Characterizing the toxicity of oil sands process-affected water

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Abstract

Oil sands process-affected water (OSPW) is produced during extraction of bitumen in the surface-mining oil sands industry in Alberta, Canada. Due to observed acute and chronic toxicity of the dissolved organic fraction of OSPW, it is currently stored in tailings ponds to be remediated and eventually returned to the surrounding environment. To this end, endpit lakes are a proposed passive remediation strategy for OSPW related toxicity, of which BaseMine Lake (BML) is the first full scale commercial test. The purpose of this thesis was to identify and characterize toxic chemical classes in the dissolved organic fraction of OSPW collected from BML to aide the development of water quality objectives and release guidelines for the oil sands industry. Using a bioassay-effect directed analysis approach and ultrahigh resolution mass spectrometry, the dissolved organic fraction of OSPW was iteratively fractionated and biologically tested to identify acutely toxic chemical classes. This work was further built upon by the development of a predictive acute aquatic toxicity model for embryos of fathead minnow (*Pimephales promelas*) exposed to OSPW related chemicals to support the development of release guidelines in the oil sands. To investigate potential mechanisms of acute toxicity of dissolved organic chemicals in OSPW, an *Escherichia coli* whole genome reporter system was used and provided evidence to support narcosis as the critical mechanism of OSPW related acute toxicity. Furthermore, the fathead minnow short term 21-day reproductive bioassay was used to investigate the potential for OSPW related chemicals to disrupt reproduction.

Results of this thesis support earlier works and confirmed a role for naphthenic acids in the acute toxicity of OSPW. However, novel acutely toxic chemical classes containing oxygen, sulphur or nitrogen were also identified. An acute aquatic toxicity model was developed which is sufficiently robust to predict toxicity of dissolved organic chemicals in OSPW within biological variation associated with toxicity tests and provides an alternative to *in vivo* test methods typically applied to complex environmental mixtures. In addition, hepatosomatic index was identified as an indicator of chronic exposure of male fathead minnow to OSPW. Overall, this work provides important toxicity and chemical information that will aide in the development of reclamation and monitoring programs in the oil sands region of Alberta, Canada.

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17-β estradiol (E2)
Acid extractable fraction (F1-AE)
Acid extractable fraction blank (b-F1-AE)
Adenosine triphosphate (ATP)
Analysis of variance (ANOVA)
Androgen receptor (*ar*)
Anhydrous ethanol (EtOH)
Aquatic species specific Critical body burden (*b*)
Aromatase (*cyp19a*)
Atmospheric pressure chemical ionization (APCI)
Barrels per day (bpd)
Base extractable (F1-BE)
Base extractable blank (b-F1-BE)
BaseMine Lake (BML)
Billion barrels of oil (bbl)
Bioassay-effect directed analysis (EDA)
Calculated toxic unit for sample *j* (TUm_j)
Calculated toxic unit for species *i* of sample *j* ($TU_{i,j}$)
Canadian Council of Ministers of the Environment (CCME)
Canadian Water Quality Guidelines (CWQG)
Centimetre (cm)
Chemical class specific correction factor (Δc)
Chironomus dilutus (*C. dilutus*)
Complimentary DNA (cDNA)
Compound-specific bioaccumulation potential (BP_i)
Concentration equivalent to the twenty-percent inhibition of growth (IC20)
Concentration required to reduce luminescence by 50% (IC50)
Concentrations required to reduce survival by fifty- percent (LC50)
Condition factor (K)
Confidence interval (CI)
Critical body burden (C_{bb})
Cubed metres (m^3)
Cumulative number of eggs produced per female (Cumulative no. eggs/female)
Degrees Celsius (^oC)
Deoxyribonucleic acid (DNA)
Dichloromethane (DCM)
Double bond equivalent (DBE)

Early-life stages (ELS)
Endpit lake (EPL)
Escherichia coli (*E. coli*)
Estrogen receptor alpha (*er*α)
Estrogen receptor beta (*erß*)
Fathead minnow (FHM)
Fluoride-ion chemical ionization mass spectrometry (FIC-MS)
Fold (×)
Fourier transform infrared spectroscopy (FTIR)
Fructose-6-phosphate (F6P)
Gene encoding for phospholipase A (*pldA*)
Gene encoding for the alcohol/acetaldehyde dehydrogenase *adhE* (*adhE*)
Gene encoding for the cAMP-activated global transcriptional regulator *crp* (*crp*)
Gene encoding for the chaperone protein *clpB* (*clpB*)
Gene encoding for the C-reactive protein *crp* (*crp*)
Gene encoding for the DNA-binding transcriptional regulator *bolA* (*bolA*)
Gene encoding for the enzyme octaprenyl diphosphate synthase *ispB* (*ispB*)
Gene encoding for the enzyme phosphoglucose isomerase *pgi* (*pgi*)
Gene encoding for the enzyme Trans-aldolase B (*talB*)
Gene encoding for the oxido-redutase enzyme *ykgE* (*ykgE*)
Gene encoding for the putative biofilm regulator protein *yceP* (*yceP*)
Gene encoding for the surface protein *ybjE* (*ybjE*)
Gene encoding for the transcriptional regulator *bolA* (*bolA*)
Gene encoding for β-galactosidase *lacZ* (*lacZ*)
Gene ontology (GO)
Gene Ontology (GO)
Glucose-6-phosphate (G6P)
Gonadosomatic index (GSI)
Grams (g)
Gravimetric mass of organics in sample *j* (Mo_j)
Green fluorescent protein (GFP)
Hepatosomatic index (HSI)
Hierarchical cluster analysis (HCA)
High performance liquid chromatography (HPLC)
Hour (hr)
Hydrochloric acid (HCl)
Intensity of species *i* in sample *j* ($I_{i,j}$)
Kyoto Encyclopedia of Genes and Genomes (KEGG)
LC50 of a species *i* ($LC50_i$)
Linear free energy relationship (LFER)

Litre (L)
Mass to charge ratio (m/z)
Mean absolute deviation (MAD)
Methanol (MeOH)
Microgram per mililitre (ug/mL)
Microlitre (μL)
Microlitre (uL)
Micrometre (μm)
Micromole per gram (μmol/g)
Mililitre (mL)
Milimetre (mm)
Milimol per litre (mmol/L)
milligram per litre (mg/L)
Minute (min)
Molecular mass of species *i* in sample *j* ($MM_{i,j}$)
Nanogram per litre (ng/L)
Nanometer (nm)
Naphthenic acid fraction components (NAFC)
Naphthenic acids (NAs)
Negative electrospray ionization carboxylic acid species (O_2^-)
Negative ion fast atom bombardment- mass spectrometry (FAB-MS)
Neutral extractable fraction (F1-NE)
Neutral extractable fraction 1 (F2-NE1)
Neutral extractable fraction 1 blank (b-F2-NE1)
Neutral extractable fraction 2 (F2-NE2)
Neutral extractable fraction 2 blank (b-F2-NE2)
Neutral extractable fraction 2 early eluting fraction (F3-NE2a)
Neutral extractable fraction 2 early eluting fraction blank (b-F3-NE2a)
Neutral extractable fraction 2 late eluting fraction (F3-NE2b)
Neutral extractable fraction 2 late eluting fraction blank (b-F3-NE2b)
Neutral extractable fraction blank (b-F1-NE)
Nicotinamide adenine dinucleotide phosphate (NADP+)
Nitrogen (N)
Octanol-water partition coefficient (K_{OW})
Oil sands process-affected waters (OSPW)
Optical density (OD)
pH dependent octanol-water distribution ratio (D_{OW})
Phospholipid membrane-water distribution ratios (D_{MW})
Poly(dimethyl)siloxane (PDMS)
Polycyclic aromatic hydrocarbons (PAH)

Pooled primary fractions (F1-Pool)
Pooled secondary fractions (F2-Pool)
Pooled tertiary fractions (F3-Pool)
Positive/ negative electrospray ionization (ESI +/-)
Primary fractions (F1)
Principal component analysis (PCA)
Quantitative real-time polymerase chain reaction (qPCR)
Reactive oxygen species (ROS)
Relative intensity of species *i* of sample *j* ($RI_{i,j}$)
Ribonucleic acid (RNA)
Root-mean square deviation (RMSD)
Secondary fractions (F2)
Sodium hydroxide (NaOH)
Solid phase extraction (SPE)
Solid-supported lipid membrane (SSLM)
Square kilometres (km^2)
Standard error mean (SEM)
Stir-bar sorptive extraction (SBSE)
Sulfuric acid (H_2SO_4)
Sulphur (S)
Target Lipid Model (TLM)
Tertiary fractions (F3)
Testosterone (T)
Time to hatch (TTH)
Toxic unit (TU)
Ultrahigh resolution mass spectrometry (uHRMS)
Unit volume/ unit volume (v/v)
Universal narcosis slope (*m*)
Vitellogenin 5 (*vtg5*)
Water concentration of species *i* in sample *j* ($Cw_{i,j}$)
Water quality based effluent limits (WQBEL)
Water quality guideline (WQG)
West In-pit (WIP)

Note to readers

This thesis is organized and formatted to follow the University of Saskatchewan College of Graduate and Postdoctoral Studies guidelines for a manuscript-style thesis. Chapter 1 is a general introduction and literature review, including project goals and objectives. Chapter 6 contains a general discussion and overall conclusions. Chapters 2, 3, 4 and 5 of this thesis are organized as manuscripts for publication in peer-reviewed scientific journals. Chapters 2 and 3 were published in the journal, Environmental Science & Technology, Chapter 4 was published in the journal Chemosphere, and Chapter 5 is in preparation for submission and publication. Full citations for the research papers and a description of author contributions are provided following the preface of each chapter. As a result of the manuscript style format, there is some repetition of material in the introduction and material and methods sections of the thesis. The tables, figures, appendices, and references cited in each chapter have been reformatted here to a consistent thesis style. References cited in each chapter are presented at the of the thesis. Appendices are presented at the end of this thesis.

Chapter 1: Introduction

1.1 Preface

Chapter 1 is a general introduction and literature review regarding the oil sands, associated environmental issues, chemicals of concern, their toxicities, detection and identification. Chapter 1 also includes the overall goals and objectives of the project, and includes null hypotheses.

1.2 Athabasca oil sands

Encompassing 141, 000 square kilometres (km^2) of boreal forest and peat bog in northern Alberta, the oil sands are the world's third largest proven reserve of crude oil.[1,2] It is estimated that 165.4 billion barrels (bbl) of oil are recoverable given current technologies and economic conditions.[1] Producing in excess of 2.5 million barrels per day (bpd) (2016), receiving approximately $16.6 billion in investments and as the largest source of resource royalties in Alberta (2016), the upstream energy sector in Alberta is economically important to both the province and the nation.[2,3] Predominantly in the form of bitumen, oil in the oil sands region is considered an unconventional source. Bitumen is a highly viscous mixture of hydrocarbons that differs from conventional petroleum sources as it does not naturally flow and requires manipulation before transportation.[3] The Athabasca region is the largest and most developed deposit of oil sands, covering 40, 000 km^2 and containing an estimated 65 bbl of extractable oil. In this region, open-pit mining is the predominant method of bitumen extraction. This method however, requires a great amount of energy, removes large tracts of land, and produces great volumes of process affected materials.

As production is projected to continue growing, the oil sands are expected to be the primary source of crude oil in Canada.[2] As Canadian oil production continues to transition to more energy intensive methods of extraction, investment and development will continue in Alberta. Given concerns about potential effects of mining activities on the natural environment, including freshwater, the development of water quality guidelines (WQG), environmental quality benchmarks, and an understanding of the potential human and environmental health effects of this industry is integral to the risk assessment and management processes.[4]

1.3 Oil sands process-affected waters

Following removal by open-pit mining, bitumen laden sands are transported to refineries where separation from sand, clay and other constituents is completed.[2] By use of the Clark hot water extraction method a combination of water, caustic sodas, heat and separation vessels are used to separate the bitumen from other components. Bitumen extracted by use of this method is ultimately used for the production of crude oil and other petroleum products. However, this process produces large volumes of process-affected materials. The resulting materials are pumped to holding ponds where sufficient time is allowed for particulate matter to settle-out, producing two distinct waste media; solid mature fine tailings and oil sands process-affected water (OSPW). In 2013 there were approximately 976 million cubic metres (m^3) of fluid tailings contained in tailings ponds with a net cumulative footprint of approximately 220 km^2 including all structures (i.e. dykes, berms, beaches and in-pit ponds).[3]

1.4 Toxicity of OSPW

Oil sands process-affected water that is produced as a by-product of the open-pit mining extraction process is a complex mixture and is acutely toxic to aquatic organisms. Early-life stages (ELS) of fathead minnow (*Pimephales promelas*) exposed in-lab to fresh OSPW from the West In-pit (WIP) tailings pond exhibited lesser survival, premature hatch, and greater incidence of malformations when compared to controls.[5] When exposed to mature fine tailings, ELS of fathead minnow presented similar toxic responses, including decreased survival, greater prevalence of malformations and lesser body size and hatching success.[6] Early-life stages of yellow perch (*Perca flavescens*) and japanese medaka (*Oryzias latipes*) exposed to OSPW from the Mildred Lake settling basin exhibited similar responses as fathead minnow, including greater incidence of deformities and lesser length at hatch.[7] Oil sands process-affected water is also acutely toxic to the invertebrate *Chironomus dilutus* as shown by the lesser growth and survival of larvae exposed to WIP-OSPW.[7] Toxicity of OSPW and other process affected materials is well established in aquatic organisms, yet their mechanisms of action and the causative agents of toxicity of OSPW have not been well characterized.

There is little knowledge of the mechanism(s) of adverse effects of OSPW to aquatic organisms. It has been suggested that the mechanism of acute lethality is narcosis.[8,9] By use of

ribonucleic acid (RNA) sequencing Wiseman *et al.*,[10] demonstrated the up- and down regulation of transcripts in male fathead minnows exposed to WIP-OSPW were indicative of potential mechanisms of toxicity such as oxidative stress, apoptosis, and suppressed immune function. These results were consistent with those found by He *et al.*,[4] where embryos of fathead minnow exposed to OSPW had greater abundances of transcripts of several genes related to oxidative stress and apoptosis, and greater concentrations of reactive oxygen species (ROS) which may contribute to oxidative stress as a potential mechanism of toxicity. By use of hepatocytes from rainbow trout, Gagne *et al.*,[11] also reported greater abundances of transcripts related to oxidative stress. Exposure of larvae of the invertebrate *C. dilutus* to WIP- OSPW supports a role for oxidative stress in toxicity as abundances of transcripts of genes involved in the response to oxidative stress, and greater peroxidation of lipids were found when compared to control.[12]

In addition to nonspecific effects on the production of oxygen radicals that might lead to oxidative stress, there is evidence from *in vitro* and *in vivo* studies that OSPW affects the reproductive endocrine system. Using the human breast cancer cell linesT47D-kbluc and MDA-kb2, it has been demonstrated that agonists of the estrogen receptor and antagonists of the androgen receptor are present in the dissolved organic phase of WIP-OSPW.[13] Exposure of the human adrenocortical carcinoma cell line H295R to WIP-OSPW significantly decreased the synthesis of the sex steroid testosterone and increased concentrations of 17β-estradiol.[13] Using fathead minnow, He *et al*.,[14] further investigated the mechanism of estrogenic effects of WIP-OSPW and found that exposure resulted in altered abundance of transcripts at all levels of the brain-gonad-liver axis in both male and female fathead minnows. Other studies have found evidence of endocrine disruption in exposed yellow perch, fathead minnow and *C. dilutus*.[15–17] Exposure to OSPW has also been shown to adversely affect reproduction of fathead minnow, by altering sex steroid synthesis, and resulting in less pronounced secondary sexual characteristics of both male and female exposed minnows.[18,19] The critical mechanism(s) of toxicity of OSPW is currently unknown, however oxidative stress and effects on steroid synthesis cannot be ruled out.

1.5 Chemistry of OSPW

One of the great challenges of research in OSPW is characterizing the organic constituents of this complex mixture. It has been estimated that there are greater than 200,000 organic chemicals in OSPW, but traditional methods in mass spectrometry are not sensitive enough to allow for identification of each of these chemicals.[20,21] Naphthenic acids (NAs) are a constituent of nearly all crude oils,[22] have inherent corrosive properties, are acutely toxic,[23,24] and are present at great concentrations in OSPW.[25] This group of compounds has classically been defined as "a group of naturally occurring acyclic, monocyclic and polycyclic carboxylic acids with the general formula of $C_nH_{2n}+zO_2$, where n represents the number of carbons and Z is zero or a negative even integer related to the number of rings in the molecule" (Figure 1.1).[22] It has long been reported that NAs are the primary causative agents of acute toxicity due to their elevated concentrations and acute toxicity.[8,24–27] Due to their inherent toxicity and prevalence in OSPW the characterization and identification of NAs has been the focus of intensive research.[21,28,29]

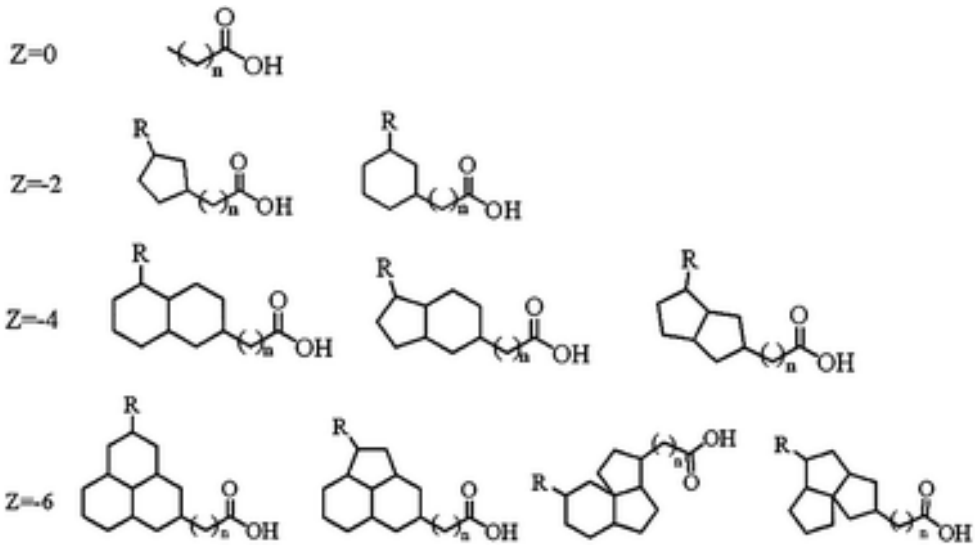

Figure 1.1 Example structures of naphthenic acids for various Z-families. R represents an alkyl group, m represents the length of the alkyl chain, and z series correspond to different ring numbers in the acids. Taken from Hao et al.[20]

Naphthenic acids are present as a mixture of which classic analytical techniques are unable to accurately identify or quantify individual constituents. Total NA have been quantified using fourier transform infrared (FTIR) spectroscopy and the carbon number distribution of the mixture was first demonstrated using fluoride-ion chemical ionization and negative ion fast atom bombardment- mass spectrometry (FIC, FAB-MS).[21,30] This work highlighted the usefulness of profiles of NAs for constructing chemical fingerprints to identify sources of NA. Improvements to this method were introduced as electron ionization emerged, offering a simpler alternative with no derivitization steps.[20,23] The application of atmospheric pressure chemical ionization (APCI) and positive/ negative soft ionization methods such as electrospray ionisation (ESI +/-) to the analysis of samples of OSPW revealed distinct chemical profiles between ESI + and ESI -. With the application of high performance liquid chromatography (HPLC) and ultrahigh resolution mass spectrometry (uHRMS), work such as that by Pereira *et al.,*[29] revealed that in addition to the "traditional NA", detected by use of ESI- as carboxylic acid containing chemical species (O_2^-), the acid extractable fraction of OSPW contained other oxygen containing species such as dihydroxy, diketo, and ketohydroxy- containing compounds and sulphur (S), nitrogen (N) and many other unidentified organic compounds. Therefore, many compounds previously identified as NA, are not classical NAs and can be classified, based on heteroatom empirical formula classes as $O_x^{+/-}$ (where x= 1-6), $NO_x^{+/-}$ (where x= 1-4), $SO_x^{+/-}$ (where x= 1-4), or $NO_xS^{+/-}$ (where x= 1-2) containing species. With the continued development of analytical methods, uHRMS has emerged as one of the most successful techniques for identifying the elemental composition of the acidic species of OSPW and NAs.[31,32]

One consequence of the advancements in analytical techniques is that the term 'naphthenic acid' in the context of OSPW has been refined. Work such as this has led to the development of the term naphthenic acid fraction components (NAFC) to classify the complex organic acid mixture of OSPW. The complexity of the dissolved organic fraction of OSPW has been a major impediment to the development of regulatory guidelines and monitoring programs for this region.[28] No single technique is able to accurately and reliably identify and quantify all of the components of such a complex mixture and therefore a battery-of-methods must be developed for the complementary characterization of OSPW and associated products.

1.6 Chemical classes contributing to the toxicity of OSPW

Identification and characterization of compounds contributing to the toxicity of OSPW is important for the development of water quality guidelines and might be useful for the development of methods for targeted remediation of process-affected materials for eventual release. By treating OSPW with activated charcoal or ozone the role of the dissolved organic fraction in driving the acute toxicity of OSPW has been demonstrated, as the prevalence of all toxic effects towards organisms were significantly lesser with its removal.[5,13,15,24] In addition, toxicity reductions have been achieved using natural microbial communities to degrade lower molecular weight NA, while higher molecular weight NA were more persistent.[33] Work by Verbeek *et al*.,[26] attributed the acute toxicity of surface tailings water to organic acid surfactants using a bioassay- directed toxicity identification and evaluation schematic. Using a fractionation technique to isolate acyclic and cyclic acids from OSPW, Scarlett *et al*.,[8] identified cyclic acid compounds to be more toxic than acyclic compounds on a per mass basis. Furthermore, some NA structures identified in OSPW share similarities with chemicals known to act as ligands of steroid hormone receptors, raising concerns for their contribution to the estrogenic and antiandrogenic properties of OSPW.[34] Klamerth *et al*.,[35] have investigated the acute lethality of ozonated fractions of OSPW by use of the Microtox® assay, and were unable to explain their results by characterization of NAs and oxidized NAs, which suggests that other chemical classes of dissolved organic chemicals in OSPW could contribute to toxicity of OSPW, consistent with the study of Verbeek *et al*.,[26] The toxicity of NAs, NA-like compounds and the NAFC of OSPW has been documented but at the initiation of this thesis program, no definitive line of evidence existed attributing the toxicity of OSPW to a group(s) of compounds.

1.7 Bioassay-effect directed analysis

Identification of compounds contributing to the toxicity of OSPW is important to the risk assessment process and the development of WQG. Bioassay-effect directed analysis (EDA) provides a framework to assess the toxicity of effluents that allows for the identification of chemicals contributing to the toxicity of the mixture. The EDA approach is an iterative process of chemical fractionation followed by bio-testing to identify active fractions and is used to

sequentially reduce the chemical complexity of a mixture.[36] Fractionation and bio-testing is repeated until toxicity can be attributed to an individual chemical or group of chemicals.[37,38]

The EDA approach is applicable to a wide range of media including air, water and sediments, and can involve various fractionation and bio-testing schemes. The specific strategy employed can be adapted to a wide range of mixtures and separations, and is dependent on the properties of the constituents.[39] Fractionation techniques include the use of solid-phase extraction by use of C18 phases, polystyrene-di-vinylbenzene, and integrated long term sampling devices and can be generated based on a broad range of chemical parameters, including polarity, carbon number, and volatility.[39] Upon fractionation, bio-analytical tools used to assess toxicity are quite variable and include both *in vitro* and *in vivo* systems with invertebrate or vertebrate species. Endpoints include biochemical and apical endpoints to a diverse range of organisms including traditional test species such as *Daphnia magna, Pimephales promelas* and *Vibrio fisheri*.[40,41] The selection of a single test system is not recommended as toxicity might not be properly characterized. A battery of bio-tests is preferred which includes organism's representative of different taxa to confidently identify toxic fractions.[41] The EDA scheme can be adapted to a variety of endpoints and organisms and can enhance chances for toxicant identification, and significance of the hazard assessment.

Without effects-related analysis, a clear assignment of toxicity to a chemical compound is hardly possible for a mixture with such great chemical complexity as OSPW. Methods implementing chemical analysis are effective for compounds known *a priori*, but cannot identify isomers nor compounds which co-elute. The EDA approach offers a method for the identification of compounds or classes of compounds contributing to toxicity. This facilitates the development of analytical methods, and the application of mixture toxicity predictions such as concentration addition employed by the Canadian Council of Ministers of the Environment (CCME).[42] The EDA approach though assumes toxicity can be assigned to a few individual anthropogenic toxicants in a mixture, and cannot assign toxicity if concentrations are too low for detection.[43,44] This method has its own limitations but does offer an approach for the assessment of hazards due to toxicants not known *a priori,* and mixtures considered too complex for current analytical methodologies.

1.8 Remediation and release of OSPW

As extraction of bitumen increases over the coming decades so too will the volume of OSPW held on-site. Release of OSPW is currently restricted to a zero discharge policy and industry is accountable for remediating all disturbed lands to an ameliorated or equal condition. As of 2012, on-site storage of OSPW exceeded 1 billion m^3 and poses a significant management issue for the companies leasing this land.[45] This has led to the introduction of endpit lakes (EPL) and the construction of wet landscapes as a remediation strategy.[45] An EPL consists of a previously mined area which has been lined with mature fine tailings, filled with OSPW and capped with freshwater or some combination thereof. These constructed landscapes are designed to attenuate the toxicity of process-affected materials by relying on the natural degradation of toxic components by microbial communities over time and are constructed to facilitate the slow reintegration of process-affected materials with the natural landscape.[46] This method repurposes previously mined areas, does not require active remediation, is more cost efficient than other remediation strategies, and provides a scheme whereby landscapes affected by the industry can be reintegrated with the natural environment.[45] However, a report by the Royal Society of Canada raised concerns that the EPL method is an untested concept as it is not known if these constructed landscapes will detoxify the process affected materials sufficiently to sustain viable aquatic ecosystems in the future.[4] With more than 25 planned EPL's over the next 60 years an understanding of their success remains a critical question to industry. As of December 31, 2012 the first commercial test of an EPL began, when Syncrude Canada Ltd. established BaseMine Lake (BML) from the tailings containment pond formerly known as WIP. The proposed project outlined in the following sections will be completed with samples collected when this EPL was established and represents time zero before biodegradation and other processes have occurred.

1.9 Regulation of effluents in Canada

The Government of Alberta and the Royal Society of Canada have released guidance documents identifying areas of concern for the oil sands region with regards to human and environmental health impacts. In 2009, the Alberta government outlined a need for performance-based environmental outcomes and the development of methods to evaluate environmental impacts of oil sands mining activities.[47] In 2010, an expert panel commissioned by the Royal

Society of Canada outlined the need for guidelines for the discharge of treated OSPW and improvements in the chemical characterization of OSPW so that effects on groundwater could be assessed.[4] These recommendations included development of measurable parameters indicative of exposure to oils sands derived materials to be used for the establishment of WQG and development of biomarkers of exposure to ensure the protection of human and environmental health in the oil sands region of Alberta.

In Canada, the release of a deleterious substance to waters frequented by fish is prohibited by the Fisheries Act.[48] Release of a deleterious substances can be authorized under federal or provincial law or is subject to federal or provincial guidelines. Guidelines for the release of a deleterious substance can be developed by use of published Canadian Water Quality Guidelines (CWQG) for the Protection of Aquatic life, CWQG guidelines for site-specific application or by use of peer-reviewed guidelines adopted by a federal or provincial body. For complex mixtures, CCME recommends the use of whole effluent toxicity tests, water quality based effluent limits (WQBEL) or technology based limits.[49] The use of WQBEL is the preferred approach for the assessment of the toxicity of effluents and employs concentration addition for prediction purposes.[50] This approach integrates quantitative chemical analysis with toxicity information to characterize mixture toxicity.

1.10 Conclusions

The organic fraction of OSPW is acutely toxic to aquatic organisms. Because establishment of strategies for the remediation of OSPW are under-way there is a need to establish guidelines for the discharge of OSPW to the receiving environment. As surface mining operations continue to expand there is a greater need to construct tailings ponds for the storage of process-affected materials. Consequently, the risk posed to the surrounding environment due to accidental release or dam failure is increasing. The research to be carried out in this Ph.D. program aims to use the EDA approach to identify organic compounds that contribute to the acute toxicity of OSPW. An acute aquatic toxicity model will be developed to predict the toxicity of OSPW samples, and to investigate the relative contribution to toxicity of chemical classes identified by use of the EDA approach. To further characterize the toxicity of produced samples of OSPW, potential mechanism(s) by which fractions of OSPW cause adverse effects will be investigated by use of

an *Escherichia coli* (*E. coli*) live cell genome reporter assay. Furthermore, the chronic toxicity of samples will be investigated by use of the short term fathead minnow reproductive bioassay. This research will contribute basic and applied research and aid in the development of WQG in the Athabasca region and will improve the evaluation of toxicity of OSPW under CCME guidelines.

1.11 Objectives

The overall objective of this research program was to produce data which identified and characterized the toxicity of chemicals classes in the dissolved organic fraction of OSPW. The research was split into 3 phases. The first phase of this research program focused on identifying the chemical classes responsible for the acute toxicity of OSPW by use of the EDA approach. The goals of this phase are reviewed in objective 1 (Chapter 2). The toxicity data and fractions produced as part of phase 1 were used throughout phase two and three and guided the fractionation method presented in Chapter 5 (objective 4). The second phase of this research program focused on developing a predictive acute aquatic toxicity model for the dissolved organic fraction of OSPW and to identify chemical classes and carbon number ranges contributing to toxicity. Specific goals of this phase are reviewed in objective 2 (Chapter 3). The third phase of this research program focused on characterizing the mechanistic (objective 3) and chronic toxicity (objective 4) of dissolved organic chemicals in OSPW. Though these three phases of research were distinct, together they constituted a comprehensive program of research which described the identities and toxicities of dissolved organic chemicals in OSPW.

Objective 1. Identify chemical classes responsible for the acute toxicity of the dissolved organic fraction of OSPW by use of the EDA approach (Chapter 2)

Due to advancements in the characterization of dissolved organic chemicals in OSPW by use of HPLC- uHRMS approaches there was a gap in knowledge regarding the toxicity of these newly identified chemical classes. Therefore, the specific objectives and associated null hypotheses of objective 1 were:

1) To identify and characterize chemical species contributing to the acute toxicity of the dissolved organic fraction of OSPW by use of the EDA approach, HPLC-Orbitrap- uHRMS and

the Microtox® and fathead minnow embryo lethality assays. For each round of fractionation, the null hypothesis is the same;

> H01: There is no statistically significant difference in the reduction of light as indicated by the Microtox® assay between fractions of OSPW and control/solvent control exposed *Vibrio fisherii.*
>
> H02: There is no statistically significant difference in the mortality or prevalence of morphological abnormalities of ELS of FHM exposed to fractions of OSPW compared to ELS of FHM exposed to control/solvent control.

Objective 2. Develop a predictive acute aquatic toxicity model by use of published bioaccumulation estimates, HPLC-Orbitrap-uHRMS data and the target lipid model for the dissolved organic fraction of OSPW to embryos of Fathead minnow

In an effort to support the development of WQG for the oil sands region, a predictive acute aquatic toxicity model will be developed. By use of previously published bioaccumulation estimates for dissolved organic chemicals in OSPW, HPLC-Orbitrap-uHRMS and toxicity data from Chapter 1, a model will be developed to investigate the contribution of specific chemical classes and to demonstrate the utility of this approach. Therefore, the specific objectives and associated null hypotheses were:

1) To develop and select a model which best predicts acute toxicity of OSPW.

> No explicit null hypotheses, developed models will be compared for their predictive performance by use of the goodness of fit statistics; mean and median residuals, mean absolute deviation (MAD) and root-mean square deviation (RMSD).

2) Identify chemical species contributing to the acute toxicity of OSPW.

> No explicit hypotheses, however by use of the best performing model, the contribution of chemical species and classes to the toxicity of OSPW will be investigated and their potency compared.

Objective 3. Characterize potential molecular mechanisms of toxicity of dissolved organic fractions of OSPW by use of the open-format *E. coli* K-12 genome reporter assay

The molecular mechanism(s) of toxicity of dissolved organic chemicals in OSPW are not well characterized. Therefore, the *E. coli* K-12 strain genome reporter assay will be used to identify and characterize potential molecular mechanisms of toxicity of fractions generated in Chapter 2. Therefore, the specific objectives and associated null hypotheses were:

H01: There is no statistically significant difference in the growth of *E. coli* K-12 wildtype following exposure to fractions of OSPW and *E. coli* exposed to the solvent control.

H02: There is no statistically significant relationship between transcriptional responses of the specified genes and time.

H03: There is no statistically significant difference in the frequency of gene ontology (GO) terms between gene lists of *E. coli* exposed to fractions of OSPW and gene lists of *E. coli* exposed to the solvent control.

