CHARACTERIZING TOXICITY AND CHEMICAL PROFILE OF ACIDIC, BASIC AND NEUTRAL FRACTIONS OF OIL SANDS PROCESS-AFFECTED WATER

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ABSTRACT

There is concern about toxicity of Oil Sands-Affected Process Water (OSPW). Results of previous studies have shown that toxicity is caused primarily by organic chemicals in the aqueous phase. Most research has focused on acute and chronic toxicity of the acid-extractable organic fraction to aquatic organisms. It has been shown that acute toxicity is caused by organic acids commonly known as naphthenic acids (NAs) as well as other sulfur- and nitrogen-containing compounds. Also, chemical analyses of OSPW have been focused only on the acid extractable fraction, however, little is known about sub-lethal effects and chemical profiles of species of chemicals other than NAs or the "acid-extractable organics (AEOs)" extracted from OSPW.

The aim of this research was to evaluate effects of acidic and non-acidic compounds in OSPW. First, a comprehensive chemical extraction method was developed to simultaneously isolate acidic, basic and neutral fractions from an end-pit lake known as Base Mine Lake (BML-OSPW; fresh OSPW) and an experimental reclamation pond known as Pond 9 or Tailings Pond Water (P9-OSPW or aged OSPW). Second, these fractions were analysed using ultrahigh resolution mass spectrometry (Orbitrap), which possess high resolution and superior selectivity and covered an extensive range of chemical components. Third, these fractions, which contain structurally different chemicals, were assessed for acute and sub-lethal toxicity of aquatic species with respect to functions and expression of genes for ABC transporter proteins as well as modulation of toxic potencies of polycyclic aromatic hydrocarbons (PAHs).

The acidic fraction of BML but not P9-OSPW was acutely toxic to larvae of Japanese medaka. However, there is a lack of information about the reasons for lesser toxic potency of P9-OSPW. Profiles of relative proportions of organic chemicals in the acidic fractions of BML and P9-OSPW were investigated in detail, by use of ultrahigh resolution mass spectrometry (Chapter

2). In both ionization modes, mass spectra were similar between the acidic fractions of BML and P9 extracts. However, relative abundances of chemicals between the two fractions were different. The profile of homologs containing heteroatoms in each fraction revealed that the profile of nitrogen, sulfur, and oxygen (NSO) containing chemicals was not very different. Acidic fractions were dominated by Ox chemicals. Sulfur and nitrogen-containing chemicals were detected in the acid fraction with high abundances in ESI^+.

The ATP-binding cassette (ABC) superfamily of transporter proteins are essential for detoxification of xenobiotic. Effects of acidic, basic and neutral compounds from BML-OSPW and P9-OSPW on activities of ABC transporters in Japanese medaka (*Oryzias latipes*) at the fry stage of development were investigated (Chapter 3). The neutral and basic fractions, but not the acidic fraction, of BML-OSPW, and neither fraction of P9-OSPW, caused inhibition of ABC proteins in fry of Japanese medaka. Neutral and basic fractions of BML-OSPW contained relatively greater amounts of several oxygen-, sulfur, and nitrogen-containing chemical species. Naphthenic acids (O_2^-), which were dominant in the acidic fraction, did not appear to be the cause of inhibition.

Solubility, bioavailability, and toxicity of PAHs might be modulated in the presence of surfactants. Effects of organic compounds extracted from BML-OSPW or P9-OSPW on toxicity of the model alkyl-PAH, retene, to early life-stages of Japanese medaka was determined (Chapter 4). Effects of retene were greater when larvae were co-exposed to OSPW compared to retene alone. However, those effects would not be expected to occur at current concentrations of OSPW and is attenuated by aging of OSPW.

Detailed studies on effects of OSPW on functions of ABC transporters (specifically P-gp) were performed *in vitro* by use of Caco-2 cells, and *in vivo* with larvae of Japanese medaka

(Chapter 5). Neutral and basic fractions of BML-OSPW inhibited activity of ATP binding cassette protein ABCB1 (permeability-glycoprotein, P-gp) in Caco-2 cells, while the acidic fraction had the least effect. Co-exposure to chlorpyrifos (a substrate of P-gp), but not malathion (not a substrate of P-gp) and an extract of OSPW containing basic and neutral compounds reduced survival of larvae, increased the internal concentration, bioconcentration and terminal elimination half-life compared to survival of larvae exposed only to chlorpyrifos.

Effects of acidic, basic and neutral compounds from BML-OSPW on expression of genes encoding biotransformation enzymes and proteins were quantified (Chapter 6). Abundances of transcripts of phase I, II and III genes were significantly different in larvae of medaka exposed to basic and neutral compounds compared to acidic compounds. Changes in abundance of transcripts in response to the pooled sample and the acidic fraction were correlated, whereas changes in abundance of transcripts in larvae exposed to the basic fraction and the neutral fraction were correlated.

From results presented in this thesis, it can be concluded that besides acute toxicities of acidic compounds (i.e. NAs) in OSPW, other compounds in basic and neutral fractions from OSPW can have adverse effects at sub-lethal levels, and may interact with the different molecular target in fish that can cause specific endpoints of toxicity. These results provide new insights into the sub lethal effects of non-acidic compounds regarding the detoxification mechanisms such as ABC transporters, which can decrease the effective protection mechanism against xenobiotic in aquatic species. Also, the toxicity of PAHs is expected to increase in the presence of OSPW, however, aging of OSPW was shown to be effective in attenuating the adverse effects of not only the acutely toxic acidic fraction but also the effects of non-acutely toxic basic and neutral fractions.

This thesis explores new concepts, methods and approaches that contribute to the risk assessment of OSPW.

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Figure A1: Survival of embryos of Japanese medaka exposed to A) 0.5, 1, 2.5, or 5× equivalent of the acidic fraction of BML-OSPW or a 5× concentration of basic and neutral of BML-OSPW, and 5× equivalent of the acidic, basic and neutral fraction of P9-OSPW. Survival is expressed as mean ± standard deviation of 3 independent studies in which there were 3 replicate exposures with 10-12 eggs per replicate. Significant differences in survival compared to control were determined by use of one-way ANOVA followed by Dunnett's post-hoc test and are designated by an asterisk. (**** = $P \leq 0.0001$). 221

LIST OF ABBREVIATIONS

°C	degree Celsius
ABC	ATP-binding cassette subfamily b member 4 (abcb4), subfamily c members 1 ,2 ,3 , and 5 (abcc1, abcc2, abcc3, abcc5), subfamily g member 2 (abcg2)
ANOVA	analysis of variance
ATRF	Aquatic Toxicology Research Facility
BCF	Bio-concentration factor
BML	Base Mine Lake
Caco-2	human colon adenocarcinoma cell line
cDNA	complimentary deoxyribonucleic acid
cm	centimetre
cyp 1a	cytochrome P450, family 1a
cyp 2a	cytochrome P450, family 2a
cyp 2c	cytochrome P450, family 2c
cyp 3a	cytochrome P450, family 3a
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EDA	effect directed analysis
FBS	fetal bovine serum
g	gram
GC/MS	gas chromatography/mass spectrometry
h	hour
HRMS	high resolution mass spectrometry
kg	kilogram
L	litre
LC/MS/MS	liquid chromatography-mass spectrometry/mass spectrometry
mg	milligram
mg l^{-1}	milligram per litre
min	minute
mL	millilitre
mRNA	messenger ribonucleic acid
MDR	Multi-Drug Resistance Proteins
MRP	Multi-Drug Resistance Associated Proteins
n	sample size
ng l^{-1}	nanogram per litre
ng g^{-1}	nanogram per gram
nM	nanomolar
NAs	Naphthenic acids
NSERC	Natural Sciences and Engineering Research Council of Canada
NSO	Nitrogen, Sulfur, Oxygen
OSPW	oil sands process affected water
P9	Pond 9
PAHs	polycyclic aromatic hydrocarbons
P-gp	permeability-glycoprotein

PXR	pregnane X receptor
qPCR	quantitative polymerase chain reaction
RNA	ribonucleic acid
rpm	revolutions per minute
S.E.M	standard error of the mean
sult1a	sulfotransferase, family 1a
$T_{1/2}$	half life
$\mu g\ L^{-1}$	microgram per liter
ugt1a	uridine diphosphate glucuronsyltransferase, family 1a
ugt2a	uridine diphosphate glucuronsyltransferase, family 2a

PREFACE

Chapter 1 of this thesis is a general introduction. Chapters 2, 3, 4, 5, and 6 are organized as manuscripts for publication in scientific journals. Thus, there is some repetition between the introduction and the materials and methods sections in each chapter. Some of these chapters have been previously published:

Chapter 3:

Alharbi, H.A., Saunders, D.M., Al-Mousa, A., Alcorn, J., Pereira, A.S., Martin, J.W., Giesy, J.P. and Wiseman, S.B., 2016. Inhibition of ABC transport proteins by oil sands process affected water. Aquatic Toxicology, 170, 81-88.

Chapter 4:

Alharbi, H.A., Morandi, G., Giesy, J.P. and Wiseman, S.B., 2016. Effect of oil sands process-affected water on toxicity of retene to early life-stages of Japanese medaka (*Oryzias latipes*). Aquatic Toxicology, 176, 1-9.

Chapter 5:

Alharbi, H.A., Alcorn, J., Al-Mousa, A., Giesy, J.P. and Wiseman, S.B., 2017. Toxicokinetics and toxicodynamics of chlorpyrifos is altered in embryos of Japanese medaka exposed to oil sands process-affected water: evidence for inhibition of P-glycoprotein. Journal of Applied Toxicology, 37(5), 591-601.

1 CHAPTER 1: GENERAL INTRODUCTION

PREFACE

Chapter 1 is a general introduction and literature review in the chemistry and toxicity of crude and sands oil as well as oil sands process affected water. This chapter includes the goals and objectives of each study in this thesis.

1.1 Petroleum-derived polar organic compounds: an overview

1.1.1 Chemistry

Water soluble compounds in petroleum, heavy oil, and bitumen are diverse and contain a greater proportion of higher-boiling point, more aromatic, and heteroatom-containing nitrogen, sulfur, or oxygen (NSO) containing groups, or non-hydrocarbon constituents than do other lighter oils. For example, individual alkyl homologues of nitrogen, oxygen and sulfur-and oxygen containing compounds (NSO compounds), including alkylated carbazoles, quinolones, pyridines, thiophenes, and dibenzothiophenes have been identified in several oils (Wang et al., 2005). More polar, water-soluble compounds are found primarily in fractions of crude oil containing aromatics, resins or asphaltnes (Speight, 2001; 2007). Although more polar hetero-atom, cyclic compounds are present in oil at relatively small concentrations (less than 15%) (Table 1.1), weathering increases their relative proportions. Oxygen-containing compounds mainly include ketones, furans, phenols, aldehydes, esters, ethers, and carboxylic acids (commonly known naphthenic acids; NAs), which is the most diverse group of oxygenated compounds in the polar water-soluble fraction of crude oil. Nitrogen-containing compounds in petroleum can be classified as basic, such as pyridine homologues, or non-basic, such as pyrrole, indole, and carbazole, but these are less abundant in crude oil (Speight, 2001). Sulfur-containing compounds are the predominant heteroatomic constitutes of petroleum. Sulfur-containing compounds might be present in several forms, including elemental sulfur, sulfide, sulfoxide, disulfide, thiols, and thiophene (and its alkylated homologues), benzothiophene (and its alkylated homologues), dibenzothiophene (and its alkylated homologues), and napthobenzothiophenes (Speight, 2001; Wang et al., 2005). Percent contribution of elements in conventional and unconventional crude oil is given in Table 1.1.

Table 1.1: Chemical composition of heavy crude oil and oil sand bitumen (Speight, 2001)

Element	Percent contribution	
	Conventional petroleum (heavy crude oil)	Unconventional petroleum (oil sand bitumen)
Carbon	83.0 to 87%	83.4 ± 0.5%
Hydrogen	10.0 to 14.0%	10.4 ± 0.2%
Nitrogen	0.1 to 2.0%	0.4 ± 0.2%
Oxygen	0.05 to 1.5%	1.0 ±0.2%
Sulfur	0.05 to 6.0%	5.0 ± 0.5%
Metals (Ni, V)	> 1000 ppm	> 1000 ppm

1.1.2 Occurrence and fate of water-soluble compounds in produced water

Produced water (or oilfield wastewater) is generated during production of oil and gas. This water contains most of the more polar, water-soluble compounds that are present in crude oil (Fakhru-Razi et al., 2009; Neff et al., 2011). Produced water can be generated when fresh water and production chemicals are injected into a reservoir to enhance recovery rates; these surface waters and chemicals sometimes penetrate to the production zone and are recovered with oil and gas (Veil et al. 2004; Neff et al., 2011).

The profile of petroleum-derived chemicals in produced water is illustrated in Figure 1.1. In general, produced water contains mainly non-hydrocarbon components of crude oil. These can be dispersed oil compounds, which are oil in the form of small droplets, inorganic dissolved components, such as heavy metals, anions, or cations, as well as water dissolved hydrocarbons such as organic acids and lower molecular weight polycyclic aromatic hydrocarbons (PAHs) that have greater solubility in water (Fakhru-Razi et al., 2009; Neff et al., 2011). Several 2-3 rings PAHs (such as naphthalene), and their alkylated analogues, have been detected in produced water (Meier et al., 2010), while PAHs with more than 3 rings are rarely detected in produced water

because of lesser solubility in water (Neff et al., 2011). Alkyphenol or alkyphenol ethoxylate surfactants (APE) such as octylphenols and nonylphenols also occur in produced water because of their relatively great solubility (Getliff and James, 1996; Neff et al., 2011). In general, compositions and concentrations of chemicals that occur in produced water are site specific as they depend mainly on characteristics of the oil deposit. Concentrations of organic chemicals in produced water from oilfield sites can lead to increases in the concentration of several water-soluble petro-genic compounds in the environment (Neff et al., 2011).

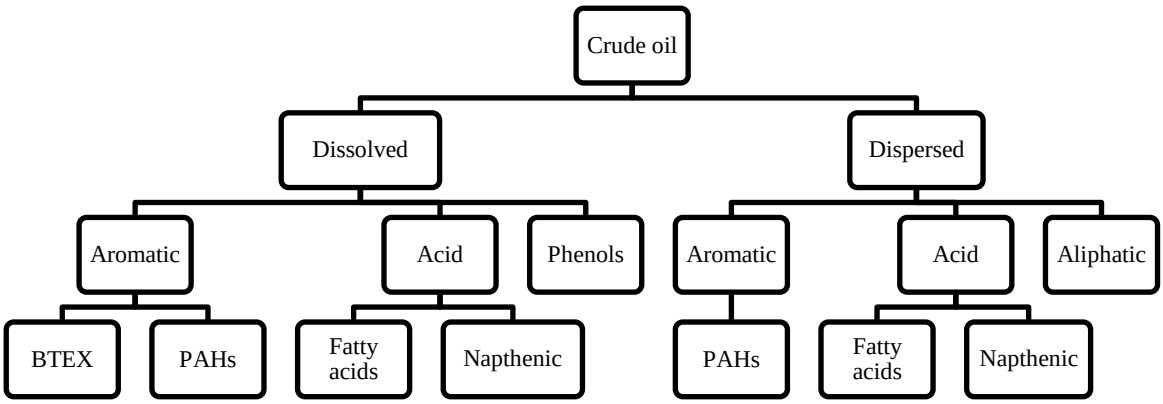

Figure. 1.1 : Classification of petroleum derived chemicals in produced water (Yang, 2011)

1.1.3 Chemical analysis of petroleum-derived polar organic compounds

Identification and quantification of petroleum-derived polar organic compounds can be performed on either crude oil (Stanford et al., 2007) or produced water (Utvik, 1999). However, methods for analysis of both matrices are different, mainly with respect to preparation of samples. Because their corrosiveness can damage refineries (Stanford et al., 2007), and concerns regarding toxicity to aquatic organisms (Clemente and Fedorak, 2005), most studies have focused on analysis

of NAs. Ultrahigh-resolution and ultrahigh-accuracy Fourier Transform Ion Cyclotron Resonance Mass Spectrometry (FT-ICR MS) have been used to analyse polar compounds, such as NAs and NSO groups in crude oil. These compounds are easily ionized by atmospheric pressure ionization (API) techniques, such as electrospray ionization (ESI) in both positive (ESI^+) and negative (ESI^-) modes, which allows for characterization of acidic, basic, and neutral compounds (or heteroatom-containing organic components of crude oils) with unambiguous assignment of thousands of distinct elemental compositions (Rodgers and Mckenna, 2011; Stanford et al., 2007).

1.1.4 Toxicity of petroleum-derived polar organic compounds

Complex mixtures of chemicals, both polar- and mid-polar in produced water, can be toxic to aquatic organisms. Although the toxicity of produced water is mainly attributed to polar compounds, other non-polar compounds such as PAHs might be responsible for some of the toxicity of produced water (Neff et al., 2011). For example, greater incidences of DNA adducts that are known to be caused by PAHs were found in fish exposed to produced water in long term laboratory exposure (Holth et al., 2009; 2014) and in fish sampled from areas near oilfields (Bakke et al., 2013). In a study of toxicity of crude oil, fractionation was performed to determine the most potent fraction. The greatest potency for toxicity was attributed to the most polar fractions that accounted gravimetrically for more than 70% of the total organic fraction of the water-soluble fraction (Melbye et al., 2009;Tilseth et al., 1984).

1.2 Oil sand process affected water (OSPW)

1.2.1 Occurrence of OSPW

In Canada, most crude oil is in the form of oil sands deposits that are located in the Athabasca Basin of northeastern Alberta and northwestern Saskatchewan, which are one of the largest reserves of oil in the world (Hunt, 1979). Because the oil sands industry has significant economic benefits for the province of Alberta, and Canada, development of oil sands is expected to continue with addition of several new projects, as well as improvements in the technology of oil extraction (Giesy et al., 2010). Extraction of oil sands (unconventional oil) is different from extraction of crude oil (or conventional oil), which makes the oil sand industry controversial due to the usage of large volumes of fresh water. Due to its viscosity and thickness, bitumen hydrocarbons are separated from other constitutes of oil sands such as water, clay, sand and heavy metals by heating processes. There are two methods of oil sands extraction - surface mining and *in situ* techniques (Government of Alberta, 2011).

During surface mining of oil sands, the extraction is based on the Clarke hot water, wherein hot water and caustic soda (NaOH) are added to bitumen which can increase the solubility of oil sand asphaltic acids (partly aromatics containing oxygen functional groups such as phenolic and carboxylic acids) to promote their release (reviewed in Kannel and Gan, 2012). The amount of water consumed during this process is estimated to be 1.25 m^3 from river and groundwater to produce 1 m^3 of oil (Suncor, 2012). Water that is a by-product of the extraction process is called "oil sand process water (OSPW)", and is a complex mixture of suspended solids, dissolved organic and inorganic compounds, including salts and various metals (Allen, 2008).

1.2.2 Characterization of the chemical composition of OSPW

Chemical characterization of the water-soluble fraction from OSPW is quite similar to that used to characterize the water-soluble fraction from crude oil and/or produced water from extraction of crude oil. OSPW contains a variety of nitrogen, sulfur and oxygen-compounds but concentrations of these NSO-compounds are different from other produced water. The main difference between OSPW compared to any other produced water from recovery of oil is that OSPW contains relatively great concentrations of water-soluble organic acids, known as (NAs), which makes OSPW a unique matrix. Naphthenic acids are a mixture of all acidic organic compounds in crude oil, such as, aliphatic, alicyclic, and mono- and poly-alkanes, containing a functional carboxyl group, and can be described by the general formula $C_nH_{2n+z}O_2$, where n is the number of carbon atoms in the molecule and Z is a negative, even integer, which specifies hydrogen deficiency (Brient et al., 1995; Clemente and Fedorak, 2005; Grewer et al., 2010; Figure 1.2). More recently, results of studies that used various accurate mass-platforms have indicated that OSPW contains classical NAs, which are nominally those containing two oxygen atoms (C-NAs), as well as oxidized NAs, referred to as oxy-NAs, which contain three or more oxygen atoms. However, both classical and oxy-NAs comprised less than 50% of the peaks detected in extracts of OSPWs analyzed by use of FT-ICR-MS analysis (Grewer et al., 2010; Kannel and Gan, 2012). Results of some studies indicate that structures of NAs identified in OSPW are different from NAs in conventional petroleum (Rowland et al., 2011a, b, c). Because of differences in NAs between OSPW and conventional petroleum, and because of evidence of other compounds in OSPWs that have not been yet characterized, it has been suggested that the term of NAs should be replaced with “oil sand tailings water acid-extractable organics (OSTWAEO)” (Grewer et al., 2010). Moreover, the dissolved organic fraction of OSPW might contain other organic compounds such

as (PAHs) as well as their alkylated forms, benzene, toluene, ethylbenezene, xylene, referred to as BTEX, and polyphenols (Zhao et al., 2012).

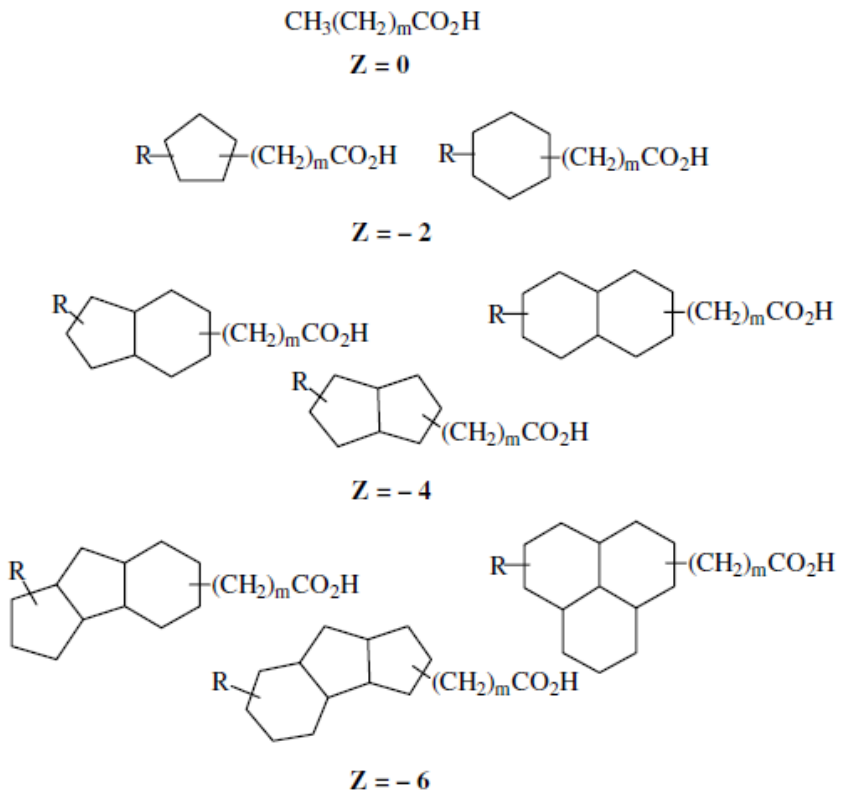

Figure. 1.2. Examples of structures of naphthenic acids. R is an alkyl chain, Z the hydrogen deficiency, and m is the number of CH_2 units (Clemente, and Fedorak., 2005).

Several analytical approaches have been used for characterization of OSPW. Fourier-Transform Infrared Spectroscopy (FTIR) (Jivraj et al., 1995), Synchronous Fluorescence Spectroscopy (SFS), and Absorption and Fluorescence Emission Spectrophotometry (UV-Vis) have been used for the determination of concentrations of NAs in OSPW (Zhao et al., 2012).

Characterization of NAs in OSPW has relied primarily on mass spectrometry either alone or attached to a separation instrument (GC or LC) (reviewed in detail by Headley et al., 2016). Recently, ultra-high-resolution FTICR-MS with electro-spray ionization (ESI) has been used to characterize the compositional structure of NAs as well as other nitrogen and sulfur-containing compounds in OSPW (Kannel and Gan, 2012). Ultra-high resolution mass spectrometry (Orbitrap) has also been used to distinguish the acid fraction of OSPW from co-extracted plant components (Headley et al., 2011a), and for characterization of acidic and non-acidic compounds (Pereira et al., 2013a,b; Morandi et al., 2015; Zhang et al., 2015).

Chemical analysis of OSPW has focused mainly on characterization of the most predominant classes of chemicals (i.e. NAs). Procedures for extraction that were developed and applied were focused on isolation and characterization of total organic acids from OSPW, and the most used methods are based on liquid-liquid extraction using dichloromethane as a solvent (reviewed in detail in Zhao et al., 2012). Although most of these methods of extraction provide relatively good results in terms of the efficiency of extraction of NAs, they are time, cost, and labour consuming. Due to the complexity of OSPW, to date, an analytical method to adequately separate components based on their physicochemical properties has not been developed, although several selective chromatographic methods have been applied in analyses of OSPW (Zhao et al., 2012). Moreover, pure standards of NAs in OSPW are not commercially available, thus chemical analysis of OSPW is classified as semi-quantitative. Most previous studies have used commercial mixtures of NAs (such as Merrichem and Kodak) as a common standard for calibration. However, the commercial mixtures of NAs cannot replace the need for individual compounds and might not be useful as calibration standards for quantifying NAs in OSPW. Depending on the analytical method, a considerable difference in the individual isomer group assignments is expected. These

differences might lead to overestimation or underestimation of the actual concentrations of NAs, and misclassification of the structure of NAs. Concentrations of NAs can be reported as total NAs for all isomer groups, or for individual isomer groups or percentage of the total NAs in each isomer group (Zhao et al., 2012), while information about the identity and concentration of nitrogen and sulfur containing compounds is absent.

1.2.3 Aging of OSPW

Toxicity of OSPW decreased with natural aging where the microbial populations indigenous to the oil sands region can degrade the most toxic components (i.e. NAs), however, natural aging is relatively slow and complete degradation might not be achievable (Herman et al.,1994; Holowenko et al., 2002; Scott et al., 2008). Monitoring degradation of organic chemicals in OSPW is based on experimental reclamation ponds and settling basins using the molecular weight and number of rings as markers of the total NAs. In general, NAs with lesser MW and fewer rings (least cyclic) are the most susceptible to biodegradation (Lai et al., 1996; Clemente and Fedorak, 2005; Del Rio et al., 2006; Frank et al., 2008; Han et al., 2009). Toxicity of aged OSPW has been shown to be lesser than fresh OSPW (OSPW derived from end pit lake or Base Mine Lake-BML) (Anderson et al., 2012a, b; Wiseman et al., 2013a). However, reproductive capacity of fathead minnows (*Pimephales promelas*) was impaired when exposed to OSPW that had been aged in experimental reclamation ponds for close to 20 years (Kavanagh et al., 2011), which indicates that long term exposure to aged OSPW might still exert some toxicity.

1.2.4 Ecotoxicology of OSPW

Toxicity of OSPW has been the subject of intense research (reviewed in detail in Mahaffey and Dubé., 2017; Li et al., 2017). Fresh OSPW is acutely toxic to a variety of aquatic organisms,

and it has been suggested that toxicity is due primarily to NAs (Allen, 2008; Frank et al., 2008; Mornadi et al., 2015). It has been suggested that the mode of action of NAs is narcosis (Frank et al., 2008), which is a mechanism that demonstrates additive toxicity; in other words, several organic compounds that cause toxicity via narcosis can be added together to give the observed total amount of toxicity in a mixture of such chemicals (Di Toro et al., 1991; Di Toro and McGrath., 2000b). Also, both fresh and weathered OSPWs that are not acutely toxic in standard bioassays inhibited growth and development of the non-biting midge, *Chironomus diutus*, (Anderson et al 2012a, b), which might be due to oxidative stress and disruption of endocrine processes (Wiseman et al., 2013a). Fresh and weathered OSPW resulted in lesser concentrations of sex steroid hormones (testosterone and estradiol) in blood plasma of fathead minnows (Kavanagh et al., 2011), and impaired reproduction and development of secondary sex characteristics of fathead minnows (Kavanagh et al., 2012). Moreover, some histological changes have been reported in gills and livers from yellow perch (*Perca flavescens*) and goldfish (*Carassius auratus*) (Nero et al., 2006). Incidences of deformities and lesser length at hatch in early life stages of fathead minnows (He et al., 2012b), yellow perch and Japanese medaka have been observed (Peters et al., 2007). In rainbow trout (*Oncorhynchus mykiss*), exposure to OSPW impaired the immune function (MacDonald et al., 2013;McNeill et al., 2012*).*

In general, mechanism(s) of either acute or chronic toxicity of OSPW are not well-known. Several studies have reported greater concentrations of reactive oxygen species (ROS) and greater abundance of transcripts of genes that are important for the response to oxidative stress, and drug metabolizing enzymes in early life stages and adults of fathead minnows, and in primary cultures of hepatocytes exposed to OSPW (Gagne et al., 2012; He et al., 2012b;Wiseman et al., 2013a). Oxidative stress can damage mitochondria and promote activation of caspase enzymes and

apoptotic cell death (He et al., 2012b; Wiseman et al., 2013a). Moreover, alterations of cell wall proteins has been observed on green algae which are consistent with the effects of surfactants, and indicate a possible role for classic NAs in the NAFC mixture to cause surfactant-mediated toxicity (Goff et al., 2013). Recently, a bioassay effects-directed analysis (EDA) of OSPW demonstrated that in addition to NAs, several organic species (i.e., O^-, SO_2^-, O^+, O_2^+, SO^+, and NO^+) contribute to the acute toxicity of OSPW (Mornadi et al., 2015), however, in depth investigation about mode of action is yet to be determined.

1.2.5 Chemical activity and bioavailability of water soluble constituents in OSPW

The chemical activity concept is derived from thermodynamics for relating exposure to acute toxicity endpoints (Mackay et al., 2011). The chemical activity concept can also be useful as exposure parameters for investigation of the environmental fate and distribution of chemicals, which is also termed as "fugacity" (Di Toro et al., 1991; Mackay et al, 2011). In general, chemical activity, which is based on the "equilibrium criterion", has been defined mainly for non-polar neutral organic chemicals that act as acute lethal baseline toxicity. The equilibrium quantifies the potential of organic chemicals for natural physicochemical processes, such as sorption into soil, and partitioning into cell membranes (Di Toro et al., 1991). So, in the case of sediments contaminated by PAHs, chemical activity determines their equilibrium partitioning concentration in sediment-dwelling organisms and differences in chemical activity determine the direction and extent of diffusion between environmental compartments (Di Toro et al., 1991).

In environmental toxicology research, toxicity, biodegradability, and efficacy of organic chemicals are dependent on their bioavailability in various media, including, soil, sediment and water. The definition of bioavailability depends on the use of the term. For example, in mammalian toxicology, bioavailability is defined as the fraction of an ingested dose of a drug or xenobiotic

that crosses the gastrointestinal epithelium and becomes available for distribution to internal target tissues and organs (van deWaterbeemd and Testa., 2007; Hurst et al., 2007). In environmental toxicology, the bioavailability is defined as the potential of a fraction of chemical, which must be the freely dissolved concentration or non-bound fraction, for entry into biological receptors (Katayama et al., 2010).

Chemical activity and bioavailability of chemical constitutes of OSPW to aquatic species have not been fully addressed. This is mainly because toxicity has been attributed solely to NAs and these organic compounds are considered bioavailable and water soluble at physiological pH (i.e. less hydrophobic). However, water solubilities of NAs are expected to be based on the degree of ionisation, which is a function of pH and p*K*a. Ionisable organic chemicals (IOCs) in OSPW might exhibit different partitioning action compared to neutral organic chemicals, which makes assessments of bio-concentration and toxicity by use of the chemical activity concept challenging for IOCs (He and Yalkowsky, 2004; Jain et al., 2006; Armitage et al., 2013; 2017). In OSPW, NAs do not bioaccumulate to the same degree as some non-acidic chemical classes, such as SO and NO, by use of stir-bar sorptive extraction (SBSE) to poly(dimethyl)siloxane (Zhang et al., 2015). The SBSE used are similar to C_{18} reverse phase which was shown to correlate with hydrophobicity (log K_{ow}) of the organic compounds (Dorsey and Khaledi, 1993).

In general, the assumption that the primary mechanisms of acute toxicity of dissolved organic chemicals in OSPW is narcosis is because of steep dose−response relationships similar to other narcotic chemicals (Frank et al., 2008; Mornadi et al., 2015). Narcosis depends on the use of equilibrium partitioning (EqP) theory for chemical portioning between media (Di Toro e al., 1991) and target lipid model (TLM) to predict exposure and toxicity (Di Toro et al., 2000a; Di Toro and McGrath., 2000b). The TLM was developed for PAHs, or neutral organic compounds, and

sediment-dwelling organisms, where the toxicity to sediment organisms is directly proportional to the amount of unbound PAH dissolved in sediment pore water.

The theory of equilibrium partitioning (EqP) cannot be applied directly to IOCs in OSPW such as NAs without taking into consideration the ionization constant (p*Ka*) in the model (He and Yalkowsky, 2004; Jain et al., 2006; Ingram et al., 2011; Boström, and Berglund., 2015). The relative amount of ionized species in IOCs can be determined using the Henderson−Hasselbach equation based on the ionization constant (p*K*a) and the pH of the environment. According to pH-partition theory, only the un-ionized form of substances preferentially traverses the lipid membranes by passive diffusion. However, while pH-partition theory describes the vast majority of cases, it should be emphasized that this is not an absolute; i.e., a small amount (largely unquantifiable) of ionized species can permeate membranes passively (Manallack et al., 2013). Some studies have attempted to investigate the bioaccumulation and/or bioconcentration factor of ionisable compounds (mainly chlorinated phenols) (Erickson et al., 2006a, b). However, the K_{ow} of all tested compounds ranged from 2.75-5.12 at physiological pH, which makes great hydrophobicity for these compounds for uptake despite the presence of phenolic groups (acidic groups). Uptake of these compounds was maintained at pH 6.2-8.4 but uptake was lesser at pH 9.2. In general, uptake and elimination rates varied among chemicals as a function of both chemical ionization and hydrophobicity.

