pH 11 and the second at pH 2 (Chapter 2). The method successfully produced three fractions with increasing polarity as verified by chemical characterization described in the following section. The aged OSPW fractions were produced using methods described previously (Chapter 2). In brief, 180 L of unaltered aged OSPW was adjusted to pH 11 ± 0.5 using sodium hydroxide (NaOH; Sigma Aldrich®, Oakville, ON) with thorough mixing. Following a 12-hour settling period, the pH-adjusted OSPW was pumped through a conditioned preparative solid phase extraction column (Isolute® ENV+ resin; Biotage®, Charlotte, NC) at a rate of 110 ± 10 mL/min. The resin was then removed from the column, dried and soxhlet extracted for 12 hours sequentially using ethyl acetate (Fisher Scientific, Mississauga, ON) to produce Fraction 1 (F1) and methanol (MeOH; Fisher Scientific, Mississauga, ON) to produce Fraction 2 (F2). The column filtrate was then adjusted to pH 2 ± 0.5 using hydrochloric acid. The aged OSPW at pH 2 was then pumped through a second column filled with freshly conditioned ENV+ resin. The resin from the second column was then soxhlet extracted using MeOH to produce Fraction (F3). All fractions were filtered through a bed of anhydrous sodium sulfate to remove residual water and stored in amber glass bottles at 4°C until chemical characterization and preparation for bioassays.

3.3.3. Chemical Characterization

The chemical composition of each fraction was characterized using a variety of analytical techniques (Chapter 2). The relative polarities of each fraction were verified using both liquid chromatography – quadrupole time-of-flight mass spectrometry (LC-QToF) and gas

chromatography – tandem mass spectrometry (GC-MS/MS). All chemical analyses were run in duplicate with respective solvent blanks and internal standards. A suite of chemical analyses and validation experiments identified that F1-F3 displayed an increase in relative polarity (Chapter 2). Associated with an increase in polarity, class distribution data and synchronous fluorescence spectroscopy (SFS) identified increased degree of oxygenation and degree of aromaticity, respectively (Chapter 2). Although F3 contained the bulk of AEO (79.5%), the majority of naphthenic acids (O_2 ions) were contained in F1.

The relative aromaticity of each fraction was determined using SFS, as outlined previously (Kavanagh et al., 2009). Fluorescence spectra were collected in the 200-400 nm excitation wavelength range using a Perkin–Elmer Luminescence Spectrometer LS50B and data were collected using FL WinLab 3 software (Perkin–Elmer, Norwalk, CT). Excitation and emission monochromator slit widths were set at 5 nm, scan speed at 50 nm min^{-1} and resolution at 0.5 nm. The spectra were blank-corrected with 0.05 M $NaHCO_3$ and then smoothed with a 5-point averaging adjacent method using Origin software ver. 7.5 (OriginLab Corp., Northampton, MA).

Additional chemical properties were analysed using electrospray ionization high-resolution mass spectrometry (ESI-HRMS) including concentrations, O_2 double-bond equivalents (DBE), ion class distribution, and mass-to-charge ratio (m/z). The ESI-HRMS analysis was conducted using an LTQ Orbitrap Elite (Thermo Fisher Scientific); methods are described in detail (Chapter 2).

Major ions were analyzed by chemical suppression ion chromatography, and dissolved metals (Ca, Mg, Na, K, Si) were analysed by inductively coupled argon plasma system (ICP-OES) by ECCC's National Laboratory for Environmental Testing (NLET) in Burlington, ON

(NLET, 2003). All other 35 dissolved metals were analyzed by NLET using ion chromatography plasma optical emissions spectrometry (ICP-MS) (NLET, 2003).

3.3.4. Bioassays

3.3.4.1. Treatment Preparation

Bioassays were exposed to an unaltered aliquot of aged OSPW (referred to as whole water) as well as the isolated organic fractions described previously. All fraction aliquots were diluted in order to bring concentrated fractions to whole water equivalents (v/v) of the original aged OSPW. Additionally, because the fractions generated were dissolved in organic solvents (Chapter 2), aqueous test solutions were prepared with solvent proportions diluted to 0.1% of bioassay exposure solutions to avoid solvent-associated toxicity. This was achieved by preparing 10-L whole water equivalent stocks for each fraction, concentrated to 100 mL. Each 10-L equivalent (170-mL aliquot from 3-L fraction) was transferred to a 500-mL round-bottom flask and solvent was removed using a rotary evaporation unit at 60°C with vacuum set to ~340 mbar. The remaining residue was then re-dissolved in 10 mL of MeOH. To aid in dissolution of organic compounds, 90 mL of 0.01 M NaOH was prepared by dissolving 36 mg of NaOH pellets into 90 mL of deionized water. The NaOH solution was then added to the 10-mL equivalent fraction to a total of 100 mL, vortexed and then sonicated for 5 minutes. These stocks solutions were then stored at 4°C until further use.

For bioassays, the fractions were brought back to environmentally relevant concentrations (e.g. 100% whole water equivalent of original aged OSPW) by pipetting 10 mL of a stock into 990 mL of deionized water, with a final MeOH concentration of 0.1% solvent by volume. The "Recombined" treatment represented a solution containing a combination of all three fractions.

As a result, 10 mL of each of the 3 fraction stocks was dissolved in 970 mL of control water, with a final MeOH concentration of 0.3% solvent by volume. The solvent controls were prepared identical to the fraction treatment stocks (1 mL MeOH, 9 mL 0.01M NaOH) and "Recombined" treatment (3 mL MeOH, 7 mL 0.01M NaOH) stock without dissolved organic compounds, and diluted to achieve a final MeOH concentration of 0.1 % and 0.3% solvent by volume, respectively. The solvent controls were prepared to account for solvent effects in fractions and the Recombined treatment will be referred to hereafter as Solvent 100 and Solvent 300, respectively.

3.3.4.2. Bioassays Methods

A total of six acute toxicity bioassays were conducted: *P. promelas* (5 days post-fertilization), *O. latipes* (10 days post-fertilization), *V. fischeri* (15-min), *D. magna* (48-hour), *L. cardium* (24-hour), and *H. azteca* (7-day). All bioassays included exposure to aged OSPW whole water, three organic fractions (F1, F2, and F3), and a Recombined treatment (Chapter 2). All tests also included water controls, and two solvent controls (Solvent 100 and Solvent 300) described previously. Bioassays were conducted with approval from respective animal care committees at ECCC (Burlington, ON) or University of Waterloo (Waterloo, ON; *O. latipes*). All bioassay parameters and procedures are described in detail in Appendix B (B1)

3.3.5. Statistical Analysis

Data were analyzed using R version 3.3.3 (R Core Team, 2017) and RStudio version 1.0.136 (RStudio Team, 2016). Except for the *V. fischeri* data set, an initial analysis used one-way analysis of variance (ANOVA) to compare each endpoint across the relevant control groups

(Control, Salt Control, Solvent 100, and Solvent 300). Bioassay method procedures for *V. fischeri* utilize control water as a reference level and it is not considered an actual treatment. Therefore, only the Solvent control treatments were used as a control group. Model assumptions were assessed via residual plots, Shapiro-Wilk's Test, and Levene's Test. Comparisons where the model assumptions appeared to have been violated were re-assessed using the non-parametric Kruskal-Wallis test. For species in which no evidence of a difference in the mean endpoint across control and solvent control groups was found, data from all relevant control groups were pooled into a single group for comparison with the remaining five treatment groups. All statistical comparisons described in the results are comparisons across treatments, where the control represents a pooled control group (control water, Solvent 100, and Solvent 300), unless otherwise stated.

All endpoints were again compared with one-way ANOVA across six treatment groups (pooled control (where applicable), whole water, Recombined treatment, and fractions F1, F2, and F3), followed by Tukey's method for pairwise comparisons when significant evidence ($p \leq 0.05$) of a difference among treatment means was identified. Assumptions of normality and constant variance were assessed as before, and comparisons in which these assumptions were not satisfied were re-assessed with the Kruskal-Wallis test, followed by Wilcoxon-Mann-Whitney tests with a Bonferroni adjustment for pairwise comparisons.

Results from the hatch success and developmental abnormalities endpoints for *P. promelas* showed evidence of a difference among controls such that the Solvent 300 control displayed a significant reduction in hatch success and increase in abnormalities ($p \leq 0.05$) compared to the water Control and Solvent 100. The Recombined treatment could not be assessed with confidence as results were likely confounded by solvent effects. Control and Solvent 100

treatments were not significantly different and, therefore, pooled and compared to the whole water treatment and three fractionated treatments (F1, F2, and F3) (Figure 3.1a). An arcsine square-root transformation was applied to the percent hatched in order to satisfy assumptions of a one-way ANOVA. A one-way ANOVA was performed on data for both endpoints and Tukey's method was used for post-hoc comparisons.

The *D. magna* bioassay only contained two control groups (control water and Solv300). As such, the Welch Approximate *t* Procedure was used to compare the control groups (controls and solvent controls), and no evidence of a significant difference between the mean percent survival between these two groups was found ($p > 0.05$). While this procedure does not require an assumption of constant variance, due to the low variability (i.e. high survival rates) of observations in both groups, it is difficult to verify the assumption of normality. However, as survival in both control groups was at (or near) 100%, it is reasonable to assume that there was in fact no effect of the solvent control group, and observations from Control and Solvent 300 were pooled.

3.4. Results

3.4.1. Bioassays

3.4.1.1. Pimephales promelas

The acute toxicity of *Pimephales promelas* was assessed with a hatch success endpoint. This analysis identified that F3 displayed a significant reduction in hatch success ($p < 0.05$; hatch success 63%) compared to pooled controls (96%), whole water (100%), and all other organic treatments (95-100%).

Four sub-lethal endpoints were assessed: time to hatch, embryonic heart rate, hatch length, and developmental abnormalities at hatch. Larval abnormalities at hatch were significantly higher ($p \leq 0.004 – 0.01$) in F3 (28%) compared to the pooled control group (9%), F1 (2%), and F2 (3%) (Appendix B, B2). No other evidence of significant differences was found. The hatch length endpoint appeared to be confounded by solvent effects from both Solvent 100 and Solvent 300 when compared to control water (Appendix B, B3) negating our ability to derive results at this endpoint. Nonetheless, because the range in average hatch lengths across all treatments was only 0.3 mm, regardless of the capacity to identify statistical significance, our observations likely have little environmental relevance. There were no significant differences among any of the control and treatment groups for the time to hatch endpoint (5.2 - 5.5 days, Appendix B, B4), while for the embryonic heart rate, there was a significant difference ($p = 0.034$) in the mean response between the whole water treatment (139 beats/min) and F2 (160 beats/min) (Appendix B, B5).

3.4.1.2. Oryzias latipes

The acute endpoint, percent hatched, displayed no significant difference among any of the control and treatment groups (92-100% hatched, Figure 3.1b).

Three sub-lethal endpoints were assessed for *O. latipes*: time-to-hatch, hatch length, and abnormalities at hatch. The mean hatch length endpoint differed significantly between the pooled controls (4.8 mm) and F1 (4.6 mm, $p = 0.037$), F3 (4.6 mm, $p = 0.024$), and the Recombined treatment (4.6 mm, $p = 0.021$) (Appendix B, B6). For abnormalities at hatch, incidences of abnormalities in all treatments were so low, all abnormalities from each replicate were pooled for each treatment. All treatments were >95% normal (no abnormalities) and of the 450 individual larvae assessed only 3.6% displayed any form of abnormality (Appendix B, B7). There were

significant differences in time-to-hatch between controls (7 days) and F1 (6.4 days, $p = 0.004$) and F3 (6.2 days, $p < 0.001$), as well as between the Recombined treatment (7.1 days) and F1 and F3 ($p = 0.017$ and 0.0004, respectively; Appendix B, B8). Compared to controls, F1 and F3 delayed average hatch time by 0.6 and 0.8 days, respectively. Time-to hatch was also significantly different ($p = 0.002$) between the whole water treatment (7 days) and F3 (6.2 days).

3.4.1.3. Vibrio fischeri

Vibrio fischeri displayed no acute effects to any treatments at 100% whole-water equivalents. However, significant toxicity was observed when the treatments were concentrated to 3x whole water equivalents. In the concentrated (3x) exposures, bioluminescence was significantly different between F3 (68%) and the Recombined fraction (51%, $p = 0.018$), while both were significantly different ($p < 0.02$) from all other treatment groups (95-110%, Figure 3.1c). Fraction F1 (95%) was also significantly different ($p < 0.027$) from controls (110%).

3.4.1.4. Daphnia magna

Mean survival of *D. magna* was > 98% for all controls and treatments, and there were no significant differences ($p > 0.05$) among any of the treatment groups (98-100%, Figure 3.1d).

3.4.1.5. Lampsilis cardium

Mean percent viability for the whole water-exposed larval mussels was significantly lower ($p < 0.001$, 51%) than all other treatment groups (91-93%) and the pooled controls (92%) ($p < 0.001$, Figure 3.1e).

3.4.1.6. Hyalella azteca

Survival was significantly lower ($p < 0.05$) in both the whole water (44%, $p = 0.001$) and the Recombined treatment (70%, $p = 0.004$) compared to controls (98%, Figure 3.1f).

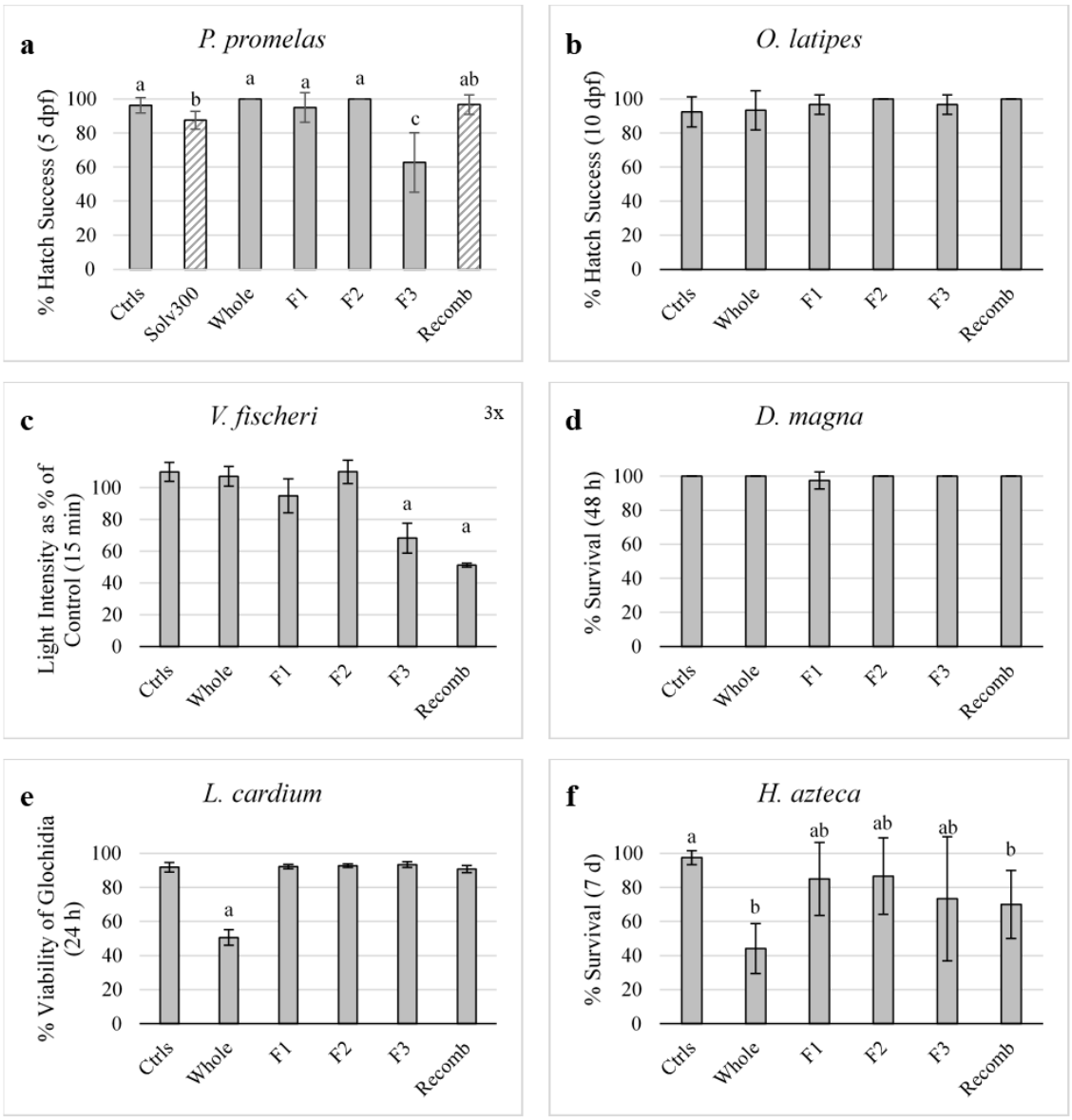


Figure 3.1. Percentage survival/viability of organisms (mean ± standard deviation) exposed to pooled controls (Ctrls), whole aged OSPW (Whole), fractions (F1-F3), and a Recombined treatment (Recomb, fractions F1-F3 combined). Test species included fish (*P. promelas* (a) and *O. latipes* (b)), marine bacteria (*V. fischeri* (c)), and invertebrates (*D. magna* (d), *L. cardium* (e), and *H. azteca* (f)). Letters (a, b, c) indicate a significant difference ($p \leq 0.05$). Hashed bars for *P. promelas* indicate treatment groups that were compared statistically, due to solvent effects. Dpf = days post fertilization.

3.4.1.7. Fraction Toxicity

Fraction F3 (most polar) displayed the greatest toxic potency overall in cases where any significant toxicity was attributable to the organic fractions. This occurred in *O. latipes* time-to-hatch, *V. fischeri* assay (3x equivalent), *P. promelas* survival, hatch length, and abnormalities. It is important to note that although F3 significantly decreased hatch length and increased embryonic abnormalities in *P. promelas* (Appendix B; B2, B3), these endpoints may have been affected by the solvent. *H. azteca* also showed decreased survival after exposure to F3, although this was not significantly different from the controls.

3.4.2. Chemical Characterization

Chemical characterization of the three fractions (F1-F3) was performed with analysis using ESI-HRMS, LC-QToF, GC-MS/MS, and SFS, as described previously (Chapter 2). Additionally, metals and major ions were analysed using ICP-MS and ICP-OES methods described herein. The chemistry results displayed an increase in oxygenation of ions, and increase in degree of aromaticity concurrent with increased polarity of the fractions. As the fractionation method excluded inorganics (metals and major ions), their concentrations were only determined for the whole aged OSPW sample (Table 3.1). Concentrations of dissolved organic compounds were determined using ESI-HRMS for F1-F3 and the final filtrate (Table 2.2). The majority of AEO was captured in F3 (79.5%), while F1 contained the bulk of the remainder (15.6%).

Table 3.1. Water chemistry for unaltered aged OSPW presented as total dissolved metals and major ions (43) determined by ICP-MS. Those elements measured but below detection limit: Al, Be, Bi, Cd, Ce, Cs, Co, Ga, Fe, La, Mn, Nb, Pt, Se, Ag, Sn, Ti, and Zn.

	Aged OSPW
pH	8.55
Conductivity (µS/cm)	2387
Hardness (mg/L $CaCO_3$[a])	71.1
Metals / Major Ions	**µg/L**
Antimony	0.186
Arsenic	4.39
Barium	28.3
Boron	2220
Chromium	1.3
Copper	8.79*
Lead	0.29
Lithium	85.2
Molybdenum	2.86
Nickel	5.96
Rubidium	1.29
Strontium	158
Thallium	0.1
Tungsten	0.148
Uranium	2.06
Vanadium	1.57
	mg/L
Calcium	10.6
Chloride	294
Fluoride	3.2
Magnesium	10.8
Potassium	8.25
Silica	1.44
Sodium	689
Sulfate	86.8

[a] Calculated based on concentrations of Ca, Mg, Fe, Sr, and Mn.
* indicates exceedance of CCME and USEPA water quality guidelines

Table 2.2. Concentration of dissolved organics in fractions and filtrate of aged OSPW determined by ESI-HRMS. Values represent concentrations based on original 180 L water sample.

	Dissolved Organics	
	Concentration (mg/L)	**Contribution (%)**
Fraction 1	5.4	15.6
Fraction 2	0.4	1.1
Fraction 3	27.7	79.5
Post-Stage 2 filtrate	1.3	3.8
Total	34.8	100.0

[a] represents a derived theoretical recovery based on all other measured values

3.5. Discussion

The present study assessed the toxicity of whole water and organic fractions from a test pond containing a tailings source that was left to undergo biodegradation and aging for approximately 18 years. Generally, toxicity varied depending on the test species. For example, vertebrates were more sensitive to the organic fractions, while invertebrates displayed greater sensitivities to the whole water, which contained inorganics and organics within the tailings. Of the organic fractions assessed, F3 appeared most toxic overall, with significant effects noted for *P. promelas* (hatch success, abnormalities, and hatch length) and *O. latipes* (hatch length, time-to-hatch), while F2 displayed no toxicity across all bioassays. F1 caused significant effects in *O. latipes* (hatch length, time-to-hatch); however, these effects were small and of questionable ecological importance, and no effects were observed in any other test species. This presents strong evidence that NAs, mostly present in F1, are not responsible for the acute toxicity observed in an aged tailings source. The present study identified significant acute toxicity of

aged OSPW (≥18 years) whole water to two of the six test species (*L. cardium* and *H. azteca*), where *L. cardium* was not sensitive to organic fraction exposures. This suggests that in an aged tailings source, inorganic components may pose a persistent concern to some invertebrate species.

3.5.1. Bioassay Comparison

A benefit to the development this preparative fractionation procedure (Chapter 2) is that it generated large fraction volumes, and thus afforded the ability to test a suite of organisms to identical sample treatments from an identical source. We assessed six different bioassays representing aquatic organisms at different trophic levels in order to compare their relative responses.

Overall, comparison of bioassays revealed that *P. promelas* is more sensitive than *O. latipes*, which was expected as similar results have been observed previously (Bauer et al., 2017). Additionally, compared to the other test species in the current study, *P. promelas* and *H. azteca* were most sensitive overall. These findings are in agreement with two previous studies which assessed the toxicity of fresh OSPW NA extracts from the same two sources to *H. azteca*, *V. fischeri*, *L. cardium*, and *P. promelas* (Marentette et al., 2015a; Bartlett et al., 2017). In these evaluations *P. promelas* embryo and *H. azteca* displayed a much greater sensitivity than both *V. fischeri* and *L. cardium*, with *P. promelas* displaying the greatest sensitivity and *L. cardium* displaying the least sensitivity overall. Interestingly, in the same two studies, when exposed to an aged OSPW NA extract, *P. promelas* and *H. azteca* were still more sensitive than *V. fischeri*, but *L. cardium* was most sensitive overall (Marentette et al., 2015a; Bartlett et al., 2017). This is contrary to observations in the present study, which show *L. cardium* being insensitive to organic

fractions (containing NA), but sensitive to the whole aged OSPW. Because OSPW has been shown to display variability in both chemistry and toxicity across sample sites and collection times (Marentette et al., 2015a; Bartlett et al., 2017; Frank et al., 2016), the observed differences may be due to a combination of the difference in OSPW source material, OSPW age, NA extraction method, as well as the presence of soluble organic compounds in addition to NA.

The commonly used *V. fischeri* bioassay was assessed herein, but displayed low sensitivity to aged OSPW components. When organic treatments were concentrated to 3x whole water equivalent, organic fractions F1 and F3 displayed the greatest reductions in viability and no toxicity was associated with the whole water. Because of comparable results to *P. promelas* acute toxicity endpoints and minimal material and labour requirements, the authors recognize the utility of the *V. fischeri* (Microtox®) assay as a screening tool to identify and prioritize toxic components that warrant more in depth investigation. Nonetheless, we recommend using caution when applying the Microtox® bioassay as a screening tool for whole waters as it has been shown to be relatively insensitive to aged OSPW (LC_{50}: 83.9 mg/L) compared to *P. promelas* (LC_{50}: 12.4 mg/L) , and *H. azteca* (LC_{50}: 18.4 mg/L) (Bartlett et al., 2017).

3.5.2. Organic Fraction Toxicity

Previous chemical characterization of fractions showed an increase in degree of aromaticity and oxygenation which contributed to an overall increase in polarity from F1 to F3 (Chapter 2). This suite of bioassays was conducted, in part, to determine whether the difference in chemical composition of the fractions contributed to differences in toxicity.

The higher toxicity associated with F3 in some species and endpoints may be due to the relatively high abundance of oxygenated groups and degree of aromaticity present, which

contributed to greater polarity in F3. Greater oxygen content and aromaticity within AEO fractions has been previously associated with greater toxicity to fish (Scarlett et al., 2013; Bauer et al., 2015; Bauer et al., 2017), which has been linked to narcosis, electrophilic reactivity, and oxidative stress (Bauer et al., 2017). It is also quite likely that the toxicity of F3 is simply due to the high concentration of AEO in this fraction (27.7 mg/L) and that the concentration present in F1 and F2 (5.4 mg/L and 0.4 mg/L, respectively) were below a toxicity threshold (Table 2.2). For comparison, studies which have assessed toxicities associated with fresh OSPW from various sources to *H. azteca*, *V. fischeri*, *L. cardium*, *S. vitreous*, and *P. promelas*, have observed LC_{50}s greater than 5 mg/L and the majority of cases greater than 10 mg/L (Marentette et al., 2015a; Marentette et al., 2015b; Bartlett et al., 2017).

In all cases, the most neutral isolation F1 was statistically similar in relative toxicity to Controls and F2, except *P. promelas* heart rate where F1 was similar to the most toxic F3. In a similar comparative study assessing the toxicity of OSPW fractions to *P. promelas*, a greater toxicity was observed for a more polar fraction compared to a polar-neutral fraction (Morandi et al., 2015), in agreement with observations herein. However, in a recent study (Morandi et al., 2015), O_2 species (naphthenic acids) caused the majority of toxicity observed compared to other fractions. In the present study the bulk of O_2 species (naphthenic acids) were present in F1 which displayed low toxicity overall. The main difference between these studies is that the aged OSPW source utilized herein displayed a lower contribution and concentration of O_2 species than other polyoxygenated compounds (Chapter 2). Therefore, this discrepancy may be simply associated with the concentrations of organics. These observations are noteworthy because NAs are generally considered the main drivers of toxicity (Morandi et al., 2015; Hughes et al., 2017). The

present study has identified that in the absence of potentially more potent O_2 organics, polyoxygenated species may be driving toxicity.

Interestingly, in the *P. promelas* assay where F3 was significantly most toxic, no toxicity was present in the Recombined treatment (Figure 3.1a). It is unclear why the toxicity of the Recombined treatment was lower, but it is possible that interactions between fraction components may have affected bioavailability of toxic compounds. Conversely, the *H. azteca* bioassay presented a case where no significant toxicity was observed in individual fractions compared to controls, but significant toxicity was observed in the Recombined treatment (Figure 3.1f). The Recombined treatment displayed no significant difference from any of the fractions, suggesting that compounds that were distributed between these fractions surpassed a toxicity threshold when recombined.

It should be noted that previous quantitative analysis of the three fractions reported that treatments F1, F2, and F3 contained approximately 15.6%, 1.1%, and 80% of total AEO, respectively (Table 2.2; Chapter 2). This has the potential to significantly contribute to the observations that F3 was most toxic while F2 was least toxic overall. The authors do caution however, that because no analytical chemical standards exist for the quantitation of organics present in OSPW, the chemical results cannot be stated with certainty. Therefore, it is difficult to ascertain to what degree toxicity is driven by polarity rather than simply concentration.

3.5.3. Whole Water Toxicity

The whole water treatments for aged OSPW represented unaltered and unfractionated samples. These treatments therefore, contained all contaminant classes including dissolved organic and inorganic components such as metals and salts. By comparing the whole water

toxicity to that of the toxicity from dissolved organic treatments, inferences could be made regarding possible contaminant interactions.

One case revealed possible contaminant interactions resulting in a reduction in dissolved organic toxicity in whole water. Specifically, *P. promelas* survival was significantly reduced ($p < 0.05$) by organic fraction F3, but no sensitivity to the whole water treatment was observed (Figure 3.1a). Also, the toxicity of F3 was significantly different from controls to some sub-lethal endpoints for *P. promelas* and *O. latipes* (Appendix B; B2, B6, B8), while whole water treatments displayed no significant toxicity. A possible explanation is that inorganic species present in the whole water had a buffering effect on the toxicity of organics present in F3 to some fish endpoints. Studies have found that the presence of high quantities of salt in water have the ability to precipitate naphthenic acids from the water column, reducing their bioavailability (Headley et al., 2011a; Celsie et al., 2016). However, both of these studies found salting out to occur at much higher salinities (e.g. >3000 mg/L NaCl) than in the present study (Table 3.1). Because the unaltered aged OSPW sample contained up to 689 mg/L of Na^+ and 294 mg/L of Cl^- (Table 3.1), the reduction in organics-associated toxicity observed is possibly also the result of additional mixture interactions and binding to larger humic or fulvic acids.

Although the toxicity of inorganic components was not tested in the present study, it is possible that inorganics were partially responsible for some of the toxicity observed in the whole water treatments. No metals or major ion concentrations for aged OSPW were above available Canadian Council of Ministers of the Environment (CCME) or United States Environmental Protection Agency (USEPA) water quality guidelines for protection of aquatic freshwater species (USEPA, 2004; CCME, 2017), except for Cu (Table 3.1). Only *H. azteca* and *L. cardium* displayed sensitivities to aged OSPW whole water (44% and 51% viability, respectively), but

unlike *H. azteca*, *L. cardium* was not sensitive to any organic components. Heightened sensitivity to salts and metals have been observed for mussel glochidia (Gillis et al., 2008; Gillis et al., 2011), but due to the complexity of the OSPW whole water mixture, it is difficult to determine specific elements responsible for toxicity. In a number of studies that have assessed metals toxicity to various species of freshwater unionid mussels (family *Unionidae*), EC_{50} values derived for B, Cd, Cr, Cu, Ni, Pb, and Zn (Hansten et al., 1996; Milam et al., 2005; Wang et al., 2010, 2017; Liu et al., 2016, Soucek et al., 2011) were all greater than water concentrations observed in the present study. Similarly, the concentrations of Cl^-, K^+, SO_4^{2-}, assessed in our study were lower than EC_{50}s observed in another study that evaluated toxicity to five different mussel species (Wang et al., 2017). However, notable exceptions for Cu and Cl^- toxicity to mussels implicate their possible role as inorganics contributing to the toxicity of the whole water to *L. cardium* observed herein. Two different studies, which assessed acute toxicity to mussel glochidia for up to 9 different species exposed to Cu, observed mean EC_{50}s in the range of 6.9 – 48 μg/L (Gillis et al., 2008; Wang et al., 2017). These values bracket the Cu concentrations measured for aged OSPW in our study (8.79 μg/L). Similarly, another study (Gillis et al., 2011) identified EC_{50}s from four different mussel species exposed to Cl^- in the range of 113 – 1430 mg/L, of which four out of six displayed EC_{50}s that were lower than the Cl^- concentration in the aged OSPW (294 mg/L) assessed herein. Although the study identified a significant reduction in toxicity associated with increased water hardness, the observations were made using reconstituted water (100 mg $CaCO_3$/L) (Gillis et al., 2011) which is similar to the water hardness of aged OSPW in our study (71.1 mg $CaCO_3$/L). Collectively, these data suggest that some whole water toxicity observed for *L. cardium* may be attributable to Cu and Cl^- at the concentrations present in this study.

3.5.4. Aged Tailings Toxicity

One of the major objectives of the toxicological assessments within this study was to determine the overall toxicity of an aged tailings source and the efficacy of aging tailings as part of the wet landscape reclamation strategy. The toxicity of the aged OSPW whole water treatment was not significantly different ($p \leq 0.05$) from controls, except in the *L. cardium* and *H. azteca* assays. In both cases, the whole water was more toxic than the organic fractions, which seems to indicate that the inorganic component of the whole water contributed some of the observed toxicity. The test pond assessed herein was aged 18 years at the time of collection and represents a tailings source that has changed from its depositional state, both in organic and inorganic composition.

With regard to organic compounds, when comparing aged OSPW (Chapter 2) to fresh tailings sources studied previously (Bauer et al., 2015; Marentette et al., 2015a), the most notable difference is the reduction of O_2 ions and relative increase of O_x ions (where x is 3 – 8). The estimated total AEO concentration for aged OSPW was ~35 mg/L and NA concentrations herein account for less than 2% of the AEO (Chapter 2). Because different analytical methods and standards were used to derive the NA values in this study than those in earlier studies, data are not directly comparable. However, it is interesting that much lower NA concentrations are reported here for OSPW aged 18 years (<1 mg/L) than was reported in the same pond after 6 years of aging (45.6 mg/L) (Siwik et al., 2000). In the *P. promelas* larval bioassays conducted with the same aged OSPW used herein, but aged for only 6 years, no survival endpoints were significantly different from controls (Siwik et al., 2000). The acute embryo-larval *P. promelas* bioassays conducted herein, which have been shown to be more sensitive than larval *P. promelas* bioassays (Kavanagh et al., 2012), also provide no evidence of significant toxicity for the same

aged OSPW whole water, aged for 18 years. As stated earlier, it is difficult to attribute the low toxicity to a shift to a greater proportion of oxygenated compounds or an overall reduction in the concentration of total organics. In general, there does not appear to be any considerable toxicity associated with aged OSPW to fishes assessed in this study.

There is, however, still reason for concern with regard to invertebrate toxicity as the (whole) aged OSPW, containing both organics and inorganics, reduced survival/viability to 44% and 51% in *H. azteca* and *L. cardium*, respectively (Figure 3.1e, f). However, no significant toxicity to organic fractions was observed for either of these two species suggesting contributions by inorganic components to observed toxicity. A comparison of aged OSPW water chemistry data herein (Table 3.1) with that conducted by Siwik et al. (2000) on the same OSPW source, aged only 6 years, reveals some differences in water chemistry from analysis performed 12 years later. Although some inorganic chemicals (Ca, SO_4^{2-}, Al, Ba, B, and Cr) appeared to decrease in concentration, increases occurred in concentrations of Na^+ (~15x), K^+ (~1.6x), Mg^{2+} (~1.1x), Cl^- (~1.3x), As (~1.5x), and Cu (~2.9x). Additionally, overall conductivity also increased (2387 µS/cm) from that measured in the study by Siwik et al. (2000) (1977 µS/cm). As discussed earlier, an increase in chemicals such as Cu and Cl^-, as well as conductivity, can be detrimental to the survival of some species such as *L. cardium* tested in our study.

It is important to note that the present study assessed wetland viability based on water tests alone which do not account for biotic processes that would be present in a natural site. For example, an increase in photosynthetic rate of cattail and invertebrate community biomass and diversity in effluent-impacted wetland sites has been observed when compared to reference sites (Bendell-Young et al., 2000). These results suggested that scenarios where plants uptake and sequester inorganics (Hozhina et al., 2001; Kamal et al., 2004; Mahdavi et al., 2013) potentially

increase the survivability of invertebrate communities that are sensitive to these anthropogenic contaminants. Therefore, it appears that after 18 years of aging this particular OSPW source poses an acute toxicity risk to lower trophic levels relevant to the Athabasca watershed and that represent a common prey base in an aquatic food web. In order to mitigate the effects on the whole system, it is likely that further reduction in inorganics is necessary.

Further investigation into the toxic drivers associated with OSPW currently involves assessing additional water sources. Current research in this regard comprises the fractionation of groundwaters known to be influenced by tailings pond seepage using methods outlined herein. As in the present study, the bulk isolation of organic fractions would allow generation of sufficient material to conduct a toxicological evaluation using a suite of aquatic bioassays. Beyond groundwater, there is merit in evaluating toxicities associated with fractions from fresh OSPW, and OSPW from different industry operators. Following an effects-directed analysis approach, the identification of toxic fractions will likely warrant further sub-fractionations based on other chemical parameters.

3.6. Conclusions

The objective of the current study was to toxicologically assess the organic components present in OSPW from an aged tailings source, which was achieved by exposing a suite of organisms to previously generated organic fractions (Chapter 2). General comparisons between species identified *P. promelas* and *H. azteca* as the most sensitive to dissolved organics within aged OSPW, while whole aged OSPW was most toxic to *L. cardium* and *H. azteca*. Three cases were observed for possible contaminant interactions within whole water treatments. Possible additive toxicity was observed for *H. azteca* in which a significant ($p \leq 0.05$) reduction in

survival was observed for both the Recombined and whole water treatments. Based on the sensitivity of freshwater mussels to inorganics, the reduced survival of *L. cardium* ($p \leq 0.05$) in whole water treatments and lack of sensitivity to dissolved organic components indicates that toxicity was likely due to elevated metals or salts in the OSPW. Amelioration of dissolved organic toxicity by whole water was observed for *P. promelas* which was only sensitive to F3 and not whole water treatments. Dissolved organics in F3 displayed the greatest overall potency compared to F1 and F2. Overall, organic components within the aged OSPW displayed very low toxicity. Drivers of toxicity appeared to be dependent on the species assayed and associated with dissolved organic and inorganic concentrations. Although, naphthenic compounds (O_2) have been recently identified as drivers of toxicity to *P. promelas* and *Oncorhynchus mykiss* in OSPW (Morandi et al., 2015; Hughes et al., 2017), O_2 concentrations which predominated in F1 were relatively low. The results of this investigation suggest that in the absence of sufficient O_2 concentrations, polyoxygenated species can elicit considerable toxicity. Thus, in an aged OSPW source, polyoxygenated organic species at sufficiently high concentrations may pose the greatest threat to aquatic species sensitive to dissolved organics. Moreover, the persistence of inorganic components within aged OSPW may pose a considerable risk to organisms sensitive to metals and salts.

Chapter 4. Preparative Isolation and Fractionation of the Soluble Organic Mixtures of Bitumen-Influenced Groundwater from the Athabasca River Watershed.

4.1. Overview

Recent developments in advanced separation and high resolution mass spectrometry have allowed for the differentiation of groundwaters exposed to natural bitumen-containing formations (oil sands) and those influenced by oil sands process-affected water (OSPW) from tailings ponds. Using these technological advances, seepage of OSPW-influenced groundwater to the Athabasca River has been reported; however, the environmental and toxicological significance of this seepage is currently unknown and needs to be assessed. To address these data gaps, an effects-directed analysis was initiated using groundwater sources previously identified as having a significant bitumen influence from the natural bitumen landscape alone (Drive-point (DP)-1 & DP-2) and two sites beside a tailings pond with evidence of OSPW influence (DP-4 & DP-5). The soluble organic compounds were then isolated and fractionated using a method recently developed to isolate the soluble organics from a large volume of aged OSPW. Analyses by ESI-HRMS, LC-QToF/MS, GC-MS/MS, and SFS indicated that DP-1 did not contain a significant presence of bitumen-derived organic compounds and, therefore, could not be used for further comparison. Analyses of DP-2, DP-4, and DP-5 indicated that the methodology was successful in isolating dissolved organics from industrial and natural sources into chemically distinct fractions, which allowed for subsequent toxicological assessments. The similarity in chemical compositions between sources reinforces the need for advanced targeted analyses for use in source discrimination. Comparison between fractions demonstrated that F3 contained compounds with greater polarity than F2, which in turn was more polar than F1. However, the abundance of soluble organics were captured in F1, including the majority of O_2 species, which include naphthenic acids. This result is consistent with those of aged OSPW and other extraction methods and suggests that additional factors other than molecular weight and the presence of

acid moieties must play a prominent role in defining compound polarities within complex bitumen-derived organic mixtures.