Objective 4. Reproductive success of fathead minnow exposed to OSPW or fractions of OSPW as determined by use of the 21-day short term reproductive assay

The reproductive toxicity of OSPW has previously been demonstrated however, an assessment of reproductive toxicity of refined fractions of OSPW has never been completed. By use of fractions identified to contain novel, previously un-characterized chemicals (Chapter 2), the aim of this research chapter will be to assess the reproductive performance of fathead minnow exposed to OSPW and fractions of OSPW. Therefore, specific objectives and associated null hypotheses were:

H01: There is no statistically significant difference in the cumulative number of eggs produced per female (Cumulative no. eggs/female) between fathead minnow breeding trios exposed to OSPW or fractions of OSPW and fathead minnow breeding trios exposed to control/ solvent control.

H02: There is no statistically significant difference in gonadosomatic (GSI) or hepatosomatic index (HSI) between male or female fathead minnow respectively exposed

to OSPW or fractions of OSPW and male or female fathead minnow respectively exposed to control/ solvent control.

H03: There is no statistically significant difference in circulating plasma 17β-estradiol or testosterone concentrations between female or male fathead minnow exposed to OSPW or fractions of OSPW and fishes exposed to control/ solvent control.

H04: There is no statistically significant difference in the expression of genes of interest between male or female fathead minnow exposed to OSPW or fractions of OSPW and male or female fathead minnow exposed to control/ solvent control.

H05: There is no statistically significant difference in time to hatch or presence of malformations of embryos collected from breeding trios exposed to OSPW or fractions of OSPW and embryos collected from breeding trios exposed to control/ solvent control.

Chapter 2: Effects-directed analysis of dissolved organic compounds in oil sands process-affected water (OSPW).

2.1 Preface

At the initiation of this research program, chemical species responsible for the acute toxicity of the dissolved organic fraction of OSPW were little understood. At the time, naphthenic acids were thought to be responsible for most of the observed toxicity in exposed organisms however advancements in the analytical characterization of oil sands related chemicals had recently demonstrated the presence of novel chemical species. Therefore, this work was completed in collaboration with the research group of Dr. Jon Martin of the University of Alberta due to their expertise in the analytical characterization of OSPW related chemical species. By use of a bioassay- effect directed analysis approach, the dissolved organic fraction of OSPW was sequentially fractionated and biologically tested three times to elucidate chemical species responsible for the acute toxicity of the dissolved organic fraction of OSPW. The biological assays applied were embryotoxicity assays of fathead minnow and the Microtox® assay. In addition, work in this chapter developed the basis for work in all subsequent chapters as the fractions produced in this chapter were used throughout this work and guided the fractionation presented in Chapter 5.

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Author contributions:

Garrett D. Morandi (University of Saskatchewan) conceived, designed, and managed experiments, generated and analyzed data, prepared all figures, and drafted the manuscript.

Dr. Alberto Pereira (University of Alberta) provided laboratory support, completed fractionation and chemical analysis of OSPW and related fractions.

Dr. Jonathan W. Martin, and Ian Gault (University of Alberta), and Drs. Steve B. Wiseman, Rishikesh Mankidy and John P. Giesy (University of Saskatchewan) provided inspiration, scientific input, and guidance, commented on and edited the manuscript, and provided funding for the research.

2.2 Abstract

Acute toxicity of oil sands process-affected water (OSPW) is caused by its complex mixture of bitumen-derived organics, but the specific chemical classes that are most toxic have not been demonstrated. Here, effects-directed analysis was used to determine the most acutely toxic chemical classes in OSPW collected from the world's first oil sands endpit lake. Three sequential rounds of fractionation, chemical analysis (ultra-high resolution mass spectrometry), and acute toxicity testing (96 hour (hr) fathead minnow embryo lethality, and 15 minute (min) Microtox® bioassay) were conducted. Following primary fractionation, toxicity was primarily attributable to the neutral extractable fraction (F1-NE), containing 27% of original organic mass. In secondary fractionation, F1-NE was sub-fractionated by alkaline water washing, and toxicity was primarily isolated to the ionizable fraction (F2-NE2) containing 18.5% of the original organic mass. In the final round, chromatographic sub-fractionation of F2-NE2 resulted in two toxic fractions, with the most potent (F3-NE2a, 11% of original organic mass) containing predominantly naphthenic acids (O_2^-). The lesser toxic fraction (F3-NE2b, 8% of original organic mass) contained predominantly non-acid species (O^+, O_2^+, SO^+, NO^+). Evidence supports naphthenic acids as among the most acutely toxic chemical classes in OSPW, but non-acidic species also contribute to acute toxicity of OSPW.

2.3 Introduction

The surface-mining oil sands industry in the Athabasca Region of Alberta, Canada, produces oil sands process-affected water (OSPW) as a by-product during extraction of bitumen. This water is a mixture of residual bitumen, silts, clays, and other inorganic and organic constituents that are naturally present in bitumen.[51,52] OSPW is pumped to tailings ponds for temporary storage and recycling, and as of 2011, greater than 1 billion m^3 of OSPW was already contained in tailings ponds, covering approximately 170 km^2.[52,53] Although recycling of OSPW reduces demand for freshwater, this process concentrates salts, metals, and other inorganic and organic constituents, resulting in water that is acutely toxic. If allowed to age, the acute toxicity of OSPW lessens because of degradation and or transformation of organic compounds, but both acute and chronic toxicity persists.[21,25,54–56]

Tailings ponds are temporary structures that hold OSPW during the active life of the mines, and evidence indicates that OSPW seeps from these structures.[57–59] When surface mines close, or OSPW is no longer required for extraction of bitumen, tailings ponds must be reclaimed and the OSPW detoxified before hydraulic connectivity is re-established with the natural environment.[46] Endpit lakes are a major strategy that has been proposed to this end and the first full-scale test of this approach was established in late 2012 with commissioning of BaseMine Lake.[46] Nevertheless, there is great uncertainty about the rate at which BaseMine Lake will detoxify.[4] A more thorough understanding of which chemicals in OSPW impart most of its toxicity would allow better monitoring and projections of BaseMine Lake detoxification.

OSPW has long been known to contain a complex mixture of naphthenic acids (NAs), classically defined as cyclic and alkyl-substituted carboxylic acids fitting the general formula $C_nH_{2n+Z}O_2$, where n is the number of carbons and Z is zero or an even negative integer representing the hydrogen deficiency due to rings or double bonds.[60] Many papers today attribute the toxicity of OSPW to NAs based on the early work of Mackinnon and Boerger,[24] and Verbeek *et al.*;[26,61] however, we argue that the results from these important pioneering studies do not specifically implicate classical NAs, but rather implicate a broader group of polar organic acids. Mackinnon and Boerger used two treatments to ameliorate the acute toxicity of OSPW, their findings suggested that surfactants within the polar acidic fraction were responsible.[24] They go on to indicate that "compounds with properties similar to naphthenic acids" are present in this fraction, but their discussion is well nuanced and does not directly attribute NAs to OSPW toxicity, and in fact they state that confirmation of the toxic component is still needed. Similarly, Verbeek *et al.*,[26,61] looked at how detoxification can be accomplished through extracting "surfactants" from OSPW, not classical NAs specifically. Thus, any claim of toxicity identification in these seminal studies attributes the toxicity generally to polar organic acids and surfactants, and to the best of our knowledge there is still no direct evidence in the literature that classical NAs are the toxic component of any OSPW sample. Much work is already underway to determine which OSPW NAs might be most toxic and there is also a proposal to add NAs to the Canadian National Pollutant Release Inventory,[62–65] thus experimental confirmation that classical NAs are indeed the toxic component of OSPW would be timely.

Since 2010, a deeper understanding of the complexity of dissolved organics in OSPW has come from applications of multidimensional chromatography, and of ultra-high resolution mass

spectrometry. The polar acidic organic fraction of OSPW is now understood to contain not only a complex mixture of NAs, but other co-occurring complex mixtures of mono- and poly-oxygenated compounds, for example di-acids,[66] and many unidentified species containing sulphur and nitrogen atoms.[29,31,67–69] Equally as important, it has also been demonstrated that major classes of organics in OSPW are not acids, but are instead polar neutral substances; only observable when mass-spectral characterization is conducted in positive ionization mode with ultrahigh resolving power.[29,31] Overall, the structure of the majority of dissolved organic constituents of OSPW remain uncharacterized, and their associated toxic potencies remain unknown. By ultrahigh resolution mass spectrometry these complex mixtures of chemicals can be broadly assigned to empirical formula classes (i.e. binning, based on accurate mass measurements). For example, NAs belong to the O_2 heteroatomic class observed in negative ion mode (i.e. O_2^- class). It therefore becomes theoretically feasible to determine which of these empirical formula classes contribute most to the acute toxicity of OSPW, be it NAs or some other group of organic acids or polar organic neutrals.

A common approach for toxicity identification in complex mixtures is effects-directed analysis (EDA).[44,70,71] In the earliest EDA of OSPW acute toxicity, Verbeek *et al.*[61] determined that the acute toxicity of several different samples of OSPW was consistently and fully attributable to an organic fraction that could be removed with C18 adsorbent. This is generally consistent with more modern studies showing that ozonation or activated charcoal can significantly attenuate or remove all toxic effects to fish and aquatic midges.[5,13,14,35,72–74] Verbeek also showed in some cases that the acute toxicity of OSPW could be fully removed by acidification and centrifugation (to precipitate organic acids, including NAs), consistent with the earlier findings of MacKinnon and Boerger.[24] Nevertheless, for some OSPW Verbeek *et al.*,[61] showed that the acidic organic fraction only explained 55-60% of the toxicity, with semi-volatile organic compounds (i.e. neutrals) explaining the balance (e.g. 20-35% of toxicity). Thus, it could be broadly concluded that the toxicity of OSPW was mostly attributable to organic acids, but that semi-volatile neutral substances were sometimes important. In a subsequent study,[75] anion-exchange chromatography was used to generate fractions of OSPW to investigate their acute toxicity, but the analytical focus was only on classical NAs. Moreover, the results of this study were largely inconclusive, in part because the low resolution infusion-electrospray mass spectrometry method lacks the necessary resolving power to differentiate NAs from isobaric compounds containing nitrogen, sulfur, or multiple oxygen atoms.[76] Klamerth *et al.*,[35] recently investigated the acute toxicity of ozonated

fractions of OSPW, but were unable to explain their results by characterization of the NAs and oxidized NAs, which suggests that other chemical classes of organic chemicals in OSPW could be important for toxicity of OSPW, consistent with the pioneering study of Verbeek *et al*.[61]

The goal of the current study was to use best available analytical technology and an EDA approach to identify the most acutely toxic dissolved organic chemical classes in OSPW. A 96 hr fathead minnow (*Pimephales promelas*) embryo-lethality assay, and a 15 min Microtox® toxicity bioassay (*Vibrio fischerii*) were used to assess acute toxicity, and the composition of chemicals in each fraction, generated by liquid-liquid extraction or chromatographic separation, was determined by HPLC-Orbitrap ultra-high resolution mass spectrometry. Moreover, the source of OSPW for the current study was BaseMine Lake, the Alberta oil sands first endpit lake.

2.4 Materials and methods

2.4.1 Chemicals and reagents

Acetic acid, methanol, dichloromethane (DCM) and water (HPLC grade) were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Anhydrous ethanol (EtOH) was purchased from Fischer Scientific (Edmonton, AB, Canada). Sulfuric acid (H_2SO_4) 98% and sodium hydroxide (NaOH), and reagents for the Microtox® assay were purchased from Osprey Scientific Inc. (Edmonton, AB, Canada).

2.4.2 Sample collection

One hundred liters (100 L) of OSPW was collected in September 2012 from the West In-Pit (WIP) tailings pond by manual insertion of 20 litre (L) high-density polyethylene pails from a permanent sampling barge (Syncrude Canada Ltd). This body of water was soon-after commissioned as BaseMine Lake in December 2012, after which all new inflow of fresh OSPW from the extraction plant was terminated. Therefore, the current sample of OSPW approximates a time zero sample of OSPW from BaseMine Lake. The OSPW was stored in the dark at 4 ^{o}C for two months prior to extraction.

2.4.3 Fractionation

Three stages of fractionation were conducted on a total of 100 L of OSPW. This is shown schematically in Appendix A (Figure A.1) along with the nomenclature and concentrations of extractable organics milligram per liter (mg/L) in each primary (F1), secondary (F2) and tertiary fraction (F3). Fractionation in stage 2 was not initiated until toxicity tests had been completed on primary fractions. Likewise, fractionation in stage 3 was not initiated until toxicity tests had been completed on the secondary fractions. Thus extracts were kept at -20 degrees Celsius (°C) until proceeding to the subsequent fractionation stages. Details of the fractionation procedures in each step are described below.

2.4.4 Primary fractionation methods

Suspended particulate matter was removed from 100 L of OSPW (in 2 L batches) by use of vacuum filtration through Grade 4 glass fiber filters with a 1.2 μm nominal particle retention size (Fisher Scientific, Nepean, ON, Canada). The major objective in the primary fractionation step was to achieve fractionation by empirical formula class, and ideally to isolate most of the naphthenic acids from other organic acids. A few solvent and pH combinations were investigated, but only the optimal method that was used is described here. After filtration, the OSPW was sequentially liquid-liquid extracted at three different pHs to yield 3 primary fractions (Figure A.1). First, the pH was adjusted from 8.0 to 7.0 with concentrated H_2SO_4 and extracted twice with 200 mL of DCM. The DCM was combined and dried, yielding the "neutral extractable" fraction (F1-NE). Then, the pH of the residual water was lowered to pH 2 with concentrated H_2SO_4 and extracted in the same manner as above to yield the acid extractable fraction (F1-AE). Finally, the pH of the residual water was raised to pH 11 with concentrated NaOH and extracted as above to yield the base-extractable (F1-BE) fraction. After drying by rotary evaporation and nitrogen evaporation, the average mass per liter of OSPW was 50 mg/L for F1-NE, 103 mg/L for F1-AE, and 14 mg/L for F1-BE. Based on total organic carbon analysis of the original filtered OSPW, and of the extracted OSPW (i.e. after extraction at all three pH's), the 3 fractions accounted for 90% of total organic carbon in the original OSPW. More details of this analysis are provided in Appendix A. It is important to note that the nomenclature for these three primary fractions are operationally defined, and as discussed later the "neutral-extractable fraction" (F1-NE) actually contains most of the

NAs, but not the more polar acids. For quality control, a blank was created for each fraction (e.g. b-F1-NE) by subjecting control water to the same fractionation methods. The gravimetric concentration of material in the blanks was <1% of real samples (Table A.1).

2.4.5 Secondary fractionation methods

The toxic F1-NE fraction was subfractionated in the second stage. The theoretical strategy was to separate non-ionizable neutral substances from acids, including the NAs which were prominent in F1-NE. Thus, twenty equivalent litres (i.e. the mass of organics of F1-NE following extraction of 20 litres of OSPW) of F1-NE fraction were dissolved in 100 mL of DCM and extracted 3 times with 300 mL of alkaline water (pH 12). The combined alkaline water (900 mL) was then washed two times with 100 mL of DCM, and this DCM (200 mL) was recombined with the original 100 mL of DCM (total 300 mL DCM) and dried to yield NE-fraction 1 (F2-NE1). The alkaline water extract was then acidified to pH 2 with concentrated H_2SO_4 and extracted three times with 200 mL of DCM which were combined (600 mL) and dried to yield NE-fraction 2 (F2-NE2). After drying, masses of F2-NE1 and F2-NE2 were 15.7 mg/L and 34.3 mg/L, respectively, and the gravimetric mass balance in this step was not different from 100%.

2.4.6 Tertiary fractionation methods

The toxic F2-NE2 fraction was sub-fractionated in this third and final stage. The theoretical strategy was to separate remaining NAs from all other compounds by use of chromatography. Eight equivalent litres (i.e. mass of organics of F2-NE2 contained in 8L of unfractionated OSPW) of F2-NE2 were dissolved in acetonitrile/ EtOH (95/5; unit volume/ unit volume (v/v)) at a concentration of 10 mg/mL, and 10 µL aliquots were repeatedly injected to isocratic HPLC. Separation was by two coupled Zorbax-Sil columns (250 mm x 4.6 mm and 5 µm particle diameter, Agilent Technologies, Santa Clara, CA, USA) at 25 °C with a mobile phase of acetonitrile/water (50/50; v/v) at a flow rate of 1 mL/min. The first eluting fraction (termed F3-NE2a) was collected for a broad peak eluting between 4 and 7 min, at which time a valve was automatically switched to divert the eluent to a second fraction (termed F3-NE2b) which eluted between 7 and 11 min (Figure A.2). Acetonitrile was removed by rotary evaporation at 40 °C. One liter of the residual water solution from each fraction was extracted three times with 200 mL of DCM and dried by

rotary evaporator. After drying, the masses of F3-NE2a and F3-NE2b were 20.0 mg/L and 14.0 mg/L, respectively, giving a gravimetric mass balance of 99%.

2.4.7 Chemical characterization of fractions

Reverse-phase high-pressure liquid chromatography (HPLC) was paired to a hybrid linear ion trap-orbitrap mass spectrometer (Orbitrap Elite, Thermo Fisher Scientific, San Jose, CA, USA) operating in electrospray mode using the methods of Pereira *et al*.[29] Nominal resolution of the Orbitrap was set to 240,000 at m/z 400. Species detected in each fraction were binned according to heteroatom empirical formula classes in negative ($^-$) or positive ($^+$) electrospray modes: $O_x^{+/-}$ (where x= 1-6), $NO_x^{+/-}$ (where x= 1-4), $SO_x^{+/-}$ (where x= 1-4), or $NO_xS^{+/-}$ (where x= 1-2). Concentrations of polycyclic aromatic hydrocarbons (PAH) in F1-NE, F1-AE and F1-BE fractions were determined by gas chromatography mass spectrometry (model 5973, Agilent, Santa Clara, CA, USA) using authentic standards. Further details of the methodologies are provided in Appendix A.

2.4.8 Sample preparation for acute toxicity tests

For assays of acute toxicity, pure material from each fraction (including fractionation blanks) was dissolved in 2 ml of 100% ethanol. "Pooled" samples were prepared after each round of fractionation by recombining equal volumes of each fraction. These pooled samples are referred to as F1-Pool (primary fractions), F2-Pool (secondary fractions), and F3-Pool (tertiary fractions).

2.4.9 96h Fathead minnow embryo-lethality assay

Exposures of embryos to fractions were conducted according to the protocol of He *et al*.[5] Details of animal husbandry and the exposure protocol are provided in Appendix A. Acute toxicity of each fraction was assayed at nominal concentrations of 10-fold (×), 5×, 2.5×, 1× and 0.5× the concentration of the original sample of OSPW. For clarity, all concentrations of fractions are reported as equivalents of the original OSPW (multiples of 100% full strength OSPW); e.g. 1× is 100% full strength, 0.5× is 50% full strength, etc. Any fractions that were toxic at 0.5× were also assayed at 0.1×. Concentrations required to reduce survival by 50% (LC50) were determined so potency of fractions could be compared.

2.4.10 Microtox® assay

The Microtox® assay was performed by use of a Microtox® Model 500 analyzer and the Basic Test procedure (AZUR environmental, 1995). The assay allows for rapid determination of acute toxicity by quantifying reductions in bioluminescence of the marine bacterium *Vibrio fisherii*. Stock solutions of fractions were prepared at a concentration of 10×, pH was adjusted to 7-8 by use of HCl, and 10% osmotic adjusting solution was added. Assays were completed by use of 6 serial dilutions with nominal concentrations of fractions of 10×, 5×, 2.5×, 1.25×, 0.63× and 0.31×. A solution of 0.1% EtOH was included as a solvent control and a solution of Microtox® diluent with 10% osmotic adjusting solution was used as a blank. The concentration required to reduce luminescence by 50% compared to the blank solution after 15- min (IC50) was calculated by use of the Microtox® Omni software, which is based on the Gamma distribution function.

2.4.11 Statistics

Statistical analyses were performed by use of SPSS software (SPSS Inc. Chicago, IL, USA). For the fathead minnow embryo-lethality assay, LC50 values were calculated by use of probit analyses to describe the steepest section of dose-response curves. The 95% confidence intervals (CI) were calculated as the standard error of 4 replicates multiplied by 1.96. Fathead minnow LC50 and Microtox® IC50 values were considered significantly different if there was no overlap of 95% CI. Data were tested for normality by use of the Kolmogorov- Smirnov test and homogeneity of variance was determined by use of Levene's test. Data that did not meet assumptions for parametric statistical procedures were $\log_{10}$ transformed (survival) but non-transformed data are shown in all figures. Differences that were statistically significant were determined by one-way Analysis of variance (ANOVA) with Dunnett's post-hoc test. In both the fathead minnow assay and the Microtox® assay there were no significant effects of the solvent control compared to the control or blank, therefore all data are expressed relative to the solvent control. Data are presented as mean ± standard error mean (SEM) and differences were considered significant at $\rho \leq 0.05$.

2.5 Results and discussion

2.5.1 Acute toxicity and characterization of primary fractions

Primary fractions showed a range of relative acute toxicity towards embryos of fathead minnow and *Vibrio fisheri*. Toxicity was not caused by the fractionation method as neither of the fractionation blanks (b-F1-NE, b-F1-AE, b-F1-BE) was acutely toxic to *Vibrio fisheri* (Table 2.1). Based on the 96 hr LC50 of F1-NE, it was significantly more potent than F1-AE or F1-BE, and more potent than F1-Pool (Table 2.1). The latter result suggests chemical antagonism in the whole mixture. The dose-response curves of embryos of fathead minnow (Figure 2.1A) further demonstrated that F1-NE was the most potent primary fraction. For example, concentrations of F1-BE as great as 10× did not cause lethality of embryos. F1-AE caused lethality of embryos, but only at the greatest concentration of 10×. In contrast, mortality of embryos exposed to 1× of F1-NE (i.e. the equivalent concentration present in the original OSPW) was 100%. Therefore, based on the 96 hr fathead minnow embryo-lethality assay, F1-NE was more potent than any other primary fraction. In the 15-min Microtox® assay, the IC50's of F1-NE and F1-AE were not significantly different from each other, and not different from the pooled fraction (F1-Pool). However, F1-BE was significantly less potent than all other samples. Thus in both acute toxicity assays F1-BE was the least potent fraction and was ruled out for further fractionation and testing. Based on the greater potency of F1-NE in the embryo lethality assay, the greater environmental relevance of this freshwater species compared to the marine bacterium, *Vibrio fisheri*, and the longer exposure time of the embryo lethality test, which allows for hydrophobic chemicals to be absorbed, F1-NE was selected for sub-fractionation. Furthermore, as described below, F1-NE contained most of the NAs, and it has been shown previously for commercial NAs that fathead minnows are a more sensitive model organism than *Vibrio fisheri* .[77]

Table 2.1 Effects of fractions of OSPW on survival of embryos (LC50) of the fathead minnow (*Pimephales promelas*) and on bioluminescence (IC50) of the marine bacterium (*Vibrio fisheri*).

Round of fractionation	Specific Fraction	LC50 ± CI (×)* (FHM embryos)	IC50-15min ± CI (×) (Microtox)
F1	F1-NE	0.72 ± 0.95 (a)**	8.85 (6.85–10.9) (g)
	F1-AE	8.32 ± 2.68 (b)	8.45 (7.92–8.98) (g)
	F1-BE	>10.0 (b)	14.1 (11.41–17.06) (h)
	F1-Pool	7.95 ± 4.04 (b)	7.92 (4.74–11.1) (g)
	b-F1-NE	N/A***	>10
	b-F1-NE	N/A	>10
	b-F1-NE	N/A	>10
F2	F2-NE1	>10 (c)	15.3 (9.30–21.3) (i)
	F2-NE2	1.93 ± 1.55 (d)	5.41 (5.04–5.78) (j)
	F2-Pool	1.79 ± 1.07 (d)	8.50 (5.56–11.44) (i,j)
	b-F2-NE-1	N/A	>10
	b-F2-NE-2	N/A	>10
F3	F3-NE2a	0.73 ± 0.97 (e)	1.23 ± (0.89–1.57) (k)
	F3-NE2b	2.18 ± 0.36 (f)	5.05 (2.58–7.52) (l)
	F3-Pool	0.68 ± 0.34 (e)	0.83 (0.8–0.86) (m)
	b-F2-NE-2a	N/A	>10
	b-F2-NE-2a	N/A	>10

*Units of "×" indicate a concentration relative to the original concentration in the unfractionated OSPW. For example, 1× means equivalent to full-strength OSPW.
**Values followed by a different letter are significantly different, as defined by a lack of overlap between 95% confidence intervals (CIs). Comparisons were made within the different fractionation steps and not among.
***Not available. Toxicity of blanks was not determined by use of the fathead minnow embryo toxicity assay.

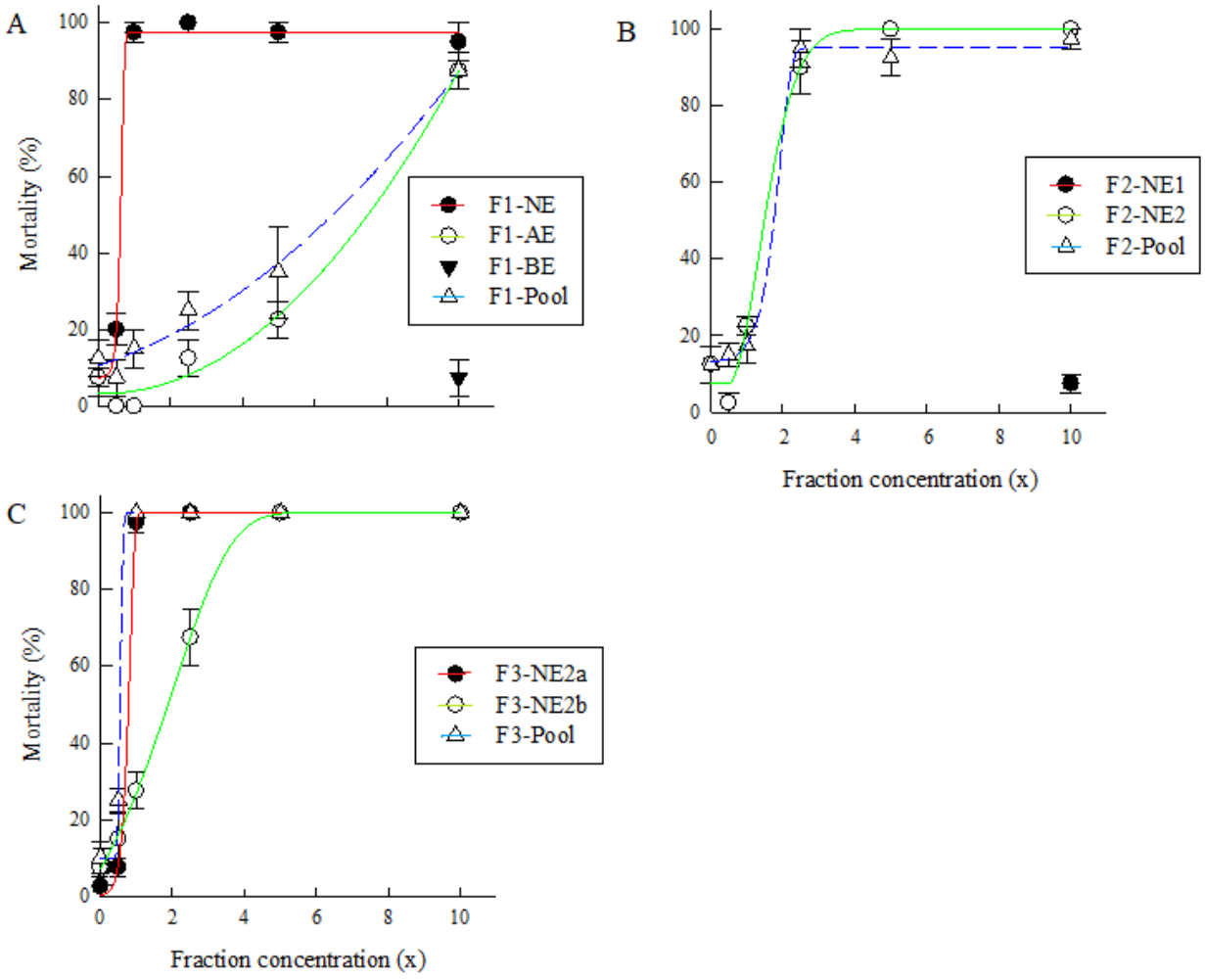

Figure 2.1 Dose-response curves for A) primary, B) secondary and C) tertiary fractions of OSPW on survival of embryos of fathead minnows. Embryos were exposed to EtOH as the solvent control, a pooled sample of fractions (F1-Pool, F2-Pool, F3-Pool), or fractions of OSPW for 96 h. Numbers represent the mean ± SEM of 4 independent replicate exposures.

Distribution of chemicals by empirical formula class determined by HPLC-Orbitrap in negative ($^-$) and positive ($^+$) ion mode are shown in Figure 2.2. In negative ion mode, which detects organic acids, the mono- and di-oxygenated classes were concentrated in F1-NE, whereas poly-oxygenated classes were concentrated in F1-AE (Figure 2.2). The relative distribution of SO_x^- classes among fractions was similar to the O_x^- classes, with SO^- and SO_2^- classes concentrated

primarily in F1-NE, and SO_4^- species concentrated primarily in F1-AE. The base-extractable fraction (F1-BE) contained very little organic mass and contained only residual acids (Figure 2.2).

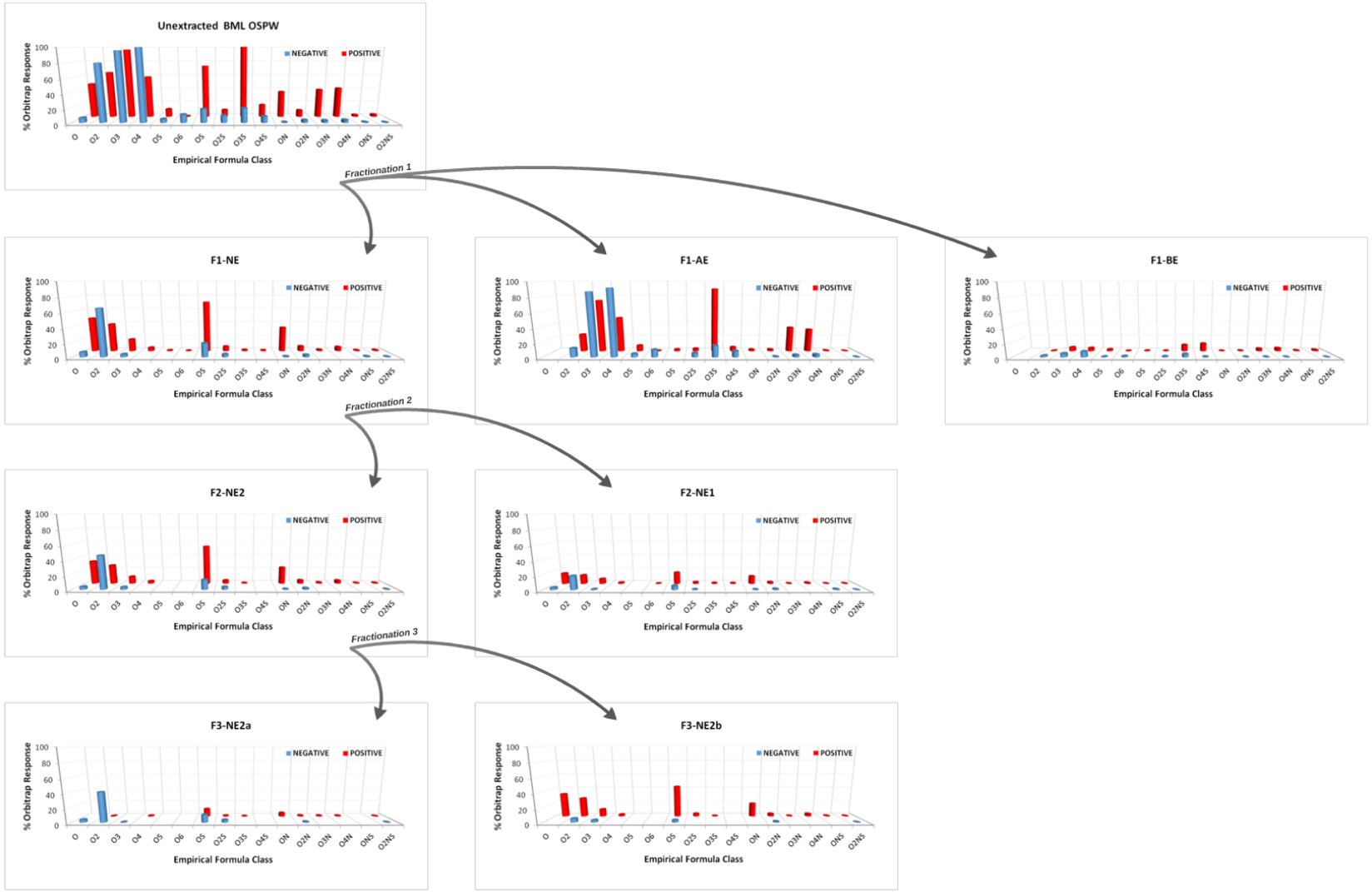

Figure 2.2 Total and relative abundance of species by heteroatom class in negative and positive ionization modes in unfractionated OSPW, and each primary (F1), secondary (F2) and tertiary (F3) fractions. Arrows indicate the sample subfractionated at each stage.