Similar to organic acids; basic and neutral compounds in OSPW are bioavailable and can be classified as “water-soluble organic compounds” in OSPW, for which the pH ranges from 7 to 9). However, neither toxicity nor chemical analysis were investigated for these compounds in OSPW. Basic and neutral compounds have the ideal physicochemical properties for permeability of biological membranes at physiological pH. Both can traverse cell membranes via passive

diffusion transport through the cell bilayer as both meet membrane permeability requirements such as hydrophobicity at physiological pH. Using partitioning to solid supported lipid membranes (SSLM) as well as whole fish tissue of Japanese medaka. It has been shown that some non-acidic species (considered as polar basic and neutral compounds) in OSPW, such as O^+, O_2^+, SO^+, and NO^+ that are detected in positive ionization mode in high resolution mass spectrometry, had phospholipid membrane-water distribution ratios (D_{MW}) similar to and correlated with octanol−water distribution ratio (D_{OW}). The value of D_{MW} has been shown to be greater than octanol−water distribution ratios (D_{OW}) for organic acids and those authors suggested the importance of electrostatic interactions of these ionisable organic acids with the lipid membrane. Interestingly, beside SO^+ and NO^+, O_2^- species (organic acids) were detectable in Japanese medaka exposed to 10% OSPW (OSPW diluted by a factor of 10 in freshwater), and had bioconcentration factors (BCFs) similar to predicted values. The authors indicated the importance of phospholipid partitioning in explaining the bioconcentration mechanism (Zhang et al., 2015; 2016). For O^+, O_2^+, SO^+ and NO^+, the BCF was shown to be lesser than predicted values, and the authors suggested the role of biotransformation of O^+, O_2^+, SO^+ and NO^+ substances (mainly hydrophobic) in fish. In OSPW, the concentration of O^+, O_2^+, SO^+ and NO^+ is expected to be lesser compared to O_2^- species, which had similar calculated and predicted values of BCF. However, it should be emphasized that accurate measurements of any chemical constitute of OSPW is not possible due to the complexity of OSPW mixture and lack of any representative standards.

Overall, organic acids, basic and neutral compounds in OSPW are considered as “dissolved organic compounds” however, basic and neutral compounds have a greater tendency than acidic compounds for bioaccumulation in aquatic organisms at the ambient pH of OSPW.

1.3 Biotransformation in fish

1.3.1 Phases I and II

Fish are exposed to a variety of anthropogenic and natural compounds that enter the aquatic ecosystem via different routes, including direct/indirect inputs of effluents, atmospheric deposition, storm-water runoff (Celander., 2011) and seepage from industrial activities such as oil sands (Ohiozebau et al., 2016). The capability of environmental pollutants to cause acute toxicity (lethality) or chronic toxicity such as neurotoxicity or reproductive and developmental effects depends on the ability of a chemical to traverse the biological membranes and accumulate in tissues. Accumulation is dependent on absorption, distribution, metabolism and excretion (Schlenk., 2008). In fish, uptake of chemicals can occur through gills and skin from the ambient water or through the gastro-intestinal tract from the diet. Once a chemical is absorbed it undergoes metabolism (or biotransformation). Biotransformation, or detoxification, processes in fish involve several enzymes of phase I such as cytochrome P450 (CYP450) monooxygenases, which comprise a highly-conserved superfamily of enzymes localized in the membrane of the endoplasmic reticulum (Nelson., 2006). Phase I biotransformation enzymes such as CYP monooxygenase, flavoprotein monoxygenase, monoamine oxidase, and epoxide hydrolase and reductase increase water-solubility via oxidation, reduction or hydrolysis of the xenobiotic, which facilitates clearance of hydrophobic compounds from the organism (Ortiz de Montellano., 2010). In phase II, metabolites generated from phase I become hydrophilic and biotransformation reactions are more readily excreted via the bile, urine or gills in fish as a conjugated. Conjugation reactions are catalyzed by Glutathione-S-transferases, UDP-glucuronosyltransferases, sulphonyl-transferases, N-acetyltransferases, and methyltransferases (conjugation enzymes) (Livingstone, 2001; Schlenk et al., 2008). Several enzymes involved in phase I and II biotransformation and conjugation are

regulated by ligand activated transcription factors. For example, the aryl-hydrocarbon receptor (AhR) regulates expression of phase I enzymes such as cytochrome *cyp1a,* while expression of *cyp3a* is largely controlled by the pregnane-X-receptor (*pxr*) (Kliewer., 2003; Pascussi et al., 1999; Synold et al., 2001).

Molecular and biochemical markers, such as abundances of mRNAs or proteins, or activities of enzymes, are responsiveness biomarkers of exposure to environmental pollutants, which can be used as good indicators of contaminant exposure in aquatic organism (Van der oost et al., 2003; Regoli et al., 2011). For example, phase I biotransformation proteins, such as *cyp1a*, *cyp2c*, and *cyp3a* have been found to be significantly induced by water-accommodated fraction (WAF) of crude oil (Rhee et al., 2013; Holtha et al., 2009; 2014; Han et al., 2015), and benzo(*a*)pyrene (Kim et al., 2014). The enzyme *cyp1a*, in particular, is known to biotransform and be induced by highly hydrophobic environmental pollutants such as polycyclic and polyhalogenated aromatic hydrocarbons (Hawkins et al., 2002), while *cyp3a* and *cyp2* biotransform and can be induced by a suite of structurally diverse chemicals, including aromatic hydrocarbons (Hegelund et al., 2004; Wassmur et al., 2010; Corcoran et al., 2012; Kubota et al., 2015). Overall, biotransformation of hydrophobic compounds can affect bioaccumulation, persistence, tissue-distribution and the toxicity of a chemical in fish (Livingstone, 2001; Schlenk et al., 2008).

1.3.2 Phase III Reactions - ATP-Binding Cassette (ABC) transporters

Excretion of xenobiotic and their metabolites occurs via specific transporters that actively pump a variety of endogenous and exogenous compounds, or their metabolites, out of the cell, which are called “efflux-transporters” which are members of ATP-Binding Cassette (ABC)

transporters. ABC transporters are considered as either phase 0 (in the case of efflux of parent compounds) or phase 3 (in the case of efflux of products of phase II).

Transmembrane proteins, known as the adenosine triphosphate (ATP)-binding cassette transporters (ABC transporters), are a highly conserved superfamily of proteins that actively shuttle substrates across cellular membranes "efflux-transporters" (Higgins, 1992). Several unchanged parent exogenous chemicals and/or their metabolites are substrates for ABC-transporters (Epel., 1998; Epel et al, 2008). Historically, the ABC proteins were identified first as biochemical factors conferring multidrug resistance (MDR) in cancer (or the resistance of tumour cells to structurally and functionally unrelated cancer drugs) (Roninson et al., 1984; Gros et al., 1986; Luckenbach et al., 2014). ABC-transporters include multidrug resistance protein 1 (MDR1), also known as permeability-glycoprotein (P-gp); the breast cancer resistance protein (BCRP), and multi-drug resistance-associated proteins (MRP1-5) (Chang, 2003; Leslie et al, 2005). However, the most well-characterized ABC transporters protein is P-gp.

In contrast to the role of ABC proteins in cancer, the transport activity, within organisms, modulates the toxic effects of xenobiotic by manipulating their disposition and bioavailability (Fromm, 2000; Kurata et al, 2002). In response to xenobiotic, the expression of genes encoding ABC proteins is increased, which increases proteins associated with xenobiotic transport (Schuetz et al, 1996). This change in protein abundance is considered as adaptive response whereby xenobiotic can cause up-regulation of specific proteins to confer a tailored cellular response (Sarkadi et al, 2006).

The function of ABC drug transporters depends on the tissues in which they are expressed. Expression of these proteins is greatest in tissues involved in excretion (e.g. kidney, liver) or that acting as barriers (e.g. gut epithelium, capillary endothelia forming the blood–brain barrier) (Fojo

et al., 1987; Thiebaut et al., 1987). Most ABC proteins, such as P-gp and MRP2 are localized on the apical side of polarised epithelial cells, which explains their role in limiting chemical accumulate and enhancing chemical elimination by pumping substrates from blood into urine (Leslie et al., 2005). However, other ABC drug transporters, such as MRP-1, are localised on the basolateral side.

Most studies of ABC proteins have been performed with mammalian models, and has focused on the role of these proteins in cancer. However, during the past decade there has been a significant increase in our knowledge of these proteins in non-mammalian animals. The mechanism whereby ABC proteins transport substrates across cellular membranes and affect bioaccumulation in fish are comparable to those in mammals (Tan et al., 2010). There is evidence that teleost fish might share overlapping in the function of MDR/MXR" multixenobiotic resistance -associated processes with other species of vertebrates, including humans (Luckenbach et al., 2014). Induction of ABC transporters was first reported in ecotoxicology research by Kurelec in which populations of the marine invertebrate from polluted areas had high levels of P-gp (Kurelec and Pivcevic, 1991). The term "multixenobiotic resistance (MXR) proteins" was coined to describe the role of ABC drug cellular pumps or efflux transporters in aquatic animals as protective factors against toxicity of chemical stressors (Kurelec, 1997; Kurelec et al, 2000). P-glycoprotein and other ABC proteins have been identified in a variety of aquatic invertebrates such as *Daphnia magna* (Campos et al., 2014) and vertebrates, including fish (Fischer et al, 2013; Luckenbach et al., 2014; Hemmer et al, 1995). The function of P-gp in embryos of zebrafish (*Danio rerio*) was investigated using either siRNA to knock-down P-gp, or a pharmacological approach with inhibitors of P-gp, such as cyclosporine A and valspodar (PSC 833). Results showed an increased accumulation of the P-gp fluorescent dye, rhodamine B in knocked down fish, compared to non-

knocked down fish or non-exposed fish to P-gp inhibitors. Moreover, zebrafish embryos in which expression of P-gp was knocked down or that were co-treated with inhibitors of P-gp exhibited greater sensitivity to P-gp substrates, such as doxorubicin, vinblastine, vincristine and to the ecotoxicological-relevant PAH, phenanthrene (Fischer et al, 2013). Inhibition of P-gp or gene knock down of P-gp reduce the clearance of various P-gp substrates, while greater expression of P-gp can increase the cellular clearance of the P-gp substrate rhodamine 123 (R123) (Yumoto et al., 1999; Kageyama, 2006).

Genes encoding ABC proteins have been identified in several species of aquatic organisms (reviewed in detail in Luckenbach et al., 2014). However, P-glycoprotein, which is encoded by the ABCB1 (MDR1) gene, is the most studied ABC-transporter protein (Leslie et al., 2005; Klaassen and Aleksunes., 2010). Expression of *ABCB1* gene can be induced and/or inhibited by a variety of structurally-unrelated compounds, which are often substrates of enzymes that catalyse phase I biotransformation reactions, such as *cyp3a* (Schuetz et al., 1996). In general, these compounds are moderately hydrophobic or amphipathic molecules ranging in size from 250 to 1250 kDa (Broccatelli et al., 2011; Ferreira et al., 2012).

Recently, chemosensitization has been proposed as an environmentally relevant mechanism of toxicity involving ABC proteins. Chemosensitization is a process whereby environmental contaminants inhibit or decrease activities of ABC transporters, leading to an increase in accumulation of ABC transporter protein substrates within cells (Luckenbach et al., 2014). Environmental contaminants such as endosulfan, and Dichloro-diphenyl-dichloroethylene (DDE) can inhibit ABC transporter proteins (Zaja et al., 2011).

It is expected that activity of P-gp plays a significant role in the toxicokinetics - toxicodynamics fate of xenobiotic (substrates of P-gp), and any modifications in P-gp expression,

structure, and function can significantly affect efficiencies of efflux of P-gp substrates. Although the function of P-gp prevents bioaccumulation of various drugs and environmental toxicants in cells (Gottesman and Pastan, 1993; Bard, 2000), it also plays an important role in normal cellular processes since it can mediate the transport of several endogenous molecules such as lipids, bile salts, and steroid hormones (van Helvoort et al, 1996; Wang et al, 2006; Eckford and Sharom, 2008).

Overall, protection to cells and tissues against accumulation of cytotoxic substances is a result of sequence of actions that involve phase I and phase II biotransformation and ABC-transporter-mediated efflux, which all encompass mechanisms of decrease bioaccumulation and toxicity of hydrophobic contaminants. The ecotoxicological relevance of the specific interactions of contaminants with membrane transporter proteins (such as ABC transporters), and the use of ABC transporters as molecular biomarkers for ecological biomonitoring and exposure assessments might be promising toxicity end point for ecological risk assessment.

1.4 Problem formulation

1.4.1 Rationale

Oil sands process-affected water contains a variety of constituents such as inorganic ions (e.g., sodium, potassium, chloride, sulfate, and bicarbonate), metals and a variety of sulfur and nitrogen and oxygen-containing organic species, including NAs (Allen, 2008; Barrow et al., 2010; Headley et al., 2013; Pereira et al., 2013a). Strategies for remediation of OSPW have focused mainly on the acid extractable organic fraction likely due to the observed toxicity for this chemical class (Wang et al., 2013; Pereira et al., 2013a, b). Although the acute and chronic toxicity of OSPW has been reported for mainly acid extractable fraction, other non-acidic species such as sulfur and

nitrogen-containing species in OSPW have been reported for years (Headley et al., 2011a, b; Barrow et al., 2010), but toxicity of these compounds has not been characterized.

Identification of the oxygen, sulfur and nitrogen containing compounds was performed without explaining if they are acidic, or non-acidic species. Selective extraction and isolation of oxygen, sulfur and nitrogen containing compounds prior to instrumental analysis was not performed but rather, identification was heavily dependent on advances in mass spectrometry instrumentation. The chemical analysis investigation using the most advanced high-resolution MS of the oxygen, sulfur and nitrogen containing compounds or their transformation products in OSPW is less informative if their toxicity is not known.

Although the acid-extractable fraction of OSPW has been shown to be the most potent fraction in OSPW, little is known regarding the sub-lethal effects of exposure to other polar organic water-soluble compounds such as basic and neutral compounds. Also, OSPW that has been aged in experimental reclamation ponds has been shown to exert less acute toxicity compared to relatively fresh OSPW, but parallel information regarding the toxicity and chemical profiles for acidic, basic, and neutral fractions of aged OSPW are not available. Some studies have addressed the chemical composition and toxicity of OSPW with respect to NAs, however, there is gap in the data with respect to the toxicity and identities of compound(s) other than NAs that cause the toxicity of OSPW, and which mode(s) of action lead to toxicity.

This research will utilise integrated chemical and biological approaches, in which a combination of fractionation procedures, toxicity testing, and instrumental chemical analyses will be used to characterize toxic constituents in OSPW. The organic fraction of OSPW will be subjected to fractionation to separate chemicals in the complex mixture by means of physical-chemical properties (ionization) into acidic, basic and neutral fractions so that the chemical profile

and toxic action of can be determined. Although identification of a single toxic agent might be difficult or even impossible, it will likely be possible to operationally define a "fraction" of the total mixture that is responsible for the majority of the toxicity based on physical-chemical properties (i.e. acidic, basic and neutral fractions). The toxic potency of these fractions can be determined, and in the future, the potential for toxicity of OSPW could be predicted by quantification of fractions, rather than individual compounds. Therefore, the development a generic fractionation scheme and then develop methods to quantify the total mass of chemicals in the fraction is needed.

1.4.2 Objectives

There is a need for objective methods to elucidate and compare the effects of acidic and non-acidic compounds in OSPW on aquatic organism's. Therefore, the objectives of this research are to use a selective fractionation method to test and compare the acute and sub-lethal effects of acidic, basic and neutral fractions derived from fresh and aged OSPW. The specific research objectives and associated null hypotheses, and the experimental approaches employed are outlined below:

1. Detailed characterization and comparison of acidic NSO compounds in fresh and aged oil sands process affected water using liquid chromatography-high-resolution electrospray ionization mass spectrometry (Chapter 2).

Toxicity of OSPW has been attributed to the acid extractable fraction, mainly NAs, which can lead to several acute and chronic toxicities, as described above. It has been shown that toxicity of OSPW decreased when treating OSPW by aging or ozonation, but there is little known about the relationship between toxicity and the chemical profile of acid fractions of fresh and aged OSPW.

Therefore, the main objective of this chapter was to perform an in-depth investigation of the chemical profile of the acidic fraction from fresh and aged OSPW by using extraction/isolation methodology that can selectively isolate the acidic compounds from a complex mixture and analysing the fractions using ultrahigh resolution mass spectrometry.

2. Inhibition of ABC transport proteins by oil sands process affected water (Chapter 3).

The ATP-binding cassette (ABC) superfamily of transporter proteins is important for detoxification of xenobiotic. Exposure to OSPW has been shown to impact abundances of mRNAs of ABC-transporter proteins in freshwater fish species. However, it is not known if OSPW and its acidic, basic and neutral fractions impact the functional activity of ABC transporter proteins in freshwater fish. Therefore, effects of the main three fractions (acidic, basic and neutral) derived from relatively fresh OSPW from Base Mine Lake (BML-OSPW) and aged OSPW from Pond 9 (P9-OSPW) on the activity of MRP transporters were investigated *in vivo* by use of early life-stages of Japanese medaka. Activities of MRPs were monitored by use of the lipophilic dye calcein, which is transported from cells by ABC proteins, including MRPs. The testable hypotheses include the following:

H01: There is no statistically significant difference in survival between early life-stages of Japanese medaka exposed to acidic, basic and neutral fractions of fresh and aged OSPW and freshwater/solvent control fish.

H02: There is no statistically significant difference in ABC transporters activity in early life-stages of Japanese medaka between control Japanese medaka and Japanese medaka exposed to acidic, basic and neutral fractions of OSPW.

3. Effect of oil sands process-affected water on toxicity of retene to early life-stages of Japanese medaka (*Oryzias latipes*) (Chapter 4)

Effects of co-exposure to OSPW and polycyclic-aromatic hydrocarbons (PAHs), which are an important class of chemicals in tailings ponds used to store OSPW, has not been investigated. The goal of the current study was to determine if organic compounds extracted from the aqueous phase of relatively fresh OSPW from Base-Mine Lake (BML-OSPW) or aged OSPW from Pond 9 experimental reclamation pond (P9-OSPW) modulated toxic potency of the model alkyl-PAH, retene, to early life-stages of Japanese medaka. Exposure was based on partition controlled delivery (PCD) system made of polydimethylsiloxane (PDMS). Survival, number of eggs hatched, incidences of pericardial edema, and transcript abundance of a battery genes related to phase I, II and III biotransformation were investigated. The testable hypotheses include the following:

H01: There is no statistically significant difference in survival between early life-stages of Japanese medaka exposed to retene and retene co-exposed to either fresh or aged OSPW and freshwater/solvent control fish.

H02: There is no statistically significant difference in number of eggs hatched between early life-stages of Japanese medaka exposed to retene and retene co-exposed to fresh or aged OSPW and freshwater/solvent control fish.

H03: There is no statistically significant difference in incidences of pericardial edema between early life-stages of Japanese medaka exposed to retene and retene co-exposed to fresh or aged OSPW and freshwater/solvent control fish.

H04: There is no statistically significant difference in transcript abundance of genes related to phase I, II and III biotransformation between early life-stages of Japanese medaka exposed to retene and retene co-exposed to fresh or aged OSPW and freshwater/solvent control fish.

4. Toxicokinetics and toxicodynamics of chlorpyrifos is altered in embryos of Japanese medaka exposed to oil sands process-affected water: evidence for inhibition of P-glycoprotein (Chapter 5)

Chapter 3 demonstrated that basic and neutral fractions of OSPW inhibit the function of ABC transporters. In chapter 5, in depth characterization of adverse effects of inhibition of P-gp. Studies were performed *in vitro* by use of Caco-2 cells, and *in vivo* with larvae of Japanese medaka to determine if OSPW modulates the toxicity of substrates of P-gp. The aqueous phase of OSPW was separated into acidic, basic and neutral fractions. Survival, accumulation and bioconcentration of the organophosphate pesticide chlorpyrifos (a substrate of P-gp) and malathion (not a substrate of P-gp) was determined. The testable hypotheses include the following:

H01: There is no statistically significant difference in cytotoxicity in caco-2 assay between control cells and cells exposed to acidic, basic and neutral fractions of OSPW.

H02: There is no statistically significant difference in ABC transporters activity in caco-2 assay between control cells and cells exposed to acidic, basic and neutral fractions of OSPW.

H03: There is no statistically significant difference in survival between fish exposed to either chlorpyrifos or malathion, OSPW and freshwater/solvent control fish.

H04: There is no statistically significant difference in survival between fish exposed to either chlorpyrifos or malathion and chlorpyrifos or malathion co-exposed to OSPW.

H05: There is no statistically significant difference in internal concentrations of either chlorpyrifos or malathion between fish exposed to the mixture of chlorpyrifos or malathion co-exposed to OSPW and chlorpyrifos or malathion co exposed to freshwater/solvent control fish.

H06: There is no statistically significant difference in BCF of chlorpyrifos between fish exposed to chlorpyrifos co-exposed to OSPW and chlorpyrifos co-exposed to freshwater/solvent control fish.

5. Differential expression of genes encoding biotransformation enzymes in Japanese medaka (*Oryzias latipes*) exposed to acidic, basic, and neutral compounds from the aqueous phase of oil sands process affected water (Chapter 6)

Evidence from a small number of studies suggest that exposure to OSPW modulates expression of genes encoding xenobiotic metabolizing enzymes and proteins, therefore greater expression of these genes might be a response that is important for coping with adverse effects of exposure to OSPW. The hypothesis tested in this study is that the most toxic fraction (i.e. acidic), and the non-toxic fractions (i.e. basic, and neutral) of OSPW affect expression of genes encoding xenobiotic metabolizing proteins differently. This hypothesis was investigated by quantifying abundances of transcripts of phase I and phase II drug-metabolizing enzymes and phase III ABC proteins in fry of Japanese medaka exposed to acidic, basic, and neutral fractions of OSPW. The testable hypotheses include the following:

H01: There is no statistically significant difference in transcript abundance of the phase I, II, and ABC transporters genes between fish exposed to the acidic, basic and neutral fractions of OSPW and freshwater/solvent control fish.

2 CHAPTER 2: DETAILED CHARACTERIZATION AND COMPARISON OF ACIDIC NSO COMPOUNDS IN FRESH AND AGED OIL SANDS PROCESS AFFECTED WATER USING LIQUID CHROMATOGRAPHY –HIGH RESOLUTION ELECTROSPRAY IONIZATION MASS SPECTROMETRY

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Author Contributions:

Hattan A. Alharbi (University of Saskatchewan) conceived, designed, managed and did the experiment, generated and analyzed the data, wrote, and drafted the manuscript.

Alberto S. Pereira (University of Alberta) analysed the samples on the mass spectrometry.

Jonathan W. Martin (University of Alberta) provided scientific input on chemical analysis data, guidance, commented on and edited the manuscript.

Drs. Steve B. Wiseman and John P. Giesy (all at University of Saskatchewan) provided inspiration, scientific input, and guidance, commented on and edited the manuscript, and provided funding for the research.

2.1 ABSTRACT

Oil sands process affected water (OSPW) that is fresh is acutely toxic to aquatic organisms but the cause of the toxicity remains unknown. As OSPW is aged acute toxicity disappears and the profile of organic chemicals changes, however, there is a lack of information about the reasosn behind the lower toxicity in aged OSPW. Mixed mode solid phase extraction (MM-SPE) was used for fractionation of the polar constituents of OSPW from an end-pit lake known as Base Mine Lake (BML-OSPW; fresh OSPW) and an experimental reclamation pond known as Pond 9 or Tailings Pond Water (P9-OSPW or aged OSPW), into acidic, basic, and neutral fractions. In this study, the profile of chemicals in acidic fraction was characterized by use of liquid chromatography combined with ultra-high resolution (orbitrap) mass spectrometry (MS) in both negative (ESI^-) and positive (ESI^+) electrospray ionization modes. Since acidic fraction was the only acutely toxic in BML-OSPW, the goal of the study was to characterize and compare in detail the organic chemicals profiles in the acidic fractions of BML and P9. In both ionization modes, the mass spectra range of fractions of BML were very similar (*m/z* 100-400) to that of fractions of P9-OSPW, the relative abundances of chemicals among fractions was different. The profile of homologs containing heteroatoms in each fraction of BML-OSPW or P9-OSPW was unique but comparison of the same fraction from the two samples revealed that the profile of nitrogen, sulfur, and oxygen (NSO) containing chemicals was not very different. When analysed by use of ESI^- mode, acidic fractions were dominated by O_x^- ($x = 4 > 2 > 3$) chemicals. Sulfur-containing chemicals were detected in the acidic fraction by use of either ESI^- or ESI^+, but the abundance of SO_3^+ chemicals was greatest when fractions were analysed by use of ESI^+. Nitrogen-containing chemicals were not detected in either acidic fractions analysed by use of ESI^-, with low intensity of NO_3^+ in ESI^+. Overall, the relative abundances are lower for chemicals species detected in ESI^-

, but SO_3^+ detected in ESI^+ is probably a good candidate group for monitoring the degree of aging of OSPW.

2.2 Introduction

It is estimated that 1.7 trillion barrels of bitumen exists in deposits of oil sands in Northern Alberta, Canada, and that 173 billion barrels are recoverable given current economic conditions (Burrowes et al., 2008). In the surface mining industry, extraction of bitumen from oil sands is done by use of a modification of the Clark caustic hot water method (Schramm et al., 2000). Extraction of bitumen by use of this process results in the generation of oil sands process affected water (OSPW) that is retained on-site in tailings ponds and settling basins that, as of 2009 covered an area of approximately 170 km^2 (Environmental management of Alberta's oil sands., 2009). Because oil sands mining companies operate under a policy that does not allow for the discharge of OSPW to the environment at large, to date, no OSPW has been released to the receiving environment or been moved off-site (Government of Alberta., 2011). Therefore, since as surface mining operations expand the volume of OSPW stored in tailings ponds will increase, methods to remediate OSPW are needed. One strategy that companies operating in the oil sands are exploring for the remediation and reclamation of OSPW is the construction of end pit lakes (EPLs), which are man-made lakes constructed by filling mined-out pits with products of the extraction of bitumen, including OSPW (Allen., 2009). Because acute toxicity of OSPW decreases with time because of the biodegradation of organic chemicals dissolved in OSPW (Anderson et al., 2012a, b; Wiseman et al., 2013a), the hope is that EPLs will eventually be capable of sustaining life and OSPW might be suitable for release to the environment. Part of the process in remediation or eventual releases to the wider environment is to have chemical and or biological methods to monitor the toxicity of OSPW before it is released.

The mixture of organic constituents of OSPW is complex. Organic chemicals dissolved in OSPW cause acute toxicity, but identities of chemicals that cause acute toxicity is very limited (Anderson et al., 2012a, b; He et al., 2012a). Naphthenic acids (NAs; $C_nH_{2n+z}O_2$) are cited as causing the toxicity of OSPW (Wiseman et al., 2013a; Clemente et al., 2005; Kavanagh et al., 2011; Toor et al., 2013), and more recently, there is evidence supporting this (Morandi et al., 2015). However, other organic chemicals containing nitrogen or sulfur can also cause acute toxicity, but they are significantly less potent organic chemicals than NAs in OSPW (Morandi et al., 2015). In general, there might be greater than 200,000 organic chemicals dissolved in OSPW, including chemicals containing oxygen (O_x), sulfur (SO_x), or nitrogen (NO_x) heteroatoms, many of which are present at small concentrations. It has been difficult to identify which individual chemicals or even classes of chemicals cause acute toxicity (Barrow et al., 2010; Headley et al., 2011b; 2013; Pereira et al., 2013a, b). One approach that can be used is to combine assays of acute toxicity with fractionation and chemical profiling using ultra-high resolution mass spectrometry of OSPW.

Different methods have been used to prepare samples of OSPW for characterization of acid-extractable organics (AEO). For example, preconcentration (enrichment) by use of ENVI+-SPE (Headley et al., 2011a), Oasis HLB (Bataineh et al., 2006), and C_{18} (Gagné et al., 2013) have been used for isolation of total organic compounds in OSPW. Mixed-modes sorbents for SPE has been used for preparation of OSPW for isotopic characterization of acid extractable organics (AEO) from OSPW (Ahad et al., 2012). Mixed-mode sorbents enhance the capacity for extraction of organic compounds from complex matrices, including water, because they can selectively extract charged and uncharged constituents. Mixed-mode chromatography uses polymeric sorbents/multi-model phases or layers that combine an anionic or cationic (strong or weak) ion-exchange with reverse phase chromatography (González-Mariño et al., 2012).

It has been shown that acid extractable fraction is what derive the acute toxicity of OSPW (Morandi et al., 2015), however, detail information about the chemical profiles differences in acid extractable fraction between ponds is not available. The objective of this study was to characterize the composition of organic chemicals in acidic fractions of OSPW from Base Mine Lake (BML-OSPW) and from an experimental reclamation pond called Pond 9 or tailings pond water (P9-OSPW). Base Mine Lake was commissioned in December of 2012 and is the first end-pit lake constructed in Alberta by the oil sands industry. Acid extractable fraction shown to be non-acutely toxic in P9-OSPW compared to BML-OSPW, therefore, there is great interest in determining whether toxicity of OSPW in BML decreases and if so the rate at which this detoxification occurs. Identification of organic chemicals dissolved in BML-OSPW that cause acute toxicity would be useful for developing analytical tools to monitor the detoxification of this lake or potentially treating OSPW to reduce or remove toxic constituents. Because of the complexity of the mixture, the organic chemicals dissolved in BML-OSPW and P9-OSPW were fractionated into acidic, basic, and neutral fractions by use of mixed-mode sorbents. Comparisons of the profiles of chemicals in acidic fraction by use of ultra-high resolution orbitrap mass spectrometry (MS) in both positive (ESI^+) and negative (ESI^-) electrospray ionization modes were made to characterize and compare chemicals that of environmentally concern.

Several studies have shown the chemical profiles of fresh OSPW from the West-in-pit (WIP), which is the settling basin that was converted to BML-OSPW, but detailed comparison the profile of organic chemicals in fractions from fresh BML-OSPW with aged P9-OSPW have not deeply investigated. This information is important for regulation of OSPW as it should facilitate identification of organic chemicals in BML-OSPW that are easy for monitoring.

2.3 Materials and Methods

2.3.1 Chemicals and Reagents

All solvents and reagents were HPLC grade and purchased from Fisher Scientific (Fair Lawn, NJ, USA). Ultrapure water was prepared by use of a Milli-Q Gradient A10 System (Millipore, Billerica, MA, USA). SPE cartridges containing Strata-X-A (500 mg) and Strata-X-C sorbents (500 mg) were purchased from Phenomenex® (Milford, MA, USA).

2.3.2 Samples of OSPW

Two samples of OSPW were collected on the site of Syncrude Canada, Ltd. (Fort McMurray, Alberta, Canada). One sample was collected from Base Mine Lake (BML-OSPW) which is an end-pit-lake constructed from the West-In-Pit (WIP) settling basin that received input of tailings from the main extraction facility. The other sample of OSPW was collected from an experimental reclamation pond called Pond 9 or tailings pond water (P9-OSPW) that was constructed in 1993. Both samples were collected in September of 2012, shipped to the University of Saskatchewan, and organics extracted and fractionated immediately upon arrival.

2.3.3 Fractionation of OSPW by Mixed Mode Sorbents (MDSPE)

Both samples of OSPW were fractionated into acidic, base, and neutral fractions of polar organic compounds by use of mixed-mode sorbents (Alharbi et al., 2016a, c), however, only acidic fractions were analysed and reported here. Briefly, prior to fractionation 500 ml of each sample of OSPW was passed through a glass microfiber filter (GF/D 0.47 mm, Whatman) to remove any particulate matter and then the samples were acidified to pH 2 by use of concentrated HCL (37%). Next, for isolation of basic fractions, pre-concentration was performed in one step by use of 500

mg of mixed-mode Strata®-X Polymeric-C solid-phase sorbent in plastic cartridges (Phenomenex, Milford, MA, USA). This matrix is a porous copolymer with a weak mixed-mode cation that provides dual modes for the retention and adsorption of lipophilic and hydrophilic compounds was well as ionic compounds. Before addition of OSPW, cartridges were conditioned with 6 ml of methanol and 6 ml of acidified water. 500 mL of filtered and acidified OSPW was passed through the cartridges under vacuum. Next, cartridges were washed with 2% formic acid and were allowed to dry under vacuum for 30 min. The first elution was performed with methanol and this extract contains acidic and neutral compounds. The second elution was performed with 5% (v/v) of NH_4OH in methanol and this fraction contains basic compounds. To generate fractions of acidic compounds and neutral compounds a pre-concentration of samples was performed by use of Strata-X-A 500 mg solid-phase matrix in plastic cartridges (Phenomenex). Strata-X-A is a weak mixed-mode anion exchanger, water wetable, polymeric sorbent that provides dual modes of retention – anion exchange and reversed phase – on a single, high surface area, organic polymer that is stable from pH 0 to 14. Prior to use the cartridge was conditioned by washing with 100% methanol followed by 5% (v/v) of NH_4OH (aq). Next, elute I from the Strata®-X Polymeric-C sorbent was evaporated to approximately 2 mL, adjusted to a pH of 10-11, and then passed through the cartridge without vacuum. The cartridges were washed with 5% (v/v) of NH_4OH (aq) and left to dry under vacuum for 30 min. Finally, the fraction containing neutral compounds was eluted with 100% of methanol and a fraction containing acidic compounds was eluted with 2% (v/v) of formic acid in methanol. Extracts were evaporated under nitrogen to dryness, re dissolved in methanol, then 50 μL of the extract was subsequently filled up to 1 mL with the mobile phase.