4.2. Introduction

Surface mining in Canada's oil sands region of northern Alberta employs an adaptation of the Clark extraction process for the isolation of bitumen, which utilizes a mixture of hot water and NaOH (FTFC, 1995; FTFC, 1995). Water is recycled throughout the extraction process, which results in oil sands process-affected water (OSPW) accumulating inorganic and organic constituents from the oil sand material. The OSPW is then stored in large settling basins (Mahaffey and Dube, 2017), often termed tailings ponds. Rapid advancement in the chemical characterization of OSPW and its associated extracts has greatly improved our understanding of these complex bitumen-derived organic mixtures. A wide range of constituents has been revealed, including various diamondoid mono-aromatic acids (Rowland et al., 2011; Rowland et al., 2011; Rowland et al., 2011; Rowland et al., 2012), bicyclic acids (Wilde and Rowland, 2015), and varying abundances of different chemical species (Headley et al., 2013; Pereira et al., 2013; Sun et al., 2017). Each of these compound classes is under further investigation for their diagnostic potential when attempting to differentiate bitumen-influenced waters from natural and industrial sources. Laboratory bioassays have demonstrated that OSPW and extracts of the chemicals within are toxic to several different classes of aquatic organism (Marentette et al., 2015; Bartlett et al., 2017; Mahaffey and Dube, 2017). While the relative toxicities of each of the different chemical classes may not be presently known, extracts of the soluble organic constituents, namely the acid extractable organics (AEOs) which include naphthenic acids, have

long been considered to be among the principal toxic components of OSPW (MacKinnon and Boerger, 1986; Brown and Ulrich, 2015; Mahaffey and Dube, 2017).

Natural groundwater in the oil sands region can have a strong bitumen influence as well (Ross et al., 2012; Frank et al., 2014; Sun et al., 2017), with a chemical composition very similar to OSPW (Frank et al., 2014; Sun et al., 2017). Recent investigation (Frank et al., 2014) of natural bitumen-influenced groundwater identified concentrations of AEOs quite comparable to groundwater that had influence from OSPW seepage in combination with natural bitumen input. This previous work was successful at identifying the presence of OSPW seepage in nearby groundwater samples outside of containment systems, however, it did not address the potential toxicity of this seepage relative to the natural input of bitumen-derived organics in the region. In addition to similarities in chemical profiles, recent work (Frank et al., 2018) has shown similarities in toxicity between groundwater samples influenced by OSPW and natural sources.

The current study applied isolation and fractionation techniques (Chapter 2) to reduce the overall mixture into less complex mixtures and allow for more detailed chemical and toxicological characterization of the bitumen-derived organic mixtures in groundwater influenced by natural and OSPW sources. Two sets of groundwaters from previously investigated sites were compared: one set having natural bitumen-influence alone (DP-1 and DP-2) and another set from beside a tailings pond with an identified OSPW influence (Frank et al., 2014; Hewitt et al., 2018) and possibly a natural bitumen-influence as well (DP-4 and DP-5). The objectives of this study included 1) determining if the method developed for aged OSPW could be applied to fractionate, in large volume, other bitumen-influenced water sources; 2) determining if the chemical composition differed substantially between the isolated fractions within a single groundwater sample source; 3) determining if the chemical composition differed

substantially between the same isolated fractions from different sample sources; and 4) determining if there were source-related differences between the chemical compositions. The generated fractions would then be investigated in an effects-directed analysis approach using a complement of bioassays and chemical characterization techniques (Chapter 3).

4.3. Methods and Materials

4.3.1. Sample Collection

Shallow riparian groundwater was collected in September 2013 from sites previously determined to be influenced by OSPW and/or natural bitumen sources. Drive-point (DP)-1 and DP-2 sites were identified as having input from the natural bitumen landscape only, while DP-5 and DP-6 sites were identified as being influenced by OSPW from a nearby tailings pond (Frank et al., 2014; Hewitt et al., 2018). Due to the low water level of the Athabasca River during the 2013 sampling period, the locations for DP-5 and DP-6 were up to 15 m closer to the middle of the river than the previous sample locations (Frank et al., 2014). Groundwater was extracted at depths of 50-90 cm below the riverbed with a stainless steel drive-point system (Roy and Bickerton, 2010). To accommodate the large volume collection, groundwater was pumped slowly over several hours (4-24 hr) from a series of drive-points (3 - 5, spaced over < 3 m along the bank) into multiple 18-L stainless steel vessels fitted with Viton seals. Sample collections from each drive point commenced following the equilibration of field-measured parameters (electrical conductivity, pH, dissolved oxygen). Once collected, the groundwater samples were maintained at 4°C during transport to the Canada Centre for Inland Waters in Burlington, ON and until sub-sampling and extraction was completed, within 7 days of arrival.

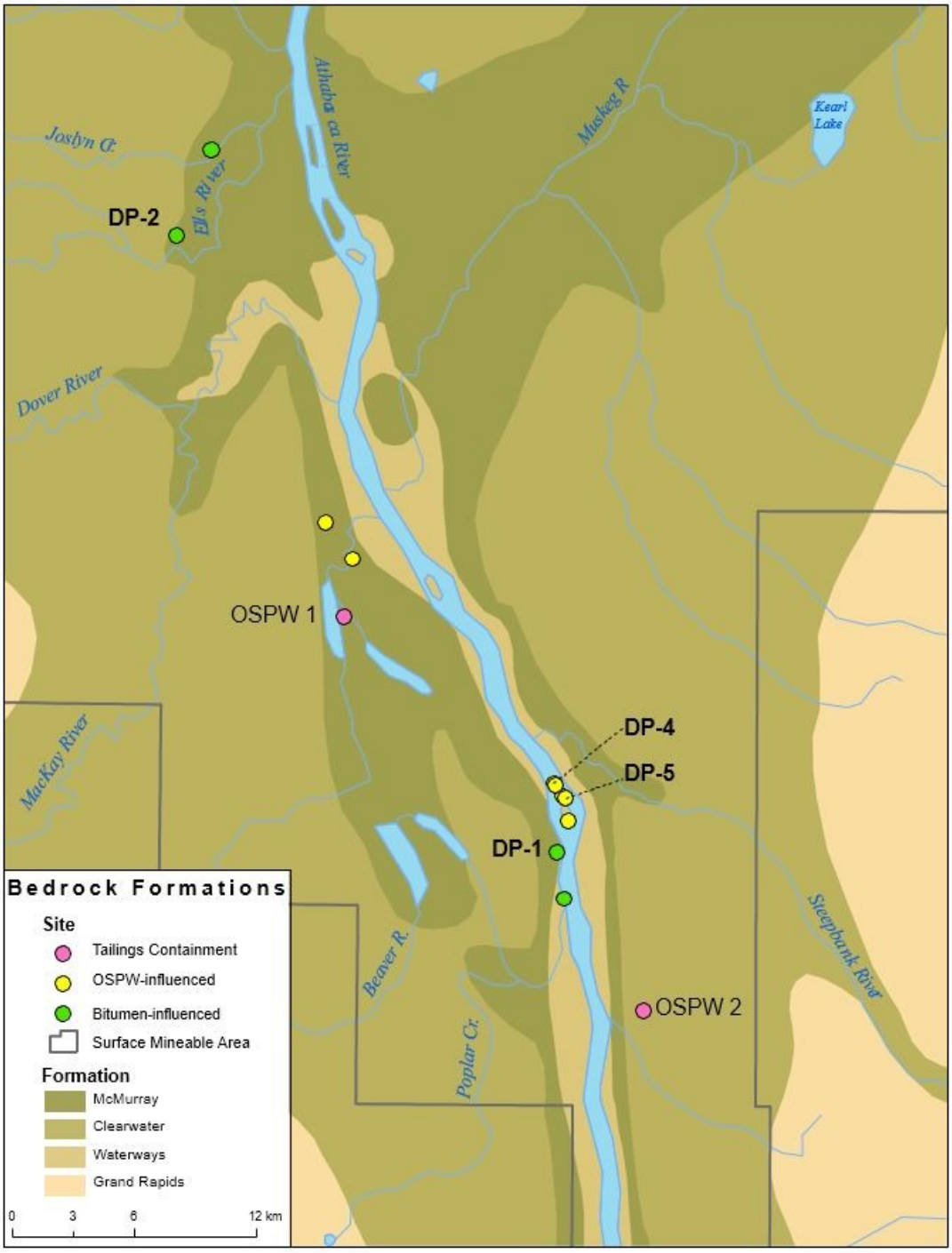


Figure 4.1. Map depicting sampling locations of groundwater sites, OSPW-influenced and natural bitumen-influenced locations and their proximity to anthropogenic OSPW sources.

4.3.2. Centrifugation of Groundwater Samples Prior to Extraction

Through the drive-point collection process, sediments could be introduced to the collected groundwater. As these particulates would not naturally flow with the groundwater, and could also slow filtration through the extraction column, they were removed. A continuous flow centrifuge (Westfalia Model KA 2-06-075) at a rotational speed of 9470 rpm was used to remove >90% of the suspended sediments, which were collected in a stainless steel bowl (Droppo et al., 2009).

4.3.3. Isolation and Fractionation of Soluble Organics

Soluble organics from the groundwater samples were isolated into three fractions, using differences in polarity, via a preparative scale solid phase extraction protocol that demonstrated excellent recovery from aged OSPW (Chapter 2). The preparative fractionation apparatus consisted of a glass column with plunger (10 cm ID x 30 cm height, Spectrum Chromatography, Houston, TX), two 200-L HDPE barrels, and a controller and motor (Cole-Palmer) with a rotary vane pump head (Procon Pumps). The column was operated as a solid phase extraction (SPE) cartridge where feedstock flow was directed onto the resin bed using an adjustable plunger. A water pump was used to pull the initial filtered sample from the sample barrel through the resin in the first column (SPE-1, Figure 4.2) with negative pressure and transfer of the filtrate to a second barrel.

In brief, ~180 L of centrifuged groundwater were filtered through two consecutive 120 g columns of ENV+ (hydroxylated polystyrene divinylbenzene; Biotage®, NC, USA), followed by a total of 3 stages of solvent extraction. Prior to filtering through SPE-1, the centrifuged groundwater sample was adjusted to pH 11.0 ± 0.5 with 10 M sodium hydroxide (NaOH), mixed

for approximately 1 hour with a hand drill fitted with a PTFE mixing rod, and allowed to equilibrate for 12 h. The pH was then re-tested, adjusted accordingly, and allowed to equilibrate for 6 h or until the pH was stable at 11.0 ± 0.5. The ENV+ resin was then preconditioned with ethyl acetate (EtOAc), then methanol (MeOH), and then pH 11 de-ionized (DI) water. A barrel containing the 180 L of centrifuged groundwater was then plumbed upstream into the pre-conditioned ENV+ column and a second empty barrel downstream of the column was also plumbed in. Here and throughout all conditioning and filtering steps, the solvent/water in the column was maintained at a height of 10 cm above the resin bed and the plunger at a height of 1 cm above the solvent/water to avoid disturbance of the resin, and the filtration rate was maintained at 100 ± 10 mL/min. Following filtration of 180 L of groundwater, the column was disassembled, the resin carefully transferred into a 4-L glass beaker covered with a large Kimwipe®, and allowed to dry in a fume hood for 12-24 hrs. Once dry, the analytes from SPE-1 were soxhlet extracted for 12 h using two soxhlet assemblies, each with 60 g ENV+ resin packed between 500 g measurements of sodium sulfate ($NaSO_4$) and 1.5 L of EtOAc. Following the 12 h extraction, the 3 L of EtOAc was pooled and filtered 4 times through 400 g $NaSO_4$ and 8 µm pore-size filter paper (Whatman grade 40 ashless, Sigma-Aldrich®, Oakville, ON) to remove any water. The final extract in EtOAc is hereafter referred to as Fraction 1 (F1), and is expected to contain the least polar soluble organics based on previous OSPW extraction using this approach (Chapter 2). The resin was then removed from the thimbles, allowed to dry, re-placed in new thimbles with fresh $NaSO_4$, and the extraction process was repeated using a total of 3 L of MeOH. Following filtration through $NaSO_4$, the final extract in MeOH is hereafter referred to as Fraction 2 (F2) and is expected to contain soluble organics with intermediate polarity relative to the other fractions.

The groundwater filtrate that was collected following passage through SPE-1 was acidified to pH 2 using 12 M HCl in the second barrel, in a manner similar to the initial adjustment to pH 11. For preparation of the SPE-2 stationary phase, 120 g fresh ENV+ resin was placed into the cleaned column, conditioned, and equilibrated as described previously for SPE-1, with the exceptions that only MeOH was used and the final conditioning was with pH 2 DI water. Following SPE-2 conditioning, the acidified SPE-1 filtrate was pumped from the second barrel, through the conditioned column, and back into barrel 1, ensuring that the solvent/water in the column was maintained at a height of 10 cm above the resin bed and the plunger at a height of 1 cm above the solvent/water to avoid disturbance of the resin, and the filtration rate was maintained at 100 ± 10 mL/min. Following filtration of the 180 L of groundwater through SPE-2, the resin was collected and dried as for SPE-1, with subsequent soxhlet extraction using a total of 3 L MeOH split between two soxhlet assemblies. Following filtration through $NaSO_4$, the final extract in MeOH is hereafter referred to as Fraction 3 (F3) and is expected to contain the most polar soluble organics relative to the other fractions.

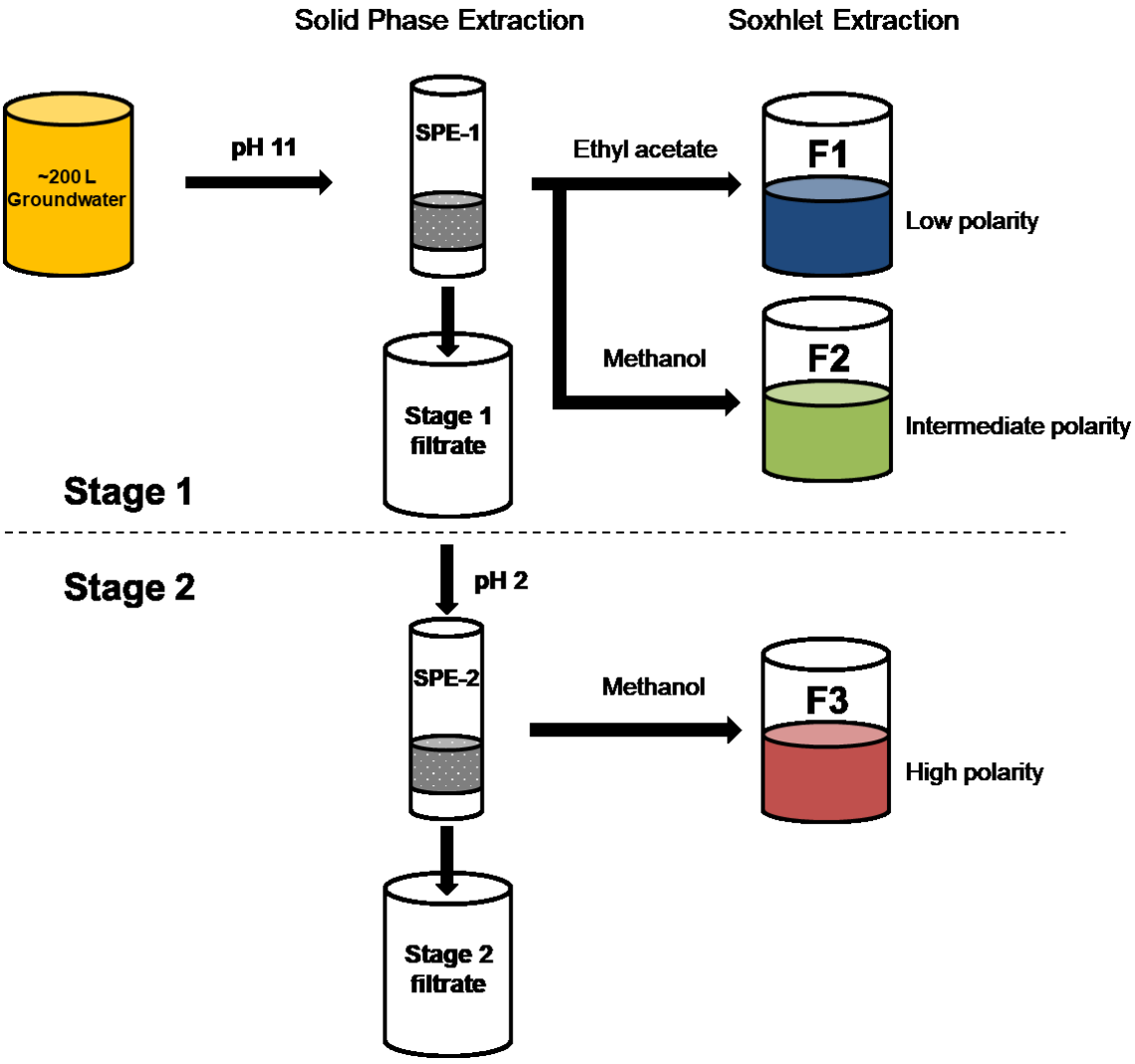


Figure 4.2. Fractionation method schematic displaying Stage 1 and Stage 2 SPE loading followed by soxhlet extraction using solvents indicated. The fractionation resulted in the generation of fractions containing dissolved organic constituents of relative lower polarity (F1), intermediate polarity (F2), and higher polarity (F3).

4.3.4. Synchronous Fluorescence Spectroscopy (SFS)

Synchronous fluorescence spectra were recorded with a Perkin–Elmer Luminescence Spectrometer LS50B, as previously described (Kavanagh et al., 2009; Frank et al., 2016). Samples were filtered through washed disk filters (PES, 25 mm GD/X, O.2 mm pore size; GE Healthcare UK Ltd., Buckinghamshire, UK) before fluorescent analysis to remove particulates and were then scanned in a 1 cm quartz cuvette with PTFE stopper (Hellman, Concord, ON, Canada) at 20±2 °C. All data were collected using FL WinLab 3 software (Perkin–Elmer, Norwalk, CT). The wavelength difference between the excitation and emission monochromators (Dk) was optimized by measuring the spectra of dilute AEO at various offset values (10–60 nm), with a Dk of 18 nm selected, and synchronous fluorescence spectra were collected in the 250–400 nm excitation wavelength range. Excitation and emission monochromator slit widths were set at 5 nm, scan speed at 50 nm min^{-1} and resolution at 0.5 nm. The spectra were blank corrected with Milli-Q water and then smoothed with a 5-point averaging adjacent method using Origin software ver. 7.5 (OriginLab Corp., Northampton, MA). Detected maxima at 272, 307, and 323 were depictive of bitumen influence (Frank et al., 2016).

4.3.5. Electrospray Ionization High-Resolution Mass Spectrometry (ESI-HRMS)

An LTQ Orbitrap Elite (Thermo Fisher Scientific) instrument was used for ESI-HRMS analysis with a pre-defined 5-point regression of OSPW-derived organic acids at known concentrations used to determine resulting dissolved organic concentrations. Operating in full scan negative-ion mode, the mass spectrometer ran at a m/z scan range of 100-600. Achieved resolution at m/z 120 = 240000, m/z 210 = 185000, m/z 300 = 150000, and m/z 400 = 130000, and all of the ions were in the m/z 100 to 300 range in which the resolution ranged from 240000

to 150000. The mass accuracy was <2 ppm error for all mass assignments. Operating parameters were as follows; sheath gas flow rate 25 (arbitrary units), spray voltage 2.90 kV, auxiliary gas flow rate 5 (arbitrary units), S lens RF level 67%, heater temperature 50°C, and capillary temperature 275°C. Infusion solvent used was 50:50 acetonitrile:water containing 0.1% ammonium hydroxide at a flow rate of 200 µL/min. Software used for molecular analysis was Xcalibur v 2.1 (Thermo Fisher Scientific) and Composer v 1.0.2 (Sierra Analytics, Inc.).

4.3.6. Liquid Chromatography Quadrupole Time-of-Flight Sass Spectrometry (LC-QToF/MS) for AEO Quantification

Detailed description of this analysis has been previously published (Brunswick et al., 2015; Brunswick et al., 2016; Brunswick et al., 2016), however a brief summary is provided here. Groundwater samples were adjusted to ~pH 10-11 with ammonium hydroxide to ensure dissolution of the AEOs. The pH-adjusted samples were aliquoted and spiked with the internal standard, decanoic-d3 acid. Reverse phase liquid chromatography was then used to separate the AEOs in the sample, together with detection by an Agilent 6550 iFunnel quadrupole time-of-flight mass spectrometer (LC/QToF). The AEOs were ionized in electrospray negative mode and data were acquired by total ion scan (TIC). The QToF uses accurate mass detection, thus reducing interferences. The instrument qualitative software was able to screen the total ion scan for accurate peak matching using the formula of O2:O3:O4 AEO species. It is noted that, due to the presence of isomers, there may be different AEO peaks in the reference material compared to the samples. Where individual isomer peaks attained acceptable mass accuracy (preferably <5ppm), reached quantitation limits, and were free of interferences, the results were transferred to the quantitative software program for integration. Final analysis employed a weighted 1/x

regression standard curve of pooled AEO responses in ratio to the internal standard. The calibration range was dependent upon the reference standard employed, with in-house validated methods using either Merichem Technical mix or a validated extract of OSPW AEOs. System suitability standards, blanks, and calibration standards were analyzed at the beginning and end of each analytical sequence with Quality Control samples included within each analytical batch.

4.3.7. LC-QToF/MS for Qualitative Assessment

All LC-QToF/MS analyses utilized a methanol mobile phase and required that all samples were dissolved in methanol. Therefore, all water samples and EtOAc fraction (F1) aliquots were rotary evaporated and subsequently brought to just-dryness with a N2 bath. Samples were then brought back up to appropriate volumes in MeOH. The analysis was carried out in full scan negative ion mode (mass range 100-980) using an LC-QToF 6520 (Agilent Technologies, Santa Clara, California, USA) under these conditions: Gas temp 350°C, drying gas 10 L/min, nebulizer 35 psi, VCap 3000 V, Fragmentor 130 V, Skimmer 65 V, reference mass recalibration enabled. The LC conditions were as follows: Column Poroshell 120 EC-C18, 3.0 x 50 mm 2.7 µm, Solvent A Water (0.1 % formic acid), Solvent B Methanol (0.1% formic acid), initial conditions 95% A for 2 minutes, to 100 % B at 20 minutes, hold until 30 minutes. Samples were injected with 1 µL of labelled internal standard (9-anthracene-d9-carboxylic acid, 84.4 pg/µL and Decanoic-d19 acid, 390 pg/µL).

4.3.8. Gas Chromatography Tandem Mass Spectrometry (GC-MS/MS)

All GC-MS/MS analyses were conducted with samples that were methylated using diazomethane and then dissolved in toluene. Therefore, all fraction extracts were solvent-

exchanged in toluene. The analysis was carried out in EI full scan mode (mass range 50-500) using a GC 7000 MSMS (Agilent Technologies, Santa Clara, California, USA). A 1 μL injection was made into a multimode inlet at 270°C into a 30 m DB5 column (Agilent). Oven temperatures were programmed at 90°C for 0.5 minutes, ramped to 300°C at 40°C/minutes with a 5 minute hold.

4.3.9. Inductively Coupled Plasma-Sector Field Mass Spectrometry (ICP-MS) for Metals and Major Ions

Total and dissolved metals were analyzed at Environment and Climate Change Canada's National Laboratory for Environmental Testing (NLET) (Burlington, ON) using Inductively Coupled Plasma-Sector Field Mass Spectrometry. (SOP 2003 - Standard Operating Procedure for the Analysis of Dissolved, Extractable and Total Trace Metals in Water by "Direct Aspiration" or "In Bottle Digestion" Inductively Coupled Plasma-Sector Field Mass Spectrometry (ICP-SFMS; NLET 2008). The analysis of anions was performed by ion exchange chromatography with conductivity detection (NLET Method 01-1080). The analysis of cations was performed by direct aspiration using atomic absorption (NLET Method 01-1061).

4.4. Results and Discussion

4.4.1. Isolation and Chemical Fractionation of Soluble Organics

For each of the 4 groundwater samples, 3 fractions of soluble organic compounds were collected, with expected increasing polarity from F1 to F2 to F3. Following extraction, the fractions were stored in their respective solvents (F1 in ethyl acetate; F2 and F3 in methanol). Samples were solvent-exchanged, if necessary, prior to each chemical analysis.

4.4.2. Aromaticity of Fractions (SFS)

The aromaticity of the water samples collected throughout the extraction process, as well as the final fractions, was assessed using SFS (Figure 4.3). For DP-4, the initial groundwater did not exhibit an SFS spectra representative of the characteristic maxima at 272, 307, and 323 nm for bitumen-influenced waters (Frank et al., 2016), where increased excitation wavelength is representative of increased aromaticity (Kavanagh et al., 2009; Rowland et al., 2011). Likewise, none of the collected fractions from DP-4 exhibited spectra consistent with bitumen-derived organics. SFS analysis of water collected throughout the extraction methodology (Figure 4.2) for the other 3 samples revealed a consistent pattern of the original whole water and pH 11 adjusted groundwater sample having the characteristic triple maxima for bitumen-derived organics, reduced aromaticity in the intermediate stages (Stage 1 filtrate, Stage 2 input), and no aromaticity in the final Stage 2 filtrate (Figure 4.3a). These results indicate that the bulk of aromatic constituents, previously demonstrated to consist of acidic compounds (Rowland et al., 2011), are removed during the first extraction stage at pH 11. Analysis of the collected fractions indicated that Fraction 2 had much lower relative signal intensity. In Fractions 1 and 3 for DP 1, DP-2, and DP-5, there was a range of aromatic compounds detected.

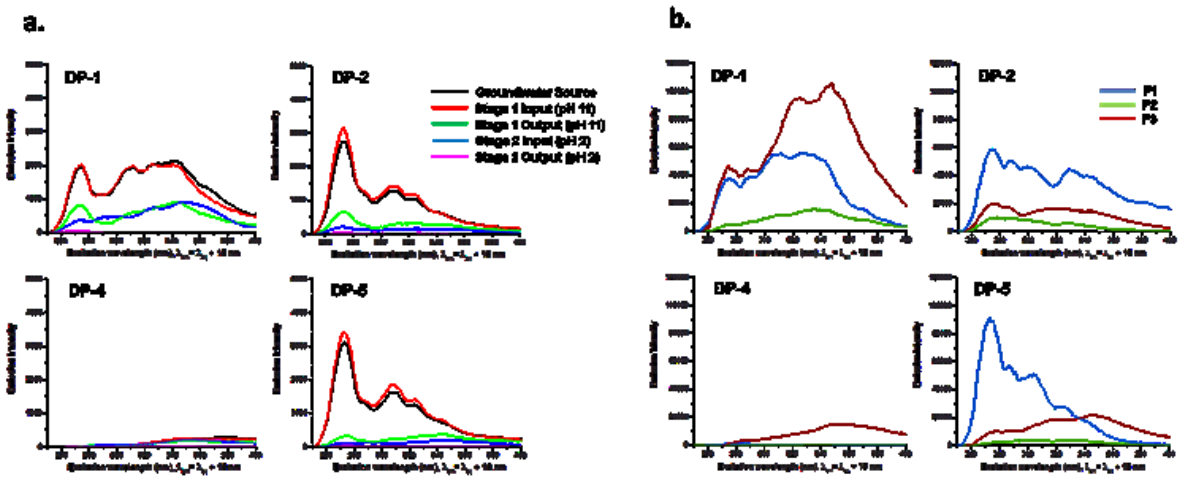


Figure 4.3. Synchronous fluorescence spectroscopy displaying aromaticity of groundwater sites. Presented are aromaticity profiles of fractionation method water sample inputs and output filtrates at each stage (a) and aromaticity of individual fractions (b). Maxima at 272, 307, and 323 nm are characteristic of bitumen-influenced waters (Frank et al., 2016).

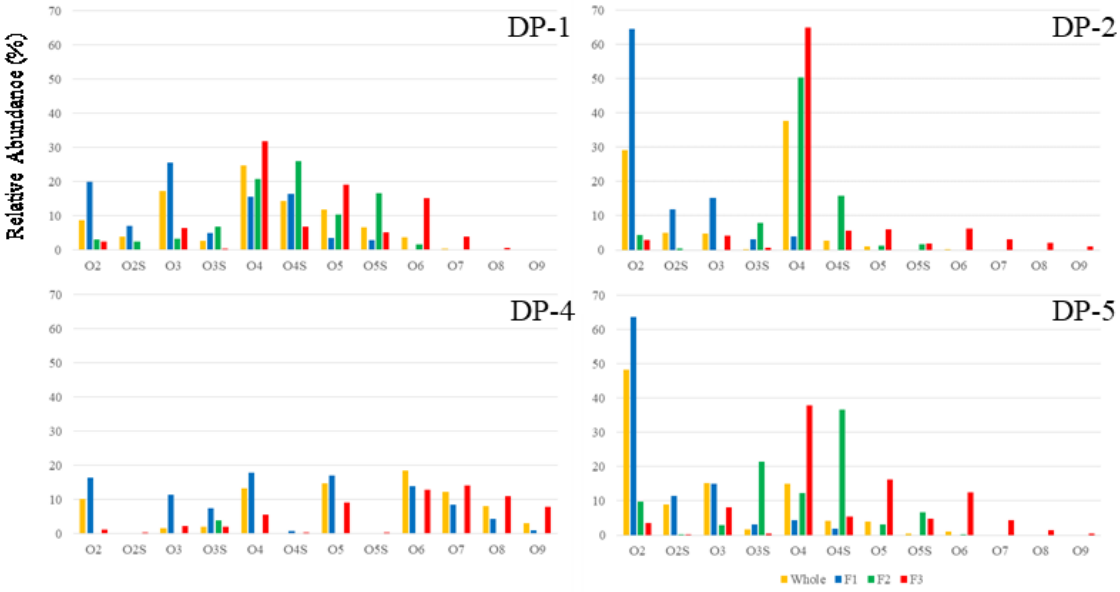


Figure 4.4. Class distribution of whole water and dissolved organic fractions for groundwater collected at sites DP-1, DP-2, DP-4, and DP-5 as determined by ESI-HRMS. Graphs present ion classes (x-axis) versus percent relative abundance of ions (y-axis).

4.4.3. Organic Ion Class Distributions (ESI-HRMS)

The class distributions of organic ions were evaluated for each fraction at all sites. Although nitrogen, sulfur, and oxygen-containing heteroatomic species were detected in each site, only species present above 5% in at least one site were presented for comparison (Figure 4.4a-d).

The composition of oxygen-containing ions in the original whole water varied between sites (Figure 4.4). Site DP-4 contained similar composition of O_2, O_4, O_5, O_6, and O_7-containing species relative to DP-5 and DP-2, with 10.1%, 13.3%, 14.8%, 18.6%, and 12.2%, respectively, however the cumulative range of oxygenated species (O_2 – O_9) comprises an overall greater abundance of more oxygenated species in DP-4, relative to the other sites. For DP-5, whole water predominantly contained O_2 species (57.4%) with lesser contributions from $>O_3$-containing ions (41.1%). The components present in site DP-2 contained similar composition of O_2 and O_4-containing ions, 35.1% and 40.5% respectively, with minor contribution from other species (6.3%). Finally, DP-1 displayed an increase in oxygenated species to a maximum at O_4 (39%) with a concurrent decrease in O_5-O_8.

At all sites, when comparing between fractions within a site, there was a shift to a relative increase in oxygenated compounds from F1 – F3 (Figure 4.4b-d). For example, in DP-5, F1 is composed of predominantly O_2-containing ions (75.2%) with lesser contributions from $>O_2$-containing ions (24.4%). Conversely, the distribution of ions in F3 is dominated by O_4 (43.3%) with contributions from O_3 and $\geq O_5$ (8.6% and 39.8%, respectively), while O_2 ions comprised only 3.7% (Figure 4.4d). Similarly, when comparing oxygenation of components in DP-2 for F1, F2, and F3, contributions by O_2-containing ions were 27%, 5.5%, and 2.4%, respectively (Figure

4.4b-d#). This trend is reversed when comparing >O_2-containing ion contributions for F1, F2, and F3 with 69.1%, 85.3%, and 89.1%, respectively (Figure 4.4b-d).

Analysis of double bond equivalent (DBE) data, also provided by ESI-HRMS, indicates varying degrees of unsaturation due to hydrogen deficiency which can be in the form of carbon-carbon double-bonds, and rings, whether they are alicyclic or aromatic. Typically, the DBE of O_2 organic acid species are representative of cyclicity, where DBE-1 indicates the number of rings present (DBE=1 is present as the carboxyl group). For example, DBE = 2 indicates a compound with a carboxyl group and one saturated ring (DBE = 3 contains 2 rings, etc.) It is quite likely that hydrogen deficiencies herein result from some degree of aromaticity. As such, a simple benzene carboxylic acid (1-ring with 3 double-bonds, 1 carboxyl group) presents a DBE of 5. As the degree of aromaticity observed using SFS (Figure 4.3) is qualitative data, any correlations to DBE must be cautiously applied. For simplicity, DBE data will mainly be interpreted as degree of cyclicity. In the present analyses, only the DBE of O_2 dissolved organic species were examined and are presented as percent abundance relative to the total abundance of O_2 species (the total percent DBE equals the percent O_2 for class distribution) (Figure 4).

Groundwater sites DP-2 and DP-5 contained the greatest abundance of O_2 species (Figure 4.4). The highest value at DBE=3 in the whole water and F1 for these two sites is speculated to be due to substances with functionalities other than aromaticity that translate to hydrogen deficiencies (i.e., hydroxyl, double bond, etc.), while the second highest value at DBE=4 is speculated to be due to mono-aromatic acids, a result supported by the SFS maxima at 272 nm (Figure 4.3). These results are consistent with previous DBE and SFS analyses of acid-extractable organics isolated from fresh OSPW (Bauer et al., 2015) and are also consistent with compound distributions between fractions observed from spiking experiments (Chapter 2). When

analyzing the isolated fractions, the majority of O_2 species in groundwater sites were present in F1, which was supported by class distribution data (Figure 4.4). The DBE in F1 displayed a bimodal distribution, albeit very minor at sites DP-1 and DP-4. These F1 data indicate a predominance of 2- and 3-ring organic acids, with lesser contributions from 4- to 8-ring acids. As noted above, DBE > 5 may also indicate the presence of low cyclicity of which one ring may be aromatic. Fraction 2 displayed a low overall abundance of O_2 species at all sites (Figure 4.4), with DP-5 being the only sample exhibiting any contribution above 5%, in which 6.3% of compounds are alicyclic. Similar to F2, F3 exhibited very low relative abundance of O_2 ions, with no contributions greater that 2% at any site. Nonetheless, sites DP-1, DP-2, and DP-5 exhibited a distribution maximum at DBE = 3 and 4, indicating predominance of 2- and 3-ring organic acids.

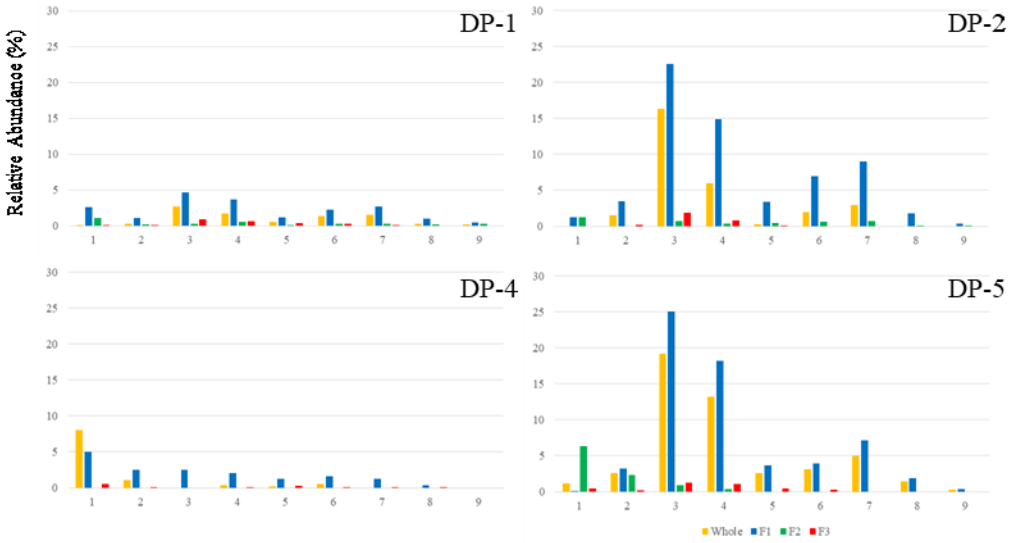


Figure 4.5. Double-bond equivalents for whole water and dissolved organic fractions of the O_2 ion class (classical naphthenic acids) in sites DP-1, DP-2, DP-4, and DP-5 as determined by ESI-HRMS. Graphs present double-bond equivalents as a function of hydrogen deficiencies (x-axis) versus percent relative abundance of the total O_2 ion class (y-axis).

4.4.4. Total Acid-Extractable Organics Concentrations (LC-QToF/MS)

Quantitative analysis of the total concentration of acid-extractable organics (AEOs), a subset within the mixture of soluble organic compounds, revealed that F1 consistently had the greatest abundance relative to the other fractions, and F3 was the fraction with the next greatest abundance (Table 4.1). It is worth noting the higher proportion of organics detected in F3 of DP-2, indicating the abundance of polar compounds at this naturally influenced site, relative to the other sites. While F1 and F3 also had greater abundances of AEOs in DP-4 relative to F2, it is worthy to note that very little AEO composition was detected for any of the DP-4 fractions, as well as the original whole water groundwater sample (Table 4.1). The final Stage 2 exhibited

AEO concentrations below detection limit for all groundwater samples (Table 4.1), indicating the efficiency of the extraction and fractionation method to capture the AEOs.

Table 4.1. Concentration of acid-extractable organics in fractions and filtrate of groundwater sites determined by LC-QToF/MS. Values represent concentrations present in original volumes of respective water samples.

	Dissolved Organics (mg/L)			
	DP-1	**DP-2**	**DP-4**	**DP-5**
Whole	1.6	5.9	<DL[a]	9.5
F1	10.5	17.1	0.1	34.0
F2	0.5	1.1	<DL	0.1
F3	1.7	6.2	<DL	1.1
Filtrate	<DL	<DL	<DL	<DL

[a] <DL = less than detection limit (0.05 mg/L)

4.4.5. Fraction Profiles by GC-MS/MS

Each fraction was profiled for all sites using GC-MS/MS. The relative abundances for each fraction are only comparable between sites, as the fractionation method did not generate equivalent fraction concentrations. Comparison of profiles for each fraction across sites showed greater abundances of unresolved organics in F1 and F3, except at site DP-4, which displayed a relatively low abundance of organics in all fractions (Figure 4.6). Sites DP-1, DP-2, and DP-5 exhibited a similar broad distribution of organics with only slightly different abundances, as displayed by the peak maxima. For the most polar fraction (F3), DP-1 exhibited the highest peak maxima, while DP-2 and DP-5 showed lower maxima indicating lower relative abundance of organics (Figure 4.6). The more polar organics in F3 displayed a broad distribution in DP-1, while DP-2 and DP-5 had reduced distributions in comparison.