The partitioning of chemical species among the three primary fractions was surprising. It was not anticipated that NAs would be extracted into organic solvent at pH 7 because the pKa of carboxylic acids is generally in the range of 4-5, and they should therefore be >99% ionized at pH 7. Nevertheless, as shown in Figure 2.3 the majority of NA species were recovered in F1-NE, with only trace amounts detected in the less toxic F1-AE or F1-BE (Figure 2.1 & Figure 2.3). In addition to NAs, only minor signals of other organic acids were detected in F1-NE, including the SO^-, SO_2^- and NO_2^- classes (Figure 2.2). Importantly, most other organic acid species were recovered in the relatively non-toxic F1-AE fraction. This is important, because a large proportion of the overall organic acid signal in HPLC-Orbitrap (e.g. including the prominent O_3^- and O_4^- classes, Figure 2.2) was able to be classified as not acutely toxic to embryos of fathead minnow. Therefore, if organic acids are the source of acute lethality to embryos in F1-NE, the majority of this toxicity must be attributable to NAs (O_2^- species) or the minor classes of sulfur and nitrogen containing acids present in F1-NE (Figure 2.2).

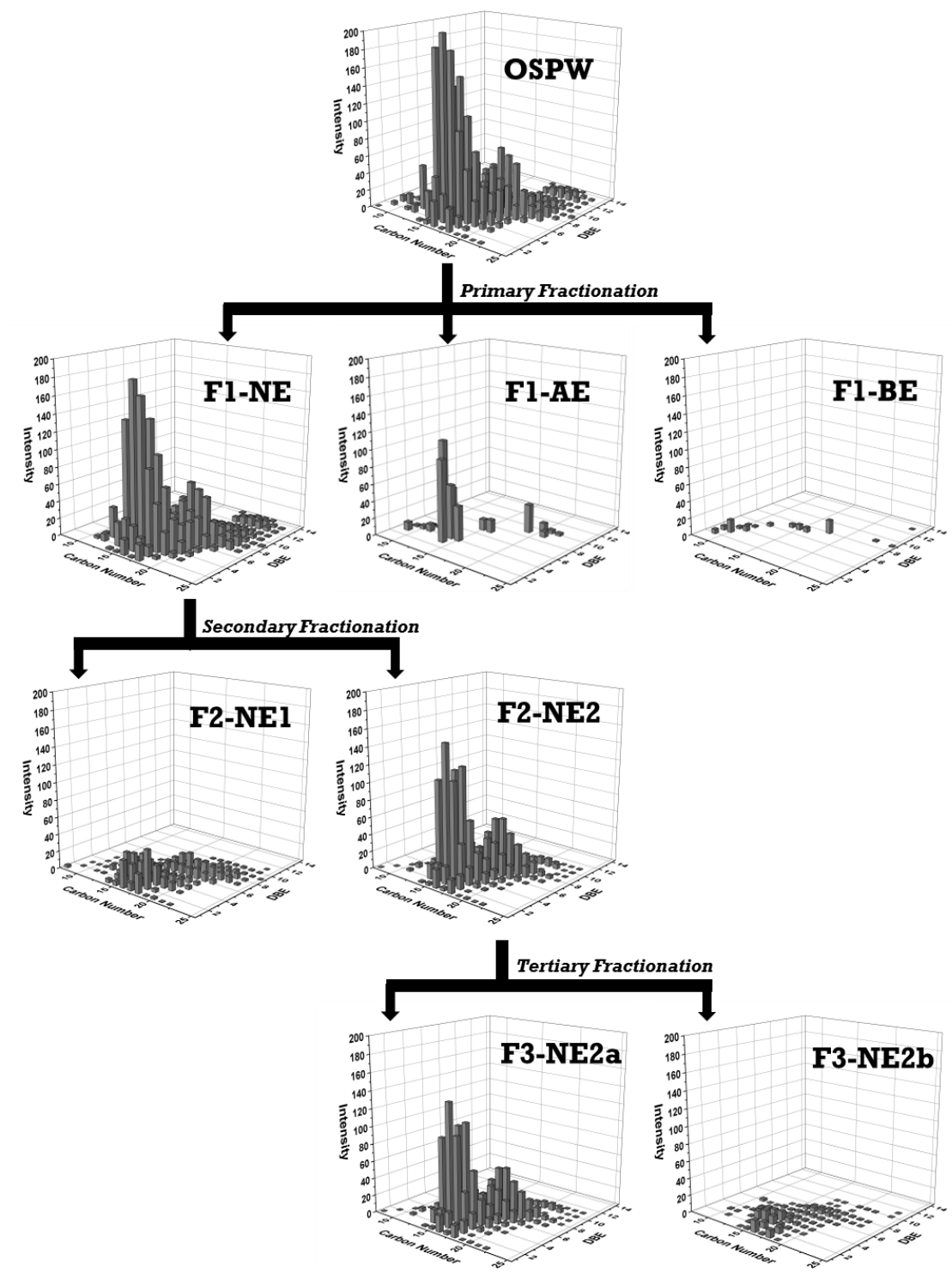

Figure 2.3 Profiles of naphthenic acids (O_2^- class) by carbon number and double bond equivalents in each OSPW fraction, compared to unfractionated OSPW. Intensity has been normalized to the response in unfractionated OSPW.

In positive ion mode, which is best for the detection of polar organic neutral compounds and organic bases, HPLC-Orbitrap also confirmed distinct fractionation of chemical classes to the primary fractions. Among the most abundant species in F1-NE was the O_2^+ species (Figure 2.2) that were first described by Pereira *et al*.[29] as diketo, dihydroxyl, or keto/hydroxyl containing species, possibly similar in structure to sex steroids. Monooxygenated species (i.e. the O^+ class) were also concentrated in F1-NE (Figure 2.2). In contrast, the more oxygenated (and likely more hydrophilic) species (O_3^+, O_4^+, and O_5^+) were primarily concentrated in F1-AE. Similar differences between F1-NE and F1-AE fractions were evident when comparing the distribution of the SO_x^+ and NO_x^+ species. Specifically, SO^+, SO_2^+, NO^+, and NO_2^+ species were concentrated in F1-NE, whereas the more oxygenated (and likely more hydrophilic) species (SO_3^+, SO_4^+, NO_3^+, and NO_4^+) were concentrated in F1-AE. NOS^+ species were detected in relatively low abundance in each of the three fractions, but because concentrations of these compounds were greater in the neutral-extractable fraction, their role in the toxicity of OSPW cannot be eliminated. Concentrations of selected PAHs (Appendix A) in the primary fractions of OSPW were all below the limit of detection of 0.2 nanogram per litre (ng/L).

2.5.2 Acute toxicity and chemical characterization of secondary fractions

The most potent primary fraction (F1-NE) was sub-fractionated into two fractions by dissolving it in DCM and liquid-liquid extracting the ionizable compounds into alkaline water, yielding F2-NE1 (residual) and F2-NE2 (extracted into alkaline water). Briefly, the F2-NE2 fraction demonstrated the greatest potency by use of both acute toxicity assays. For embryos of fathead minnow, the 96 hr LC50 of F2-NE2 was at least 5-fold lesser than F2-NE1. In fact, the greatest concentration of F2-NE1 tested (10×) did not cause any embryo lethality and therefore was not tested at lesser concentrations (Figure 2.1B). The 96h LC50 of F2-Pool was not different from F2-NE2 (Figure 2.1B), thus it was clear that F2-NE1 had no significant toxicity to embryos of fathead minnow. When assayed by use of Microtox®, the 15-min IC50 of F2-NE2 was 3-fold lesser than F2-NE1, and the IC50 of F2-Pool was intermediate of F2-NE2 and F2-NE1 (Table 2.1). Therefore, F2-NE2 was most potent by use of both assays, and it was sub-fractionated in stage three. As observed for the first round of fractionation, neither of the round 2 fractionation blanks (b-F2-NE1, b-F2-NE2) was acutely toxic to *Vibrio fisheri* (Table 2.1).

In negative ion mode, the bulk of the organic acid signal partitioned into alkaline water (as expected) and was recovered in the toxic F2-NE2 fraction (Figure 2.1). For example, approximately 75% of the NA class was recovered in the toxic F2-NE2 fraction (Figure 2.2, see O_2^- class). A more detailed examination of the NA species (Figure 2.3) demonstrates that the majority of species recovered in the toxic F2-NE2 fraction were those with higher numbers of double-bond equivalents. This is consistent with previous work which demonstrated that aromatic NAs have greater toxicity to embryos of zebrafish compared to alicyclic NAs.[63]

Similar to negative ionization mode, characterization by HPLC-Orbitrap-MS in positive ionization mode showed that the majority of the chemical signal partitioned into the toxic F2-NE2 fraction. This is consistent with the fact that ~69% of the gravimetric mass in F1-NE was recovered in F2-NE2. At the level of empirical formula class, however, there was little difference in the relative distribution of formula classes between F2-NE1 and F2-NE2, both being primarily composed of $O_2^+ > O^+ > SO^+ > NO^+$ in descending order of intensity (Figure 2.2). The mode of partitioning controlling the concentration of these presumed polar neutral substances into the alkaline water is not clear, but is likely related to weakly acidic functional groups; for example the neutral hydroxyl or phenoxy groups in the O_2^+ species structures described by Pereira *et al*.[29] would become negatively charged at highly alkaline pH.

2.5.3 Acute toxicity and chemical characterization of tertiary fractions

The most potent secondary fraction (F2-NE2) was sub-fractionated by HPLC with fraction collection (Figure A.2) to yield F3-NE2a (4-7 min) and F3-NE2b (7-11 min). Briefly, both of these tertiary fractions were toxic, but the potency of F3-NE2a was greater than that of F3-NE2b. In the Microtox® assay, the 15 min IC50 of F3-NE2a was approximately 4-fold less than the IC50 of F3-NE2b (Table 2.1). In the embryo toxicity assay the LC50 of F3-NE2a was 3-fold lesser than the LC50 of F3-NE2b (Table 2.1). The isolation of the majority of acute toxicity to F3-NE2a was remarkable considering that it contained only 10.8% of the original organic mass present in the OSPW. Moreover, the 96 hr LC50 of F3-NE2a was less than full strength OSPW (i.e. LC50 < 1×) demonstrating the environmental relevance of this fraction. Acute toxicity was not caused by the fractionation methods as neither of the round 3 fractionation blanks (b-F3-NE2a, b-F3-NE2b) was acutely toxic to *Vibrio fisheri* (Table 2.1).

Nevertheless, the pooled F3 sample had greater potency than either individual F3 fraction, indicating that both fractions contributed significantly to the toxicity. Specifically, the IC50 of F3-Pool was significantly less than the IC50 of either F2-NE2a or F3-NE2b (Table 2.1). Additionally, although the fathead minnow dose response curve was very steep for F3-Pool and F3-NE2a, the F3-Pool dose-response curve was shifted left relative to the two individual fractions, an indication of its greater toxicity at the 0.5× concentration.

Obvious differences in potency of the two F3 fractions were evident from the dose-response curves of acute lethality to embryos (Figure 2.1C). The dose-response curve of F3-NE2a was very steep. For example, at concentrations of 0.1× or 0.5× of F3-NEF2a embryo lethality was not significantly different from the solvent control, but at 1× the mortality was 97.5%, and reached 100% mortality at all concentrations greater than 1×. The dose-response curve of F3-NE2b was much less steep, but the toxic threshold was similar to F3-NE2a. Mortality of embryos exposed to 0.1× or 0.5× of F3-NEF2b was not significantly different from solvent control, but at 1× and 2.5× the mortality was 27.5% and 67.5%, respectively, and achieved 100% mortality at concentrations of 5× or greater. Although F3-NE2b was less toxic than F3-NE2a at equivalent concentrations, it was more potent than any primary (i.e. F1-AE, F1-BE) or secondary (i.e. F2-NE1) fraction that was not sub-fractionated; none of which caused lethality at concentrations of 5× or less.

Differences in the slopes of the dose-response curves for the two F3 fractions likely indicates differences in the mechanism of acute-lethality and is likely related to the different chemical classes present in these fractions.[78] The steep dose-response curve of acute-lethality caused by F3-NE2a is similar to that caused by surfactant–like compounds that act via a narcosis mode of action.[79,80] Because NAs are surfactants, it has been suggested that narcosis is the mode of acute toxicity of OSPW.[79,81] Acute toxicity of fractions from the esterifiable and acid-extractable fraction of OSPW also displayed a steep dose response, similar to characteristics of surfactants.[63] Therefore, F3-NE2a most likely causes acute lethality via narcosis, and based on the more shallow slope, toxicity of F3-NE2b is likely due to a different mode of action. Previous evidence suggested that oxidative stress played a role in the toxicity of OSPW.[5,10,12] For example, He *et al*.,[5] quantified greater concentrations of reactive oxygen species (ROS) in embryos of fathead minnow exposed to OSPW, while Wiseman *et al*.,[12] showed greater peroxidation of lipids in larvae of C*hironomus dilutus* exposed to OSPW. In addition, abundances of transcripts of glutathione-*S*-transferase or catalase, whose enzyme products are important for clearance of ROS,

were greater in embryos of fathead minnow and larvae of *C. dilutus* exposed to OSPW.[5,12] The current data indicate that the acute toxicity of OSPW is likely caused by more than one mode of action, but definitive studies are required to confirm the mode(s) of toxic action for the fractions isolated here.

The toxic potency of the F1-NE sample was maintained throughout the fractionation steps, allowing partial conclusions to be drawn linking chemical profiles of the tertiary fractions to the acute toxicity of OSPW. In general, the majority of chemical species detected in the more potent F3-NE2a were organic acids (detected in negative ion mode), whereas the majority of species detected in less potent F3-NE2b were polar neutral compounds (detected in positive ion mode). For example, the O^- class was detected exclusively in F3-NE2a (Figure 2.2), whereas the O^+ class was detected exclusively in F3-NE2b (Figure 2.2). Naphthenic acids (O_2^- class) were the most prominent class of compounds in F3-NE2a (Figure 2.2), whereas only traces of NAs were detected in F3-NE2b. The O_2^+ species, which are polar neutral compounds, were the most prominent species in the lesser potent tertiary fraction F3-NE2b, and were not detected in F3-NE2a. After the O_2^+ species, the most prominent classes of compounds in F3-NE2b were O^+, OS^+, and NO^+ species. Interestingly, these four classes of compounds are also the four most hydrophobic classes of compounds in OSPW and therefore have a high potential to accumulate in aquatic organisms.[82] For this reason, sub-chronic and chronic toxicological endpoints may yield different results than what are shown here for acute toxicity. For example, OSPW is known to have endocrine activity and impairs fish fecundity.[12–14,83–86] Certain NAs have been shown to be endocrine disruptors, but Pereira *et al*.,[29] suggested that OSPW O_2^+ species may resemble sex steroids, although this remains to be confirmed. Yue *et al*.,[86] recently reported an effects-directed analysis study of the estrogenic response in OSPW, but unfortunately they did not analyze their fractions in positive ion mode, thus even the source of the estrogenic response still remains uncertain.

2.6 Environmental significance

Naphthenic acids have long been proposed to be the primary toxic constituent of OSPW,[24,60] and the current study provides new systematic evidence by use of an EDA to support this. Nevertheless, results of the current study provide additional evidence that polar neutral organic compounds also contribute to the acute toxicity, which is consistent with the pioneering work of Verbeek *et al*.[61]

For the first time, these toxicologically relevant non-carboxylic acid chemical classes were shown to include O_2^+, O^+, OS^+, and NO^+ species. More EDA testing with other aquatic species, and by use of other relevant endpoints (e.g. growth, reproduction, development), will increase confidence for generalizing of these data. These data should be considered going forward in evaluating the endpit lake strategy in the oil sands region of Alberta, and also for environmental water monitoring around oil sands end-pit lakes and tailings ponds.

Chapter 3: Predicting acute toxicity of dissolved organic fractions of OSPW to embryos of fathead minnow (*Pimephales promelas*).

3.1 Preface

The use of WQBEL is the preferred approach in the toxicity assessment of complex environmental mixtures, and employs concentration addition for prediction purposes. Without a clear assignment of toxicity to a group(s) of compounds, current regulatory schemes for assessing the toxicity of mixtures cannot be utilized for the dissolved organic fraction of OSPW. Chapter 2 demonstrated the contribution of NA in addition to nonpolar neutral chemical classes to the acute toxicity of the dissolved organic fraction of OSPW. Although we have identified classes of compounds contributing to the acute toxicity of OSPW, the identities and toxicities of specific chemical species remain little understood. Work in Chapter 3 builds on that of Chapter 2 by further investigating the contribution of individual chemical species and chemical classes to the toxicity of OSPW by development of a predictive acute aquatic toxicity model for the dissolved organic fraction of OSPW to embryos of fathead minnow. Recently published bioaccumulation estimates for chemical species of the dissolved organic fraction of OSPW, HPLC-Orbitrap-uHRMS and toxicity data generated in Chapter 2 will be used.

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Author contributions:

Garrett D. Morandi (University of Saskatchewan) conceived, designed, and managed model development, generated and analyzed data, prepared all figures, and drafted the manuscript.

Drs. Jonathan W. Martin, and Kun Zhang (University of Alberta), and Drs. Steve B. Wiseman, Rishikesh Mankidy and John P. Giesy (University of Saskatchewan) provided inspiration,

scientific input, and guidance, commented on and edited the manuscript, and provided funding for the research.

3.2 Abstract

Dissolved organic compounds in oil sands process-affected water (OSPW) are known to be responsible for most of its toxicity to aquatic organisms, but the complexity of this mixture prevents use of traditional bottom-up approaches for predicting toxicities of mixtures. Therefore, a top-down approach to predict toxicity of the dissolved organic fraction of OSPW was developed and tested. Accurate masses (i.e. m/z) determined by ultrahigh resolution mass spectrometry in negative and positive ionization modes were used to assign empirical chemical formulas to each chemical species in the mixture. For each chemical species, a predictive measure of lipid accumulation was estimated by stir-bar sorptive extraction (SBSE) to poly(dimethyl)siloxane, or by partitioning to solid-supported lipid membranes (SSLM). A narcosis mode of action was assumed and the target-lipid model was used to estimate potencies of mixtures by assuming strict additivity. A model developed using a combination of the SBSE and SSLM lipid partitioning estimates, whereby the accumulation of chemicals to neutral and polar lipids was explicitly considered, was best for predicting empirical values of LC50 in 96-hr acute toxicity tests with embryos of fathead minnow (*Pimephales promelas*). Model predictions were within 4-fold of observed toxicity for 75% of OSPW samples, and within 8.5-fold for all samples tested, which is comparable to the range of inter-laboratory variability for *in vivo* toxicity testing.

3.3 Introduction

The oil sands regions of northern Alberta, Canada, contain among the largest proven reserves of petroleum in the world. Oil sands process affected water (OSPW) is a by-product of extraction of bitumen from oil sands and is a mixture of residual hydrocarbons, silts, clays and dissolved organic and inorganic constituents.[51,52] OSPW is acutely and chronically toxic to a range of organisms and is stored in tailings ponds during the active life of oil sands surface mines.[52,87] When surface mines are closed, or when the OSPW is no longer required for extraction of bitumen, OSPW must be remediated, and ultimately must be hydraulically reconnected with the natural environment.[88] To this end, Alberta's endpit lake (EPL) strategy designates previously mined-out areas for long-term storage and remediation of process affected materials, including OSPW.[87] Over time, natural degradation within EPLs is hoped to attenuate toxicity of OSPW. BaseMine Lake (BML) was established in 2012 and is the first commercial test of the EPL strategy.

OSPW is a complex mixture of organic compounds containing naphthenic acids (NAs), oxidized NAs and related organic acids containing sulphur or nitrogen, as well as non-acidic polar neutral substances.[29,31,66–69] Because natural *in situ* aging or treatment of OSPW by activated charcoal adsorption or ozonation significantly attenuates, or removes, all toxic effects of OSPW, it is accepted that the dissolved organic fraction is responsible for most acute toxicity to aquatic organisms.[13,35,72,74,83,89] Advances in identification of dissolved organic compounds in OSPW, by ultrahigh resolution mass spectrometry (uHRMS), has improved understanding of its composition. By measurement of accurate mass (i.e. m/z) in both positive ($^+$) and negative ($^-$) ionization modes, this technique facilitates the identification of chemical "species" based on empirical formulas, and the binning of these species into broader "heteroatomic classes" sharing the same numbers of heteroatoms (i.e. oxygen, sulfur and nitrogen).[29,31,68]

The specific organic compounds responsible for the acute toxicity of OSPW has been the focus of much research.[9,35,61,75] It has long been reported that NAs (O_2^- empirical formula class, general formula $C_nH_{2n+z}O_2$ where z represents the number of hydrogen atoms absent due to rings or double bonds) are the primary agents causing acute toxicity.[24,26,60,61,63,90] This assumption is further supported by their elevated concentrations in OSPW and in collected fractions with acute toxicity.[63,91] However, recently by use of a bioassay effects-directed analysis (EDA) for BML OSPW it was demonstrated that in addition to NAs, other heteroatomic classes also contribute to acute toxicity of OSPW (i.e. O^-, SO_2^-, O^+, O_2^+, SO^+ and NO^+).[92] Some of these non-acidic chemical classes also showed high predicted propensity to accumulate in storage lipid.[82,93]

Narcosis, a reversible mode of toxic action, has been suggested as the mode of acute toxicity of OSPW, in part because organic extracts of OSPW demonstrate steep dose-response relationships similar to other narcotic chemicals.[9,63,80,92] It is accepted that the onset of toxic effects of narcotic chemicals is related to an aquatic species-specific concentration in lipid, termed the critical body burden (C_{bb}). Toxic potencies of narcotic chemicals are related to their potential to accumulate in lipids, specifically, it is the volume of molecules dissolved in lipid, especially the phospholipid bilayer of membranes. Once dissolved in the membrane narcotic molecules disrupt a range of processes including membrane fluidity, gap-junction cell-cell communication and activities of membrane-bound enzymes. Traditionally the tendency of a molecule, specifically a neutral molecule to partition into these lipids has been described by use of the chemical independent

parameter, octanol-water partition coefficient (K_{OW}).[94–97] To this end, the Target Lipid Model (TLM) has been developed to predict toxicity of narcotic chemicals to a wide range of species by use of a linear free energy relationship (LFER) that relates toxicity to K_{OW} and a species' C_{bb} (Equation 1).[98] In this equation, *LC50* is the concentration required to cause 50% mortality in milimol per litre (mmol/L), *m* is the universal narcosis slope of Van Leeuwen *et al.*,[99] BP_i is a compound-specific bioaccumulation potential, traditionally termed K_{OW}, Δc is a chemical class specific correction factor and *b* is an aquatic species specific C_{bb} (Equation 1).

$$Log\ (LC50) = m\ log(BP_i) + \Delta c\ + log\ (b) \qquad (1)$$

It is now recognized that the C_{bb} of a narcotic chemical is a function of accumulation into both neutral and polar lipids of fish.[100] Although K_{OW} accurately describes the accumulation of narcotic chemicals into neutral storage lipids, it cannot be used to accurately assess accumulation of narcotics, particularly polar chemicals, into polar lipids, such as phospholipids.[100,101] Improvements in predicting the aquatic toxicity of polar chemicals acting by a narcosis mode of action have been made by accounting for the accumulation of polar chemicals to phospholipids in addition to neutral storage lipids.[100,102]

Biomimetic approaches using solid sorbents facilitate prediction of the potential for compounds to be accumulated into lipids. Uptake from water by a surrogate lipid material, such as poly(dimethyl)siloxane (PDMS), measures the fraction of neutral organic compounds that is freely available for uptake into an organism, and by use of previously defined relationships can be used to predict K_{OW} and accumulation potential into neutral storage lipids.[103,104] In addition to neutral lipids which make up approximately 6% of the tissues of fish, polar lipids account for up to 1.25% of total lipids in fish and are known to be a target for polar organic chemicals acting by a narcosis mode of action.[100–102] Extending surrogate lipids such as PDMS to assess accumulation potential of polar organic chemicals can result in under predictions of accumulation because it does not account for interactions of chemicals with relatively polar (and charged) phospholipids.[101,105] Solid-supported lipid membranes (SSLM), composed of a phospholipid bilayer, offer an approach which improves estimates of bioaccumulation for ionic

and polar organic chemicals. Analogous to K_{OW}, the partition coefficient of a chemical between the SSLM and water, known as membrane affinity (D_{MW}), can be derived to more accurately predict the bioaccumulation potential of such chemicals.[106,107]

Acute toxicities of mixtures of petroleum hydrocarbons are best described by a narcosis mode of action.[108–110] Toward this end, the PETROTOX model has been developed to predict toxicities of mixtures of petroleum hydrocarbons from knowledge of the relative proportions of hydrocarbons and use of a multi-component fate and effect model.[110] That approach has been used previously by identifying constituents by use of two-dimensional gas chromatography in combination with flame ionization detection, followed by classification of constituents into hydrocarbon blocks based on carbon number and compound class. Representative structures are assigned to hydrocarbon blocks by use of chemical characterization data (Ex: carbon number and hydrocarbon class) for calculation of predicted environmental fates and distribution, and inherent toxicity of individual hydrocarbons in the aqueous phase is estimated by use of the TLM. Mixture effects are assessed by use of the toxic unit (TU) approach, assuming strict additivity of hazard. By use of this model, the relative hazard of a variety of petroleum mixtures can be assessed in a risk assessment framework.

The purpose of the present study was to develop a model to predict acute lethality of the extractable organic fraction of OSPW to embryos of the model fish, fathead minnow (*Pimephales promelas*). In contrast to previous models that predict toxic potencies of mixtures, here an approach was taken whereby identification of chemical species and their potentials to accumulate in neutral and polar lipids were determined experimentally for the complex mixture. Identification was by use of accurate masses from Orbitrap-uHRMS in both positive and negative ionization modes. The propensities of chemical species to accumulate in lipids were assigned based on previous results from stir-bar sorptive extraction (SBSE) and SSLM in BML-OSPW samples. Predictions of toxic potencies of individual species were then made by use of the TLM, and mixture toxicity was predicted by use of the TU approach, assuming strict additivity. By use of the two available empirical data sets for determining potentials of organic compounds to accumulate, four models were developed and compared. To assess performance of models and make comparisons among their predictions over a range of extracts and fractions,

toxicity predicted by the various models was compared to empirical data from acute toxicity tests with embryos of fathead minnow.

3.3.1 Model rationale

Previous petroleum mixture toxicity models require the classification of mixture constituents to specific hydrocarbon blocks by use of gas chromatography, and assign associated structures and chemical properties. Due to the complexity of organic constituents and lack of authentic standards, it is currently unfeasible to assign structures to specific compounds identified in OSPW. Therefore, HPLC-Orbitrap-uHRMS will be used to 'label' compounds based on empirical formulae derived from accurate masses, by use of previously published bioaccumulation measurements.[82] In this way, values of accumulation potential can be assigned to individual species detected in the mixture. Using some assumptions, water concentrations of species can be established and the acute toxicity of each species can be estimated by use of the TLM. Using the toxic unit approach, the acute toxicity of the dissolved organic fraction of OSPW can be assessed.

3.4 Materials and methods

3.4.1 Model development

Estimates of toxic potencies of mixtures by use of chemical composition require three basic elements; identification of mixture constituents, an assessment of their concentration in the mixture, and the inherent toxic potency of each constituent for a defined endpoint. The approach taken in this work is described below, including assumptions made in development of the model. An illustration of steps involved in model toxicity predictions and verification also are outlined in Figure 3.1.

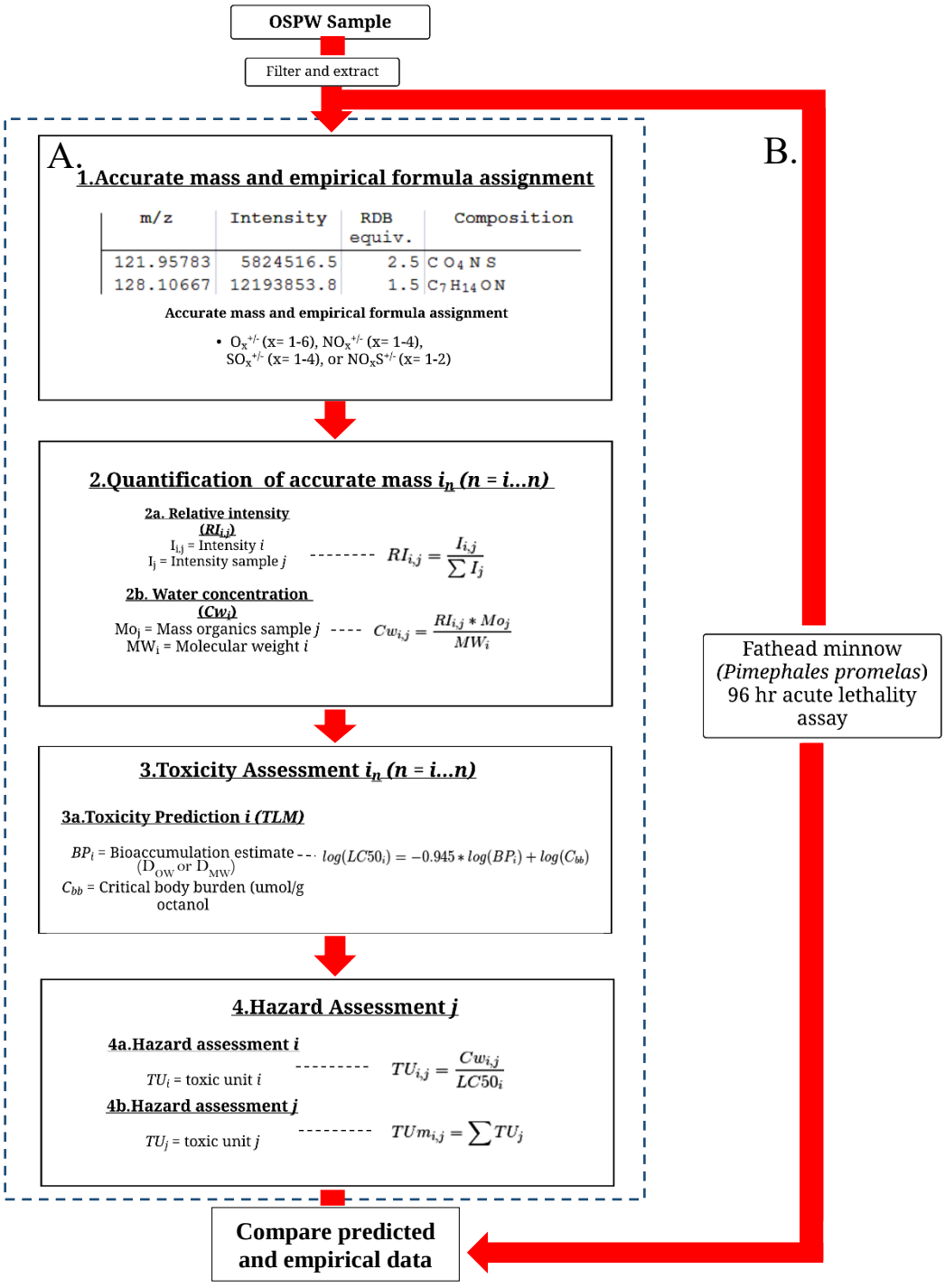

Figure 3.1 Flow chart outlining A) steps for predicting the 96 hr lethal concentration to elicit a 50% response (LC50) and, B) the verification of the aquatic toxicity model, for embryos of fathead minnow exposed to extractable organics from OSPW.

3.4.2 Data compilation

For model development, empirical acute toxicity, estimated potential to accumulate in lipids, and chemical characterisation data of samples were compiled from Morandi *et al*.,[92] and two studies by Zhang *et al*.[82,93] Because the primary goal of the EDA approach is to isolate active compounds or chemical classes, it inherently produces samples (fractions) which can be used to test the accuracy and specificity of the model. Therefore, the model presented here is complimentary to the EDA approach and can be used to further understand contributions of certain chemical classes to the acute toxicity of OSPW, to assess potential critical mechanisms of toxic action of OSPW, and make predictions of acute lethality to aquatic organisms. The EDA fractions produced previously were used here for development of the model.[92] Estimates of pH dependent octanol-water distribution ratio (D_{OW}) and phospholipid membrane-water distribution ratios (D_{MW}) for each species in OSPW (i.e. for each distinct species) were taken from Zhang *et al*.[82,93] Where a D_{OW} or D_{MW} was not reported for a certain species detected in OSPW, estimates were made by use of chemical class-specific regressions for groups of chemical species, separated into double bond equivalent (DBE) groups, relating log D_{OW} or log D_{MW} to molecular mass.

3.4.3 Sample characterization

The profile of dissolved organic chemicals in samples was determined by use of high pressure liquid chromatography with Orbitrap uHRMS detection (Orbitrap Elite, Thermo Fisher Scientific, San Jose, CA, USA) as described by Pereira *et al*.[29] Details of the analytical method are provided in Appendix B. In this study, individual species identified by use of Orbitrap uHRMS were tracked and referred to as individual chemical species by use of their accurate mass and molecular formula in each ionization mode as described by Pereira *et al*.,[29] for the O_2^- and O_2^+ formula classes (i.e. a distinct empirical formula detected in negative mode was named separately from the same empirical formula detected in positive mode). Detected species were assigned to bins based on heteroatomic empirical formula class in negative (-) or positive (+) ionization modes: O_x (where x= 1-6), NO_x (where x= 1-4), SO_x (where x= 1-4), or NO_xS (where x= 1-2).