2.3.4 Profiling of Fractions of OSPW

The profile of chemicals in fractions of BML-OSPW and P9-OSPW was analysed by use of reversed-phase liquid chromatography paired with a linear ion trap-orbitrap mass spectrometer (Orbitrap XL, Thermo Fisher Scientific, San Jose, CA, USA) in both ESI^- and ESI^+ as described previously (Alharbi et al., 2016a). Briefly, the HPLC instrument was an Accela System (Thermo Fisher Scientific, San Jose, CA), consisting of a degasser, a 600 bar quaternary pump, an autosampler, and a column oven. Chromatographic separation was performed on a Cosmosil C18 MS-II column (100 × 3.0 mm, 2.5 μm particle size, Nacalai USA, San Diego, CA) at 40 °C. The flow rate was 0.4 mL/min and the injection volume was 5 μL. The mobile phases were 0.1% (v/v) acetic acid in water (Solvent A) and 100% methanol (Solvent B). 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.

In this study, mass values of electrospray ionization coupled with mass spectrometry (ESI-MS) was used to monitor a molecular mass of 100-500 Da with singly charged and signal to charge ratios greater than 3. The magnitude of each peak divided by the sum of magnitudes of all identified peaks represents the relative intensity, excluding the isotopic peaks. The elemental composition tool within the Xcalibur software (Thermo Scientific, Bremen, Germany) was used to calculate the elemental composition (i.e., C*c*H*h*N*n*O*o*S*s*) with the following parameters: 0-40 ^{12}C, 0-2^{13}C, 0-100 ^{1}H, 0-2 ^{14}N, 0-8 ^{16}O, 0-2 ^{34}S, and 1 ^{32}S, and the molecular formula was confirmed by the presence of ^{13}C. Furthermore, the most common adduct ions such as anionized adducts (negative mode), and cationized adducts (positive mode) were investigated and detected. The data was analysed based on the distribution of heteroatom classes, type, and number of carbon atoms. Relative intensity of each chemical (i.e. O_x, SO_x, NO_x) in each fraction was summed over all

masses detected. Only those chemicals in the total ion mass spectrum that had a peak threshold > 600, were present at relative intensity of at least 2%, and that produced a discernible extracted ion chromatographic peaks (i.e., S/N > 3) are reported. The mass accuracy of the assigned formula better than 2 ppm, and any background spectra (if any) were subtracted by analyzing Saskatoon municipal tap water (blank), which were processed following the same SPE procedures to rule out any compounds resulting from the extraction procedure. All samples were analyzed at the same time. Data visualization and analysis was performed using OriginPro 9.1 (OriginLab, Northampton, MA) and Mzmine 2.16 (Pluskal et al., 2010). MZmine 2 is an open-source software that is used for mass-spectrometry data processing, statistical analysis, and visualisation. In this work, the processing of the data generated from BML and P9 has been done separately for the positive and the negative ionization mode, and were identical for each set of data (Table 2.1).

Table 2.1: MZmine 2.16 parameters

	Criteria	**Parameter**
Mass detection	Noise level	1.0×10^3
	MS level	1
Chromatogram building	Minimum time span (min)	0.2 min
	Minimum area	5×10^4
	m/z tolerance	2 ppm
Chromatogram deconvolution	Algorithm	Local minimum search
	Chromatographic threshold	1 %
	Search minimum in RT range (min)	0.2 min
	Minimum relative area	5 %
	Minimum absolute area	5×10^4
	Minimum ratio of peak top/edge	5
	Peak duration range (min)	0.1-3
Isotopic peaks grouper	*m/z* tolerance	2 ppm
	Retention time tolerance	0.1 absolute (min)
	Maximum charge	2
	Representative isotope	Most intense
Peak list alignment	Algorithm	Join aligner
	m/z tolerance	2 ppm
	Retention time tolerance after correction	0.2 min
	Retention time tolerance	0.2 min
	Minimum number of points	20 %
	Threshold value	2
	Linear model	No
Gap filling	Algorithm	Same *m/z* and RT range gap filler
	m/z tolerance	2 ppm
Peak list filtering	Algorithm	Rows filter
	Minimum peaks in a row	45
	Minimum peaks in an isotope pattern	1
	m/z range	100-500 *m/z*
	RT range	2– 20 min
	Peak duration range	0.1 – 6 min
Adduct search	Algorithm	Adduct search
	RT tolerance	0.2 absolute (min)
	Adducts	Positive mode: Na, K, NH_4 and ACN+H Negative mode: HCOO and ACN+H
	m/z tolerance	2 ppm

	Maximum relative adduct peak height	50 %
Peak complex search	Algorithm	Peak complex search
	Ionization method	ESI positive: M-H^+ ESI negative: M-H^-
	RT tolerance	0.2 absolute (min)
	m/z tolerance	2 ppm
	Maximum complex peak height	50 %

2.4 Results and Discussion

2.4.1 Comparison and molecular characterization of the acidic fraction of BML-OSPW and P9-OSPW

Ion exchange sorbent was used as an extraction mechanism for isolation of organic acids and for desalting to enhance the ionization and minimize the ion suppression for electrospray ionization mass spectrometry to provide elemental compositional information on molecules within OSPW samples. In this sorbent, molecules which can be adsorbed on the anion exchange portion of the mixed mode extraction via primary interaction are expected to be isolated and analyzed by mass spectrometry, thus, chemicals isolated are mainly acidic in nature. Samples analysed in both $ESI^{-/+}$ and showed that no clear evidence of a possible significant distinction between fresh and aged OSPW as both samples very close in terms of elemental composition (Figure 2.1).

The mass spectra of both acidic fractions analysed by use of ESI^- exhibited a pseudo Gaussian distribution with intense signal clusters dominated by odd *m/z* values with mass range from 100-400 *m/z* with a maximum of the peak distribution of ions ranged from *m/z* 250-350 (Figure 2.2). This is similar to the broadband spectra of BML-OSPW that was not fractionated as determined by use of Orbitrap in ESI^- (Pereira et al., 2013a, b). The number of peaks resolved in the acidic fraction of BML-OSPW and P9-OSPW was approximately 4000 and 3000, respectively,

with the identification of isotope peaks (such as containing ^{13}C, or ^{34}S) in both ionization modes. The chemicals detected were singly charged (based on the observed *m/z* unit difference between the $^{12}C_c$ and $^{13}C_1$-$^{12}C_{c-1}$) and the average molecular mass of chemicals in the acidic fraction of BML-OSPW and P9-OSPW was 272 and 269, respectively, which does indicate similar *m/z* distribution between the two samples. In general, the assigned elemental composition formula identified in ESI^- is expected to different from that in ESI^+, which is because of different ionization mechanism in the gas phase of the analytes in both modes (Cech and Enke., 2001).

Although there were differences in the abundance of ions in the mass spectra of each fraction, the total ion chromatographs (TIC) of the acidic fraction of BML-OSPW and P9-OSPW generated by use of ESI^- indicated that there were no differences in the distribution of *m/z* versus time between the acidic fraction of BML-OSPW and P9-OSPW (Figure 2.3). Total ion chromatogram (TIC) of both fractions indicated no change in retention time but the magnitude of TIC abundance in 12-14 minutes' range was greater in the acidic fraction of BML-OSPW compared to P9-OSPW. A 3D plot of the *m/z* ratio, ion intensity, and retention time of the acidic fraction of BML-OSPW and P9-OSPW shows that chemicals with an *m/z* from 225-350 were more abundant in the acidic fraction of BML-OSPW compared to P9-OSPW (Figure 2.4, and 2.5, respectively).

In Figure 2.3, the window of chromatogram at retention time of 11-13 and 13-14 minutes "the hump" contains likely hundreds or thousands of compounds and their isomers making the use of liquid chromatography for separation and identification of individual species in OSPW extremely difficult. However, this duration of peaks showed the unique feature between the two acidic fractions that is the higher intensity of compounds isolated at this time range in BML-OSPW compared to P9-OSPW. For example, the number of peaks (ions) isolated at the range 13-14

minutes was approximately doubled in BML-OSPW compared to P9-OSPW with 160, and 85, respectively. Detailed of the elemental composition is given in section 3.4.1.

Moreover, the number of peaks separated in the range of 160-340 *m/z* was higher by around 1.5-fold in BML-OSPW compared to P9-OSPW (Figure 2.6). The number of peaks eluted early was similar for both samples but the most compounds were eluted when the methanol increased in the mobile phase in BML-OSPW compared to P9-OSPW (Figure 2.7). This indicates that the unique feature of chemicals in BML-OSPW compared to P9-OSPW is the higher quantity (and abundant) of late eluted compounds that are easily degraded during aging. These compounds in BML-OSPW might drive the observed acute toxicity in BML but not P9 of OSPW (Alharbi et al., 2016a). The acute toxicity data is presented in Figure 3.1 (Chapter 3), and in Figure A1 in Appendix A.

In BML-OSPW, more than 89% of the detected peaks contain CHO while around 10% contain CHOS, and in P9-OSPW, the same trend of the total abundance of the elemental composition was observed for CHO, and CHSO with 88, and 10%, respectively. No nitrogenous-containing compounds detected at the relative abundance cut off ≥2% for both fractions. Acidic nitrogenous compounds have been detected in crude oil (Hughey et al., 2001; 2002; 2007), and OSPW samples (Pereira et al., 2013a, b). The high concentration of carboxylic acids compounds in acidic fractions might compete with other classes chemical ions presented in the acidic fraction in the sprayed solution for access to the droplet surface for gas-phase emission (Tang et al., 2004). As a result, nitrogenous compounds were not detected in the acidic fraction but not necessarily an absent of acidic nitrogen compounds in OSPW samples. Moreover, the low contribution of compounds containing atoms different from CHO such as CHOS and CHON could be explained,

in part due to the threshold at ≥ 2% of the relative intensities performed, and small molecules signals with heteroatoms have not been considered in the final assigned formulas.

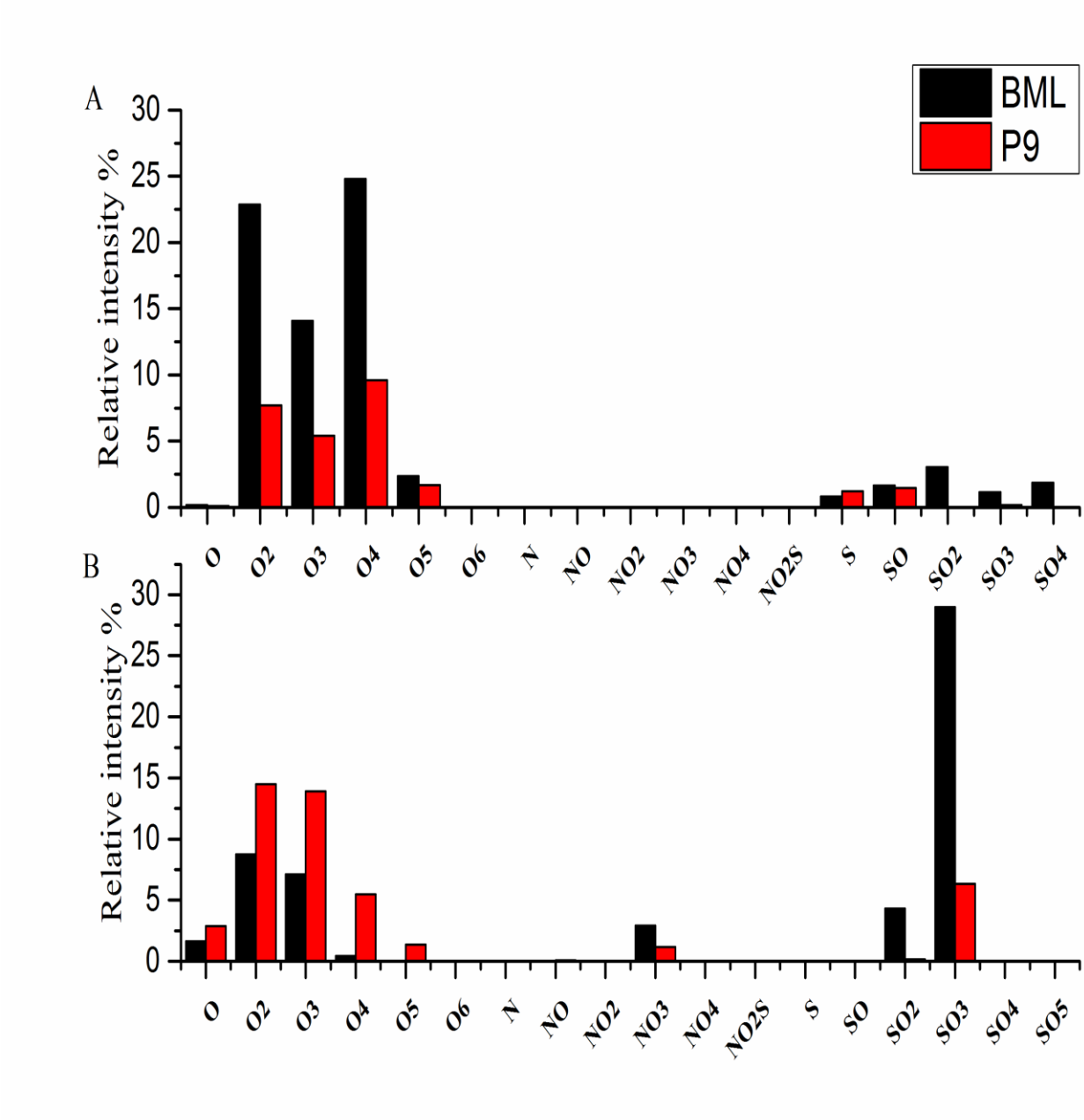
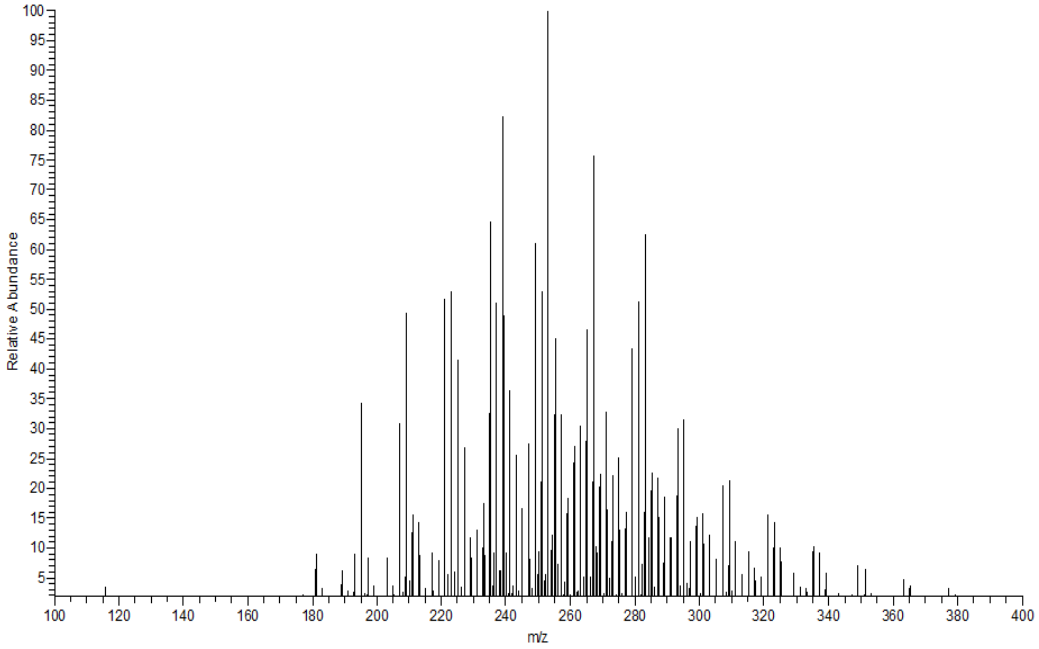


Figure 2.1: Elemental composition and relative abundances of water-soluble compounds in acidic fraction of BML-OSPW and P9-OSPW acquired in A) ESI^- and B) ESI^+.

A



B

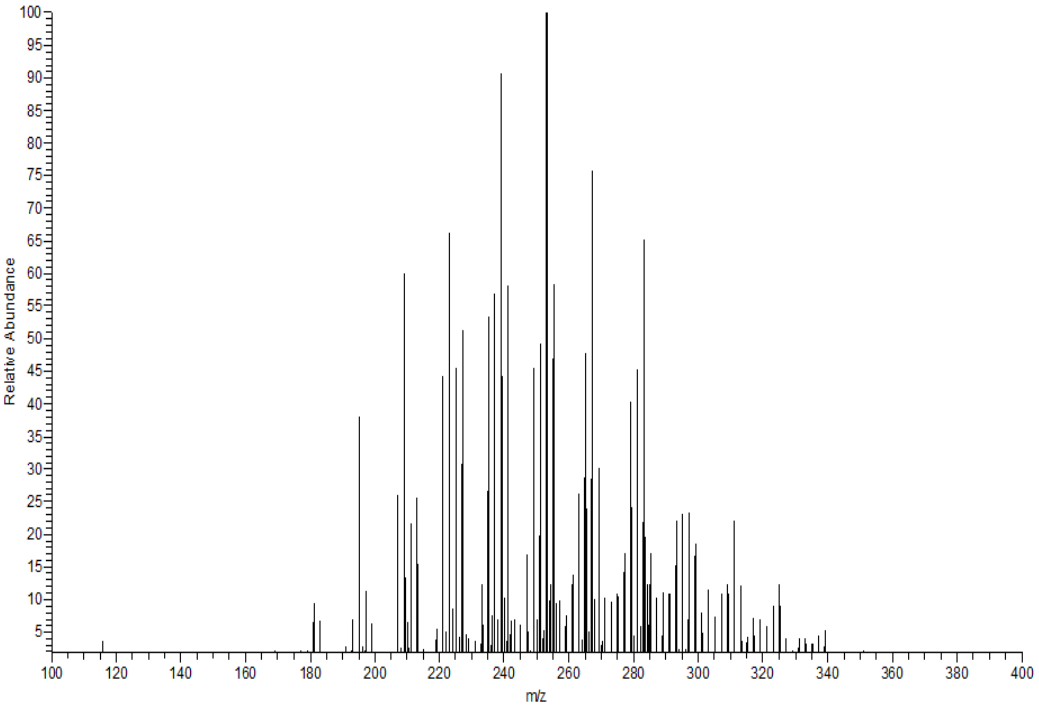


Figure 2.2: Negative ion Orbitrap mass spectra of the acidic fraction of A) BML-OSPW and B) P9-OSPW.

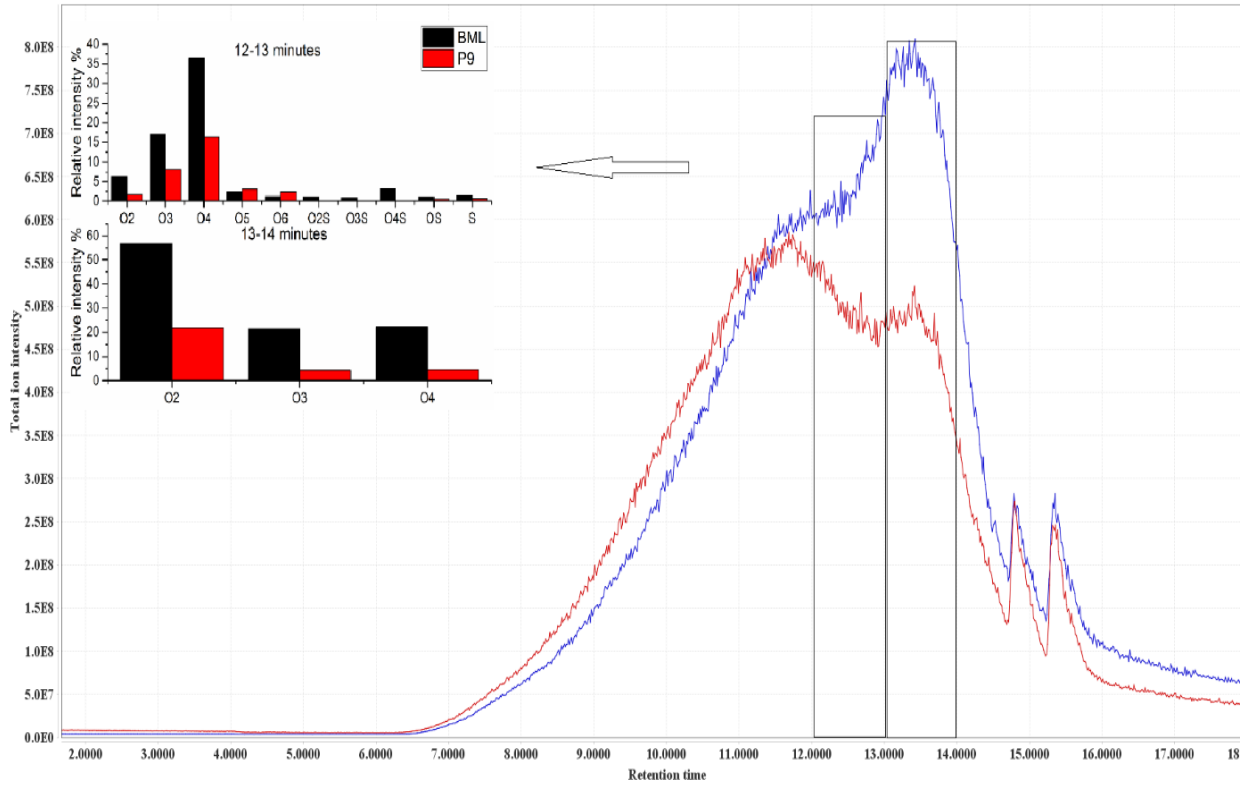


Figure 2.3: Total ion chromatograms (TICs) of the acidic fraction of BML (in blue) and P9 (in red) of OSPW acquired in ESI^-. The insert shows the intensity of the elemental compositions of compounds eluted at time range from 12-13, and 13-14 min.

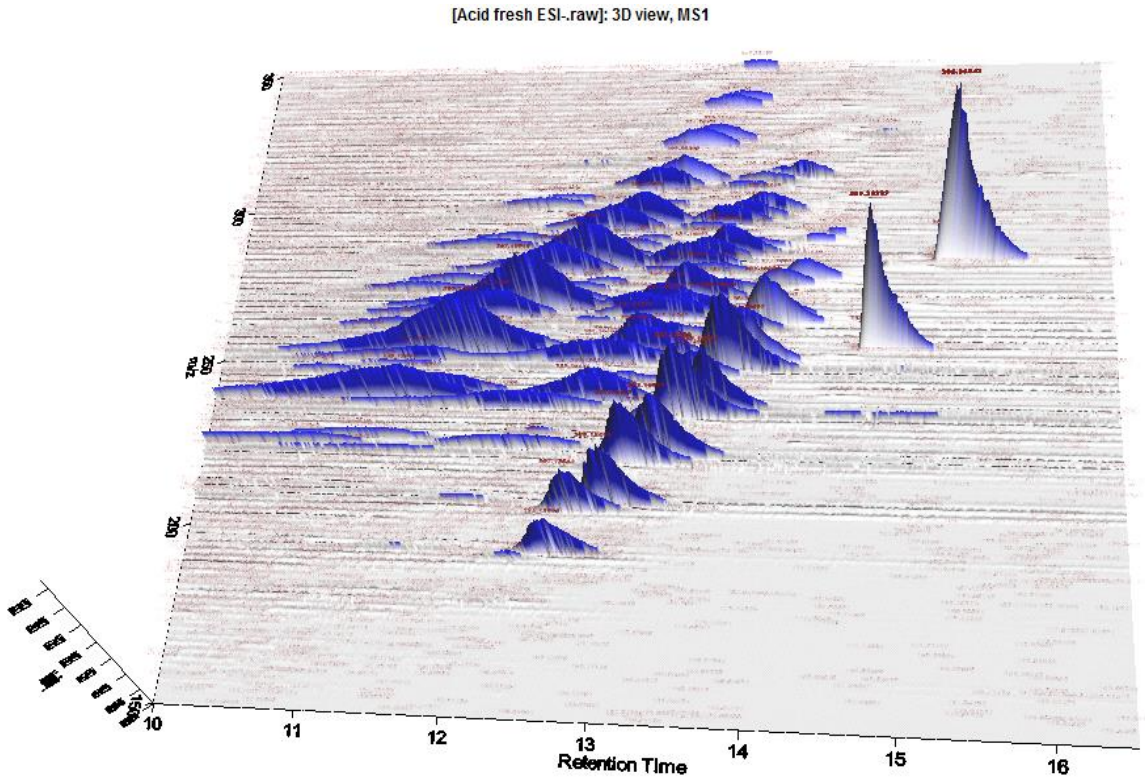


Figure 2.4. 3D total ion chromatograms (TICs) of the acidic fraction of BML-OSPW acquired in $ESI^{-\cdot}$

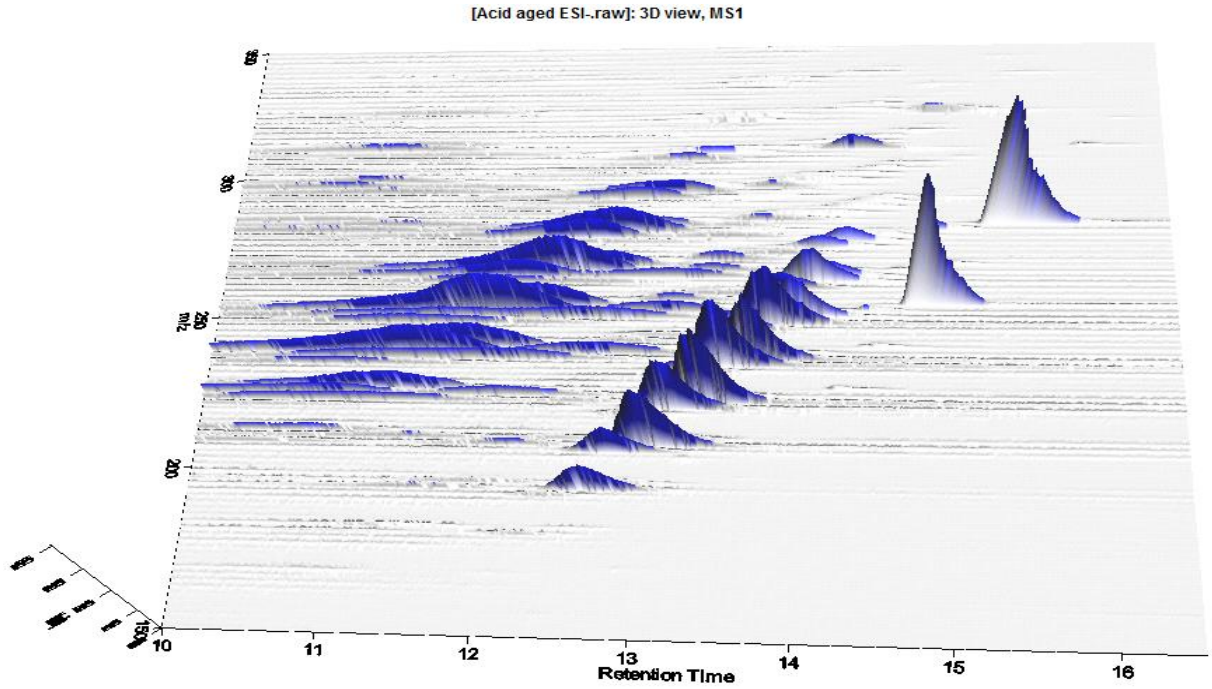


Figure 2.5 3D total ion chromatograms (TICs) of the acidic fraction of P9-OSPW acquired in ESI^-

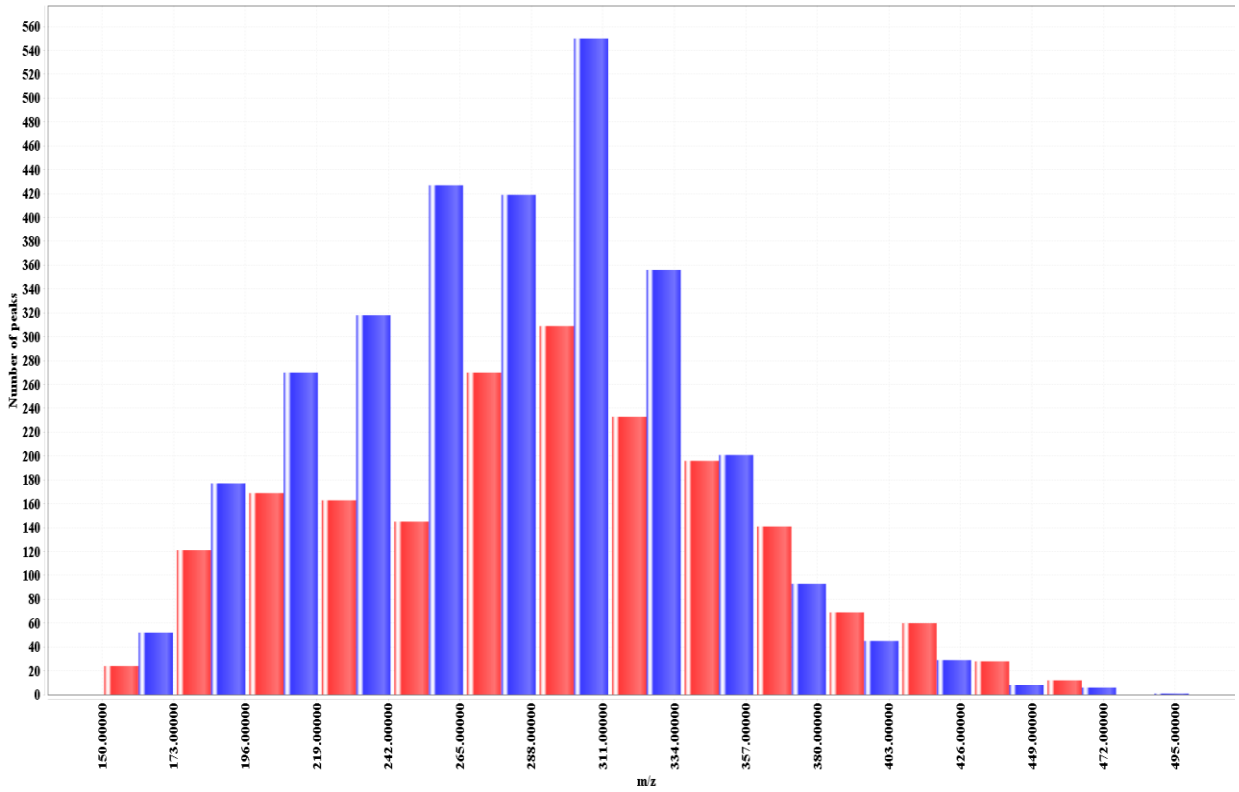


Figure 2.6: Number of peaks eluted from acidic fractions of BML-OSPW (in blue) and P9-OSPW (in red) at *m/z* range 150-500 acquired in ESI^-.