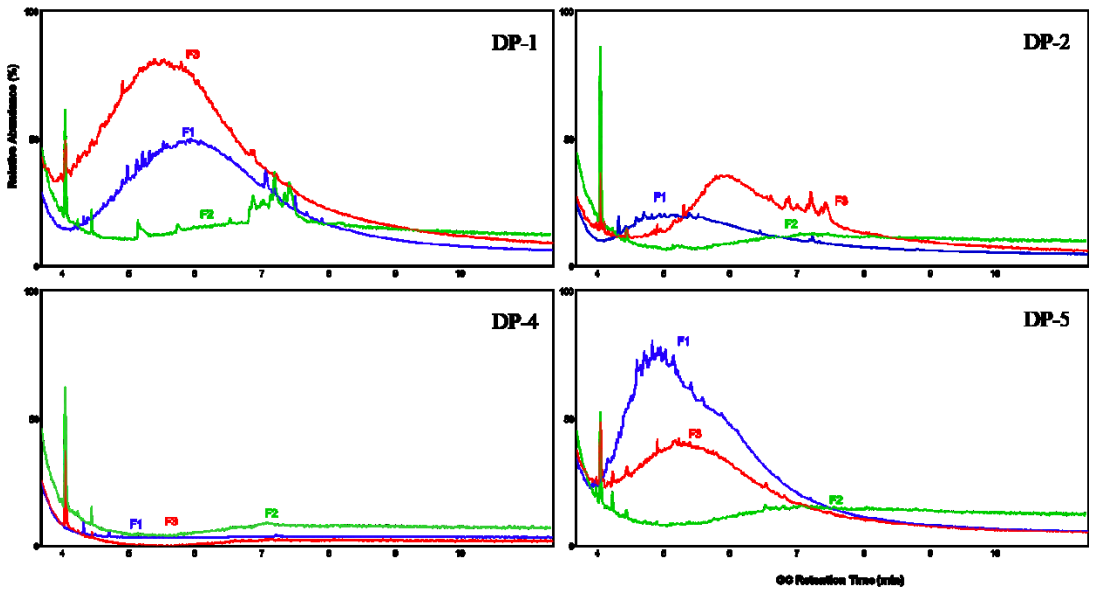


Figure 4.6. GC-MS/MS ion chromatograms of relative percent abundance vs. retention time for F1 (blue), F2 (green), and F3 (red) for each groundwater site DP-1, DP-2, DP-4, and DP-5.

4.4.6. Fraction Profiles by LC-QToF/MS

In order to elucidate relative abundance of polar organic components present in each fraction, LC-QToF/MS was utilized. As with GC-MS/MS, the relative abundance of each fraction is only comparable between sites and not between fractions, as the fractionation method did not generate equivalent fraction concentrations. The retention time for LC-QToF/MS can be interpreted such that components which are identified earlier indicate a greater compound polarity than those retained longer (Figure 4.7).

For all sites, F3 contained the most abundant organics profiled using this method (ESI-), which is expected based on the polarities of the fractions and the ionization method used (Figure 4.7). The differences in the F3 profiles between sites are indicative of the source differences and consistent with differences noted above for ion class distributions (Figure 4.4) and DBE (Figure 4.5). In general, when observing the maxima for each fraction, there is a shift to earlier retention

times from the least polar (F1) to the most polar (F3) fractions. The peaks at 14 min and 17 min are internal labelled standards (9-anthracene carboxylic acid and decanoic acid). For the least polar fraction, F1, sites DP-1 and DP-5 displayed the broadest distribution of organics compared to DP-2 and DP-4 (Figure 4.7). Site DP-2 displayed a reduced distribution of organics with a similar peak retention time as DP-5. Fraction 2 showed low abundance of organics at all sites except DP-1, which displayed a broad distribution of compound polarities. Similarly in F3, DP-1 and DP-5 exhibited broad distributions of compound polarity, while DP-2 and DP-4 displayed a relatively reduced range in polarity. Worthy of note, DP-2 displayed a unique, bimodal distribution for F3.

As with GC-MS/MS analysis, DP-1 and DP-5 exhibited a distribution of organics in F1 and F3 which encompassed a broad range in retention times. The LC-QToF/MS analysis revealed that DP-2 contained organics which exhibited a more polar distribution of organics at all three fractions. DP-4 displayed a relatively low abundance of organic components in all fractions, with a minor peak in F3.

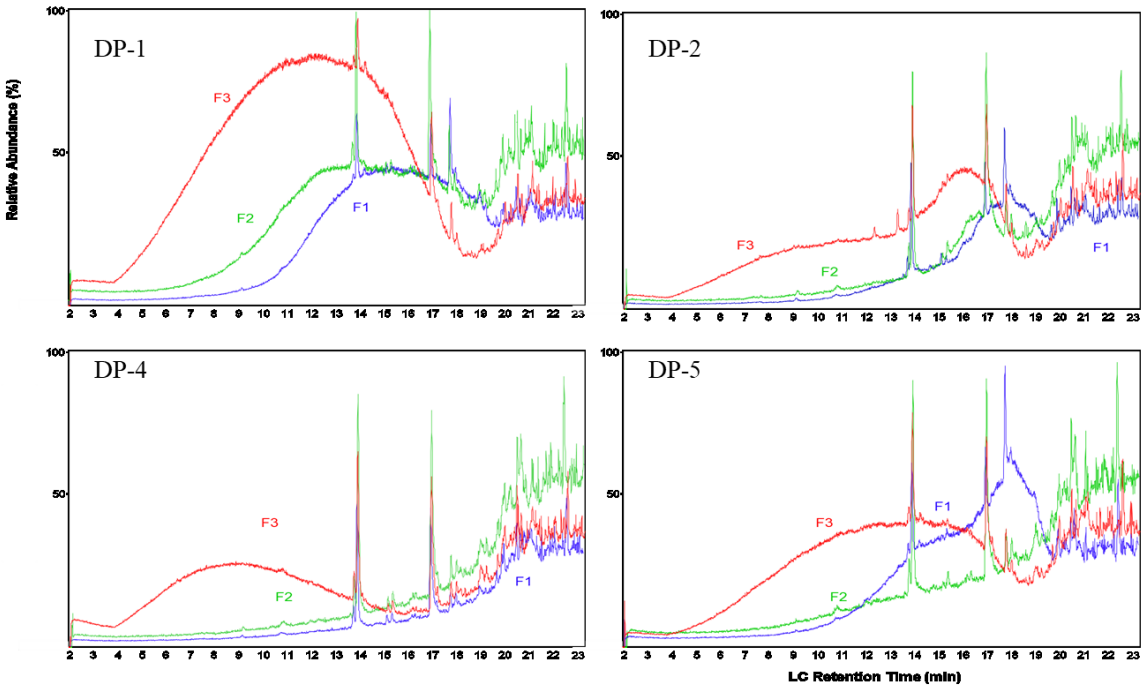


Figure 4.7. LC-QToF/MS ion chromatograms of relative percent abundance vs. retention time for F1 (blue), F2 (green), F3 (red) for each groundwater site DP-1, DP-2, DP-4, and DP-5.

4.4.7. Metals and Major Ion Analysis

The fractionation method employed in this study (Chapter 2) isolated only water soluble organic compounds in F1-F3. While all inorganic components remained in the water fraction following fractionation, necessary pH adjustments using HCl and NaOH modified the inorganic profile relative to the initial groundwater source. Therefore, an unaltered sample of whole groundwater from each site was analyzed, and Table 4.2 displays the unaltered pH, total hardness (mg/L $CaCO_3$), and metal ions for each site. The total water hardness was calculated based on concentrations of calcium, magnesium, iron, strontium, and manganese. Of the 45 metal ions investigated, only those present at concentrations greater than 10 µg/L, in at least one site, are presented.

Table 4.2. Water quality parameters for unaltered groundwaters. Presented as total dissolved metals and major ions determined by ICP-MS. Elements measured but not present were below detection limit at all sites: Be, Bi, Ce, Ga, La, Nb, Pt, Se, Ti, and Y.

	DP-1	DP-2	DP-4	DP-5
Hardness (mg/L $CaCO_3$[a])	499.4	129	541.7	173.2
Metals / Major Ions		**µg/L**		
Aluminum	1.4	BDL	4.7	43.8
Antimony	0.074	0.142	0.171	0.07
Arsenic	0.23	1	0.82	1.13
Barium	26.1	1120	78.3	33.6
Boron	1520	7030	133	1370
Cadmium	0.007	BDL	0.018	0.312
Cesium	0.028	0.122	0.102	0.038
Chromium	0.66	1.82	0.91	0.71
Cobalt	0.332	0.502	2.17	1.07
Copper	4.16*	17.1*	27.2*	4.41*
Iron	3	27.1	16.9	8.6
Lead	0.144	0.434	0.319	0.911
Lithium	252	665	36.7	119
Manganese	22.9	2.65	89.7	9.65
Molybdenum	1.19	0.481	2.7	4.94
Nickel	2.9	2.53	16.1	3.31
Rubidium	3.07	9.59	3.04	3.24
Silver	0.034	0.172	0.352	0.048
Strontium	1020	1680	1040	395
Thallium	0.001	0.161	0.005	0.001
Tin	0.119	BDL	0.273	0.182
Tungsten	0.013	0.101	0.022	0.022
Uranium	0.356	0.159	11.3	0.56
Vanadium	0.2	1.86	0.23	0.14
Zinc	0.7	BDL	1.9	0.6
		mg/L		
Calcium	120	9.03	64.5	17.1
Chloride	93.8	999.0*	4.51	15.2
Fluoride	0.23	0.64	0.06	1.59
Magnesium	48.3	25.4	92.2	31.6
Potassium	8.24	16	5.68	9.8
Silica	18.7	13.2	26.3	25
Sodium	185	2290	26.4	222
Sulfate	303	3.39	85.1	18.8

[a] Calculated based on concentrations of Ca, Mg, Fe, Sr, and Mn.

* indicates exceedance of CCME and USEPA water quality guidelines

BDL = Below detection limit

Concentrations of most inorganic constituents were not in exceedance of Canadian Council of Ministers of the Environment (CCME) or United States Environmental Protection Agency (USEPA) freshwater quality thresholds at any of the sites assessed (USEPA, 2004; CCME, 2017), with the exception of copper (Cu) which was in exceedance at all of the sites (Table 4.2). Comparison between groundwater samples indicated that DP-5 had the highest concentration of zinc, and a high concentration of iron. DP-4 displayed the greatest water hardness ($CaCO_3$) compared to other sites due to a relatively high concentration of calcium. This site also displayed the lowest overall concentrations of sodium, potassium, boron, and iron. Site DP-1 was most similar to DP-5 in that it displayed the highest concentration of iron while all other ions displayed similar concentrations. DP-2 displayed the highest concentrations of sodium, potassium, aluminum, barium, boron, and strontium, while having the lowest concentrations for calcium, magnesium, manganese, zinc, and overall water hardness.

4.5. Conclusions

This investigation applied a recently developed extraction method (Chapter 2) to isolate and fractionate the soluble organic compounds within groundwater samples previously identified as having significant bitumen influence (Frank et al., 2014; Hewitt et al., 2018). Two samples (DP-1 and DP-2) were selected due to previous determination of their bitumen influence being solely natural, and two samples (DP-4 and DP-5) were selected due to previous determination of being influenced by OSPW (and possibly natural bitumen also).

The first objective of this investigation was to determine if the method developed for aged OSPW could be applied to fractionate, in large volume, other bitumen-influenced water sources. The method created 3 distinct fractions with little observed loss in the final Stage 2 filtrate

(Figure 4.3, Table 4.1), thus indicating its success at isolating the soluble organic compounds from all investigated bitumen-influenced groundwater samples. It was not possible to add surrogate spikes to each groundwater sample to quantify total recovery as all generated fractions were to be subjected to toxicological assays (Chapter 3). It should be noted that DP-4, which had previously been identified as having influence from OSPW seepage (Frank et al., 2014; Hewitt et al., 2018), had little to no signal detected with several of the methodologies previously demonstrated as being diagnostic of bitumen influence (Figures 4.3, 4.6, and 4.7; Table 4.1). We conclude that the water collected from DP-4 did not contain appreciable amounts of bitumen, and it was therefore not possible to make comparisons to the other sites.

The second objective of this study was to determine if the chemical composition differed substantially between the isolated fractions within a single groundwater sample source. Analyses by GC and LC for the isolated fractions from DP-1, DP-2, and DP-5 indicated that F1 contained the greatest abundance of organic compounds, and F2 had the least. This result is different from analyses of fractions isolated from aged OSPW in which F3 had the greatest abundance of organic compounds, followed by F1 (Chapter 2). Assessment of chemical speciation revealed increased oxygenation through F1 to F2 to F3, consistent with increases in polarity that were expected given the extraction protocol. Previous analyses of fractions isolated from an aged OSPW source revealed that compounds captured in F3 are earlier eluting by reverse phase LC, indicating that F3 retains the most polar compounds (Chapter 2). However, analyses of F1 in this previous OSPW fractionation, as well as in the current groundwater investigation, revealed that the majority of O_2 species, which include organic acids such as naphthenic acids, are collected in F1. Therefore, the polarity of bitumen-derived soluble organic compounds appears to be a function of factors other than just protonation and deprotonation of carboxylic acid moieties,

including functional groups, water solubility, molecular size, and molecular structure. Spiking experiments with the fractionation method utilized here also suggest that additional factors other than molecular weight and the presence of acid moieties must play a prominent role in differentiating polarity of the compounds present within complex bitumen-derived organic mixtures (Chapter 2). This observation is also consistent with the results of another investigation that used liquid-liquid extractions of fresh OSPW (Morandi et al., 2015). While these studies employed two different methods (SPE, liquid-liquid) and used different sources of bitumen-influenced waters (fresh OSPW, groundwater, aged OSPW), they both resulted in the abundance of dissolved organics being isolated in the first high pH extraction, and not in the fraction expected to contain the most polar components. Furthermore, this result of larger, more complex, compounds being more polar than smaller, simpler acids is also consistent with HRMS analyses of a previous fractionation of fresh OSPW by distillation (Frank et al., 2008; Frank et al., 2009; Bauer et al., 2015). These same functionalities that are affecting compound polarity may very well play a role in their relative bioavailability and toxicological properties.

The final objectives of this study were to determine if the chemical composition differed substantially between the same isolated fractions from different sample sources and also to determine if there were source-related differences between the chemical compositions. Using advanced separation and high resolution analytical methodologies, previous investigations had identified an OSPW influence at DP-5, and solely natural bitumen influence at the sites of DP-1 and DP-2 (Frank et al., 2014; Hewitt et al., 2018). In many ways, the analytical profiles for DP-2 and DP-5 closely resemble each other, including SFS maxima, DBE plots of O_2 species, LC-QToF profiles, and speciation plots; an interesting result given previous determination of solely natural bitumen influence in DP-2 and OSPW influence in DP-5. The lack of noticeable

differences in organic and inorganic compound abundance, chemical speciation, aromaticity, and double bond equivalents demonstrate the chemical similarity of bitumen-influenced groundwaters regardless of the natural or OSPW origin of the bitumen source, and reinforces the need for more advanced targeted analyses in source differentiation (Frank et al., 2014; Hewitt et al., 2018; Milestone et al., 2018).

Further characterization of the generated fractions from these groundwater sources using advanced separation and high resolution analytical methodologies may allow for the identification of compounds unique to OSPW and/or natural sources. In addition, toxicological assessment of the isolated fractions may help to identify principal drivers of toxicity in bitumen-influenced groundwaters and may also help to identify sensitive species and endpoints; information that is vital for monitoring and remediation research initiatives.

Chapter 5. Toxicity of Bitumen-Influenced Groundwater Fractions to a Suite of Aquatic Organisms

5.1. Overview

The extraction of surface mined bitumen from oil sands deposits in northern Alberta, Canada produces large quantities of waste tailings, termed oil sands process-affected water (OSPW). Due to the toxic nature of some OSPW constituents, industry operators in the region must store OSPW on site in large mined-out pits called tailings ponds. Unfortunately, recent evidence that seepage from tailings reservoirs is entering into surrounding groundwaters poses a concern for potential surface water contamination. The present study investigated dissolved organic toxicity from two OSPW-influenced groundwater sites (DP-4, DP-5) and compared their toxicity to two natural bitumen-influenced groundwater sites (DP-1, DP-2). This comprehensive analysis involved exposing previously fractionated bitumen-influenced groundwater samples to a suite of bioassays: *P. promelas, O. latipes, V. fischeri, H. azteca, D. magna,* and *L. siliquoidea*. The fractionation method isolated three fractions from each groundwater site (F1-F3) using differences in polarity. By exposing the suite of organisms to original whole groundwater, F1, F2, F3, and a Recombined treatment (all fractions) from all sites, comparison of fraction constituents, naturally- and OSPW-influenced sites, and relative species sensitivities was achieved. In general, *P. promelas* and *H. azteca* were the most sensitive to organic components, while *V. fischeri* and *L. siliquoidea* appeared least sensitive. Invertebrate species *D. magna* and *L. siliquoidea* bioassays suggested sensitivity to inorganic components within bitumen-influenced groundwater. The overall absence of toxicity observed for F2 was possibly due to the low concentration of detectable organics present in F2. Comparison of bitumen-influenced groundwater sites indicated that those containing appreciable amounts of dissolved organics (DP-1, DP-2, DP-5) presented similar toxicities to sensitive species regardless of the source. It is likely that the transport of tailings seepage through substrate and mixing with groundwaters

affects toxicity associated with tailings contaminants. These findings should be taken into account with respect to tailings pond construction material, design, location, seepage reclamation, and tailings pond decommissioning.

5.2. Introduction

Oil sands mining in northern Alberta, Canada produces an estimated 2.37 million barrels of crude oil per day (Canadian Association of Petroleum Producers, 2016). The oil sands deposits contain roughly 165 billion barrels of remaining bitumen reserves (Canadian Association of Petroleum Producers, 2016). The extraction and separation of bitumen from oil sand produces large quantities of waste tailings and oil sands process-affected water (OSPW). To mitigate industry-related waste contaminants entering the natural environment, oil sands operators currently store tailings in pits remaining from surface mining, termed tailings ponds. Tailings ponds contain a variety of concentrated metals (i.e. Al, Fe, Cu, Pb, Zn etc.), major ions (i.e. Na^+, Cl^-, SO_4^{2-}, CO_3^{2-}, etc.), neutral organics (i.e. polycyclic aromatic hydrocarbons: PAHs), and polar organic compounds (acid-extractable organics: AEO) (Allen, 2008). Of these, a subgroup of AEO called naphthenic acids (NA) have been identified as the primary toxic components (Allen, 2008; Brown and Ulrich, 2015). More recent findings have noted toxicity can be attributed to the suite of AEO present in OSPW, which contain not only classical NA (O_2-containing compounds) but also polyoxygenated compounds ($>O_2$) (Bauer et al., 2017). Moreover, both the inorganic and organic constituents of OSPW display some degree of acute toxicity to aquatic organisms (Allen, 2008; Royal Society of Canada Expert Panel, 2010). Although much of the tailings contaminants are present in the natural bitumen ore, those present in tailings ponds are much more concentrated. For example, polar organic NAs make up roughly 2% of bitumen by weight,

but comprise up to 50% of AEO within tailings (Brown and Ulrich, 2015). Additionally, tailings pond sediments have been found to contain several hundred times the hydrocarbon abundance as local riverbed sediments (Wang et al., 2014). Finally, the high salinity present in tailings is largely a result of the caustic hot water solutions (NaOH) employed for the separation of bitumen (FTFC, 1995a), which becomes concentrated as a result of water recycling.

Due to the toxic nature of oil sands tailings, industry operators have invested considerable effort to prevent tailings from leaching into underlying soils and contaminating groundwaters. Tailings pond construction methods include perimeter dykes, low-permeability clay-till dyke material, internal dyke drainage, and tailings interceptor ditches (Ferguson et al., 2009; Yasuda et al., 2010; Holden et al., 2011). Additionally, as tailings begin to settle and dewater, the consistency changes to a more dense material which migrates to the bottom of the ponds during construction and filling (Ferguson et al., 2009). Over decades this material, termed mature fine tailings, effectively creates a less permeable barrier at the bottom of the tailings ponds (Ferguson et al., 2009). Nonetheless, despite efforts to moderate pond seepage, recent evidence suggests OSPW infiltration into underlying groundwaters is occurring (Ferguson et al., 2009; Oiffer et al., 2009; Yasuda et al., 2010; Ross et al., 2012; Frank et al., 2014; Roy et al., 2016, Sun et al., 2017). A particular study estimated seepage from a pond bottom into underlying substrate at a rate of 2.0 L/s (Ferguson et al., 2009).

A major concern with tailings pond seepage is the potential for groundwaters to transport contaminants into nearby rivers, posing a risk to aquatic organisms. In addressing this concern recent research has focussed on chemically profiling OSPW sources and developing methods to differentiate OSPW from natural surface and groundwaters. Specifically, chemical fingerprinting has been achieved using parameters such as molecular charge/weight ratio (Barrow et al., 2015;

Holowenko et al., 2002; Ross et al., 2012), AEO fluorescence spectra (Frank et al., 2014; Kavanagh et al., 2009), heteroatom proportions (Barrow et al., 2015; Frank et al., 2014; Headley et al., 2011b; Sun et al., 2017), and double-bond equivalents (Barrow et al., 2015; Headley et al., 2011b). Using chemical diagnostics fingerprinting, studies have identified that OSPW can change chemically due to interaction with underlying substrate. For example, total polar organic concentrations (including NAs) decrease in seepage plume samples further away from the source (Ahad et al., 2013), likely due to sorption by soils containing some degree of organic matter (Janfada et al., 2006). Groundwater in the oil sands region displays a wide range in salinity with Cl^- concentrations in some shallow groundwater discharges of up to 50 mg/L (Jasechko et al., 2012). This is noteworthy because salinity also has the potential to alter the chemistry of seepage plumes. With regard to major ions, the persistence of Na^+ and Cl^- in OSPW has been proposed to be dependent upon the underlying substrate, and where Na^+ persists the precipitation of existing ions (SO_4^{2-}, Ca^{2+}, Mg^{2+}) will likely occur (Holden et al., 2011). Additionally, there is evidence to suggest that high Cl^- concentrations can preferentially precipitate certain AEO classes, changing the composition of organics within OSPW (Headley et al., 2012). Overall, tailings pond seepage becomes less distinguishable from groundwaters the further they are sampled from the plume source due to transport through substrate and mixing with natural groundwaters.

There is ample research which has evaluated the toxicity of OSPW and the organic components therein, but few studies have addressed the toxic effects of groundwater contaminant mixtures. There is a significant lack of knowledge with regard to seepage toxicity as a source of surface water contamination. A recent investigation assessed the acute toxicity of freshwater mussel, *H. azteca*, and fathead minnow to several bitumen-influenced groundwaters, using sites previously identified as having OSPW influence or having influence from natural bitumen

deposits only (Frank et al., 2018). The results of this previous study indicated that toxicity was associated with all of the bitumen-influenced groundwaters, regardless of source, and suggested that additional research was needed to identify the compounds that were the primary cause of the observed responses.

The current study was designed as an extension of this previous work, using several of the same sample locations, however collected in much larger volume to allow for fractionation followed by a suite of chemical and toxicological assessments. The primary objective of this study was to evaluate the toxicity of anthropogenically-derived groundwater contamination and compare it to natural bitumen-influenced groundwaters. Shallow riparian groundwater sites were selected from two sites which were identified previously as having input from a mixture of natural bitumen and OSPW sources (DP-4, DP-5) (Frank et al., 2014; Frank et al., 2018; Hewitt et al., 2018). For comparison to a reference, two groundwater sites which were identified previously as having input from natural bitumen only (DP-1, DP-2) were selected (Frank et al., 2014; Frank et al., 2018; Hewitt et al., 2018). A secondary objective involved assessing drivers of toxicity and the influence of organic and inorganic groundwater components. This was achieved by utilizing a fractionation method developed for the separation and isolation of contaminants within an aged tailings pond water (Chapter 2), which was applied to the four bitumen-influenced groundwater sites (Chapter 4). The produced fractions were then exposed to a suite of bioassays which evaluated toxicity to aquatic organisms at multiple trophic levels.

5.3. Materials and Methods

5.3.1. Sample Acquisition

Methods for acquisition of bitumen-influenced groundwater samples have been described in detail (Chapter 4). Briefly, water samples from shallow riparian groundwaters were collected in September 2013 from four sites (Figure 4.1). Two sites were previously identified as having input from natural bitumen alone (bitumen-influenced; DP-1, DP-2), while the other two sites were identified as having input from OSPW (OSPW-influenced; DP-4, DP-5) (Frank et al., 2014; Frank et al., 2018; Hewitt et al., 2018). The groundwater was extracted at depths of 50-90 cm over several hours (4-24 hr) using a stainless steel drive-point system (Roy and Bickerton, 2010). Samples were collected in 18-L stainless steel collection vessels and stored at 4°C.

5.3.2. Groundwater Fractionation

Prior to fractionation, all groundwater samples were centrifuged, removing potentially introduced suspended sediments (>90%) from the groundwater samples with a continuous flow centrifuge (Westfalia Model KA 2-06-075) at a rotational speed of 9470 rpm, with the suspended sediments collected in a stainless steel bowl (Droppo et al., 2009). Fractionation of whole, centrifuged groundwaters has been previously described in detail (Chapter 4), utilizing an adapted method for the isolation of soluble organics from OSPW (Chapter 2). In brief, a preparative scale solid phase extraction method was employed to fractionate soluble organic mixtures from collected samples into three fractions, using differences in polarity. By design, the fractionation method isolated fractions using differences in polarity. Fractionation involved using a water pump (controller and motor: Cole-Palmer, rotary vane pump head: Procon Pumps) to direct ~180 L of feedstock flow into a glass column (10 cm ID x 30 cm height, Spectrum

Chromatography, Houston, TX) containing 120 g columns of ENV+ resin (hydroxylated polystyrene divinylbenzene; Biotage®, NC, USA). Fractionation involved two subsequent stages, where the resulting filtrate was stored in a 200-L HDPE barrel after each filtration. In order to achieve separation of organics by polarity, whole water in Stage 1 was pH-adjusted to pH 11 and filtered at a rate of 100 ± 10 mL/min through the preconditioned ENV+ resin contained in the fractionation apparatus. In Stage 2, the resulting filtrate was then acidified to pH 2 and filtered through a fresh batch of newly conditioned ENV+. The ENV+ resin containing trapped organics was removed from the column at each stage, and dried in a fume hood for 12-24 hrs. Resin from Stage 1 was soxhlet extracted with 3 L of ethyl acetate (fraction 1: F1) followed by a separate extraction with 3 L of methanol (MeOH, fraction 2: F2), while resin from Stage 2 was soxhlet extracted with 3 L of MeOH (fraction 3: F3). Soxhlet extractions were performed over a 12 h period, and solvent from each of the three isolations was filtered through sodium sulfate 4 times to remove any water. The result was three fractions (F1-F3) contained in 3 L each of respective solvent.

5.3.3. Chemical Characterization

Chemical analysis of organic fractions and whole water was performed using a suite of analytical instruments including liquid chromatograpy quadrupole time-of-flight mass spectrometry (LC-QToF) in negative-ion mode, gas chromatography tandem mass spectrometry (GC-MS/MS), electrospray ionization high-resolution mass spectrometry (ESI-HRMS) in negative-ion mode, and synchronous fluorescence spectroscopy (SFS). All instrument methods and parameters have been described in detail (Chapter 2; Chapter 4). Major ions were analyzed by chemical suppression ion chromatography, and dissolved metals (Ca, Mg, Na, K, Si) were

analysed by an inductively coupled argon plasma system (ICP-OES) by ECCC's National Laboratory for Environmental Testing (NLET) in Burlington, ON (NLET, 2003). All other 35 dissolved metals were analyzed by NLET using ion chromatography plasma optical emissions spectrometry (ICP-MS) (NLET, 2003).

5.3.4. Bioassay Treatment Preparation

Bioassays were exposed to unaltered whole groundwater (hereafter referred to as whole water), the three generated fractions, and a "Recombined" treatment which represented all three organic fractions combined. All treatments (except whole water) were subject to preparation prior to bioassay exposure. Because fractions represented a concentrated sample in solvent, dilutions were necessary to bring fractions to whole water equivalents and reduce the amount of solvent used in exposures to 0.1% solvent by volume. The Recombined treatment was reduced to 0.3% solvent by volume. Treatment preparation has been previously described in detail (Chapter 3). Briefly, an aliquot from each 3-L fraction, equivalent to 10-L of original whole water sample volume, had solvent removed using a rotary evaporation unit at 60°C with vacuum set to ~340 mbar. The remaining residue was then re-dissolved into 100 mL of 10% MeOH and 90% 0.01 M NaOH. The 100-mL stock solution (representing 100x concentrated whole water equivalent) was vortexed, sonicated for 5 minutes, and stored at 4°C until further use. For bioassay fraction treatments, 10 mL of each stock solution were dissolved into 990 mL of control water while the Recombined treatment involved adding 10 mL of each fraction stock to 970 mL of control water. Two different solvent controls were prepared such that they contained 0.1% (Solv100) and 0.3% (Solv300) MeOH by volume dissolved in control water, to account for potential solvent effects present in fractions and the Recombined treatment, respectively.

5.3.5. Bioassays

Bioassays were exposed to eight treatments: whole water, three fractions, a Recombined treatment, a water control, and two solvent controls. The only modification to whole groundwater was that it was centrifuged prior to fractionation in order to remove particulates as it was a concern that some may have been introduced during collection of samples. The "Recombined" treatment represented all three organic fractions combined. Control water varied slightly depending on bioassay requirements. Two separate solvent controls consisted of MeOH dissolved into control water at identical concentrations to that present in the isolated fractions (0.1% MeOH) and the Recombined treatment (0.3% MeOH). All bioassays are described in detail previously (Chapter 3) and herein in Appendix B (B1).

5.3.6. Statistical Analysis

Data were analyzed using R version 3.3.3 (R Core Team, 2017) and RStudio version 1.0.136 (RStudio Team, 2016). For each site, an initial analysis comparing each endpoint across the relevant control groups (Control, Solvent 100, and Solvent 300) using one-way analysis of variance (ANOVA) tests was conducted to determine possible solvent effects present in the bioassay. Model assumptions were assessed via residual plots, Shapiro-Wilk's Test, and Levene's Test. Comparisons where the model assumptions appeared to have been violated were re-assessed after a transformation of the data. In cases where a transformation of the data still resulted in an apparent violation of the model assumptions, data on the original scale were re-assessed using the non-parametric Kruskal-Wallis test. For species in which no evidence of a difference in the mean endpoint across control groups was found, data from all relevant control groups was pooled into a single group for comparison with the remaining five treatment groups.

All endpoints within sites were again compared with one-way ANOVA across six treatment groups (pooled control (where applicable), whole water, fractions F1, F2, and F3, and Recombined treatment), followed by Tukey's method for pairwise comparisons when significant evidence ($p \leq 0.05$) of a difference among treatment means was identified. Assumptions of normality and constant variance were assessed as before, and comparisons in which these assumptions were not satisfied were re-assessed either after a transformation of the data, or with the Kruskal-Wallis test followed by Wilcoxon-Mann-Whitney tests with a Bonferroni adjustment for pairwise comparisons. Additionally, each treatment was analyzed for evidence of a difference across the four sampled locations (DP-1, DP-2, DP-4, and DP-5) within each bioassay using the same approach described for analysis within sites.

Some of the statistical analyses presented herein using parametric methods were subject to mild violations of the assumptions required for ANOVA tests. Specifically, *H. azteca, O. latipes,* and *P. promelas* at sites DP-4 and DP-5, and *P. promelas* at sites DP-1 and DP-2. In all cases, even after a transformation of the data, the assumption of normality was not satisfied according to Shapiro-Wilks test. However an assessment of a normal quantile-quantile plot of the model residuals in each analysis suggested that the violation of normality was mild. According to Levene's Test, there was no evidence of a violation of the constant variance assumption in any of these cases. However, at each site for *H. azteca, O. latipes,* and DP-1 and DP-2 for *P. promelas* one treatment had no variability (all replications had the same percent hatched, either 0% or 100%). Since ANOVA is relatively robust to mild violations of normality assumption, and violations of constant variance where sample sizes are identical, as was the case herein, parametric methods were used. Nonetheless, although we are confident in the statistical interpretations, the results of these analyses should be interpreted with caution.

5.4. Results

5.4.1. Pimephales promelas

A comparison of all bioassays showed that fathead minnow (*P. promelas*) was among the most sensitive species tested (Figure 2a). The *P. promelas* bioassay presented a case where the Solv300 solvent control was significantly different from control water. Because solvent effects were assumed to have an effect on toxicity, only Solv100 was pooled with control water and the Recombined treatment was assessed separately. Because the Recombined treatments were significantly more toxic than the Solv300 control, the data were still presented graphically and discussed.

The natural bitumen-influence site DP-1 displayed significant ($p < 0.001$) acute toxicity compared to pooled controls (control water and Solv100, hatch success 96%) for the whole water (hatch success 8%) and F1 treatments (3%). These two treatments displayed hatch success below 10% and were also significantly more toxic than all other treatments ($p < 0.001$), but were statistically similar ($p = 0.93$) to each other. Sample DP-2 was also very toxic, displaying 100% mortality in the whole water treatment. Similar to DP-1, at DP-2 the whole water (hatch success 0%), and F1 (27%) treatments were significantly ($p < 0.001$) more toxic than pooled controls. In this case, whole water and F1 were significantly different from each other ($p < 0.001$). At the OSPW-influenced site DP-4, whole water and F3 were significantly different from pooled controls and F1 ($p < 0.001$). The other OSPW-influenced site DP-5, displayed a significant reduction ($p < 0.004$) in hatch success for F1 (hatch success 32%) compared to controls and all other treatments. Whole water was also significantly more toxic than pooled controls, F2, and F3 ($p < 0.04$), but less toxic than F1.

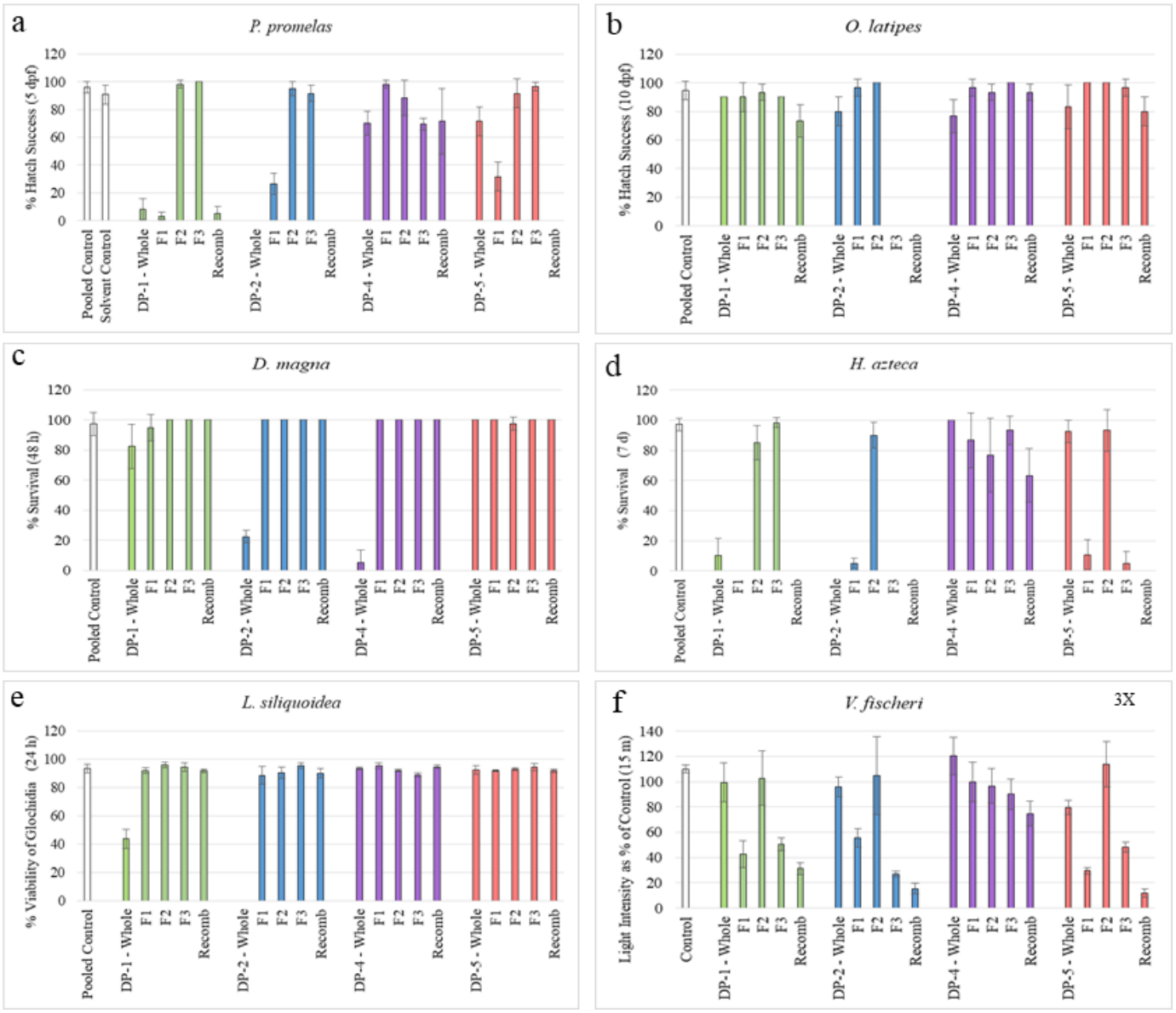


Figure 5.1. Percentage survival/viability of organisms (mean ± standard deviation) exposed to control water, solvent control, whole OSPW (Whole), fractions (F1-F3), and a Recombined treatment (Recomb, fractions F1-F3 combined). Vertebrate organisms are represented by *P. promelas* (a) and *O. latipes* (b). Invertebrate organisms are represented by the *D. magna* (c), *H. azteca* (d), and *L. siliquoidea* (e). The Microtox® assay (f) represents a prokaryotic marine bacterium, *V. fischeri*. For the *V. fischeri* assay, exposure to F1-3 and the Recombined fraction

test solutions was 3x whole water concentration of the OSPW source. Exposure OSPW was from groundwater sites, DP-1 (green), DP-2 (blue), DP-4 (purple), and DP-5 (red).

5.4.2. Oryzias latipes

The Japanese medaka (*O. latipes*) bioassay displayed relatively low sensitivity compared to other bioassays (Figure 5.1b). DP-1 displayed significant ($p = 0.048$) acute toxicity in only the Recombined treatment (hatch success 73%), which differed from pooled controls (95%). At site DP-2, no survival was observed for F3 and the Recombined treatment, which were observed to be significantly different from pooled controls ($p = 0.027$). Only the whole water treatment (hatch success 77%) displayed a significant toxic effect for DP-4, and was significantly different from all other treatments ($p \leq 0.05$). Finally, at DP-5, hatch success for the Recombined treatment was significantly different (80%; $p \leq 0.05$) from the pooled controls as well as F1 (100%) and F2 (100%).

5.4.3. Daphnia magna

Results for the *Daphnia magna* bioassay indicate that they were not very sensitive to the organic fractions within each site (Figure 5.1c). DP-1 whole water displayed a slightly greater toxicity than other treatments (survival 83%), but was not significantly different ($p > 0.05$) from the water control (98%). The whole water treatments for DP-2 (survival 23%) and DP-4 (survival 5%) significantly reduced survival ($p = 0.015$ and 0.00084, respectively) compared to pooled controls. DP-5 displayed no toxic effects at any of the treatments.