3.4.4 Constituent concentrations

Models of toxicity for exposure of aquatic organisms to petroleum hydrocarbons employ a multi-compartment fate model to predict the environmental distribution of constituents of mixtures, followed by an assessment of potential effects of the aqueous phase.[108,110] However, because the mixture of interest was the extractable dissolved organic phase of OSPW filtrate (1.2 µm), the distribution of chemical species that were detected was assumed to be 100% in the aqueous phase, thus simplifying model assumptions. Therefore, concentrations of each species were calculated by use of equations 2 and 3. Where $RI_{i,j}$ is the relative intensity of species *i* of sample *j* calculated as the intensity of species *i,j* ($I_{i,j}$) over the sum of the responses of all species detected in sample *j* (Equation 2). Where $Cw_{i,j}$ is water concentration of species *i,j* (mmol/L) calculated as the $RI_{i,j}$ multiplied by the gravimetric mass of organics in the sample (Mo_j) over the molecular mass of the species $(MM_{i,j})$ (equation 3). It was assumed that the total response of a sample was accounted for in the measured gravimetric mass of the dried organic fraction and that individual chemical species had a response factor of one (1.0) in the mass spectrometer. Thus, the molar concentration of each species was defined only by its relative intensity, its molecular mass and the total gravimetric mass of dissolved organics.

$$RI_{i,j} = \frac{I_{i,j}}{\sum I_j} \qquad (2)$$

$$Cw_{i,j} = \frac{RI_{i,j} * Mo_j}{MW_i} \qquad (3)$$

3.4.5 Predicted toxic potencies

Acute toxic potency of each chemical species was predicted assuming a narcosis mode of action by use of the TLM (equation 1).[111] The C_{bb} for fathead minnow, 105 µmol/g octanol, has been derived from a dataset of 182 data points, consisting of a variety of chemical classes including halogenated and non-halogenated aliphatic and aromatic hydrocarbons, polycyclic aromatic hydrocarbons (PAHs), alcohols, ethers, furans, and ketones.[111] The LC50 of a species *i* ($LC50_i$) was estimated using equation 4. Where BP_i is the chemical species specific bioaccumulation potential (D_{OW} and/ or D_{MW}) and C_{bb} is the fathead minnow specific critical body burden

(equation 4). Chemical class corrections (Δc) were not applied because where only the molecular formula is known it is not feasible to assign the individual species to functional group chemical classes (e.g. alcohol, ether, furan, ketone etc.). The TLM has been developed by use of K_{OW} to describe the accumulation of narcotic chemicals. However, because there are recognized deficiencies in using K_{OW} to predict the accumulation and thus toxicity of polar organic chemicals, it was instructive to investigate the use of D_{MW} in predicting the toxicity of chemical species in OSPW, many of which are known to be acidic.

$$Log\ (LC50)_i = -0.945 log\ (BP)_i + log\ (C_{bb}) \quad (4)$$

3.4.6 Prediction of toxic potencies in mixtures

Contribution of chemical species to mixture toxicity was assessed by use of toxic units (TU) with lethality as the endpoint predicted. This approach normalizes the aqueous concentration of a chemical by its endpoint specific toxicity, the LC50 in this work (equation 5). Therefore, the relative hazard ($TU_{i,j}$) of species i,j was calculated by use of equation 5, as $Cw_{i,j}$ over $LC50_i$ (Equation 5). Toxicity of the sample (TUm_j) was calculated as the sum of $TU_{i...n,}$ of sample j (Equation 6). When TUm_j was equal to or greater than 1, samples were expected to elicit 50% mortality or greater.

$$TU_{i,j} = \frac{Cw_{i,j}}{LC50_i} \quad (5)$$

$$TUm_j = \sum TU_j \quad (6)$$

3.4.7 Model parameterization

Because the choice of partition coefficient can affect interpretation of toxicity,[101] it was of interest to investigate the effect of BP_i on estimates of toxic potency. Therefore, four models were developed, each of which used different estimates of distribution between water and

organisms. The first model (Model I) estimated toxicity of mixtures by use of measured a D_{OW} for each chemical species. The second model (Model II) estimated toxicity by use of D_{MW} only, and any chemical species which showed no significant partitioning ($D_{MW} < 1$) in membrane partitioning experiments were ignored by the model. The third model (Model III) was developed by use of both Model I and Model II, whereby toxicity estimates were made by use of D_{MW} when measured data was available, in preference of D_{OW}. A fourth model (Model IV) was developed by use of all available data, assuming that chemical species with both a measured D_{OW} and D_{MW} partition into neutral and polar lipids and contribute to toxicity.

3.4.8 Statistical analysis

Statistical analyses were performed by use of SPSS software (SPSS Inc. Chicago, IL, USA). LC50 values from Morandi *et al*.,[92] were normalized to the gravimetric mass (Table 3.2) of the respective samples. Spreadsheet models were developed by use of Excel (2013) (Microsoft Excel, Microsoft, Redmond, WAS, USA). Predictions of the four models were compared to empirical data for lethality. Goodness-of-fit statistics, mean and median residuals were calculated as the mean or median difference between observed and predicted LC50 values, the mean absolute deviation (MAD) was calculated as the mean of the absolute value of the residuals, and the root-mean square deviation (RMSD) was calculated as the square root of the mean of the residuals squared.

3.5 Results and discussion

3.5.1 Model verification

The distribution of heteroatom classes in samples are presented (Figure B.1). Values of LC50 predicted by the various models are compiled (Table 3.1) and compared to observed LC50 values, with a line showing one-to-one correspondence (Figure 3.2). Observed toxicity spanned two orders of magnitude, which was similar to the ranges of predictions made by use of Model II, III, and IV. Predictions made by use of Model I were more variable, spanning three orders of magnitude (Table 3.1). The mean, median, log residual error, MAD and RMSD were also compiled (Table 3.2). The log residual plot of Model I, II, III and IV demonstrated no obvious deviations from the mean observed LC50 (Figure B.2) and were log normally distributed (Figure B.3). Because no significant lethality was observed for the F1-BE and F1-NE1 samples, they

were not included in the residual analysis. In general, predictions of acute lethality from each model compared well with observed toxicity and the goodness- of- fit statistics of Model II, III and IV were similar, and were better relative to Model I.

Table 3.1 Comparison of model predicted LC50, and empirical LC50 values for embryos of fathead minnow exposed to samples of the extractable dissolved organic fraction of OSPW. The measured gravimetric mass of the samples corresponding to its equivalent in 100% OSPW are presented as well.

Sample	Gravimetric mass, 100% effluent equivalent (mg/L)	Predicted LC50 Model I (mg/L)	Predicted LC50 Model II (mg/L)	Predicted LC50 Model III (mg/L)	Predicted LC50 Model IV (mg/L)	Observed LC50 (mg/L)
F1-NE	50.0	32.0	76.0	76.0	23.2	36.0
F1-AE	103	1.64 E4	1.05 E3	1.05 E3	990	857
F1-BE	14.0	4.14 E3	990	1.08 E3	280	>140
F1-Pool	167	737	242	239	159	1.33 E3
F2-NE1	15.7	17.7	64.2	65.5	16.2	>157
F2-NE2	34.3	38.4	97.1	98.1	29.7	66.2
F2-Pool	50.0	32.7	32.0	78.0	22.6	89.5
F3-NE2a	20.0	852	104	104	92.8	14.6
F3-NE2b	14.0	31.0	110	108	28.3	30.5
F3-Pool	34.0	50.2	10.5	110	32.3	23.1
Maximum		1.64 E4	1.05 E3	1.08 E3	990	1.33 E3
Minimum		17.7	10.5	65.5	16.2	14.6

Table 3.2 Calculated mean residual, median residual, mean absolute deviation (MAD) and root mean square deviation (RMSD) between predicted and observed LC50 for Model I, II, III and IV.

	Mean residual	Median residual	MAD	RMSD
Model I	0.69	-0.05	3.74	3.44
Model II	0.13	0.29	4.30	1.35
Model III	0.53	0.57	3.90	1.45
Model IV	-0.31	-0.26	4.74	1.35

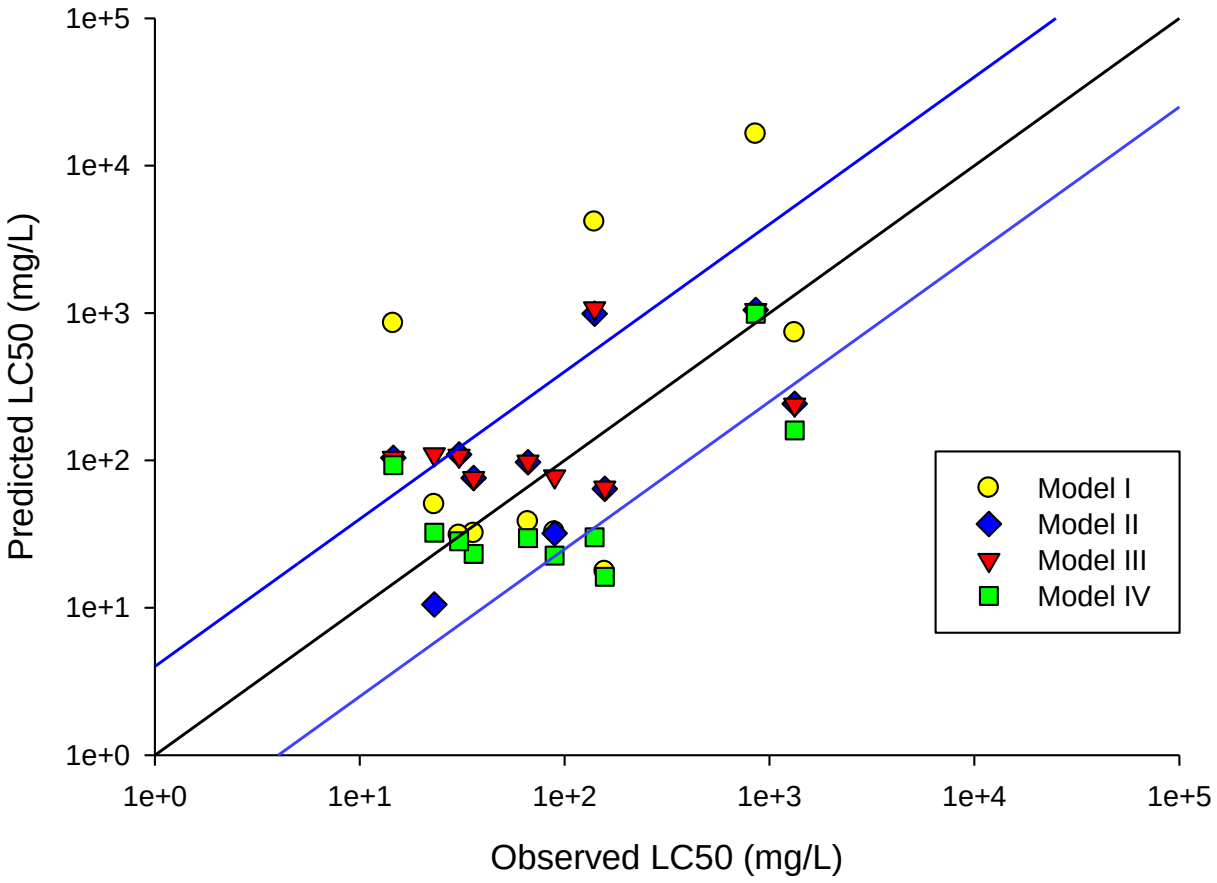

Figure 3.2 Comparison of model predicted LC50 to observed LC50 for embryos of fathead minnow exposed to samples of OSPW for 96 hr. Yellow circles represent predictions made by use of Model I, blue diamonds represent predictions made by use of Model II, red triangles represent predictions made by use of Model III and green squares represent predictions made by use of Model IV. The black solid line represents the line of perfect agreement and blue solid lines represent a 2-fold error.

Analysis of toxicity databases has demonstrated significant variations among acute aquatic toxicity tests for the same chemical, experimental design and species. Deviations from the geometric mean for a given chemical and endpoint, of a factor-of 2, 5, and 10 were found to encompass 57, 86 and 94% of acute lethality results respectively.[112] Furthermore, work by Baas *et al*.,[113] demonstrated deviations for acute lethality of narcotic chemicals among acute toxicity tests from different laboratories of 2- to 8-fold. A 2-fold difference from empirical data encompassed 50, 37.5, 25.0 and 50% of predictions by use of Model I, II, III and IV respectively. These results compared well with the performance of the PETROTOX model which was 42.9% for petroleum products, and is similar to the 2-fold range associated with the TLM.[109,110] In addition, 75.0, 62.5, 75.0 and 75.0% of predictions were within a 4-fold difference of observed toxicity for Model I, II, III and IV respectively and results compared well with the 69.4% value observed by Redman *et al*.[110] The F1-BE sample was predicted to be non-toxic within the range of tested concentrations, agreeing with the observed lack of lethality in the assay. By use of a 5-fold safety factor, the false positive rate for all models was 1 in 9, the F2-NE1 sample. The F2-NE1 sample did not cause acute lethality, but each of the four models predicted this fraction would cause acute lethality within the range of concentrations tested. Using the highest tested concentration for comparison, Models I, II, III and IV were greater than 8, 2, 2 and 9-fold different, respectively (Table 3.1). Similarly, the false negative rate for Models I, II, III and IV were 2, 1, 1, 1 of 9 predictions, respectively. Toxic potency of sample F3-NE2a was underpredicted by use of all four models, but the difference from the empirical dataset was less than 8.5-fold for Model II, III and IV while Model I was different by greater than a factor-of 58. Furthermore, the toxicity of sample F1-AE was under-predicted by a factor of 19 by Model I.

A plot of predicted *TUm* and observed mortality (Figure 3.3) can be used to identify model inadequacies. In general, predictions of Model II, III and IV were accurate to within a factor-of 5 compared to the empirical dataset. Significant acute lethality occurred at 0.19 TU (F3-NE2a), and the 50% effect level spanned 0.140- 5.54 TU (Figure 3.3). By use of Model II, III, and IV toxicity of the F1-Pool sample was over-predicted by a factor of 5.56, 5.48, and 8.32 respectively, while toxicity of F3-NE2a was under-predicted by a factor of 7.14 for Model II and III and 6.36 by use of Model IV. When a safety factor of 8.5-fold was applied, all predictions of toxicity were protective, as well as being within inter-laboratory variation, or accuracy of empirical tests.[112,113] Predictions made with Model I were less accurate, since two samples

exceeded a 10-fold difference from observed effects (F1-AE and F3-NE2a). Significant acute lethality occurred at 0.023 TU (F3-NE2a), and the 50% effect level spanned 0.017- 2.963 TU.

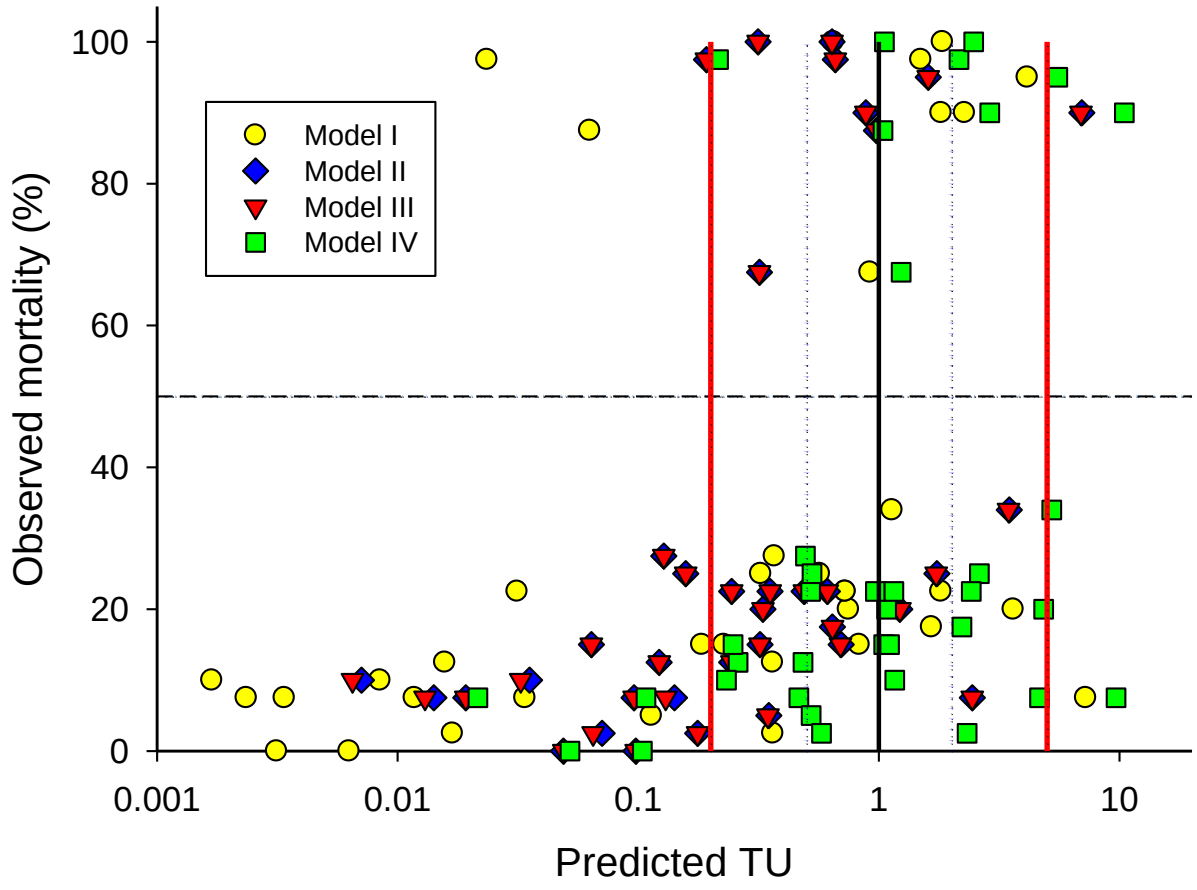

Figure 3.3 Comparison of model predicted toxic units (TU) to observed mortality for embryos of fathead minnow exposed to samples of OSPW. Yellow circles represent predictions made by use of Model I, blue diamonds represent predictions made by use of Model II, red triangles represent predictions made by use of Model III and green squares represent predictions made by use of Model IV. The black solid line corresponds to 1 toxic unit, the dotted black line represents 50% mortality, blue dotted lines area 2-fold error and red solid lines are 5-fold error.

3.5.2 Model selection

As demonstrated above, differences in predicted toxicity were observed among models, and some discrepancies were found when comparing each to empirical data. For chemicals causing

lethality via a narcotic mode of action, toxic potency is directly proportional to the fraction that is accumulated into the body of a particular species.[114] Therefore, the C_{bb} is aquatic species-specific and chemical independent, and the toxic potency of a compound acting through this mechanism of action is dependent solely on its distribution from water to the body.[95,96] To exert a toxic effect, chemicals need to be accumulated into the body, or at least interact with membranes of the gill. The lipid accumulation of polar organic chemicals is not well predicted by use of D_{OW}, and D_{MW} is known to better describe the behaviour of these chemicals.[115] Because OSPW is composed of both polar and neutral polar organic chemicals, it was instructive to investigate the effect that potential to accumulate in phospholipids had on prediction of toxicity and how explicit consideration of chemical distribution among lipid types affected accuracy of predictions of toxicity. Model II, III and IV incorporated D_{MW} and had better goodness-of-fit statistics, accuracy, specificity and robustness when compared to Model I (Table 3.2, Figure B.2, B.3), which was based solely on D_{OW}. In addition, Model IV was developed assuming narcotic chemicals distribute among polar and neutral lipids, and the improved performance of this model, demonstrated the utility of explicitly considering the differential accumulation among lipid types in predicting the acute toxicity of the dissolved organic fraction of OSPW.

OSPW is a mixture and its composition, to some extent, is known to be variable spatially and temporally.[116] Therefore, a model to predict toxicity in a given sample must be sufficiently robust to describe toxicity of varying mixtures. Although goodness of fit statistics for Model II and IV were similar, inclusion of all available D_{OW} and D_{MW} data into Model IV resulted in the model having greater robustness. Due to this, there is greater confidence in predictions of toxic potencies made by use of Model IV because of its increased domain of applicability and explicit assessment of chemical accumulation in polar and neutral lipids.

3.5.3 Contribution of chemical classes to the acute lethality of BML-OSPW

Incorporation of hazard assessment frameworks into the EDA approach has previously been used to assess relative contributions of chemical classes to the toxic potency of mixtures.[109,117] The F1-Pool sample contains all dissolved organic compounds in BML-OSPW.[118] In the work of Morandi *et al.*,[92] NAs (i.e. the O_2^- class) were highlighted for their contribution to acute toxic potency of OSPW due to their large relative abundance in the most toxic tertiary fraction. In

addition, a few other chemical classes $O^{+/-}$, O_2^+, SO^+, NO^+ and SO_2^- were cited for their contributions to acute toxic potency of BML-OSPW.[92] Therefore, it was of interest to investigate the predicted contribution of these previously identified chemical classes to the acute lethality of the F1-Pool sample. By use of Model IV, the sum of TUs for the chemical classes: $O^{+/-}$, $O_2^{+/-}$, SO^+, NO^+ and SO_2^-, accounted for 97.3% of total calculated TUs (i.e. for all detected chemicals) in the F1-Pool sample, while representing less than 43.4% of total mass spectral intensity. The chemical classes $O^{+/-}$, $O_2^{+/-}$, SO^+, NO^+ and SO_2^- are predicted to account for a disproportionate amount of toxicity, thereby demonstrating their combined potency relative to other chemical classes, which accounted for less than 3% of total calculated TUs and greater than 56% of total mass spectral intensity. Contribution of specific chemical classes (i.e. O_2^-, O_2^+, NO^+ and SO^+) to total TU_m is displayed in Figure 3.4. Normalization of total calculated TU of each chemical class by its percent relative mass spectral response separates chemical classes based on their relative toxic potencies (Table 3.3). By use of this approach, although $O_2^{+/-}$, NO^+ and SO^+ chemical classes contributed the majority of total TU of the mixture, based on their relative intensities, the SO^+ and SO_2^- chemical classes are suggested by this result to be among the most potent toxic chemical classes in OSPW. Interestingly, the SO^+ chemical class was among the most hydrophobic chemical classes in OSPW, based on its partitioning to PDMS and SSLM, thus this class may be relatively bioaccumulative if not metablized.[82,93] Evidence presented here agrees with previous results that the $O^{+/-}$, $O_2^{+/-}$, SO^+, NO^+ and SO_2^- classes are together responsible for the majority of acute toxicty of OSPW,[82,92,93] but further suggests potential contributions from SO^+ and SO_2^-. It is also important to note the utility of the proposed model in improving our understanding of the contribution of chemical classes to the toxicity of a mixture. Previous work has identified NAs as the most toxic chemical species in OSPW, whereas evidence here suggests that $SO^{+/-}$ and NO^+ compounds may be more potent and in some cases, contribute more to toxicity.

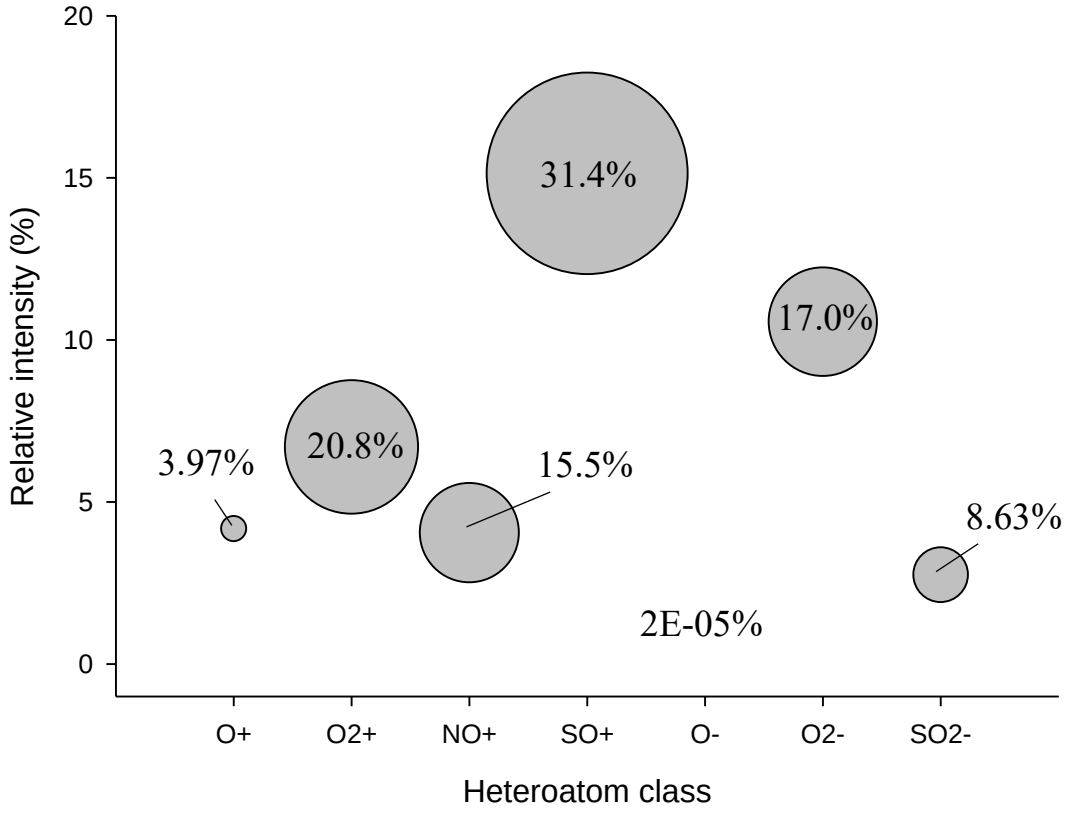

Figure 3.4 Percent contribution of total toxic units (TU) of the chemical classes in the F1-Pool sample identified by Morandi *et al*.,[25] plotted as a function of relative intensity. Relative intensity was calculated as the total mass spectral intensity of a chemical class over the sum of the responses of all chemical species detected in sample.

Table 3.3 Relative potencies of the chemical classes identified as acutely toxic by Morandi *et al*.[92] Values were calculated as the total TU of a chemical class over its percent relative response by use of Model IV.

Chemical Class	Potency (TU per unit percent relative intensity)
SO^+	8.13
SO_2^-	3.28
NO^+	2.43
$O2^-$	1.68
$O2^+$	1.44
O^+	0.99
O^-	4.60E-4

3.5.4 Limitations of the model

Deviations of predicted toxicity from observed toxicity occurred and might be related to assumptions made in development of the model. In the current study, concentrations of individual chemical species were calculated assuming a mass spectral response factor of one (Equation 3). It is known that the response of chemicals in the OSPW matrix differ from their responses in a more simple solution,[119] and it is an oversimplification to assume that each species has the same mass spectral response per unit mass injected. Nevertheless, this approach was the only reasonable assumption that could be made with available data. Due to the complexity of OSPW (e.g. hundreds of thousands to millions of individual isomers),[119,120] identities of the majority of compounds were not known and authentic standards cannot be synthesized or purchased for the chemicals in the dissolved organic fraction.

In addition, a narcosis mode of action was assumed for toxicity predictions. This assumption might not accurately represent the potencies of all components of the mixture, since both neutral and polar organic compounds are known to act via a number of different modes of action.[78] Previously, comparisons of observed and model predicted toxicity have been used to classify chemicals by their mode of action.[78] However, because the identities and specific chemical

characteristics of the majority of chemicals in OSPW are unknown, this approach could not have been taken and comparisons can solely be drawn between whole mixture toxicity predictions and observed toxicity. The TLM assumes equilibration of chemicals between the aqueous and lipid phases and ignores the effect of metabolism on chemical accumulation. Previously, it has been suggested that the SO^+ and NO^+ chemical classes might be metabolized,[93] this might affect the accumulation of these chemicals into the organism, resulting in less than predicted toxicity as observed with the F1-Pool sample. Furthermore, deviations observed might be related to chemical class specific inadequacies of the TLM in describing toxicity and as demonstrated in previous works the application of correction factors might improve model predictions.[96,111] Due to limitations from a lack of knowledge of identities of individual chemicals in OSPW, corrections of LC50 values, such as those suggested by McCarthy *et al*.,[96] cannot be applied. Despite assumptions made in the development of the presented predictive aquatic toxicity model, predicted LC50 values did not differ by greater than 8.5-fold from empirically derived toxicity data including the most complete mixture, representing that in BML.

3.6 Environmental relevance

Currently it is not practical to chromatographically separate and identify the structure of each organic chemical compound in OSPW. For example, recent applications of supercritical fluid chromatography demonstrate the utter complexity of isomers that can be present for various chemical species.[119] Nevertheless, models for assessment of hazards of petroleum mixtures by use of its chemical composition require identification, categorization and structural assignment for all components of the mixture, followed by prediction of their environmental fate and subsequent effects. So, even if all of the compounds could be separated and structures elucidated, the assessment would require toxicological information on each of those species. Here, a novel approach was developed whereby mixture components are not identified explicitly, but rather, characterized and labeled by use of their accurate masses under both negative and positive ionization. In this way, the chemicals can be binned into classes based on empirical formulas derived from the identified accurate mass. Furthermore, this approach can be used to operationally define distribution coefficients for each of the identified accurate masses using biomimetic approaches, which when combined with a simple extraction method and Orbitrap-uHRMS can be used to develop a model to predict the 96 hr LC50 of the dissolved organic

fraction of OSPW to embryos of fathead minnow. This approach allowed prediction of the toxicity for complex mixtures with accuracies well within the range of empirical measures. In addition, the developed model was used to assess the contribution of previously defined chemical classes to the toxicity of OSPW,[118] complimentary to the EDA approach, and highlighted the potential contribution of SO^+ and SO_2^+ chemical classses to toxicity. We propose that the model developed during this study is sufficiently accurate and robust to make predictions of potential acute lethality. These predictions, along with application of an appropriate safety factor of 10 can allow calculation of the dilution required to allow releases of the complex mixture to the environment while protecting aquatic organisms from acute toxicity. Furthermore, if the primary mode of toxic action is narcosis, application of a relatively small acute to chronic ratio will allow the model to be expanded to protect environments from chronic effects of diluted OSPW.

Chapter 4: Characterization of the mechanism of action of acutely toxic fractions of OSPW by use of the *E. coli* genome reporter assay.

4.1 Preface

The molecular mechanism(s) of toxicity of OSPW are not well characterized. Chapter 2 demonstrated the contribution of NAs in addition to nonpolar neutral chemical classes to the acute toxicity of the dissolved organic fraction of OSPW. Although chemical classes contributing to the acute toxicity of OSPW have been identified, their mechanisms of toxicity remained little understood. Work in Chapter 4 builds on that of Chapter 2 by further investigating the mechanisms of toxicity of chemical species and chemical classes by use of an open format gene reporter system.

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Author contributions:

Garrett D. Morandi (University of Saskatchewan) conceived, designed, and managed the experiment, generated and analyzed the data, prepared all figures, and drafted the manuscript.

Miao Guan (University of Nanjing) demonstrated lab techniques, aided with data analysis and advised on the manuscript.

Dr. Jonathan W. Martin (University of Alberta), Dr. Xiaowei Zhang (University of Nanjing), and Drs. Steve B. Wiseman, and John P. Giesy (University of Saskatchewan) provided inspiration, scientific input, and guidance, commented on and edited the manuscript, and provided funding for the research.

4.2 Abstract

Oil sands process-affected water (OSPW) is generated during extraction of bitumen in the surface-mining oil sands industry in Alberta, Canada, and is acutely and chronically toxic to aquatic organisms. It is known that dissolved organic compounds in OSPW are responsible for most toxic effects, but knowledge of the specific mechanism(s) of toxicity, is limited. Using bioassay-based effects-directed analysis, the dissolved organic fraction of OSPW has previously been fractionated, ultimately producing refined samples of dissolved organic chemicals in OSPW, each with distinct chemical profiles. Using the *Escherichia coli* K-12 strain MG1655 gene reporter live cell array, the present study investigated relationships between toxic potencies of each fraction, expression of genes and characterization of chemicals in each of five acutely toxic and one non-toxic extracts of OSPW derived by use of effects-directed analysis. Effects on expressions of genes related to response to oxidative stress, protein stress and deoxyribonucleic acid (DNA) damage were indicative of exposure to acutely toxic extracts of OSPW. Additionally, six genes were uniquely responsive to acutely toxic extracts of OSPW. Evidence presented supports a role for sulphur- and nitrogen-containing chemical classes in the toxicity of extracts of OSPW.