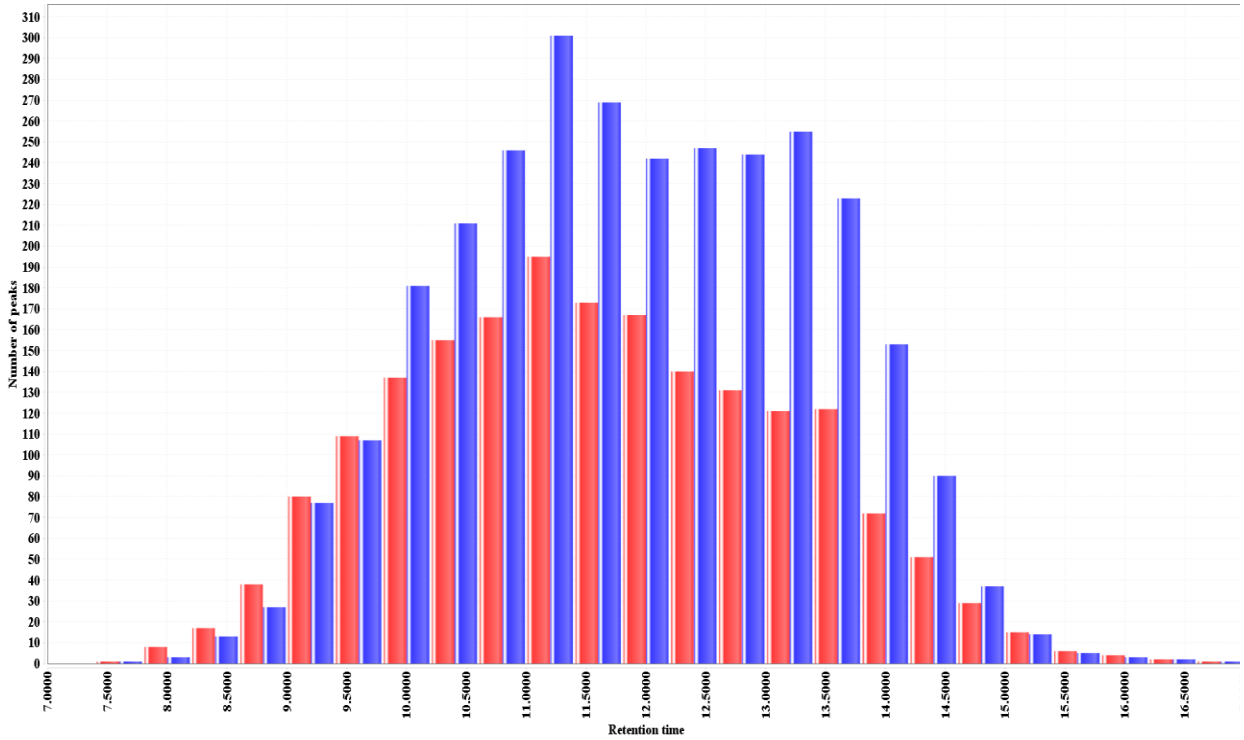


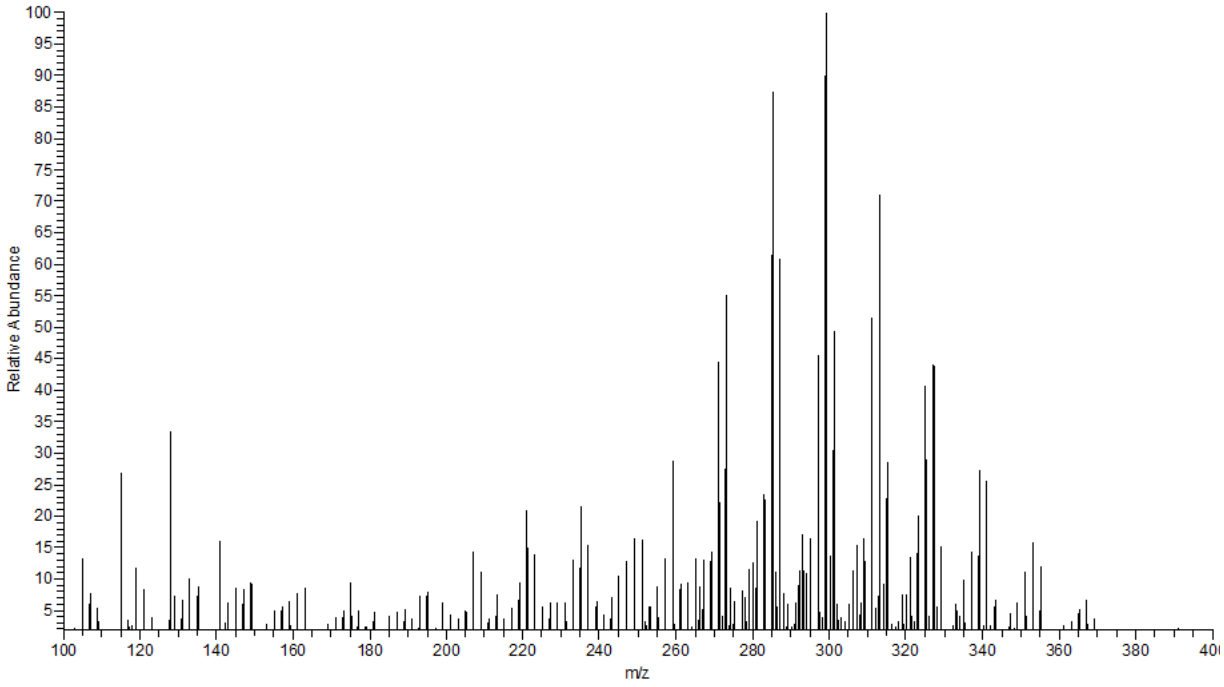
Figure 2.7: Number of peaks acquired in ESI^- eluted as a function of retention time (7-17 minutes) for both BML-OSPW (in blue) and P9-OSPW (in red). More peaks in BML-OSPW eluted when the methanol in the mobile phase increased, while no difference in the number of peaks eluted at early time.

The mass spectra of each fraction analysed by use of ESI^+ had a mass range from 100-400 *m/z*, and the greatest number of signals from *m/z* 260-360 in BML-OSPW (Figure 2.8A), but it is more broad in P9-OSPW (Figure 2.8B). The mass spectra of acidic fraction of BML-OSPW is similar to the broadband spectra of whole BML-OSPW that was not fractionated as determined by use of Orbitrap in ESI^+ (Pereira et al., 2013a). Moreover, there were differences in the abundance of ions in the mass spectra as well as differences in the distribution of *m/z* between the acidic fraction of BML-OSPW and P9-OSPW in the range from *m/z* 210-340 (Figure 2.8). However, the number of peaks resolved in the acidic fraction of BML-OSPW was slightly higher at *m/z* 275-325 compared to lower molecular weight compounds (Figure 2.9). The average molecular mass of all chemicals in the acidic fraction of BML-OSPW and P9 was 275 and 269, respectively, which also indicates similarity in ions distribution between the two samples of OSPW. In addition, the chromatographic separation showed that the number of peaks eluted early was similar for both acidic fractions but as the methanol increased in the mobile phase; more peaks were eluted in BML-OSPW compared to P9-OSPW (Figure 2.10). The higher number of peaks eluted and separated at higher methanol concentration of mobile phase is a common feature in BML-OSPW compared to P9-OSPW despite the ionization mode.

The total ion chromatographs (TIC) of the acidic fraction of BML-OSPW and P9-OSPW generated by use of ESI^+ are given in Figure 2.11. There was no change in retention time but the magnitude of TIC abundance at 10-15 minutes was lesser for the acidic fraction of P9-OSPW compared to BML-OSPW. A 3D plot of the *m/z* ratio, ion intensity, and retention time of the

acidic fraction of BML-OSPW and P9-OSPW shows that chemicals with an *m/z* from 220-370 were more abundant in the acidic fraction of BML-OSPW compared to P9-OSPW as well as increase in the abundances of low *m/z* in P9-OSPW (Figure 2.12 and 2.13, respectively). The total abundance of peaks contain CHO was around 33% and CHSO 61%, while CHNO had the lowest abundance with 7% in BML-OSPW. In P9-OSPW, the contribution of CHSO was lower than CHO with 14 and 83%, respectively, with almost the same as in BML-OSPW for CHNO with 6%. The presence of nitrogenous species in ESI^+ is not surprisingly, as it has been shown that only ESI^+ can detect nitrogenous species more efficiently than ESI^- (Barrow et al., 2010).

A



B

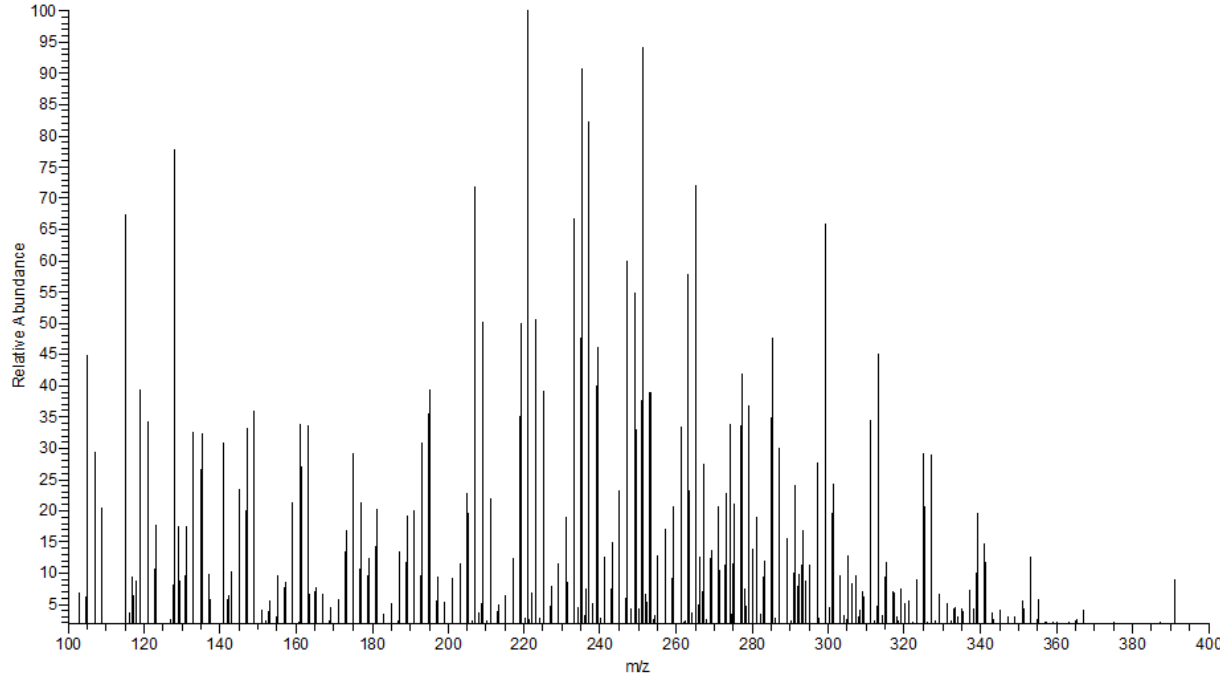


Figure 2.8: Positive ion Orbitrap mass spectra of the acidic fraction of A) BML-OSPW and B) P9-OSPW.

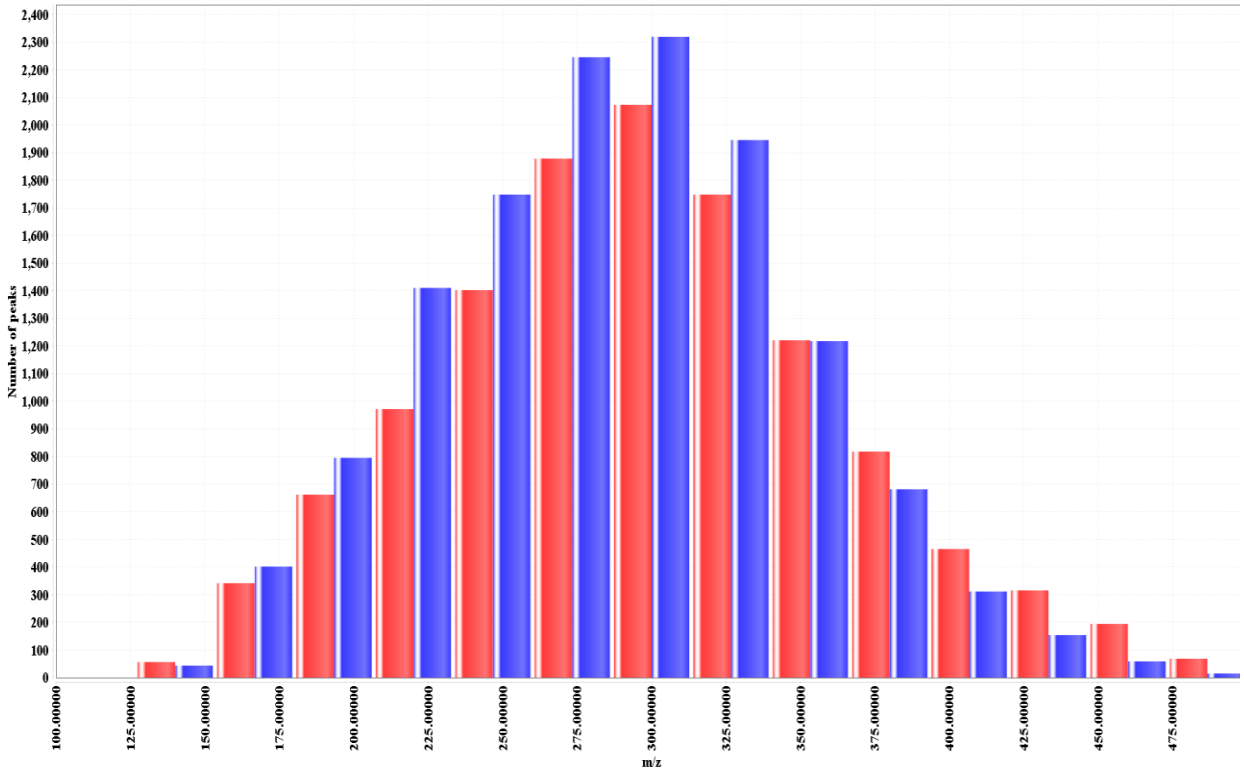


Figure 2.9: Number of peaks eluted from acidic fractions of BML-OSPW (in blue) and P9-OSPW (in red) at *m/z* range 100-500 acquired in ESI^+.

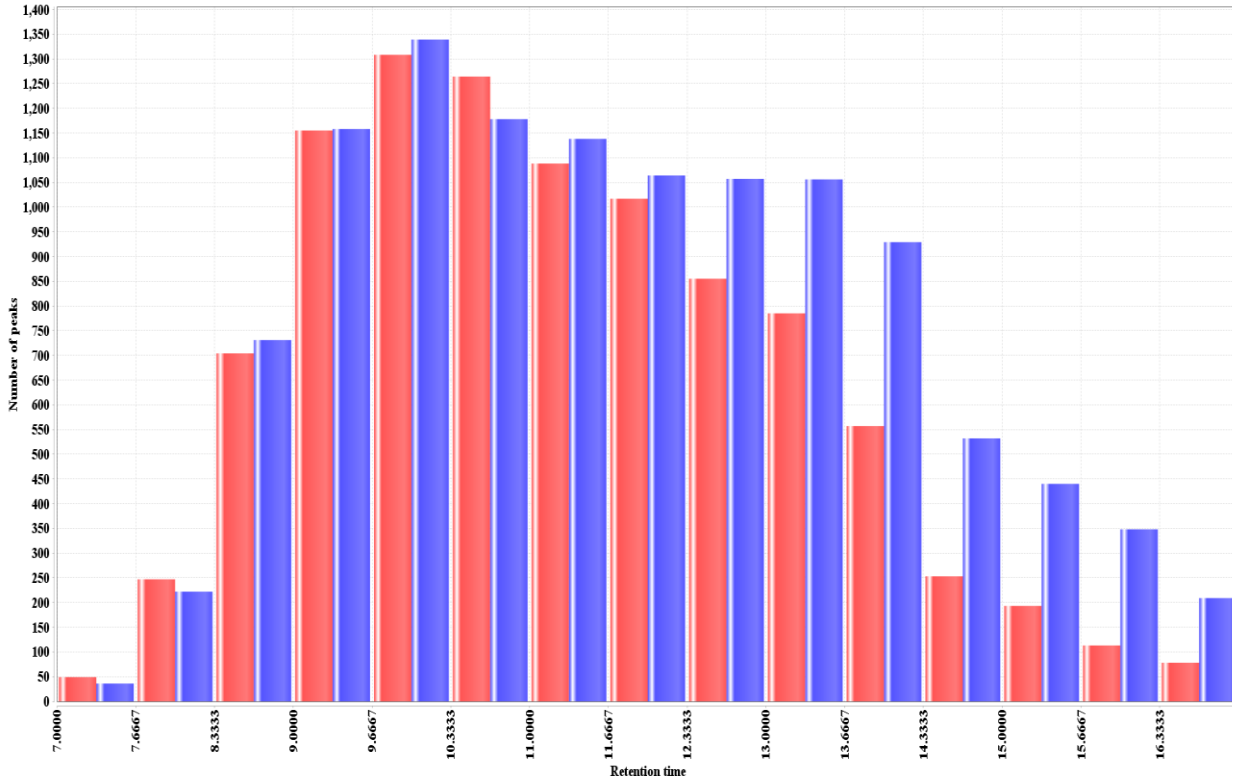


Figure 2.10: Number of peaks acquired in ESI^+ eluted as a function of retention time (7-17 minutes) for both BML-OSPW (in blue) and P9-OSPW (in red). As in ESI^-, the same trend of elution was observed where more peaks in BML-OSPW eluted when methanol in mobile phase increased, while no difference in the number of peaks eluted at early time.

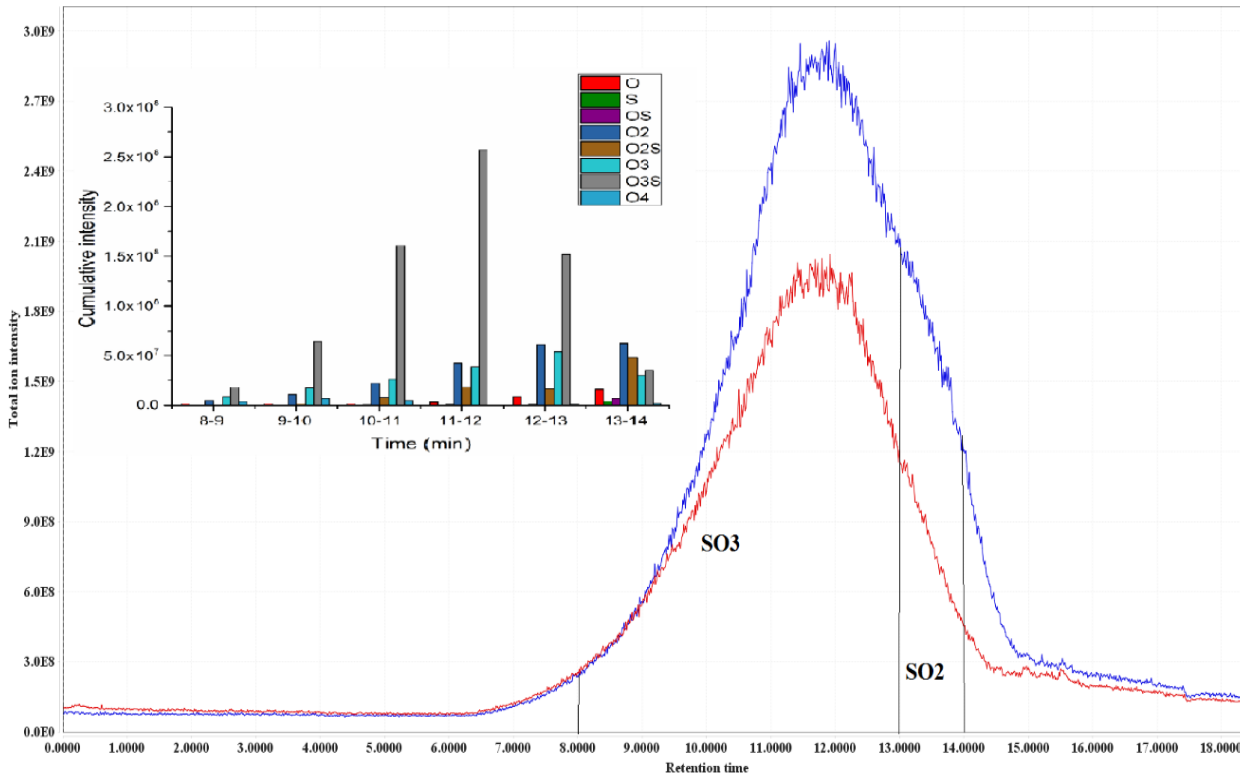


Figure 2.11: Total ion chromatograms (TICs) of the acidic fraction of BML-OSPW (in blue) and P9-OSPW (in red) acquired in ESI^+. The insert shows the intensity of the elemental compositions of compounds eluted at time range from 8-14 min in BML-OSPW.

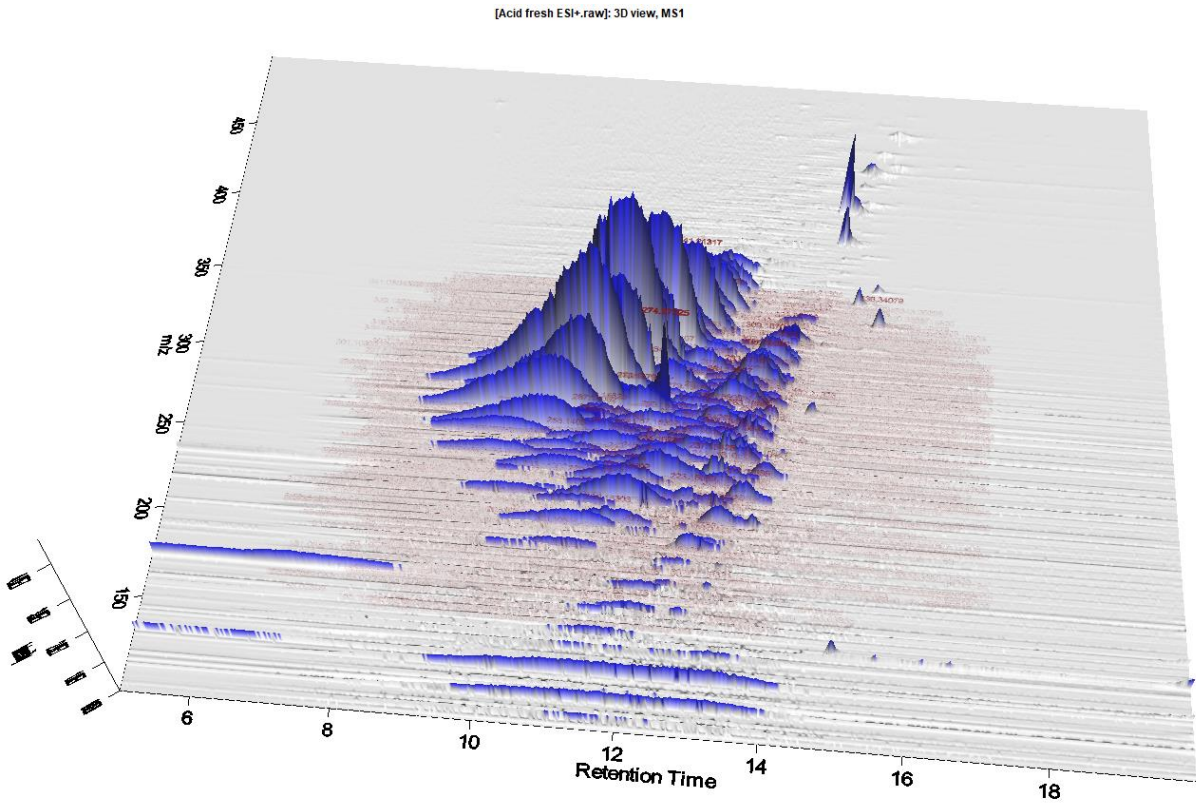


Figure 2.12 3D total ion chromatograms (TICs) of the acidic fraction of BML-OSPW acquired in ESI^+

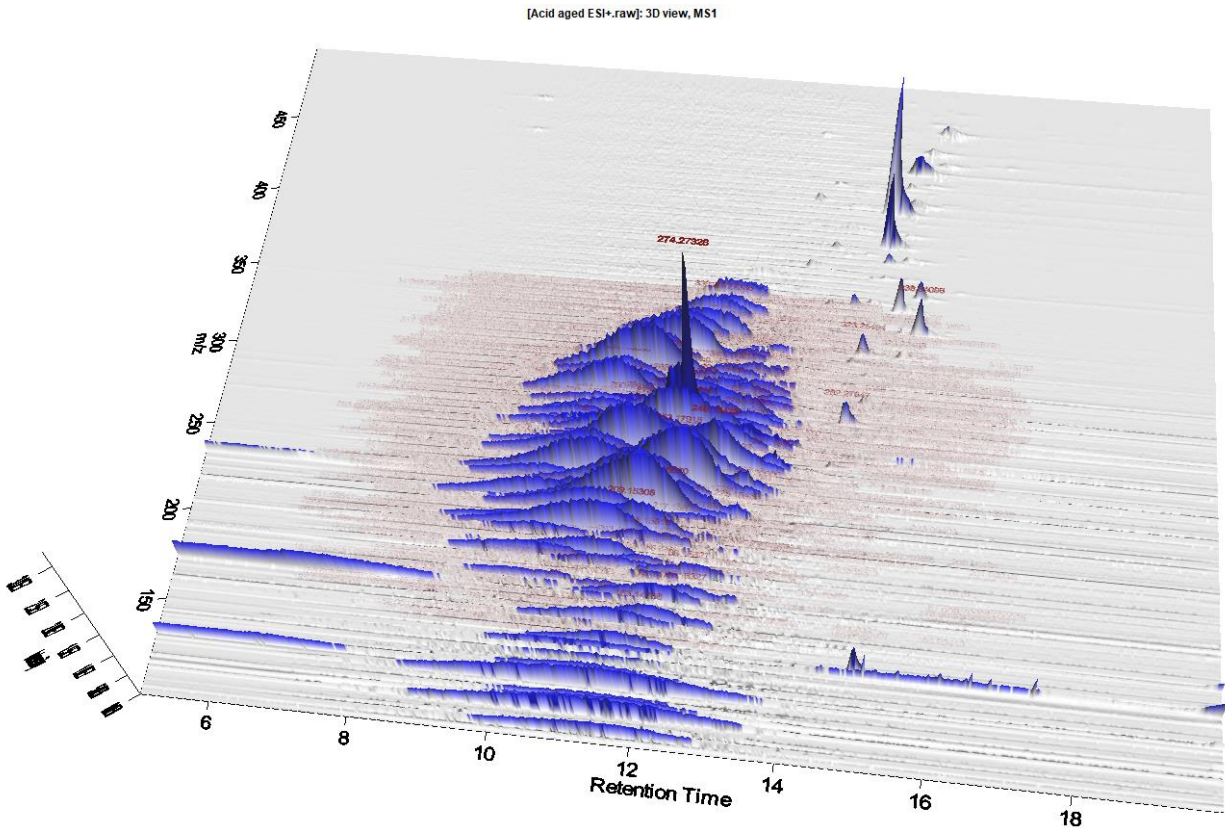


Figure 2.13 3D total ion chromatograms (TICs) of the acidic fraction of P9-OSPW acquired in ESI^+

2.4.2 Comparison of oxygen-containing chemicals

The profile of chemicals containing oxygen in the acidic fraction of BML-OSPW and P9-OSPW were similar. Although both fractions contained O^-, O_2^-, O_3^-, O_4^-, and O_5^- chemicals, and these accounted for approximately 89% of all chemicals detected in each fraction, the relative abundance of each chemical was greater in the acidic fraction of BML-OSPW compared to P9-OSPW (Figure 2.1A). Identities of O^-, O_3^-, O_4^-, and O_5^- chemicals are not known (Pereira et al., 2013a). O_4^- chemicals are likely di-carboxylic acids (diacids), which are generated via microbial biodegradation of hydrocarbons and monocarboxylic acids (monoacids) (Lengger et al., 2013). The most abundant chemicals in both fractions were O_2^-, O_3^-, and O_4^-. Relative abundances of O^-, O_2^-, O_3^-, O_4^-, and O_5^- in the acidic fraction of BML-OSPW was 0.15, 22.6, 13.9, 24.5 and 2.3%, respectively, whereas aging decrease the relative abundances of O^-, O_2^-, O_3^-, O_4^-, and O_5^- in the acidic fraction of P9-OSPW to 0.08, 7.8, 5.3, 9.8, and 1.7%, respectively. This indicates that aging is unlikely to produce more oxidized compounds that can be detected in ESI^-. In both fractions, all these chemicals totally had double bond equivalents (DBE) from 1-9 and number of carbon atoms from ~C_{11}-C_{21} (Figure 2.14), and the relative abundance of individually O_2^-, O_3^-, and O_4^- was lesser in P9-OSPW compared to BML-OSPW (Figure 2.15, 2.16, and 2.17). O_2^- compounds are likely naphthenic acids which were approximately 3-fold less in P9-OSPW compared to BML-OSPW However, naphthenic acids, (O_2^-) with number of carbon atoms from C_{15}-C_{19} and DBE from 5-8 (1–6 naphthenic rings) were detected in the acidic fraction of BML-OSPW but were almost absent from the acidic fraction of P9-OSPW (Figure 2.15). It has been shown that the O_2^- species with 15–18 carbons and DBE = 4 was positively correlated with toxicity and suggested to be the more toxic class (Yue et al., 2014; 2015), and more recent studies have proven the O_2^- is of environmental concern compounds compared to all other oxygen containing compounds (Morandi

et al., 2015). BML-OSPW was almost 2-fold acutely toxic compared to P9-OSPW, thus; the profile of O_2^- in both fractions might explain the lack of acute toxicity of BML-OSPW compared to P9-OSPW. However, it has been shown that NAs with lower carbon number of 11-16 and DBE 3-4 are more biodegradable compared to higher carbon number NAs (Toor et al., 2013). The chromatographic separation indicated that the majority of O_2^- compounds eluted at 13-14 minutes, while O_4^- compounds eluted at 12-13 minutes and almost equal intensity was observed for O_3^- compounds at time spanned from 11-14 minutes (Inserted panels in Figure 2.3). Since O_4^- species were generally eluted earlier, this indicates their higher hydrophilicity compared to O_2^- compounds. However, overlap of O_2^-, O_3^- and O_4^- compounds in the elution time is unavoidable.

There have been several reports on the detailed analysis of naphthenic acids in OSPW, which are a very diverse group of acyclic, alicyclic and aromatic carboxylic acids (Grewer et al., 2010; Rowland et al., 2011a, b, c; Jones et al., 2012; Lengger et al., 2015). Although the presence of high carboxylic acids concentration in OSPW is mainly related to the biodegraded bitumen, it has been shown the concentration of acids in oils decreased with increasing degrees of biodegradation (Behar and Albrecht., 1984), however the type of acids compounds that are more or less resilient to biodegradation is not known. Greater abundance of O_2^- in the acidic fraction of BML-OSPW compared to P9-OSPW is consistent with results from analysis of OSPW by use of Fourier transform infrared spectroscopy (FTIR) where the concentration of O_2^- was 60 mgL^{-1} in WIP-OSPW compared to approximately half 31 mg L^{-1} in P9-OSPW (Han et al., 2008;2009).

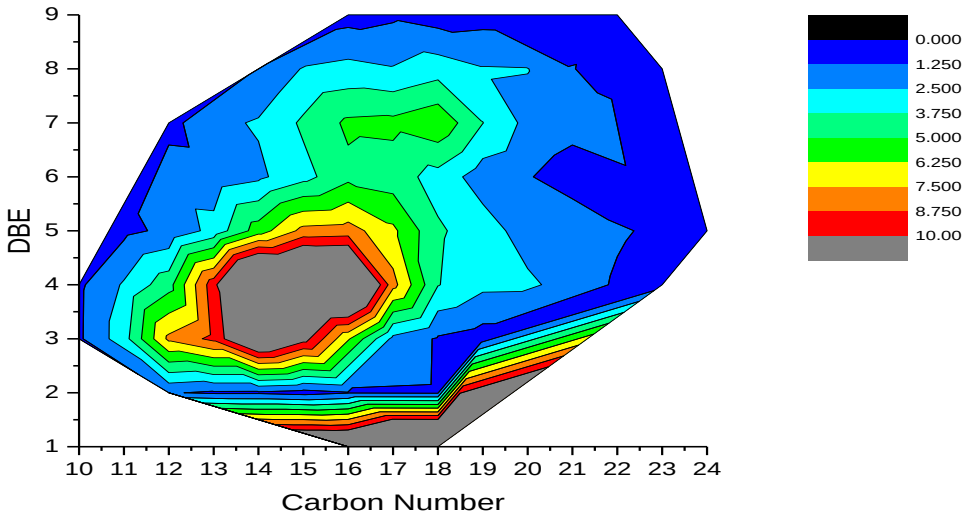
The presence of the fatty acids (DBE=1, and C_{16}-C_{18}) in acidic fractions is likely due to unresolved separation from the neutral fraction (Alharbi et al., 2016a). The distribution of molecular mass of all oxygen-containing chemicals detected by use of ESI^- indicates the abundance of compounds with MW 150-375 Da is greater in the acidic fraction of BML-OSPW

compared to P9-OSPW (Figure 2.18). The plot of H/C vs. O/C atomic ratio (Kim et al., 2003) of ionisable oxygenated compounds (CHO_x) from ESI-MS corresponded to O/C and H/C was similar between the BML-OSPW and P9-OSPW with 0.19 and 1.45 for BML-OSPW and 0.19 and 1.46 for P9-OSPW, respectively, which indicates similar degree of lower oxygenation and a higher degree of hydrogen saturation for both samples but the average intensity was approximately 2-fold higher in BML-OSPW compared to P9-OSPW. Moreover, chemicals with O/C and H/C at range 0.1-0.4 and 1-1.4, respectively were higher in BML-OSPW compared to P9-OSPW (Figure 2.19).

Although aromatic compounds that do not contain a heteroatom or organic compounds that cannot ionize are not detected by negative-ion mode ESI, the majority of the toxicity has previously been shown to be associated with the acidic NA fraction of OSPW (MacKinnon and Boerger, 1986; Lo et al., 2006). The profile of chemicals containing oxygen in the acidic fraction of BML-OSPW and P9-OSPW were similar when analysed by use of ESI^+ (Figure 2.1B). The O_x^+ chemicals accounted for approximately 33% and 83% of all chemicals in the acidic fraction of BML-OSPW and P9-OSPW, respectively. Both fractions contained O^+, O_2^+, O_3^+, and O_4^+ chemicals, but O_5^+ chemicals were detected only in the acidic fraction of P9-OSPW (Figure 2.1B). Relative abundances of O^+, O_2^+, O_3^+, O_4^+, and O_5^+ were 2.27, 13.91, 9.87, 5.21 and 0.61% in the acidic fraction of BML-OSPW. Relative abundances of O^+, O_2^+, O_3^+, O_4^+, and O_5^+ chemicals in the acidic fraction of P9-OSPW were 2.49, 7.50, 7.20, 6.85, and 0.71%, respectively. The distribution of numbers of carbon atoms versus DBE for O_x^+ chemicals showed that chemicals with C_{12}-C_{18} and DBE 4-9 were most abundant in BML-OSPW and had an equal distribution (Figure 2.20A), and the abundance of chemicals with C_{12}-C_{18} were most abundant in P9-OSPW but had different distribution pattern (Figure 2.20B). The distribution of molecular masses of all chemicals detected by use of ESI^+ indicates that the abundance of compounds with MW 175-250

Da is greater in the acidic fraction of P9 -OSPW compared to BML-OSPW, but the abundance of compounds with MW 250-400 Da was greater in BML-OSPW (Figure 2.21). However, the distribution of molecular masses of individual chemicals was similar but the differences in relative abundances among fractions. Greater abundances of O_x^+ chemicals and chemicals of lesser molecular mass in P9-OSPW might be explained by a greater abundance of organic chemicals that had been oxidized.