5.4.4. Hyalella azteca

The *Hyalella azteca* bioassay was one of the most sensitive to OSPW-influenced groundwaters for the organisms assessed herein (Figure 5.1d). At DP-1, data showed that whole water (survival 10%), F1 (0%), and Recombined (0%) treatments were not found to be statistically different ($p > 0.05$) from each other but were significantly more toxic ($p = 0.003$ in all cases) than pooled controls. Similar to DP-1, site DP-2 displayed significant acute toxicity ($p = 0.0024$ in all cases) for whole water (survival 0%), F1 (5%), Recombined (0%), and F3 (0%), compared to pooled controls (97%). Groundwater site DP-4 displayed acute toxicity such that the pooled controls and whole water were significantly different ($p \leq 0.01$) from F2 (survival 77%) and the Recombined treatment (63%). Additionally, F3 was significantly less toxic (survival 93%) than the Recombined treatment ($p < 0.004$). For DP-5, treatments F1 (survival 11%), F3 (5%), and the Recombined treatment (0%) were significantly more toxic ($p < 0.001$) than whole water (93%), F2 (93%), and controls, but were not different from each other.

5.4.5. Lampsilis siliquoidea

Mussel (*L. siliquoidea*) glochidia was relatively insensitive to the organic components from all exposure sites (Figure 5.1e). Whole water treatments for DP-1 (viability 44%) and DP-2 (0%) sites were significantly more toxic than all other treatments ($p < 0.001$) and displayed a substantial reduction in *L. siliquoidea* glochidia viability compared to pooled controls. Whole water for DP-2 displayed 100% mortality, was significantly more toxic than DP-1 ($p = 0.006$), and was the most toxic treatment overall. Site DP-4 displayed no significant toxicity ($p > 0.05$) resulting from exposure to any treatments. At site DP-5 treatments F1 and Recombined were significantly more toxic that pooled controls ($p = 0.02$ and 0.01, respectively).

5.4.6. Vibrio fischeri (Microtox® assay)

Bioassay method procedures for *V. fischeri* utilize control water as a reference level and it is not considered an actual treatment. Therefore, only the solvent control treatments were used as a control group. At 100% whole water equivalents, the Microtox® bioassay displayed no toxicity at any treatments across all sites (data not shown). Therefore, exposures were concentrated to 300% whole water equivalents in order to assess potential toxicity trends.

At concentrated exposures *V. fischeri* displayed sensitivity to mainly organic components within sites (Figure 5.1f). DP-1 treatment exposures showed that the Recombined treatment (31%) was most significantly toxic overall ($p < 0.001$), while F1 (viability 43%) and F3 (51%) were significantly more toxic ($p < 0.001$) than all of the remaining fractions. Similarly, DP-2 exposures showed that F1 (viability 56%), F3 (27%) and Recombined (15%) were significantly more toxic ($p < 0.001$) than all other treatments. However, F3 and the Recombined treatment were significantly more toxic ($p < 0.001$) than F1, while the Recombined treatment was significantly more toxic than F3 ($p < 0.001$), and most toxic overall. At the DP-4 site, the Recombined treatment (viability 75%) was significantly different ($p < 0.012$) from all but F3 (90%), while F3 was more toxic than only solvent control (110%, $p = 0.049$) and whole water (120%, $p = 0.01$). For DP-5, whole water (viability 80%), F1 (30%), F3 (48%), and Recombined (12%) treatments were significantly more toxic ($p < 0.001$) compared to solvent controls. The whole water treatment was significantly less toxic ($p < 0.001$) than F1 and F3, which were significantly less toxic ($p < 0.001$) than the Recombined treatment. Similar to all other sites, the Recombined treatment was most toxic overall ($p < 0.001$).

5.4.7. Whole Water Site Comparisons

In order to compare the relative toxicities of each site, whole water toxicity was evaluated within each bioassay. In three of the six bioassays, both natural bitumen influenced sites were found to be significantly more toxic ($p < 0.05$) than at least one of the OSPW-influenced sites. The other three bioassays found no significant differences between groundwater sources or similar toxicities ($p > 0.05$). For the *P. promelas* and *L. siliquiodea* bioassays, natural bitumen-influenced sites (DP-1 and DP-2) were significantly more toxic ($p < 0.004$) than OSPW-influenced sites (DP-4 and DP-5. The *H. azteca* bioassay displayed a reduction in survival at both natural bitumen-influenced sites DP-1 and DP-2 that was significantly different from OSPW-influenced site DP-5 ($p = 0.04$-0.045). The *D. magna* bioassay displayed similar toxicities between natural- and OSPW-influenced sites DP-2 and DP-4, which were both significantly more toxic that sites DP-1 and DP-5 ($p < 0.001$). Finally, *O. latipes* displayed no significant differences in whole water toxicity across all sites.

5.5. Discussion

In general, fraction toxicity, and therefore site toxicity, was related to sensitivity of the organism assayed. *P. promelas* and *H. azteca* were the most sensitive species overall, while *V. fischeri* and *L. siliquoidea* appeared least sensitive. Invertebrate species *D. magna* and *L. siliquoidea* displayed significantly reduced survival/viability ($p \leq 0.05$) to only whole waters at some sites indicating sensitivity to inorganic components within bitumen-influenced groundwater or some level of contaminant interaction. Where species displayed significant sensitivity ($p < 0.05$) to organic fractions, it was always as a result of exposure to F1 and/or F3. The only exceptions was for *H. azteca* at site DP-4, where F2 was statistically different ($p <$

0.02) from pooled controls and whole water, but similar ($p > 0.3$) to F1, F3, and the Recombined treatment. Comparison of bitumen-influenced groundwater sites indicated that those containing appreciable amounts of dissolved organics (DP-1, DP-2, DP-5, Table 4.1) presented similar toxicities to sensitive species regardless of whether the source was solely naturally-influenced or additionally had OSPW influence.

5.5.1. Bioassay Comparison

When toxicity data across bioassays are qualitatively compared, *P. promelas* and *H. azteca* were most sensitive overall (Figure 5.1). Both bioassays were particularly sensitive to the organic components within bitumen-influenced groundwaters. This is noteworthy because other bioassays such as *L. siliquoidea* and *D. magna* were relatively insensitive to the organic fractions, but showed some significant sensitivity to the whole water treatments. Previous studies which exposed multiple organisms to OSPW or commercial NAs validated our observations that *P. promelas* was the most sensitive species when compared to aquatic invertebrates (including *D. magna*) (Swigert et al., 2015; Kinley et al., 2016; Bartlett et al., 2017; McQueen et al., 2017). In fact, much of the relative species sensitivities remain consistent across these studies when compared to results herein. Similar to our observations, *H. azteca* were observed to display sensitivity to OSPW that was similar to *P. promelas* (Kinley et al., 2016), while *H. azteca* were also found to be more sensitive to AEO than *Lampsilis cardium* (freshwater mussel) and *V. fischeri* (Bartlett et al., 2017). With respect to relative species sensitivities, it was found that sensitivity ranked: fish ≥ aquatic invertebrates > macrophytes, when exposed to OSPW (Kinley et al., 2016; McQueen et al., 2017). More specifically with exposure to commercial NA, sensitivity ranked: *P. promelas* > *Ceriodaphnia dubia* > *H. azteca* > *Chironomus dilutus* > *Typha*

latifolia (Kinley et al., 2016). In the present study it is difficult to assign general species sensitivities as the study design emphasized toxicity due to organic components, and therefore whole water toxicity was assessed as a single concentration (100%) parameter. Although no median lethal concentrations (LC_{50}) were derived, an average response to whole water treatment across all bitumen-influenced groundwater sites provides simple insight into relative species responses. With respect to unaltered groundwater samples across all sites species sensitivities were such that *P. promelas, H. azteca, D. magna,* and *L. siliquiodea* displayed the greatest sensitivies to whole waters (Figure 5.2). With respect to only the organic components across sites, represented by the Recombined treatement, general species sensitivities were observed such that *P. promelas, O. latipes,* and *H. azteca* displayed the greatest sensitivities (Figure 5.2). Although this represents a simplistic analysis and there is a high degree of variability, *P. promelas* and *H. azteca* remain similar and considerably more sensitive than *V. fischeri*. It is interesting to note that the overall sensitivities changed when only organics were being compared. This disparity is likely due to the diversity of sensitivities that individual species possess to organic versus inorganic compound classes, a point that is discussed later.

Although at 1x environmentally equivalent concentrations *V. fischeri* displayed no acute toxicity, the Microtox® bioassay may still be useful as a screening tool to assess organic fractions. When treatments were exposed at 3x environmental concentrations, considerable toxicity was observed, and for those bioassays that displayed sensitivity to organic components (*P. promelas* and *H. azteca*), *V. fischeri* displayed comparable relative sensitivities. This is exemplified by the relative insensitivity to F2, and the relative significantly high toxicities for F1 and F3 exposures at sites DP-2, DP-4, and DP-5. Ideally, the Microtox® assay could be used as a screening tool for OSPW-influenced waters known to contain organic contaminants, but for

which quantitative analysis has not been performed. Because it is a cost-effective, rapid assessment with minor sample material requirements, the Microtox® assay can be utilized as a tool to direct further toxicological analyses. Nonetheless, caution is necessary when interpreting results from the Microtox® bioassay, even when used as a screening tool, as the results are upon concentration of sample material, and its utility for whole OSPW is unproven.

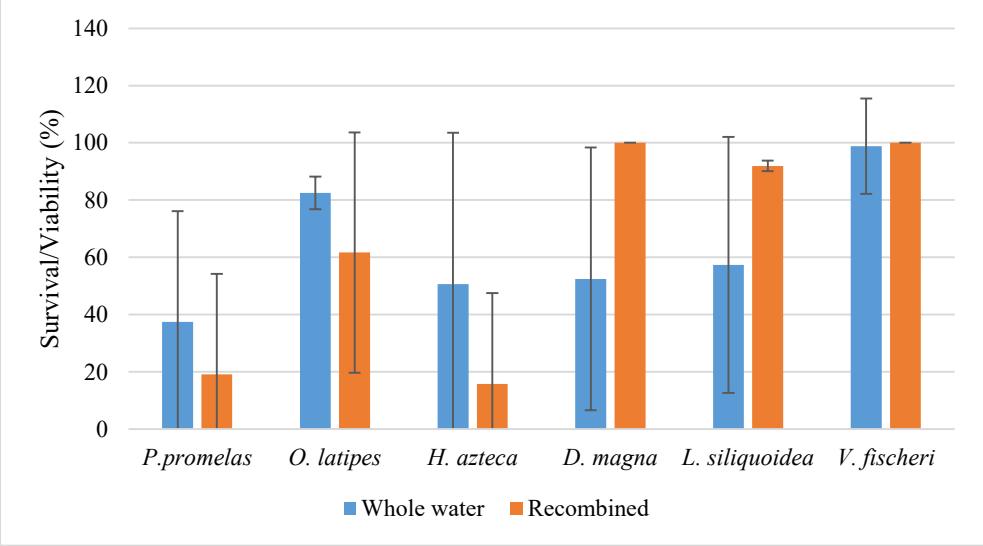


Figure 5.2. Mean (± standard deviation) survival/viability of organisms exposed to whole water (blue) and Recombined treatments (orange) from all sample sites combined.

5.5.2. Toxicity of Organics

A comparison of toxicity from all bioassays across sites revealed that *P. promelas* and *H. azteca* were particularly sensitive to organic components within groundwaters. Both organisms displayed significant ($p < 0.001$) acute toxicity to F1 exposures at sites DP-1, DP-2, and DP-5

(Figure 5.1a, d) compared to controls. *H. azteca* additionally displayed toxicity to F3 at DP-2 and DP-5 and F2 at DP-4, while *P. promelas* displayed toxicity to F3 at DP-4, compared to controls. At each site where fraction toxicity was observed, the Recombined treatment also displayed toxicity. Although *P. promelas* appeared to have solvent affects associated with Solv300, the Recombined treatment was consistently more toxic than Solv300 ($p < 0.03$). Like the Recombined treatment, at each site where fraction toxicity was observed, whole water toxicity was also observed. Therefore, it appears that the main drivers of whole water toxicity were present in F1 and F3, for those organisms sensitive to the organic components.

The relative toxicity of the organic fractions can be explained primarily by the concentrations of dissolved organics. Unsurprisingly, the most consistently toxic fraction F1 always contained the highest dissolved organics concentration (>10 mg/L) at sites where toxicity was observed (Table 4.1). Conversely, the dissolved organics concentration for F2 was < 2 mg/L across all sites and showed significant ($p \leq 0.05$) toxicity to only *H. azteca* at DP-4. With respect to F3, at site DP-2 where the dissolved organic concentration was 6.2 mg/L (highest across sites), significant toxicity to *O. latipes* and *H. azteca* was observed.

There were some notable exceptions to this association between dissolved organic concentration, such that toxicity was observed in organic fractions which displayed relatively low concentrations. It is possible that aquatic species are differentially sensitive to certain organic ion classes relative to others. With respect to F3, toxicity was observed at DP-2 for *H. azteca* and *O. latipes*, and DP-5 for *H. azteca* and (Figure 5.1b, d). At both DP-2 and DP-5, the concentrations of dissolved organics for F3 were 6.2 mg/L and 1.1 mg/L, respectively, but contributed > 95% mortality (Table 4.1). Additionally, although dissolved organic concentration at DP-4 was below instrumental detection limits, exposure to *P. promelas* resulted in 30%

mortality for F3 (Figure 5.1a). In comparison, at the same sites F1 had higher dissolved organics concentrations of >17 mg/L and contributed lower mortality (95 – 13%) to both organisms. The analysis of ion class distribution generated previously for F3 (Chapter 4, SI #2) indicated a predominance of O_4 ions (65.1%) at site DP-2, and predominance of O_4 and $\geq O_5$ ions (38.4% and 33.6%, respectively) at site DP-5. These data suggest that *H. azteca* and *O. latipes* may be more sensitive to polyoxygenated organic constituents present in F3.

With respect to *P. promelas*, at three of the four sites tested (DP-1, DP-2, and DP-5), F1 displayed significant toxicity, and lower sensitivity to polyoxygenated ions present in F2 and F3. According to ESI-HRMS class distribution data, F1 for sites DP-2 and DP-5 contained predominantly O_2 ions (64.6% and 63.8%, respectively) (SI #2). As has been previously outlined (Chapter 2, Chapter 4), classical NAs (O_2 ions) are predominantly present in F1, and F3 is predominantly polyoxygenated compounds. This is particularly noteworthy as research has implicated O_2 class organics (containing NA) as principal drivers of toxicity in OSPW (Allen, 2008; Brown and Ulrich, 2015). The relative sensitivity by *P. promelas* to O_2 organic compounds (including classical NA), and insensitivity to polyoxygenated compounds, is paralleled by a previous study which assessed dissolved organic fractions to *P. promelas*, but used a slightly different fractionation method (Morandi et al., 2015). In general, the toxicity of F1 to *P. promelas* may be a result of the relatively higher concentration of organics in this fraction, as discussed earlier, as well as a greater sensitivity to the O_2 ion class present.

The breadth of these data suggest, first, that in order to confer measureable toxicity, dissolved organics concentrations likely must be greater than some threshold level. In most cases the organic acid concentrations above 8 mg/L resulted in significant toxicity to *P. promelas* and *H. azteca*. Second, the data suggest that this threshold may be governed by organic acid ion

classes present in OSPW. Specifically, waters containing a greater proportion of polyoxygenated compounds may have a lower threshold concentration, depending on the species assayed. In other words, although classical NAs (O_2 ions) may be driving toxicity, due to their greater abundance in some bitumen-influenced groundwaters, polyoxygenated ions ($>O_2$) appear to present considerable potencies to other species or at different sites.

Table 4.1. Concentration of acid-extractable organics in fractions and filtrate of groundwater sites determined by LC-QToF/MS. Values represent concentrations present in original volumes of respective water samples.

	Dissolved Organics (mg/L)			
	DP-1	**DP-2**	**DP-4**	**DP-5**
Whole	1.6	5.9	BDL	9.5
F1	10.5	17.1	0.1	34.0
F2	0.5	1.1	BDL	0.1
F3	1.7	6.2	BDL	1.1
Filtrate	BDL	BDL	BDL	BDL

BDL = below detection limit (0.05 mg/L)

Table 4.2. Water quality parameters for unaltered groundwaters. Presented as total dissolved metals and major ions determined by ICP-MS. Elements measured but not present were below detection limit at all sites: Be, Bi, Ce, Ga, La, Nb, Pt, Se, Ti, and Y.

	DP-1	DP-2	DP-4	DP-5
Hardness (mg/L $CaCO_3$[a])	499.4	129	541.7	173.2
Metals / Major Ions		**µg/L**		
Aluminum	1.4	BDL	4.7	43.8
Antimony	0.074	0.142	0.171	0.07
Arsenic	0.23	1	0.82	1.13
Barium	26.1	1120	78.3	33.6
Boron	1520	7030	133	1370
Cadmium	0.007	BDL	0.018	0.312
Cesium	0.028	0.122	0.102	0.038
Chromium	0.66	1.82	0.91	0.71
Cobalt	0.332	0.502	2.17	1.07
Copper	4.16*	17.1*	27.2*	4.41*
Iron	3	27.1	16.9	8.6
Lead	0.144	0.434	0.319	0.911
Lithium	252	665	36.7	119
Manganese	22.9	2.65	89.7	9.65
Molybdenum	1.19	0.481	2.7	4.94
Nickel	2.9	2.53	16.1	3.31
Rubidium	3.07	9.59	3.04	3.24
Silver	0.034	0.172	0.352	0.048
Strontium	1020	1680	1040	395
Thallium	0.001	0.161	0.005	0.001
Tin	0.119	BDL	0.273	0.182
Tungsten	0.013	0.101	0.022	0.022
Uranium	0.356	0.159	11.3	0.56
Vanadium	0.2	1.86	0.23	0.14
Zinc	0.7	BDL	1.9	0.6
		mg/L		
Calcium	120	9.03	64.5	17.1
Chloride	93.8	999.0*	4.51	15.2
Fluoride	0.23	0.64	0.06	1.59
Magnesium	48.3	25.4	92.2	31.6
Potassium	8.24	16	5.68	9.8
Silica	18.7	13.2	26.3	25
Sodium	185	2290	26.4	222
Sulfate	303	3.39	85.1	18.8

[a] Calculated based on concentrations of Ca, Mg, Fe, Sr, and Mn.

* indicates exceedance of CCME and USEPA water quality guidelines

BDL = Below detection limit

5.5.3. Toxicity of Whole Water

Although inorganic toxicity was not specifically evaluated, it is reasonable to attribute the whole water toxicity to the contaminant mixture and possibly inorganics in some cases. Specifically, the invertebrates *D. magna* and *L. siliquoidea* exhibited >50% mortality to whole water treatments, where <10% survival was observed in all organic treatments (DP-2 / DP-4 and DP-1 / DP-2, respectively) (Figure 5.1c, e). Research has identified that *D. magna* possesses a greater sensitivity to cationic metals than fish (McQueen et al., 2017), while freshwater mussels have displayed heightened sensitivity to inorganic contaminants (Gillis et al., 2008; Gillis et al., 2011). The only inorganics that exceeded Canadian Council of Ministers of the Environment (CCME) or United States Environmental Protection Agency (USEPA) freshwater quality thresholds (CCME, 2017; USEPA, 2004) were copper (Cu) at all of the groundwater sites and Cl at site DP-2 (Tables 4.2). Therefore, understating that toxicity is likely a result of the whole mixture, contributions by individual inorganics warrant further discussion with respect to *L. siliquiodea* and *D. magna*.

In a number of studies that have assessed metals toxicity to various species of freshwater unionid mussels (family *Unionidae*), EC_{50}s derived for B, Cd, Cr, Ni, Pb, and Zn (Hansten et al., 1996; Milam et al., 2005; Wang et al., 2010, 2017; Soucek et al., 2011; Liu et al., 2016) were all greater than water concentrations observed in the present study. Specifically, for *L. siliquiodea*, EC_{50}s were 137 mg B/L, >227 µg Cd/L, 266 µg Cr/L, 506 µg Ni/L, >299 µg Pb/L, 576 and 2,685 µg Zn/L (Milam et al., 2005; Wang et al., 2010, 2017; Soucek et al., 2011). Additionally, although Cu exceeded CCME freshwater thresholds (2 - 4 µg Cu/L at 50-600 mg/L $CaCO_3$ water hardness; CCME, 2017) at all sites, we did not expect it to contribute greatly to whole water toxicity to *L. siliquiodea*. Studies which have assessed the toxicity of Cu to *L. siliquoidea*

observed EC_{50}s in the range of 36.1 – 130 µg/L (Milam et al., 2005; Gillis et al., 2008; Wang et al., 2017), while the highest Cu concentration in our study was 27.2 µg/L in site DP-4, which did not display whole water toxicity. Based on CCME and USEPA water quality thresholds as well as observations from the previous studies, contribution from individual metals to whole water toxicity to *L. siliquiodea* was likely minor. This suggests that the toxicity observed in sites DP-1 and DP-2 may be a result of contributions from other contaminants such as major ions. In particular, Na^+ and Cl^- concentrations were highest in site DP-2, while DP-1 contained the second highest Cl^- concentrations compared to the other groundwater sites. Moreover, at site DP-2 (999 mg Cl/L), Cl^- exceeded CCME and USEPA water quality thresholds (640 and 860 mg/L, respectively; CCME, 2017; USEPA, 2004). However, research assessing Cl^- exposure to *L. siliquiodea* has observed EC_{50}s in the range of 1430 – 1962 mg/L, which is higher than concentrations observed at both DP-1 and DP-2. Collectively, these data suggest that inorganic contaminants such as Cu and Cl^- may not be present in sufficient concentrations to individually affect *L. siliquiodea* viability, but that toxicity is likely a result of the combined inorganic mixture present in whole water.

The invertebrate *D. magna* displayed significant mortalities ($p = 0.015$ and 0.00084, respectively) for only whole water at sites DP-2 and DP-4 (Figure 5.1). Literature has identified LC_{50} concentrations for *D. magna* exposed to various inorganic elements including Al, As, Ba, Co, Cd, Fe, K, Mg, Mn, Ni, Pb, Sn, SO_4, Sr, and Zn (Biesinger and Christensen, 1972; Gostomski, 1990; Yim et al., 2006; Davies and Hall, 2007; Traudt et al., 2017), which were higher than concentrations present in the whole waters herein. However, previous investigations exposed *D. magna* neonates to Cu and recorded LC_{50}s of 9.8 µg/L (45 mg/L $CaCO_3$ hardness) (Biesinger and Christensen, 1972) and 12 µg/L (150 mg/L $CaCO_3$ hardness) (Yim et al., 2006),

which are very similar to CCME water quality thresholds (2 - 4 µg Cu/L at 50-600 mg/L $CaCO_3$ water hardness; CCME, 2017). Moreover, in whole water treatments for sites DP-2 and DP-4, where Cu concentrations were highest (17.1 µg /L and 27.2 µg/L, respectively) compared to other sites, *D. magna* exhibited 23% and 5% survival, suggesting a substantial influence to whole water toxicity by Cu. It is unclear whether the relatively high ion concentrations in DP-2 (2290 mg Na/L, 999 mg Cl/L) contributed to *D. magna* mortality at that site, as mortality was much greater in DP-4 which displayed the lowest Cl, F, K, and Na concentrations compared to other sites. If we use NaCl as an example for salt content, the sites DP-2 and DP-4 would contain 1637.7 mg/L and 7.4 mg/L, respectively (assuming NaCl composition: ~39% Na and ~61% Cl, and complete binding of Na and Cl). In comparison to literature which observed *D. magna* LC_{50} for NaCl in the range of 2182.4 - 6034 mg/L (Cowgill and Milazzo, 1991; Lilius et al., 1995), it is unlikely that these ions contributed substantially to whole water toxicity. Therefore, considering the likelihood of Cu toxicity, and that both sites where whole water toxicity was observed typically contained the highest concentrations of metals, it possible that *D. magna* toxicity was a result of the combined metals mixture.

It is important to note that the observed toxicity of whole waters was undoubtedly a result of contaminant mixtures and cannot be solely attributed to a specific chemical or chemical class. As such, sensitivities of *L. siliquiodea* and *D. magna* to inorganic components in OSPW warrants further investigation.

5.5.4. Groundwater Sites

One of the main objectives of the present study was to compare the toxicity of OSPW-influenced groundwaters to natural bitumen-influenced groundwaters (Figure 4.1). Sites DP-4

and DP-5 represent the OSPW-influenced groundwaters and were chosen because they were previously documented to have received inflow indicative of oil sands tailings seepage (Frank et al., 2014; Frank et al., 2018). Conversely, DP-1 and DP-2 represent natural bitumen-influenced groundwaters and were chosen as sites within the natural Athabasca bitumen deposit but situated far outside of operation lease sites and having no industrial influence (Frank et al., 2014; Frank et al., 2018). However, more recent data revealed that at the time of collection site DP-4 was not in the path of the tailings pond seepage plume. As was discussed earlier, site toxicity differed between species and was dependent on contaminant type and concentration.

Comparison of sites showed some variability, but natural-bitumen influenced sites generally displayed greater whole water toxicities compared to OSPW-influenced sites. It is important to re-iterate that more recent chemistry data revealed that site DP-4 did not appear to contain any bitumen influence from any source (Chapter 4) and, therefore, toxicity at this site should be interpreted with caution. When comparing only whole water toxicities across sites, within bioassays, three out of six bioassays (*P. promelas, H. azteca, L. siliquiodea*) found both natural bitumen-influenced sites (DP-1 and DP-2) to be significantly more toxic ($p < 0.05$) than either both OSPW-influenced sites DP-4 and DP-5 or only DP-5 (no sites significantly different from only DP-4). The other three bioassays (*O. latipes, D. magna, V. fischeri*) found no significant differences between groundwater sources or similar toxicities ($p > 0.05$) between sources. The only exception was the Microtox® assay, in which a significant difference was found between sites previously identified as having OSPW-influence. More broadly, when all treatments were observed, natural bitumen-influence site DP-2 appeared to be most consistently toxic as it displayed toxicity in at least one treatment to all bioassays. The organic constituents of F1 and F3 appeared to be at concentrations (17.1 mg/L and 6.2 mg/L, respectively) sufficient to cause

significant toxicity to *P. promelas*, *O. latipes*, and *H. azteca* at DP-2. Likewise, DP-2 whole water appeared to possess inorganics at quantities capable of causing significant toxicities to *L. siliquoidea* and *D. magna*. In all but *O. latipes*, DP-2 whole water toxicity was significantly ($p < 0.05$) greater than control water regardless of which chemical class may have been driving toxicity.

Some tests suggested that constituents present in whole water may elicit protective effects to those organisms sensitive to dissolved organics, due to contaminant interactions. OSPW-influenced site DP-5 presented toxicities to those organisms sensitive to organic contaminants (*P. promelas*, *O. latipes*, and *H. azteca*), but in all cases whole water toxicity was less than that caused by organic fractions. This suggests that DP-5 whole water constituents buffered the toxic effects of organic components to some organisms. This was also observed for *O. latipes* at site DP-2, where high survival rates in whole water (77%) were observed while organic treatments (F1 and Recombined) elicited 100% mortality (Figure 5.1b). Literature assessing the interaction of salts with AEO have reported a general reduction in bioavailability with increasing salt content (Headley et al., 2011a). Specifically, high salt concentrations have been shown to alter the proportions of AEO species through salting-out effects, resulting in a general enhancement of 2-ring species proportions and a loss in 4 – 7-ring species (Headley et al., 2011a) and relative reduction in O_3 species (Headley et al., 2012). This AEO proportional shift and salting-out of organics may be responsible for the reduction in toxicity of DP-2 and DP-5 whole water as they contained the highest Na^+ content across sites.

Site DP-4 displayed low toxicity overall, which was not surprising given that it contained very low concentrations of dissolved organics (Table 4.1). Moreover, according to chemical analyses performed previously, ESI-HRMS, LC-QToF/MS, GC-MS/MS, and SFS indicated little

to no evidence of OSPW or natural bitumen influence due to dissolved organics at this site (Chapter 4). In fact, more recent data revealed that at the time of collection site DP-4 was not in the path of the tailings pond seepage plume. Although some bioassays indicated toxicity in the whole water treatment at DP-4, only *P. promelas* (F3), *H. azteca* (F2) displayed toxicity due to organic treatments at whole water equivalents (Figure 5.1). Thus, the toxicity data observed herein is largely in agreement with the chemistry data observed previously (Chapter 4).

In summary, these data propose that both OSPW-influenced and natural bitumen-influenced groundwaters are highly toxic if they possess high concentrations of dissolved organics, metals and salts, individually or in combination. Furthermore, in some cases natural bitumen-influenced groundwaters were more toxic than those groundwaters influenced by oil sands tailings pond seepage. In another case, the low toxicity observed for DP-4 may indicate that when OSPW-influenced groundwaters are no longer influenced by tailings seepage, it may result in a reduction in toxicity. It is important to remember that groundwaters represent contaminant mixtures which can possess varied compound interactions. As was the case herein, toxicity as a result of dissolved organics may be reduced in whole water exposures due to interactions with inorganic components. Bitumen-influenced groundwater chemistry appears to be affected by contaminant interactions, and therefore, toxicity is contingent on location, regardless of parent source.

It is important to note that results derived herein were based on assessments of only 4 samples total, of which two were known to be influenced by only natural bitumen (DP-1 and DP-2), one was influenced by both natural bitumen and OSPW (DP-5), and one appeared to contain neither natural bitumen nor OSPW (DP-4). The low dissolved organic nature of DP-4 chemically (Chapter 4) was also observed toxicologically in the present chapter. It is, therefore,

recommended that more work is necessary to compare natural and OSPW influenced groundwater toxicity.

5.6. Conclusions

This study utilized dissolved organic fractions (Chapter 4), generated according to a previously developed fractionation procedure (Chapter 2), to assess the potential drivers of toxicity in oil sands bitumen-influenced groundwaters. Groundwater sites were chosen to evaluate the potential influence of OSPW-derived constituents and to compare toxic outcomes to those observed from natural bitumen-derived constituents. Whole water and organic fractions were exposed to a suite of bioassays to compare relative species sensitivities.

Results from these exposures indicate that species sensitivities are quite varied, and as such, so are likely the drivers of toxicity. Some species were more sensitive to organics while others appeared more sensitive to inorganics, even within contaminant types drivers of toxicity varied. While *P. promelas* appeared most consistently sensitive to O_2 class organic compounds and *O. latipes* was sensitive to polyoxygenated ($>O_2$) organic compounds, *H. azteca* displayed sensitivity to all ion classes. For those organisms sensitive to whole water treatments, *D. magna* and *L. siliquoidea* appeared to be particularly sensitive to inorganic elements. It was evident that toxicity was dependent on contaminant concentration, but thresholds were difficult to determine for whole waters which contained chemical mixtures that were quite variable. Nonetheless, there is evidence to suggest that inorganic constituents present in whole bitumen-influenced groundwaters (ie. DP-2 and DP-5) have the ability to ameliorate toxicity associated with organic constituents. A general comparison of groundwater sites, containing OSPW-derived constituents vs. natural bitumen-derived constituents, revealed that whole water toxicities were quite variable.

Overall, natural bitumen-influenced sites were more consistently toxic than DP-5, which was observed to have been influence by both OSPW and natural bitumen sources. Therefore, it is possible that toxicity associated with tailings seepage into groundwater is mitigated by chemical changes as a result of soil composition. Through processes such as salting-out of organics, metal ion exchange, and contaminant sorption to organics and clays, tailings seepage can more closely resemble natural groundwaters flowing through the McMurray Formation of oil sand the further it migrates from the source. These findings should be taken into account with respect to tailings pond construction material, design, location, seepage reclamation, and tailings pond decommissioning. The variability in species sensitivities should be further investigated as it will aid in developing thresholds for the protection of aquatic life in the oil sands region.

Chapter 6. Environmental Risk Assessment of Oil Sands Acid-Extractable Organics in Tailings Waters to Aquatic Organisms

6.1. Overview

The Canadian oil sands region in northern Alberta contains one of the largest petroleum deposits worldwide. Oil sands industry operators in this region have generated vast amounts of tailings waste, termed oil sands process-affected water (OSPW), from surface mining of bitumen. The toxicity within OSPW tailings waters has been primarily attributed to water-soluble acid-extractable organics (AEO). A comprehensive review of oil sands toxicity literature was conducted to identify comparable articles relating to the acute toxicity of acid-extractable organics (AEO) exposure to aquatic organisms. Selection criteria aimed to incorporate only articles in which AEO were extracted from fresh tailings ponds and exposed by comparable means. The evaluated articles contained individual assessments of 11 different organisms including; *V. fischeri, H. azteca*, *D. magna*, *L. stagnalis*, *L. cardium*, *P. promelas, D. rario, O. latipes, S. vitreus, P. flavascens,* and *O. mykiss*. From the data obtained, a species sensitivity distribution was generated which revealed that, with the exception of *O. latipes*, fish species were generally more sensitive to AEO than invertebrates. The most sensitive species overall was *P. flavascens* while the least sensitive was *L. cardium*. It was found that a high hazard quotient of 61.2 (where >1 suggests possible risk) was associated with toxicity to aquatic organisms exposed to AEO at measured tailings pond concentrations. However this hazard only exists in a scenario where tailings containments were immediately connected to surface waters or a tailings dyke breach. A probabilistic approach revealed that the 90th centile for AEO exposure was 87.1 mg/L, which is a predicted concentration present in 10 percent of tailings environments. Predicted concentrations protective of 90 percent (10th centile) of fish and invertebrates were 19.6 mg/L and 5.5 mg/L, respectively. Furthermore, a joint probability curve predicted that the probability of exceeding the 10th centile for fish and invertebrates was 100% and 97.7%, respectively. In the

case of fish species, the Area Under the Curve was 43.7, while 73.2 for invertebrates. Aggregate data all strongly suggest low survivability for aquatic organisms exposed to AEO from fresh tailings. The presence of low levels of natural bituminous input into surface waters in the region has likely resulted in some tolerance to AEO by organisms indigenous to the oil sands region. Therefore, sensitivities observed for lab-reared organisms potentially overestimate the level of risk posed to native taxa. Nonetheless, the high predicted risk stresses the need for monitoring in this region. Monitoring should account for current anthropogenic AEO input from tailings seepage, and its effect on particularly sensitive fish species. For future efforts regarding tailings reclamation strategies, monitoring should account for changes in AEO concentration over time. In particular, reclamation efforts need to account for the time-dependent reduction of risk associated with biodegradation of anthropogenic AEO, and interaction with naturally-derived AEO.

6.2. Introduction

The Canadian oil sands region in northern Alberta contains one of the largest petroleum deposits worldwide. Oil sands industry operators in this region produce an estimated 2.37 million barrels of crude oil per day (Canadian Association of Petroleum Producers, 2016). In order for oil sand to be upgraded to a marketable product it is first extracted from underlying substrate and in surface mining procedures bitumen is separated from sand using the Clark hot water extraction method. This process produces large quantities of waste tailings and oil sands process-affected water (OSPW). The extraction of the mineable bitumen has increased significantly in the last 4 decades (Royal Society of Canada Expert Panel, 2010) and as a result, the volume of associated OSPW in containments has also increased. OSPW has displayed considerable acute toxicity to a

variety of aquatic biota (Clemente and Fedorak, 2005; Brown and Ulrich, 2015). The Alberta government's Environment Protection and Enhancement Act, prohibits the release of substances that may cause adverse effects to the environment and requires that Crown-leased land must be reclaimed (Government of Alberta, 2017, FTFC, 1995a). Industry has complied with this requirement by using the bitumen-depleted open pits from surface mining as storage reservoirs for OSPW termed tailings ponds.

Tailings pond OSPW can be defined as any water that has been subject to industrial processes including extraction, separation, upgrading, etc. OSPW contains a variety of toxic organic and inorganic components. Specifically, these are concentrated metals, major ions (i.e. Na, Cl, SO_4, CO_3), neutral organics (i.e. polycyclic aromatic hydrocarbons: PAHs), and polar organic acids (Allen, 2008). High salinities present in tailings are largely a result of the caustic hot water solutions (sodium hydroxide) employed during extraction and separation of bitumen (FTFC, 1995a), which can be exacerbated with industrial water recycling. Much of the tailings contaminants is present in the natural bitumen ore, but those present in tailings ponds are enriched from what is present naturally. For example, polar organic NAs make up roughly 2% of bitumen by weight, but comprise up to 50% of acid extractable organics (AEO) within tailings (Brown and Ulrich, 2015). The most toxic component within OSPW tailings waters has been attributed to be the water-soluble acid-extractable organics (AEO) (Brown and Ulrich, 2015). A well-studied subgroup of AEO called naphthenic acids (NA) have been historically identified as the primary drivers of toxicity (Allen, 2008; Brown and Ulrich, 2015). Toxic effects in a variety of aquatic species exposed to OSPW have been observed at between 8 mg/L to 65 mg/L NA concentration for various endpoints (Kinley et al., 2016). However, more recent findings have noted toxicity can be attributed to the suite of AEO present in OSPW and not simply classical

NA (O_2-containing compounds) but also polyoxygenated compounds (O_2+) (Grewer et al., 2010; Bauer et al., 2017; Li et al., 2017). Moreover, AEO have been shown to possess additional heteroatomic species, including sulfur and nitrogen, as well as aromatic rings (Grewer et al., 2010; Rowland et al., 2011b; Bauer et al., 2015), further expanding their chemistry beyond classical NA-like structures.

Investigations involving characterization of polar organics have described these organics using terms including naphthenic acids (NA), naphthenic acid fraction components (NAFC), naphthenic acid extracts (NAE), the OSPW organic fraction (OSPW-OF), the acid extractable fraction (AEF), and acid extractable organics (AEO), among others. The abundance of terms is primarily due to the lack of knowledge regarding the compositions of the organics present. Many of these terms are derived from the fact that analytical methods can only confidently identify classical NA in solution (NAFC, NAE). These analyses, therefore, only regard organics in narrow terms and in relation to NA. Others define organics with regard to extraction method (AEF, AEO), recognising that current extraction methodologies capture a broad suite of dissolved organics irrespective of analytical capabilities. Considering that most oil sands organic extraction methods incorporate an acid-precipitation step, it is reasonable to group all of these terms under "AEO". Therefore, all reference to dissolved organics which have been extracted from oil sands waters will be referred to herein as AEO.

6.2.1. Objectives

There is currently no meta-evaluation, or risk assessment, of toxicity associated with oil sands dissolved organics. There are a number of factors why this is the case. These include the inconsistent definition of the chemical mixtures that comprise OSPW, a lack of methodological

standardization for extraction and analysis of AEO, a poor understanding of AEO compositions, and the absence of chemical standards for instrument calibration. The result is a high degree of variability in reported AEO compositions and toxicological responses between studies using dissimilar methodologies (Grewer et al., 2010).Comparisons between studies are further compounded by the variability in the chemical profiles themselves between OSPW sources and the inherent sensitivities of the organisms assayed (Frank et al., 2014; Marentette et al., 2015a; Frank et al., 2016; Bartlett et al., 2017; Chapter 5).

To address this gap, the focus of the present risk assessment is to evaluate current literature regarding the acute toxicity of industrially derived oil sands AEO. In particular, the aim is to provide a prediction of risk associated with exposure and effects concentrations derived from studies reported in the literature. This will be achieved by comparison of exposure and effects cumulative distributions.