4.3 Introduction

Oil sands process-affected water (OSPW) is a complex mixture produced as a waste stream of the open-pit surface mining oil sands industry where hot water is used to extract bitumen from the oil sands. Consisting of high concentrations of dissolved bitumen-derived organic chemicals, dissolved salts and metals, and suspended particulate matter, OSPW exhibits both acute and chronic toxicity to a range of organisms.[15,20,118] Because of concerns about adverse effects in the environment, the oil sands industry follows a no-release policy and OSPW is stored on-site in large tailings ponds where it is recycled back into the extraction process. Eventually, all process-affected materials must be remediated and returned to the surrounding environment. However, acceptable remediation techniques and guidelines for evaluating toxic potency prior to release of treated OSPW remain to be established.

Currently it is known that the dissolved organic fraction of OSPW is responsible for most toxicity, however the specific compounds and mechanisms by which these chemicals cause toxicity remains an active field of research.[15] Recently, by use of a bioassay effects-directed

analysis approach, and ultrahigh resolution mass spectrometry (uHRMS) for characterization of OSPW organic fractions, it was demonstrated that acute toxicity of OSPW was due primarily to naphthenic acids (NA), oxygenated-NAs, but also other neutral and basic chemical species containing sulphur and nitrogen.[118,121] It has been suggested that the mechanism of acute toxicity is due to non-polar narcosis.[34,81,118,121] In addition, genotoxicity has been observed in Rainbow trout (*Oncorhynchus mykiss*) hepatocytes exposed to a synthetic OSPW extract.[122] Application of the test systems SOS Chromotest and Ames test have identified concentrated whole extracts of OSPW as genotoxic and mutagenic.[123] However, evidence exists to support the role of other mechanisms of OSPW toxicity.[10]

High throughput open format investigations into the mechanisms of action of chemicals have gained popularity over recent years as new technologies have emerged.[124,125] Previous application of RNAseq to investigate mechanisms of toxicity of OSPW collected from Wet In-pit tailings pond (WIP) following 7-days of exposure of male fathead minnow (*Pimephales promelas*; FHM) demonstrated response of genes related to oxidative metabolism, oxidative stress, apoptosis and immune function.[10] These results are consistent with those observed previously during exposures of embryos of FHM to OSPW which resulted in differential expression of several genes related to oxidative stress and apoptosis, and resulted in greater peroxidation of lipids in exposed embryos.[14] Additional evidence exists to support oxidative stress playing a role in toxicity of WIP-OSPW following exposure of the midge *Chironomus dilutus* (*C. dilutus*) since changes to abundances of transcripts of genes involved in responses to oxidative stress, and peroxidation of lipids were observed in larvae compared to control.[12]

Naphthenic acids contribute to acute toxicity of OSPW, and NA standards and commercial NA mixtures have been used as surrogates to represent OSPW derived NAs.[118,125–128] The live cell genome reporter assay has previously been used to identify a number of novel molecular mechanisms of toxicity of a commercial NA mixture. Differentially expressed genes could be identified in the Pentose Phosphate Pathway, adenosine triphosphate (ATP)-binding cassette transporter complex and SOS response pathway.[125] In this work, the live cell array genome reporter system was used to screen acutely toxic organic extracts of OSPW produced in the previous work of Morandi *et al*.,[118] that were prepared from BaseMine Lake OSPW (formerly WIP) collected in 2012, to elucidate molecular mechanisms of toxic action.

4.4 Materials and methods

4.4.1. Data compilation

Recently, an effects directed analysis of dissolved organic fraction of OSPW provided better understanding of chemical classes that contribute to acute lethality of exposed aquatic organisms.[118] In that study, three rounds of sequential fractionation and toxicity testing were completed, ultimately producing five fractions with observed acute toxicity to embryos of FHM and *Vibrio fisheri*; a pooled sample representative of the whole dissolved organic fraction of OSPW (F1-Pool), round one neutral extractable fraction (F1-NE), round two acidic fraction (F2-NE2) and round three early and late eluting fractions by use of HPLC (F3-NE2a and F3-NE2b) and a second round fraction with no observed LC50 (F2-NE1). Additionally, the chemical profile of each extract was determined by use of uHRMS. Therefore, fractions produced as part of the effects-directed analysis were screened to investigate potential molecular mechanisms of toxicity of the whole mixture, represented by the complete mixture of OSPW (F1-Pool), and how its toxicity is related to toxicity of refined extracts of OSPW. To the knowledge of the authors, this is the first application of open format investigations of molecular mechanisms of toxicity to be conducted in conjunction with effects-directed analysis.

4.4.2 Sample collection and chemical fractionation

A brief overview of the chemical characterization procedure is presented in Appendix C and fractionation methodology in Morandi *et al*.[118] Gravimetric mass of each extract was measured following extraction to establish the concentrations of extracts. Chemical characterization data of each extract was compiled from Morandi *et al*.,[118] and are presented in Figure C.1.

4.4.3 *Escherichia coli* K-12 strain MG1655 gene reporter system

The gene reporter system was purchased from Open Biosystems Thermo Fisher Scientific (Huntsville, AL, USA) and was developed at the Weizmann Institute of Science. Assays were conducted as outlined in Zhang *et al*. (2011). Briefly; 1855 promoter clones (out of 2500 in the whole genome) were grown at 37 °C in a 1× LB-Lennox media with 25 ug/mL kanamycin for 24 h prior to assay. Individual wells are used for each promoter to facilitate the measurement of 1855 individual genes over time. Therefore, this assay facilitates the measurement of promoter activity

by use of fluorescence to monitor gene expression. Assays of cytotoxicity were performed prior to performing the genome reporter assay. Cytotoxicity assays were performed in 96-well plates by use of the parent strain, and exposures were performed in triplicate to four concentrations of fractions for 24 h. For completion of the genome reporter assay, concentrations equivalent to the twenty-percent inhibition of growth (IC20) for cytotoxicity, were used for the F1-Pool, F1-NE, F2-NE2, F3-NE2a and F3-NE2b samples. The F2-NE1 sample was assayed at a concentration of 392.5 mg/L, which was the highest tested concentration, due to lack of observed toxicity. Black 384-well optical bottom plates (NUNC, Rochester, NY, USA), were prepared with 71.25 uL of LB medium per well and incubated for 3.5 h prior to exposure. Following incubation, the optical density (OD) of each well was measured at 600 nanometres (nm) by use of a Synergy H4 hybrid microplate reader (BioTek Instruments Inc., Winooski, VT). Following the initial reading, 3.75 uL of nanopure water (control) or sample of OSPW was added to each well to a final volume of 75 uL. The intensity of green fluorescent protein (GFP) of each well was quantified consecutively every 10 min during the 4 h exposure (excitation/emission: 485 nm /528 nm).

4.4.4 Statistical analysis

Statistical analyses were completed by use of R 2.3.0 (R 2.3.0, Vienna, Austria). IC20 was calculated by use of the probit model. To assess responses of the gene reporter system, linear regressions were used to assess effects of time ($\rho \leq 0.001$) for the response of each gene. Details on the statistical analyses applied have been previously published (Jung *et al.*,[124]; Zhang *et al.*,[125]). Effects on expression were expressed as fold-change relative to control. Fold-changes of 1.5- and 2-fold change were used as cut-off values for gene selection for downstream analysis described below.

4.4.5. Network visualization, pathway analysis and clustering analysis

Lists of genes were developed by use of cutoffs of 1.5- or 2.0-fold changes. The transcriptional network was constructed by use of the ClueGO plug-in of Cytoscape v2.3.3, an open source bioinformatics software platform by use of 1.5-fold gene lists. The ClueGO v2.2.3 enrichment/depletion two-sided hypergeometric test was conducted by use of the Bonferroni step-down correction and used ($\rho < 0.05$) to identify enriched Gene Ontology (GO) terms/ Biological

Processes and Kyoto Encyclopedia of Genes and Genomes pathways (KEGG). Hierarchical cluster analysis (HCA), and Principal component analysis (PCA) were completed by use of R.2.3.0. For PCA, lists of genes identified by use of biological or KEGG pathway analyses were linearly combined as suggested by Ma & Dai prior to analysis.[129] For analysis of uHRMS data, chemical classes were limited to those accounting for a minimum of 5% of total ion count in at least one sample, resulting in a list of 27 chemical classes (Figure C.1). To facilitate easier interpretation of score and loadings plots following PCA analysis, identified biological processes and KEGG pathways, and chemical classes were labeled with identifiers (Table C.1 & C.2).

4.5 Results and discussion

4.5.1. Cytotoxicity of OSPW fractions

Fractions of OSPW were cytotoxic within the range of tested concentrations (Figure C.2) and IC20 values are presented (Table 4.1). In general, *E. coli* were less sensitive to extracts of OSPW than the marine bacterium, *Vibrio fisheri* or embryos of fathead minnow.[118] However, *E. coli* exhibited sensitivity previously observed following exposure to a technical mixture of NAs (Sigma Aldrich # 70340).[125] Maximum inhibition of growth of E. coli exposed to F1-Pool, F1-NE, F2-NE2, F3-NE2a and F3-NE2b was 100, 72.9, 22.9, 82.5, 88.8 and 52%, respectively. In contrast, no significant toxicity was observed following exposure to F2-NE1. The rank-order of toxicity was similar to that observed by Morandi *et al*.,[118] however, in this study, fraction F2-NE2 exhibited the greatest toxic potency, whereas it was previously observed that fraction F3-NE2a exhibited the greatest toxic potency. Nevertheless, exposure of *E. coli* to the F1-Pool, F1-NE, F2-NE1, F2-NE2, F3-NE2a and F3- NE2b extracts of OSPW resulted in similar classification of samples as toxic (i.e. observed acute lethality) and non-toxic (i.e. no observed acute lethality) as previously observed and demonstrated the utility of this *E.coli* cell line for screening the toxicity of dissolved organic chemicals from OSPW.[118]

Table 4.1 Concentrations of dissolved organic chemicals in OSPW required to inhibit growth of *E.coli* wild-type strain by twenty percent (IC20).

Sample	IC20 (mg/L) (95% CI)
F1-Pool	1608 (786 - 2429)
F1-NE	1057 (993 – 1057)
F2-NE1	N/A
F2-NE2	152 (87.82 – 217.9)
F3-NE2a	212 (170 - 254)
F3-NE2b	372 (306 - 438)

4.5.2 Changes to gene expression following exposure of *E.coli* to extracts of OSPW

Exposure of *E.coli* to fractions of OSPW resulted in differential expression of 263 genes when a 1.5-fold cut-off was applied (Table C.3). When a 2-fold cut-off was applied, 115 genes were identified as being differentially expressed (Table 4.2, Figure 4.1).

Table 4.2 Numbers of genes differentially expressed ≥ 1.5- and 2- fold in *E.coli* exposed to fractions of OSPW relative to *E. coli* exposed to a solvent control.

Sample	1.5-fold		Total	2-fold		Total
	Up	Down		Up	Down	
F1-Pool	2	121	123	0	27	27
F1-NE	20	98	118	6	23	29
F2-NE1	14	78	92	8	19	27
F2-NE2	32	88	120	16	29	45
F3-NE2a	0	121	121	0	37	37
F3-NE2b	6	111	117	2	22	24

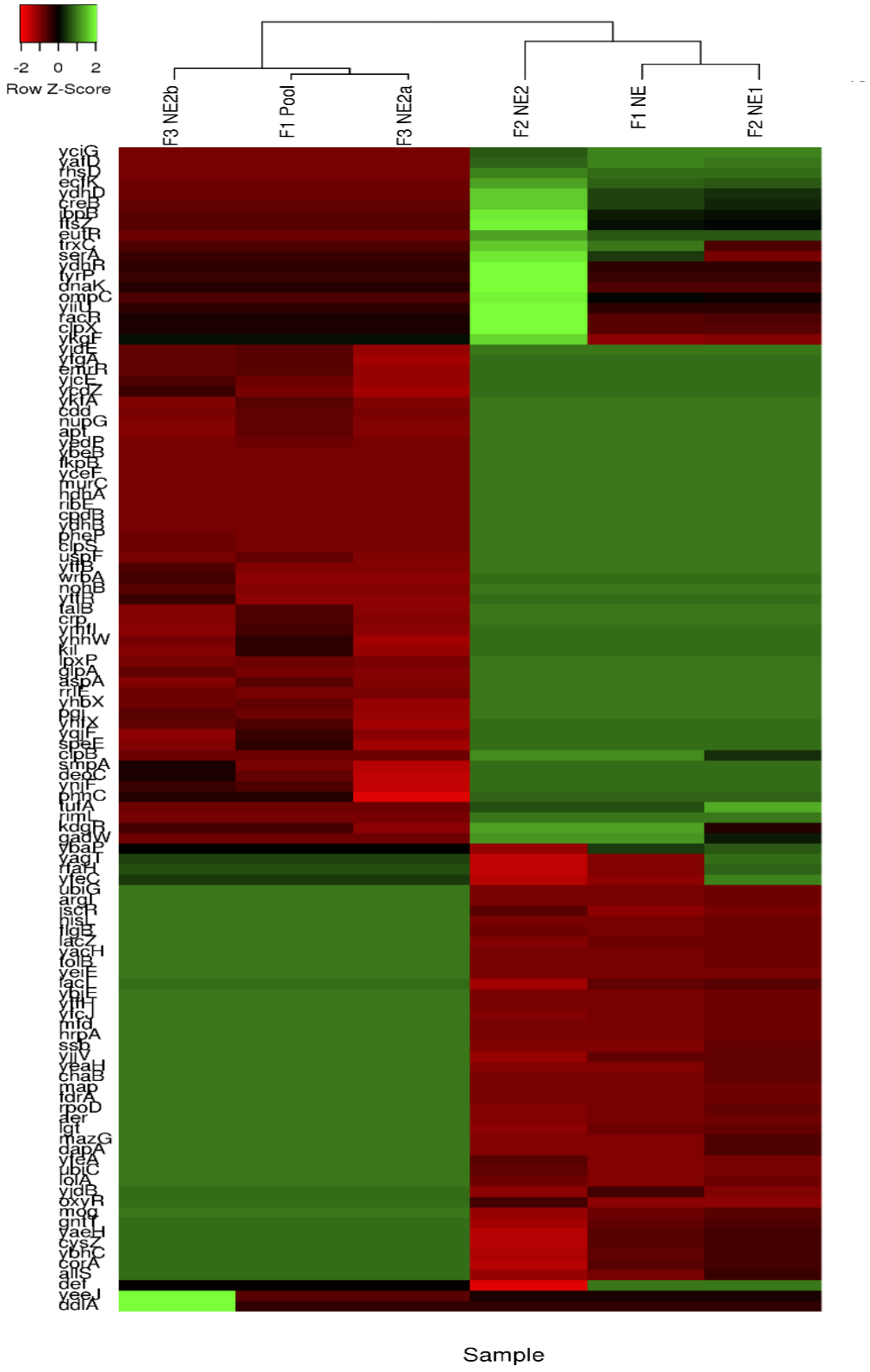
-2
0
2
Row Z-Score
F3 NE2b
F1 Pool
F3 NE2a
F2 NE2
F1 NE
F2 NE1
yciG
yafD
rhsD
eclK
ydhD
creB
ibpB
ftsZ
eutR
trxC
serA
ydbR
tyrP
dnaK
ompC
yiiU
racR
clpX
ykgF
yidE
yfgA
emrR
yicE
ycdZ
yktA
cdd
nupG
apt
yedP
ybeB
fkpB
yceF
murC
hdpA
ribE
cpdB
ydhB
pheP
clpS
uspF
yfiB
wrbA
nohB
yifB
talB
crp
yrhH
yhhW
kil
lpxP
gipA
aspA
rnlF
yhbX
pgi
yhiX
yqiF
speF
clpB
smpA
deoC
ynIF
pmnC
tufA
rimL
kdgR
gadW
ybaP
yagT
rfaH
yfeC
ubiG
argI
iscR
nisI
flgB
lacZ
yacH
tolB
yeiE
lacI
ybiE
yfiH
yicJ
mfd
hrpA
ssb
yjiV
yeaH
chaB
map
rdrA
rpoD
aer
lgt
mazG
dapA
yfeA
ubiC
lolA
yidB
oxyR
mqo
gntT
yaeH
cysZ
ybhC
corA
allS
def
yeeJ
ddlA
Sample

Figure 4.1 Hierarchical cluster analysis and heat map for gene differentialy expressed ≥ 2-fold in *E. coli* exposed to extracts of OSPW relative to *E. coli* exposed to a solvent control. Z-score represents standardized data, red infers down-regulation and green infers up-regulation.

Six differentially expressed genes were unique to the five fractions of OSPW that were acutely toxic (i.e. F1-Pool, F1-NE, F2-NE2, F3-NE2a, F3-NE2b) (Table 4.3 and Figure 4.2). The six genes could be classified into four broad categories based on their biological functions; transcriptional regulators (*yceP, clpB*), enzyme or putative enzymes (*adhE, ykgE*), putative surface protein (ybjE), and outer membrane phospholipase (pldA). The gene y*ceP* was down-regulated greater than 2-fold following exposure to all five acutely toxic fractions of OSPW. Down-regulation of expression of *yceP* is associated with the general stress response of *E.coli* resulting in biofilm formation, increased motility, and catabolite repression.[130] The gene *clpB* encodes for a chaperone protein that has been associated with proteotoxicity following exposure to heat stress.[131] The enzyme, alcohol/acetaldehyde dehydrogenase (*adhE*) is involved in maintenance of cellular redox homeostasis.[132] Additionally, the enzyme (*ykgE*) has an oxido-reductase function and is involved in maintenance of the cellular redox environment.[133] The surface protein (*ybjE*) is an important transmembrane transporter of lysine.[134] Phospholipase A (*pldA*) is important in maintaining homeostasis of cell functions, and down-regulation of the *pldA* gene might indicate reduced requirements for precursors for production of cyclooxygenases and lipoxygenases that ultimately are utilized for production of prostaglandins, and which are important for control of multiple biological processes.[135] Only one gene, *lacZ,* which encodes for the protein, β-galactosidase, and is involved in metabolism of lactose was similarly down-regulated among all six fractions of OSPW.[136]

Table 4.3 Genes differentially expressed in *E. coli* exposed to each of the acutely toxic fractions of OSPW.

Gene name	Description	Biological function
yceP	Transcription repressor	Biofilm formation
clpB	Transcription repressor	Chaperone protein
ykgE	Enzyme	Putative surface protein
pldA	Enzyme	Outer-membrane phospholipase
adhE	Enzyme	Alcohol dehydrogenase
ybjE	Putative surface protein	L-lysine transmembrane transporter

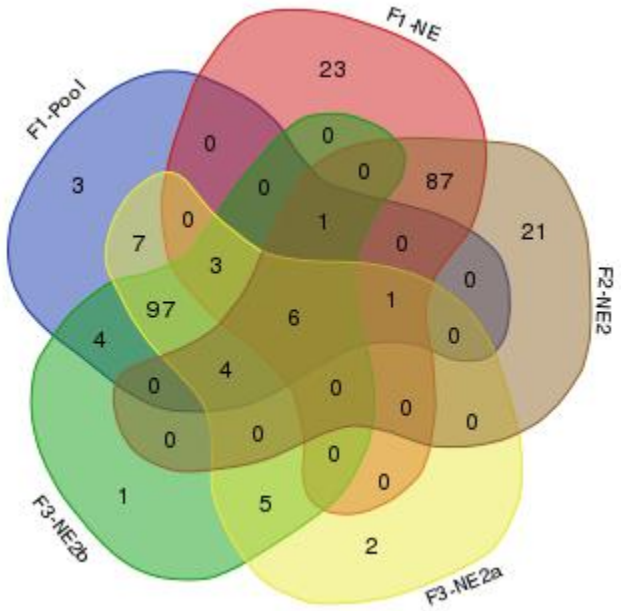

Figure 4.2 Venn diagram comparing genes expressed in *E. coli* exposed to acutely toxic fractions of extracts of OSPW.

4.5.3 Stress responsive genes affected by exposure to fractions of extracts of OSPW

Lists of differentially expressed genes of *E. coli* exposed to each fraction of OSPW were compared to a set of 93 genes previously identified as related to the general stress response of *E. coli*.[137] Overall, 11 of the stress-response genes were found to be responsive to extracts of OSPW (Table C.2). Genes related to energy stress (*bola, crp*) and protein stress (*clpB*) were differentially expressed in *E. coli* exposed to F1-Pool (Table C.2). Additional stress pathways included cell death, detoxification, redox stress, drug resistance and DNA repair, and are similar to results of Zhang *et al*.[125]

4.5.4 Mechanisms of toxic action of extracts of OSPW

Gene enrichment analysis identified 20 responsive biological and KEGG pathways in *E. coli* exposed to fractions of OSPW (Table C.1, Figure 4.3). Among samples, 6, 5, 3, 8, 7 and 7 biological processes or KEGG pathways were identified as responsive to F1-Pool, F1-NE, F2-NE1, F2-NE2, F3-NE2a and F3-NE2b fractions, respectively ($\rho<0.05$). Because F1-Pool contains the whole extractable organic fraction of OSPW, it was instructive to identify mechanisms of toxicity for this sample. Negative regulation of cellular processes (GO: 0048523) was identified as a biological process that was responsive in *E. coli* exposed to F1-Pool and likely representative of non-specific toxicity of extracts as suggested previously.[124] This conclusion was supported by the greater than 2-fold down-regulation of the global transcriptional regulator *crp,* which has been identified as responsive to general stress. Additional changes in gene expression were associated with regulation of anabolic and catabolic processes in cells. Of particular interest were changes to regulation of metabolism of organic hydroxyl compounds (GO: 1901615) and the KEGG pathway Pentose Phosphate Pathway (KEGG id: 00030), which might suggest response to changes in redox status of cells. A number of genes associated with responses to oxidative stress and anabolism were identified in the two pathways. Alcohol dehydrogenase, which is encoded by the gene *adhE,* has a putative role under aerobic conditions as a member of the antioxidant defense system and is a putative helicase.[132] Octaprenyl diphosphate synthase (*ispB*) is essential for growth and catalyzes a reaction in the production of ubiquinone, an important component of the antioxidant defense system.[138]

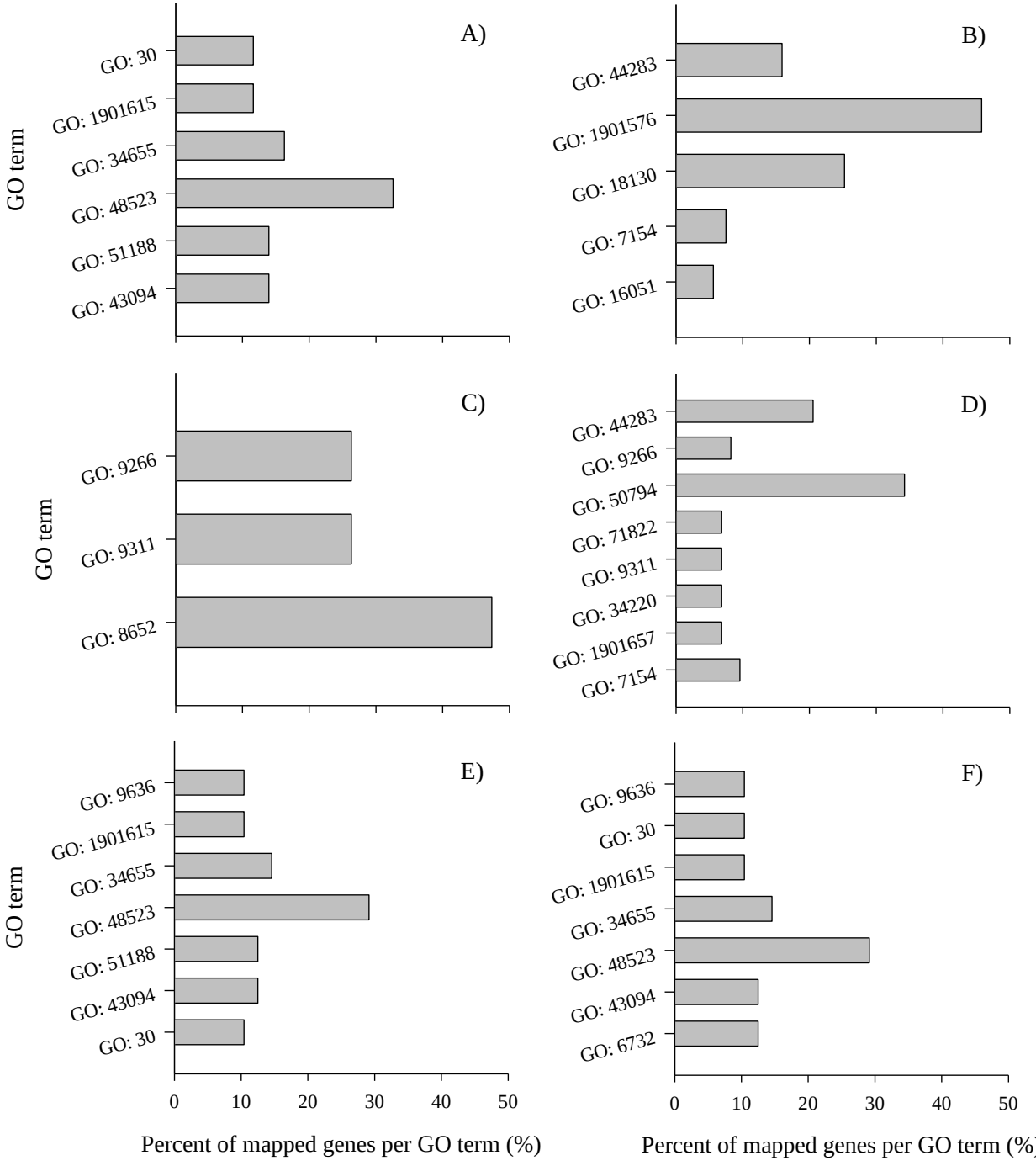

Figure 4.3 Proportions of differentially expressed genes mapped to GO biological processes or KEGG pathway for *E. coli* exposed to: A) F1-Pool, B) F1-NE, C) F2-NE1, D) F2-NE2, E) F3-NE2a and F) F3-NE2b. Biological processes identified include: carbohydrate biosynthetic process

(GO: 0016051), cell communication (GO: 0007154), cellular amino acid biosynthetic process (GO: 0008652), cellular metabolic compound salvage (GO: 0043094), coenzyme metabolic process (GO: 0006732), cofactor biosynthetic process (GO: 0051188), glycosyl compound metabolic process (GO: 1901657), heterocycle biosynthetic process (GO: 0018130), ion transmembrane transport (GO: 0034220), negative regulation of cellular process (GO: 0048523), nucleobase-containing compound catabolic process (GO: 0034655), oligosaccharide metabolic process (GO: 0009311), organic hydroxy compound metabolic process (GO: 1901615), organic substance biosynthetic process (GO: 1901576), Pentose Phosphate Pathway (KEGG id: 00030), protein complex subunit organization (GO: 0071822), regulation of cellular process (GO: 0050794), response to temperature stimuli (GO: 0009266), response to toxic substance (GO: 0009636), small molecule biosynthetic process (GO:0044283).

Previously, the Pentose Phosphate Pathway of *E. coli* MG1655 was identified as being responsive to a commercial mixture of NAs.[125] Exposure of *E. coli* to the F1-Pool resulted in greater than 2-fold down-regulation of *pgi* and *talB*. Phosphoglucose isomerase (*pgi*), catalyzes interconversion of glucose-6-phosphate (G6P) and fructose-6-phosphate (F6P) as part of the oxidative branch of the Pentose Phosphate Pathway and is important in control of metabolite flux between glycolytic and Pentose Phosphate Pathways.[139] Down-regulation of *pgi* has been associated with greater production of reducing equivalents, such as nicotinamide adenine dinucleotide phosphate (NADP+) in response to oxidative stress.[139] Trans-aldolase B (*talB*) catalyzes a reversible reaction of the non-oxidative branch of the Pentose Phosphate Pathway, where down-regulation coincides with general negative regulation of cellular processes, as the non-oxidative branch of the Pentose Phosphate Pathway produces important intermediates for normal metabolic function.[140] These results were interesting because several studies suggested that oxidative stress likely is a mechanism of toxicity of OSPW.[12,141]

4.5.5 Hierarchical cluster and principal component analysis of genomic data

No biological or KEGG pathway was identified as responsive to all fractions of OSPW that exhibited acute toxicity (Figure C.3). PCA was used to investigate relationships between toxicity ranking of fractions (i.e. most toxic fraction has rank equal to one) and identified mechanisms of

toxicity (Figure 4.4). Cell communication (GO: 0007154) and negative regulation of cellular processes (GO: 0048523) were associated with a lesser toxicity rank (i.e. more toxic extracts) (Figure 4). HCA and PCA clustered samples similarly, grouping extracts into two groups: 1) F1-Pool, F3-NE2a and F3-NE2b; 2) F1-NE, F2-NE1 and F2-NE2 (Figure 4.1, Figure C.4).

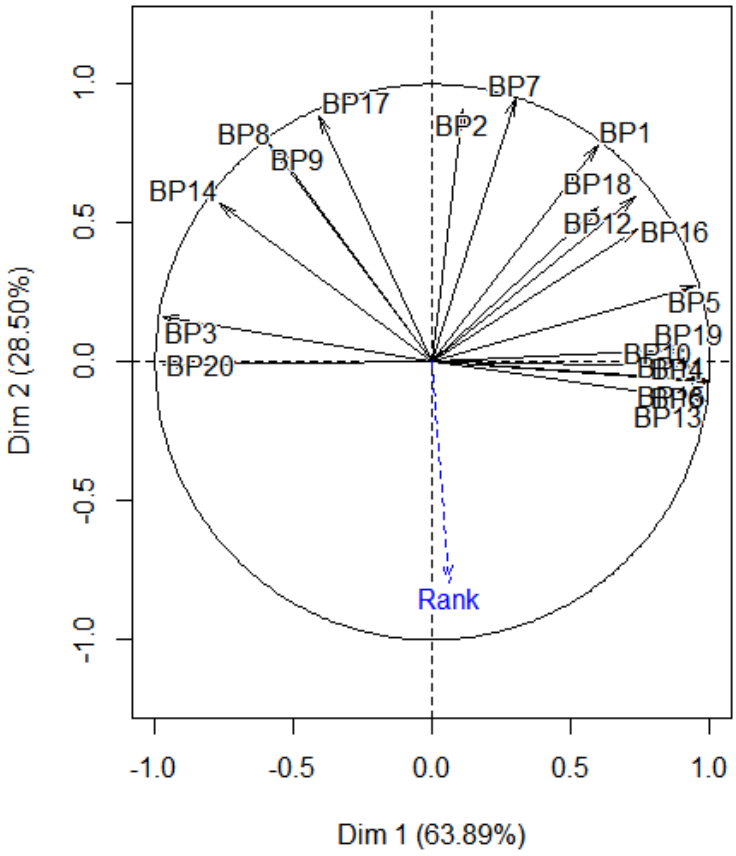

Figure 4.4 Variable factor map of differentially expressed biological pathways in *E. coli* following exposure to fractions of extracts of OSPW. Biological process (BP) labels are presented in Table C.1.

Clustering of samples was instructive, since F1-Pool is representative of the whole dissolved organic fraction of OSPW, whereas F3-NE2a and F3-NE2b are the most refined and toxic fractions of OSPW produced by Morandi *et al.*,[118] accounting for less than 11 and 8% of the organic mass in the F1-Pool, respectively. Because dose-response curves for acute lethality of embryos of FHM

to F3-NE2a and F3-NE2b were different, it has been suggested that these fractions differ in their mechanisms of acute toxicity.[118] However, as demonstrated in Figure C.2, dose-response curves were similar for *E. coli* and the number of common genes (Figure 4.2), biological pathways (Figure 4.3, Figure C.3) and clustering by use of HCA (Figure 4.1) and PCA (Figure C.4) suggest similar mechanisms of toxicity in *E. coli* exposed to these fractions.

4.5.6 Effect of chemical composition on mechanisms of toxicity of extracts of OSPW

Identification of chemical species and chemical classes responsible for toxicity of OSPW has received much attention recently.[8,118,142,143] Therefore, it was instructive to investigate if profiles of relative proportions of chemicals in extracts could be used to classify samples as toxic or non-toxic, and if clustering was similar to the results of biological pathway analysis. Extracts of OSPW had differing chemical profiles (Figure C.1). PCA could be used to describe the majority of observed variation (>73%) among samples by use of 2 components (Figure C.5). Classification of samples as 'toxic' (i.e. observed IC20) or 'non-toxic' (i.e. no observed IC20) revealed no significant structure in the data since there was no distinct clustering of the two groups (Figure C.6). Additionally, the data revealed an association of chemical classes O_4^+, SO_3^+, SO_4^+ and NO_3^+ with a greater overall toxicity rank (i.e. less toxic samples). Naphthenic acids were detected in all samples of OSPW, however the non-toxic extract F2-NE1 had relatively low abundances of NAs. Similarly, the F3-NE2b extract had low abundances of NAs but has been demonstrated to contain chemical classes which are bio-accumulative and toxic.[118,127,144] This evidence supports a role for NAs in the toxicity of the dissolved organic fraction of OSPW as demonstrated previously. [63,118,126] The chemical classes SO_3^-, SO_4^-, NO^+, O_2NS^+, SO^+, ONS^+ and NO_3^- were identified as correlated with lesser toxicity ranking, consistent with our previous results identifying the SO^+ and NO^+ chemical classes in the toxic extracts.[118,127] Samples F1-NE, F1-NE1 and F2-NE2 were clustered, which was consistent with previous results using biological responses (Figure 4.1& C.3). Regardless, the inability of PCA to cluster chemicals in a similar way as biological pathway is not surprising since it is known that chemicals behave different chemically when present as mixtures compared to when they exist independently.[145,146]

4.6. Conclusions

The dissolved organic fraction of OSPW is responsible for the majority of toxicity of OSPW, yet mechanistic studies investigating molecular mechanisms of OSPW have focused on a limited number of endpoints or results might have been confounded by potential interactions of the complex mixture e.g. high salinity. Therefore, it was instructive to screen extracts of OSPW re-suspended in laboratory control water by use of the LCA system to gain a greater understanding of molecular mechanisms of toxicity and to identify a profile of gene expression indicative of exposure to acutely toxic extracts of OSPW.