A



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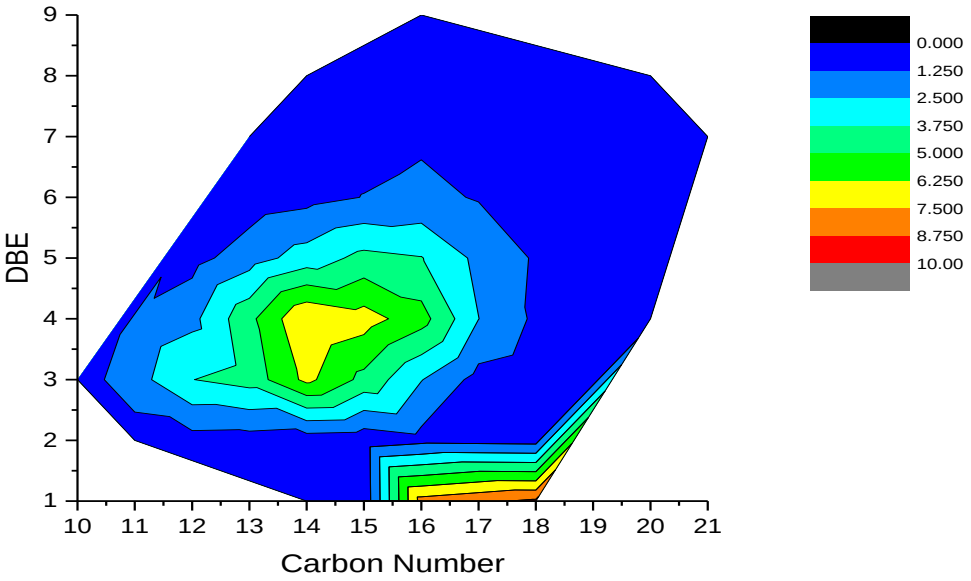
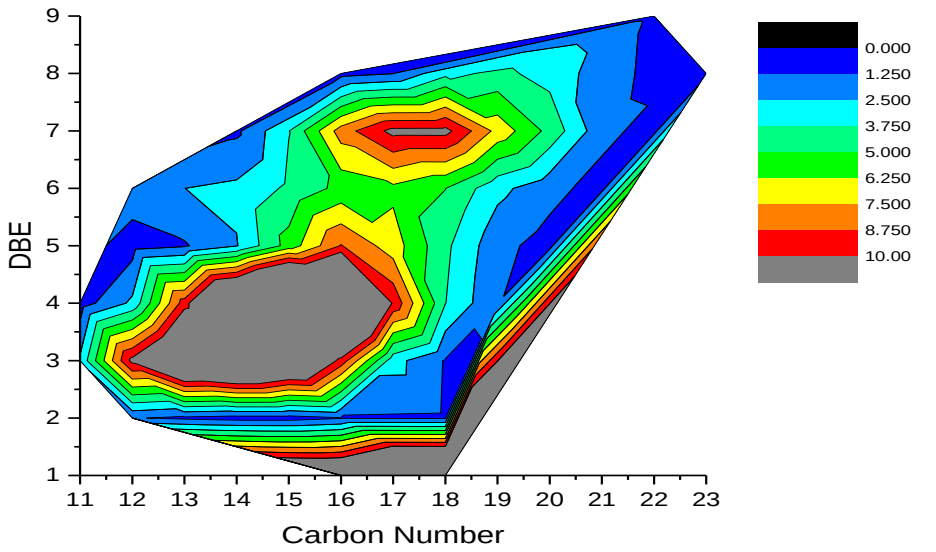


Figure 2.14: Relative abundances of all oxygen O_x^- containing chemicals in acidic fraction (A) BML-OSPW and (B) P9-OSPW based on DBE and number of carbon atoms acquired in ESI^-

A



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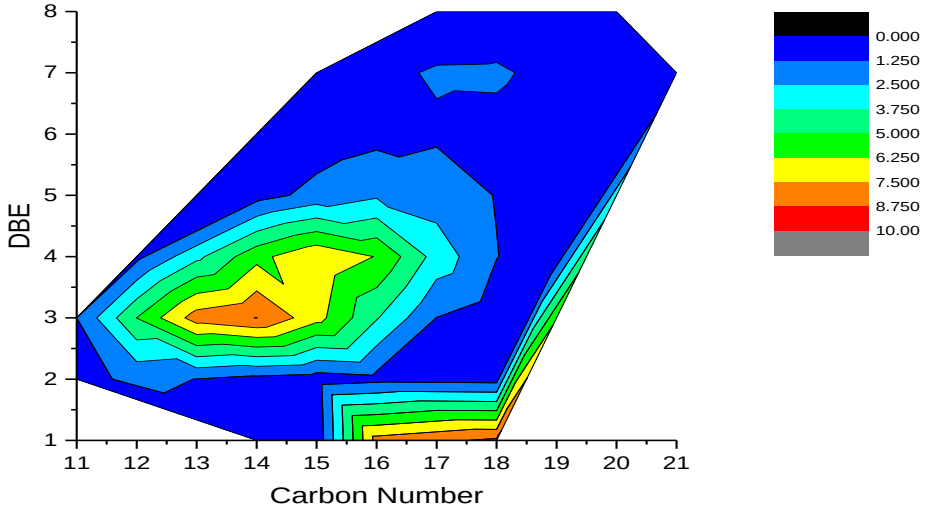
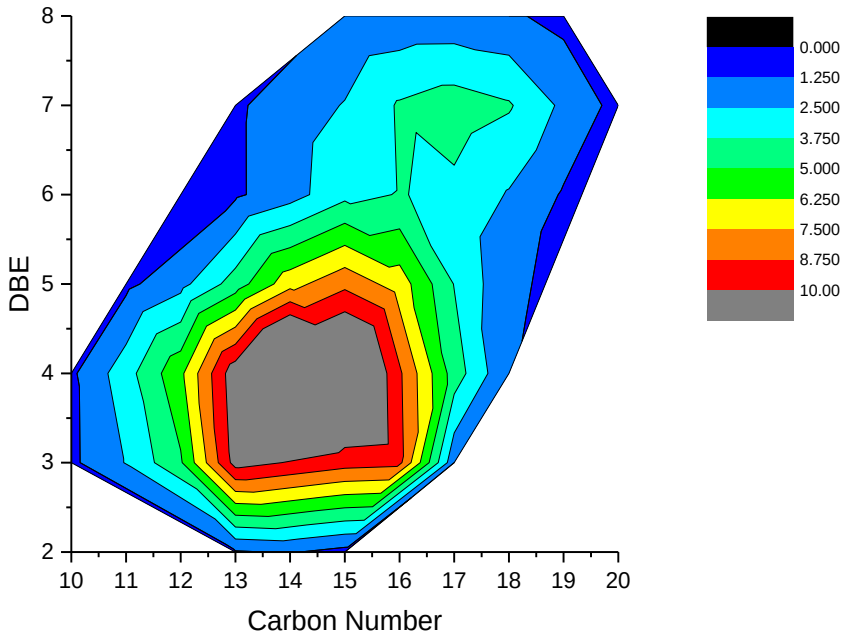


Figure 2.15: Relative intensity of O_2^- in acidic fraction (A) BML-OSPW and (B) P9-OSPW based on DBE and number of carbon acquired in ESI^-

A



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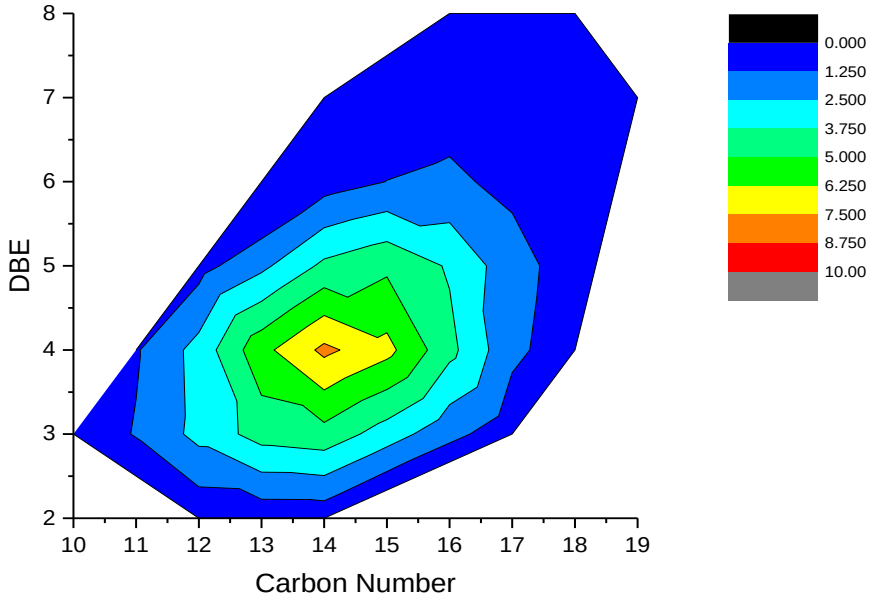
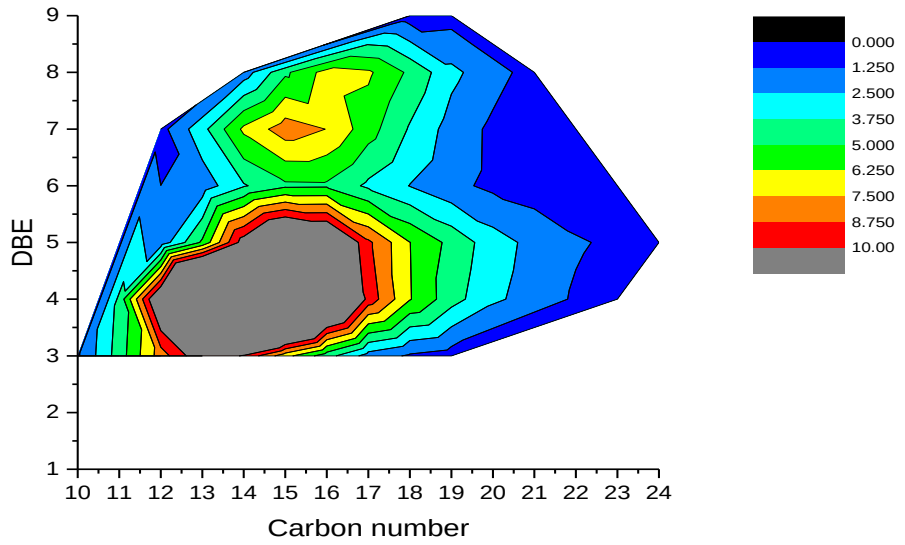


Figure 2.16: Relative abundances of O_3^- in acidic fractions (A) BML-OSPW and (B) P9-OSPW based on DBE and number of carbon acquired in ESI^-

A



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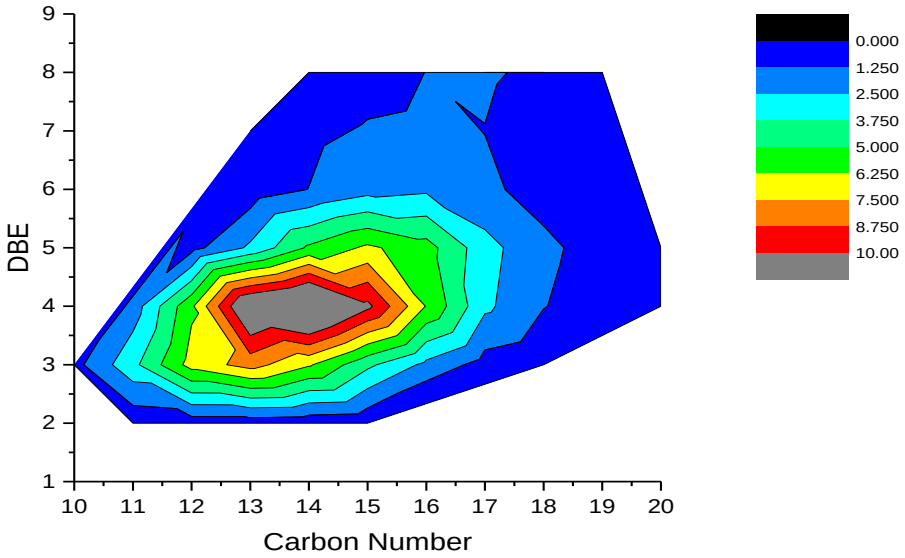


Figure 2.17: Relative abundances of O_4^- in acidic fraction (A) BML-OSPW and (B) P9-OSPW based on DBE and number of carbon acquired in ESI^-.

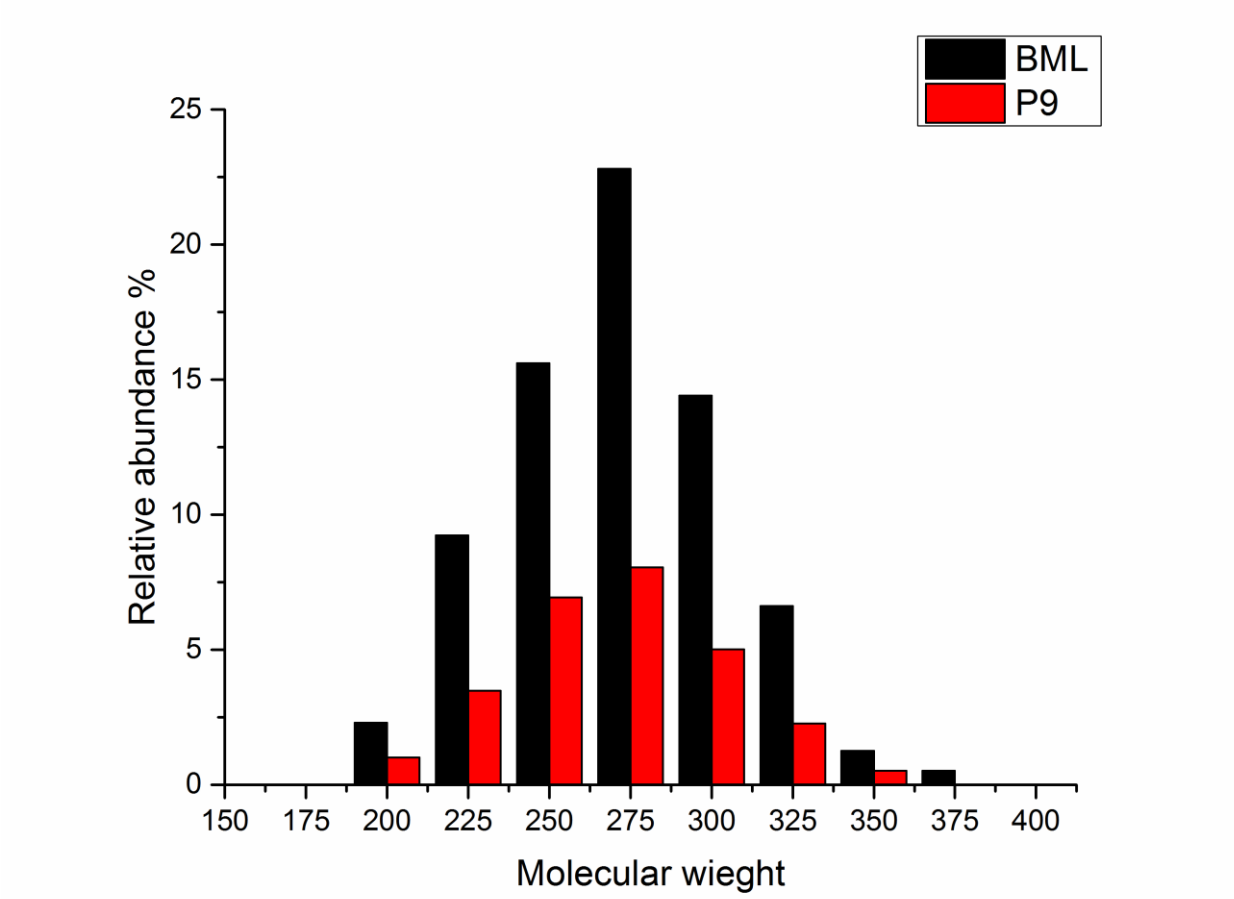
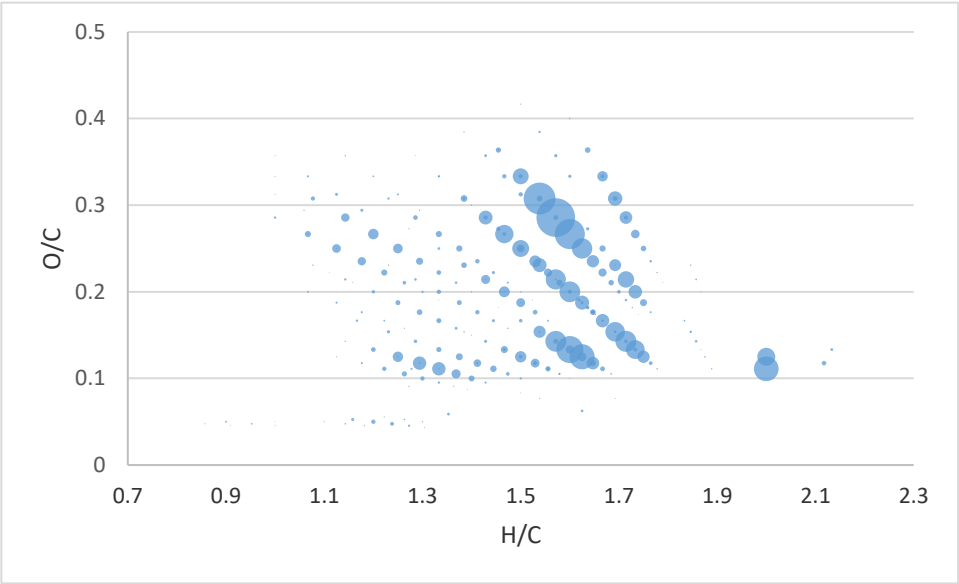


Figure 2.18: Molecular weight versus relative abundances of all oxygen-containing compounds in the acidic fraction of BML-OSPW and P9-OSPW acquired in ESI^-. Same trend but different abundance between the two fractions.

A



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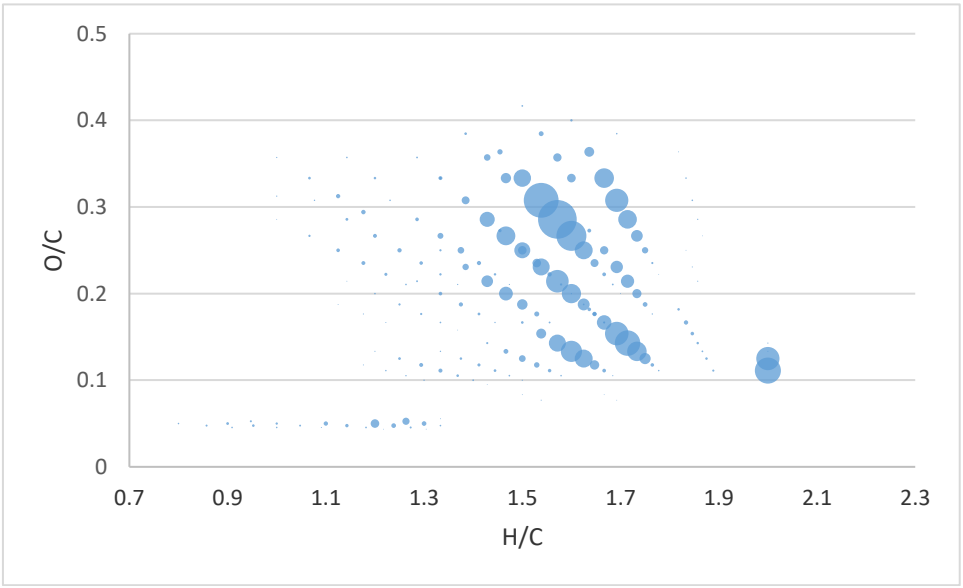
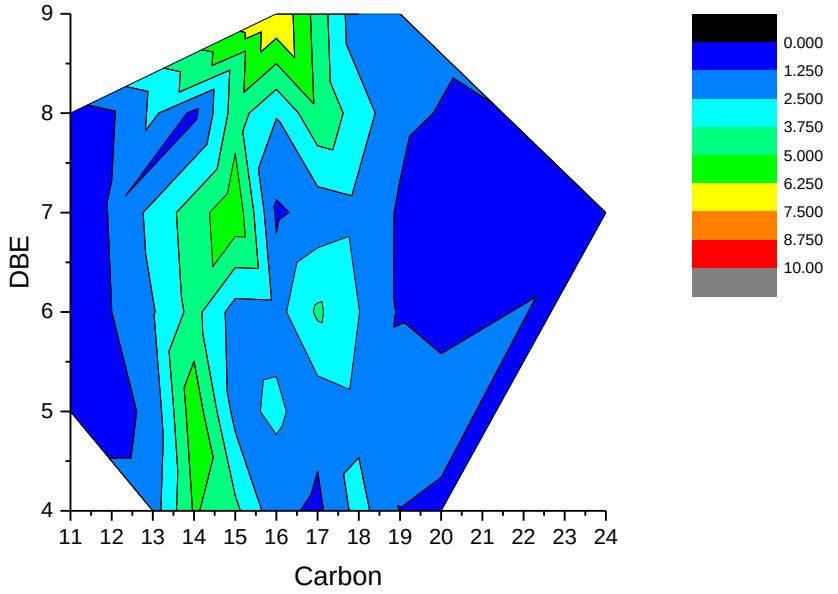


Figure 2.19: Relative abundance of O_2^- compounds acquired in ESI^- as a function of DBE value in (A) BML-OSPW, and (B) P9-OSPW. The difference can only be observed of chemicals with O/C and H/C at range 0.1-0.4 and 1-1.4, respectively which are higher in BML-OSPW compared to P9-OSPW. The size of circles indicates the relative abundance of O_2^- species in each fraction.

A



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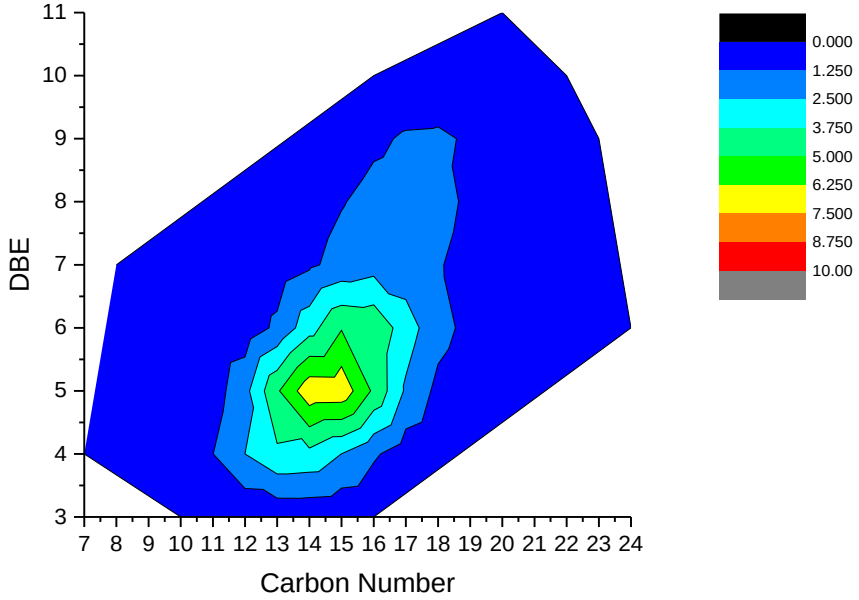


Figure 2.20: Relative abundances of all oxygen O_x^+ in acidic fraction (A) BML-OSPW and (B) P9-OSPW based on DBE and number of carbon acquired in ESI^+

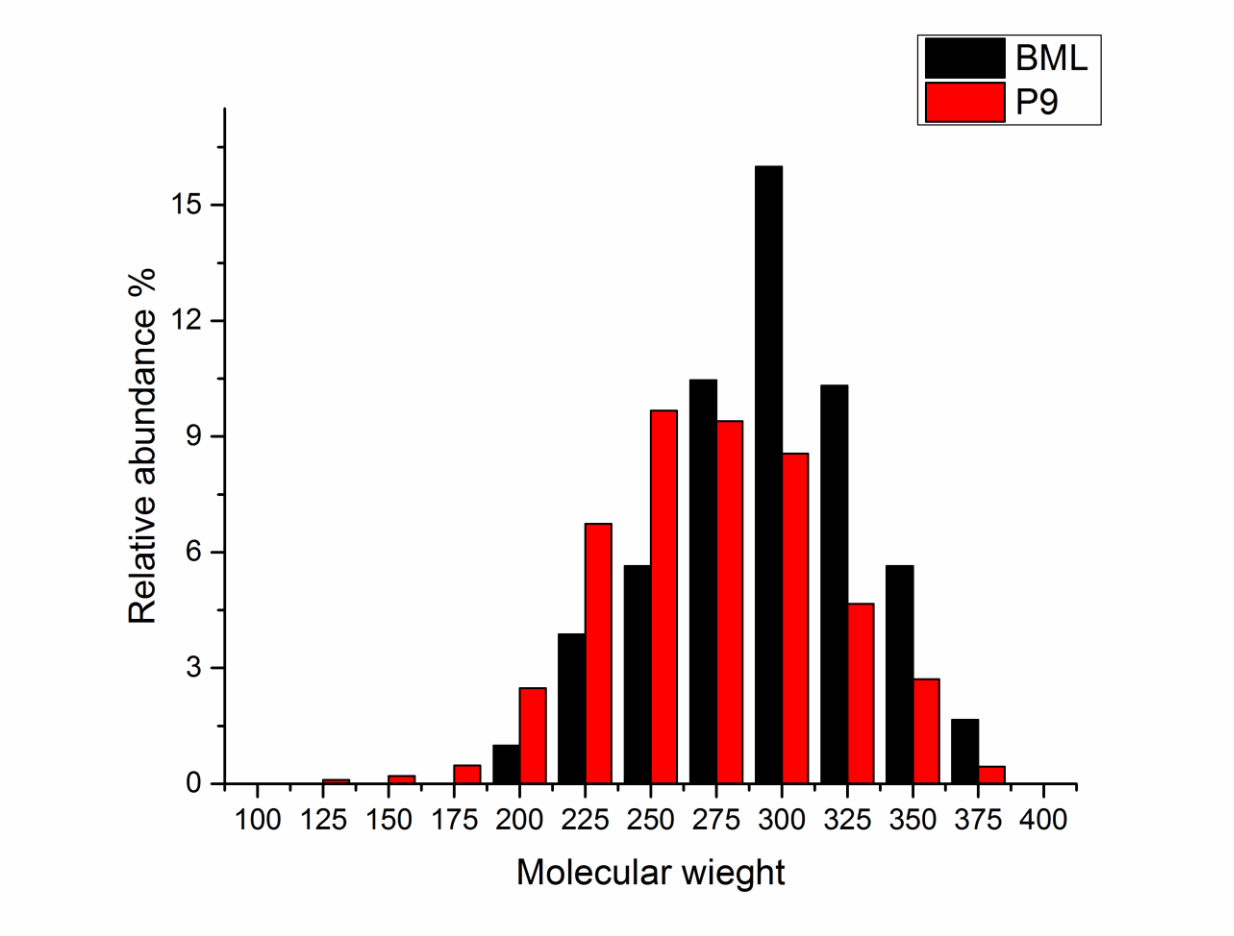


Figure 2.21: Molecular weight distribution vs relative abundances for chemicals in acidic fractions of BML-OSPW and P9-OSPW acquired in ESI^+. Same trend abundance between the two fractions.

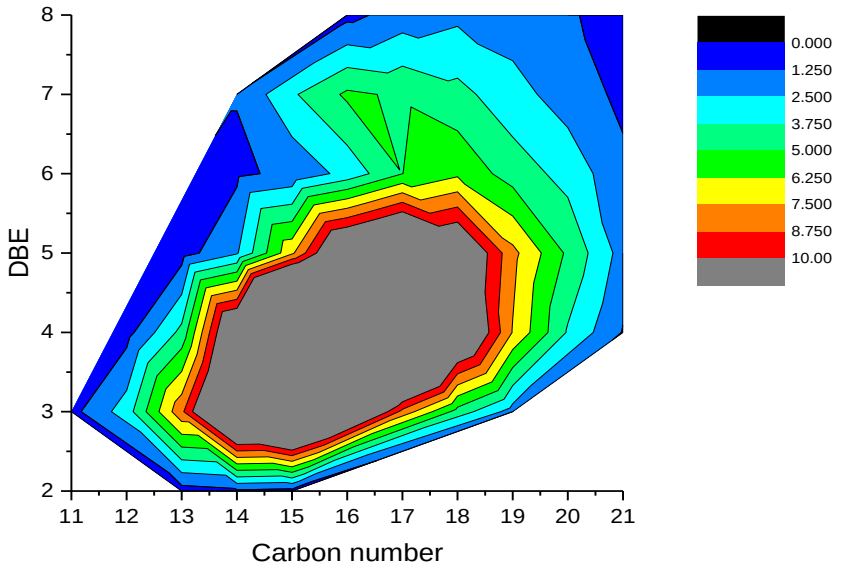
2.4.3 Comparison of chemicals containing sulfur

Chemicals containing sulfur were detected in the acidic fraction of BML-OSPW and P9-OSPW, but the profile of these chemicals was different between the two fractions when analysed by use of ESI^-. Species containing sulfur accounted for approximately 10% of all chemicals in each fraction. Relative abundances of S^-, SO^-, SO_2^-, SO_3^-, and SO_4^- in the acidic fraction of BML-OSPW was 0.7, 1.6, 2.99, 1.13, and 1.82%, respectively (Figure 2.1A), and the relative abundances of S^-, SO^-, and SO_3^- chemicals in the acidic fraction of P9-OSPW was 1.2, 1.50, and 0.18%, respectively (Figure 2.1A). The number of carbon atoms and distribution of DBE of chemicals containing sulfur in the acidic fraction of BML-OSPW was different from chemicals containing sulfur in the acidic fraction of P9-OSPW. Chemicals containing sulfur in the acidic fraction of BML-OSPW had C_{11}-C_{23}, whereas chemicals containing sulfur in P9-OSPW ranged from C_{16}-C_{23}. The majority of chemicals in the acidic fraction of BML-OSPW that contained sulfur had a DBE from 3-13, and chemicals with a DBE from 3-6 or 8-10 were the most abundant. In contrast, the DBE of the majority of chemicals containing sulfur in the acidic fraction of P9-OSPW ranged from 7-11.

Most of the sulfur-containing chemicals in BML-OSPW and P9-OSPW, detected by use of ESI^+, were present in the acidic fraction of each, but relative abundances of each sulfur-containing chemicals much lesser in the acidic fraction of P9-OSPW. In contrast to the variety of chemicals detected by use ESI^-, the only chemicals detected by use of ESI^+ were SO_2^+ and SO_3^+ with trace amount for SO^+ (Figure 2.1B). The abundance of SO_2^+ and SO_3^+ chemicals in the acidic fraction of BML-OSPW was 5% and 40%, respectively whereas in acidic fraction of P9-OSPW the relative abundances was 0.08 and 7%, respectively. In both fractions, the SO_3^+ chemicals of

greatest abundance had C_{13}-C_{19} and DBE 2-6 (Figure 2.22). Among all chemicals identified in the acidic fraction of BML-OSPW and P9-OSPW by use of ESI+, the difference in the relative abundance of SO_3^+ in the acidic fraction of BML-OSPW compared to P9-OSPW was greater than for any of the other chemicals identified in OSPW fractions in either ionization mode. The analysis of the plot of H/C vs. O/C atomic ratio of SO_3^+ in the acidic fraction shows similarity between the BML-OSPW and P9-OSPW with range of 0.15-0.3 and 1.2-2 for H/C and O/C, respectively. This indicates identical degree of oxygenation and of hydrogen saturation for both samples but also the average intensity was approximately 10-fold higher in BML-OSPW compared to P9-OSPW. SO_3^+ compounds had a range of molecular weight from 275-400 for both samples and the average intensity ranged from 3-6-fold higher in BML-OSPW compared to P9-OSPW. The chromatographic separation of SO_3^+ chemicals in the acidic fraction of BML-OSPW did not show any difference in the elution time with Gaussian distribution from 8-14 min with highest peak of intensity at 11-12 min (Inserted panel in Figure 2.11). This elution time profile might indicate similarity in the physico-chemical properties among all chemicals. The presence of chemicals containing sulfur in OSPW has been reported previously (Pereira et al., 2013a; Grewer et al., 2010). The presence of SO_3^+ in the acidic fraction had been extensively characterised and suggested to be sulphur-containing alicyclic and aromatic hydroxy carboxylic acids. The origin of SO_3^+ has been suggested to be via biotransformation (hydroxylation) of the related S-containing carboxylic acids (Rowland et al., 2014), however SO_3^+-containing compounds are unlikely to be of environmentally risks compared to the most abundant naphthenic acids (Morandi et al., 2015). The relative abundances of sulfur-containing species had been suggested as useful indicator for distinguishing OSPW sources (Headley et al., 2011b). Based on these results, SO_3^+ might be a useful indicator species for monitoring the degree of anaerobic/ bio-degradation of OSPW.