6.3. Methods

6.3.1. Exposure Assessment

A literature search was conducted in order to obtain environmental exposure data for oil sands tailings ponds constituents. Specifically, AEO and NA concentrations from fresh tailings were compiled from a broad search using University of Waterloo Library (Primo) and Web of Science online databases. This was accomplished using keyword combinations including “oil sands”, “OSPW”, “naphthenic acid”, and “acid extractable organics”. Publications were screened such that those reporting concentrations from active tailings ponds (at the time of collection) were accepted. As such, AEO concentrations from groundwaters, seepage dykes/ponds, experimental ponds, and natural waters were excluded. These criteria were necessary in order to

compare environmental exposures to the observed effects from only AEO extracts from fresh OSPW (in a separate literature review) described below. It is important to note that extraction and quantitation of AEO incorporated a variety of methods, of which their disparities are contrasted in greater detail below. Nonetheless, in order to provide a comprehensive dataset, all data reporting concentrations of dissolved organics which were derived through acid extraction ("NA", "AEO", "AEF", etc.) were included.

6.3.2. Effects Assessment

6.3.2.1. Literature Review

In order to compile the breadth of literature regarding oil sands dissolved organics the University of Waterloo Library (Primo) and Web of Science online databases were used. Searches were conducted using keyword combinations including "oil sands", "OSPW", "naphthenic acid", "acid extractable organics", "naphthenic acid fraction components", "naphthenic acid extract", "acid extractable fraction", "organic acid", "dissolved organics", or "toxicity". Of the articles returned, those selected were studies which involved aquatic toxicity assessments where dissolved organic measurements were made. It was important to recognize that tailings treatment studies (biodegradation, UV/Gamma irradiation, activated carbon adsorption, chemical oxidation, etc.) and chemical characterization studies occasionally incorporate toxicity assays. As a result, a pool of 123 articles were selected for a Tier 1 assessment.

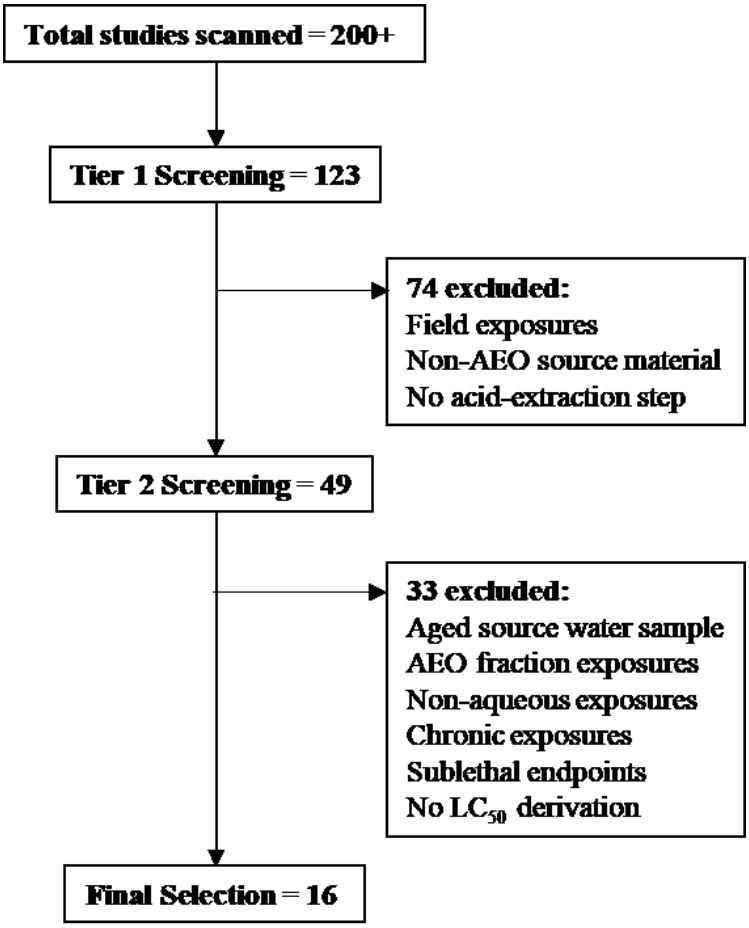


Figure 6.1. Article screening tiers and their exclusion parameters

6.3.2.2. Tier 1 Selection Criteria

The goal of this first tier screening was to arrive at a list of studies that assessed anthropogenic AEO exposure to aquatic organisms in the lab. The pool of 123 articles were further screened based on parameters including:

1) exposure location: lab
2) source material: AEO
3) extraction/fractionation methods: acid extraction

First, exposure type was assessed, and all studies carried out in the field were omitted. The justification for this screening parameter was twofold: i) no concentration-response design can be incorporated (therefore, no EC_{50} derived), and ii) the potential for confounding abiotic factors relating to toxicity exist. The source of original oil sands material was important for assessing anthropogenic impact, and data assessing oil sands organic extracts generated from natural waters were excluded. Additionally, many other studies used commercial NA mixtures (Merichem, Acros, Fluka, Kodak) as surrogates for oil sands bitumen-derived acids, likely due to the difficulty in acquiring an OSPW sample and then generating an extract. Ample research has identified the drawbacks of using commercial NAs that are chemically and toxicologically distinct from oil sands-derived NAs. Not only do commercial mixtures approximate only a subset of dissolved organics within tailings, but they also exhibit less complexity (Bataineh et al., 2006; Smith et al., 2008; Grewer et al., 2010; Rowland et al., 2011b; Brown and Ulrich, 2015) and greater toxicities (Lai et al., 1996; Marentette et al., 2015a; Bartlett et al., 2017). As a result, all data from studies using commercial NA mixtures were excluded. Finally, there are extraction/fractionation methods for isolation of organics from OSPW including; liquid-liquid extraction, centrifugation, solid-phase extraction, and liquid chromatography, with combinations and variations among these. The sample source, sample preparation, extraction methodology, and procedural order all contribute to the composition of the final extract. Although extraction method comparison would likely yield AEO proportional differences, no standard method has been adopted by researchers. Data were, therefore, selected based simply on whether the exposed treatment involved an acidification step (pH~2) prior to extraction/fractionation. This selection criteria resulted in retaining only toxicological literature that exposed oil sands industry-derived AEO to aquatic organisms in lab.

6.3.2.3. Tier 2 Selection Criteria

The first level of screening, outlined above, identified 49 articles as candidates for the second tier of screening which focussed on comparability between studies. The secondary screening was more stringent than the first and assessed the following parameters:

1) source water age: fresh
2) treatment type: unfractionated AEO
3) exposure method: whole organism, aqueous
4) measured endpoints: acute/lethal
5) statistical derivation: median lethal concentration (LC_{50})

Specific justification for the Tier 2 selection criteria are outlined as follows. Source material for AEO extraction is typically performed on fresh or aged tailings, and subsequently an active tailing pond or a test pond. Aged tailings have been shown to be chemically and toxicologically distinct from fresh tailings, where a reduction in recalcitrant AEO by biodegradation and photodegradation is associated with lower toxicity (MacKinnon and Boerger, 1986; Herman et al., 1993; Lai et al., 1996; Marentette et al., 2015a; Bartlett et al., 2017). For aged tailings experiments, fresh tailings are either artificially "aged" in lab by subjection to microbial processes, aeration, or UV irradiation, or naturally aged samples were collected from test ponds. Finally, aged experiments were not internally comparable due to differences in ages between sample sources. Therefore, in order to account for toxicity from all organics initially present and reduce variability associated with aging, studies assessing fresh tailings were selected. Frequently, AEO extractions were a result of a fractionation, or subject to a subsequent fractionation. In the former case, only data presented for fractions resulting from an acid

extraction were used. In the latter case, only data presented for a whole, pre-fractionated extract were used. This criteria prevented the misrepresentation of AEO toxicity due to presence of more neutral compounds, and underrepresentation of AEO toxicity from exposure to only a fraction of the original suite of dissolved organics. AEO route of exposure to organisms can be administered as aqueous, subcutaneous injection, dietary, *in vitro*, etc. Because some exposure methods bypass an organism's natural metabolic (and potential detoxification) pathways, to ensure comparability between studies, selection based on similar exposure method was necessary. To that end, only exposures involving whole organism, aqueous exposures were selected. Exposure endpoints were also examined, as some studies assessed sub-lethal toxicity effects such as growth parameters or longer term chronic toxicity. Only studies which presented data regarding acute toxicity were retained. The previous two requirements for acute toxicity via aqueous exposure are related to the statistical endpoint screening parameter which required the presentation of an LC_{50}. Many of the Tier 2 studies statistically derived No-observed effect concentration (NOEC), lowest-observed effect concentration (LOEC), effect concentration (EC_x), or lethal concentration (LC_x; where x = 1-100 and denotes a percentage response at a given concentration). Because EC_x generally denotes an effects response other than mortality (LC_x), only LC_x data was considered. The exception is the EC_x endpoint derived from the Microtox® bioassay which uses luminescence intensity from bioluminescent bacteria as an indicator of survival/viability. As with EC/LC_x, derivation of NOEC and LOEC values involves exposure using a dilution series, but is typically presented where a partial response (some value between 0% and 100% mortality) is lacking or 100% mortality is not observed. Conversely, EC/LC_x values are typically derived only where more than one partial response is observed. Although the binomial method can be used to derive an LC_{50} where one partial response and

100% response is observed, this method is not commonly used. For best comparability, only data presenting derivation of lethal concentrations where 50% mortality occurs (LC_{50}) were screened in. It is common in toxicology to derive LC_5, LC_{10}, and LC_{25} values especially when presenting some ecological limit for the protection of a species. Nonetheless, derivation of an LC_{50} is preferred for better understanding of an organism's response to a toxicant. This is because it is the point along a statistical regression where the variability is lowest, resulting in contracted 95% confidence intervals (Motulsky and Christopoulos, 2004). This allows for better statistical comparison against other LC_{50}s and greater likelihood of determining a statistically significant difference (Motulsky and Christopoulos, 2004).

The exposure pH and life-stage of the exposed organism were criteria which were considered but did not result in the removal of any assessments. Exposure pH was considered because it can greatly affect the solubility, and therefore, bioavailability of the dissolved organics. Because low pH can cause organic acids to precipitate out of solution, only environmentally relevant exposure pH between 7- 9 were considered. Although none of the studies reported exposures of pH 9, exposures up to 8.6 were used and within the pH range of tailings ponds receiving fresh tailings (Allen, 2008). For exposure life-stage, where multiple studies exposed AEO to the same organism at either embryo, embryo-larval, or larval life-stages, a separation in the data was required. The studies were evaluated on a case-by-case basis and data were grouped with the life-stage for which exposure duration was longest.

As a result of two tiers of screening, 16 studies were identified as comparable candidates to assess acute toxicity of industrially derived AEO to aquatic organisms. In order to account for the limited number of studies available for review following the screening, variability in certain parameters was accepted including: chemical characterization methods, measured vs. nominal

concentrations, variability of inorganics composition, and exposure duration. In some cases endpoint measurements were reported as millimolar concentration (mM), % whole water (v/v) or a concentration factor "x" (where x = some multiple of the whole water concentration). For these studies if whole water concentration of dissolved organics was provided (in mg/L units), the appropriate derivations were made in order to convert LC_{50}s to a comparable unit (mg/L).

6.3.3. Hazard Quotient

The Hq is derived from the following equation; **Hazard = [Exposure]/[Effect]**, where exposure concentration (highest reported concentration) /effect concentration (level at which no species are affected; NOEC) (USEPA, 1989; Solomon, 1996). An Hq is interpreted such that values > 1 represent a scenario where adverse effects are possible. It is understood that a hazard quotient indicating adverse effects may not necessarily parallel observations in the field due to abiotic factors, temporal/spatial variability, exposure type (pulsed or episodic), etc.

6.3.4. Probabilistic Risk Assessment

The probabilistic risk assessment allows for the prediction of risk, where risk is the joint probability of exposure and effects concentrations (Solomon, 1996). This assessment requires the generation of probability distributions of exposure and effects data. A compilation of relevant concentrations from literature was conducted; measured values for exposure and LC_{50} for effects. Exposure and effects probability distribution data were treated and plotted as follows. The Log_{10} of exposure values and mean LC_{50} for each species was calculated and ranked by concentration value. Ranks were then transformed to Probit and a regression of Log_{10} mean vs Probit was

plotted. The Weibull equation **P = 100*i/(n+1)** was used for generation of an empirical cumulative probability.

In the present study, the 10th centile of sensitivity was determined as an indicator of effects concentration. The 10th centile concentration can be interpreted as the concentration at which 10 percent of species are effected or the concentration at which 90 percent of species are protected. As an indicator of exposure concentration, the 90th centile was determined and can be interpreted as the AEO concentration present in 10 percent of tailings environments. Comparing probability distributions for exposure and toxicological effect of AEO allows for prediction of percent probability of *n* percent species (exceeding the *n*th centile of the effects distribution) affected at a predicted exposure concentration (Solomon, 1996). This ultimately enables the establishment of a level of protection for the receiving ecosystem. Additionally, the Area Under the Curve (AUC) was calculated in order to rank the risk to fish and invertebrates. The AUC can be described as the mathematical equivalent to the mean risk (Aldenberg et al., 2002), where 100 is the maximum value indicating greatest risk. All calculations and transformations were performed using Microsoft Excel and data was then plotted using Sigma Plot v.11.

6.4. Results and Discussion

6.4.1. Exposure Characterization

Organic toxicants in tailings ponds are present due to their separation from bitumen, and are, therefore, also present in natural bitumen deposits in the oil sands region. The relatively shallow depth of these deposits results in bitumen seepage along river banks and in riparian groundwater due to natural hydraulic erosion. As a result, toxicants contained in bitumen deposits can be dissolved into surface waters, representing a natural input into the environment. Monitoring of

water chemistry from natural bitumen-influenced surface waters and groundwaters reveals that they can contain up to 2 mg/L of NAs (Sun et al., 2017). Aquatic life in the Athabasca region has adapted to low-level natural inputs, but are likely intolerant to the elevated levels of NA present in tailings ponds (up to 70 mg/L; Allen, 2008). Although spatial and temporal variabilities of AEO compositions and concentrations have been observed (Frank et al., 2016), the present literature assessment of fresh OSPW represents an initial "unmodified" AEO composition considered not affected by microbial and environmental degradation pathways that would be expected to occur within tailings containments.

Much of the physicochemical properties of AEO are based on the well-studied subgroup of NAs. NA sodium salts are soluble in water with pKa of 5-6 (Headley and McMartin, 2004; Brown and Ulrich, 2015). As a result, this group of weak acids are soluble at pH >7 and are present in both OSPW and natural waters in the region (Allen, 2008). In their soluble, protonated form (sodium naphthenates), NA remain in the water phase and are, therefore, bioavailable to aquatic organisms (Clemente and Fedorak, 2005). Due to their surfactant nature, AEO encompass a broad suite of compounds which are thought to function in a narcotic pathway (Brown and Ulrich, 2015). Nonetheless, studies have revealed other modes of toxic action including those consistent with oxidative stress (Wiseman et al., 2013), endocrine disruption (Kavanagh et al., 2012) and effects on cardiac development (Mohseni et al., 2015).

Table 6.1. Measured fresh tailings AEO concentrations from literature grouped by site and ordered chronologically by collection year. The value "n/a" refers to values that were not presented in the study.

Site	AEO (mg/L)	Collection Year	Reference
WIP (Syncrude)	67	2004	Penner and Foght, 2010
	69		
	69		
	77	2006	Han et al., 2009
	20.7	2009	Lu et al., 2013
	23.6	2009	Pourrezaei et al., 2011
	25.5	2009	Lu et al., 2013
	71.7	2009	Anderson et al., 2012
	41.7	2010	Reichert et al., 2017
	45.3	2010	Islam et al., 2014
	70.2	2010	Anderson et al., 2012
	61	2012	Pourrezaei et al., 2014b
	36	n/a	Grewer et al., 2010
	52	n/a	McKenzie et al., 2014
	60	n/a	Grewer et al., 2010
	60.3	n/a	Pourrezaei et al., 2014a
	75	n/a	El Din et al., 2011
MLSB (Syncrude)	41	2004	Penner and Foght, 2010
	42		
	84		
	85		
	50	2006	Han et al., 2009
	28	n/a	Grewer et al., 2010
	44		
	49	n/a	Holowenko et al., 2002
	71	n/a	Peters et al., 2007
	84.7		
SEP (Syncrude)	77	2006	Han et al., 2009
AURTP (Syncrude)	60		
Suncor South (Suncor)	56.5	n/a	Sohrabi et al., 2013
Shell	39.8	2014	Leshuk et al., 2016
Muskeg R. tailings (Shell)	98	n/a	McQueen et al., 2017
	104		
n/a	54	2009	Frank et al., 2014
	60		
	17.4	2011	Frank et al., 2016
	24.4		
	25.5		
	32.2		
	35.3		
	37.6		
	41.2		
	41.8		
	43.3		
	51.5		
	67.5	2011	Islam et al., 2015

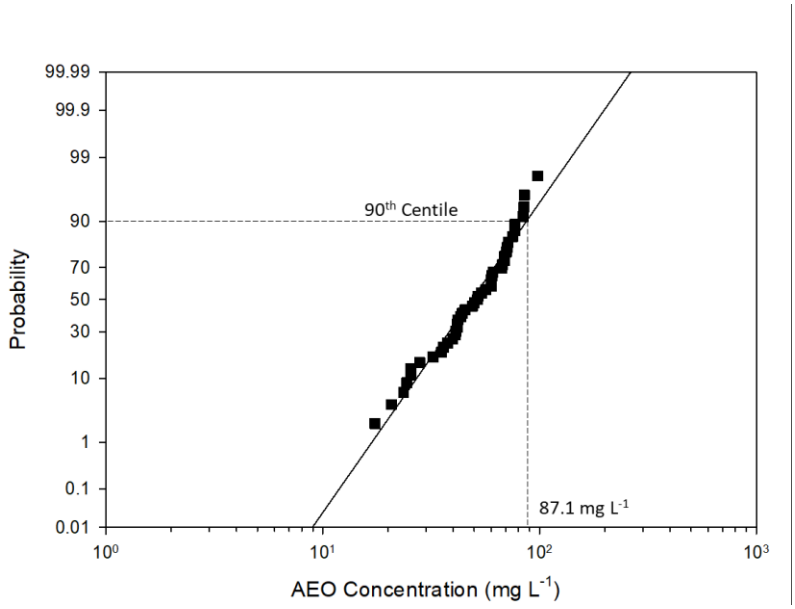


Figure 6.2. Percent rank distribution for environmental exposure concentrations of oil sands AEO. Dashed lines indicate the 90th centile of exposure concentrations, which predicts AEO concentrations present in 10 percent of a tailings environment.

A literature review of tailings AEO concentrations from >114 articles resulted in suitable data from 23 articles (Table 6.1). Compiled literature covered OSPW AEO concentrations in a 10-year period from 2004 to 2014, and was comprised of 46 individual data points. Collected data incorporated spatial and temporal ranges from at least 3 major industry operators, Suncor, Syncrude, and Shell. Tailings OSPW concentrations encompass a range between 17.4 – 104 mg/L (Table 6.1). Many early studies reported NA concentrations which contrast recent studies which report the whole acid-extractable fraction (AEF or AEO). This discrepancy can largely be attributed to the state of knowledge at the time which placed emphasis on only O_2 acid species, believing AEO to consist of "classical" NA compounds. However, some data undoubtedly

included other non-O_2 species of AEO in their assessment of NAs. For the purposes of this assessment, reported concentrations in which and acid extraction was performed were included.

The exposure distribution plot (Figure 6.2) presents the 90th centile for AEO concentration from fresh OSPW and predicts an exposure of 87.1 mg/L. The prediction indicates the level present in 10 percent of fresh tailings environments. In comparison to < 2 mg/L concentrations present in the natural environment (Allen, 2008), a tailings breach may be beyond the current assimilative capacity of the receiving environment.

With respect to environmental relevance, AEO exposure is not likely to occur at concentrations present in tailings ponds. Current industry-derived AEO exposure is expected to be from tailings pond seepage. The amount and composition of AEO that seep into groundwater (Frank et al., 2014) is largely unknown and would be modified by underlying substrate (Janfada et al., 2006; Ahad et al., 2013). A particular study modelled seepage from the bottom of a single tailing pond into underlying substrate at a rate of 2.0 L/s (Ferguson et al., 2009). The resulting bioavailability of AEO in groundwater has been shown to be highly variable and likely a result of groundwater chemistry and target species sensitivity (Chapter 4; Chapter 5). In addition to the potential for berm breaching leading to unintentional OSPW release, groundwater transport of AEO into surface waters has the potential to increase local concentrations to a level beyond the current tolerable capacity of system. This largely unexplored area of groundwater contamination by OSPW-derived AEO warrants further investigation, and will provide information required for planned releases of OSPW in the next 5 years.

Future AEO exposure must also be considered with regard to the wet landscape reclamation strategy and assessments herein are more applicable to this scenario. To address the growing containments of OSPW on industrial leases, operators have begun development and testing of

large-scale landscape reclamation strategies. The long-term objective of this strategy involves the detoxification of OSPW, which will be accomplished by converting tailings storage ponds into viable wetland areas called end-pit lakes. The strategy relies on the reduction in toxicity associated with biodegradation of organic components within aged tailings (MacKinnon and Boerger, 1986; Herman et al., 1993; Lai et al., 1996). However, the long period of time required (>10 yrs) to reduce AEO contents in tailings ponds to non-toxic levels has challenged the viability of this strategy (Quagraine et al., 2005). Nonetheless, the low cost associated with natural biodegradation supports oil sands industry operators' interest in this approach. Future AEO exposure will likely occur as part of the wet landscape reclamation strategy, particularly when end-pit lakes are connected to natural waterways.

6.4.2. Evaluation of Effects Literature

6.4.2.1. Current State of the Literature

The 49 Tier 2 articles were published between 1994 and mid-2017. In 23 years, 61% of those were published in the last 5 years. Of the articles that passed Tier 2 screening, 47% were published in the last 5 years. This indicates a more recent interest on the subject of toxicity associated with acid extractable organics. It also identifies a general trend toward better reporting and more in-depth toxicological analysis. However, the most common reasons articles were excluded during Tier 1 and Tier 2 evaluation were because commercial acids were used for exposure or no acute toxicity was generated. Commercial NA and AEO have been shown to be both chemically and toxicologically dissimilar (Marentette et al., 2015a; Bartlett et al., 2017), and therefore, not relevant to this evaluation. As such, the data also reflect a greater, more recent, use of AEO in toxicological assessments. Assessments were also excluded if chronic, sub-lethal,

in vitro, or in silico exposures were conducted with no associated acute data, and if only LOECs were generated. Although these types of assessments are useful, they do not provide adequate data to evaluate acute risk to taxa.

Literature for acute toxicity of dissolved oil sands organics have assessed 11 different aquatic species. Of the 11 assessments, 6 were conducted using the Microtox® assay (*Vibrio fischeri*), 4 utilized a *P. promelas* bioassay, while other species were only represented by 1 assessment each (Figure 6.3). This indicates the need for more stringent assessment of AEO using a wider variety of species in toxicological analysis. Single-celled organisms of the kingdom Bacteria are represented by *V. fischeri*. Invertebrates were represented by *H. azteca*, *D. magna*, *L. stagnalis*, and *L. cardium*, and vertebrates represented by *P. promelas, D. rario, O. latipes, S. vitreus, P. flavascens,* and *O. mykiss*. Specific evaluation of results from each group are expanded upon in the following sections.

The final selection of literature consisted of 16 articles of which 10 are primarily toxicological in nature. Of the remaining 6, 3 are fractionation or extraction methods studies and 3 were remediation or degradation studies. These 6 studies utilized toxicological analyses to provide validation for method development. The Microtox® assay (*V. fischeri*) was used in all but 1 of these method development studies. These findings indicate a predominance toward use of *V. fischeri* as a species for method development.

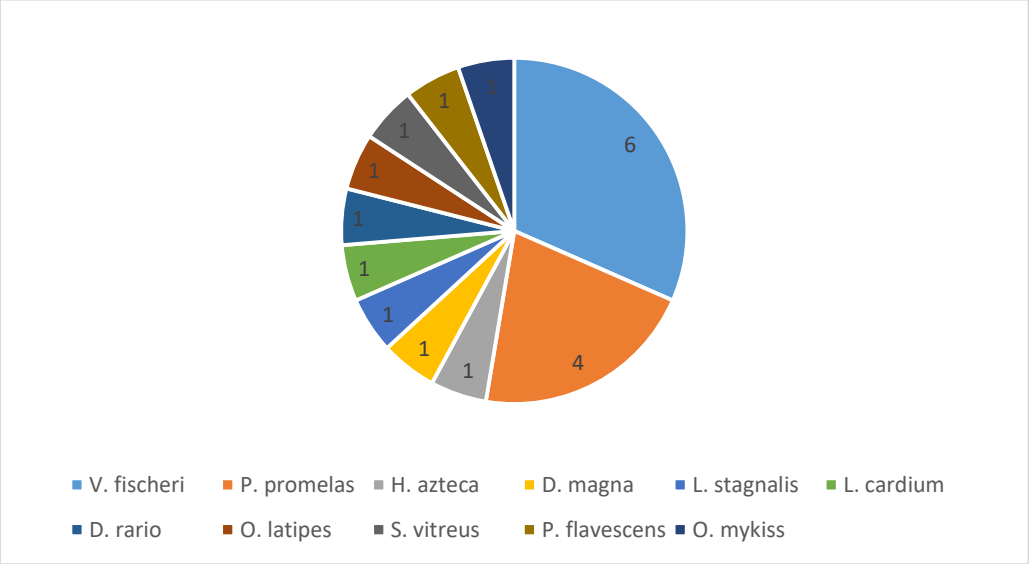


Figure 6.3. Number of assessments grouped by organism from 16 studies.

6.4.2.2. Extraction Methods

The plethora of terms used for dissolved organics in OSPW indicates the variety of extraction methods used in this area of research. Commonalities between all extractions are that they involve pH adjustment of OSPW followed by solvent extraction. The most common extraction method involves acidification of OSPW (pH 2), followed by centrifugation to consolidate precipitated solids (Frank et al., 2006). The majority of toxicological assessments have isolated AEO using this method (Farwell et al., 2006; Nero et al., 2006; Kavanagh et al., 2012; Woodworth et al., 2012; Leclair et al., 2013; Scarlett et al., 2013; Bauer et al., 2015; Marentette et al., 2015a; Marentette et al., 2015b; Bartlett et al., 2017; Bauer et al., 2017) or slight variation thereof (Lo et al., 2006; Quesnel et al., 2011; Klamerth et al., 2015; Quesnel et al., 2015; Johnston et al., 2017). The resulting supernatant is removed and the precipitated solids are

reconstituted in an alkaline solution (pH 12). Humic acids are removed via cellulose filtration, and neutral organics are removed with a DCM liquid-liquid extraction. The resulting alkaline filtrate containing AEO is then concentrated by acidification with collection of precipitate on a filter, after which the precipitate is reconstituted in the desired solvent. An alternate method employs a liquid-liquid wash in DCM, following sequential neutral, alkaline, or acidic adjustments of OSPW in an order that isolates the fraction of interest (Madill et al., 2001; Holowenko et al., 2002; Rogers et al., 2002; Janfada et al., 2006; Armstrong et al., 2008; Armstrong et al., 2009; Mishra et al., 2010; Sohrabi et al., 2013; Morandi et al., 2015; Wang et al., 2015). This results in base-neutral fractions and acid-organic fractions without previous centrifugation to isolate the precipitate.

As part of a concentration or clean-up step, typically for chemical analysis, the water containing dissolved organics is subject to solid-phase extraction through a C_{18} (octadecyl carbon chain) or PSDVB (polystyrene divinylbenzene) resin packed in a column (Frank et al., 2008; Gagné et al., 2011). This adaptation utilized pH adjustment, solid-phase extraction, and then soxhlet extraction with an organic solvent (MeOH) for isolation of AEO.

Slight variations in methods employed for extraction of AEO have the potential to change the constituents within the sample. Across extraction methods, some minor differences between studies include OSPW acidification limits (pH 1-3), alkalinisation limits (pH 10-12), extraction solvent (DCM, acetonitrile, MeOH), and number of solvent washes (3-4x). The solvent used for liquid-liquid extraction, solid-phase extraction, and even storage or concentration of sample has been shown to affect the composition of dissolved organics (Barrow et al., 2010; Headley et al., 2013a).

6.4.2.3. Sample Preparation and Analytical Methods

A variety of methods have been used for the analysis of AEO profiles and concentration in water samples. There is no current analytical standard for quantification of oil sands dissolved organics. From only the final 16 studies assessed herein, each utilized at least one of the following methods: ESI-MS (Frank et al., 2006; Frank et al., 2008; Armstrong et al., 2009; Mishra et al., 2010; Kavanagh et al., 2012), ESI-HRMS (Scarlett et al., 2013; Marentette et al., 2015a; Morandi et al., 2015; Bartlett et al., 2017; Bauer et al., 2017), GC/MS (Nero et al., 2006; Scarlett et al., 2013), GC-MS/MS (Scarlett et al., 2013), LC/MS-QToF (Sohrabi et al., 2013; Marentette et al., 2015a; Marentette et al., 2015b; Bartlett et al., 2017), and FTIR (Nero et al., 2006; Kavanagh et al., 2012; Sohrabi et al., 2013).

There are several inconsistencies between analytical methods with regard to determination of AEO concentration, as has been reviewed (Brown and Ulrich, 2015). For example, FTIR and GC low res-MS have been shown to produce false high concentrations of AEO compared to other high resolution analyses (Yen et al., 2004; Martin et al., 2008; Han et al., 2009; Headley et al., 2009a). Generally, higher resolution methods produce lower, more accurate concentrations (Brown and Ulrich, 2015). Detection of AEO classes also differs depending on whether an instrument operated in negative- or positive-ion mode (Headley et al., 2013b). Although negative-ion mode is better suited for analysis of NAs with a greater detection of oxygenated species, positive-ion mode has been shown to better detect species containing sulfur heteroatoms (Barrow et al., 2010; Barrow et al., 2015). This likely makes positive-ion in complement to negative-ion mode useful in identifying the suite of compounds present in AEO extracts. A final area for potential discrepancy during analysis of AEO is with regard to the standards used for calibration of instruments. For analysis of NAs in OSPW samples, commercial preparations

under five trade names are typically used as standards; Acros, Merichem, Aldrich, Kodak, and Fluka. Studies have shown that these commercial mixtures are of dissimilar composition to each other (Hindle et al., 2013; Lu et al., 2013). Therefore, AEO ion composition derived from analysis is largely dependent on standards used, which inevitably produce unique distributions due to their inherent variability.

Chemical analysis of dissolved organics involved a variety of methods. As a result, reported AEO concentrations may differ, and may not be fully characterized in studies that used commercial NA standards for instrument calibration. These analyses must therefore be considered semi-quantitative (Martin et al., 2008). Because there are currently no oil sands reference standards available, LC_{50} concentrations were regarded as "comparable" regardless of the analytical methods employed. This approach was taken in order to retain a more comprehensive assemblage of organisms to assess in this review.

6.4.2.4. Bioassay Exposure Water

Following AEO extraction, many studies required sample preparation prior to exposure. The extracted sample was typically dissolved in an organic solvent or at alkaline pH in water, depending on extraction method employed. If dissolved in an organic solvent that is immiscible with water, AEO required reconstitution in water or a water-miscible solvent. Solvents such as dimethyl sulphoxide (DMSO), ethanol, or methanol are water miscible, and AEO samples were therefore concentrated in these solvents as a carrier and transferred to exposure water (Scarlett et al., 2013; Morandi et al., 2015; Wang et al., 2015; Johnston et al., 2017). Although acetonitrile is also a water-miscible organic solvent often used for AEO extraction, it is not used as a delivery solvent because in vertebrates exposure can generate toxic metabolites such as hydrogen

cyanide, formaldehyde, and formic acid (Pozzani et al., 1959). Alternatively, in other studies solvents were evaporated and organics re-dissolved into an alkaline pH for delivery into exposure water (Madill et al., 2001; Lo et al., 2006; Sohrabi et al., 2013). Most studies simply dissolved organics into alkaline water for storage (Frank et al., 2006; Nero et al., 2006; Armstrong et al., 2009; Kavanagh et al., 2012; Woodworth et al., 2012; Marentette et al., 2015a; Marentette et al., 2015b; Bartlett et al., 2017; Bauer et al., 2017), and was the most common method used. The advantage to extracting and storing AEO in alkaline water is that no evaporation and reconstitution in a solvent is necessary, reducing possible loss of organic material. Regardless of the method employed, the pH of the exposure water required adjustment. In general, exposure water was brought to a pH slightly higher than neutral, which was in the range pH 7.9-8.6 for the studies assessed. This pH range is similar to that found in tailings ponds OSPW and natural lakes and rivers in the region (pH 7-8.6; (Allen, 2008)) and, therefore, is environmentally relevant.

6.4.3. Effects Characterization

6.4.3.1. Bacteria

Studies have overwhelmingly utilized the Microtox® assay likely due to low sample volume requirements, relative ease of use, and rapid results (Clemente and Fedorak, 2005). In fact, many of the Tier 1 selection studies utilised the Microtox® assay to assess OSPW remediation, fractionation, or analytical methods, where dissolved organics toxicity was not the primary objective. The 6 Microtox® studies represent 32% of total assessments and indicate the utility of the assay. Researchers should be cautious when using *V. fischeri,* as recent investigation has identified their low sensitivity to AEO relative to other aquatic organisms (Chapter 5). However,

the authors also indicate its utility as a rapid and predictive screening tool for further toxicological assessment, provided samples are concentrated (Chapter 5). Regardless of its sensitivity, its use as an indicator species will likely foster its continued use for assessments of oil sands toxicity.

Of the 6 studies which used the Microtox® assay (Table 6.2), there were 10 individual assessments with a mean acute toxicity ranging from LC_{50} of 30.5-78.9 mg/L. While these values encompass a wide range, it is likely a reflection of variability between OSPW-derived AEO sources. OSPW variability studies have previously identified that AEO composition can vary both temporally and spatially within a tailings pond (Frank et al., 2016), and also be quite variable across tailings ponds resulting in observed differences in toxicity (Marentette et al., 2015a; Bartlett et al., 2017). Within these studies, at least 2 different OSPW sources from at least 2 different industry operators were sampled, accounting for inherent variability.

6.4.3.2. Invertebrates

From the 3 studies that utilized invertebrate bioassays (Armstrong et al., 2009; Bartlett et al., 2017; Johnston et al., 2017), there were 8 individual assessments representing 4 different species (Table 6.2). These included *H. azteca* (amphipod crustacean), *D. magna* (water flea), *L. stagnalis* (pond snail), and *L. cardium* (freshwater mussel). Invertebrate LC_{50}s encompassed a range from 16.7-123 mg/L, and with the exception of *L. cardium* (LC_{50} 34.8-123.0 mg/L), across-species variability for acute toxicity was relatively low (LC_{50} 16.7-37.5 mg/L). The freshwater mussel (*L. cardium*) displayed the greatest within-species variability, and was generally less sensitive to AEO than *H. azteca, D. magna,* and *L. stagnalis*. Observations from the study by Bartlett et al. (2017) showed that *L. cardium* displayed the lowest sensitivity compared to the 3 other species

assessed therein (*P. promelas, H. azteca,* and *V. fischeri*) (Bartlett et al., 2017). This is possibly due to greater sensitivity to inorganic components, which has been observed for a similar species in a comparative study of AEO toxicity (Chapter 5). Despite the diversity of the invertebrates evaluated, representing various Phyla, life-histories, and behaviours, the range in LC_{50}s are within an order of magnitude.

6.4.3.3. Fish

Evaluations for freshwater fish included Japanese medaka (*O. latipes*), fathead minnow (*P. promelas*), walleye (*S. vitreus*), rainbow trout (*O. mykiss*), zebrafish (*D. rerio*), and yellow perch (*P. flavascens*). Fish acute embryo bioassay mean LC_{50} ranged from 4.4-51.8 mg/L (Table 6.2), encompassing 1 order of magnitude. *P. promelas* was the most studied fish with 18 individual assessments from 4 studies (Kavanagh et al., 2012; Marentette et al., 2015a; Marentette et al., 2015b; Bauer et al., 2017). Although *P. promelas* was one of the least sensitive fish species assessed, it is a well-studied organism, common bioassay and environmentally relevant. Furthermore, it is a small-bodied fish with lower toxicant exposure volume requirements which make it well suited for assessing oil sands extracts. Important factors in the utility of the *P. promelas* bioassay are the ease of husbandry and embryo acquisition and the fact that they are indigenous to much of North America including the Athabasca region. These reasons likely contributed to the high representation of this species in the present literature. The sensitivity of the *P. promelas* bioassay is dependent on the life-stage assessed as the embryonic stage was ~2.4x more sensitive than the larval stage (Table 6.2; (Kavanagh et al., 2012)). This observation is likely similar for other fish species and exemplifies the importance of exposures that target the most sensitive life-stage of an organism. *S. vitreus*, *O. mykiss*, *D. rerio*, and *P. flavascens*

embryo displayed similar sensitivities to AEO with LC_{50}s in the range of 4.4-11.0 mg/L (Table 6.2). Conversely, *O. latipes* embryo were much less sensitive compared to all other fish species (LC_{50} 37.6 mg/L) and was most similar to *P. promelas* larval exposures (LC_{50} 46.8-51.8 mg/L) (Table 6.2).

Table 6.2. Individual toxicological assessments grouped by organism and ordered chronologically by publication date.