Genes indicative of general stress, protein damage and DNA damage were identified as uniquely responsive to acutely toxic extracts of OSPW. A general down-regulation of catabolic and anabolic processes was observed and are indicative of general non-specific toxicity. Changes to the expression of multiple genes and biological processes/ KEGG pathways were indicative of changes to the redox state of the cell, response to oxidative stress and are consistent with previous results across a range of extracts and species.[5,10,74,125] Additionally, findings here support previous work that sulphur and nitrogen containing chemical classes are responsible for toxic potencies of extracts of OSPW.[118,121,147] Interestingly, responsive genes, biological and KEGG pathways did not demonstrate a clear distinction among fractions, this was especially apparent for the tertiary fractions (F3-NE2a and F3-NE2b) which had previously been hypothesized to have differing mechanisms of action.

Chapter 5: Assessing reproductive performance of fathead minnow exposed to OSPW and fractions of OSPW by use of the 21-day reproductive bioassay.

5.1 Preface

Previously it has been demonstrated that OSPW can disrupt the reproduction of laboratory or field exposed fathead minnow. However, due to advances in the analytical characterization of OSPW related chemicals and results of Chapter 2, there was a lack of information as to the potential for novel chemical classes to disrupt reproduction of exposed fathead minnow. Therefore, work in Chapter 5 builds on that of Chapters 2- 4 by investigating the potential of these novel chemical classes to disrupt the reproduction of laboratory raised fathead minnow. OSPW used in Chapter 5 differs from that of previous chapters as it was collected in the fall of 2015 from BML.

Author contributions

Garrett D. Morandi (University of Saskatchewan) conceived, designed, and managed the experiment, generated and analyzed the data, prepared all figures, and drafted the manuscript.

Chrisitie Miller (University of Lethbridge) performed qPCR for identified genes.

Drs. Jonathan W. Martin and Chenxing Sun (University of Alberta), and Drs. Steve B. Wiseman, and John P. Giesy (University of Saskatchewan) provided inspiration, scientific input, and guidance, commented on and edited the manuscript, and provided funding for the research.

5.2 Abstract

Oil sands process-affected water (OSPW) is a byproduct of bitumen extraction in the surface-mining oil sands industry in Alberta, Canada. Organic compounds in OSPW are acutely and chronically toxic to aquatic organisms, and this water is contained in large tailings ponds where it is continuously recycled over the lifetime of the mine. One strategy for the long-term remediation of OSPW is ageing of water in artificial lakes, termed end-pit lakes. BaseMine lake (BML) is the first oil sands end-pit lake, commissioned in 2012. At the time of its establishment an effects-directed analysis of BML-OSPW showed that naphthenic acids and polar organic chemical species containing sulfur or nitrogen contributed to its acute lethality. However, the chronic toxicity of these same chemical fractions has not yet been investigated. In this work, the short-term fathead minnow reproductive bioassay was used to assess endocrine-system effects of two fractions of BML-OSPW collected in 2015. One of the fractions (F1) contained predominantly naphthenic acids, while the other (F2) contained non-acidic polar organic chemical species. Exposure of minnows to F1 or F2 at concentrations equivalent to 25% (v/v) of the 2015 BML-OSPW sample (5-15% of the 2012 BML-OSPW sample) did not alter reproductive performance, fertilization success, or concentrations of sex steroids in exposed female or male minnows. Additionally, there were no significant differences in fertility, hatching success or incidence of morphological indices of embryos collected on day 7 or 14 from exposed breeding trios. However, exposure of male fathead minnow to 25% (v/v) intact 2015 BML-OSPW resulted in a significantly greater hepatosomatic index. Exposure of fathead minnow to refined fractions of dissolved organic chemicals in 2015 BML-OSPW, or a 25% (v/v) of the intact mixture did not affect fertility or fecundity as measured by use of the 21-day reproductive bioassay. These data might be useful in setting future threshold guidelines for OSPW reclamation and treatment.

5.3 Introduction

As a complex mixture of salts, metals and dissolved organic compounds, oil sands process-affected water (OSPW) is a byproduct of bitumen extraction by the surface mining oil sands industry in Alberta, Canada.[20] OSPW is toxic to aquatic organisms and is stored on-site in large tailings ponds where it is continuously recycled back into the extraction operations.[17,26,63,118]

OSPW toxicity may be actively treated, for example it has been demonstrated that ozonation or adsorption with activated charcoal can diminish the dissolved organic fraction which is responsible for most of the acute toxic potency.[13,74,148] Alternatively, endpit lakes are one of the existing yet unproven strategies for the passive, and longer-term remediation of OSPW. Consisting of previously mined-out areas which have been hydraulically disconnected from the surrounding environment and filled with OSPW or other oil sands related materials, endpit lakes are designed to retain oil sands materials over a prolonged period of time to facilitate natural degradation of related chemicals of concern.[149] However, due to uncertainties related to the identity, toxicity and degradation potential of chemical species in OSPW, the effectiveness of the endpit lake strategy is uncertain.[4] As a result, BaseMine lake (BML) was established as the first commercial test of the oil sands endpit lake strategy in 2012 and represents a critical step forward in the evaluation of this remediation strategy.

Naphthenic acids (NAs) are a complex mixture of organic carboxylates that are known to contribute to observed acute and chronic toxicity of OSPW.[9,18,63,118,126] In OSPW, NAs may be composed of a range of carbon atoms (typically less than 20 carbon atoms), a range of double-bond equivalents (due to rings, double bonds or aromatic complexes) and countless structural isomers that can be detected using negative ion electrospray (ESI^-) and ultrahigh resolution mass spectrometry (uHRMS).[29] Together, NAs are therefore also termed 'O_2^- species', as each is composed only of carbon, hydrogen and two oxygen atoms, and are detected as negative ions. In addition to using ESI^-, it has been demonstrated that important polar chemical classes can be detected in positive ion electrospray (ESI^+) in OSPW, and that many of these contain sulfur or nitrogen heteroatoms in their structures.[29] In particular, it has been shown that NO^+ species and SO^+ species have the potential to bio-accumulate, and that these contribute to the acute lethality and other sub-lethal effects of OSPW.[118,143,144,147]

Several studies have demonstrated potential for oil sands related materials to affect the endocrine system.[12,13,18,86,148] Using T47-D or MDA cell lines, effects on receptor signaling related to production of the sex steroids17-β estradiol (E2) and testosterone (T) have been observed following exposure to OSPW.[13] Similarly, exposure of goldfish (*Carassius auratus*) or fathead minnow (*Pimephales promelas*) to fresh OSPW, or OSPW that has been aged in a small scale demonstration pond for 20 years (i.e. Pond 9), and an extract of West In-pit settling basin OSPW

results in changes to the concentration of circulating plasma sex steroids and altered expression of key regulatory genes associated with the endocrine system of fathead minnow.[14,17–19,84] In addition, lesser fecundity and frequency of spawning, as well as less pronounced secondary sexual characteristics have been observed in fathead minnow exposed to OSPW, aged OSPW or OSPW extracts.[5,14,15] By use of the yeast estrogenic screening bioassay and an effects-directed analysis approach, Yue *et al*.,[86] demonstrated the contribution of a fraction containing O_2^-, O_3^- and O_4^- chemical classes, which includes NAs, to the estrogenic activity of OSPW.

Previously, using a fractionation and effects-directed analysis approach it was demonstrated that NAs are responsible for most of the observed acute and chronic toxicity of the dissolved organic fraction of OSPW collected from BML in 2012 (BML-OSPW), which includes effects on the endocrine system of exposed fishes.[13,91,143] In addition, it is now known that chemical species in BML-OSPW, containing sulfur and nitrogen, contribute to acute lethality and might bioaccumulate.[82,91] However, there is currently limited information for the contribution of these chemical species to chronic or sub-lethal toxicity endpoints. Therefore, the purpose of the present study was to gain a greater understanding of the potential for oil sands related chemicals to disrupt the reproductive performance and endocrine system of hatchery raised fathead minnow. BML-OSPW collected in 2015 and two fractions containing chemical species known to cause acute toxicity in BML-OSPW collected in 2012 were prepared and assessed by use of the 21-day fathead minnow reproductive bioassay.

5.4 Materials and methods

5.4.1 Chemicals and reagents

Acetic acid, methanol, dichloromethane, diethyl ether and water (HPLC grade) were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Anhydrous ethanol was purchased from Fischer Scientific (Edmonton, AB, Canada). Sulfuric acid 98% and sodium hydroxide were purchased from Osprey Scientific Inc. (Edmonton, AB, Canada).

5.4.2 OSPW sample collection

Approximately five hundred liters of surface water was collected from BML using a permanent sampling barge (Syncrude Canada Ltd) in August 2015, from which two hundred liters (200 L)

was subsampled and stored in ten 20 L high-density polyethylene pails. The water was stored in the dark at 4 ^{o}C for two months prior to extraction.

5.4.3 Fractionation

We previously demonstrated that fractions of BML-OSPW collected in 2012, one containing naphthenic acids and another containing non-acidic polar organic species, were acutely toxic to embryos of fathead minnow at their native concentrations at the time.[118] To achieve the greater quantities of test-substance fractions needed for longer-term tests in the current work, the goal of the fractionation method below was to generate two fractions by a single technique, rather than three step-wise techniques used in our previous fractionations. The objective was to end up with two fractions similar to those final fractions in previous work, one fraction containing predominantly O_2^- species, and one containing predominantly O^+, O_2^+, SO^+, and NO^+ polar organic species.

Suspended particulate matter was removed from 200 L of BML-OSPW collected in 2015 by use of vacuum filtration through Grade 4 glass fiber filters (1.2 µm nominal particle retention, Fisher Scientific, Nepean, ON, Canada). Solid phase extraction (SPE) of BML-OSPW was completed by use of multiple Oasis HLB SPE columns (35 cc, 6 g sorbent per cartridge, 60 µm particle size, Waters Limited, Ontario, Canada). In detail, each SPE cartridge was conditioned with 20 mL MeOH and equilibrated by use of 20 mL HPLC grade water. The SPE procedures were carried out using a 20-position extraction manifold (Waters Limited, Ontario, Canada) with the exit valve connected to the vacuum pump with inline flask as liquid trap. Each cartridge was then slowly loaded with approximately 2.1 L filtered BML-OSPW over 12 hrs. The cartridges were then washed in turn with 40 mL MeOH:H_20 (50:50, v/v) and 20 mL MeOH: 2% acetic acid (55:45, v/v). The corresponding eluents were Wash-1 and Wash-2, and were discarded after HPLC- Orbitrap MS analysis indicated no analytes of interest. Next, an eluent was collected by adding 40 mL of MeOH: 5% NH_4OH (65:35, v/v) to each cartridge (Eluent-1). The eluents from ten SPE cartridges were combined, methanol was evaporated by rotary evaporator and the remaining aqueous phase was adjusted to pH 12 with concentrated NaOH. This aqueous phase was extracted 3 times using 40 mL DCM that was then evaporated to dryness (Eluent 1-Basic). The pH of the remaining sample was adjusted to pH 2 with concentrated H_2SO_4 and extracted 3

times using 30 mL DCM to produce fraction 1(F1). Eluent-2 was generated by using 25 mL of pure MeOH to elute the remaining analytes from each SPE cartridge, and then combined with Eluent 1-Basic to produce fraction 2 (F2). The F1 and F2 fractions were blown to dryness by use of a rotary evaporator and nitrogen gas. The dry weight of fractions were measured and are presented as mg of dried extract per L of the original filtered BML-OSPW. F1 and F2 were dissolved in methanol for Orbitrap MS analysis, and in ethanol for biological assays.

5.4.4 Characterization of fractions by HPLC-Orbitrap

Profiles of organic compounds in fractions were determined by use of high performance liquid chromatography coupled to an ultrahigh-resolution Orbitrap mass spectrometer (HPLC-Orbitrap). The HPLC (Transcend, Thermo Fisher Scientific) consisted of a degasser, a 1250 bar quaternary pump, an auto-sampler, and a column oven. Separation was performed on a Hypersil Gold C18 analytical column (50×2.1mm, 1.9 µm particle size, Thermo Fisher Scientific) at 40 °C. A flow rate of 0.5 mL/min and an injection volume of 2 µL was used in all analyses. Mobile phases consisted of (A) 0.1% acetic acid in water, and (B) 100% methanol. The mobile phase composition was 5% B for 1 min, followed by a linear gradient ramp to 90% B at 9 min, to 99% B over 5 min, and returning to 5% B in 1 min followed by a 4 min hold prior to the next injection. The Orbitrap MS was operated with an ESI source in either positive or negative mode, with separate injections of the same sample for each analysis. Ionization voltage was set at ± 4 kV, while the sheath, aux, and sweep gas flows were set to 40, 25 and 2 (arbitrary units), respectively. Vaporizer and capillary temperature were 325 °C and 300 °C, respectively. Acquisition was performed in full scan mode (*m/z* 100 to 500) at 1.2 Hz with resolving power set to a nominal value of 240,000 at full width half-maximum at *m/z* 400. For data presentation in the current work, each chemical species detected was binned according to its corresponding heteroatom class in either ESI^+ or ESI^-, and heteroatomic class profiles are presented as relative abundance compared to unextracted BML-OSPW.

5.4.5 Fathead minnow 96-hr exposure

Studies with fathead minnows were conducted at the Aquatic Toxicology Research Facility in The Toxicology Centre, University of Saskatchewan. Expression of genes along the brain-gonad-

liver axis that are key regulators of reproduction were quantified in male and female minnows exposed to OSPW and fractions of OSPW for 96-hr because previous work has demonstrated rapid changes in abundances of endocrine-related transcripts following exposure to endocrine disrupting chemicals.[150] Two female and one male fathead minnow were acclimatized in 10-litre aquaria by use of a static renewal system for three weeks prior to exposure (n=4). A 50% water renewal was completed daily and each third day 75% of waters were replaced. Light: dark cycle was maintained at 16:8 and temperature of water was 26 ±1 °C. Fish were fed bloodworms twice daily to satiety and detritus was removed from tanks approximately one-hour post-feeding. Upon test initiation, fathead minnows were exposed to city of Saskatoon municipal water, solvent control (0.002% ethanol, S. Control), 25% (v/v) OSPW, 25% (v/v) equivalent (equ.) F1, or 25% (v/v) equ. F2 diluted in city of Saskatoon municipal water. On the day of test termination, fish were anaesthetized by use of aquacalm and their brain stem was cut by use of dissecting scissors. Phenotypic sex determined by use of presence of secondary sexual characteristics, mass (grams; g) and length (centimetres; cm) were recorded for each fish. Liver, brain (including pituitary) and gonad from each fish were stored in pre-weighed vials, weighed and immediately snap frozen by use of nitrogen and stored at -80°C.

5.4.6 Fathead minnow 21-day exposure

Effects on reproduction were determined according to USEPA test method *Short-term test method for assessing the reproductive toxicity of endocrine-disrupting chemicals using the fathead minnow (Pimephales promelas)*.[151] To establish baseline fecundity and fertility data, two female and one male fathead minnow were acclimatized in 10-litre aquaria, as outlined above, with two breeding tiles, for a one month period prior to initiation of exposure. Following the pre-exposure period, tanks producing a minimum of 10 eggs/ female/ day were exposed for 21-days to one of; Saskatoon municipal water, S. Control (0.002% EtOH), 25% (v/v) BML-OSPW, 25% (v/v) equ. F1, or 25% (v/v) equ. F2. Exposures were static renewal, and 50% of the exposure solution was replaced in each tank daily, except for each third day when 75% of the solution was renewed. Tanks were checked for eggs twice daily approximately 45 minutes subsequent to feeding. Eggs were removed from breeding tiles and placed into 25 ml petri dishes containing dechlorinated city of Saskatoon municipal tap water. The number of eggs and success of fertilization were recorded by use of a dissecting microscope. A subset of 10 eggs was collected

from each tank on day 7 and 14 following the first spawning event. Embryos were placed in 25 mL petri dishes with 20 mL dechlorinated, Saskatoon municipal water, maintained at 26±1°C and half of the water solution was replaced daily. Embryos were checked for survival daily and dead embryos were removed. Following hatch, fathead minnow hatchlings were checked by use of a dissecting microscope to assess the presence/ absence of deformities including; spinal curvature, hemorrhaging, pericardial and yolk sac edema, and craniofacial malformations. On the day of test termination, fish were anaesthetized and blood was collected by use of heparinized tubes and caudal vein incision, and immediately stored on ice. Blood plasma was separated within 6 hrs of test termination as described in the next section. Phenotypic sex determined by use of presence of secondary sexual characteristics, mass (g), length (cm) and presence or absence of secondary sexual characteristics (e.g. tubercles) were recorded. Liver, brain and gonad were weighed immediately in pre-weighed vials, snap frozen by use of liquid nitrogen, and stored at -80°C. Condition factor (K), hepatosomatic (HSI), and gonadosomatic (GSI) indices were calculated.[151]

5.4.7 Measurement of plasma 17-β estradiol and testosterone concentrations

The E2 and T enzyme immunoassay kits (Cayman Chemical, Ann Arbor, MI) were used to measure concentrations of E2 and T in plasma of female and male fish, respectively. Due to limited plasma volumes, concentrations of E2 were determined at the tank level by pooling blood plasma from all females (n=2) in one tank. Blood plasma was collected by centrifugation of blood for 15 min @ 2600 rpm, and immediately stored at -80 °C. Samples were extracted three times by use of 0.5 mL DCM or diethyl ether for E2 and T, respectively. Extracts were combined, blown-down to near dryness by use of nitrogen gas, and immediately suspended in the assay media. Assays were performed as described by the manufacturer.

5.4.8 Quantification of gene expression

Abundances of transcripts of several genes (androgen receptor, *ar*; estrogen receptor alpha, *erα*; estrogen receptor beta, *erß*, vitellogenin 5, *vtg5*; aromatase, *cyp19a*) that are important for regulation of reproduction in fathead minnows were quantified. Total RNA was isolated from

livers and gonads of male and female minnow by use of the RNeasy Plus kit, according to the protocol provided by the manufacturer (Qiagen, Mississauga, ON, Canada). Concentration and purity of RNA was determined by use of a NanoDrop spectrophotometer (Fisher Scientific). Complementary DNA (cDNA) was synthesized using 1ug of total RNA by use of the QuantiTect® reverse transcript kit, according to the protocol provided by the manufacturer (Qiagen). Quantitative real-time polymerase chain reaction (qPCR) was performed in 96-well plates by use of an CFX96 Real-Time PCR System (BioRad, Mississauga, ON, Canada). A 35 µL reaction mixture of 2x concentrated SsoFast™ EvaGreen® Supermix , 2.5 µL of cDNA, 10 pmol gene-specific primers, and nuclease free water was prepared for each cDNA sample and primer combination. Each sample of cDNA was analyzed in duplicate with 10 µL reaction volumes per well. The reaction mixture for qPCR was denatured at 95 ^{o}C for 10 min followed by a thermal cycle profile consisting of denaturing at 95 ^{o}C for 10 s and extension for 1 min at 60 ^{o}C for a total of 40 cycles. Abundances of transcripts were quantified by normalizing to the housekeeping gene B-actin and the analysis was performed using CFX Manager™software. The analysis software corrects for differences in reaction efficiencies. Reaction efficiencies were determined by performing qPCR on serial dilution of a pool of cDNA that was created by combining equal volumes of cDNA from male and female fathead minnows exposed to freshwater, the S. Control, OSPW, and fractions of OSPW.

5.4.9 Statistical analysis

All statistical comparisons were made at the 5% significance level ($\rho < 0.05$). Data were tested for normality by use of the Kolmogorov-Smirnov test, while homogeneity of variance was tested by use of Levine's test. If data did not meet the assumptions of normality they were log-transformed. When data met the assumptions of normality and homogeneity, effects of OSPW and fractions of OSPW on mean eggs produced per female per day, percentage fertilization, percentage hatching, time to 50% hatch (TTH), morphometric indices and concentrations of sex steroids in blood plasma were compared to the S. control and were determined by use of one way analysis of variance (ANOVA) followed by Dunnett's test, with aquariums as experimental units of replication.

5.5 Results

5.5.1 Characterization of BML-OSPW and fractions

Distributions of chemical species by heteroatom class in BML-OSPW and fractions are shown in Figure 5.1. Similar to our previous study on the fractionation of BML-OSPW collected in 2012, the F1 fraction accounted for 86% of the original mass spectral intensity of O_2^- species detected in unfractionated BML-OSPW, demonstrating high recovery for naphthenic acids, as well as only minor $O_3^{+/-}$, O_4^- and SO_2^- species. The F2 fraction accounted for 70% of the original O_2^+ class content, 95% of the SO^+ and NO^+ content, and 65% of the O^+ content based on mass spectral intensity. To provide context and comparison to our previous work, the organic content of BML-OSPW collected in 2015 (41.5 mg/L, measured by total organic carbon analysis of filtered BML water) was lesser than BML-OSPW collected in 2012 (150 mg/L, measured by total organic carbon analysis of filtered BML water) and used for our previous acute toxicity identification study. Similarly, the organic mass content of F1 and F2 (12.9 and 2.2 mg/L, respectively) were less than the corresponding fractions from our previous work using 2012 BML-OSPW (20.0 and 14.0 mg/L), owing to the different water and to some extent the different fractionation procedure. Compared to the original mass of organics in 2015 BML-OSPW, these two fractions accounted for 31% (F1) and 5% (F2) of the organic mass of BML.

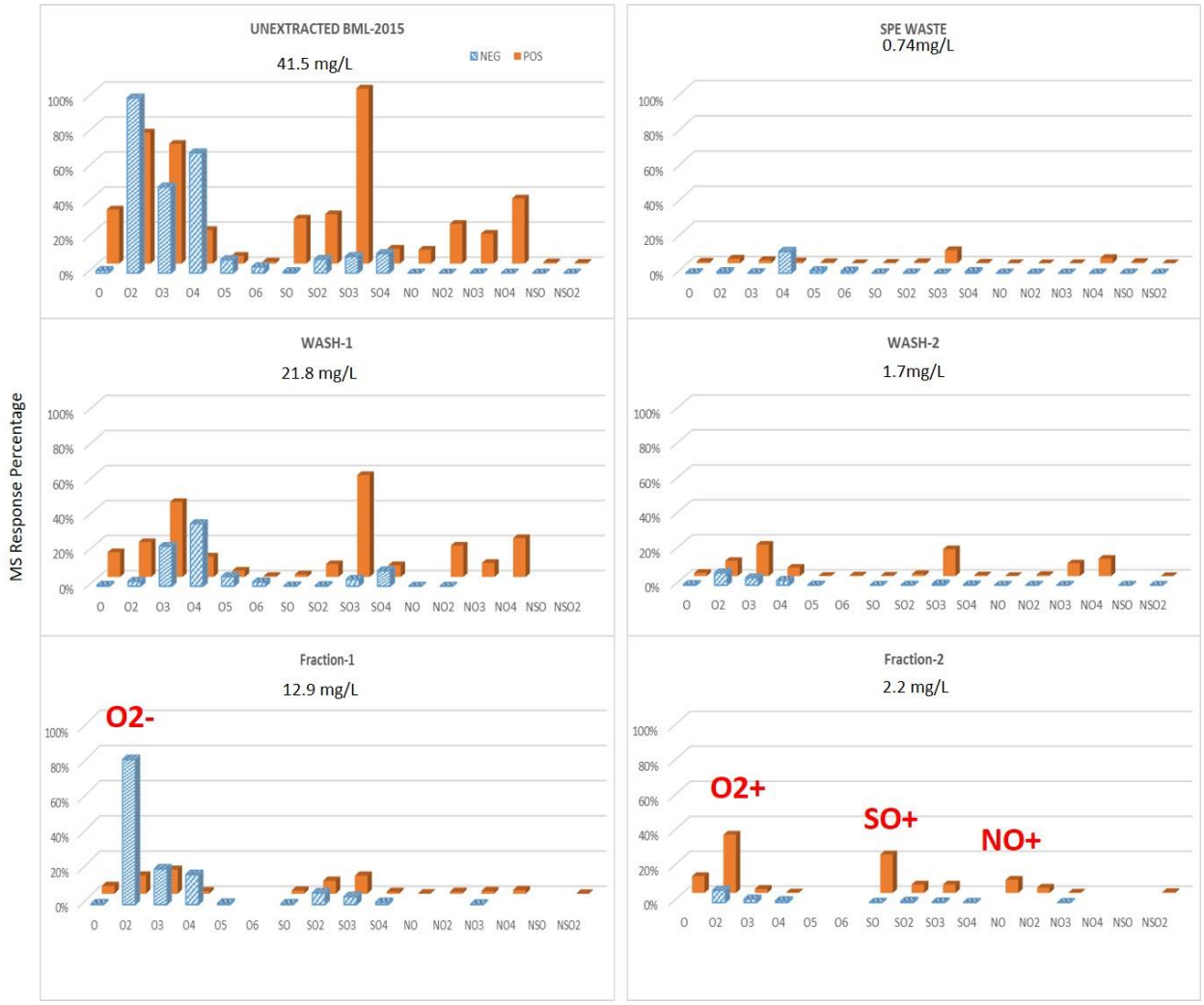

Figure 5.1 Total and relative abundance of species by heteroatom class in positive (orange) and negative (blue) electrospray ionization modes in unfractionated BML-OSPW collected in 2015 and fractions produced by SPE: waste, Wash-1, Wash-2, F1 and F2 by use of HPLC-Orbitrap MS. The organic content (based on gravimetric weight for SPE fractions and total organic carbon analysis of filtered BML before SPE fractionation) is presented as the average mass per liter of filtered BML-OSPW.

5.5.2 Reproductive performance of fathead minnow exposed to OSPW and fractions

Exposure of fathead minnow breeding trios to 25% (v/v) BML-OSPW, 25% (v/v) equ. F1 and 25% (v/v) equ. F2 for 21-days did not affect fecundity when compared to control fish (Figure 5.2). Similarly, morphometric analysis demonstrated no significant effects on K, GSI, or concentrations of E2 or T in blood plasma of female and male fish, respectively. However, exposure of male fathead minnow to 25% (v/v) BML-OSPW resulted in significantly greater HSI compared to that of unexposed, control fish (Table 5.1).

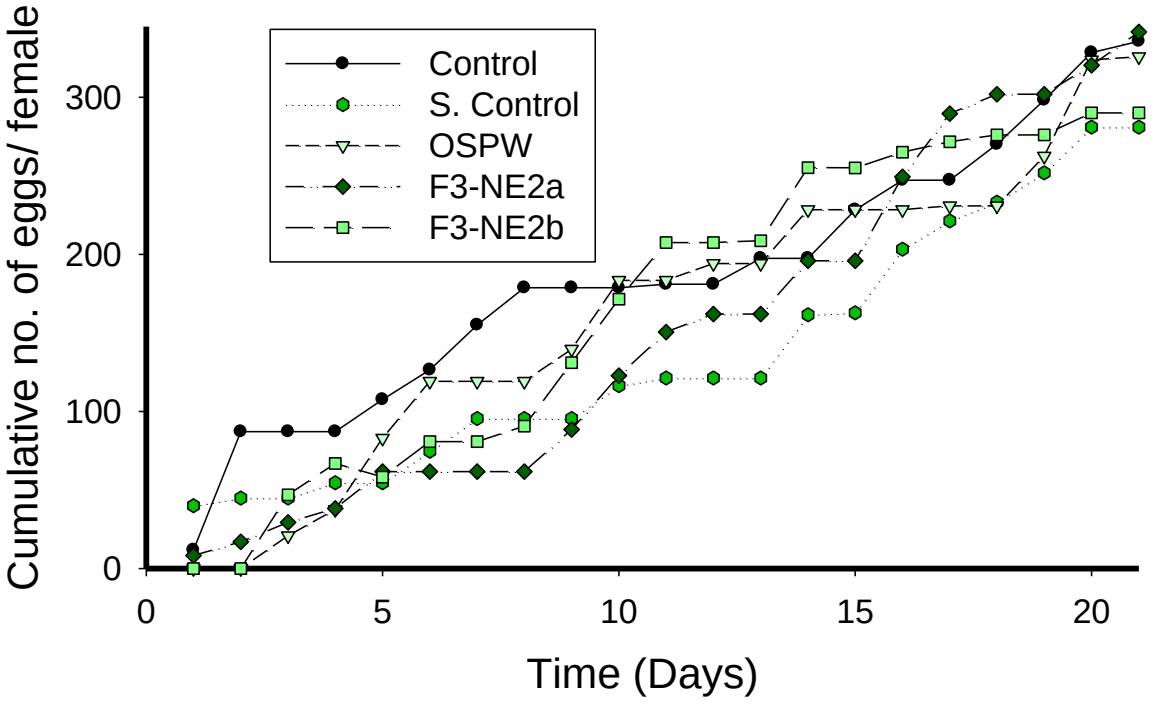

Figure 5.2 Cumulative production of eggs by fathead minnows exposed to 25% (v/v) BML-OSPW, 25% (v/v) equ. F1 and 25% (v/v) equ. F2. Cumulative number shown as eggs/ female.

Table 5.1 Condition factor (K), hepatosomatic index (HSI), gonadosomatic index (GSI) and plasma concentrations of testosterone or estrogen for male and female fathead minnows respectively following 21-days of exposure to 25% (v/v) BML-OSPW, 25% (v/v) equ. F1 or 25% (v/v) equ. F2. Data are shown as mean ± SEM. Asterisk denotes significant difference from control ($p < 0.05$, one-way ANOVA followed by Dunnett's test).

		K		HSI		GSI		Testosterone (ng/mL)	
Exposure	Gender	mean	sem	mean	sem	mean	sem	mean	sem
Control	Male	1.93	0.06	2.50	0.45	3.3	0.66	5.35	1.58
S. Control	Male	1.72	0.16	2.04	0.52	2.52	0.51	4.18	1.56
25% (v/v) OSPW	Male	2.24	0.20	4.20*	0.31	1.7	0.49	3.66	1.75
25% (v/v) equ. F1	Male	1.93	0.25	2.50	0.31	2.5	0.46	5.42	1.16
25% (v/v) equ. F2	Male	2.15	0.37	2.04	0.17	2.23	0.35	4.20	1.49
		K		HSI		GSI		Estradiol (ng/mL)	
Exposure	Gender	mean	sem	mean	sem	mean	sem	mean	sem
Control	Female	3.41	0.24	3.66	0.74	13.3	1.53	7.78	2.31
S. Control	Female	3.18	0.38	4.18	0.62	8.34	2.01	4.34	1.29
25% (v/v) OSPW	Female	3.23	0.14	2.84	0.40	9.11	1.36	4.55	1.65
25% (v/v) equ. F1	Female	3.30	0.18	3.44	0.32	8.19	1.32	6.36	3.64
25% (v/v) equ. F2	Female	3.17	0.26	2.87	0.24	13.6	0.93	3.40	0.86

5.5.3 Embryo-larval indices

Cumulative percent fertilization of embryos collected from fathead minnow breeding trios exposed over 21-days to BML-OSPW and fractions of BML-OSPW did not significantly differ between exposed and control exposed fish. Time to 50% hatch and incidence of malformations of embryos collected on day 7 or 14 did not significantly differ between breeding trios of fathead minnow exposed to BML-OSPW, fractions of BML-OSPW or control (Table 5.2).

Table 5.2 Cumulative percent fertilization of embryos collected throughout 21-day exposure, time to 50% hatch and percentage incidence of malformations of embryos (n=10) collected on days 7 and 14 (n=4) from fathead minnow breeding trios exposed to 25% (v/v) BML-OSPW, 25% (v/v) equ. F1, 25% (v/v) equ. F2, control or S. Control (0.002% EtOH).

	Cumulative fertilization		TTH (days) Day 7		TTH (days) Day 14		Incidence malformations	
Exposure	mean (%)	sem	mean (%)	sem	mean (%)	sem	mean (%)	sem
Control	97.5	0.86	5.00	0.5	5.25	0.29	7.00	0.70
S. Control (2E-03% EtOH)	97.8	1.68	4.50	0.35	4.33	0.29	6.00	1.20
25% (v/v) OSPW	95.2	0.55	5.33	0.57	4.75	0.25	5.00	0.50
25% (v/v) equ. F1	96.9	0.97	5.40	0.25	4.20	0.35	4.00	1.00
25% (v/v) equ. F2	95.7	1.59	5.25	0.29	4.8	0.63	7.00	1.10

5.5.4 Effects on gene expression

Abundances of transcripts of *vtg5, ar, era, erb,* and *cyp19a* were not significantly different in male or female minnows exposed to BML-OSPW or fractions of BML-OSPW compared to unexposed, control.