A



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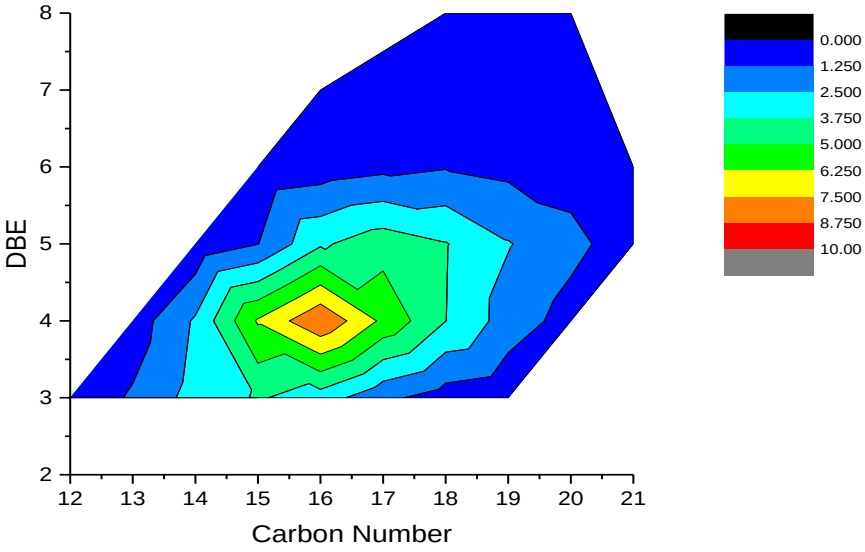

Figure 2.22: Relative abundances of SO_3^+ in acidic fraction (A) BML-OSPW and (B) P9-OSPW based on DBE and number of carbon in $ESI^{+\cdot}$

2.4.4 Comparison of chemicals containing nitrogen

Few chemicals containing nitrogen were detected in the acidic fraction of BML-OSPW and P9-OSPW. Nitrogen-containing chemicals have been detected in OSPW from WIP (Pereira et al., 2013a; Grewer et al., 2010), and were present in the basic and neutral fractions of BML-OSPW and P9-OSPW (Alharbi et al., 2016a). Nitrogen containing chemicals were not detected in either fraction by use of ESI^-. This suggests that the nitrogen-containing acidic chemicals at very low abundant in OSPW samples. When analysed by use of ESI^+ the only chemicals containing nitrogen that were detected were NO^+ and NO_3^+. The relative abundance of NO_3^+ chemicals was 3.45% in BML-OSPW and 0.6% in P9-OSPW. The acidic fraction of P9-OSPW had a low abundant of NO^+ chemicals 0.04% but these were not detected in the acidic fraction of BML-OSPW. Based on these findings it is unlikely that the nitrogen-containing chemicals of the acidic fraction of BML-OSPW is a remarkable species of acidic fraction. However, the relative abundances of nitrogen-containing species (i.e. NO_n, and N_2O_n) within OSPW has been shown to be a potential species for distinguishing natural from industrial sources of petrogenic derived compounds from oil sands (Headley et al., 2011b).

2.5 Conclusion

Aging of OSPW in end-pit lakes is one potential method to reduce the environmentally risks of dissolved organic compounds in OSPW that is generated during the extraction of bitumen from oil sands, but the effectiveness of this strategy is not known. Identification of chemicals in fresh and aged OSPW would allow for the development of methods to monitor the detoxification of OSPW in end-pit lakes by monitoring concentrations of specific chemicals. The acidic fraction

of BML-OSPW had greater abundance of O_2^-, O_3^-, O_4^-, and SO_3^+ chemicals than the acidic fraction of P9-OSPW, however, SO_3^+ abundant reduced significantly in P9-OSPW compared to naphthenic acids. Although the role of NAs (O_2^-) in the acute toxicity of OSPW confirmed in several studies, the concentrations of low molecular weight NAs (O_2^-) that causing acute toxicity must be considered. The results suggest that monitoring of other species than NAs such as SO_3^+ might be more useful for identification of the source and age of oil sands related aqueous samples.

3 CHPTER 3: INHIBITION OF ABC TRANSPORT PROTEINS BY OIL SANDS PROCESS AFFECTED WATER

Preface:

This chapter has been published in Aquatic Toxicology (2016) Volume 170, Pages 81-88, under joint authorship with David M.V. Saunders, Jane Alcorn, Ahmed Al-Mousa (University of Saskatchewan), Alberto S. Pereira, and Jonathan W. Martin (University of Alberta), John P. Giesy, and Steve B. Wiseman (University of Saskatchewan). The tables, figures and references cited in this article have been re-formatted here to the thesis style. References cited in this chapter are listed in the reference section of this thesis.

Author Contributions:

Hattan A. Alharbi (University of Saskatchewan) developed, designed, managed and did the experiment, generated and analyzed the data, wrote, and drafted the manuscript.

David Saunders (University of Saskatchewan) provided laboratory assistance with the multi drug resistance assay systems.

Ahmed Al-Mousa (University of Saskatchewan) provided laboratory assistance with the multi drug resistance assay systems.

Jane Alcorn (University of Saskatchewan) provided scientific input on multi drug resistance assay systems data, guidance, commented on and edited the manuscript.

Alberto Pereira (University of Alberta) analysed the samples on the mass spectrometry.

John Martin (University of Alberta) provided scientific input on chemical analysis data, guidance, commented on and edited the manuscript.

Drs. Steve Wiseman and John P. Giesy (all at University of Saskatchewan) provided inspiration, scientific input, and guidance, commented on and edited the manuscript, and provided funding for the research.

3.1 ABSTRACT

The ATP-binding cassette (ABC) superfamily of transporter proteins is important for detoxification of xenobiotic. For example, ABC transporters from the multidrug-resistance protein (MRP) subfamily are important for the excretion of polycyclic aromatic hydrocarbons (PAHs) and their metabolites. Effects of chemicals in the water soluble organic fraction of relatively fresh oil sands process affected water (OSPW) from Base Mine Lake (BML-OSPW) and aged OSPW from Pond 9 (P9-OSPW) on the activity of MRP transporters were investigated *in vivo* by use of Japanese medaka at the fry stage of development. Activities of MRPs were monitored by use of the lipophilic dye calcein, which is transported from cells by ABC proteins, including MRPs. To begin to identify chemicals that might inhibit activity of MRPs, BML-OSPW and P9-OSPW were fractionated into acidic, basic, and neutral fractions by use of mixed-mode sorbents. Chemical compositions of fractions were determined by use of ultrahigh resolution orbitrap mass spectrometry in ESI^+ and ESI^- mode. Greater amounts of calcein were retained in fry exposed to BML-OSPW at concentration equivalents greater than 1× (i.e. full strength). The neutral and basic fractions of BML-OSPW, but not the acidic fraction, caused greater retention of calcein. Exposure to P9-OSPW did not affect the amount of calcein in fry. Neutral and basic fractions of BML-OSPW contained relatively greater amounts of several oxygen-, sulfur, and nitrogen-containing chemical species that might inhibit MRPs, such as O^+, SO^+, and NO^+ chemical species, although secondary fractionation will be required to conclusively identify the most potent inhibitors. Naphthenic acids (O_2^-), which were dominant in the acidic fraction, did not appear to be the cause of the inhibition. This is the first study to demonstrate that chemicals in the water soluble organic fraction of OSPW inhibit activity of this important class of proteins. However, aging of OSPW

attenuates this effect and inhibition of the activity of MRPs by OSPW from Base Mine Lake does not occur at environmentally relevant concentrations.

3.2 Introduction

In the surface mining oil sands industry, the extraction of bitumen from oil sands generates oil sands process affected water (OSPW) that is retained on-site in tailings ponds and settling basins that, as of 2009, covered an area of approximately 170 km^2 (Government of Alberta, 2011). Because oil sands mining companies do not discharge OSPW to the wider environment, the volume of OSPW stored in tailings ponds will increase as surface mining operations expand. Therefore, methods to remediate OSPW are needed. One strategy companies are exploring for remediation and reclamation of OSPW is use of end pit lakes (EPLs) constructed by filling mined-out pits with products of the extraction of bitumen, including OSPW (Gosselin et al., 2010). The expectation is that toxicity of OSPW in EPLs will decrease because of biodegradation of chemicals in the water soluble organic fraction of OSPW (Del Rio et al., 2006; Han et al., 2008, 2009), and that EPLs will eventually be capable of sustaining life.

The chemistry of tailings ponds is complex. Liquid tailings are a mixture of water, residual bitumen, sand, silt, and inorganic and organic compounds. Over time particulates (silt and clay fractions) settle to form a layer of mature fine tailings (MFTs), leaving behind an aqueous layer of OSPW. Two constituents of tailings ponds that have the potential to cause toxicity to aquatic organisms are polycyclic aromatic hydrocarbons (PAHs) and acid-extractable organic compounds, including naphthenic acids (NAs; $C_nH_{2n+z}O_2$), in the water soluble organic fraction of OSPW. Concentrations of individual lower molecular mass PAHs range from 10 – 330 ng/L in pore water of MFTs (Madill et al., 1999) and total concentrations of PAHs range from 1150 - 1600 ng/L in

the upper clarified zone of OSPW (Rogers et al., 2002; Galarneau et al., 2014). The water-soluble organic fraction of OSPW has been described as a "super-complex mixture" (Jones et al., 2011). Much of the characterization of the water-soluble organic fraction has focused on NAs, but advances in ultra-high-resolution mass spectrometry have identified a variety of oxygen-, sulphur- and nitrogen-containing compounds in this mixture (Barrow et al., 2010, Pereira et al., 2013a, b; Morandi et al., 2015).

Several mechanisms by which OSPW could cause toxicity have been identified. OSPW that is fresh causes acute lethality, and it has been proposed that the mechanism of this effect is narcosis (Frank et al., 2009; Scarlett et al., 2013; Morandi et al., 2015). Also, OSPW causes a variety of sub-lethal effects, including endocrine disruption (Lister et al., 2008; He et al., 2010, 2011, 2012a; Van den Heuvel et al., 2012; Kavanagh et al., 2011, 2012, 2013; Leclair et al., 2015), oxidative stress (He et al., 2012b; Wiseman et al., 2013a, b), and alterations to immune function (Garcia-Garcia et al., 2011; McNeill et al., 2012; MacDonald et al., 2013; Hagen et al., 2014). In many of these studies effects were caused by the water-soluble organic fraction of OSPW.

A variety of natural and synthetic chemicals can inhibit members of the ATP (energy-dependent efflux pumps)-binding cassette (ABC) superfamily of transporter proteins. These inhibitors might not be toxic themselves, but might cause toxicity of other chemicals by inhibition of these transporter proteins, a process known as chemosensitization (Smital and Kurelec, 1997; Kurelec et al., 2000; Ferreira et al., 2012; Kurth et al., 2015). ABC proteins are important for detoxification of xenobiotic because they actively transport a variety of structurally diverse chemicals, and their metabolites, from cells thereby protecting organisms from adverse effects (Leslie et al., 2005; Klaassen and Lauren, 2010; Hessel et al., 2013). In teleost fishes, PAHs and their metabolites are transported from cells by multidrug resistance-associated proteins (MRP) 1–

6 (ABCC1–6) (Bard, 2000; Ferreira et al., 2012; Luckenbach et al., 2014). Although it is not known if constituents of OSPW inhibit ABC proteins, water-soluble fractions of crude oil inhibit ABC transporters in larvae of the marine invertebrate, the fat innkeeper (*Urechis caupo)* (Hamdoun et al., 2002). If constituents of OSPW inhibit activity of MRPs it could exacerbate accumulation and effects of PAHs or their bio-activated metabolites on aquatic organisms. Therefore, the objective of this study was to determine if the water soluble organic fraction of OSPW affects the activity of MRPs by use of a model species of teleost fish, the Japanese medaka (*Oryzias latipes*). Also, semi-quantification of chemicals in fractions by use of ultrahigh resolution orbitrap mass spectrometry, were performed to identify classes of chemicals in OSPW that might cause effects on activity of MRPs.

3.3 Materials and methods

3.3.1 Chemical, reagents, and OSPW

MK-571, an inhibitor of MRPs (Fischer et al., 2013; Zaja et al., 2007), was purchased from Cayman Chemical Company (Anne Arbor, MI, USA) and calcein-AM was from AAT Bioquest (Sunnyvale, CA, USA). Dimethyl sulfoxide (DMSO) and trypan blue were from the Sigma Chemical Company (St. Louis, MO, USA). All solvents used were of analytical grade. Two samples of OSPW were collected on the site of Syncrude Canada, Ltd. (Fort McMurray, AB, Canada). One sample was from Base Mine Lake (BML-OSPW), which is an end-pit-lake constructed from the West-In-Pit settling basin that received input of tailings from the main extraction facility. The other sample was from an experimental reclamation pond called Pond 9 (P9-OSPW) that was constructed in 1993 and has not received input of OSPW since that time.

Both samples were collected in September of 2012, shipped to the University of Saskatchewan (Saskatoon, SK, Canada), and used for fractionation immediately upon arrival.

3.3.2 Fractionation of OSPW

Both samples of OSPW were fractionated into acidic, basic, and neutral fractions of polar organic compounds by use of mixed-mode sorbents (MMS). Prior to fractionation 500 ml of each sample of OSPW was passed through a glass microfiber filter (GF/D 0.47 mm, Whatman) to remove any particulate matter, then acidified to pH 2 by use of concentrated HCl (37%). Next, for isolation of basic fractions, pre-concentration was performed in one step by use of 500 mg of mixed-mode Strata®-X Polymeric-C solid-phase sorbent in plastic cartridges (Phenomenex, Milford, MA, USA). This matrix is a porous copolymer with a weak mixed-mode cation that provides dual modes for the retention and adsorption of lipophilic and hydrophilic compounds was well as ionic compounds. Before addition of OSPW cartridges were conditioned with 6 ml of methanol and 6 ml of acidified water. The 500 mL of filtered and acidified OSPW was passed through the cartridges under vacuum. Next, cartridges were washed with 2% (v/v) of formic acid and were allowed to dry under vacuum for 30 min. The first elution was performed with 100% of methanol and this extract contained acidic and neutral compounds. The second elution was performed with 5% (v/v) of NH_4OH in methanol and this fraction contained basic compounds. To separate acidic and neutral compounds a pre-concentration of samples was performed by use of Strata®-X-A 500 mg solid-phase matrix in plastic cartridges (Phenomenex). This polymeric sorbent is water wetable and provides dual modes of retention – anion exchange and reversed phase. Prior to use the cartridge was conditioned by washing with 100% methanol followed by 5% (v/v) of NH_4OH (aq). Next, elutant I from the Strata®-X Polymeric-C sorbent was evaporated to approximately 0.5 mL, adjusted to a pH of 10-11 with NaOH, and then passed through the

cartridge without vacuum. Cartridges were washed with 5% (v/v) of NH_4OH (aq) and left to dry under vacuum for 30 min. Finally, the fraction containing neutral compounds was eluted with 100% of methanol and a fraction containing acidic compounds was eluted with 2% (v/v) of formic acid in methanol. Fractions were dried, and reconstituted in ethanol for bioassays. A pooled sample representative of the organics fraction was generated by pooling equal volumes of the acidic, neutral, and basic fractions. Blank samples, which were city of Saskatoon municipal tap water, were extracted by use of this method.

3.3.3 Profiling of fractions

The profile of chemicals in fractions of BML-OSPW and P9-OSPW was analysed by use of reversed-phase liquid chromatography paired with a linear ion trap-orbitrap mass spectrometer (Orbitrap Elite, Thermo Fisher Scientific, San Jose, CA, USA) in both negative (ESI^-) or positive (ESI^+) electrospray according to the method described by Pereira et al. (2013a, b). Chemical species detected in each fraction were grouped according to heteroatom empirical formula classes in ESI^- or ESI^+ electrospray: $O_x^{+/-}$ (where x = 1-5), $N^{+/-}$, $NO_x^{+/-}$ (where x = 1-4), $S^{+/-}$, $SO_x^{+/-}$ (where x = 1-5), or $NO_xS^{+/-}$ (where x = 2). Briefly, the HPLC instrument was an Accela System (Thermo Fisher Scientific, San Jose, CA), consisting of a degasser, a 600 bar quaternary pump, an autosampler, and a column oven. Chromatographic separation was performed on a Cosmosil C_{18} MS-II column (100 × 3.0 mm, 2.5 μm particle size) (Nacalai USA, San Diego, CA) at 40 °C. The flow rate was 0.5 mL/min and the injection volume was 3 μL. The mobile phases were 0.1% (v/v) acetic acid in water (Solvent A) and 100% methanol (Solvent B). 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 at 14 min, and returning to 5% B in 1 min, followed by a 4 min hold prior to the next injection. In this study,

mass values were restricted to singly charged ions with a molecular mass of 100-500 Da and signal to noise ratios greater than 3. Elemental compositions (i.e., C*c*H*h*N*n*O*o*S*s*) were calculated with the elemental composition tool within the Xcalibur software (Thermo Scientific, Bremen, Germany), and by use of the following restrictions: 0-40 ^{12}C, 0-2 ^{13}C, 0-100 ^{1}H, 0-2 ^{14}N, 0-8 ^{16}O, 0-2 ^{34}S, and 1 ^{32}S. Molecular formulas were also confirmed by the presence of ^{13}C isotopes. Determination of elemental composition was based on the accurate mass *m*/*z* by matching the theoretical mass with the observed mass of each ion to within 5 ppm (typically <2 ppm). Data was qualitatively analysed based on the distribution of heteroatom classes, type, and number of carbon atoms. Only those chemicals in the total ion mass spectrum that had a peak threshold > 600, a mass spectral signal-to-noise ratio (S/N) > 3, were present at relative abundances of at least 2%, and that produced discernible extracted ion chromatographic peaks (i.e., S/N > 3) were reported.

3.3.4 Acute lethality

Effects of OSPW on survival of fry of Japanese medaka were quantified to determine sub-lethal concentrations that could be used to determine effects on activity of MRPs. Japanese medaka (*Oryzias latipes*) were cultured in the Aquatic Toxicology Research Facility at the University of Saskatchewan. Eggs were collected daily from the culture system and maintained in embryo rearing medium (ERM) (1 g/L NaCl, 0.030 g/L KCl, 0.040 g/L $CaC_{l2}\cdot H_2O$, 80 mg/L MgSO4, and 1 mg/L Methylene Blue in distilled water) until hatch. All culturing of adult fish and rearing of embryos was conducted at a water temperature of 28 ˚C with a photoperiod of 16 h:8 h (light: dark). Acute lethality caused by BML-OSPW and P9-OSPW were determined first at an enrichment equivalent to 5× the concentration in OSPW of pooled sample. Next, if toxicity was observed, dose-response relationships were established by determining toxicity at 0.5, 1, 2.5, and

5× equivalents. Lethality was determined after 6 h and 24 h of exposure. The concentration of ethanol in exposure solutions did not exceed 0.1% (v/v). Lethality was determined with 3 batches of fry, and each time exposures were conducted in triplicate with 10 fry per exposure. Experiments with fish was approved by the University of Saskatchewan's Animal Research Ethics Board, and adhered to the Canadian Council on Animal Care Guide to the Care and Use of Experimental Animals (Protocol #20090108).

3.3.5 Effect of OSPW on accumulation of calcein by Japanese medaka

Japanese medaka at the fry stage of development (developmental stage 40, which extends from hatching until appearance of fin rays in the caudal and pectoral fins) were used for the assay. Accumulation of calcein in fry was quantified by modification of a method described by Fischer et al. (2013). Briefly, a single fry was transferred to each well of a 24 well plate with a clear bottom (VisiPlate-24 Black, Perkin Elmer, Woodbridge, ON, Canada) and was exposed to either 5 µM of MK-571 (positive control), 5× equivalent of the pooled fraction of BML-OSPW or P9-OSPW, 5× equivalents of fractions of BML-OSPW, or 0.5, 1, 2.5 and 5× equivalents of the pooled organic fraction of whichever OSPW might have caused inhibition of activity of ABC transporters. Fry were exposed to DMSO or ethanol at 0.1% (v/v) to control for effects of solvents. After addition of chemicals, plates were incubated at 28°C for 15 min, after which calcein-AM was added to a final concentration of 1 µM. Next, the plate was incubated for 60 min at 28°C and then placed on ice to slow activity of esterase enzymes, and each well was washed three times with dechlorinated water warmed to 28 °C. Accumulation of calcein was determined by quantifying fluorescence inside fry by use of a Zeiss Axio ObserverZ1 inverted microscope (Zeiss, Toronto, Ontario, Canada) with an AxioCam ICc1 camera (excitation 470, emission 525 nm at 5×

magnification). All fish were imaged from a dorsal viewpoint. Images were captured by use of AxioVision software V.4.8 (Zeiss, Toronto, Ontario, Canada) and analyzed by use of ImageJ software V.1.48 (NIH, Bathesda, Maryland, USA). Corrected total fluorescence (CTF), a measure which was corrected for size of the sample, was used as the measure of accumulation of calcein (Formula 1). CTF was a function of integrated density (an extensive quantity and product of area and mean gray value), area of sample (the size of the selected fish), and mean fluorescence of background (calculated by determining the mean of intensities of fluorescence of the background of the image). Two independent experiments were conducted and each exposure was replicated 4 times per experiment.

CTF = Integrated Density – (Area of Selection × Mean Grey Value) (Formula 1)

3.3.6 Analysis of data

Effects of OSPW on survival of embryos and accumulation of calcein in fry was determined by use GraphPad Prism 5 software (San Diego, CA, USA). Normality of data was assessed by use of the Kolmogorov Smirnov one-sample test and homogeneity of variance was determined by use of Levene's test. If necessary, data were $\log_{10}$ transformed to ensure normality and homogeneity of variance. Effects of treatments relative to controls were evaluated by use of one-way ANOVA followed by Dunnett's post-hoc test. Differences were considered significant at a ρ-value < 0.05. Effects of samples of OSPW on survival of fry were compared to effects of the solvent. Effects of samples of OSPW on accumulation of calcein were compared to effects on fry exposed to calcein-AM without inhibitors. Fluorescence of fry exposed to calcein-AM dissolved in DMSO was not significantly different from fluorescence of fry exposed only to DMSO.

3.4 Results

3.4.1 Acute lethality

Exposure to organic chemicals extracted from the water soluble organic phase of BML-OSPW affected survival of fry of Japanese medaka. No effects on survival were observed at 6 h of exposure to the pooled, acidic, neutral, or basic fraction of BML-OSPW (data not shown). Survival of fry exposed for 24 h to a 5× equivalent of the pooled fraction of BML-OSPW was significantly lesser (17.5 ± 8.5%) compared to the solvent control (96.55 ± 1.99%) (Figure. 3.1A). Neither 0.5, 1, nor 2.5× equivalent of the pooled organic fraction of BML-OSPW affected survival of fry. Survival of fry exposed to 2.5 or 5× equivalent of the acidic fraction of BML-OSPW was 71.44 ± 4.5% and 10 ± 4.2%, respectively (Figure. 3.1B). Neither the neutral nor basic fraction of BML-OSPW affected survival of fry at either of the equivalent concentrations tested. Survival of fry exposed to a 5× equivalent of the pooled organic fraction of P9-OSPW was not different from the solvent control (Figure. 3.1A) at the equivalent concentrations tested.

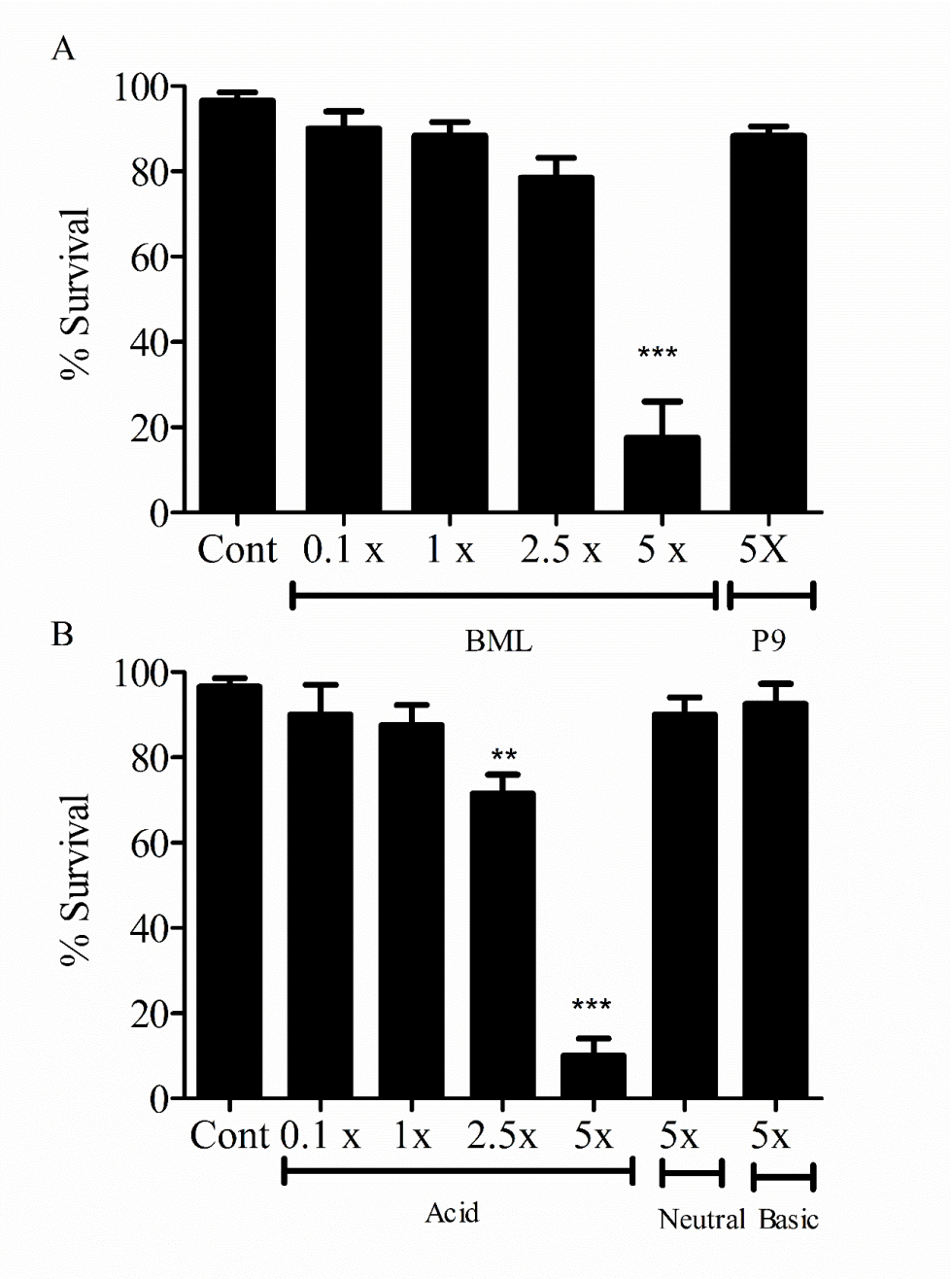

Figure 3.1. Survival of fry of Japanese medaka exposed to A) 0.1, 1, 2.5, or 5× equivalent of the organic fraction of BML-OSPW or a 5× concentration of the organic fraction of P9-OSPW, and B) 0.1, 1, 2.5, or 5× equivalent of the acidic fraction of BML-OSPW or a 5× concentration of the neutral fraction or basic fraction of BML-OSPW. Survival of fry is expressed as mean ± standard deviation of 3 independent studies in which there were three replicate exposures with 10 fry per replicate. Significant differences in survival compared to control were determined by use of one-way ANOVA followed by Dunnett's post-hoc test and are designated by an asterisk. (* = $P \leq 0.05$, ** = $P \leq 0.01$, and *** = $P \leq 0.001$).

3.4.2 Effects of OSPW on accumulation of calcein in Fry

Exposure to organic compounds from the water soluble phase of BML-OSPW inhibited efflux of calcein from fry. The amount of calcein in fry exposed to MK-571 was 3.88 ± 0.34-fold greater compared to fry exposed to the solvent control (Figure. 3.2A). Amounts of calcein in fry exposed to 2.5 or 5× equivalent of the pooled fraction of BML-OSPW were 7.6 ± 1.6- and 9.7 ± 2.0-fold greater, respectively, compared to fry exposed to the solvent control (Figure. 3.2B), but amounts of calcein in fry exposed to 0.5 or 1× equivalent of the pooled fraction of BML-OSPW were not different from the solvent control. The amount of calcein was not greater in fry exposed to a 5× equivalent of the pooled fraction of P9-OSPW (Figure. 3.2B). Amounts of calcein in fry exposed to 5× equivalent of the neutral or basic fraction of BML-OSPW were 8.2 ± 4.4- and 7.5 ± 0.25-fold greater, respectively, compared to solvent controls (Figure. 3.2C). The amount of calcein in fry exposed to the acidic fraction of BML-OSPW was not different from the solvent control (Figure. 3.2C).

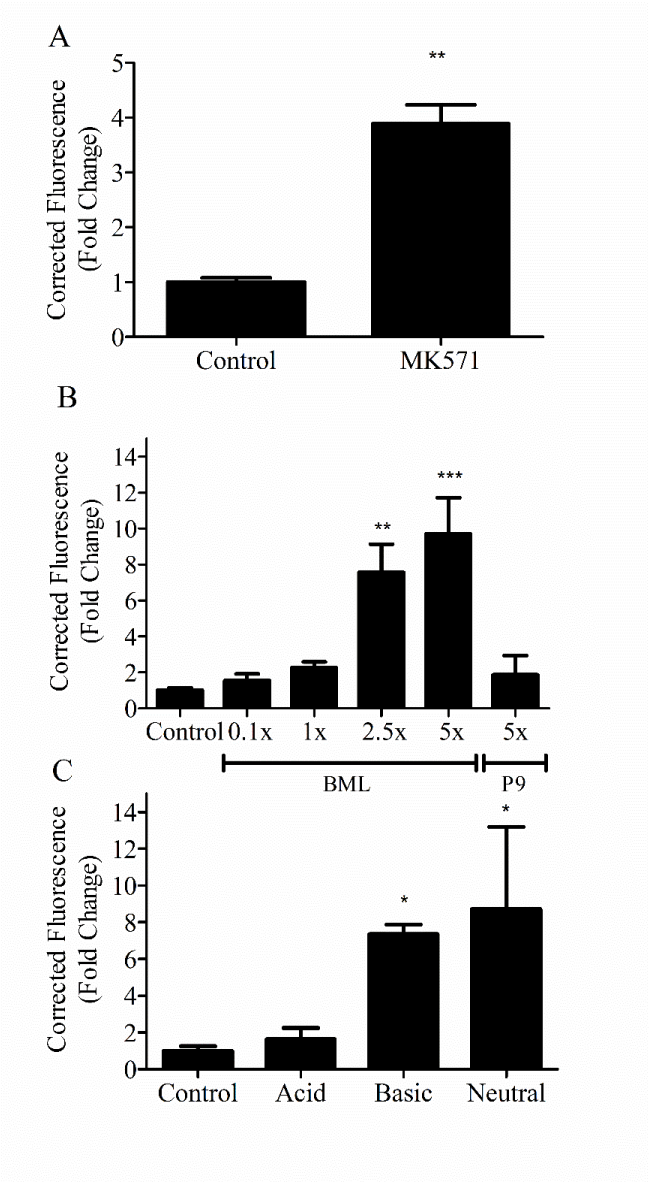

Figure 3.2. Accumulation of calcein in fry of Japanese medaka exposed to (A) MK-571 (B) 5×, 2.5×, 1×, and 0.5× equivalents of the pooled organic fraction of BML-OSPW and 5× equivalent of the pooled organic fraction of P9-OSPW, and (C) 5× equivalents of acidic, basic and neutral fractions of BML-OSPW. Fry were exposed to (5µM) MK-571 and OSPW for 15 min prior to co-exposure to calcein-AM for 1h. Fry used as controls were exposed to embryo rearing medium containing 0.1% v/v DMSO. Accumulation of calcein was measured as fluorescence and data is expressed as mean ± standard deviation of 2 independent experiments in which the number of replicates per experiment was four. Differences that are statistically significant from the control are indicated by an asterisk. (* = $p \leq 0.05$, ** = $p \leq 0.01$, and *** = $p \leq 0.001$).

3.4.3 Comparison of chemicals in fractions of BML-OSPW

As a qualitative first step to identify chemicals in BML-OSPW that might have inhibited activity of MRPs, the profile and relative intensities of chemicals in neutral and basic fractions, which inhibited efflux of calcein from fry, were compared to relative intensities of chemicals in the acid fraction, which did not inhibit efflux of calcein. In ESI^- mode, which detects organic acids, chemical species containing oxygen and chemical species containing sulfur were more abundant in the acidic fraction than in the neutral or basic fraction, while chemical species containing nitrogen were not detected in either fraction (Figure. 3.3A). Intensity of NAs, which are O_2^- chemical species detected in the acidic fraction by use of ESI^- mode, was 3.0- and 8.9-fold greater in the acidic fraction compared to the neutral and basic fraction, respectively. Also, intensity of O_3^- and O_4^- chemical species was much greater in the acidic fraction than in the neutral or basic fraction, and O_5^- chemical species were detected only in the acidic fraction.