OSPW Source	Test Organism	Life-stage	Duration	Exposures	LC/EC_{50} (mg/L)	Reference
		Fish				
Syncrude, WIP	*P. promelas*	embryo	9-d	NAE (350mg salts)	32.8	Kavanagh et al., 2012
				NAE+Cl	30.3	
				NAE+SO4	32.4	
Syncrude, WIP	*P. promelas*	embryo	9-d	NAE (700mg/L salts)	32.6	Kavanagh et al., 2012
				NAE+Cl	27	
				NAE+SO4	29.5	
Syncrude, WIP	*P. promelas*	larval	96-hr	NAE (700 mg/L salts)	51.8	Kavanagh et al., 2012
				NAE+Cl	47.4	
				NAE+SO4	46.8	
Indusrty A fresh1	*P. promelas*	embryo	96-hr	AEO	13.2	Marentette et al., 2015a
Industry A fresh2				AEO	7.5	
Industry B fresh				AEO	13.8	
Indusrty A fresh1	*P. promelas*	embryo	to hatch	AEO	10.6	Marentette et al., 2015a
Industry A fresh2				AEO	5	
Industry B fresh				AEO	9.3	
Industry A fresh	*P. promelas*	embryo-larval	to hatch	AEO	21.8	Marentette et al., 2015b
Industry B fresh				AEO	24.5	
Syncrude, WIP	*P. promelas*	embryo-larval	6-d	AEO	18.9	Bauer et al., 2017
Syncrude WIP	*D. rerio*	larval		AEO	8.4	Scarlett et al., 2013
NR	*D. rerio*	embryo-larval	?	AEO	7.4	Wang et al., 2015
Syncrude, WIP	*O. latipes*	embryo-larval	9-d	AEO	37.6	Bauer et al., 2017
Industry A fresh	*S. vitreous*	embryo-larval	to hatch	AEO	11	Marentette et al., 2015b
Industry B fresh				AEO	9.5	
Sy ncrude, WIP	*P. flavascens*	YOY	96-h	AEO	4.4	Nero et al., 2006
Syncrude, MLSB	*O. mykiss*	YOY	96-h	AEO	10	Verbeek et al., 1994
		Invertebrates				
Syncrude, WIP	*V. fischeri*		15 min	pre-DEAE filtration	31.4	Frank et al., 2006
				post-DEAE	30.5	
ND	*V. fischeri*		15 min	particulate NA	37	Lo et al., 2006
				aqueous NA	35	
Syncrude, WIP	*V. fischeri*		15 min	AEO	52.7	Frank et al., 2008
ND	*V. fischeri*		15 min	AEO	22.9	Mishra et al., 2010
Suncor, South TP	*V. fischeri*		10 min	AEO	44	Sohrabi et al., 2013
Indusrty A fresh1	*V. fischeri*		15 min	AEO	71.9	Bartlett et al., 2017
Industry A fresh2				AEO	78.9	
Industry B fresh				AEO	71.8	
Indusrty A fresh1	*H. azteca*	neonates	7-d	AEO	16.7	Bartlett et al., 2017
Industry A fresh2				AEO	25	
Industry B fresh				AEO	27.4	
ND	*D. magna*	neonates	48 h	AEO	37.5	Armstrong et al., 2009
ND	*L. stagnalis*	embryo	28-d	AEO	32	Johnston et al., 2017
Industry A fresh1	*L. cardium*	glochidia	48-h	AEO	97.4	Bartlett et al., 2017
Industry A fresh2				AEO	123	
Industry B fresh				AEO	34.8	

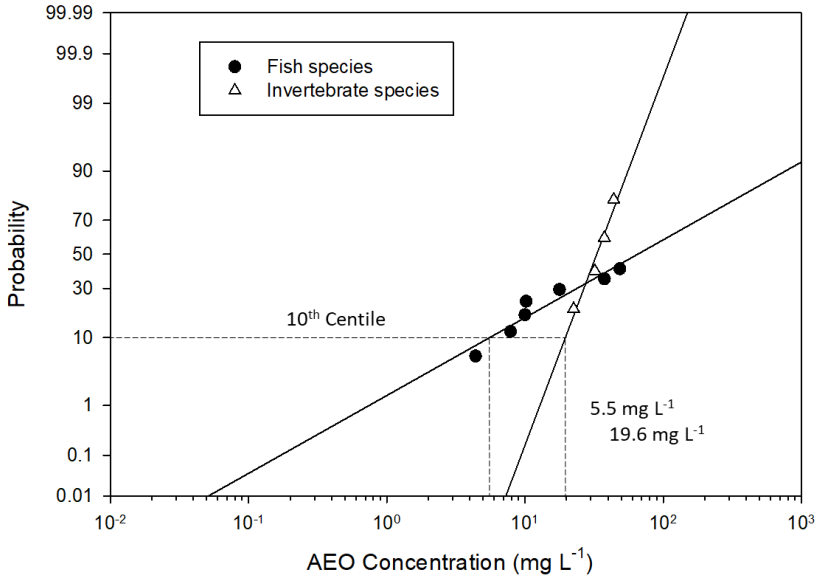


Figure 6.4. Percent rank distribution for toxicological effects concentrations of oil sands AEO. Dashed lines indicate the 10th centile of effect concentrations, which predicts AEO concentrations required to produce an effect in 10 percent of organisms. Fish and Invertebrate effect concentrations are 5.5 mg/L and 19.6 mg/L, respectively.

6.4.3.4. Relative Species Sensitivities

Effects concentration data from selected literature were grouped by species and a mean LC_{50} was derived (Table 6.3). The range in lethal responses for all organisms was 4.4 – 74.7 mg/L. These data indicate that embryonic fish display a greater sensitivity to AEO than invertebrate organisms. Relative toxicities expressed by different organisms are likely dependent on a number of factors, including the species' metabolic capacity and mode of action driving toxicity.

The generated probability distribution for invertebrate and fish species exposed to AEO predicted 10 percent of species would be affected at 19.6 mg/L and 5.5 mg/L, respectively (Figure 6.4). The distribution displays a distinction in sensitivities such that invertebrates are less

sensitive to AEO than fish species. The exception to this observation is larval *P.promelas*. Larval *P.promelas* appeared to be much less sensitive than their embryological cohorts, likely due to developmental stage and improved ability to detoxify organic compounds. The greater sensitivity of fish species compared to invertebrates when exposed to AEO, OSPW, or commercial NAs has been observed previously (Kinley et al., 2016; Bartlett et al., 2017; McQueen et al., 2017; Chapter 5). A study that exposed a suite of organisms to isolated components of OSPW identified that invertebrates are more sensitive to inorganic components (metals and salts) and less sensitive to dissolved organics (Chapter 5). Nonetheless, it has been proposed that although invertebrates were more sensitive to inorganics than fish, these components were within a tolerable range in OSPW exposures and toxicity was likely driven by AEO (McQueen et al., 2017).

Table 6.3. Evaluated species grouped into fish and invertebrates and ranked by mean AEO LC_{50} concentrations.

Species	Mean LC_{50} (mg/L)
Fish	
P. flavascens	4.4
D. rerio	7.9
O. mykiss	10.0
S. vitreus	10.2
P. promelas	17.8
O. latipes	37.6
P. promelas larval	48.6
Invertebrates	
H. azteca	22.5
L. stagnalis	32.0
D. magna	37.5
V. fischeri	43.9
L. cardium	74.7

6.4.4. Risk Characterization

6.4.4.1. Hazard Approach

Given that industrial tailings ponds displayed AEO concentrations between 17.4 - 104 mg/L (Table 6.1) and natural lakes and rivers in the region can contain concentrations of 1-2 mg/L (Allen, 2008), one expects some level of hazard associated with industrially-derived AEO. This assertion is made clear when generating a hazard quotient (Hq) for anthropogenic AEO exposure. We derive an Hq from the highest measured exposure concentration and the effects concentration at which no species are affected (NOEC from most sensitive species; *P. flavascens*) where **Hazard = [Exposure]/[Effect]** (Solomon, 1996). According to data presented herein, exposure is constant at 104 mg/L and the effect concentration is 1.7 mg/L. Thus, the Hq = 61.2 which strongly suggests low survivability for aquatic organisms exposed to raw fresh tailings.

6.4.4.2. Probabilistic Approach

A probabilistic risk assessment was conducted in order to assess the risk of tailings AEO exposure to aquatic organisms. This assessment was based on tailings concentrations from a range of samples that differed spatially and temporally reported in the literature that passed screening criteria. Effects data were also acquired from suite of aquatic species, including fish and invertebrates, for which AEO acute toxicity concentrations were screened from reported values in literature. Effects data were plotted separately as fish species and invertebrate species and compared to the probability distribution for exposure (Figure 6.5). By plotting the exposure and effects distributions for AEO together, risk can be predicted as a joint probability which allows for the determination of the percent probability of affecting *n* percent of the species

(exceeding the *n*th centile of the effects distribution). The 10th centiles for fish and invertebrates were 19.6 mg/L and 5.5 mg/L (Figure 6.4, Table 6.4), respectively, while the 90th centile for AEO exposure was 87.1 mg/L (Figure 6.2, Table 6.4). The probability of exceeding the 10th centile for fish and invertebrates was 100% and 97.7%, respectively (Table 6.4). These data indicate a very high risk for both evaluated organism groups exposed to undiluted tailings AEO. In order to quantify the level of risk present, exceedance profiles were plotted (Figure 6.6) and the AUC was calculated for both fish and invertebrates. The AUC can be described as the mathematical equivalent to the mean risk (Aldenberg et al., 2002). In the case of fish species the AUC was 43.7, while the invertebrates displayed an AUC of 73.2 (Table 6.4).

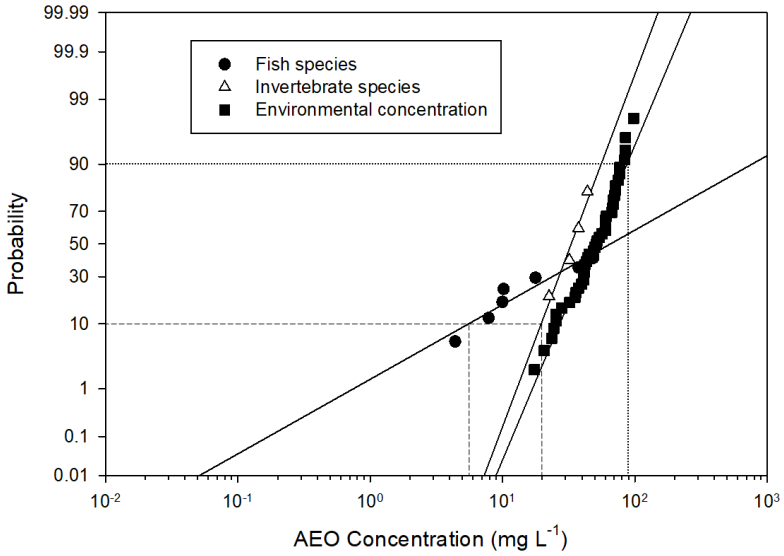


Figure 6.5. Percent rank distribution for exposure and effects concentrations of oil sands AEO. Dotted lines indicate the 90th centile of exposure concentrations, which predicts AEO concentrations present in 10 percent of a tailings environment. Dashed lines indicate the 10th centile of effect concentrations, which predicts AEO concentrations required to produce an effect in 10 percent of organisms.

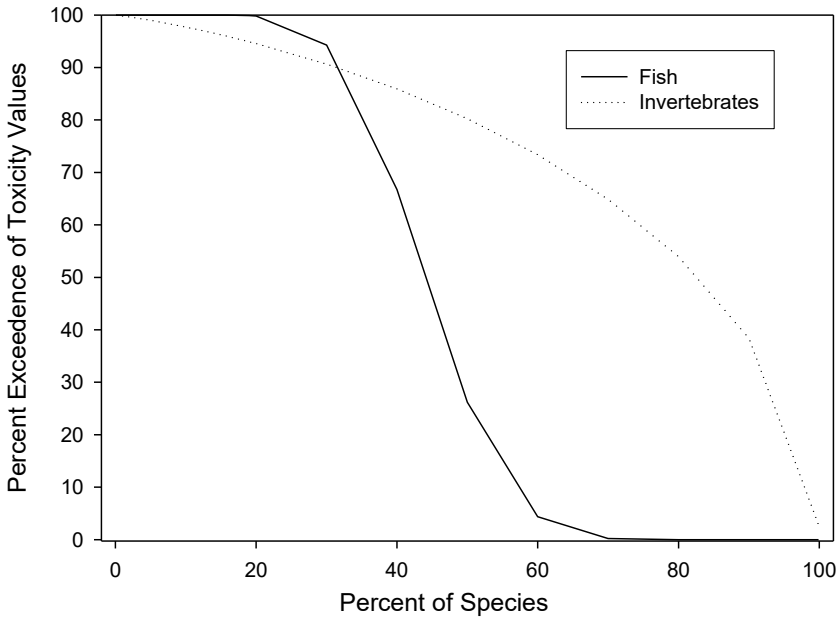


Figure 6.6. Exceedance profile of fish and invertebrate organisms for exposures of tailings AEO.

Table 6.4. Linear regression equations and derived centile intercepts, 10th centile exceedance, and area under the curve from probability distributions of exposure and effects.

	Distribution Equation	Centile Intercepts (mg/L) 10%	90%	10th Centile Exceedance (%)	Area Under Curve
Fish	$y = 1.2x - 2.17$, $r^2 = 0.91$	5.536		100	43.7
Invertebrates	$y = 5.66x - 8.6$, $r^2 = 0.96$	19.608		97.7	73.2
AEO Exposure	$y = 5.06x - 8.54$, $r^2 = 0.96$		87.073		

Because risk assessments incorporate a level of exposure relating to frequency, spatial and temporal distribution of exposure, one must consider these with respect to the degree of hazard present. Specifically, because most AEO extracts are derived from active tailings ponds, the AEO concentration and composition within these ponds must be considered. Spatially within a tailings pond, and across industrial sites, AEO composition can be quite different (Frank et al., 2016). This spatial variability was captured in the SSD as studies therein assessed multiple sources of AEO. The exposure distribution that wasn't captured herein was temporality. Studies have shown that as tailings age (without constant input) the composition of AEO shifts toward a greater proportion of higher molecular weight compounds (Herman et al., 1993; Lai et al., 1996). Concurrent with this shift is a reduction in overall toxicity in OSPW (Marentette et al., 2015a; Bartlett et al., 2017) which inherently alters the hazard and risk associated with AEO. One of the oil sands industry's main reclamation strategies involves aging tailings through natural biological processes. Therefore, it is likely that the level of risk present will decrease over time.

According to the above risk characterization, industrial sources of AEO present a risk to aquatic organisms. Although this may be the case, it is important to note that those taxa currently present in the natural system have likely developed tolerance to low levels of AEO. Adaptation to natural bitumen input by indigenous organisms may have decreased overall sensitivity compared to lab-reared organisms used in most studies. Unlike point-source or episodic contamination, AEO input in the natural environment represents a semi-constant low-level exposure, providing greater likelihood for natural selection of more tolerant communities. The caveat being AEO dilution by snow and ice melt during the spring freshet, and possible increased input from river banks during warmer months due to reduction in bitumen viscosity. Regardless, it is unlikely that indigenous organisms could tolerate AEO present at tailings concentrations.

6.4.4.3. Uncertainty Analysis

There is some degree of uncertainty associated with the effects assessment herein. First, in the generated SSD, *L. stagnalis*, *D. magna*, *O. latipes, O. mykiss, P. flavascens* were only represented by one assessment from one AEO source. All other species incorporated multiple AEO sources with >1 assessment. Thus, there is a greater degree of confidence for those species represented by multiple assessments. Additionally, because effects data were collected from the literature, it is difficult to determine whether the most sensitive species or a keystone species (a species that plays a crucial role in ecosystem function) in the natural ecosystem was represented in the literature. It may be that the assessment underestimates the level of risk because of the absence of data from a more sensitive indigenous organism. Uncertainty associated with risk may also arise from the fact that all effects data were derived from controlled laboratory bioassays. This experimentation does not account for potentially confounding abiotic or indirect effects present in the natural environment. It is possible that AEO compounds interact with abiotic factors in the natural environment, modifying toxicity to affected organisms, and also, the potential that toxicity to a keystone species has some greater indirect effect on the larger system.

Uncertainty is also present as a result of exposure data. As discussed earlier, derivation of anthropogenic AEO concentrations may have utilized analytical techniques and standards which did not account for the whole suite of dissolved organics in sample waters. Alternatively, some analysis may provide overrepresentation or misclassification of some AEO ion classes. Until a standard method for analysis of AEO is developed to a high degree of accuracy, there is no guarantee that analyses are either comparable or represent true concentrations. Therefore, derived values herein are not wholly representative, but serve to present a general trend.

6.5. Conclusions

Review of oil sands toxicity literature (123 articles) identified 16 comparable articles relating to acute toxicity of AEO exposure to aquatic organisms. The relatively few useable articles indicates a general lack of comprehensive experimental design, leading to the exclusion of potentially useful data and derivation of an LC_{50}. In all, 11 organisms were subject to individual assessments. Evaluation of 16 selected articles revealed that *V. fischeri* was the most commonly used bioassay followed by *P. promelas*. Although most of the evaluations using *V. fischeri* were for the purposes of validating novel methodologies, the use of this organism is not environmentally relevant nor particularly sensitive. Rather, use of a more sensitive, indigenous organism like *P. promelas* is encouraged. Additionally, exposures using a greater variety of taxa would provide a more comprehensive overview on the potential risk to the ecosystem.

The most sensitive species overall in response to AEO exposure was *P. flavascens* while the least sensitive was *L. cardium*. In general, fish embryo were more sensitive than invertebrate taxa with the exception of *O. latipes*. Using a hazard approach an Hq of 61.2 was derived. A probabilistic approach revealed that concentrations protective of 90 percent (10^{th} centile) of fish and invertebrates were 5.5 mg/L and 19.6 mg/L, respectively. The 90^{th} centile for AEO exposure was 87.1 mg/L, which is a predicted concentration present in 10 percent of tailings environments. Furthermore, the probability of exceeding the 10^{th} centile for fish and invertebrates was 100% and 97.7%, respectively. In the case of fish species the AUC was 43.7, while the invertebrates displayed an AUC of 73.2. The aggregate data all strongly suggest low survivability for aquatic organisms exposed to AEO from fresh tailings.

The presence of low levels of natural bituminous input into surface waters in the region has likely resulted in some tolerance by indigenous organisms to AEO. Therefore, sensitivities

observed for lab-reared organisms potentially overestimate the level of risk posed to native taxa. Nonetheless, due to the high exposure level associated with tailings AEO, indigenous organisms would likely still be at a very high risk.

The high probability of AEO predicted to present a detrimental effect to aquatic life, stresses the need for monitoring in this region. Monitoring should account for current anthropogenic AEO input from tailings seepage, and its effect on sensitive fish species. The challenge in this regard is that fish development beyond an embryonic life-stage appears to become much less sensitive to dissolved organics. Therefore, monitoring needs to incorporate representative species which include more sensitive invertebrates such as *H. azteca*. For future efforts regarding the wet landscape strategy, monitoring should account for changes in AEO concentration over time. In particular, connection of end-pit lakes to natural systems during reclamation efforts need to account for the time-dependent reduction of risk associated with biodegradation of AEO.

Chapter 7. General Conclusions

7.1. Fractionation and Chemical Characterization

In order to investigate principal toxic components of OSPW and other bitumen sources, a novel extraction and fractionation method was developed (Chapter 2). The developed method utilized solid-phase extraction (SPE) and soxhlet extraction techniques. Small-scale SPE has been used in previous research for the isolation of organic compounds from OSPW (Verbeek et al., 1993; de Campos et al., 2006; Gagné et al., 2011; Headley et al., 2013a). The objective of our research was the adaptation of this method to preparative-scale applications. Through scale-up experimentation, successful isolation of organic compounds based on pH and polarity from 180 L of an aged OSPW source was demonstrated. Three organic fractions were generated (F1, F2, F3) at a preparative scale, resulting in 3 L of concentrated organics in each fraction (60-fold concentration). The isolation of organics was verified by analysis of inorganics (metals and major ions) at each step in the fractionation process. This analysis revealed that only the final filtrate contained inorganic components. In order to chemically characterize the organics present in the fraction, each was subject to a suite of chemical analyses. Liquid chromatography quadrupole time-of-flight mass spectrometry (LC-QToF), gas chromatography triple quadrupole time-of-flight mass spectrometry (GC-MS/MS), and synchronous fluorescence spectroscopy (SFS) analyses displayed an increase in relative polarity with subsequent fractions (F1 – F3). Synchronous fluorescence spectroscopy (SFS) additionally displayed an increase in the degree of aromaticity from F1 to F3. Results from electrospray ionization high-resolution mass spectrometry (ESI-HRMS) analysis indicated that degree of oxygenation increased from F1 to F3, and likely contributed to the relative increase in polarity.

Following the fractionation method development, which was evaluated using an aged OSPW source (Chapter 2), subsequent fractionation of bitumen-influenced groundwater sources was

conducted (Chapter 4). This was done, in part, to evaluate the method's utility for use with a variety of water sources. However, the main objective was to investigate possible differences in the chemical composition between natural bitumen-influenced groundwaters compared to OSPW-influenced groundwaters. Accordingly, groundwaters from two natural bitumen-influenced sources (DP-1, DP-2) and two groundwaters from a mixture of OSPW- and naturally-influenced sources (DP-5, DP-4) were fractionated on a preparative scale using the previously developed method. For each of the four sites, three fractions were generated; each comprised of 3 L of concentrated dissolved organics. The same chemical characterization performed for the aged OSPW was completed for fractions generated from each groundwater site sampled. Comparable to the aged OSPW, with each subsequent fraction (F1 to F2 to F3), LC-QToF and GC-MS/MS analyses displayed an increase in relative polarity. Likewise, ESI-HRMS revealed a relative increase in oxygenation of compounds from F1 to F3 for all sites.

Chemical characterization of aged OSPW and collected groundwater fractions revealed the polar compounds such as naphthenic acids (O_2 class) were isolated in the first high pH fraction (F1). This is consistent with a similar fractionation study (SPE, liquid-liquid) in which an abundance of dissolved organics were isolated in a high pH fraction (Morandi et al., 2015). Therefore, polarity of bitumen-derived soluble organic compounds appears to be a function of several factors including functional groups, water solubility, molecular size, and molecular structure, and is not necessarily predominately controlled by protonation and deprotonation of carboxylic acid moieties. This is supported by spiking experiments with the fractionation method developed herein, which suggested that factors other than the presence of acid moieties and molecular weight contribute to the polarity of bitumen-derived organics (Chapter 2). It is very

likely that factors contributing to the observed compound polarity also affect bioavailability and toxicity of dissolved organic compounds isolated in our investigation.

Identical fractionation and chemical characterization methods between aged OSPW and bitumen-influenced groundwaters allowed for direct comparison of chemical composition (Chapters 2 and 4). It is important to note that the fractionation method resulted in the majority of O_2 ions (including NA) being isolated in F1 for all samples assessed. With respect to class distribution of ions (ESI-HRMS), direct comparison of F1 between bitumen-influenced groundwater sites and aged OSPW indicated a greater proportion of O_2 ions in groundwaters collected. Conversely, F1 for Aged OSPW contains a considerably higher proportion of O_3 ions compared to bitumen-influenced groundwaters. In general, aged OSPW contains a greater proportion of polyoxygenated compounds in F3 than collected groundwaters assessed herein. These observations were not surprising, as the reduction of less recalcitrant organics in OSPW by biodegradation has been shown to produce a similar reduction in naphthenic compounds (O_2) and a predominance of more oxygenated and alkyl-branched compounds (Bataineh et al., 2006; Smith et al., 2008; Han et al., 2009). The contaminants collected from bitumen-influenced groundwaters represented seepage flow through anaerobic conditions and were likely not exposed to the complement of indigenous aerobic bacteria responsible for biodegradation of AEO observed in the previous studies. When comparisons of O_2 double-bond equivalents (DBE) were made, aged OSPW and bitumen-influenced groundwaters displayed similar DBE profiles with bimodal distributions. The major difference was that aged OSPW was predominantly composed of compounds with DBE 4, 5, 7, and 8 while groundwaters were dominated by compounds with DBE 3, 4, 6, and 7. Because DBE can indicate the number of rings per molecule as well as aromaticity, this observation suggests that aged OSPW contains a greater

proportion of more complex O_2 ions. This is again, likely a result of biodegradation by indigenous aerobic bacteria present in tailings environments.

Comparison of bitumen-influenced sites (DP-1 and DP-2) to OSPW-influenced sites (DP-4 and DP-5) did not display any common chemical characteristics that allowed for differentiation between the two groups (Chapter 4). In fact, sites DP-5 and DP-2 displayed the most similar class distribution of ions and O_2 double-bond equivalents when compared across sites with ESI-HRMS analysis. Characterization with LC-QToF and GC-MS/MS displayed varied chemical characteristics across sites with no major commonality between sites. Similarly, analysis of metals within collected groundwaters was quite variable across sites. The lack of trends in chemical signatures across sites suggests that the organic composition within groundwaters is likely modified by not only bitumen source but substrate and groundwater mixing. It is important to note that the four bitumen-influenced groundwater sites evaluated herein may not be wholly representative of all groundwaters in the region.

7.2. Toxicological Assessment

The generation of large quantities of fractionated material from the aged OSPW and bitumen-influenced groundwater sources allowed for toxicity evaluation of each fraction with a suite of bioassays. This is important because previous work has identified that sensitivity to bitumen-derived organics and inorganics varies between organisms and endpoints (Marentette et al., 2015b; Bartlett et al., 2017). Therefore, a complement of organisms should be used when assessing toxicity associated with whole OSPW, which was explored in this study. Because samples were fractionated and prepared using the same method, direct comparison of relative toxicities across bioassays was possible. Toxicological assessments were conducted by

exposures to two fish species: *P. promelas* and *O. latipes*, four invertebrate species: *H. azteca*, *D. magna*, *L. cardium* (aged OSPW), and *L. siliquiodea* (groundwaters), and a commonly used bacterial assay: *V. fischeri* (Chapters 3 and 5). The variety of species used allowed for comparison of species sensitivities at multiple trophic levels.

Bioassay exposures consisted of controls (water control, solvent controls), whole water, organic fractions (F1-F3), and a Recombined treatment representing all three fractions combined. Because each fraction was prepared with 0.1% methanol by volume, the Recombined treatment contained 0.3% methanol by volume, therefore, solvent controls were prepared identically to reflect possible toxicity due to solvent effects. Whole water was exposed as unaltered OSPW or bitumen-influenced groundwater which had not undergone any prior manipulations. These exposures, therefore, contained both organic and inorganic constituents present in the initial sample. The organic fractions and Recombined treatment only contained dissolved organics and were prepared (by volume) to reflect concentrations present in whole water. As a result, the presence of inorganic compounds in addition to the organic constituents in the whole water samples is the main difference from the Recombined samples.

Broad comparison of species sensitivities for the tested organisms across all sources revealed that some species tested herein were generally more sensitive to organic components while some invertebrates appeared sensitive to inorganic components. The two most sensitive species to dissolved organics overall were *P. promelas* and *H. azteca*. Similar toxicological analysis has also observed comparable sensitivities of *P. promelas* and *H. azteca* exposed to AEO when compared to other species (Kinley et al., 2016). The *V. fischeri* assay displayed a general insensitivity to dissolved organics, but when exposed to 3x concentrated treatments, the observed toxicities were similar to *P. promelas* and *H. azteca*. This suggests that the utility of the

V. fischeri (Microtox®) assay is requisite on the concentration of exposure treatments, but can be predictive in identifying relative toxicities between fractions in an EDA approach. General species sensitivities for whole bitumen-influenced groundwater exposures were such that *P. promelas* and *H. azteca* were most sensitive overall . Research which has assessed oil sands organics toxicity to a suite of organisms generally showed similar trends to what we observed for relative sensitivites herein (Swigert et al., 2015; Kinley et al., 2016). These findings suggest that investigators of OSPW toxicity take into account the relative sensitivity of organisms chosen for exposure studies, and that care be taken in interpreting results from single species studies.

Comparison between organic fractions suggested that relative toxicities were partially dependent on organism sensitivity. For example, in bitumen-influenced groundwaters where *P. promelas* and *H. azteca* were sensitive to organics present in F1 (major contribution from the O_2 class), *H. azteca* and *O. latipes* were sensitive to polyoxygenated ($>O_2$) organic compounds present in F3. Toxicities resulting from whole water exposures revealed that *D. magna* and *L. siliquoidea* appeared to be sensitive to inorganic elements. Similarly, a study which assessed toxicity of OSPW to *D. magna* found that they possessed lower sensitivity to organic components in OSPW compared to fish (McQueen et al., 2017). It is, therefore, important to recognize that different bioassays may be useful in assessing toxicity to a narrow range of contaminant classes, and that ecosystem health is likely dependent on the action of multiple stressors.

The relative toxicities of organic fractions appeared to be partially due to organic ion classes as well as concentration. Studies have identified that O_2 species (naphthenic compounds) are responsible for the majority of toxicity observed in OSPW (Morandi et al., 2015; Hughes et al., 2017). Following this line of evidence, we expected F1 to be most toxic fraction, as chemical

characterization revealed that the majority of O_2 compounds were isolated therein. In bitumen-influenced groundwater sources where concentrations of O_2 compounds present in F1 were higher compared to aged OSPW, significant ($p \leq 0.05$) toxicity was observed to multiples species. However, in the aged OSPW, only F3 (polyoxygenated compounds) appeared to elicit significant toxicity, while F1 displayed little to no toxicity overall. The low abundance of naphthenic compounds (O_2 class) in F1 of aged OSPW may have contributed to low toxicity observed in that fraction. These observations strengthen the argument that O_2 organic compounds are potent toxic drivers. However, in both aged OSPW and sampled groundwaters, significant toxicity was also observed for species exposed to F3, suggesting polyoxygenated compounds may also be potent drivers of whole water toxicity. It is important to note that regardless of toxic potency, contaminant concentration is a critical factor in the relative toxicity of ion classes. For example, the absence of toxicity observed in F2 for all bitumen-influenced waters tested was likely due to the low concentration of bitumen-derived dissolved organics present (<1.5 mg/L). In fractions where significant toxicity was observed, it is difficult to ascertain whether ion class or concentration was the greater contributor to toxicity, but we suggest that both are important predictors.

Notable observations were made with regard to possible contaminant interactions in OSPW and bitumen-influenced groundwaters. Specifically, in some cases constituents present in whole water conferred a reduction in toxicity compared to organic fractions from the same site. Whole water treatments were un-fractionated and contained the suite of organic and inorganic contaminants present in the water samples, while Recombined treatments contained the complement of only isolated organics from each fraction. In general, where significant acute toxicity was observed in organic fractions and Recombined treatments, significant toxicity was

also observed in whole water. However, there were enough notable exceptions to this observation that whole water contaminant interactions warrant further investigation. For example, toxicity was observed for *P. promelas* (DP-5), *O. latipes* (DP-2), *H. azteca* (DP-4 and DP-5), and *V. fischeri* (3x concentration: DP-1, 2, 5) exposed to dissolved organic treatments, but little to no toxicity was observed in the whole water treatments (Chapters 5). Exposure of aged OSPW to *P. promelas* was the only case where significant toxicity in an organic fraction was not also observed in the Recombined fraction (Chapter 3). Therefore, although inorganic toxicity was not specifically assessed, it appeared that the inorganics present may be responsible for some of the reduction in toxicity observed in whole water treatments. It is possible that the presence of salts in the whole waters resulted in precipitation of some of the dissolved organics, reducing their overall bioavailability, as has been observed previously for NAs (Headley et al., 2011a; Celsie et al., 2016). It is also possible that contaminant interactions such as binding to larger humic/fulvic acids reduced overall bioavailability of bioactive components, resulting in a reduction in whole water toxicity.

7.3. Evaluation of Oil Sands Contaminant Source

A major concern for the oil the sands industry is the unintentional release of OSPW contaminants into the environment. This contaminant pathway and fate occurs in different forms. Unintended release can occur through seepage of OSPW from tailings impoundments. This contaminant pathway was the scenario under investigation when evaluating groundwater chemical and toxicological characteristics (Chapters 4 and 5). The results of this investigation suggest that the chemical nature of OSPW can be modified by existing hydrogeology and is highly dependent on the natural conditions in close proximity to tailings the seep. Moreover, the

OSPW- and natural-influenced sites displayed similar toxicities to those influenced only by naturally occurring bitumen deposits (Chapter 5). Also, underlying groundwater hydrology affected one of the sample sites (DP-4) which in previous collection years displayed evidence of OSPW (Frank et al., 2014; Hewitt et al., 2018). In the collection year for the present study, the sampling appeared to miss the tailings plume as no evidence of OSPW, or any bitumen organic signature, was detected. This site displayed the lowest observed toxicity to all bioassays compared to other groundwaters sites assessed (Chapter 5). Consequently, the toxicity associated with the groundwaters sampled herein appears to be a result of bitumen influence, but is not wholly dependent on bitumen source.

As part of the provincial government's mandated reclamation of oil sands lease sites (Government of Alberta, 2017), industry operators' wet landscape reclamation strategy involves decommissioning of active tailings ponds (Alberta Environment, 2007). One approach is the connection of sufficiently aged tailings ponds to natural surface water systems in the region (Allen, 2008; Dixon, 2015). This scenario represents an intended contaminant release into the ecosystem for which temporality affects the chemical and toxicological nature of OSPW. This contaminant pathway was the purpose for investigation of an aged OSPW source described herein (Chapters 2 and 3). The results of the investigation suggest that OSPW aging may be a viable strategy for the reduction of toxicity associated with some dissolved organic compounds. Various studies have identified that biodegradation of lower molecular weight, less recalcitrant organics results in a predominance of compounds with higher carbon number, cyclicity, degrees of oxygenation, and greater degree of branching (Clemente et al., 2004; Scott et al., 2005; Bataineh et al., 2006; Smith et al., 2008; Han et al., 2009). These findings are consistent with our observation that the aged OSPW source contained organics with a higher degree of oxygenation

(Chapter 2) compared to fresh tailings sources (Bartlett et al., 2017). Furthermore, we compared chemistry data from the aged OSPW source assessed herein (aged 18 years at collection) to a study which had previously characterized the same aged OSPW source (aged 12 years at collection) (Siwik et al., 2000). This comparison revealed that with an additional 6 years of aging, the chemical composition displayed a shift to more oxygenated AEO ions and a reduction in organic compound concentrations, consistent with biodegradation of OSPW (Bataineh et al., 2006; Han et al., 2009). Nonetheless, although toxicological evaluation displayed very low toxicity to all organisms in F1 and F2, toxicity was observed in F3 (*P. promelas*) which contained more oxygenated organics (Chapter 3). Additionally, two invertebrate organisms (*H. azteca* and *L. cardium*) displayed sensitivity to only whole aged OSPW, suggesting toxicity was associated with inorganic components. Therefore, the success of the wet landscape reclamation strategy is likely dependent on a reduction of some ployoxygenated organic classes as well as a reduction in inorganic components.

7.4. Risk Assessment of Tailings AEO

In order to evaluate the potential risk associated with acid-extractable organics (AEO) to the aquatic environment, an ecotoxicological risk assessment was conducted. The determination of risk requires the characterization of exposure and effects. This was accomplished by an extensive literature review and screening of relevant data (Chapter 6). Review of AEO exposure resulted in the compilation of 46 individual measurements, over a 10-year period (2004-2014) from at least 7 different fresh OSPW sources. AEO concentration encompassed a range from 17.4 – 104 mg/L. A generated exposure distribution plot predicted that an AEO concentration of 87.1 mg/L would be present in 10 percent of tailings environments. Screening of toxicological data relating

to AEO effects compiled median lethal concentration (LC_{50}) data from 11 different species. In general, fish embryo bioassays were more sensitive than invertebrate organisms, resulting in separate assessments for each. After separating fish and invertebrate data, generated effects distribution plots predicted AEO concentrations of 5.5 mg/L and 19.6 mg/L respectively, to be protective of 90 percent of species. When a combined probability distribution of exposure and effects was evaluated, the probability of effecting 10 percent fish and invertebrates was 100% and 97.7%, respectively.

According to the ecotoxicological risk assessment, AEO in fresh tailings presents a very high risk to the natural environment at the concentrations described. It is important to understand that the level of risk may only apply to scenarios where a failure in a tailings dyke occurs, a tailings pond is connected to natural surface waters without prior aging, or organisms are introduced into a tailings environment. Moreover, the predicted risk does not account for dilution of AEO from surface or groundwaters. Another important consideration is the possibility that species indigenous to the Athabasca oil sands region may have developed tolerance to bituminous compounds. Constant exposure to low levels of natural bitumen input into the environment may have selected for organisms more tolerant than lab-reared organisms to bitumen-derived AEO over time. In general, although AEO present a high risk to aquatic biota, predictions derived herein may overestimate the risk as it pertains to current tailings reclamation strategies, which likely include aging and dilution of tailings OSPW.

7.5. Future Research

Beyond groundwater and aged OSPW, there is merit in evaluating toxicities associated with fractions from fresh OSPW, OSPW from different industry operators, and OSPW-influenced

surface waters. A more comprehensive assessment of the heterogeneity between OSPW sources as well as OSPW influence in surface waters would aid in the development of overall contaminant transport and fate models.

Determining drivers of toxicity in OSPW is a primary concern for the oil sands industry operators and regulators. Results from chemical characterization presented here have identified the most bioactive components in aged OSPW and bitumen-influenced groundwaters to be present in F1 and F3. Although O_2 and O_4 dissolved organic species predominated in F1 and F3, respectively, the fractions were composed of an abundance of other species making it difficult to determine the degree to which toxicity was a result of additive interactions. Therefore, suggested future research should involve sub-fractionation of F1 and F3 to further isolate organic species therein. This could remove confounding interactions and allow for more accurate determination of toxic drivers.

The work presented in this thesis suggests that primary drivers are highly dependent on the species to which OSPW is exposed. The present thesis has identified *P. promelas* and *H. azteca* as species that are particularly sensitive to bitumen-derived dissolved organics, while *D. magna* and *L. siliquoidea* appeared to display sensitivity to inorganic components. Therefore, future research should consider further assessment of sentinel species for prediction of OSPW-associated toxicity in aquatic environments. These may include organisms particularly sensitive to organic or inorganic components, but ultimately provide for greater ecosystem protection.

In summation, for those organisms that display sensitivity to dissolved organics in OS waters, aging by natural biodegradation appears to be a viable strategy. Moreover, industrially-influenced groundwaters do not appear to pose a greater risk to aquatic organisms than groundwaters influenced by naturally-derived bitumen. Nonetheless, due to invertebrate

sensitivities to inorganic components of OSPW and toxicity observed by some organisms to polyoxygenated organics, a strategy to deal with these bio-persistent compounds warrants investigation. Furthermore, observed whole water toxicities indicate that toxicity appears to be mitigated by contaminant interactions in some cases. Therefore, investigations into bitumen-derived contaminant interactions could better predict environmental risk.

References

Ahad, J. M. E., H. Pakdel, M. M. Savard, A. I. Calderhead, P. R. Gammon, A. Rivera, K. M. Peru and J. V. Headley (2013). "Characterization and quantification of mining-related 'naphthenic acids' in groundwater near a major oil sands tailings pond." Environmental Science and Technology 47(10): 5023-5030.

Ajaero, C., D. W. McMartin, K. M. Peru, J. Bailey, M. Haakensen, V. Friesen, R. Martz, S. A. Hughes, C. Brown, H. Chen, A. M. McKenna, Y. E. Corilo and J. V. Headley (2017). "Fourier transform ion cyclotron resonance mass spectrometry characterization of Athabasca oil sand process-affected waters incubated in the presence of wetland plants." Energy & Fuels 31: 1731-1740.

Akre, C. J., J. V. Headley, F. M. Conly, K. M. Peru and L. C. Dickson (2004). "Spatial patterns of natural polycyclic aromatic hydrocarbons in sediment in the lower Athabasca River." Journal of Environmental Science and Health A39(5): 1163-1176.

Alberta Energy (2008a). Alberta's Oil Sands: Opportunity. Balance, Government of Alberta.

Alberta Energy (2008b). Alberta Oil Sands: Resourceful. Responsible. (ISBN 978-07785-7348-7). September 2008: 13.

Alberta Energy (2009). "Facts about Alberta's oil sands and its industry." http://history.alberta.ca/oilsands/resources/docs/facts_sheets09.pdf.

Alberta Energy (2013). "Oil sands tailings facts sheet."

Alberta Energy and Utilities Board (2009-2010). Alberta Ministry of Energy Annual Report.

Alberta Energy Regulator (2014). Alberta’s energy reserves 2013 and supply/demand outlook 2014-2023. ST98-2014

Alberta Environment (2007). Guidelines for Wetland Establishment on Reclaimed Oil Sands Leases, Alberta Environment.

Aldenberg, T., J. Jaworska and T. P. Traas (2002). Normal species sensitivity distributions and probabilistic ecological risk assessment. Species Sensitivity Distributions in Ecotoxicology. L. Posthuma, G. W. Suter and T. P. Trass. Boca Raton, FL, USA, CRC Press: 49-102.

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

ATSDR (1996). Polycyclic aromatic hydrocarbons (PAHs).

Anderson, J. C., S. Joudan, E. Shoichet, L. D. Cuscito, A. E. C. Alipio, C. S. Donaldson, S. Khan, D. M. Goltz, M. D. Rudy, R. A. Frank, C. W. Knapp, M. L. Hanson and C. S. Wong (2015). "Reducing nutrients, organic micropollutants, antibiotic resistance, and toxicity in rural wastewater effluent with surface filtration treatment technology." ecological engineering 84: 375-385.

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

Argonaught Technologies (2002). Step by step guide to SPE method development.

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

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

ASTM (2006). Standard guide for conducting laboratory toxicity tests with freshwater mussels. West Conshohocken, PA, ASTM International.

Azur Environmental (1995). Microtox: Acute toxicity basic test procedures. Carlsbad, CA.