5.6 Discussion

The fractionation method generated fractions with similar profiles of dissolved organic chemicals as those produced in Morandi *et al.*[118] The F1 fraction contained the majority of NAs (i.e. O_2^- class and the F2 fraction contained the majority of non-acidic species such as O^+, O_2^+, SO^+ and NO^+ (Figure 5.1). However, the organic content of BML-OSPW collected in 2015 and related fractions was less than BML-OSPW we collected in 2012. Exposure of fathead minnow breeding trios to BML-OSPW or refined fractions of BML-OSPW did not result in any significant effect on reproductive capacity or the endocrine system. Due to the large volumes of OSPW required for the single dose used in this study, we were unable to test the dose-response but the lack of significant effects at this single dose contrasts with results of other studies, which have demonstrated that OSPW, aged OSPW and NA extracts can affect the endocrine

system.[10,13,17–19,84,142,148] However, the lack of observed effects in this work might be due to the dilution of the OSPW used in this work (4-fold) whereas previous studies have mainly used 100% OSPW or equivalents thereof.[10,13,17–19,84,142,148] Furthermore, these results are interesting because exposure of fathead minnow to 100% OSPW collected from West In-pit, now BML, had previously been demonstrated to alter the abundance of transcripts at all levels of the brain-gonad-liver axis in both male and female fathead minnows.[10] Also these results were interesting because F1 contained predominantly acidic chemical species (i.e. $O_{2\text{-}4}^-$ classes)that are known to have estrogenic activity[86] and affect fecundity and sex steroid synthesis of fathead minnow.[18] The $O_{2\text{-}4}^-$ chemical classes accounted for approximately 90% of total mass spectral intensity of F1 under ESI^- (Figure 5.1). Therefore, results of this study suggest that dissolved organic chemicals in BML-OSPW at 25% (v/v), which are known to have acute and chronic toxicity when exposed at higher concentrations had no effect on reproductive performance of fathead minnows at the dose used here.[118,143,144,147] Additionally, results of this study demonstrate significant dilution of BML-OSPW between 2012 and 2015 as the concentration of organics has decreased from >150 mg/L to <42 mg/L. Dilution of BML-OSPW since 2012 is likely due to a range of effects including accumulation of surface run-off, degradation of dissolved organic compounds by natural biological communities or active pumping of water from Beaver Creek Reservoir by Syncrude Canada Ltd.[152]

Although no effects on the endocrine system were observed, exposure of male fathead minnow to 25% (v/v) intact OSPW resulted in significantly larger liver masses compared to control exposed fish. Previously, it has been demonstrated that exposure of fathead minnow for 21-days to OSPW that has been aged in an experimental reclamation pond (Pond 9) resulted in decreased spawning success and fecundity but no effect on HSI.[17] Similarly, minnows exposed under laboratory conditions to a NA extract for 21-days demonstrated decreased spawning, fecundity, and lesser concentrations of testosterone in blood plasma, but had greater HSI.[18] In contrast to these results, laboratory fish exposed to OSPW collected from Syncrude's demonstration pond, demonstrated no effect on spawning, fecundity or HSI,[18] but wild minnows collected from the same pond had larger K, HSI and GSI when compared to reference fish.[19] Although results are inconsistent among intact OSPW and fractions of dissolved organic species from OSPW, HSI might be used as an indicator of longer term exposure to OSPW and OSPW derived chemicals. However, the causative agent of increased liver masses remain unknown.

To the knowledge of the authors, this is the first assessment of reproductive performance of fathead minnow exposed to refined fractions of BML-OSPW, one containing predominantly acidic species including NAs (F1) and the other, predominantly non-acidic chemical species (F2). Furthermore, these results suggest that exposure of fathead minnow breeding trios to dissolved organic chemicals in BML-OSPW collected in 2015 at 25% (v/v) have no effect on reproductive performance. Although there has been dilution of BML-OSPW since our original study completed in 2012, as indicated by the lower concentrations of organics in BML-OSPW collected in 2015, the results of this study suggest that the intact BML-OSPW mixture is more toxic than refined fractions as identified by use of HSI. The identity of causative agents of larger HSI measured in male fathead minnow remains unknown however, HSI might be used as an indicator of exposure to intact OSPW.

Chapter 6: General discussion

6.1 History and project rationale

Petroleum is a major source of global primary energy output.[1] Containing the third largest proven oil reserves in the world, 97% of which are located in the oil sands region of Alberta, Canadian investment and development will continue into petroleum resources. Projected to increase from 3.85 million bpd in 2016 to 5.1 million bpd by 2030, Canada will continue to be a primary exporter of petroleum products globally.[149] Present as bitumen laden soils or deep underground deposits, bitumen in the oil sands region is considered an unconventional petroleum source. In the past, open-pit mining of bitumen-laden soils has been the predominant method of extraction. However, before bitumen-laden soils can be transported for refinement, bitumen must be separated from soils and other impurities. Although variations exist among operations, a thermal liquid separation technique known as the Clark Hot Water extraction process is used to isolate bitumen.[153] This process also produces a waste stream known as oil sands process-affected water (OSPW) consisting of water, clays, residual bitumen and other impurities. Although the industry recycles greater than 95% of OSPW for reuse in the extraction process, the quality of recycled OSPW deteriorates to a point where it is no longer effective in the extraction process.[149]

Because of toxicity to exposed organisms, OSPW which is no longer useful in the extraction process, is stored in large containment ponds known as tailing ponds where it is held until sufficiently remediated to facilitate release to the watershed.[15,19,26,148] However, to date no OSPW has been sufficiently remediated to facilitate its return to the watershed. To this end, identifying causative agents of toxicity in OSPW is important to inform the development of remediation techniques, release guidelines and monitoring programs for the oil sands area to ensure that treated OSPW released to the surrounding environment does not adversely effect the environment or human health. Several techniques for remediation of OSPW have been developed with construction of endpit lakes (EPL) being a cost-effective strategy that has been proposed.[73] Endpit lakes are a remediation technique which relies on the natural attenuation of process-affected material related toxicity over time in constructed landscapes before being hydraulically connected with the surrounding environment when sufficiently remediated. In

2012, the first full-scale test of this approach was established with commissioning of BaseMine Lake (BML).

At the initiation of this Ph.D. program, it was known that dissolved organic chemicals in OSPW were the primary cause of toxicity. In addition, groundbreaking work by Grewer *et al*.,[67] and Pereira *et al*.,[29] demonstrated that many chemical species previously identified as naphthenic acids (NA) were in fact co-occurring mono- and poly-oxygenated compounds, for example di-acids,[66] and many unidentified polar neutral species or those containing sulfur and nitrogen heteroatoms.[28,29,31,67] However, the specific chemical species responsible for the toxicity of OSPW, and the associated mechanisms, were unknown. Therefore, the focus of this Ph.D. program was to identify and characterize the toxicity of chemical species which contribute to the toxicity of OSPW collected from BML (BML-OSPW). This research program was designed to aide in the development of release guidelines for the oil sands industry, targeted remediation techniques and to inform the development of monitoring programs for the area.

6.2 Summary- Characterizing the toxicity of dissolved organic chemicals in BML-OSPW

6.2.1 Identification of chemical classes contributing to the acute toxicity of the dissolved organic fraction of BML-OSPW

Bioassay effects-directed analysis is a technique used to identify the causative agents of toxicity in a mixture by use of an iterative fractionation and biological testing approach.[154] In this work, BML-OSPW collected in 2012 was sequentially fractionated and biologically tested three times to identify dissolved organic compounds which contribute to acute toxicity following exposure of the bacterium, *V. fisheri,* or embryos of fathead minnow (*Pimephales promelas*) (Figure A.1). Ultimately, this work generated two refined fractions of OSPW (F3-NE2a and F3-NE2b) which had acute toxicity and differed in their dose-response curves and chemical profiles (Figure 2.1 & 2.2). The more potent fraction, F3-NE2a, contained predominantly organic acids including NA (detected in negative ion mode) (Figure 2.3). In addition, the F3-NE2a fraction was acutely toxic at environmentally relevant concentrations (<1x equivalent), and represented less than 15% of the original organic mass of the original mixture, demonstrating the potency of these compounds.

The lesser potent fraction, F3-NE2b, contained predominantly polar neutral compounds detected by use of positive ion mode, including O_2^+, O^+, OS^+, and NO^+ containing chemical species. The F3-NE2b fraction was less potent than F3-NE2a as the LC50 of this fraction was greater than a 2x equivalent. However, both fractions exhibited significant toxicity at a 1x equivalent as survival of embryos of fathead minnow exposed to the F3-NE2a (2.50%) or F3-NE2b (72.5%) fractions was significantly less than control exposed embryos (Figure 2.1). Additionally, a pooled sample of F3-NE2a and F3-NE2b (i.e. F3-Pool) had greater potency than either individual F3 fraction, indicating that both fractions contributed significantly to toxicity. These results provided the first evidence to support a role for non-carboxylic acid chemical classes identified as O_2^+, O^+, OS^+, and NO^+ containing chemical species in the acute toxicity of BML-OSPW and confirmed the contribution of NA.

6.2.2 Predicting the acute toxicity of dissolved organic chemicals in BML-OSPW

Currently, guidelines to facilitate the release of OSPW have not been developed. In Canada, the use of water quality based effluent limits is the preferred approach for regulating the release of waste streams.[155] Therefore, by use of empirically derived accumulation estimates for chemical species dissolved in OSPW and chemical characterization data, an acute aquatic toxicity model was developed to predict the acute toxicity of the organic fraction of OSPW to embryos of fathead minnow. Because the literature contains two empirical measures of accumulation potential for chemical species in OSPW,[144] membrane affinity and poly(dimethyl)siloxane partition coefficients (K_{MA} and K_{OW}), the effect of partition estimates on predictions of acute toxicity was investigated to develop a robust and accurate toxicity model. By matching measured accumulation estimates with chemical species detected in fractions of BML-OSPW (Chapter 2), LC50 estimates were made for all chemical species in the mixture. By consideration of accumulation of chemical species to neutral ($K_{OW)}$ and polar lipids (K_{MA}), a sufficiently robust model could be developed which predicted toxicity of BML-OSPW within the error associated with biological assays (Figure 3.3). Because the model that was developed was constructed considering the contribution of all chemical species detected in the dissolved organic fraction of BML-OSPW, it facilitated a detailed examination of which chemical species contributed most to its toxicity and a comparison of their potencies (Figure 3.4). This work supported results of Chapter 2 as $O^{+/-}$, $O_2^{+/-}$, SO^+, NO^+ and SO_2^- containing chemical species were predicted to

account for the majority of acute toxicty of BML-OSPW. Additionally, it was demonstrated that SO^+ and SO_2^- are among the most potent chemical species in BML-OSPW.

6.2.3 Characterizing mechanisms of acute toxicity of the dissolved organic fraction of BML-OSPW

Petroleum related chemicals are generally believed to cause their toxicity by a narcosis mode of action.[108] Additionally, previous research has suggested NA and acidic polar compounds cause their toxicity by a narcosis mode of action.[9,34] Because many of the chemical classes identified in Chapter 2 as acutely toxic had previously never been identified, there existed significant gaps in our understanding of the mechanism(s) by which these chemical species cause their toxicity. Therefore, acute toxicity and potential molecular mechanisms of toxicity of fractions of BML-OSPW were investigated by monitoring the expression of over 1800 genes in an *E. coli* gene reporter system.

Following exposure to fractions of BML-OSPW, differentially expressed genes were identified and affected biological processes and KEGG pathways mapped. Genes indicative of general stress, protein damage and DNA damage were identified as uniquely responsive to acutely toxic extracts of BML-OSPW (Table 4.3). In addition, changes to the expression of multiple genes and associated biological processes and KEGG pathways were indicative of changes to the redox state of the cell and the response to oxidative stress, and are consistent with previous results across a range of extracts and species.[10,12,125] These results provided additional evidence to support a role for narcosis as the mechanism of acute toxicity of OSPW. Additionally, principle component analysis revealed a clear separation of fractions by use of chemical profiles and demonstrated that chemical classes containing SO_3^-, SO_4^-, NO^+, O_2NS^+, SO^+, ONS^+ or NO_3^- were associated with more acutely toxic fractions of BML-OSPW, further supporting the results of the effects-directed analysis (Chapter 2) and developed model (Chapter 3).

6.2.4 Characterizing the reproductive toxicity of the dissolved organic fraction of BML-OSPW

In Chapter 2, it was demonstrated that chemical classes in BML-OSPW are acutely toxic at concentrations less than those present in the environment, i.e. acidic oxygen containing chemical

species identified in the F3-NE2a fraction. In addition, it was demonstrated that nonacidic chemical species are acutely toxic at environmentally relevant concentrations (Figure 2.2). Previous work has demonstrated that NA and sulfur containing chemical species (SO^+ and SO_2^-) have high potentials to bioaccumulate.[144] Because a number of novel chemical species with acute toxicity and potential to bioaccumulate were identified in this thesis it was important to characterize their potential to cause chronic toxicity. Specifically, the 21-day fathead minnow reproductive bioassay was used to assess the potential for these chemical species to disrupt reproduction.

For this work, BML-OSPW was collected in the fall of 2015 and fractionated to generate two fractions, one containing predominantly acidic oxygen containing species, F1 (similar to F3-NE2a), and one containing predominantly polar neutral compounds, F2 (similar to F3-NE2b) (Figure 5.1). Following 21-days of exposure of fathead minnow breeding trios to fractions of BML-OSPW or BML-OSPW at 25% equivalent dilutions, no statistically significant effects were observed on fecundity, morphometric indices, transcript abundances of genes related to the endocrine system, or sex steroid concentrations in plasma of fathead minnow (Figure 5.2; Table 5.1). However, male fathead minnow exposed to intact BML-OSPW had greater hepatosomatic index (Table 5.1). In addition, embryos collected from exposed breeding trios demonstrated no difference in cumulative percent fertilization, time to hatch or incidence of malformations from control collected embryos (Table 5.2).

6.3 Future directions

1. The focus of this Ph.D. program was to identify and characterize toxic chemical classes in BML-OSPW. A number of novel chemical classes that cause acute toxicity were identified and there exists limited information as to the degradation potential of these chemical species or the effectiveness of current remediation technologies at removing them. Therefore, it is important for future work to investigate the degradation potential of acutely toxic chemical species identified throughout this work which have the potential to bioaccumulate.

2. By use of an effects-directed analysis approach it was demonstrated that chemical species dissolved in BML-OSPW are acutely toxic at environmentally relevant concentrations. However the toxicity of these chemical classes was only observed following chemical fractionation and

refinement of the complex mixture. This suggests that when present as the intact mixture, the toxicity of acutely toxic chemical species is antagonized. Thus, there is a need for future research to investigate the mechanism(s) by which the toxicity of these chemicals is antagonized when present as the intact mixture.

3. Recent work by Hughes *et al*.,[126] has provided further evidence to support a role for acidic oxygen containing chemical species (O_2^-, O_2S^-) in the acute toxicity of OSPW. Because the composition of OSPW is highly variable, further studies investigating the contribution of chemical classes to acute toxicity of OSPW using ultrahigh resolution mass spectrometry technologies are needed to support the development of release guidelines for the industry.

4. The work presented in this thesis has focused on identifying and characterizing the toxicity of BML-OSPW related chemicals to various life stages of fathead minnow and strains of bacteria. There is a need to further characterize the toxicity of these chemical species by use of other environmentally relevant species to facilitate the risk assessment process, such as algae, invertebrates and other vertebrate species, and to extend these tests to include chronic toxicity endpoints.

6.4 Final thoughts and discussion on the toxicity of OSPW

While this Ph.D. program was underway, the Alberta Energy Regulator released *Directive 085: Tailings Management Framework*, which is designed to guide oil sands operators in the development of tailings treatment technologies,[156] benchmarks for release, and to set deadlines for the remediation of legacy tailings and future tailings. In regards to OSPW, Directive 085 outlines three main requirements:

- All produced tailings must be on a trajectory to meet long term remediation targets (i.e. ready-to reclaim) within 10 years of mine closure

(1)… fluid tailings are treated and reclaimed progressively during the life of a project and all fluid tailings associated with a project are ready-to-reclaim within 10 years of the end of mine life…

- All legacy tailings must be reclaimed before mine closure

(2)…a requirement to have all legacy tailings in a ready-to-reclaim state at the end of mine life...

- Release of new waters will be considered but subject to as of yet established requirements

(3)…Where preferred approaches are found to be insufficient to manage all of the tailings generated and stored on site, regulatory applications that seek the return of new wastewater streams to the environment may be considered …subject to supplemental requirements, which may include enhanced wastewater characterization (i.e., physical, chemical and toxicological evaluation), additional criteria and guidelines for acceptable quality of release, wastewater treatment performance standards, more comprehensive in-stream modeling, and more stringent environmental effects monitoring requirements…

As outlined above, there is a need for oil sands companies to develop remediation strategies and targets, and to submit these to Alberta Energy Regulator for approval. The work presented in this thesis provides supporting evidence to help guide the development of remediation technologies and monitoring programs. Specifically, this work has provided evidence to support a role for acidic oxygen containing chemical species, including NA, as the primary toxic agents of the dissolved organic fraction of BML-OSPW. However, nonpolar neutral chemical species present in BML-OSPW, particularly those containing sulphur and nitrogen, are acutely toxic and have among the highest potentials to bioaccumulate.

As part of this work, an aquatic toxicity model was developed which was sufficiently robust to predict the acute toxicity of the dissolved organic fraction in BML-OSPW well within error associated with biological assays among laboratories for the same chemical and species. This acute aquatic toxicity model can be used to evaluate the effectiveness of remediation techniques without the use of *in vitro* or *in vivo* test systems and might be used in the development of release guidelines for oil sands operations. Furthermore, the developed model supports Directive 085, and provides a method for the establishment of guidelines for release of treated waters.

By use of a weight of evidence approach, results of this thesis support narcosis as the critical mechanism of acute toxicity for dissolved organic chemicals in BML-OSPW, as previously suggested. Steep dose-response curves of exposed embryos of fathead minnow, identified

mechanisms of action by use of the *E. coli* live cell array, and accurate predictions of a model developed assuming a narcosis mode of action, support narcosis as the critical mechanism of action of dissolved organic chemicals in BML-OSPW. These results support the use of traditional narcosis toxicity models, such as the target lipid model used in the presented model, for predicting the toxicity of oil sands related chemicals to facilitate the risk assessment process and the eventual release of oil sands related chemicals.

To further characterize the toxicity of novel acutely toxic chemical species in BML-OSPW, the 21-day fathead minnow short term reproduction bioassay was implemented. Exposure of fathead minnow to fractions of BML-OSPW or BML-OSPW at 25% equivalent dilutions did not affect reproduction. However, exposure to 25% equ, BML-OSPW as an intact mixture resulted in greater hepatosomatic index in male fathead minnow. These results support Directive 085 and identify endpoints following prolonged exposure of a fish species relevant to the oil sands region of Alberta.

By use of a weight of evidence of approach, including *in vivo*, *in vitro*, biomimetic and modeling techniques, it has been empirically demonstrated that NA (O_2^-) and the chemical classes identified as SO_2^-, O_2^+, SO^+ and NO^+ in OSPW contribute significantly to acute toxicity and have the potential to bioaccumulate. Results of this thesis supports previous work and highlights the environmental relevance of these chemical classes to the oil sands industry as remediation techniques and release guidelines are developed. Although specific structures and associated identities of chemical species in OSPW remain unresolved, results of this thesis support the use of uHRMS approaches to characterize the organic fraction of OSPW and associated toxicity. In addition, multiple lines of evidence exist to support narcosis as the critical mechanism of acute toxicity of OSPW. By use of the 21-day fathead minnow reproductive bioassay the chronic toxicity of novel chemical classes in OSPW was assessed and provides important chemical and toxicity data as the industry prepares to release treated OSPW.

As the oil sands industry begins to lay the foundation for releases of treated process-affected waters, there remains a need for the development of remediation targets and water quality guidelines. Results of this thesis has provided valuable insights as to chemical classes and chemical species of concern in the dissolved organic fraction of BML-OSPW that should be the focus of targeted remediation technologies due to their acute toxicity and potential to

bioaccumulate. In addition, a method to assess potential adverse effects of the dissolved organic fraction of BML-OSPW without biological testing has been developed to support the establishment of release guidelines for the industry. The petroleum industry will continue to grow over the coming decades and information presented in this thesis will help to ensure the responsible development of this resource to support the protection of aquatic life in the oil sands mining region of Canada.

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Appendices

Appendix A

Measurement of total organic carbon

Total organic carbon was measured with a Shimadzu TOC 505A non-purgeable organic carbon analyzer (Columbia, MD, USA) as the average of triplicate injections of 100 µL of sample. Prior to analysis, 2mL samples were acidified to pH 1 with 1 M HCl and sparged with high purity nitrogen for 1 min immediately prior to analysis to remove inorganic carbon.

Characterization of fractions by HPLC-Orbitrap uHRMS

Profiles of relative proportions of organic compounds in fractions were determined by use of reversed-phase high pressure liquid chromatography-linear ion trap-orbitrap mass spectrometry (Orbitrap Elite, Thermo Fisher Scientific, San Jose, CA, USA) by use of the method described in Pereira *et al*.[29] Chromatographic separation was performed by use of an HPLC Transcend system (Thermo Fisher Scientific), consisting of a degasser, a 1250 bar quaternary pump, an auto-sampler, and a column oven. Separation was performed on a Cosmosil C18 MS-II column (100 x 3.0 mm, 2.5 µm particle size) (Nacalai USA, San Diego, CA, USA) at 40 °C. A flow rate of 0.5 mL/min and an injection volume of 3 µL were used in all analyses. Mobile phases consisted of (A) 0.1% acetic acid in water, and (B) 100% methanol. The mobile phase composition was 5% B for 1 min, followed by a linear gradient ramp to 90% B at 9 min, to 99% B over 5 min, and returning to 5% B in 1 min followed by a 4 min hold prior to the next injection.

Determination of PAHs by GC/MS

A mixture at 10 µg/mL of the following PAHs: Acenaphthene, acenaphthylene, anthracene, benzo[a]anthracene, benzo[b]fluorathene, benzo[k]fluorathene, benzo[gh]perylene, benzo[a]pyrene, chrysene, dibenz[a,b]anthracene, fluoranthene, fluorene, indeno[1,2,3-cd]pyrene, naphthalene, phenanthrene and pyrene were purchased from Sigma-Aldrich (St. Louis, MO, USA). Concentrations of PAHs in fractions were determined by use of gas chromatography-mass spectrometry (GC/MS) (Agilent model 5973, Santa Clara, CA, USA) by use of a capillary column (30 m × 0.25 mm × 0.25 µm) containing 5% diphenyl and 95%

dimethylpolysiloxane HP-5MS (Agilent). The temperature in the oven was held at an initial temperature of 40 °C for 2 min and was raised a rate of 8 °C $min^{-1}$to 320 °C and held there for 20 min. Carrier gas was helium at a flow rate of 2.5 mL min^{-1}. The injector was operated at 310 °C in splitless mode for 0.75 min. The mass spectrometer was operated in electron ionization mode (EI) with energy of 70 eV. The temperature of the ion source was 200 °C, and the temperature of the GC/MS interface was 310 °C. The analysis was performed in ion monitoring and a limit of detection of approximately 0.2 ng/L was achieved for all PAHs analyzed.

Fathead minnow husbandry

Fathead minnows that were sexually mature were selected from a culture that is maintained in the Aquatic Toxicology Research Facility (ATRF) at the University of Saskatchewan. Eight tanks were established to collect eggs for the embryo-lethality assay, and each tank contained one sexually mature male and two sexually mature females. These tanks contained 10 L of dechlorinated tap water that was City of Saskatoon municipal water that had been dechlorinated, and half this volume was replaced daily. Tanks were maintained at 25 ± 1 °C with a 16/8 h day/night photoperiod. Minnows were fed frozen blood worms to satiety twice daily.

Fathead minnow exposure protocol

Embryos were collected within 1 h post fertilization, rinsed 3 times in dechlorinated tap water, and eggs that were not fertilized were discarded. Exposures were conducted in 6-well cell culture plates and depending on the number of eggs available the minimum number of eggs per well was 10 and the maximum number of eggs per well was 15. Each well contained 4 mL of control water (dechlorinated municipal tap water), control water with an appropriate concentration of EtOH (to a maximum of 0.1%), or the fraction. Exposures were performed at 26 ± 1 °C with a 16/8 h day/night photoperiod, and 50% of the volume of test solutions was replaced daily. Duration of exposures was 96 h, and numbers of embryos or larvae that were alive or dead were counted each day prior to renewal of test solutions, and embryos and larvae that were dead were removed.

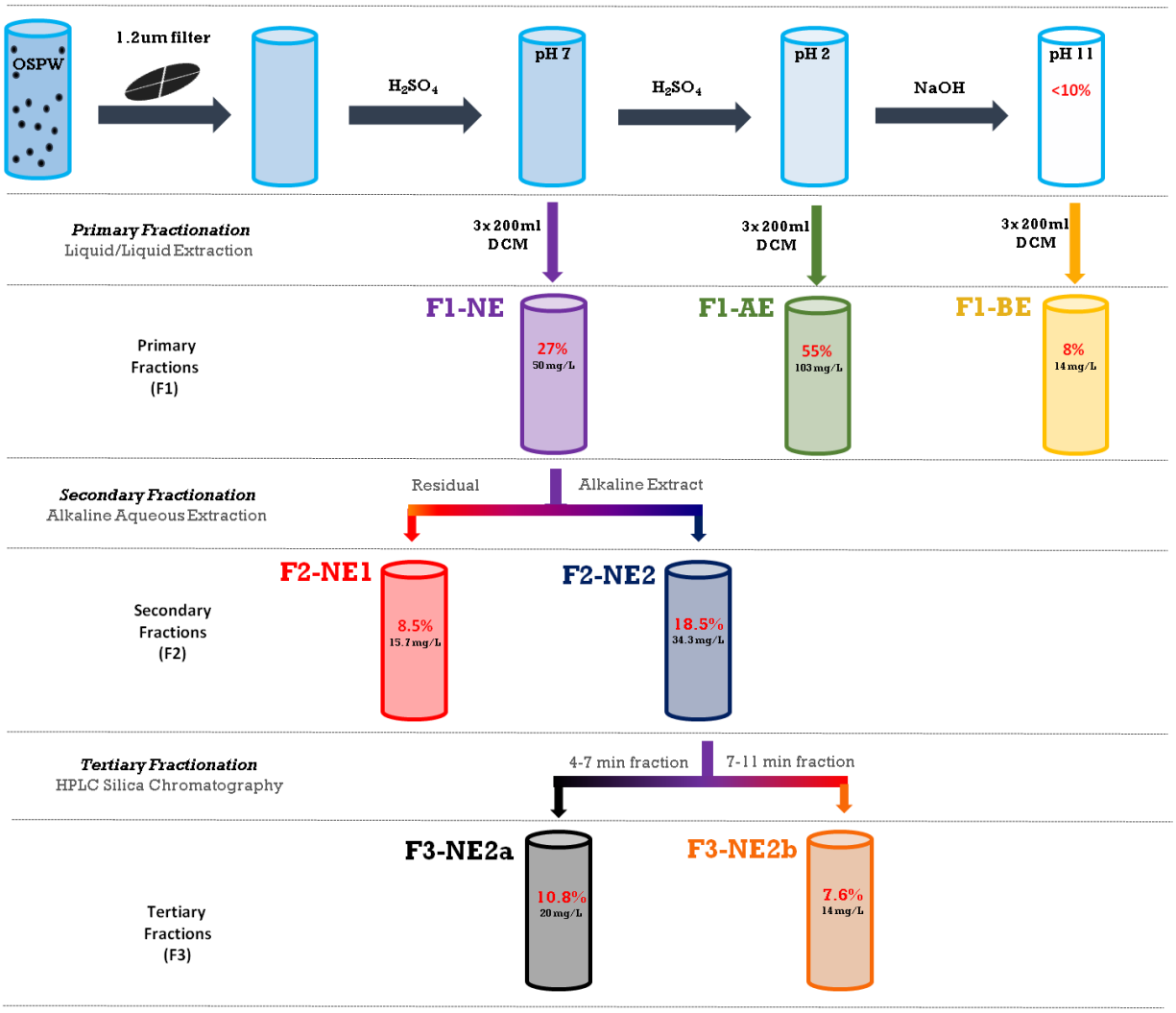

Figure A.1. Overall fractionation scheme implemented for the OSPW sample, also showing nomenclature of each primary (F1), secondary (F2) and tertiary (F3) fractions. Shown in red font is the percentages of the original OSPW dissolved organic compounds in each sub-fraction (based on gravimetric weight and TOC analysis), and in black font the gravimetric concentration of organic compounds (mg/L) for each fraction.

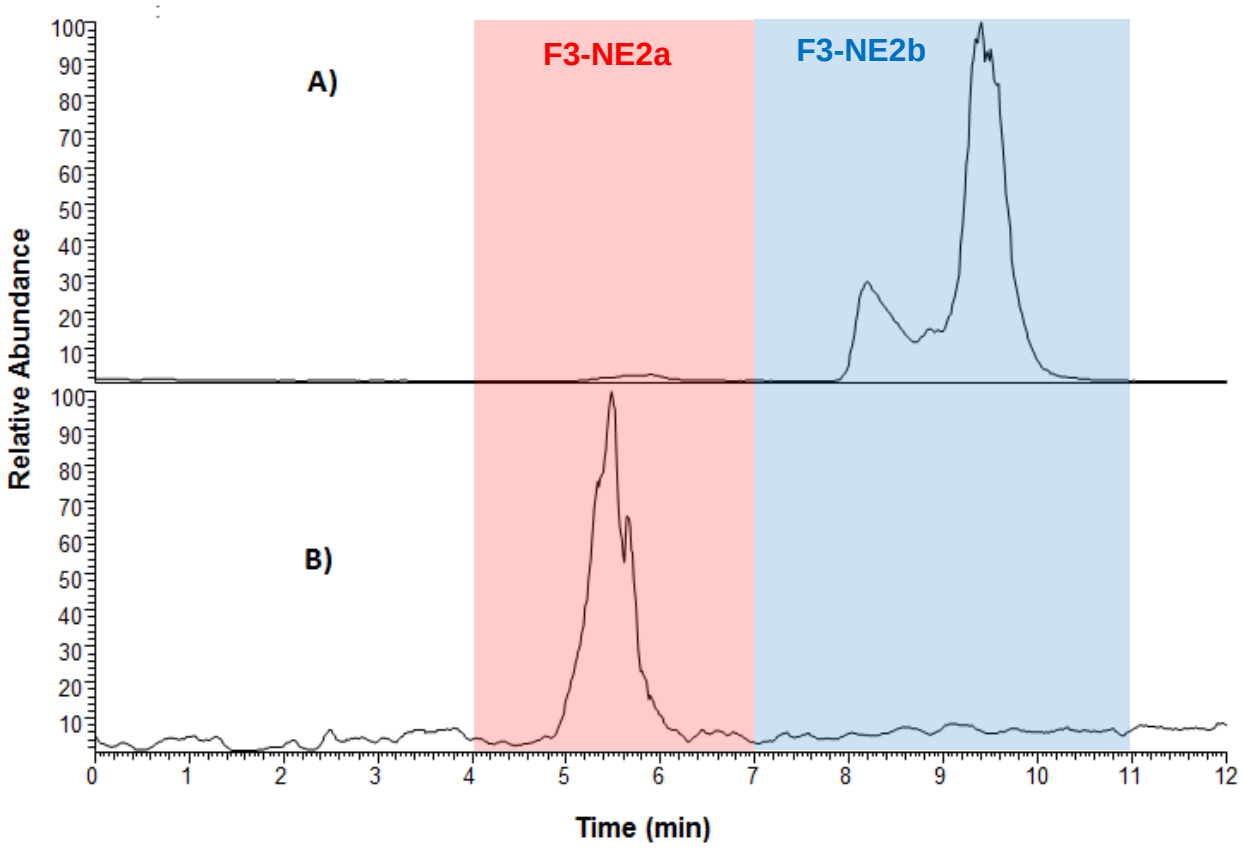

Figure A.2 Total ion chromatograms showing separation achieved for the final fractionation of F2-NE2 into sub-fractions F3-NE2A and F3-NE2B. Panel A shows the total ion chromatogram in positive ionization mode, while Panel B shows the total ion chromatogram in negative ionization mode. A fraction collection system collected the eluent from 4-7 minutes (F3-NE2a, red shaded region) and from 7-11 min (F3-NE2b, blue shaded region).

Table A.1 The gravimetric concentration of organic compounds (mg/L) for each primary fractionation blank.

Specific fraction	Concentration (mg/L)
b-F1-NE	0.08
b-F1-AE	0.07
b-F1-BE	0.12

Appendix B

Chemicals and materials

Acetic acid, dichloromethane (DCM), methanol (HPLC grade) and water (Optima grade) were purchased from Fisher Scientific (Fair Lawn, NJ, USA). The sample of OSPW was collected on the site of Syncrude Canada, Ltd. (Fort McMurray, Alberta, Canada) from the West In-Pit active settling basin, now known as BaseMine Lake (BML), in March 2011 and was stored at 4 °C until use.