Polar organic neutral and basic compounds were detected by use of ESI^+ mode (Figure. 3.3B). Chemical species containing oxygen, sulfur, or nitrogen were detected in each fraction, but there were differences in intensities among fractions. Intensities of chemical species containing oxygen was greater in the acidic fraction and neutral fraction compared to the basic fraction. The most abundant chemical species in the neutral fraction was O_2^+, and intensity was 1.4- and 51-fold greater than in the acidic and basic fractions, respectively. These chemical species are not NAs, but might be dihydroxy, diketo, or ketohydroxy compounds (Pereira et al., 2013a). The greatest intensity of any chemical species in either fraction was of SO_3^+ in the acidic fraction. S^+ and SO^+ chemical species were detected in the neutral and basic fractions but were not detected in the acidic fraction. The greatest number of chemical species containing nitrogen were detected in the basic fraction. Specifically, NO^+, NO_2^+, NO_3^+, NO_4^+, and NO_2S^+ were detected in the basic fraction.

The NO^+ chemical species was detected in neutral and basic fractions, and intensity in the neutral fraction was approximately 9-fold greater in the basic fraction. The only chemical species containing nitrogen in the acidic fraction was NO_3^+, and intensity was greater than in either the neutral or basic fraction.

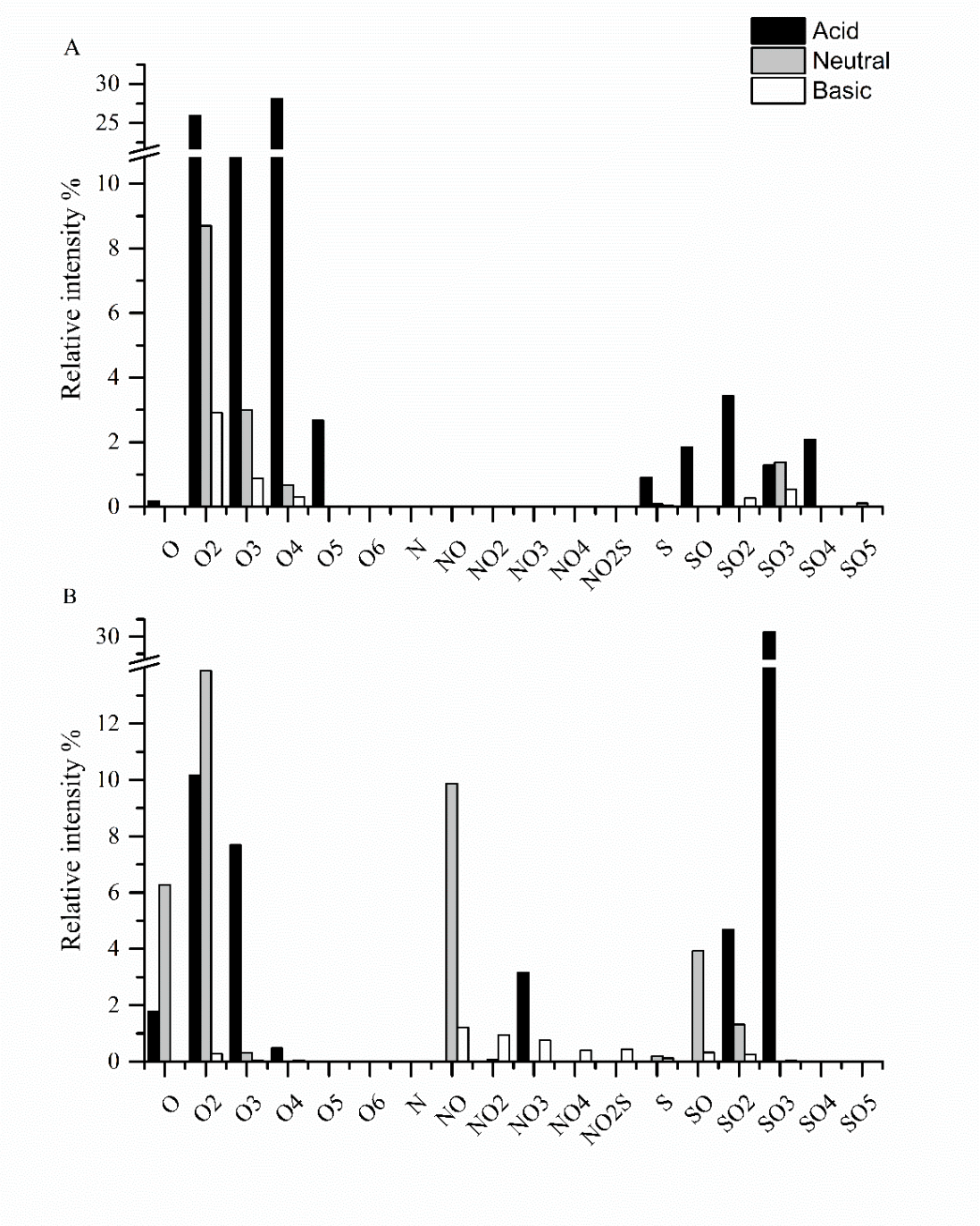

Figure 3.3. Elemental composition and relative intensity of water-soluble chemicals in acidic, basic and neutral fractions of BML-OSPW acquired in A) ESI^- and B) ESI^+.

3.4.4 Comparison of chemicals in fractions of BML-OSPW and P9-OSPW

Whereas the amount of calcein in fry exposed to either the basic or neutral fraction of BML-OSPW was significantly greater than in controls, neither the basic nor neutral fraction of P9-OSPW caused this effect. Therefore, the profile and intensities of chemicals in these basic and neutral fractions from BML-OSPW were compared to the basic and neutral fractions of P9-OSPW to qualitatively identify chemicals that might inhibit MRPs. Chemicals in the acidic fraction of BML-OSPW and P9-OSPW were not compared because calcein dye did not accumulate in fry exposed to the acidic fraction of BML-OSPW. The comparison was limited to chemical species detected by use of ESI^+ because the majority of chemical species detected by use of ESI^- were in the acidic fraction.

There were differences in intensity of chemical species containing oxygen between the neutral and basic fraction of BML-OSPW and P9-OSPW. The greatest difference was that intensity of O^+ species was approximately 8-fold greater in the neutral fraction of BML-OSPW compared to the neutral fraction of P9-OSPW. This species was detected in the basic fraction of P9-OSPW but not BML-OSPW. Intensity of O_2^+ species was greater in both fractions of BML-OSPW, but the difference was less than 2-fold. In contrast, intensity of O_3^+ was approximately 5-fold greater in the neutral and basic fraction of P9-OSPW compared to BML-OSPW.

Intensities of chemical species containing sulfur were greater in neutral and basic fractions of BML-OSPW compared to P9-OSPW (Figure. 3.4). The most abundant chemical species containing sulfur in either fraction were SO^+ and SO_2^+. Intensities of these species in the neutral fraction of BML-OSPW were 7.43- and 1.98-fold greater, respectively, than in the neutral fraction of P9-OSPW. Neither chemical species containing sulfur that was detected in the basic fraction of BML-OSPW was detected in the basic fraction of P9-OSPW (Figure. 3.4A).

The most pronounced difference between the neutral and basic fractions of BML-OSPW and P9-OSPW was the difference in the number and intensities of species containing nitrogen (Figure. 3.4). For both samples of OSPW, a greater number of chemical species containing nitrogen were detected in the basic fraction compared to the neutral fraction. Most chemical species containing nitrogen that were detected in the basic fraction of BML-OSPW were detected in the basic fraction of P9-OSPW, but the intensity was much greater in BML-OSPW. The most abundant chemical species containing nitrogen in the basic fraction of BML-OSPW was NO^+, and the intensity was 5-fold greater than in P9-OSPW. The exception was that intensity of NO_2^+ was greater in the neutral fraction of P9-OSPW than BML-OSPW, and NO_3^+ was detected in the neutral fraction of P9-OSPW but not BML-OSPW. NO_2S^+ was detected only in the basic fraction of BML-OSPW (Figure. 3.4B).

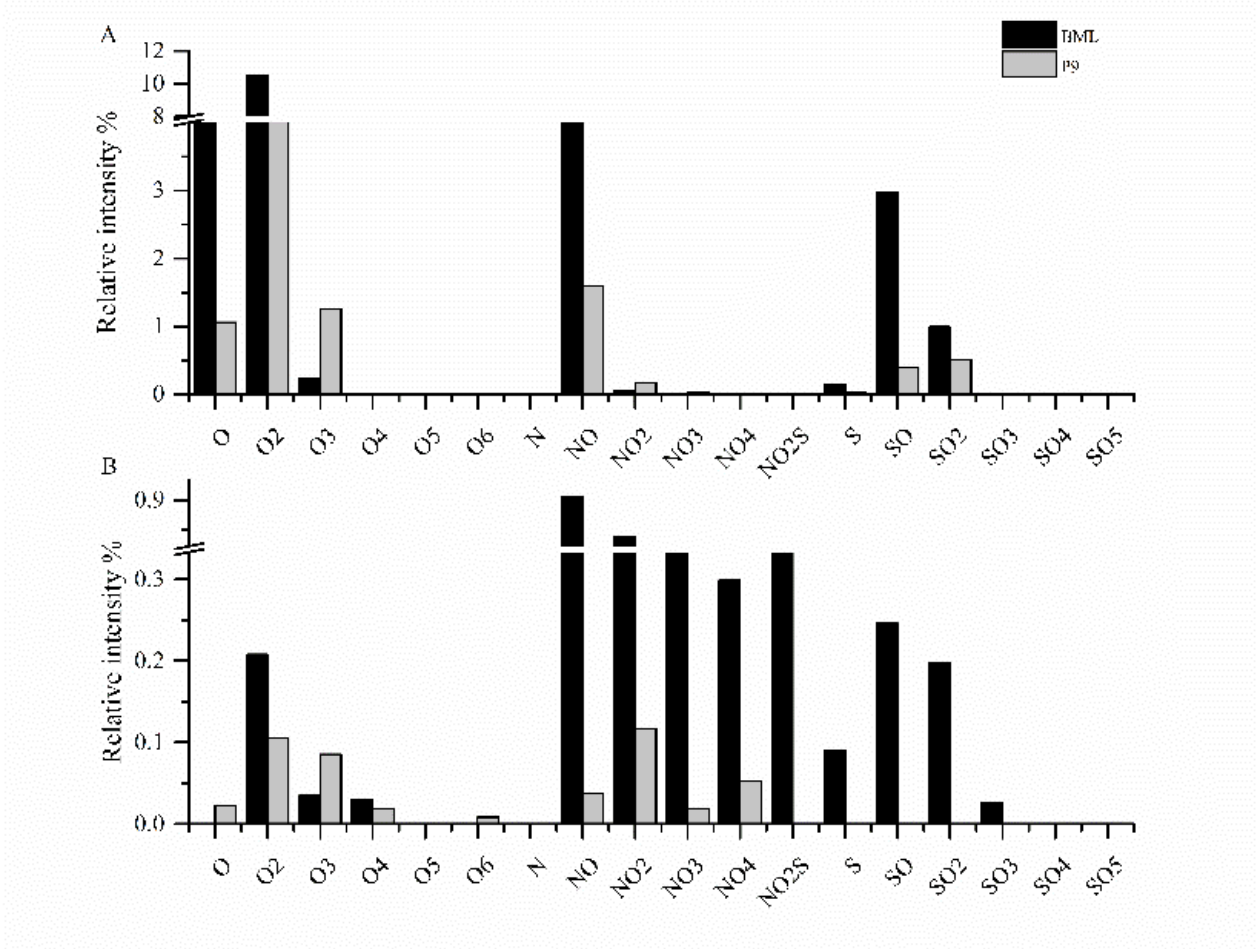

Figure 3.4. Comparison of the elemental composition and relative intensity of species containing sulfur or nitrogen in (A) Basic and (B) Neutral fractions of BML-OSPW and P9-OSPW acquired in ESI^+ mode.

3.5 Discussion

Results of this study suggest that activity of MRPs, which are members of the ATP-binding cassette (ABC) superfamily of membrane-transporter proteins, might be inhibited by organic compounds dissolved in OSPW. Previous studies have shown that OSPW affects expression of genes encoding members of the ABC superfamily of transporters (Wiseman et al., 2013b; Gagné et al., 2012). However, this is the first study to demonstrate that activity of a specific family of ABC transporters, the MRPs, is inhibited by organic compounds dissolved in OSPW.

Inhibition of MRPs by constituents of the water soluble organic fraction of OSPW could lead to accumulation of chemicals, and their metabolites, which could cause adverse effects on aquatic organisms. Numerous studies have shown that inhibition of the activity of ABC transporters might exacerbate bioaccumulation and toxicity of chemicals, a process known as chemosensitization (Smital and Kurelec, 1997; Kurelec et al., 2000; Ferreira et al., 2012; Kurth et al., 2015). As an example, cytotoxicity of the anticancer drug paclitaxel was greater in the mammalian cell line caco-2 exposed to long-chain polyunsaturated fatty acids that inhibit activity of p-glycoprotein (P-gp), which is a member of the ABC superfamily (Kuan et al., 2011). Also, inhibitors of P-gp increased the cytotoxicity of several drugs to embryos of the innkeeper worm (*U. caupo*) (Toomey and Epel, 1993). With respect to tailings ponds, inhibition of MRPs by chemicals in the water soluble organic fraction of OSPW could inhibit efflux of PAHs and their metabolites from cells, and this could result in greater toxicity. Bioaccumulation of benzo[*a*]pyrene (BaP) was greater in catfish exposed to the surfactant C-12 linear alkyl benzenesulfonate (LAS), and it was hypothesized that inhibition of P-gp by LAS might have been a mechanism of this effect (Tan et al., 2010). Mortality of embryos of zebrafish co-exposed to inhibitors of an ABCB4 protein and the PAH phenanthrene was greater compared to embryos

exposed only to phenanthrene (Fischer et al., 2013). Also, DNA damage in MCF-7 cells co-exposed to BaP and inhibitors of ABC proteins was greater than in cells exposed only to BaP (Myllynen et al., 2007). PAHs, which exist primarily in their alkylated form in oil sands materials, can exert adverse effects on fishes (Lin et al., 2015). While the metabolism and clearance of alkylated PAHs has not been elucidated, glucuronic and glutathione conjugated metabolites of PAHs are excreted from cells by MRPs (Hessel et al., 2013; Srivastava et al., 2002). Thus, inhibition of MRPs might enhance the toxicity of PAHs associated with tailings ponds and other oil sands materials, and future studies should be designed to investigate this potential mechanism of toxicity. These studies also should investigate the mechanism of chemosensitization by constituents of OSPW. Chemosensitizers can inhibit efflux of compounds either by competitive inhibition or by other mechanisms such as blocking the ATPase activity, disruption of phosphorylation of the transport protein, or alteration of membrane fluidity and permeability (Ferreira et al., 2012; Kurth et al., 2015).

Identities of specific chemicals in BML-OSPW that inhibit MRPs are not known. Activity of ABC transporters can be inhibited by structurally unrelated compounds, including non-ionic surfactants (Bogman et al., 2003), dietary fatty acids (Kuan et al., 2011), and biodegraded petroleum hydrocarbons (Hamdoun et al., 2002). Because the acidic fraction of BML-OSPW was composed primarily of chemicals containing oxygen or sulfur that were detected by use of ESI^-, which detects organic acids, results of the current study suggest that acids, including NAs (O_2^-), in BML-OSPW likely do not inhibit activity of MRPs. Several chemicals detected by use of ESI^+, which is used to detect polar organic neutral and basic compounds, were more abundant in the neutral and basic fractions of BML-OSPW compared to both the acidic fraction of BML-OSPW and the neutral and basic fraction of P9-OSPW, and therefore might cause inhibition of MRPs.

Specifically, S^+, SO^+, NO^+, and NO_2^+ were detected in the neutral and basic fraction of BML-OSPW but not in the acidic fraction of BML-OSPW. Also, these chemicals were present in the neutral and basic fractions of P9-OSPW but at lesser relative intensities compared to BML-OSPW. In addition, O^+ in the neutral fraction of BML-OSPW, and NO_3^+, NO_4^+, NO_2S^+, SO_2^+ in the basic fraction of BML-OSPW were more abundant than in P9-OSPW. Properties of some of these chemicals are consistent with several properties of chemosensitizers, including positive charge at physiological pH, and the presence of a basic nitrogen atom (Ecker et al., 1999; Kurth et al., 2015). Also, based on partitioning to polydimethylsiloxane (PDMS) coated stir bars, it was determined that O^+, SO^+, and NO^+ are among the most hydrophobic (i.e. apparent K_{ow} up to 203,000 for SO^+ species), and therefore have the greatest potential to bioaccumulate, of any chemicals in the aqueous phase of OSPW (Zhang et al., 2015). Moderate to high lipophilicity is a property of chemosensitizers (Ecker et al., 1999; Kurth et al., 2015). Additional fractionation steps are required to determine whether each, or a subset, of these compounds inhibits MRPs.

This study is the first to report inhibition of ABC transporters by organic compounds in OSPW. The data suggest that at concentrations currently occurring in BML or Pond 9, organics extracted from OSPW would not be expected to have adverse effects on fish in short-term exposures. However, indirect effects of these organics in the presence of other chemicals (i.e. PAHs) from tailings ponds have not been investigated. Also, because some chemicals identified as potentially inhibiting MRPs have the greatest potential for bioaccumulation, long-term effects on organisms that might inhabit tailings ponds under future reclamation scenarios, such as end-pit lakes, should be investigated.

4 CHAPTER 4: EFFECT OF OIL SAND PROCESS AFFECTED WATER ON TOXICITY OF RETENE TO EARLY LIFE-STAGES OF JAPANESE MEDAKA (*Oryzias latipes*)

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Author Contributions:

Hattan A. Alharbi (University of Saskatchewan) conceived, designed, managed and did the experiment, generated and analyzed the data, wrote, and drafted the manuscript.

Garrett Morandi (University of Saskatchewan) provided laboratory assistance.

Drs. Steve Wiseman and John P. Giesy (all at University of Saskatchewan) provided inspiration, scientific input, and guidance, commented on and edited the manuscript, and provided funding for the research.

4.1 ABSTRACT

Toxicity of oil sands process-affected water (OSPW) to aquatic organisms has been studied, but effects of co-exposure to OSPW and polycyclic-aromatic hydrocarbons (PAHs), which are an important class of chemicals in tailings ponds used to store OSPW, has not been investigated. The goal of the current study was to determine if organic compounds extracted from the aqueous phase relatively fresh OSPW from Base-Mine Lake (BML-OSPW) or aged OSPW from the Pond 9 experimental reclamation pond (P9-OSPW) modulated toxic potency of the model alkyl-PAH, retene, to early life-stages of Japanese medaka (*Oryzias latipes*). Embryos were exposed to retene by use of a partition controlled delivery (PCD) system made of polydimethylsiloxane (PDMS) until day of hatch. Incidences of pericardial edema and expression of *CYP1A* were not significantly greater in larvae exposed only to dissolved organic compounds from either OSPW but was significantly greater in larvae exposed only to retene. Expression of *CYP1A* and incidences of pericardial edema were significantly greater in larvae co-exposed to retene and 5× equivalent of dissolved organic compounds from BML-OSPW compared to retene alone. However, there was no effect of co-exposure to retene and either a 1× equivalent of dissolved organic compounds from BML-OSPW or 5× equivalent of dissolved organic compounds from P9-OSPW. While there was evidence that exposure to 5× equivalent of dissolved organic compounds from BML-OSPW caused oxidative stress, there was no evidence of this effect in larvae exposed only to retene or co-exposed to retene and a 5× equivalent of dissolved organic compounds from BML-OSPW. These results suggest that oxidative stress is not a mechanism of pericardial edema in early-life stages of Japanese medaka. Relatively fresh OSPW from Base Mine Lake might influence toxicity of alkylated-PAHs to early life stages of fishes but this effect would not be expected to occur at current concentrations of OSPW and is attenuated by aging of OSPW.

4.2 Introduction

During surface mining of oil sands in Northern Alberta, Canada, liquid fine tailings generated from extraction of bitumen are held in tailings ponds. Over time, particulates, such as silt and clay, settle to form a layer of mature fine tailings (MFTs), leaving behind an aqueous layer of oil sands process affected water (OSPW) (Allen, 2008). Currently, contents of tailings ponds are not discharged to the ambient environment, but rather OSPW is recycled for use in extraction of bitumen. As a result, salts, metals, and other inorganic and organic chemicals become concentrated in OSPW in tailings ponds. Currently, more than 1 billion m^3 of OSPW is held in tailings ponds (Giesy et al., 2010).

The composition of chemicals in the dissolved organic fraction of OSPW is complex, with more than 3000 elemental compositions having been detected by use of ultra-high resolution mass spectrometry (Pereira et al., 2013a). Included in this mixture are a variety of acidic, basic, and neutral species containing oxygen (O_x), sulfur (S), nitrogen (N) (Barrow et al., 2010; Grewer at al., 2010; Pereira et al., 2013a, Morandi et al., 2015; Alharbi et al., 2016a). Dissolved organic compounds in OSPW cause a variety of adverse effects, including inhibition of growth of Chironomids (Anderson et al., 2012a,b), oxidative stress (He et al., 2012b; Wiseman et al., 2013a,b) immunotoxicity (MacDonald et al., 2013; Wang et al., 2013), endocrine disruption (He et al., 2010, 2011, 2012a; Leclair et al., 2015) and impaired reproduction of fishes (Kavanagh et al., 2011; 2012), developmental malformations in early-life stages of fishes (He et al., 2012b), and acute lethality (Morandi et al., 2015).

In addition to the dissolved organic chemicals in OSPW, polycyclic aromatic hydrocarbons (PAHs) and their alkylated analogs occur in tailings ponds. However, it is thought that PAHs are associated with the sediment phase of tailings ponds and that concentrations in water are small

(Colavecchia et al., 2004, 2006, 2007). For example, concentrations of individual lower molecular mass PAHs range from 10 to 330 ng/L in pore water of MFTs (Madill et al., 1999) and total concentrations of PAHs range from 1150 to 1600 ng/L in the upper clarified zone of tailings ponds (Galarneau et al., 2014). PAHs are natural components of bitumen and there is concern about adverse effects that these chemicals might have on aquatic environments in the oil sands mining region (Madill et al., 1999; Conly et al., 2002; Kelly et al., 2009; Colavecchia et al., 2006). PAHs and alkyl-PAHs cause adverse effects to early-life stages of fishes, including oxidative stress, and blue sac disease (BSD) that is characterized by induction of expression of CYP1A and a suite of developmental malformations, including pericardial edema (Hodson et al., 2007; Billiard et al., 1999; Bauder et al., 2005; Lin et al., 2015; Mu et al., 2014; Wolinska et al., 2013; Turcotte et al., 2011).

Effects of co-exposure to dissolved organic compounds from OSPW and PAHs has not been investigated. However, there is evidence that this co-exposure scenario might cause greater effects than exposure to PAHs alone. Dissolved organic chemicals in OSPW have properties similar to those of surfactants (Clemente and Fedorak, 2005; Frank et al., 2008; Schramm et al., 1984, 1985), and bioaccumulation of PAHs is greater when fish are co-exposed to PAHs and a surfactant (Tan et al., 2010). Greater bioaccumulation of PAHs has been attributed to inhibition of members of the ATP-binding cassette (ABC) superfamily of transporter proteins. For example, concentrations of metabolites of benzo(*a*)pyrene (B*a*P) in cells are greater when activities of ABC proteins are inhibited (Hessel et al., 2013; Ebert et al., 2005; Kranz et al., 2014). Recently, it was demonstrated that dissolved organic compounds in OSPW can inhibit the function of ABC-transporter proteins in early-life stages of Japanese medaka (*Oryzias latipes*) (Alharbi et al., 2016a).

The goals of the current study were to determine if dissolved organic compounds in OSPW modulate adverse effects of exposure to PAHs, and to compare effects of relatively fresh OSPW with OSPW that had been aged in an experimental reclamation pond. Early-life stages of Japanese medaka were exposed to the alkylated-PAH, 7-isopropyl-1-methylphenanthrene (retene), and the dissolved organic fraction of OSPW. Effects of retene, and the potential for the dissolved organic chemicals in OSPW to modulate effects of retene, were determined by quantifying pericardial edema, and expression of genes important for metabolism of retene and the response to oxidative stress.

4.3 Materials and Methods

4.3.1 Chemicals and OSPW

Retene (purity = 95%) was purchased from Chem Service, Inc. (West Chester, PA, USA). Polydimethylsiloxane (PDMS) aquarium-grade sealant was purchased from Marineland (Blacksburg, VA, USA). Acetone, hexane, dichloromethane (DCM), and ethyl-acetate, each of HPLC grade, were purchased from Fisher Scientific (Ottawa, ON, Canada). Anhydrous ethanol was obtained from GreenField Ethanol Inc. (Brampton, ON, Canada). Two samples of OSPW were collected on the site of Syncrude Canada Ltd. (Fort McMurray, AB, Canada). One sample was from Base Mine Lake (BML-OSPW), which is an end-pit-lake constructed from the West-In-Pit settling basin that received input of tailings from the main extraction facility until December 2012, after which all input of tailings from the extraction plant was ceased. The other sample was from an experimental reclamation pond called Pond 9 (P9-OSPW) that was constructed in 1993 and has not received input of OSPW since that time. Samples were collected in September of 2012, shipped to the University of Saskatchewan (Saskatoon, SK, Canada), and stored in the dark

until extraction. Samples were inspected visually immediately upon arrival and were observed to be free of any residual bitumen or MFT.

4.3.2 Extraction of dissolved organics from OSPW

Basic and neutral compounds, but not acidic compounds, in the water soluble organic fraction of OSPW can modulate activity of ABC proteins in early-life stages of Japanese medaka (Alharbi et al., 2016a). Therefore, a method to isolate basic and neutral compounds into one fraction that contains lesser amount of acidic compounds was used for extraction of OSPW (Vieno et al., 2006). Briefly, 1000 mL of each sample of OSPW was passed through a glass microfiber filter (GF/D 0.47 mm, Whatman) to remove any particulate matter and the pH of the OSPW adjusted to pH 9 by use of NH_4OH. Pre-concentration of samples was performed in one generic step by use of EVOLUTE® ABN sorbent (Biotage, Charlotte, NC, USA). Before addition of OSPW, cartridges were conditioned with 6 mL of methanol followed by 6 mL of ultrapure Milli-Q water (Millipore, Mississauga, Canada). Five cartridges were used for each sample of OSPW, and 200 mL of OSPW was extracted on each cartridge. OSPW was passed through cartridges under vacuum at a flow rate of 10–15 mL min^{-1}. At a pH 9, acidic compounds will not bind the ABN sorbent. Subsequently, cartridges were washed with Milli-Q water and allowed to dry under vacuum for 30 min. Basic and neutral compounds were isolated in one step in 6 mL of methanol without use of vacuum. Samples were pooled, evaporated to dryness under a gentle stream of nitrogen, and reconstituted in 500 L of absolute ethanol. Therefore, the concentration of dissolved organic compounds in the final samples was 2000 × greater than in the original samples of OSPW

4.3.3 Chemical profiling of OSPW

The profile of organic chemicals in extracts of BML-OSPW and P9-OSPW was determined by use of an Q Exactive mass spectrometry (Orbitrap, Thermo Fisher Scientific, San Jose, CA, USA) equipped with a Dionex™ UltiMate™ 3000 UHPLC system (Thermo Fisher Scientific). Separation was achieved on a Betasil C18 column (5 μm; 2.1 mm × 100 mm; Thermo Fisher Scientific) with an injection volume of 5 μL. The mobile phase consisted of ultrapure water (A) and methanol (B). Initially, 20% B was increased to 80% in 3 min, then increased to 100% at 8 min and held static for 19.5 min, followed by a decrease to initial conditions of 20% B and held for 2 min to allow for equilibration of the column. The flow rate was 0.25 mL/min. The temperature of the column and sample chamber were maintained at 30 and 10 °C, respectively. Data were acquired using full scan mode in both positive electrospray (ESI^+) and negative (ESI^-) ion mode. Briefly, MS scans (200−2,000 *m/z*) were recorded at resolution R = 70,000 (at *m/z* 200) with a maximum of 3×10^6 ions collected within 200 ms, based on the predictive automated gain control. General mass spectrometry settings applied for positive and negative ion modes were as follows: spray voltage, 3.0 kV; capillary temperature, 400 °C; sheath gas, 46 L/h; auxiliary gas, 15 L/h; probe heater temperature, 350 °C. Chemical species were grouped according to heteroatom empirical formula classes in ESI^- or ESI^+ electrospray: $O_x^{+/-}$ (where x = 1–5), $N^{+/-}$, $NO_x^{+/-}$ (where x = 1–4), $S^{+/-}$, $SO_x^{+/-}$ (where x = 1–5), or $NO_xS^{+/-}$ (where x = 1-4). Only those chemicals in the total ion mass spectrum that had a peak threshold >600, amass spectral signal-to-noise ratio (S/N) >3, were present at relative abundances of at least 2%, and that produced discernible extracted ion chromatographic peaks (i.e., S/N > 3) were reported as described previously (Alharbi et al., 2016a).

4.3.4 Effects of co-exposure to retene and OSPW

Embryos of Japanese medaka were collected from a breeding culture that is maintained in the Aquatic Toxicology Research Facility at the University of Saskatchewan (Saskatoon, SK, Canada). Culturing of adult fish and rearing of embryos until they were required for exposures was conducted in dechlorinated City of Saskatoon municipal tap water at a temperature of 28 °C and a photoperiod of 16 h:8 h (light: dark). All protocols were approved by the University of Saskatchewan's Committee on Animal Care and Supply (UCACS Protocol #20090108).

Embryos of Japanese medaka were exposed to retene by use of a partition controlled delivery system with PDMS (Kiparissis et al., 2003; Turcotte et al., 2011; Lin et al., 2015). Briefly, retene was dissolved into a solution of DCM: hexane (15:85) at a ratio of 6 mg of retene per ml of solution. An appropriate volume of this mixture was added to PDMS to achieve a final concentration of retene of 15 $\mu g.ml^{-1}$. A negative control was prepared by use of the same procedure but without retene. Next, 2 ml was transferred into each 20 ml glass vial and vials were placed in a fume hood until all solvent was evaporated. Vials were wrapped in tin foil and all procedures were conducted in the dark to avoid any photo-modification of retene (Oris and Giesy, 1987).

Prior to exposure of fish to retene, a study was conducted to assess partitioning of retene from PDMS to OSPW or the solvent control, which was City of Saskatoon municipal tap water containing 0.25% v/v of ethanol. Sampling of solutions was performed after 24, 96, 168 and 240 h in vials free of embryos. For exposures of embryos, 15 mL of the solvent control, 5× or 1× of the dissolved organic fraction of BML-OSPW, or 5× of the dissolved organic fraction of P9-OSPW were added to vials containing PDMS with or without 15 $\mu g.mL^{-1}$ of retene. These concentrations of the dissolved organic fraction of BML-OSPW and P9-OSPW did not affect survival of embryos

(data not shown). Next, 12 embryos that were raised in freshwater until 4 dpf were added to each vial and placed on an orbital shaker (VWR Inter-national, Radnor, PA, USA) at 700–900 rpm and at 28 ˚C. Each day the number of embryos that were alive or dead or that had hatched was determined, and any dead embryos were removed. Exposures were terminated at 192 h of exposure. Samples of solutions were taken at 24 h and 192 h of exposure to quantify concentrations of retene. Solutions were not replaced during the exposure. Incidences of pericardial edema in hatched fry were evaluated by visualizing larvae with a light microscope at 10X magnification. Samples were stored at −80 ˚C for quantification of gene expression. All exposures were performed on 5 separate batches of embryos (n = 5).

4.3.5 Quantification of retene by GC/MS

Concentrations of retene in the solvent control or OSPW were determined by use of liquid-liquid extraction according to a method described by El-Amrani et al (2013). Briefly, 1 mL of solution was extracted twice with 500 µL of a 1:1 mixture of hexane: ethyl acetate. Samples were centrifuged at 9000 g for 5 min, the organic phase was removed, and the remaining supernatant was concentrated under a gentle stream of nitrogen until samples were almost dry. Next, samples were reconstituted in 95 µL of nonane: hexane (15:85) and transferred to an auto-sampler vial fitted with a glass insert and 5 µL of a solution containing 1 $\mu g.mL^{-1}$ of fluorinated-polybrominated diphenyl ether 47 (F-BDE-47 in nonane) was added to samples as an internal standard for analysis by use of an Agilent (Santa Clara, CA, USA) 7890A gas chromatograph (GC) system coupled to an Agilent 5975C mass spectrometer (MS) operated in the electron impact ionization mode (EI). Samples at a volume of 1 µl were injected at an injection port temperature of 280 ˚C in the splitless mode. Chromatographic separation was achieved by use of a DB-5MS (60 m × 0.250 mm × 0.1µm) fused silica capillary GC column (Aiglent J&W). The carrier gas was helium at a constant flow

of 1.5 mL.min^{-1}. The following GC oven temperature program was used: 100 ˚C for 1 min, 5 ˚C/min to 190 ˚C for 2 min, 20 ˚C/min to 220 ˚C for 2 min, and 40 ˚C/min to 300 ˚C for 4 min. The GC/MS transfer line was maintained at 28 ˚C. Selected ion monitoring was *m/z* 234/219 for retene and 343/235 for F-BDE-47 (IS). Recovery of retene spiked into water at 10 or 350 µg.L^{-1} was 86.5 ± (RSD < 2%) and 104 ± (RSD < 5%), respectively. The limit of detection was 0.6 µg.L^{-1} and the limit of quantification was 2.4 µg.L^{-1}.