Barrow, M. P., J. V. Headley, K. M. Peru and P. J. Derrick (2009). "Data visualization for the characterization of naphthenic acids within petroleum samples." Energy & Fuels 23: 2592-2599.

Barrow, M. P., K. M. Peru, B. Fahlman, L. M. Hewitt, R. A. Frank and J. V. Headley (2015). "Beyond naphthenic acids: environmental screening of water from the Athabasca region using APPI-FTICR mass spectrometry." Journal of the American Society of Mass Spectrometry 26(9): 1508-1521.

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

Bartlett, A. J., R. Frank, P. L. Gillis, J. Parrott, J. R. Marentette, L. R. Brown, T. Hooey, R. Vanderveen, R. McInnis, P. Brunswick, D. Shang, J. V. Headley, K. M. Peru and L. M. Hewitt (2017). "Toxicity of naphthenic acids to invertebrates: Extracts from oil sands process-affected water versus commercial mixtures." Environmental Pollution 227: 271-279.

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

Bauer, A. E., R. A. Frank, J. V. Headley, K. M. Peru, A. J. Farwell and D. G. Dixon (2017). "Toxicity of oil sands acid-extractable organic fractions to freshwater fish: *Pimephales promelas* (fathead minnow) and *Oryzias latipes* (Japanese medaka)." Chemosphere 171: 168-176.

Bauer, A. E., R. A. Frank, J. V. Headley, K. M. Peru, L. M. Hewitt and D. G. Dixon (2015). "Enhanced characterization of oil sands acid-extractable organics fractions using electrospray ionization-high-resolution mass spectrometry and synchronous fluorescence spectroscopy." Environmental Toxicology and Chemistry 34(5): 1001-1008.

Biesinger, K. E. and G. M. Christensen (1972). "Effects of various metals on survival, growth, reproduction, and metabolism of *Daphnia magna*." Journal of Fisheries Research Board of Canada 29(12): 1691-1700.

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

Borgmann, U. (1996). "Systematic analysis of aqueous ion requirements of Hyalella azteca: A standard artificial media containing the essential bromide ion." archives of Environmental Contamination and Toxicology 30: 356-363.

Borgmann, U., K. M. Ralph and W. P. Norwood (1989). "Toxicity test procedures for *Hyalella azteca*, and chronic toxicity of cadmium and pentachlorophenol to *H. azteca*, *Gammarus fasciatus*, and *Daphnia magna*." archives of Environmental Contamination and Toxicology 18: 756-764.

Borgund, A. E., K. Erstad and T. Barth (2007). "Normal phase high performance liquid chromatography for fractionation of organic acid mixtures extracted from crude oils." Journal of Chromatography A 1149: 189-196.

Brown, L. D. and A. C. Ulrich (2015). "Oil sands naphthenic acids: A review of properties, measurement, and treatment." Chemosphere 127: 276-290.

Brunswick, P., L. M. Hewitt, R. A. Frank, M. Kim, G. Van Aggelen and D. Shang (2016a). "A traceable reference for direct comparative assessment of total naphthenic acid concentrations in commercial and acid extractable organic mixtures derived from oil sands process water." Journal of Environmental Science and Health; Part A - Toxic/Hazardous Substances and Environmental Engineering 52: 1-7.

Brunswick, P., L. M. Hewitt, R. A. Frank, G. Van Aggelen, M. Kim and D. Shang (2016b). "Specificity of high resolution analysis of naphthenic acids in aqueous environmental matrices." Analytical Methods 8: 6764-6773.

Brunswick, P., D. Shang, G. van Aggelen, R. Hindle, L. M. Hewitt, R. A. Frank, M. Haberl and M. Kim (2015). "Trace analysis of total naphthenic acids in aqueous environmental matrices by LC/MS-QToF direct injection." Journal of Chromatography A 1405: 49-71.

Canadian Association of Petroleum Producers (2016). Crude oil: Forecast, markets and transportation: 43.

CCME (2017). Water quality guidelines for the protection of aquatic life, Canadian Council of Ministers of the Environment.

Celsie, A., J. M. Parnis and D. Mackay (2016). "Impact of temperature, pH, and salinity changes on the physico-chemical properties of model naphthenic acids." Chemosphere 146: 40-50.

ChemSpider. Royal Society of Chemistry. 2015 http://www.chemspider.com/ Accessed: 16 June. 2018.

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

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

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

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

Cowgill, U. M. and D. P. Milazzo (1991). The sensitivity of two cladocerans to water quality variables: Salinity <467 mg NaCl/L and hardness <200 mg $CaCO_3$/L. Archives of Environmental Contamination and Toxicology 21: 218-223.

Davies, T. D. and K. J. Hall (2007). Importance of calcium in modifying the acute toxicity of sodium sulphate to *Hyalella azteca* and *Daphnia magna*. Environmental Toxicology and Chemistry 26(6): 1243-1247

de Campos, M. C. V., E. C. Oliveira, P. J. S. Filho, C. M. S. Piatnicki and E. B. Caramão (2006). "Analysis of tert-butyldimethylsilyl derivatives in heavy gas oil from Brazilian naphthenic acids by gas chromatography coupled to mass spectrometry with electron impact ionization." Journal of Chromatography A 1105: 95-105.

de Schamphelaere, K. A. C. and C. R. Janssen (2002). "A biotic ligand model predicting acute copper toxicity for *Daphnia magna*: The effects of calcium, magnesium, sodium, potassium, and pH." Environmental Science and Technology 36: 48-54.

Dixon, D. G. (2015). Surface and groundwater management in the oil sands industry. Canadian Water Network. Waterloo, ON, Canada, University of Waterloo.

Droppo, I. G., S. N. Liss, D. Williams, T. Nelson, C. Jaskot and B. Trapp (2009). "Dynamic existence of waterborne pathogens within river sediment compartments. Implications for water quality regulatory affairs." Environmental Science and Technology 43(6): 1737-1743.

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

Energy Resources Conservation Board and Canadian Environmental Assessment Agency (2011). Report of the Joint Review Panel established by the Federal Minister of the Environment and the Energy Resources Conservation Board - Decision 2011-005: Total E&P Joslyn Ltd., Application for the Joslyn North Mine Project.

Environment Canada (1992). Report EPS 1/RM/24 Biological test method: Toxicity test using luminescent bacteria, Environment Canada.

Environment Canada (1996). Biological Test Method: Acute lethality test using *Daphnia spp*, Environment and Climate Change Canada: 57.

Environment Canada (2013). Biological test method: Test for survival and growth in sediment and water using the freshwater amphipod *Hyalella azteca*. E. P. Series, Environment Canada.

Fair, A. (2010). CONRAD - PRomoting Innovation in the Oil Sands. Edmonton, Alberta, CONRAD.

Farrell, A. P., C. J. Kennedy and A. Kolok (2004). "Effects of wastewater from an oil-sand refining operation on survival, hematology, gill histoilogy, and swimming of fathead minnows." Canadian Journal of Zoology 82: 1519-1527.

Farwell, A., V. Nero, M. Croft, P. Bal and D. G. Dixon (2006). "Modified Japanese medaka embryo-larval bioassay for rapid determination of developmental abnormalities." Archives of Environmental Chemistry and Toxicology 51: 600-607.

Ferguson, G. P., D. L. Rudolph and J. F. Barker (2009). "Hydrodynamics of a large oil sand tailings impoundment and related environmental implications." Canadian Geotechnological Journal 46: 1446-1460.

Frank, R. A., J. W. Roy, L. Grapentine, W. P. Norwood, P. L. Gillis, A. J. Bartlett, G. Bickerton, M. Brown, L. Brown, L. M. Hewitt and J. L. Parrott (2018). "Toxicity of bitumen-influenced groundwaters in the oil sands region of Alberta, Canada." In Preparation.

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

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

Frank, R. A., R. Kavanagh, B. K. Burnison, J. V. Headley, K. M. Peru, G. Van Der Kraak and K. R. Solomon (2006). "Diethylaminoethyl-cellulose clean-up of a large volume naphthenic acid extract." Chemosphere 64: 1346-1352.

Frank, R. A., C. B. Milestone, S. J. Rowland, J. V. Headley, R. J. Kavanagh, S. K. Lengger, A. G. Scarlett, C. E. West, K. M. Peru and L. M. Hewitt (2016). "Assessing spatial and temporal

variability of acid-extractable organics in oil sands process-affected waters." Chemosphere 160: 303-313.

Frank, R. A., J. W. Roy, G. Bickerton, S. J. Rowland, J. V. Headley, A. G. Scarlett, C. E. West, K. M. Peru, F. M. Conly and L. M. Hewitt (2014). "Profiling oil sands mixtures from industrial developments and natural groundwaters for source identification." Environmental Science and Technology 48(5): 2660-2670.

FTFC (1995a). Volume I: Clark hot water extraction fine tailings. In: Advances in oil sands tailings research. Alberta Department of Energy, Oil sands and research division, Edmonton, AB, Canada, FTFC (Fine Tailings Fundamentals Consortium).

FTFC (1995b). Volume II: Fine tails and process water reclamation. In: Advances in oil sands tailings research. Alberta Department of Energy, Oil sands and research division, Edmonton, AB, Canada, FTFC (Fine Tailings Fundamentals Consortium).

Gagné, F., C. André, M. Douville, A. Talbot, J. L. Parrott, M. E. McMaster and L. M. Hewitt (2011). "An examination of the toxic properties of water extracts in the vicinity of an oil sand extraction site." Journal of Environmental Monitoring 13: 3075-3086.

Gillis, P. L. (2011). "Assessing the toxicity of sodium chloride to the glochidia of freshwater mussels: Implications for salinization of surface waters." Environmental Pollution 159(6): 1702-1708.

Gillis, P. L., R. J. Mitchell, A. N. Schwalb, K. A. McNichols, G. L. Mackie, C. M. Wood and J. D. Ackerman (2008). "Sensitivity of glochidia (larvae) of freshwater mussels to copper: Assessing the effect of water hardness and dissolved organic carbon on the sensitivity of endangered species." Aquatic Toxicology 88: 137-145.

Gostomski, F. (1990). The toxicity of aluminum to aquatic species in the US, United States Environmental Protection Agency.

Government of Alberta (2017). Environmental Protection and Enhancement Act - Revised Statutes of Alberta 2000 Chapter E-12 (Current as of March 31, 2017).

Grbovic, L., K. Pavlovic, B. Prekodravac, K. Kuhajda, S. Kevresan, M. Popsavin, J. Milic and V. Cirin-Novta (2012). "Fractionation of complex mixtures of naphthenic acids, their characterization and biological activity." Journal of Serbian Chemical Society 77(2): 147-157.

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

Hall, R. I., B. B. Wolfe, J. A. Wiklund, T. W. D. Edwards, A. Farwell and D. G. Dixon (2012). "Has Alberta oil sands development altered delivery of polycyclic aromatic compounds to the Peace-Athabasca Delta?" PLoS ONE 7(9): e46089.

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

Hansten, C., M. Heino and K. Pynnonen (1996). "Viability of glochidia of Anodonta anatina (Unionidae) exposed to selected metals and chelating agents." Aquatic Toxicology 34: 1-12.

He, Y., S. B. Wiseman, N. Wang, L. A. Perez-Estrada, M. Gamal El-Din, J. W. Martin and J. P. Giesy (2012). "Transcriptional responses of the brain-gonad-liver axis of fathead minnows exposed to untreated and ozone-treated oil sands process-affected water." Environmental Science and Technology 46: 9701-9708.

Headley, J. V. and C. J. Akre (2001). "Preliminary characterization and source assessment of PAHs in tributary sediments of the Athabasca River, Canada." Environmental Forensics 2: 335-345.

Headley, J. V., M. P. Barrow, K. M. Peru and P. J. Derrick (2011a). "Salting-out effects on the characterization of naphthenic acids from Athabasca oil sands using electrospray ionization." Journal of Environmental Science and Health 46: 844-854.

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

Headley, J. V., K. M. Peru and M. P. Barrow (2009b). "Mass spectrometric characterization of naphthenic acids in environmental samples: A review." Mass Spectrometry Reviews 28: 121-134.

Headley, J. V., M. P. Barrow, K. M. Peru, B. Fahlman, R. A. Frank, G. Bickerton, M. E. McMaster, J. Parrott and L. M. Hewitt (2011b). "Preliminary fingerprinting of Athabasca oil sands polar organics in environmental samples using electrospray ionization Fourier transform

ion cyclotron resonance mass spectrometry." Rapid Communications in Mass Spectrometry 25: 1899-1909.

Headley, J. V. and D. W. McMartin (2004). "A review of the occurrence and fate of naphthenic acids in aquatic environments." Journal of Environmental Science and Health. Part A 39(8): 1989 - 2010.

Headley, J. V., K. M. Peru, B. Fahlman, A. Colodey and D. W. McMartin (2013a). "Selective solvent extraction and characterization of the acid extractable fraction of Athabasca oil sands process waters by Orbitrap mass spectrometry." International Journal of Mass Spectrometry 345-347: 104-108.

Headley, J. V., K. M. Peru, B. Fahlman, D. W. McMartin, M. M. Mapolelo, R. P. Rodgers, V. V. Lobodin and A. G. Marshall (2012). "Comparison of the levels of chloride ions to the characterization of oil sands polar organics in natural waters by use of fourier transform ion cyclotron resonance mass spectrometry." Energy & Fuels 26: 2585-2590.

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

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

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

Hewitt, L. M., J. W. Roy, G. Bickerton, A. De Silva, S. J. Rowland, J. V. Headley, A. G. Scarlett, C. E. West, K. M. Peru, J. Parrott, F. M. Conly and R. A. Frank (2018). "Chemical tracers for tracking migration of oil sands process waters in northern Alberta, Canada." In Preparation.

Hindle, R., M. Noestheden, K. M. Peru and J. V. Headley (2013). "Quantitative analysis of naphthenic acids in water by liquid chromatography-accurate mass time-of-flight mass spectrometry." Journal of Chromatography A 1286: 166-174.

Holden, A. A., R. B. Donahue and A. C. Ulrich (2011). "Geochemical interactions between process-affected water from oil sands tailings ponds and North Alberta surficial sediments." Journal of Contaminant Hydrology 119: 55-68.

Holden, A. A., K. U. Mayer and A. C. Ulrich (2013). "Biogeochemical processes controlling the mobility of major ions and trace metals in aquitard sediments beneath an oil sand tailing pond: Laboratory studies and reactive transport modeling." Journal of Contaminant Hydrology 151: 55-67.

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

Hozhina, E. I., A. A. Khramov, P. A. Gerasimov and A. A. Kumarkov (2001). "Uptake of heavy metals, arsenic, and antimony by aquatic plants in the vicinity of ore mining and processing industries." Journal of Geochemical Exploration 74: 153-162.

Huang, R., N. Sun, P. Chelme-Ayala, K. N. McPhedren, M. Changalov and M. G. El-Din (2015). "Fractionation of oil sand-process affected water using pH-dependent extractions: A study of dissociation constants for naphthenic acids species." Chemosphere 127: 291-296.

Hughes, S. A., A. Mahaffey, B. Shore, J. Baker, B. Kilgour, C. Brown, K. M. Peru, J. V. Headley and H. C. Bailey (2017). "Using ultra-high resolution mass spectrometry and toxicity identification techniques to characterize the toxicity of oil sands process-affected water: The case for classical naphthenic acids." Environmental Toxicology and Chemistry 36(11): 3148-3157.

International Sorbent Technologies (2001). "Method development in solid phase extraction using non-polar ISOLUTE SPE columns for the extraction of aqueous samples."

Islam, M. D. S., J. Moreira, P. Chelme-Ayala and M. G. El-Din (2014). "Prediction of naphthenic acid species degradation by kinetic and surrogate models during the ozonation of oil sands process-affected water." Science of the Total Environment 493: 282-290.

Janfada, A., J. V. Headley, K. M. Peru and S. L. Barbour (2006). "A laboratory evaluation of the sorption of oil sands naphthenic acids on organic rich soils." Journal of Environmental Science and Health Part A 41: 985-997.

Jasechko, S., J. J. Gibson, S. J. Birks and Y. Yi (2012). "Quantifying saline groundwater seepage to surface waters in the Athabasca oil sands region." Applied Geochemistry 27: 2068-2076.

Johnston, C. U., L. N. Clothier, D. M. Quesnel, L. M. Gieg, G. Chua, P. M. Hermann and W. C. Wildering (2017). "Embryonic exposure to model naphthenic acids delays growth and hatching in the pond snail *Lymnaea stagnalis*." Chemosphere 168: 1578-1588.

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

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

Kamal, M., A. E. Ghaly, N. Mahmoud and R. Cote (2004). "Phytoaccumulation of heavy metals by aquatic plants." Environment International 29: 1029-1039.

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

Kavanagh, R. J., R. A. Frank, B. K. Burnison, R. F. Young, P. M. Fedorak, K. R. Solomon and G. Van Der Kraak (2012). "Fathead minnow (*Pimephales promelas*) reproduction is impaired when exposed to a naphthenic acid extract." Aquatic Toxicology 116-117: 34-42.

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

Keller, A. E. and S. G. Zam (1991). "The acute toxicity of selected metals to the freshwater mussel, *Anodonta imbecilis*." Environmental Toxicology and Chemistry 10: 539-546.

Kinley, C. M., A. D. McQueen and J. H. Rodgers (2016). "Comparative responses of freshwater organisms to exposures of a commercial naphthenic acid." Chemosphere 153: 170-178.

Klamerth, N., J. Moreira, C. Li, A. Singh, K. N. McPhedran, P. Chelme-Ayala, M. Belosevic and M. G. El-Din (2015). "Effect of ozonation on the naphthenic acids' speciation and toxicity of pH-

dependent organic extracts of oil sands process-affected water." Science of the Total Environment 506-507: 66-75.

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

Leclair, L. A., G. Z. MacDonald, L. J. Phalen, B. Köllner, N. S. Hogan and M. R. van den Heuvel (2013). "The immunological effects of oil sands surface waters and naphthenic acids on rainbow trout (*Oncorhynchus mykiss*)." Aquatic Toxicology 142-143: 185-194.

Leshuk, T., T. Wong, S. Linley, K. M. Peru and J. V. Headley (2016). "Solar photocatalytic degradation of naphthenic acids in oil sands process-affected water." Chemosphere 144: 1854-1861.

Leung, S. S., M. D. MacKinnon and R. E. H. Smith (2003). "The ecological effects of naphthenic acids and salts on phytoplankton from the Athabasca oil sands region." Aquatic Toxicology 62: 11-26.

Li, C., F. Li, J. Stafford, M. Belosevic and M. Gamal El-Din (2017). "The toxicity of oil sands process-affected water (OSPW): A critical review." Science of the Total Environment 601-602: 1785-1802.

Lilius, H., T. Hastbacka and B. Isomaa (1995). A comparison of the toxicity of 30 reference chemicals to *Daphnia magna* and *Daphnia pulex*. Environmental Toxicology and Chemistry 14(12): 2085-2088.

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

Liu, H., X. Chen, Y. Su, I. J. Kang, X. Qiu, Y. Shimasaki, Y. Oshima and J. Yang (2016). "Effects of calcium and magnesium ions on acute copper toxicity to glochidia and early juveniles of the Chinese pond mussel *Anodonta woodiana*." Bulletin of Environmental Contamination and Toxicology 97: 504-509.

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

Lu, W., A. Ewanchuk, L. Perez-Estrada, D. Sego and A. C. Ulrich (2013). "Limitation of fluorescence spectrophotometry in the measurement of naphthenic acids in oil sands process water." Journal of Environmental Science and Health Part A 48(4): 429-436.

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

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

Mahaffey, A. and M. Dube (2017). "Review of the composition and toxicity of oil sands process-affected water." Environmental Reviews 25: 97-114.

Mahdavi, H., Y. Liu and A. C. Ulrich (2013). "Partitioning and bioaccumulation of metals from oil sands process affected water in indigenous *Parachlorella kessleri*." Chemosphere 90: 1893-1899.

Marentette, J. R., R. A. Frank, A. J. Bartlett, P. L. Gillis, L. M. Hewitt, K. M. Peru, J. V. Headley, P. Brunswick, D. Shang and J. L. Parrott (2015a). "Toxicity of naphthenic acid fraction components extracted from fresh and aged oil sands process-affected waters, and commercial naphthenic acid mixtures, to fathead minnow (*Pimephales promelas*) embryos." Aquatic Toxicology 164: 108-117.

Marentette, J. R., R. A. Frank, L. M. Hewitt, P. L. Gillis, A. J. Bartlett, P. Brunswick, D. Shang and J. Parrott (2015b). "Sensitivity of walleye (*Sander vitreus*) and fathead minnow (*Pimephales promelas*) early-life stages to naphthenic acid fraction components extracted from fresh oil sands process-affected waters." Environmental Pollution 207: 59-67.

Markich, S. J. and R. A. Jeffree (1994). "Absorption of divalent trace metals as anologues of calcium by Australian freshwater bivalves: An explanation of how water hardness reduces metal toxicity." Aquatic Toxicology 29: 257-290.

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

McQueen, A. D., C. M. Kinley, M. Hendrikse, D. P. Gaspari, A. J. Calomeni, K. J. Iwinski, J. W. Castle, M. Haakensen, K. M. Peru, J. V. Headley and J. H. Rodgers (2017). "A risk-based

approach for identifying constituents of concern in oil sands process-affected water from the Athabasca oil sands region." Chemosphere 173: 340-350.

Miano, T., G. Sposito, J.P. Martin. (1988). Fluorescence spectroscopy of humic substances. Soil Sci. Soc. Am. J., 52: 1016-1019.

Milam, C. D., J. L. Farris, F. J. Dwyer and D. K. Hardesty (2005). "Acute toxicity of six freshwater mussel species (glochidia) to six chemicals: Implications for *Daphnids* and *Utterbackia imbecillis* as surrogates for protection of freshwater mussels (*Unionidae*)." Archives of Environmental Contamination and Toxicology 48: 166-173.

Milestone, C. B., C. Sun, J. W. Martin, G. Bickerton, J. W. Roy, R. A. Frank and L. M. Hewitt (2018). "Untargeted profiling of bitumen influenced waters for the identification of tracers of oil sands processed water (OSPW) migrations in the Athabasca watershed of Alberta Canada." In Preparation.

Minister of Environment (2013). Decision Statement for the Jackpine Mine Expansion Project.

Mishra, S., V. Meda, A. K. Dalai, J. V. Headley, K. M. Peru and D. W. McMartin (2010). "Microwave treatment of naphthenic acids in water." Journal of Environmental Science and Health Part A 45: 1240-1247.

MOE (2014). *Daphnia magna* acute lethality testing of chemicals, Ontario Ministry of the Environment.

Mohseni, P., N. A. Hahn, R. A. Frank, L. M. Hewitt, M. Hajibabaei and G. Van Der Kraak (2015). "Naphthenic acid mixtures from oil sands process-affected water enhance differentiation of mouse embryonic stem cells and affect development of the heart." Environmental Science and Technology 49: 10165-10172.

Morandi, G. D., S. B. Wiseman, A. Pereira, R. Mankidy, I. G. M. Gault, J. W. Martin and J. P. Giesy (2015). "Effects-Directed Analysis of Dissolved Organic Compounds in Oil Sands Process-Affected Water." Environmental Science and Technology 49: 12395-12404.

Motulsky, H. and A. Christopoulos (2004). Fitting Models to Biological Data Using Linear and Nonlinear Regression: A Practical Guide to Curve Fitting. Oxford, UK, Oxford University Press.

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

NLET (2003). Standard Operating Procedure for the Analysis of Dissolved, Extractable and Total Trace Metals in Water by “Direct Aspiration” or “In Bottle Digestion” Inductively Coupled Plasma-Sector Field Mass Spectrometry (ICP-SFMS). Burlington, Ontario, Canada, Environment and Climate Change Canada.

Oiffer, A. A. L. (2006). Integrated solid phase, aqueous phase and numberical investigation of plume geochemistry at an oil sand mining facility. M.Sc., University of Waterloo.

Oiffer, A. A. L., J. F. Barker, F. M. Gervais, K. U. Mayer, C. J. Ptacek and D. L. Rudolph (2009). "A detailed field-based evaluation of naphthenic acid mobility in groundwater." Journal of Contaminant Hydrology 108: 89-106.

Penner, T. J. and J. M. Foght (2010). "MAture fine tailings from oil sands processing harbour diverse methanogenic communities." Canadian Journal of Microbiology 56: 459-470.

Pereira, A. S., S. Bhattacharjee and J. W. Martin (2013). "Characterization of Oil Sands Process-Affected Waters by Liquid Chromatography Orbitrap Mass Spectrometry." Environmental Science and Technology 47: 5504-5513.

Peters, L. E., M. D. MacKinnon, T. Van Meer, M. R. van den Heuvel and D. G. Dixon (2007). "Effects of oil sands process-affected waters and naphthenic acids on yellow perch (*Perca flavescens*) and Japanese medaka (*Orizias latipes*) embryonic development." Chemosphere 67: 2177-2183.

Peuravuori, J., R. Kiovikko and K. Pihlaja (2002). "Characterization, differentiation and classification of aquatic humic matter separated with different sorbents: synchronous scanning fluorescence spectroscopy." Water Research 36: 4552-4562.

Pourrezaei, P., A. Alpatova, P. Chelme-Ayala, L. Perez-Estrada, M. Jensen-Fontaine, X. C. Le and M. G. El-Din (2014a). "Impact of petroleum coke characteristics on the adsorption of the organic fraction from oil sands process-affected water." International Journal of Science and Technology 11: 2037-2050.

Pourrezaei, P., A. Alpatova, K. Khosravi, P. Drzewicz, Y. Chen, P. Chelme-Ayala and M. G. El-Din (2014b). "Removal of organic compounds and trace metals from oil sands process-affected water using zero-valent iron enhanced by petroleum coke." Journal of Environmental Management 139: 50-58.

Pourrezaei, P., P. Drzewicz, Y. Wang, M. G. El-Din, L. Perez-Estrada, J. W. Martin, J. C. Anderson, S. B. Wiseman, K. Liber and J. P. Giesy (2011). "The impact of metallic coagulants on the removal of organic compounds from oil sands process-affected water." Environmental Science and Technology 45: 8452-8459.

Pozzani, U. C., C. P. Carpenter, P. E. Palm and C. S. Weil (1959). "An investigation of the mammalian toxicity of acetonitrile." Journal of Occupational Medicine: 634-642.

Pubchem. National Centre for Biotechnology Information, National Library of Medicine, National Institute of Health. https://pubchem.ncbi.nlm.nih.gov/ Accessed: 16 June. 2018.

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

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

Quesnel, D. M., T. B. P. Oldenburg, S. R. Larter, L. M. Gieg and G. Chua (2015). "Biostimulation of oil sands process-affected water with phosphate yields removal of sulfur-containing organics and detoxification." Environmental Science and Technology 49: 13012-13020.

Rathore, A. S. and A. Velayudhan (2002). Scale-up and optimization in preparative chromatography: Principals and biopharmaceutical applications, CRC Press.

Reichert, M., B. Blunt, T. Gabruch, T. Zerulla, A. Ralph, M. G. El-Din, B. R. Sutherland and K. B. Tierney (2017). "Sensory and behavioural responses of a model fish to oil sands process-affected water with and without treatment." Environmental Science and Technology 51: 7128-7137.

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

Ross, M. S., A. dos Santos Pereira, J. Fennell, M. Davies, J. Johnson, L. Sliva and J. W. Martin (2012). "Quantitative and qualitative analysis of naphthenic acids in natural waters surrounding the Canadian oil sands industry." Environmental Science and Technology 46: 12796-12805.

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

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

Rowland, S. J., C. E. West, A. G. Scarlett, C. Ho and D. Jones (2012). "Differentiation of two industrial oil sands process-affected waters by two-dimensional gas chromatography/mass spectrometry of diamondoid acid profiles." Rapid Communications in Mass Spectrometry 26: 572-576.

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

Roy, J. W. and G. Bickerton (2010). "A proactive screening approach for detecting groundwater contaminants along urban streams at the reach-scale." Environmental Science and Technology 44: 6088-6094.

Roy, J. W., G. Bickerton, R. A. Frank, L. Grapentine and L. M. Hewitt (2016). "Assessing risks of shallow riparian groundwater quality near an oil sands tailings pond." Groundwater 54(4): 545-558.

Royal Society of Canada Expert Panel (2010). Environmental and Health Impacts of Canada's Oil Sands Industry.

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

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

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

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

Smith, D. F., T. M. Schaub, S. Kim, R. P. Rodgers, P. Rahimi, A. Teclemariam and A. G. Marshall (2008). "Characterization of acidic species in Athabasca bitumen and bitumen heavy vacuum gas oil by negative-ion ESI FT-ICR MS with and without acid-ion exchange resin prefractionation." Energy & Fuels 22: 2372-2378.

Sohrabi, V., M. S. Ross, J. W. Martin and J. F. Barker (2013). "Potential for in situ chemical oxidation of acid extractable organics in oil sands process affected groundwater." Chemosphere 93: 2698-2703.

Solomon, K. R. (1996). "Overview of recent developments in ecotoxicological risk assessment." Risk Analysis 16(5): 627-633.

Soucek, D. J., A. Dickinson and B. T. Koch (2011). “Acute and chronic toxicity to a variety of freshwater organisms.” Environmental Toxicology and Chemistry 30(8): 1906-1914.

Stanford, L. A., S. Kim, G. C. Klein, D. F. Smith, R. P. Rodgers and A. G. Marshall (2007). "Identification of water-soluble heavy crude oil organic-acids, bases, and neutrals by electrospray ionization and field desorption ionization Fourier transform ion cyclotron resonance mass spectrometry." Environmental Science and Technology 41: 2696-2702.

Swigert, J. P., C. Lee, B. C. L. Wong, R. White, A. G. Scarlett, C. E. West and S. J. Rowland (2015). "Aquatic hazard assessment of a commercial sample of naphthenic acids." Chemosphere 124: 1-9.

Sun, C., W. Shotyk, C. W. Cuss, M. W. Donner, J. Fennell, M. Javed, T. Noernberg, M. Poesch, R. Pelletier, N. Sinnatamby, T. Siddique and J. W. Martin (2017). "Characterization of naphthenic acids and other dissolved organics in natural water from the Athabasca oil sands region, Canada." Environmental Science and Technology 51(17): 9524-9532.

The Royal Society of Canada (2010). Environmental and Health Impacts of Canada's Oil Sands Industry. Ottawa.

Timoney, K. P. and P. Lee (2009). "Does the Alberta tar sands industry pollute? The scientific evidence." The Open Conservation Biology Journal 3: 65-81.

Tollefson, K. E., K. Petersen and S. J. Rowland (2012). "Toxicity of naphthenic acids and mixtures of these to fish liver cells." Environmental Science and Technology 46(5143-5150).

Traudt, E. M., J. F. Ranville and J. S. Meyer (2017). "Acute toxicity of ternary Cd-Cu-Ni and Cd-Ni-Zn mixtures to *Daphnia magna*: Dominant metal pairs change along a concentration gradient." Environmental Science and Technology 51: 4471-4481.

USEPA (1989). Risk assessment guidance for superfund Volume I, Human health evaluation manula (Part A), United States Environmental Protection Agency.

USEPA (1991). Guidelines for culturing the Japanese medaka, *Oryzias latipes*, United States Environmental Protection Agency. EPA/600/3-91/064

USEPA (2002). Methods for measuring the acute toxicity of effluents and receiving waters to freshwater and marine organisms. EPA-821-R-02-012

USEPA (2004). National recommended water quality criteria, United States Environmental Protection Agency.

Verbeek, A., W. McKay and M. MacKinnon (1993). Isolation and characterization of the acutely toxic compounds in oil sands process water from Syncrude and Suncor. Proceedings of Oil Sands - Our Petroleum Future Conference, Fine Tails Fundamentals Consortium. Edmonton, AB: F10,11-11.

Wang, J., X. Cao, Y. Huang and X. Tang (2015). "Developmental toxicity and endocrine disruption of naphthenic acids on the early life stage of zebrafish (*Danio rerio*)." Journal of Applied Toxicology 35: 1493-1501.

Wang, N., C. G. Ingersoll, C. D. Ivey, D. K. Hardesty, T. W. May, T. Augspurger, A. D. Roberts, E. van Genderen and M. C. Barnhart (2010). "Sensitivity of early life stages of freshwater mussels (*Unionidae*) to acute and chronic toxicity of lead, cadmium, and zinc in water." Environmental Toxicology and Chemistry 29(9): 2053-2063.

Wang, N., C. D. Ivey, C. G. Ingersoll, W. G. Brumbaugh, D. Alvarez, E. J. Hammer, C. R. Bauer, T. Augspurger, S. Raimondo and M. C. Barnhart (2017). “Acute sensitivity of a broad range of freshwater mussels to chemicals with different modes of toxic action.” Environmental Toxicology and Chemistry 36(3): 786-796.

Wang, Z., C. Yang, J. L. Parrott, R. A. Frank, Z. Yang, C. E. Brown, B. P. Hollebone, M. Landriault, B. Fieldhouse, Y. Liu, G. Zhang and L. M. Hewitt (2014). "Forensic source

differentiation of petrogenic, pyrogenic, and biogenic hydrocarbons in Canadian oil sands environmental samples." Journal of Hazardous Materials 271: 166-177.

WHO (2010). "IARC monographs on the evaluation of carcinogenic risks to humans. Some non-heterocyclic polycyclic aromatic hydrocarbons and some related exposures." World Health Organization International Agency for Research on Cancer 92.

Wilde, M. J. and S. J. Rowland (2015). "Structural Identification of Petroleum Acids by Conversion to Hydrocarbons and Multidimensional Gas Chromatography-Mass Spectrometry." Analytical Chemistry (in press).

Wiseman, S. B., J. C. Anderson, K. Liber and J. P. Giesy (2013). "Endocrine disruption and oxidative stress in larvae of *Chironomus dilutus* following short-term exposure to fresh or aged oil sands process-affected water." Aquatic Toxicology 142-143: 414-421.

Woodworth, A. P., R. A. Frank, B. J. McConkey and K. M. Müller (2012). "Toxic effects of oil sand naphthenic acids on the biomass accumulation of 21 potential phytoplankton remediation candidates." Ecotoxicology and Environmental Safety 86: 156-161.

Yasuda, N., N. R. Thomson and J. F. Barker (2010). "Performance evaluation of a tailings pond seepage collection system." Canadian Geotechnological Journal 47: 1305-1315.

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

Yim, J. H., K. W. Kim and S. D. Kim (2006). "Effect of hardness on acute toxicity of metal mixtures using *Daphnia magna*: Prediction of acid mine drainage toxicity." Journal of Hazardous Materials B138: 16-21.

Appendices

Appendix A

A1: Description of Analytical Chemistry Instrument Parameters

ESI-HR/MS parameters

An LTQ Orbitrap Elite (Thermo Fisher Scientific) instrument was used for ESI-HRMS analysis with a pre-defined 5-point regression of OSPW-derived organic acids at known concentrations used to determine resulting AEO concentrations. Operating in full scan negative-ion mode, the mass spectrometer ran at an m/z scan range of 100-600. Achieved resolution at m/z 120 = 240000, m/z 210 = 185000, m/z 300 = 150000, and m/z 400 = 130000, and all of the ions were in the m/z 100 to 300 range in which the resolution ranged from 240000 to 150000. The mass accuracy was <2 ppm error for all mass assignments. Operating parameters were as follows; sheath gas flow rate 25 (arbitrary units), spray voltage 2.90 kV, auxiliary gas flow rate 5 (arbitrary units), S lens RF level 67%, heater temperature 50°C, and capillary temperature 275°C. Infusion solvent used was 50:50 acetonitrile:water containing 0.1% ammonium hydroxide at a flow rate of 200 µL/min. Software used for molecular analysis was Xcalibur v 2.1 (Thermo Fisher Scientific) and Composer v 1.0.2 (Sierra Analytics, Inc.).

LC-QToF parameters

All LC-QToF/MS analyses utilized a methanol mobile phase and required that all samples were dissolved in methanol. Therefore, all water samples and EtOAc fraction (F1) aliquots were rotary evaporated and subsequently brought to just-dryness with a N_2 bath.

Samples were then brought back up to appropriate volumes in MeOH. The analysis was carried out in full scan negative ion mode (mass range 100-980) using an LCQToF 6520 (Agilent Technologies, Santa Clara, California, USA) under these conditions: Gas temp 350°C, drying gas 10 L/min, nebulizer 35 psi, VCap 3000 V, Fragmentor 130 V, Skimmer 65 V, reference mass recalibration enabled. The LC conditions were as follows: Column Poroshell 120 EC-C18, 3.0 x 50 mm 2.7 µm, Solvent A Water (0.1 % formic acid), Solvent B Methanol (0.1% formic acid), initial conditions 95% A for 2 minutes, to 100 % B at 20 minutes, hold until 30 minutes. Samples were injected with 1 µL of labelled internal standard (9-anthracene-d_9-carboxylic acid, 84.4 pg/µL and Decanoic-d_{19} acid, 390 pg/µL) .

GC-MS/MS parameters

All GC-MS/MS analyses required that samples were dissolved in toluene because the procedure utilized a toluene mobile phase. Therefore, all water samples were brought to just-dryness with a rotary evaporator and N_2. Samples were then brought back up to appropriate volumes in toluene. The analysis was carried out in electron impact (EI) full scan mode (mass range *m/z* 50-500) using a GC 7000 QQQ system (Agilent Technologies, Santa Clara, California, USA). A 1 µL injection was made into a multimode inlet at 270°C into a 30 m DB5 column (Agilent). Oven was at 90°C for 0.5 minutes, ramped to 300°C at 40°C/minutes with a 5 minute hold.