Stock preparation for derivation of membrane- water partition coefficients

A total of 1 L of OSPW was filtered through a 0.45 μm filter (Millipore, Billerica, MA) to remove suspended solids and then extracted with 2×200 mL of DCM. Next, the extract was evaporated to near dryness with a rotary evaporator (model R- 210, Buchi, Toronto, Ontario, Canada). The remaining volume was transferred to a 20 mL glass vial and taken to full dryness under a gentle stream of nitrogen at room temperature (Turbovap LV, Biotage, Charlotte, NC) and dissolved in 1 mL of dimethyl sulfoxide (DMSO) to make stock solutions that were 1000-fold more concentrated than the original sample of OSPW. Then, 16 μL of the 1000-fold stock solutions were diluted by use of the aqueous buffer included in the membrane affinity kit to prepare stock solutions that were 50-fold more concentrated than the original sample of OSPW.

Characterization of Fractions by HPLC-Orbitrap-uHRMS

Profiles of relative proportions of organic compounds in fractions were determined by use of LC-uHRMS according to the method described by Pereira *et al*.[29] Chromatographic separation was performed by use of an HPLC Transcend system (Thermo Fisher Scientific), consisting of a degasser, a 1250 bar quaternary pump, an auto-sampler, and a column oven. Separation was performed on a Cosmosil C18 MS-II column (100 x 3.0 mm, 2.5 μm particle size) (Nacalai USA, San Diego, CA, USA) at 40 °C. A flow rate of 0.5 mL/min and an injection volume of 3 μL were used in all analyses. Mobile phases consisted of (A) 0.1% acetic acid in water, and (B) 100% methanol. The mobile phase composition was 5% B for 1 min, followed by a linear gradient ramp to 90% B at 9 min, to 99% B over 5 min, and returning to 5% B in 1 min followed by a 4 min hold prior to the next injection.

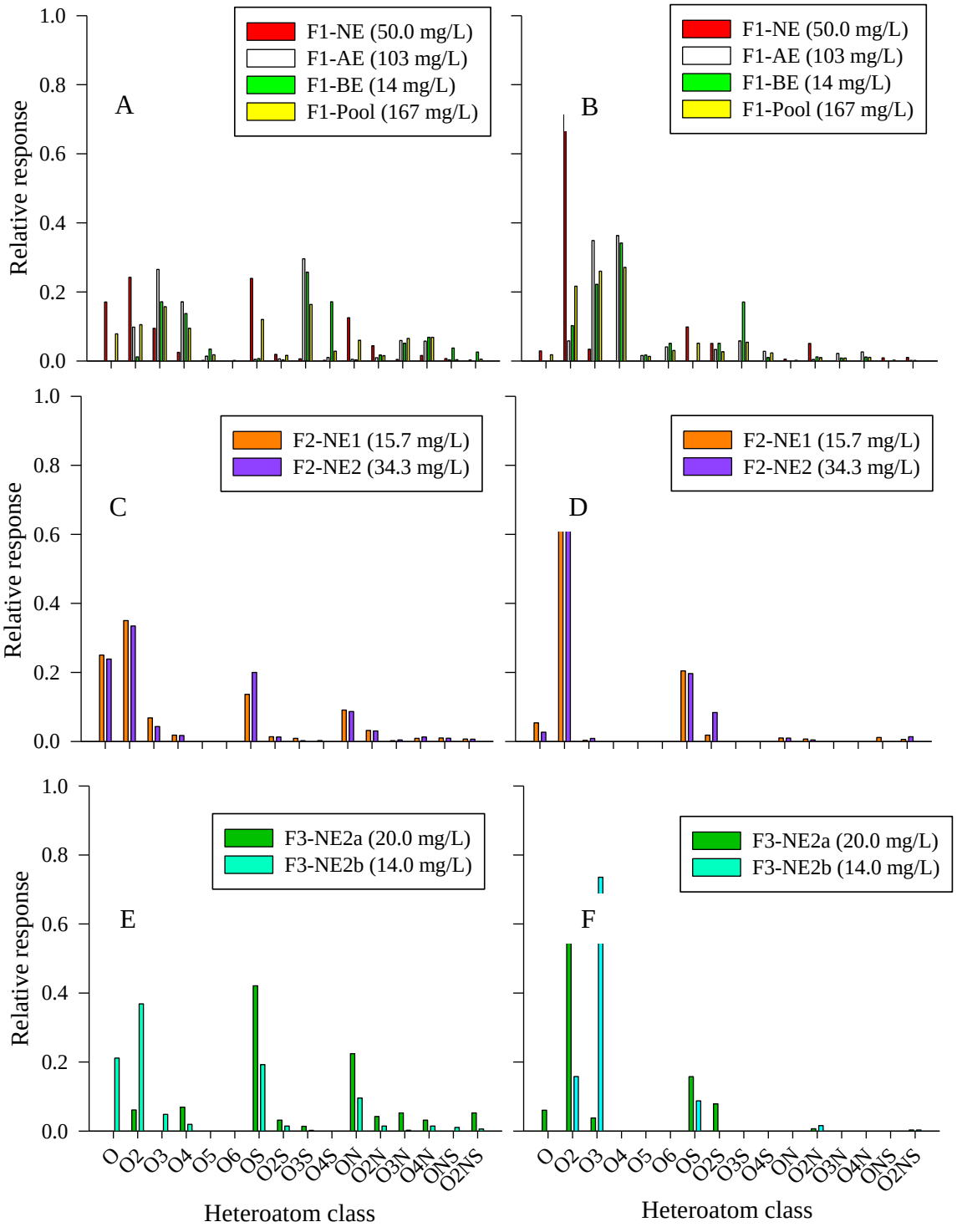

Figure B. 1 Total abundances of species by class of heteroatoms, based on sum of peak areas in chromatograms of fractions of BML-OSPW: A) Primary fractions in ESI+, B) Primary fractions in ESI-, C) Secondary fractions in ESI+, D) Secondary fractions in ESI-, E) Tertiary fractions in ESI+, F) Tertiary fractions in ESI-. Abundances were normalized to F1-Pool.

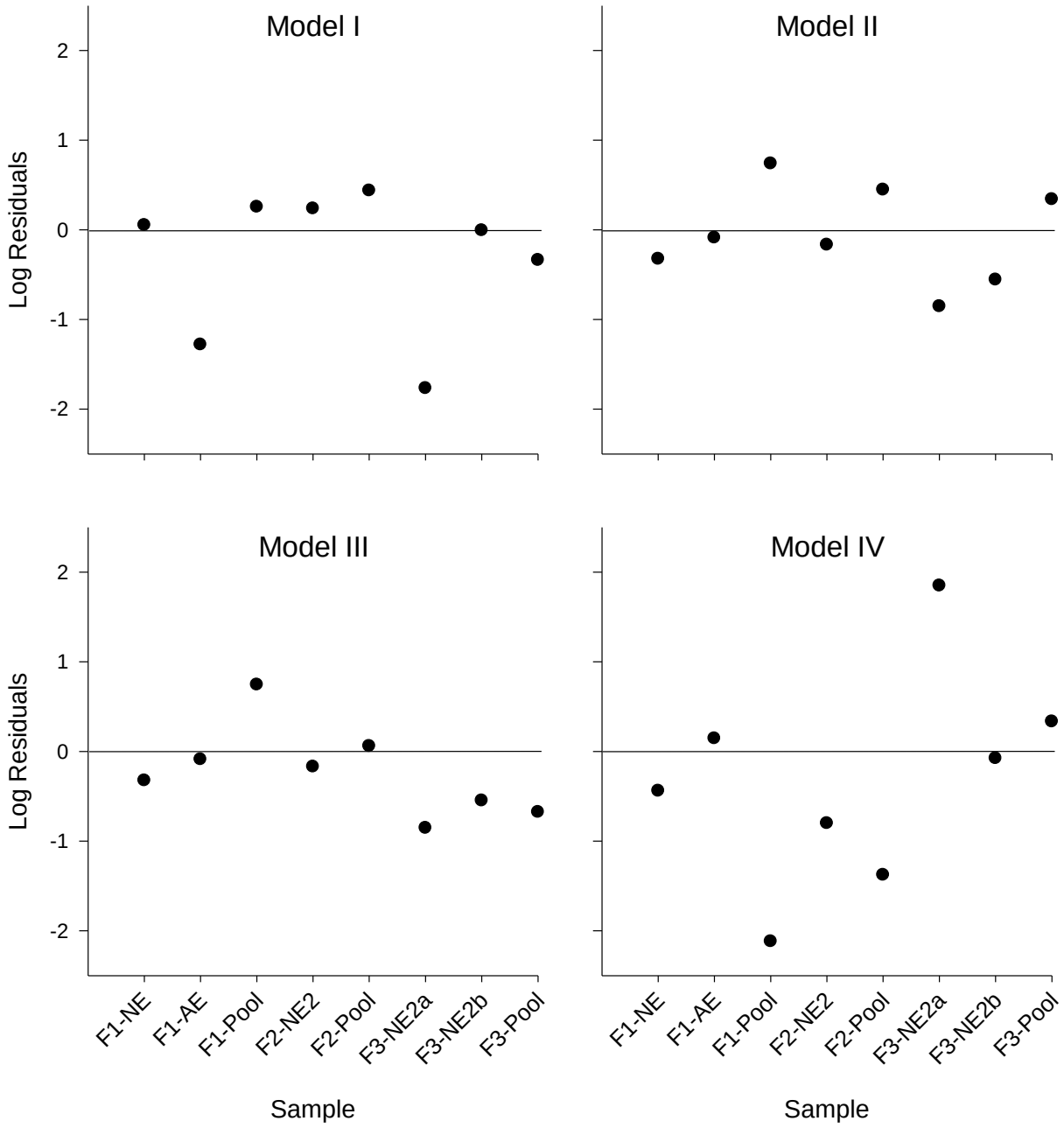

Figure B.2 Distribution of log of residuals between predicted and observed LC50 by use of Model I, II, III and IV.

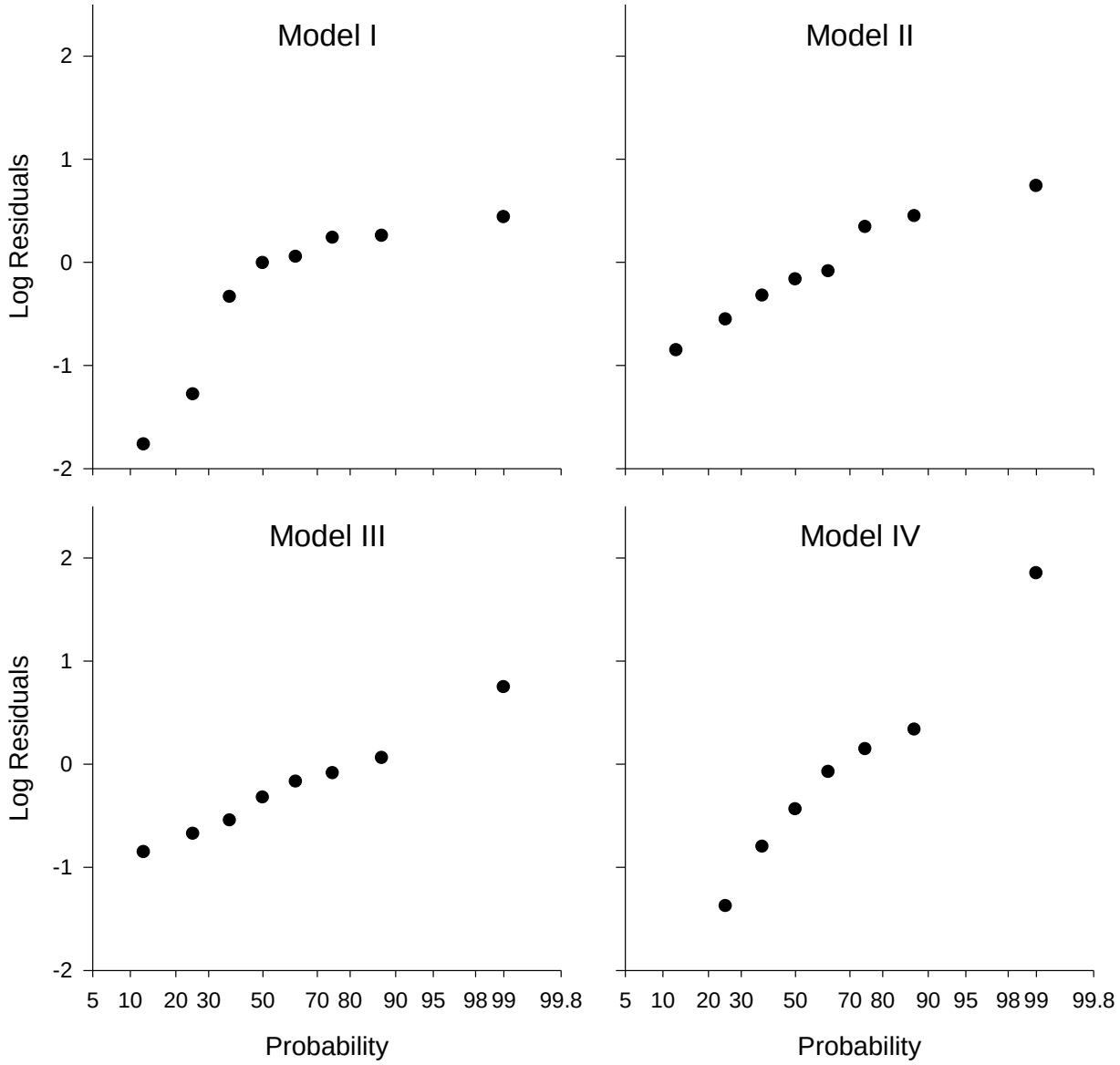

Figure B.3 Log residuals as a function of Log probability of occurring for Model I, II, III and IV.

Appendix C

Characterization of Fractions by HPLC-Orbitrap-uHRMS

Profiles of organic compounds in fractions were determined by use of LC-UHRMS according to methods described by Pereira *et al*.[29] Chromatographic separation was performed by use of an HPLC Transcend system (Thermo Fisher Scientific), consisting of a degasser, a 1250 bar quaternary pump, an auto-sampler, and a column oven. Separation was performed on a Cosmosil C18 MS-II column (100 x 3.0 mm, 2.5 µm particle size) (Nacalai USA, San Diego, CA, USA) at 40 °C. A flow rate of 0.5 mL/min and an injection volume of 3 µL were used in all analyses. Mobile phases consisted of (A) 0.1% acetic acid in water, and (B) 100% methanol. The mobile phase composition was 5% B for 1 min, followed by a linear gradient ramp to 90% B at 9 min, to 99% B over 5 min, and returning to 5% B in 1 min followed by a 4 min hold prior to the next injection. For figures and statistical analysis, total ion counts were summed for individual chemical classes.

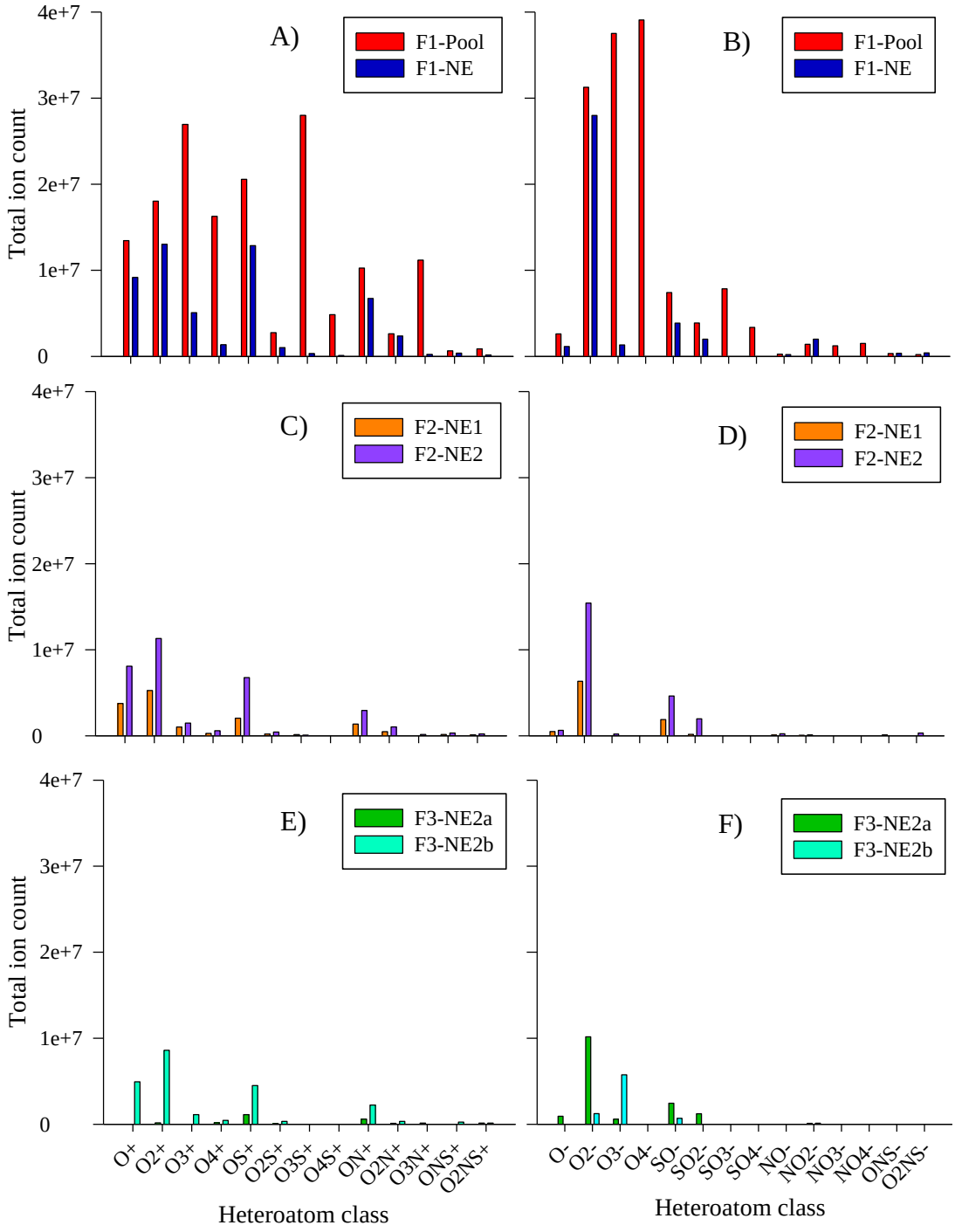

Figure C.1 Total ion count for chemical classes detected in extracts of OSPW, A) Primary fractions in ESI+, B) Primary fractions in ESI-, C) Secondary fractions in ESI+, D) Secondary fractions in ESI-, E) Tertiary fractions in ESI+, F) Tertiary fractions in ESI-.

Table C.1 Labels used to identify biological processes or KEGG pathways for PCA analysis.

PCA ID	GO ID	GO Term
BP1	GO: 16051	carbohydrate biosynthetic process
BP2	GO: 7154	cell communication
BP3	GO: 8652	cellular amino acid biosynthetic process
BP4	GO: 43094	cellular metabolic compound salvage
BP5	GO: 6732	coenzyme metabolic process
BP6	GO: 51188	cofactor biosynthetic process
BP7	GO: 1901657	glycosyl compound metabolic process
BP8	GO: 18130	heterocycle biosynthetic process
BP9	GO: 34220	ion transmembrane transport
BP10	GO: 48523	negative regulation of cellular process
BP11	GO: 34655	nucleobase-containing compound catabolic process
BP12	GO: 9311	oligosaccharide metabolic process
BP13	GO: 1901615	organic hydroxy compound metabolic process
BP14	GO: 1901576	organic substance biosynthetic process
BP15	GO: 30	Pentose phosphate pathway
BP16	GO: 71822	protein complex subunit organisation
BP17	GO: 50794	regulation of cellular process
BP18	GO: 9266	response to temperature stimuli
BP19	GO: 9636	response to toxic substance
BP20	GO:44283	small molecule biosynthetic process

Table C.2 Labels used to identify chemical classes detected in extracts of OSPW for PCA analysis.

PCA ID	Chemical Class
CC1	O^-
CC2	O_2^-
CC3	O_3^-
CC4	O_4^-
CC5	SO^-
CC6	SO_2^-
CC7	SO_3^-
CC8	SO_4^-
CC9	NO^-
CC10	NO_2^-
CC11	NO_3^-
CC12	NO_4^-
CC13	ONS^-
CC14	O_2NS^-
CC15	O^+
CC16	O_2^+
CC17	O_3^+
CC18	O_4^+
CC19	SO^+
CC20	SO_2^+
CC21	SO_3^+
CC22	SO_4^+
CC23	NO^+
CC24	NO_2^+
CC25	NO_3^+
CC26	ONS

CC27	O_2NS

Table C.3 1.5-fold differentially expressed genes of *E.coli* exposed to extracts of OSPW.

F1-Pool	F1-NE	F2-NE1	F2-NE2	F3-NE2a	F3-NE2b
add	adhE	aer	adhE	add	add
adhE	adrA	allS	adrA	adhE	adhE
aldA	aer	ansB	aer	aldA	aldA
apt	allS	araD	allS	apt	apt
aspA	ansB	atpI	araD	aspA	aspA
b0663	araD	b3007	araE	b0663	b0663
bolA	argI	bax	argI	bolA	bolA
brnQ	asd	betT	atpI	brnQ	brnQ
cdd	atpI	clpX	b3007	cdd	cdd
clpB	b3007	corA	bax	clpB	clpB
clpS	betT	cysZ	betT	clpS	clpS
cpdB	chaB	dapA	chaB	cmr	cmr
crp	clpB	dapF	clpB	cpdB	cpdB
ddlA	clpX	def	clpX	crp	crp
deoB	corA	dhaR	corA	ddlA	ddlA
deoC	creB	dnaK	creB	deoB	deoB
dksA	cysZ	ecfK	cysZ	deoC	deoC
dsbG	dapA	eutR	dapA	dksA	dksA
emrR	dapF	fdrA	dapF	dppA	dppA
fkpB	def	flgB	def	dsbG	dsbG
frdA	dhaR	ftsZ	dhaR	emrR	emrR
gadW	ecfK	galS	dnaK	fkpB	fkpB
glpA	elaA	glmU	ecfK	frdA	frdA
glyQ	eutR	glnB	eutR	gadW	gadW
gntP	fdrA	glnU	fdrA	glpA	glpA
gpt	fecI	gntT	flgB	glyQ	glyQ
hdhA	flgB	hisL	ftsZ	gntP	gntP
hemH	ftsZ	ibpB	galS	gpt	gpt
insC-4	gadW	ileV	glmU	hcaT	hcaT
insE-4	galS	ilvI	glnB	hdhA	hdhA
ispB	gatR_1	ilvY	glnU	hemH	hemH
kdgR	glmU	insC-7	gntT	insC-4	insC-4
kil	glnU	iscR	hrpA	insE-4	insE-4

lacZ	gntT	kdtA	ibpB	ispB	ispB
lipA	hisL	lacI	ilvI	kdgR	kdgR
lpxP	hrpA	lacZ	ilvY	kil	kil
mcrA	ibpB	lgt	insC-7	lacZ	lacZ
menF	ileV	lolA	insE-3	lipA	lipA
menG	ilvI	map	kdtA	lpxP	lpxP
mokB	insE-3	mazG	lacI	mcrA	mcrA
murC	iscR	mcrB	lacZ	menF	menG
nanT	kdgR	mfd	lgt	menG	mgsA
nohB	kdsB	mog	lolA	mgsA	mokB
nupG	kdtA	otsB	mcrB	mokB	murC
pdxH	lacI	oxyR	mfd	murC	nohB
pgi	lacZ	pyrH	mog	nohB	nupG
pheP	lgt	queD	ompC	nupG	pdxH
phnC	lolA	racR	otsB	pdxH	pgi
pitA	map	rfaH	oxyR	pgi	pheP
pldA	mazG	rhsD	pldA	pheP	phnC
plsB	mcrB	ribC	pntA	phnC	pitA
pntA	mfd	rpoD	prfC	pitA	pldA
potA	mog	serA	pyrH	pldA	plsB
pspF	otsB	sfmA	racR	plsB	pntA
pykF	oxyR	torT	rfaH	pspF	potA
pyrB	phoA	ubiG	rhsD	pyrB	pspF
rcsF	pldA	ucpA	ribA	ribE	pykF
ribE	pntA	yacH	ribC	rimL	rcsF
rimL	pyrH	yacL	rmuC	rluB	rluB
rluB	queD	yaeH	rpoD	rrlE	rrlE
rrlE	racR	yafD	sdaA	rumA	rumA
rumA	relB	yagG	serA	serA	sieB
serA	rhsD	yagT	sodA	sieB	smpA
sieB	ribA	ybaP	sodC	smpA	speE
smpA	ribC	ybdK	ssb	speE	sscR
speE	rmuC	ybdL	sufI	sscR	talB
sscR	rpoD	ybhC	tesB	talB	tktA
talB	sdaA	ybjL	tolB	tktA	tufA
tktA	serA	yciG	torT	tufA	tyrP
tufA	sodA	ydhD	trxC	tyrP	ubiX
tyrP	sodC	ydjN	tyrP	ubiX	uspF
ubiX	ssb	yebR	ubiC	uspF	uspG
uspF	tolB	yeiE	ubiG	uspG	wrbA
uspG	torT	yfcJ	ucpA	wrbA	xseB

wrbA	tufA	yfeA	yacH	xseB	yabN
xseB	ubiC	yfeC	yacL	yabN	ybdL
yabN	ubiG	yffH	yaeH	ybdL	ybeB
ybdL	ucpA	yfiF	yafD	ybeB	ybgD
ybeB	yacH	ygeY	yagT	ybgD	ybjE
ybgD	yacL	yhaH	ybaP	ybjE	ybjN
ybjE	yaeH	yheO	ybcW	ybjN	yccA
ybjN	yafD	yhhT	ybdK	yccA	ycdC
yccA	yagG	yjdB	ybdL	ycdC	ycdZ
ycdC	ybaP	yjjV	ybhC	ycdZ	yceF
ycdZ	ybcW	ykgF	ybiS	yceF	yceP
yceF	ybdK	ykiA	ybjE	yceP	ycjM
yceP	ybhC	ypfG	ybjL	ycfQ	ydcF
ycjM	ybiS	ypjM_3	yceP	ycjM	ydfZ
ydcF	ybjE	yqhC	yciG	ydcF	ydiY
ydfZ	ybjL	yqhD	yddA	ydcJ	yedP
ydhB	yceP	yrfF	ydhD	ydfZ	yeeJ
ydiY	yciG		ydhR	ydhB	yejL
yedP	yddA		ydiQ	ydiY	yfbR
yejL	ydhD		ydjN	yedP	yfcC
yfbR	ydjN		yeaH	yejL	yfgA
yfcC	yeaH		yebR	yfbR	ygiU
yfgA	yebR		yeeI	yfcC	ygjH
ygiU	yeiE		yeiE	yfgA	yhbX
ygjH	yfcJ		yfcJ	ygiU	yhfG
yhbX	yfeA		yfeA	ygjH	yhfX
yhfG	yffH		yffH	yhbX	yhhW
yhfX	yfiF		ygeY	yhfG	yiaG
yhhW	ygeY		ygjD	yhfX	yiaJ
yhiI	ygjV		ygjV	yhhW	yidE
yiaG	yhaH		yhaH	yiaG	yigI
yiaJ	yhbW		yheO	yidE	yjcE
yidE	yhfK		yhhT	yigI	yjeN
yigI	yhhT		yiiU	yjcE	ykfA
yjcE	yjdB		yjdB	yjeN	ykgE
yjeN	yjjV		yjjV	ykfA	ymfI
ykfA	ykgE		ykgE	ykgE	ynfM
ykgE	ykgF		ykgF	ykgJ	ynjF
ykgJ	ykiA		ykiA	ymcC	yohJ
ymcC	ypfG		ypfG	ymfI	yqfA
ymfI	ypjM_3		yqcD	ynfM	yqjF

ymjA	yqhC		yqeG	ynjF	ytfB
ynfM	yqhD		yqfA	yohJ	ytfR
ynjF	yrfF		yqhC	yqfA	
yohJ			yqhD	yqjF	
yqfA			yrfF	ytfB	
yqjF				ytfR	
ytfB					
ytfR					

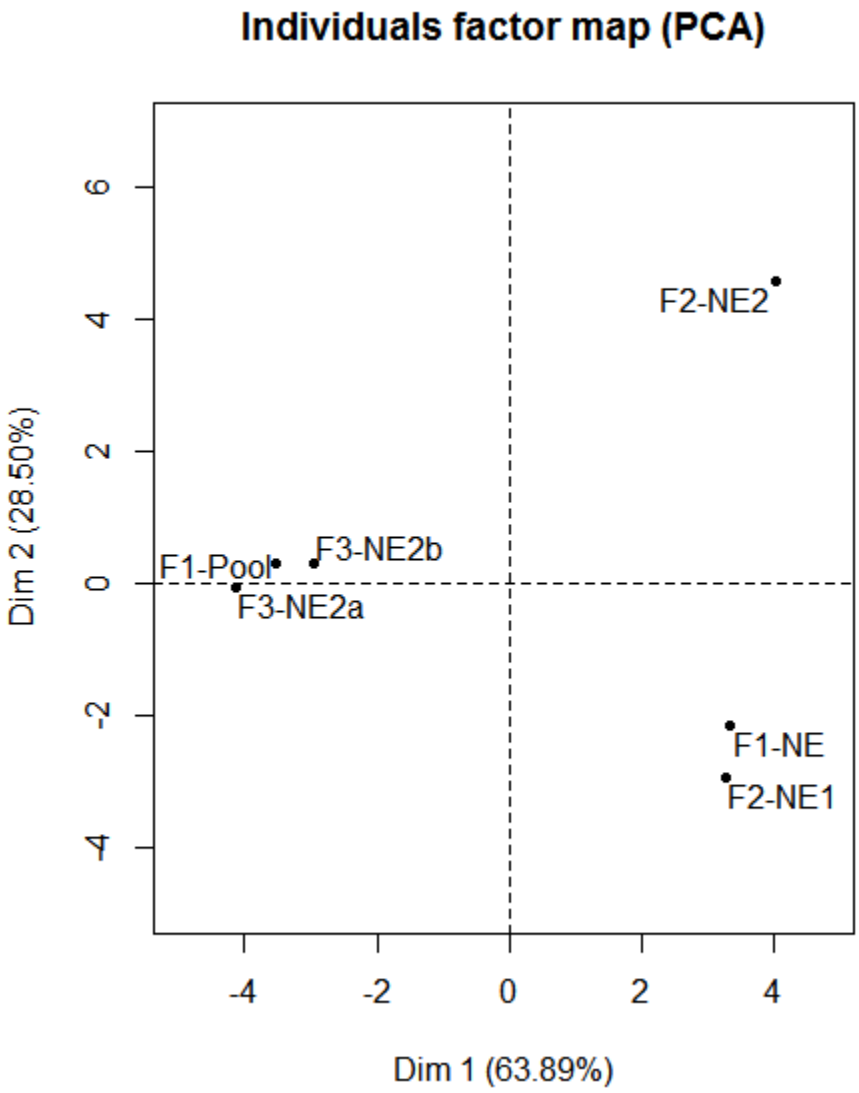

Figure C. 2 Individual factor map of extracts of OSPW, clustered by use of identified BP.

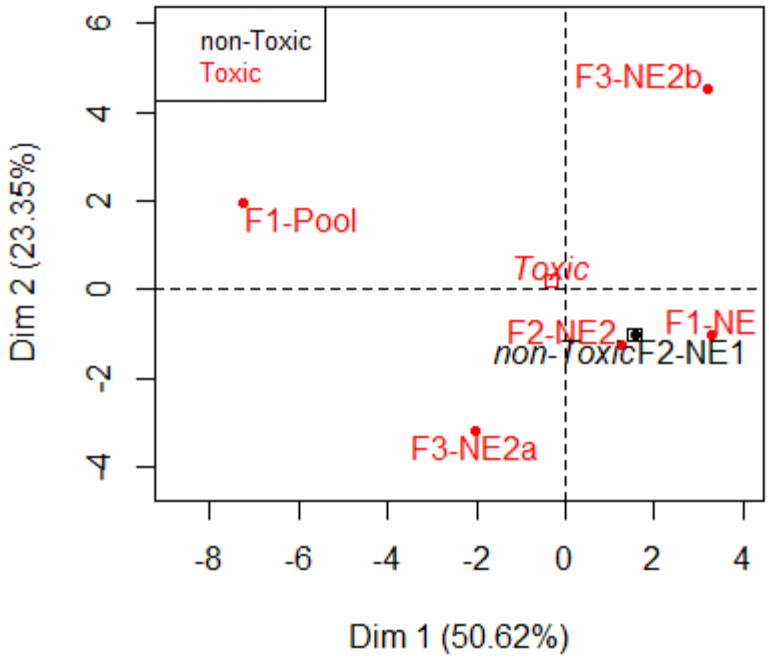

Figure C.3 Individual factor map of extracts of OSPW, clustered by use of identified chemical classes.