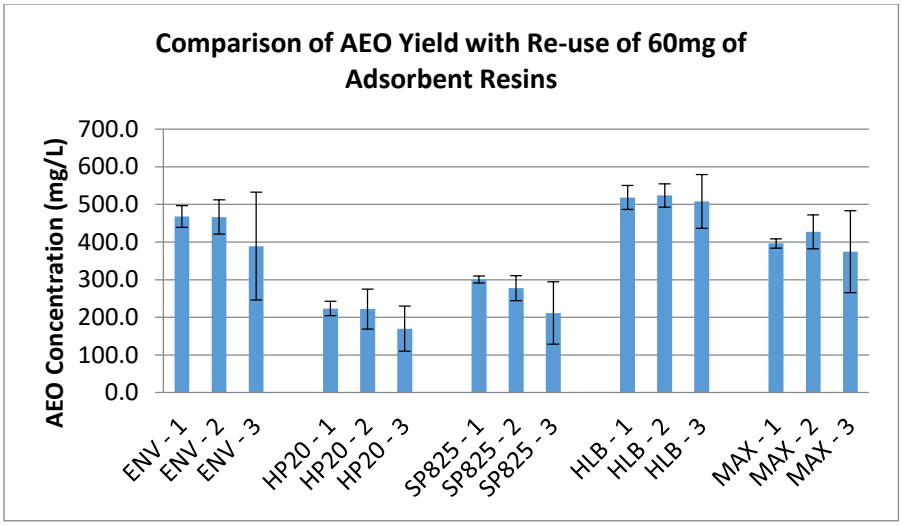
SFS parameters

Synchronous fluorescence spectra were recorded with a Perkin–Elmer Luminescence Spectrometer LS50B. Samples were filtered through a washed 0.2 µ filter (Millipore) before

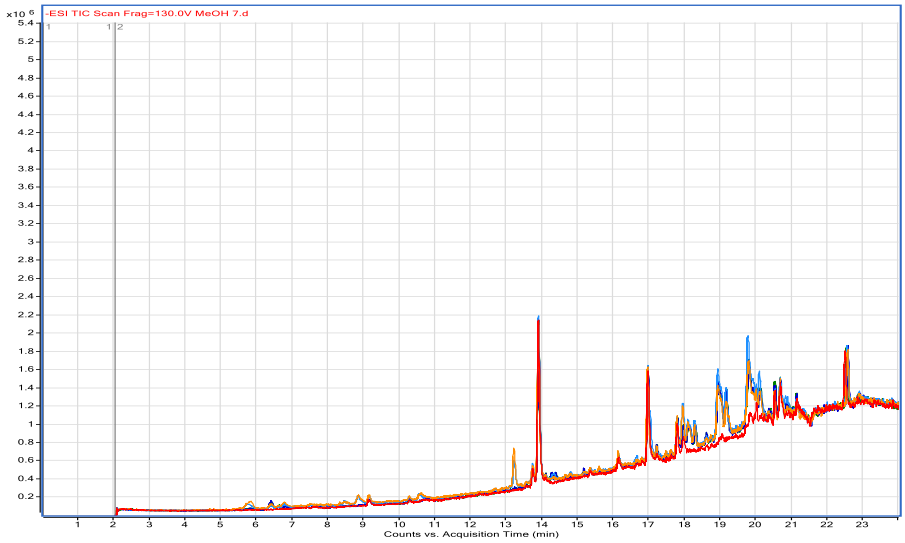
fluorescence analysis to remove particulates and were scanned in a 1 cm quartz cuvette with PTFE stopper (Hellman, Concord, ON, Canada) at 20°C. All data were collected using FL WinLab 3 software (Perkin–Elmer, Norwalk, CT). The wavelength difference between the excitation and emission monochromators (Dk) was optimized by measuring the spectra of dilute NAE at various offset values (10–60 nm). The recommended Dk of 18 nm (Miano et al., 1988; Peuravuori et al., 2002) was chosen and synchronous fluorescence spectra were collected in the 250–400 nm excitation wavelength range. Excitation and emission monochromator slit widths were set at 5 nm, scan speed at 50 nm min^{-1} and resolution at 0.5 nm. The spectra were blank corrected with Milli-Q water and then smoothed with a 5-point averaging adjacent method using Origin software ver. 7.5 (OriginLab Corp., Northampton, MA).

Metals and salts parameters

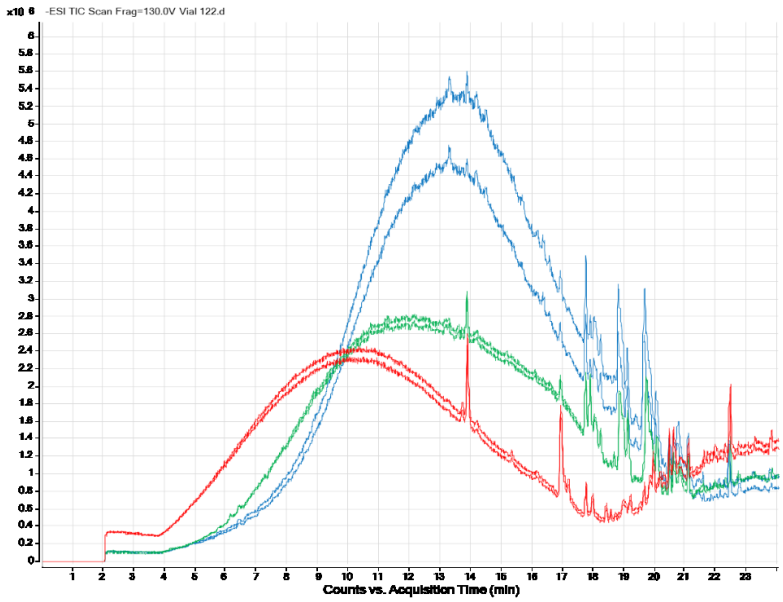
Total and dissolved metals were analyzed at Environment and Climate Change Canada's National Laboratory for Environmental Testing (NLET) (Burlington, ON) using Inductively Coupled Plasma-Sector Field Mass Spectrometry. (SOP 2003 - Standard Operating Procedure for the Analysis of Dissolved, Extractable and Total Trace Metals in Water by "Direct Aspiration" or "In Bottle Digestion" Inductively Coupled Plasma-Sector Field Mass Spectrometry (ICP-SFMS; NLET 2008). The analysis of anions was performed by ion exchange chromatography with conductivity detection (NLET Method 01-1080). The analysis of cations was performed by direct aspiration using atomic absorption (NLET Method 01-1061).



A2: AEO recovery with re-use of five different adsorbent resins from 30 mL of OSPW and 60 mg resin.



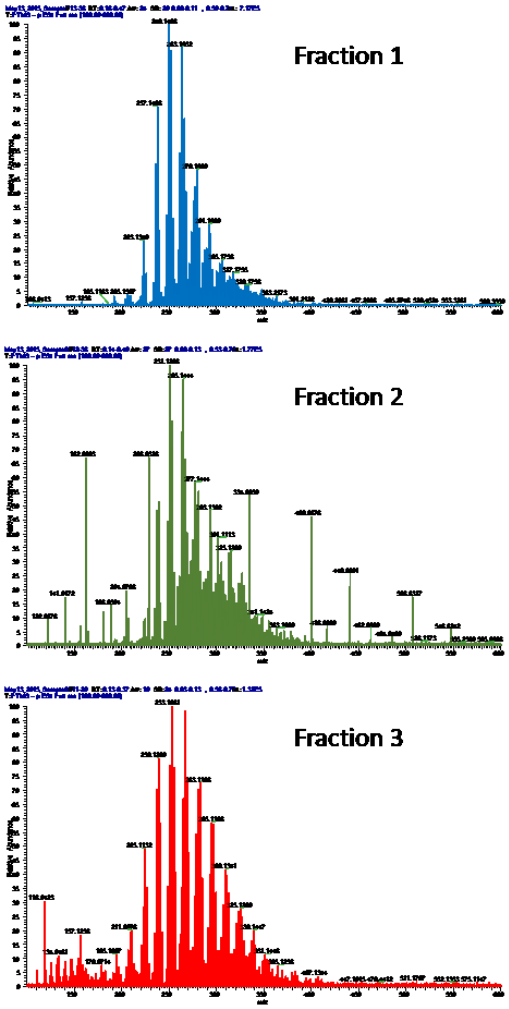
A3: LC-QToF spectra displaying full procedural method blank wherein OSPW was substituted with DI water. Fractions F1 (light blue), F2 (orange), and F3 (dark blue) show no appreciable counts about the solvent blanks (red).



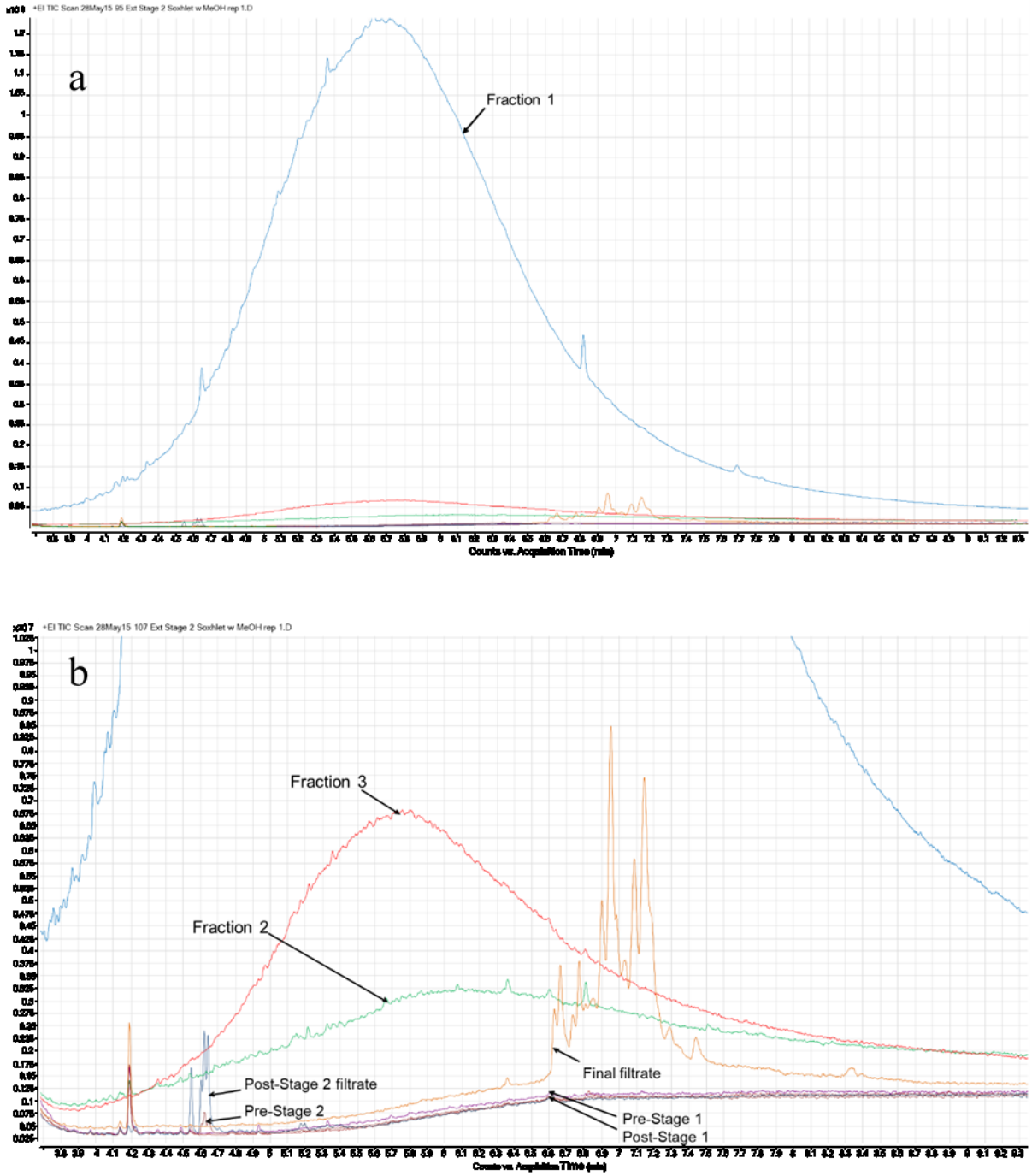
A4: Aged OSPW repeatability test displaying LC-QToF chromatograms of fractions F1 (blue), F2 (green), and F3 (red).

A5: Metals and major ions analysis of pre- and post- fraction water samples.

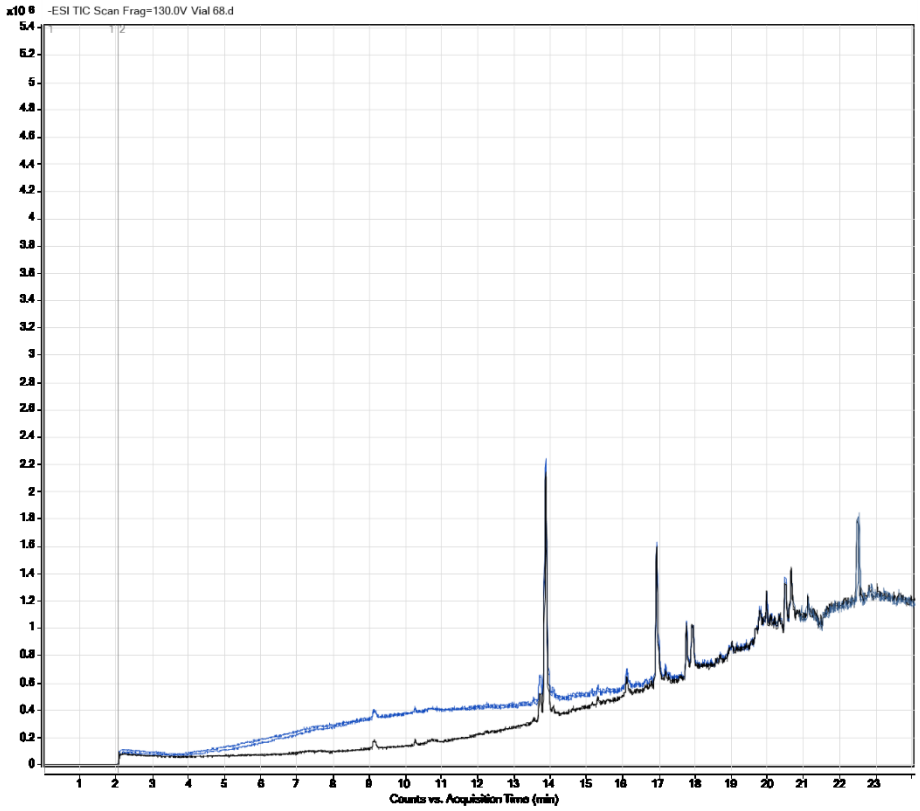
			Sample		
Metals	**unaltered**	**Pre-Stage 1**	**Stage 1 filtrate**	**Pre-Stage 2**	**Stage 2 filtrate**
Calcium	2.06	1.6	1.61	1.62	1.61
Magnesium	0.62	0.6	0.59	0.6	0.59
Sodium	12.2	18	17.6	17.6	17.4
Potassium	0.34	0.38	0.38	0.38	0.38
Silica	0.17	0.21	0.19	0.17	0.18
Major Ions					
Fluoride	0.1	0.1	0.09	0.1	0.1
Chloride	7.88	7.72	7.65	29.7	29.5
Phosphate	<0.07	<0.07	<0.07	<0.07	<0.07
Sulfate	2	2.41	2.01	1.97	1.94
Bromide	<0.07	<0.07	<0.07	<0.07	<0.07
Nitrate	0.09	0.09	0.1	0.09	0.1
Nitrite	<.006	<.006	<.006	<.006	<.006



A6: ESI-HRMS mass/charge spectra of F1-3 for aged OSPW.



A7: GC-MS/MS ion chromatograms of counts vs. acquisition time for F1 (blue), F2 (green), F3 (red), Pre-Stage 1 (purple), Post-Stage 1filtrate (dark red), Pre-Stage 2 (brown), Post-Stage 2 filtrate (dark blue), final filtrate (orange) and method blanks (black). Figure (b) is a magnified version of Figure (a).



A8: LC-QToF spectra of post-Stage 2 filtrate (dark blue) show only a slight increase in organic content compared to the method blank (black).

Appendix B

B1: Bioassay Procedures

Pimephales promelas embryo tests

Bioassays of *P. promelas* (fathead minnow) embryos were conducted at Environment and Climate Change Canada's (ECCC) Aquatic Life Research Facility (Burlington, ON) under Animal Use Protocol # 1510, approved by the Animal Care Committee (operated under the approval of the Canadian Council of Animal Care). The embryo tests were performed in environmental chambers to control temperature, light, and humidity (25 °C, 16 h light: 8 h dark, 60 % humidity). Embryos were exposed using daily static renewal methods in 24-well cell culture plates (Falcon, Becton, Dickenson and Co., New Jersey, USA), following the method of Marentette et al. (2015a).

Test solutions of OSPW or OSPW fractions were prepared 18-24 h prior to experiments starting. Fractions were prepared from stock solutions described previously and test solutions were renewed daily. Controls included water controls, as well as solvent controls for 1x fractions (1x whole water equivalents), and the Recombined treatment. Lab dilution water for the extracts was dechlorinated charcoal filtered, UV sterilized, Lake Ontario water.

Newly fertilized *P. promelas* embryos were purchased from Aquatox Laboratories (Guelph, ON). The eggs used in testing had been fertilized < 18 h before the start of the exposure. Eggs from ≥ 4 breeding groups were used to begin each replicate, with three replicate plates per treatment, 6 replicate plates for solvent controls, and at least nine replicate plates for water controls. Plates contained 24 embryos, one per 2-mL well. All embryo examinations and solution changes were performed in the 25 °C environmental chamber to ensure consistent temperatures throughout the daily manipulations. Embryos were assessed and moved to new

plates containing fresh test solutions daily. Any dead embryos were removed from plates. At 2 days post fertilization, 5 embryos per plate were removed and videotaped to count heart rates, after which they were returned to their test well. Embryos began to hatch at 4-5 days post-fertilization embryos, and time of hatch was noted for each. Hatched embryos were euthanized and abnormalities, hatch success, and length (measured on a dissecting microscope) were assessed. Abnormalities assessed at hatch included edemas and circulatory problems (necrosis, cardiac edema, yolk edema, bubbles under skin, hemorrhages, and others such as tube heart), craniofacial abnormalities (small face, eye edema, or other jaw deformities), and spinal abnormalities (lordosis or "belly out", kyphosis or "belly in", scoliosis or "bent to the side", bent tail fin, or others).

Oryzias latipes tests

A breeding culture of *O. latipes* (Japanese medaka) were established to provide the embryo necessary for toxicity testing following conditions outlined in USEPA (United States Environmental Protection Agency) protocols (USEPA, 1991). Sexed *O. latipes* adults were purchased from Aquatic Research Organisms in Hampton, New Hampshire, and held in the wetlab facility on the University of Waterloo campus. All culturing procedures were approved by the institutional animal review board and Animal Care Committee at the University of Waterloo (UW-ACC) under Animal Use Protocol #14-08.

Embryos were collected from females the morning of test initiation by gently removing them from the oviduct with blunt forceps. Embryos were then examined under a dissecting microscope and unfertilized embryos were removed. Medaka embryos at the late blastula stage were exposed to a water control, solvent control, positive control, whole Aged OSPW water,

three Aged OSPW fractions (F1 – F3), and the Recombined treatment. Culture water was used for control and to prepare solvent control, positive control and fractions. Fractions, Recombined treatment, and solvent control were prepared from stock solutions as described previously. The positive control consisted of 4 mg/L 3,4-dichloroaniline dissolved in control water. All treatment stocks were adjusted to pH 8.0 ± 0.1 and stored in amber glass bottles in the dark. Each treatment consisted of 3 replicates with 10 individuals per replicate. Embryo were placed in Falcon™ 6-well tissue culture plates and filled with 10 mL of respective treatment, which was renewed daily. Water quality parameters including dissolved oxygen, conductivity, and temperature were checked daily, and pH was adjusted if necessary. Tissue culture plates were placed on a shaker set to 100 rpm in order to synchronize and shorten hatch time (Farwell et al., 2006). The shaker was kept in a growth chamber (Conviron®, Winnipeg, MB) with temperature held at 27 ± 1°C and the photoperiod was held at 16 light: 8 dark. Assessments of hatch success, time-to-hatch, hatch length, and abnormality endpoints were made twice daily and tests were terminated upon hatch (≤10 days post-fertilization). Following inspections any dead embryo were removed. Hatch length and abnormality measurements were performed using a light microscope. Abnormalities assessed included pericardial and yolk sac edema, cranio-facial abnormalities, tube heart, hemorrhages, fin erosion, and spinal curvature.

Vibrio fischeri (Microtox®)

The Microtox® assay assesses the effect of toxicants on the bioluminescence (as an indicator of survival/viability) of the marine bacterium *Vibrio fischeri*, which was adapted from Environment Canada protocols (Environment Canada, 1992). The deviation from the standard protocol, which analyzes a serial dilution of the test mixture and results in a generated IC50: the

inhibitory concentration at which 50% in luminescence of bacteria is reduced (Azur Environmental, 1995), can be utilized to allow for time- and cost-effective screening of large sample sets (Anderson et al., 2015). In brief, *V. fischeri* bioluminescence was measured using a Microbics M500 Analyzer, before and after exposure to the aged OSPW and the isolated fractions. *V. fischeri* bioluminescence was measured in triplicate at 81% original concentration for the aged OSPW (required addition of osmotic adjusting solution to attain 2% salinity) and at 3x whole water equivalent for F1-3 as well as the Recombined treatment (fraction salinity at 2%). *V. fischeri* bioluminescence following 15 min of exposure to each test solution was compared to the control and is presented as the mean percent of control.

Daphnia magna tests

Daphnia magna culturing and test parameters were adapted from Environment Canada and Ontario Ministry of the Environment (MOE) protocols (Environment Canada, 1996; MOE, 2014). 48h acute lethality assays were conducted with seven treatments per site: a water control, solvent control, unaltered Aged OSPW, F1-3, and a Recombined fraction treatment. Fractions, Recombined treatments, and solvent controls were prepared as described previously for bioassay treatment preparation.

Each test was conducted twice with 3 replicates for controls and 2 replicates for the whole water, F1-3, and Recombined fraction test solutions (e.g. a total of 6 replicates for controls and 4 replicates for whole water and fractions). For each test vessel, 10 *D. magna* neonates, <24 h old, were placed into each 250-mL beaker containing 150 mL of control or treatment solution. After 48 h, the number of living *Daphnia*, defined as visibly mobile, and water quality parameters were recorded. Temperature was maintained at 20° ± 2° C, pH was maintained at 8.0 ±0.2, and

light intensity was maintained at 400-800 lux with a photoperiod of 16 h light: 8 h dark. Control/dilution water consisted of aerated, dechlorinated municipal water which was left to settle for >24 hours. Dissolved oxygen content of control water prior to test initiation was 8.3 mg/L.

Lampsilis siliquoidea and Lampsilis cardium (glochidia) tests

Gravid freshwater mussels (*Lampsilis siliquoidea*, fatmucket clam; *Lampsilis cardium*, plain pocketbook) were collected from a reference site (43.71775, -81.12662) and held at ECCC's Aquatic Life Research Facility (Burlington, ON) in a flow-through system with dechlorinated Lake Ontario water at 12 ± 2ºC (to prevent the glochidia release). Glochidia for toxicity tests were collected by flushing the marsupia (i.e., brooding chambers) with a water-filled syringe.

Acute toxicity tests with glochidia were modeled after the American Society of Testing Materials (ASTM) method for conducting toxicity tests with early life stages of freshwater mussels (ASTM, 2006) and have been described in detail (Gillis, 2011). In order to parasitize a fish, glochidia must be able to close their valves and clamp down on a fish's gill and encyst. Glochidia viability (i.e., the ability to close valves) was assessed after 24 h in a sub-sample (~100) of the exposed glochidia (500-1000) through the addition of a saturated salt solution (NaCl 240 g/L). Viability was calculated using the following equation:

Percent Viability = 100 x (Number of closed glochidia after addition of NaCl – Number of closed glochidia before addition of NaCl) / (Number of closed glochidia after addition of NaCl + Number of open glochidia after addition of NaCl).

Results are expressed as effective median concentrations (EC50) rather than median lethal concentrations (LC50), but as they are obligatory parasites, for practical purposes non-viable

glochidia should be considered ‘dead’ because they would be unable to attach to a host and complete their life cycle. As per the ASTM method, glochidia were pooled from three or four gravid females that exhibited >90% glochidia viability.

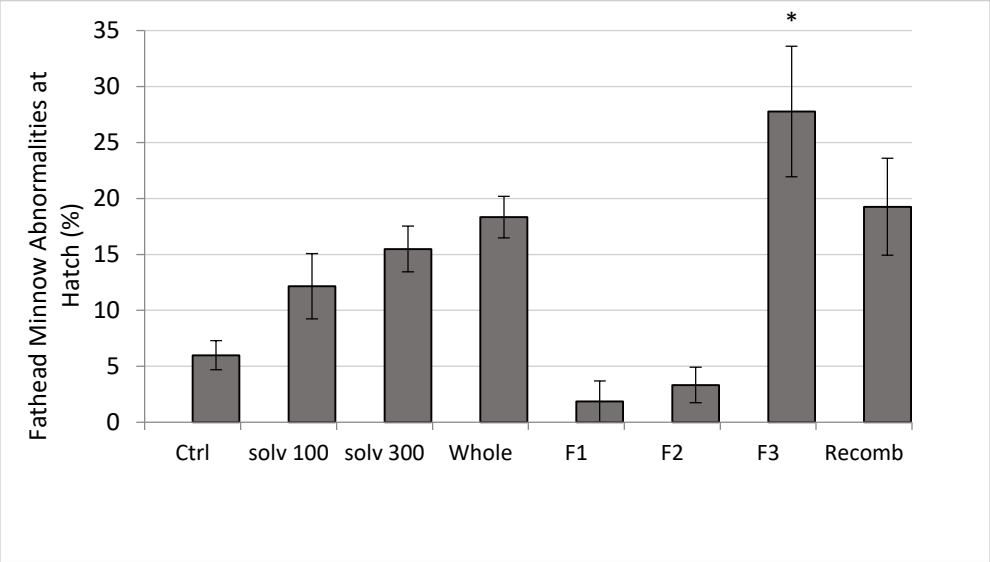
Because glochidia have a heightened sensitivity to some contaminants, in addition to a moderately-hard reconstituted water control (Gillis et al., 2008; Gillis, 2011), solvent controls at 0.1 and 0.3% methanol were included in each test to represent the concentration of solvent in a single fraction exposure and a Recombined (all three fractions) treatment, respectively. Five control replicates and four replicates for remaining treatments were conducted. Tests were conducted in 250-mL glass beakers, under a 16 h light: 8 h dark cycle at 21 ± 2ºC. Dissolved oxygen, pH, and conductivity were measured at the beginning and end of each test. Dissolved organic carbon (DOC), water hardness, and major ions concentrations (Na, K, Ca, Cl, SO_4) were assessed at the end of each test (t=48 h) in one composite sample per treatment. Water analysis (major ions, DOC, hardness) was conducted by the Canadian Association for Environmental Analytical Laboratories accredited NLET (Burlington, ON, Canada) following ECCC standard operating procedures.

Hyalella azteca tests

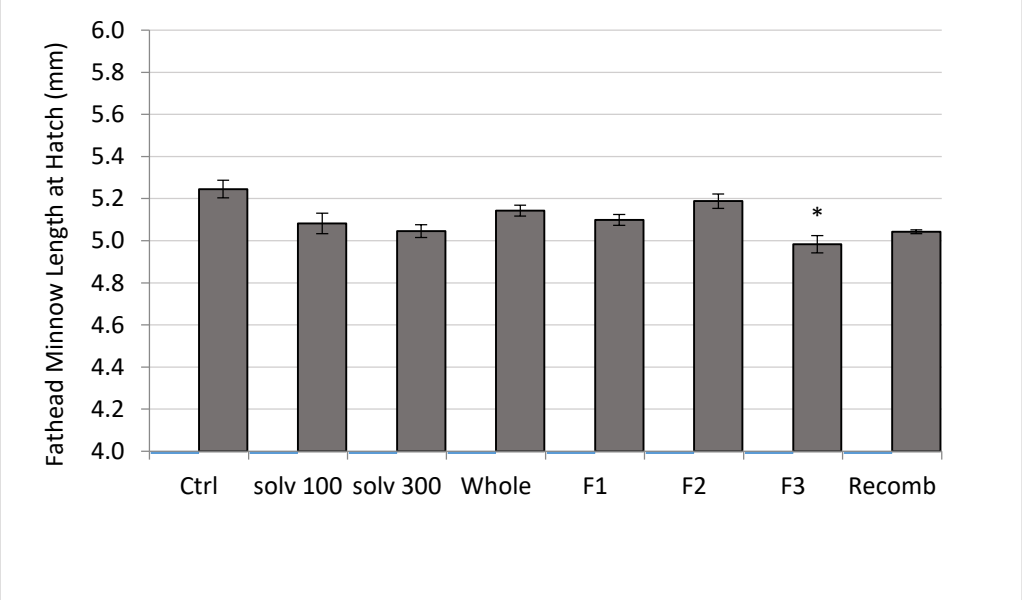
Culturing methods for *Hyalella azteca* (freshwater amphipod crustacean) have been described in detail (Borgmann et al., 1989). Both cultures and tests were maintained at 25 °C ±1 with a photoperiod of 16 h light: 8 h dark, and amphipods were fed finely ground Tetra-Min fish food flakes (Tetra GMBH, Melle, Germany). Juvenile *H. azteca* were removed from breeding containers weekly, and were 2-9 d old at initiation of tests.

One-week, static, water-only tests were conducted with aged OSPW, F1-3, and Recombined treatments. An initial set of tests was conducted with six OSPW dilutions ranging from 2.5-100%. Based on these results, additional tests were conducted with fractions of the OSPW at 1x whole water-equivalent concentrations, both as individual and Recombined fractions. Standard artificial media, a five-salt solution routinely used to culture amphipods (Borgmann, 1996), was used in all exposures as negative controls and to prepare solvent controls and test solutions. Fifteen juvenile *H. azteca* were added to 250-mL glass beakers containing 200 mL of test solution, 2.5 mg Tetra-Min, and one piece of cotton gauze (2.5 cm^2). Each test consisted of negative controls (3 replicates), solvent controls (for fraction exposures only, 3 replicates), and OSPW solutions (whole water and fractions, 2 replicates each). Each exposure was conducted twice (i.e., a total of 6 replicates for controls and 4 replicates for whole water and fractions). Water quality (dissolved oxygen, pH, conductivity, chloride, and total ammonia (NH_3/NH_4^+)) was measured at the beginning and end of each test. *H. azteca* individuals were removed from each beaker at the end of the 7-d test, and the number of surviving animals was recorded. Mean survival of controls in all tests was 93-100%, exceeding the recommended performance criteria of 90% for 96-h water-only tests (Environment Canada, 2013).

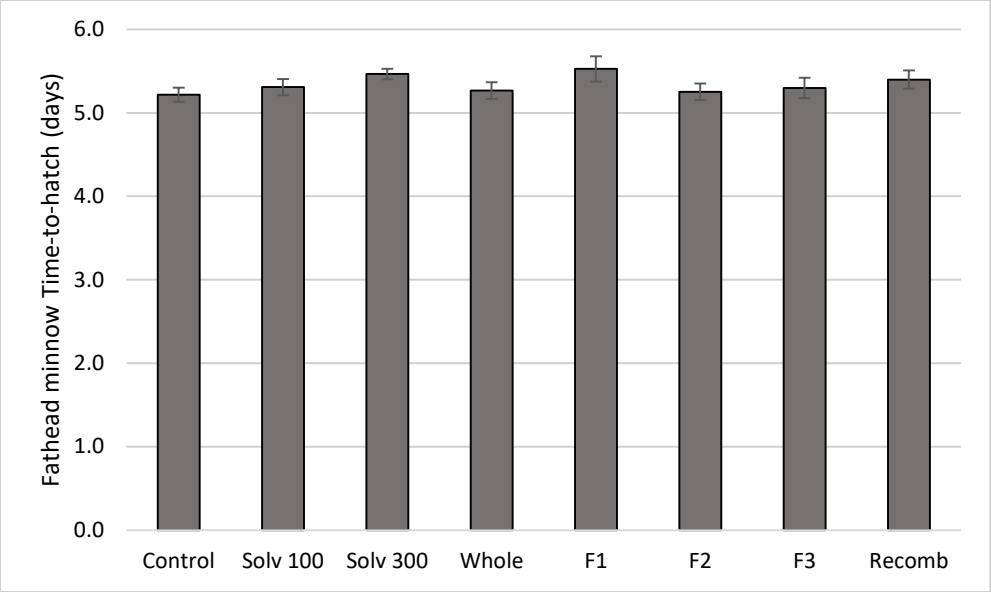
Fathead minnow sub-lethal endpoints



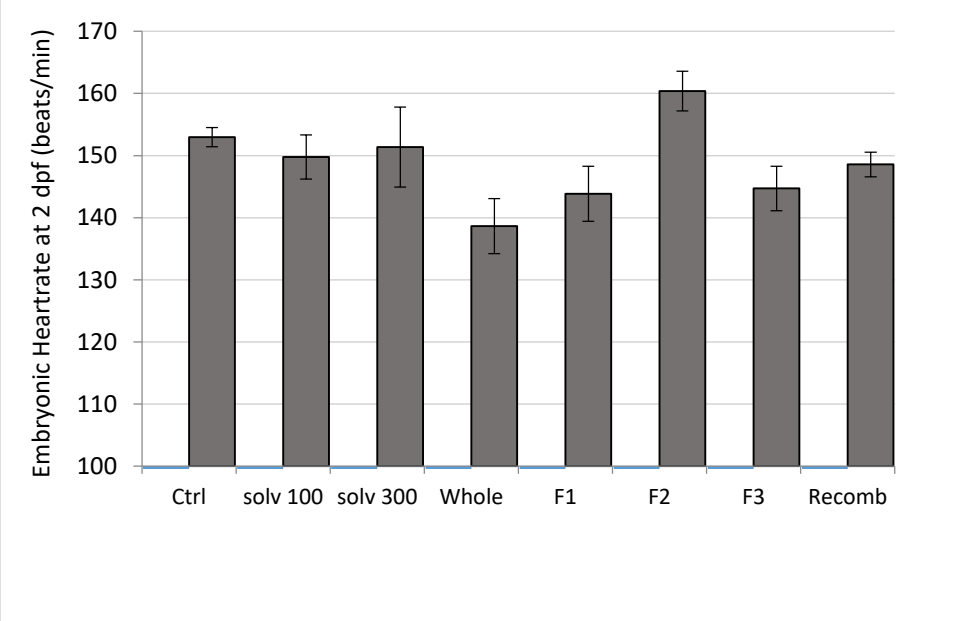
B1: Effects of aged OSPW whole water and fractions on fathead minnow percent abnormalities endpoint. Bars represent mean effect ± standard error. Asterisk (*) denotes treatments that are significantly different from pooled controls.



B2: Effects of aged OSPW whole water and fractions on fathead minnow length at hatch endpoint. Error bars represent standard error. Asterisk (*) denotes treatments that are significantly different from pooled controls.

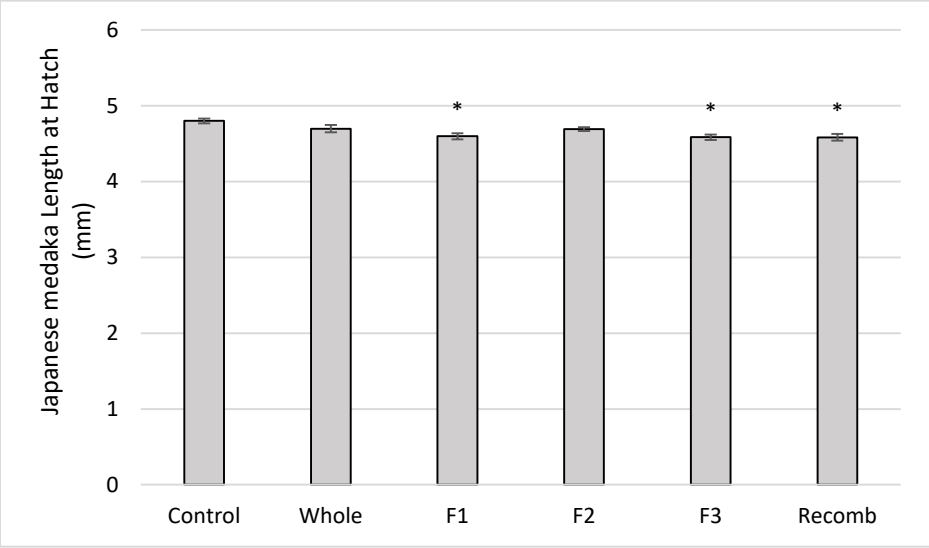


B3: Effects of aged OSPW whole water and fractions on fathead minnow time-to-hatch endpoint. Bars represent mean effect ± standard error.

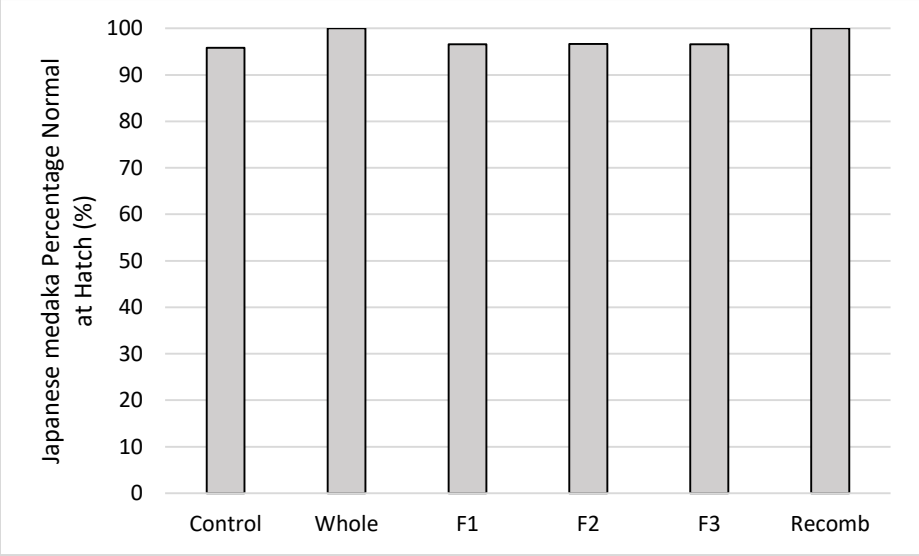


B4: Effects of aged OSPW whole water and fractions on fathead minnow embryonic heartrate endpoint at two days post-fertilization. Bars represent mean effect ± standard error. Asterisk (*) denotes treatments that are significantly different from pooled controls.

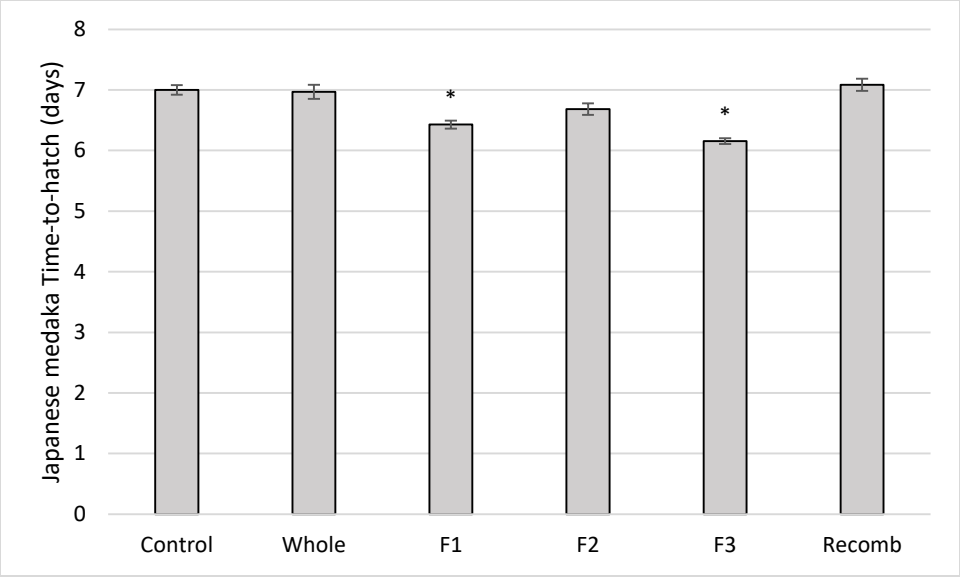
Japanese medaka sub-lethal endpoints



B5: Effects of aged OSPW whole water and fractions on Japanese medaka length at hatch endpoint. Bars represent mean effect ± standard error. Asterisk (*) denotes treatments that are significantly different from pooled controls.



B6: Effects of aged OSPW whole water and fractions on pooled percentage normal Japanese medaka at hatch.



B7: Effects of aged OSPW whole water and fractions on Japanese medaka time-to-hatch endpoint. Bars represent mean effect ± standard error. Asterisk (*) denotes treatments that are significantly different from pooled controls.

Appendix C

C1: Class distribution of whole water and dissolved organic fractions in sites DP-1, DP-2, DP-4, and DP-5 as determined by ESI-HRMS. Graphs present ion classes (x-axis) versus percent relative abundance of ions (y-axis).
Duplicate figure from Chapter 4 (Figure 4.4)