4.3.6 Quantitative real-time PCR

Total RNA was extracted from pools of 5 pools of 6 larvae by use of the Qiagen RNeasy Protect Mini Kit according to the protocol provided by the manufacturer (Qiagen, Mississagua, ON, Canada). Quantities of total RNA were determined by use of a ND-1000 Spectrophotometer (Nanodrop Technologies, Welmington, DE, USA) and integrity of RNA was determined by use of denaturing agarose gel electrophoresis. The cDNA was synthesized from 1 µg of total RNA by use of the QuantiTect Reverse Transcription Kit (Qiagen, Toronto, ON, Canada) according to the protocol provided by the manufacturer. Quantitative real-time quantitative PCR (qPCR) was performed in 96-well plates by use of an ABI 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). A 50 µl reaction mixture of 2X concentrated QuantiFast SYBR Green Master Mix (Qiagen), an optimized concentration of cDNA, 10 pmol of gene-specific qPCR primers, and nuclease free water was prepared for each cDNA sample and primer combination. All reactions were conducted in duplicate with 20 µl reaction volumes per well. The PCR reaction mixture was denatured at 95 °C for 10 min prior to the first PCR cycle. The thermal cycle profile consisted of denaturing at 95 °C for 10 s and extension for 1 min at 60 °C for a total of 40 PCR cycles. Amplification of a single PCR product was confirmed by melt curve analysis and abundances of target genes were quantified by normalizing to expression of the housekeeping gene

RPL-7 (Pfaffi, 2001). Primers to amplify cytochrome p450 1A (*cyp1a*), ATP-binding cassette subfamily c member 2 (*abcc2*) and subfamily G member 2 (*abcg2*), superoxide dismutase (*sod*), catalase (*cat*), and glutathione-s-transferase pi (*gstp*) were designed by use of Primer express 3.0 software (Applied Biosystems) and were purchased from Invitrogen (Mississauga, ON, Canada). Sequences of primers and efficiencies of PCR reactions are given in Table 4.1. Prior to qPCR, the efficiency of each primer set was established by performing qPCR with cDNA that was generated by pooling equal volumes of each sample of cDNA and that was serially diluted by 5-fold from undiluted to 1:3125. Reactions without cDNA were performed to check for contamination of reagents and check for primer-dimers. All primer sets had a coefficient of determination (R^2) of at least 0.99 and efficiencies of 84–106%

Table 4.1. Sequences of primers used to quantify expression of target genes in fry of Japanese medaka.

Target transcript	Accession #	Sense primer (5'-3')	Antisense primer (5'-3')	Efficiency (%)
cyp1a	AY297923	GAGCACCTGGTCAAAGAGATAG	AGCACATGCCACAGATAACA	96
gstp	NA*	GAGCACCTGGTCAAAGAGATAG	AGCACATGCCACAGATAACA	96
cat	NA*	GAGGAGCAAGAGCACGAATC	ATGAAGCACAGAGGCTGGAG	84
sod	EF546450	AGAGAATCCTCGCCGTGAAGTT	CCACGATTTGGTTGATGACCTC	96
abcc2	NA*	GGCGGTCACATTAGGAGAGG	ACGTCACACAGAACCAGCAA	91
abcg2	NA*	AGGGTAAGCAGGGGATGACT	GAGAGCTCCAACGATCAGGG	106
rpl-7	DQ118296	GTCGCCTCCCTCCACAAAG	AACTTCAAGCCTGCCAACAAC	94

*****Nucleotide sequence was obtained from the Ensembl database (http://uswest.ensembl.org/Oryzias_latipes/Info/Index).

4.3.7 Statistical analyses

Effects of treatments on survival, rate of hatching, incidence of pericardial edema, gene expression, and concentrations of retene were determined by use GraphPad Prism 5 software (San Diego, CA, USA). Normality of data was assessed by use of the Kolmogorov Smirnov one-sample test and homogeneity of variance was determined by use of Levene's test. If necessary, data were log transformed to ensure normality and homogeneity of variance. Significant differences among treatments were evaluated by use of a one-way ANOVA followed by Tukey post-hoc test. Differences were considered significant at $\rho \leq 0.05$.

4.4 Results

4.4.1 Profiles of organic chemicals in BML and P9

Extraction of OSPW at pH 9 resulted in samples that were enriched in basic and neutral chemicals relative to acidic chemicals. Relative abundances of species detected by use of ESI^-, which is used to detect organic acids, were lesser than abundances of species detected by use of ESI^+, which is used to detect polar organic neutral and basic chemicals (Figure. 4.1). For example, relative abundance of naphthenic acids (NAs; O_2^-) was less than relative abundance of O_2^+, which are not NAs but had been proposed to be dihydroxy, diketo or ketohydroxy chemicals (Pereira et al., 2013). However, a small amount of acidic chemicals were present in the final extracts. Classes of heteroatoms in extracts of BML-OSPW and P9-OSPWwere similar, but differences in elemental compositions and relative intensities of species of heteroatoms were observed. In both extracts, intensity of species containing oxygen (O_x^-) or sulfur (SO_x^-) were greater than intensities of chemical species containing nitrogen (NO_x^-) (Figure. 4.1A). When analysed by use of ESI^-, intensity of intensity of O_2^- was 3.5-fold greater in BML-OSPW compared to P9-OSPW, and the same was observed for the intensity of $O_3^-$and O_4^- chemical species which were 2.5- and 3.0-fold greater, respectively, in BML-OSPW compared to P9-OSPW. Intensities of SO_2^-, and SO_3^- chemicals species were 1- and 5-fold greater, respectively, in BML-OSPW compared to P9-OSPW. When analysed by use of ESI^+ mode (Figure. 4.1B). Intensities of O^+, O_2^+, O_3^+ and O_4^+chemical species were 1.5-, 1.6-, 2- and 3.1-fold greater in P9-OSPW than in BML-OSPW. Intensities of S^+, SO^+, $SO_2^+$and SO_3^+ were 4.5-, 4.9-, 26- and 4.6-fold greater in BML-OSPW compared to P9-OSPW. Intensities of N^+, NO^+, NO_2^+, NO_2S^+, NO_3^+, NO_3S^+, $NO_4^+$and NO_4S^+ were greater by 3-, 3-, 4-, 5-, 2.3-, 2.9-, 4- and 3.3 fold, respectively, in BML-OSPW compared to P9-OSPW.

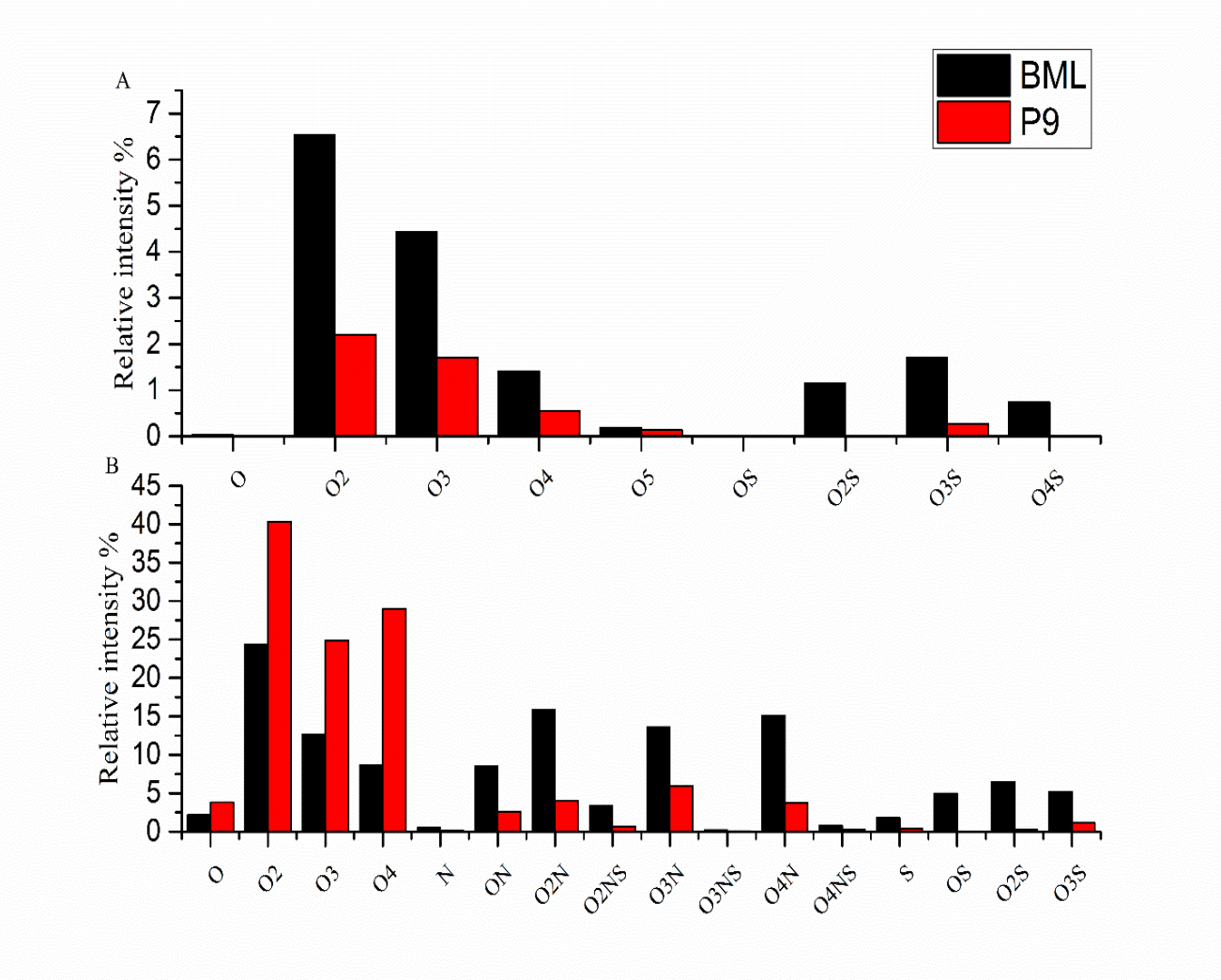

Figure 4.1: Elemental composition and relative intensity of water-soluble species of chemicals by heteroatom class in BML-OSPW and P9-OSPW acquired in (A) ESI^- and (B) ESI^+.

4.4.2 Concentrations of retene in water and OSPW

Prior to initiation of exposures a preliminary study was conducted to confirm partitioning of retene from the PDMS into freshwater or OSPW (Table 4.2). Concentrations of retene in vials containing PDMS without retene were less than the limit of quantification. In vials containing PDMS with retene at 15 $\mu g.mL^{-1}$ concentrations of retene in the solvent control ranged from 25.9 to 28.8 $\mu g.L^{-1}$ when the aqueous phase was 0.25% v/v of ethanol. Concentrations of retene were greater when the aqueous phase contained OSPW. In vials containing 1× of BML-OSPW concentrations of retene ranged from 30.2 to 46.7 $\mu g.L^{-1}$ and in vials containing 5× of BML-OSPW concentrations of retene ranged from 179.4 to 188.4 $\mu g.L^{-1}$, which is 7-fold greater compared to the concentration in 0.25% v/v of ethanol. The concentration of retene in vials containing 5× of P9-OSPW ranged from 71.8 to 83.3 $\mu g.L^{-1}$, which is 2.8-fold greater compared to 0.25% v/v of ethanol but 2-fold lesser compared to the concentration in 5× of BML-OSPW.

In vials containing embryos, concentrations of retene in the solvent control or OSPW were quantified at 24 h of exposure and upon termination of the exposure (Table 4.2). In vials containing PDMS without retene, concentrations of retene were less than the limit of quantification. In vials containing PDMS with retene at 15 $\mu g.mL^{-1}$, concentrations of retene were approximately equal at both time points (see Table 4.2). Concentrations of retene in vials containing retene and OSPW were greater than in vials containing 0.25% v/v of ethanol. However, the difference in concentrations of retene among vials containing different samples of OSPW and retene were small.

Table 4.2 Concentrations of retene (µg.L^{-1}) in aqueous phase in vials with and without larvae. The concentration of retene in PDMS was 15 µg.mL^{-1}.

Aqueous media	Vials without fish		Vials with fish
	Time (h)	[µg.L^{-1}]	[µg.L^{-1}]
0.25% v/v ethanol	24	27.8 ± 0.6	65.9 ± 7.0
	96	27.6 ± 3.9	NA
	168	25.9 ± 1.8	NA
	192	28.8 ± 1.4	77.7 ± 5.9
1×BML-OSPW	24	30.6 ± 1.9	90.7 ± 4.0
	96	30.2 ± 1.5	NA
	168	35.4 ± 1.4	NA
	192	46.7 ± 7.5	113.1 ± 5.1
5×BML-OSPW	24	180.5 ± 5.27	108.9 ± 4.5
	96	179.4 ± 5.71	NA
	168	184.3 ± 6.85	NA
	192	188.4 ± 9.81	112.4 ± 4.7
5× P9--OSPW	24	71.8 ± 4.2	97.9 ± 0.7
	96	74.7 ± 0.1	NA
	168	76.1 ± 3.7	NA
	192	83.3 ± 3.8	96.2 ± 2.8

NA = not analysed

Values represent the mean ± standard error of 3 independent replicates. In vials containing only the solvent control or OSPW the concentration of retene was less than the limit of detection.

4.4.3 Lethality, hatching, and incidences of malformations

Mortality of embryos exposed to either treatment was less than 10% and there were no significant differences among treatments (data not shown). Percentage of eggs hatched by 192 h of exposure was greater than 90% for all exposures, and there were no significant differences among treatments. However, there were differences in the time to hatch (Table 4.3). Hatching of embryos exposed only to the solvent control began at 144 h of exposure. However, hatching of embryos exposed only to either 5× of BML-OSPW or 5× of P9-OSPW did not begin until 168 h of exposure. This effect was attenuated by diluting BML-OSPW as hatching of embryos exposed to 1× of BML-OSPW began at 144 h of exposure. Hatching of embryos exposed to 15 $\mu g.ml^{-1}$ of retene began at 168 h of exposure and this effect was not modulated by exposure to either sample of OSPW.

Exposure to OSPW increased incidences of malformations caused by exposure to retene (Figure. 4.2). No incidences of malformations were observed in embryos exposed only to the solvent control or either sample of OSPW. Incidences of malformations of embryos exposed only to retene was 46.87%. Co-exposure to retene and either 1× of BML-OSPW or 5× of P9-OSPW did not modulate effects of retene as incidence of malformations were 41.14% and 49.74%, respectively. However, incidences of malformations of embryos exposed to retene and 5× of BML-OSPW was 79.98%.

Table 4.3 Effects of exposure to OSPW, retene or OSPW and retene on hatching of embryos.

Treatment	% of eggs hatched (cumulative)		
	144 h	**168 h**	**192 h**
Solvent control	27.0 ± 10.1^{a}	96.9 ± 9.6^{a}	0^{a}
5× BML-OSPW	0^{b}	36.0 ± 8^{b}	99.5 ± 9^{b}
1× BML-OSPW	16.7 ± 12^{c}	35.0 ± 1^{b}	97.0 ± 15^{b}
5× P9-OSPW	0^{b}	37.8 ± 7.2^{b}	96.2 ± 8^{b}
15 µg.mL^{-1} Retene	0^{b}	51.2 ± 23^{b}	93.4 ± 23^{b}
15 µg.mL^{-1} Retene + 5× BML-OSPW	0^{b}	31.2 ± 3.4^{b}	97.9 ± 3.8^{b}
15 µg.mL^{-1} Retene + 1× BML-OSPW	4.2 ± 4.2^{c}	33.4 ± 10.8^{b}	95.5 ± 15^{b}
15 µg.mL^{-1} Retene + 5× P9-OSPW	0^{b}	36.5 ± 7.2^{b}	96.5 ± 7.2^{b}

Embryos were reared in freshwater from fertilization until 96 h post-fertilization at which time they were transferred to vials used for the exposure. Embryos were exposed to treatments for 192 h. Significant differences in hatching were determined by use of one-way ANOVA followed by Tukey's post hoc test ($n = 5$, $p < 0.05$) and different letters indicate significant effects.

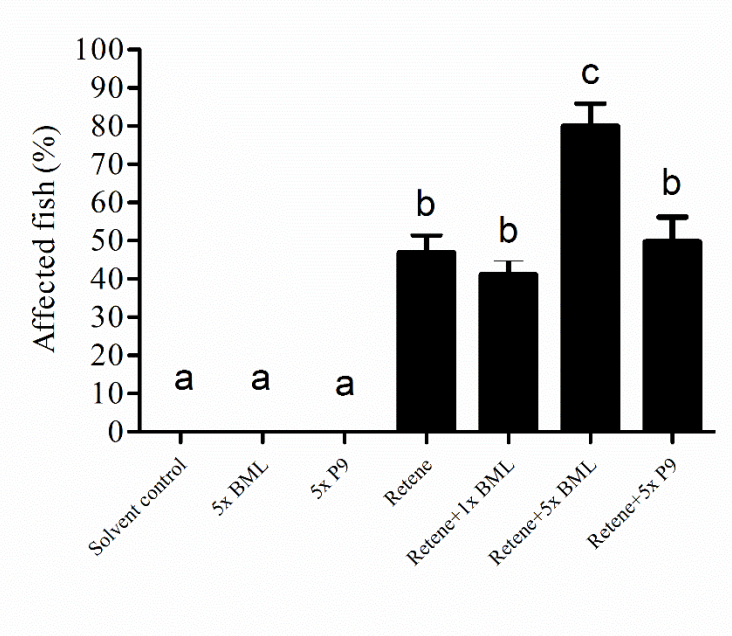

Figure 4.2: Percentage of larvae of Japanese medaka with pericardial edema. Embryos were reared in freshwater from fertilization until 96 h post-fertilization at which time they were transferred to vials used for the exposure. Embryos were exposed to treatments for 192 h. Significant differences in incidences of pericardial edema were determined by use of one-way ANOVA followed by Tukey's post hoc test ($n = 5$, $p < 0.05$) and are indicated by different letters.

4.4.4 Expression of Genes

Expression of *cyp1a* was quantified as a biomarker of exposure to agonists of the aryl-hydrocarbon receptor (AhR) (Figure. 4.3). Compared to embryos exposed to the solvent control, abundance of transcripts of *cyp1a* was 85.6-fold greater in embryos exposed only to retene. Exposure to 1× of BML-OSPW or 5× of P9-OSPW did not significantly alter the response to retene as abundance of transcripts of *cyp1a* was 81.3- and 74.9-fold greater, respectively. However, in embryos co-exposed to retene and 5 × equivalent of BML-OSPW the abundance of transcripts of *cyp1a* was 133.2- fold greater compared to the solvent control.

Expression of genes encoding proteins that are important for the response to oxidative stress were quantified (Figure. 4.4). Compared to larvae exposed to the solvent control, abundances of transcripts of *sod*, *cat* and *gstp* were 2.1-, 3.2- and 2.6-fold greater, respectively, in larvae exposed to 5 × equivalent of BML-OSPW, and abundance of transcripts of *gstp* was 2.0-fold greater in larvae exposed to 5 × equivalent of P9-OSPW. However, abundances of transcripts of *sod*, *cat* and *gstp* were significantly lesser in larvae co-exposed to retene and 5 × equivalent of OSPW compared to larvae exposed only to 5 × equivalent of OSPW.

Expression of genes encoding proteins that are important for phase III metabolism of xenobiotic were quantified (Figure. 4.5). Compared to embryos exposed to the solvent control, abundances of transcripts of *abcc2* and *abcg2* were 12.4- and 5.3-fold greater, respectively, in larvae exposed to 5× equivalent of BML-OSPW. Abundances of transcripts of *abcc2* and *abcg2* were not significantly greater in larvae co-exposed to 5 × equivalent of BML-OSPW and retene compared to controls, but were significantly less than in embryos exposed to 5 × equivalent of

BML-OSPW. Exposure to 5×of P9-OSPW did not affect abundances of transcripts of *abcc2* or *abcg2.*

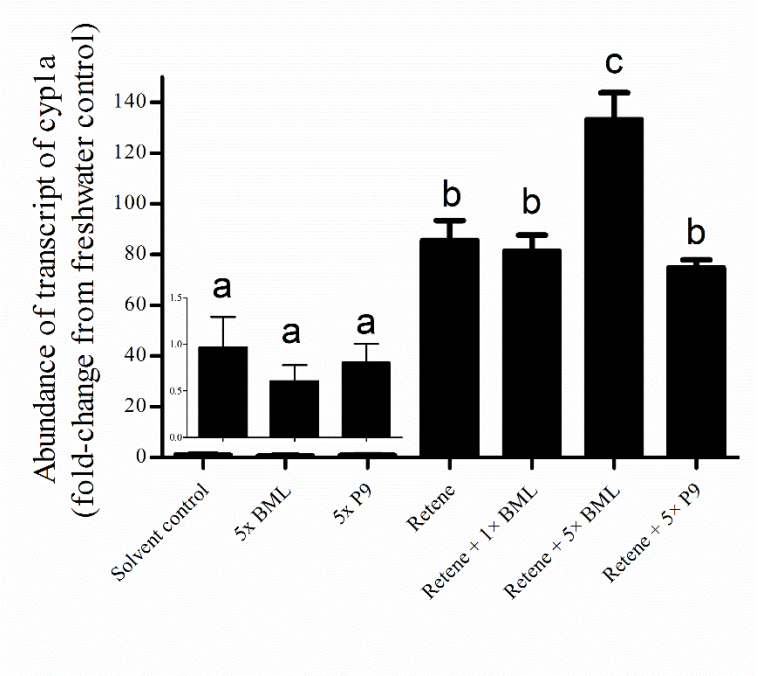

Figure 4.3: Abundances of transcripts of *cyp1a* in larvae of Japanese medaka. Embryos were reared in freshwater from fertilization until 96 h post-fertilization at which time they were transferred to vials used for the exposure. Embryos were exposed to treatments for 192 h. Significant differences in gene expression were determined by use of one-way ANOVA followed by Tukey's post hoc test ($n = 5$, $p < 0.05$) and are indicated by different letters.

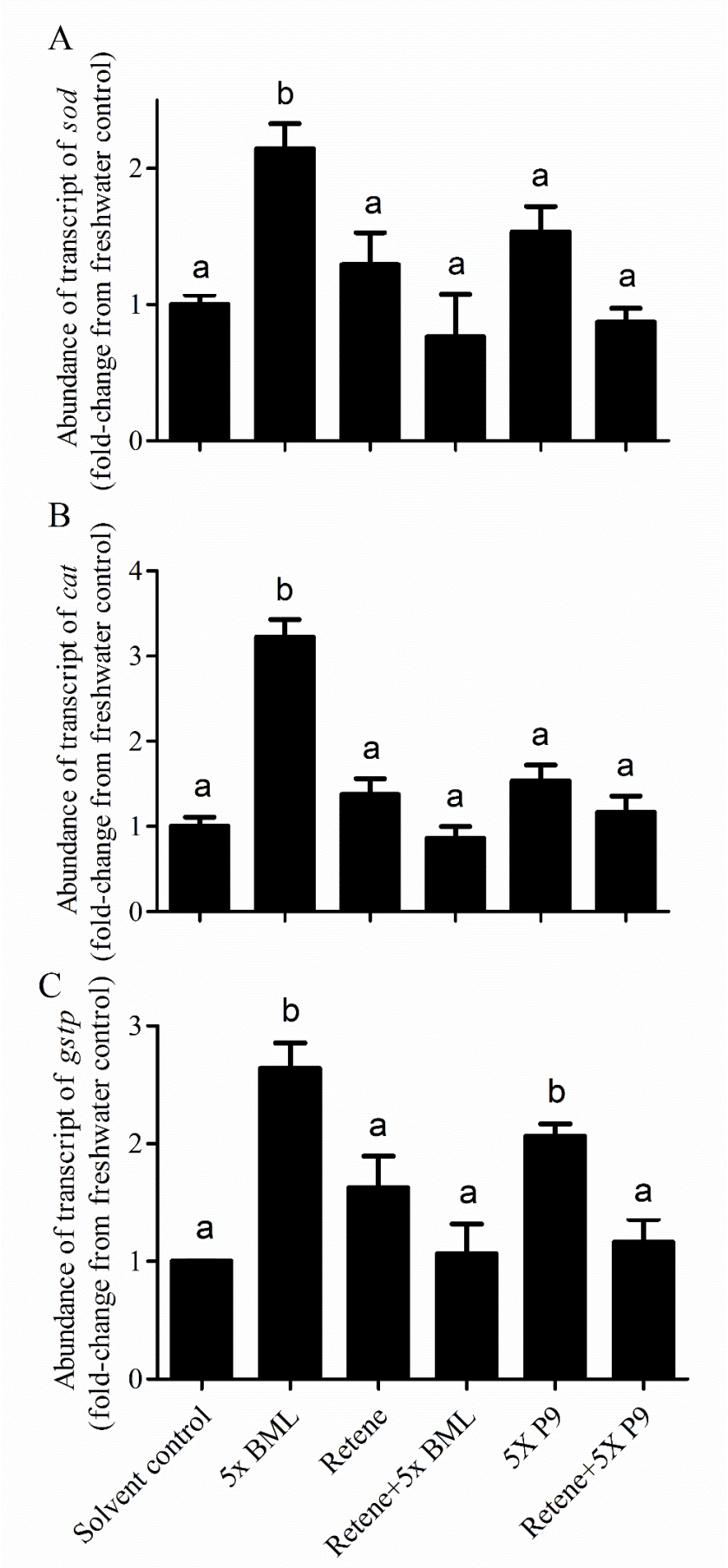
A
Abundance of transcript of sod
(fold-change from freshwater control)
0
1
2
a
b
a
a
a
a
B
Abundance of transcript of cat
(fold-change from freshwater control)
0
1
2
3
4
a
b
a
a
a
a
C
Abundance of transcript of gstp
(fold-change from freshwater control)
0
1
2
3
a
b
a
a
b
a
Solvent control
5x BML
Retene
Retene+5x BML
5X P9
Retene+5X P9

Figure 4.4: Abundances of transcripts of (A) *sod*, (B) *cat* and (C) *gstp* in larvae of Japanese medaka. Embryos were reared in freshwater from fertilization until 96 h post-fertilization at which time they were transferred to vials used for the exposure. Embryos were exposed to treatments for 192 h. Significant differences in in gene expression were determined by use of one-way ANOVA followed by Tukey's post hoc test ($n = 5$, $p < 0.05$) and are indicated by different letters.

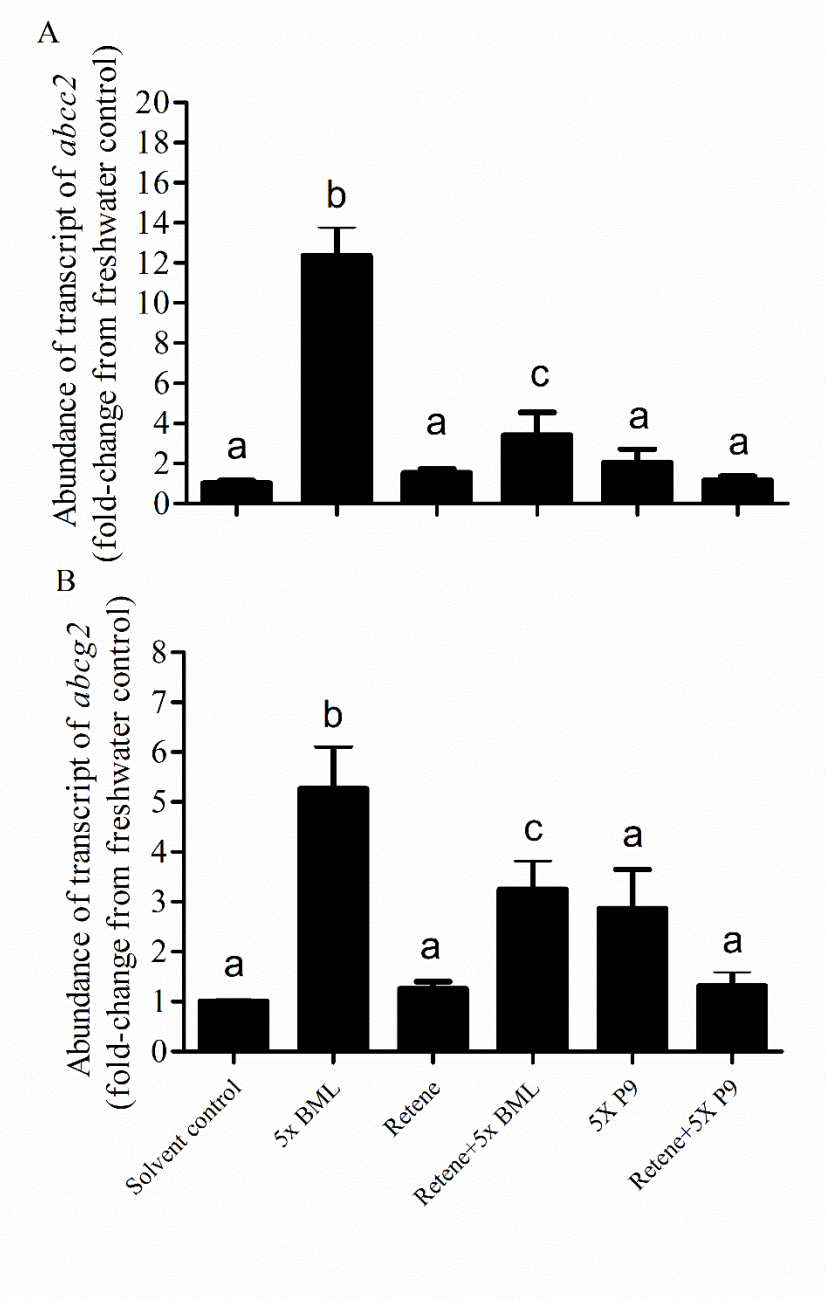

Figure 4.5: Abundances of transcripts of (A) *abcc2*, and (B) *abcg2*, in larvae of Japanese medaka. Embryos were reared in freshwater from fertilization until 96 h post-fertilization at which time they were transferred to vials used for the exposure. Embryos were exposed to treatments for 192 h. Significant differences in gene expression were determined by use of one-way ANOVA followed by Tukey's post hoc test ($n = 5$, $p < 0.05$) and are indicated by different letters.

4.5 Discussion

This study is the first to investigate whether dissolved organic compounds in OSPW affect toxicity of PAHs, which are an important component of tailings ponds used to store OSPW in the surface mining oil sands industry in northern Alberta, Canada. Exposure to retene, either sample of OSPW, or co-exposure to retene and OSPW did not cause acute lethality to embryos. The lack of acute lethality of embryos exposed to retene is consistent with the absence of mortality in marine medaka (*Oryzias melastigma*) exposed to concentrations of retene less than 200 $\mu g.L^{-1}$ (Mu et al., 2014), and larvae of rainbow trout (*Oncorhynchus mykiss*) exposed to a nominal concentration of 320 $\mu g.L^{-1}$ (Billiard et al., 1999; Bauder et al., 2005) or 100 $\mu g.L^{-1}$ (Scott and Hodson 2008).

Greater expression of CYP1A, and a greater prevalence of pericardial edema in larvae exposed to retene is consistent with results of previous studies that linked exposure to retene with blue sac disease (BSD) (Billiard et al., 1999; Brinkworth et al., 2003; Bauder et al., 2005; Scott and Hodson, 2008). Neither greater abundance of transcripts of *cyp1a* nor greater prevalence of pericardial edema were observed in larvae exposed only to OSPW. This is consistent with results of another study where abundances of transcripts were not significantly greater in larvae of fathead minnow (*Pimephales promelas*) exposed to OSPW from the west-in-pit settling basin, which was commissioned as Base Mine Lake in 2013 (He et al., 2012b). However, in that study, embryos exposed to OSPW exhibited several manifestations that are characteristic of blue-sac disease, including pericardial edema. That effect was attenuated by treating OSPW with ozone or activated carbon, both of which reduce concentrations of dissolved organic compounds in OSPW, suggesting that the dissolved organic chemicals caused pericardial edema without activating the AhR (He et al., 2012b). It is not known why BML-OSPW in the current study did not cause pericardial edema, but at the time of the study by He et al (2012b) the west-in-pit was receiving

input of fresh tailings from a bitumen extraction facility, so there might have been differences in composition of chemicals in OSPW. Also, because the extracts used in the current study were enriched in basic and neutral chemicals relative to acidic chemicals, the pericardial edema observed in the study be He et al. (2012b) might have been caused by acidic compounds. Compared to exposure only to retene, co-exposure to retene and 1× of BML-OSPW or 5× of P9-OSPW did not result in greater abundance of transcripts of *cyp1a* or greater incidences of pericardial edema. However, compared to fish exposed only to retene, or fish co-exposed to retene and 1× of BML-OSPW or 5× of P9-OSPW, abundance of transcripts of *cyp1a* and incidences of pericardial edema were greater in fish co-exposed to retene and 5× of BML-OSPW.

The mechanism(s) of greater expression of CYP1A and incidences of pericardial edema in embryos co-exposed to retene and 5× of BML-OSPW are not known. Because expression of CYP1A and induction of blue-sac disease in early-life stages of fishes, including retene, is dependent on activation of the AhR (Billiard et al. 2006; Incardona et al., 2006; Scott and Hodson et al., 2008), these effects might be a result of the dissolved organic compounds in OSPW increasing activation of the AhR by retene. An example of this effect is greater activation of the AhR by TCDD in the presence of humic acids that do not activate the AhR (Bittner et al., 2009). In that study it was proposed that humic acids increased bioavailability of TCDD by enhancing solubility. Studies have shown that lower molecular weight organic acids increase solubility of PAHs in water (Doring and Marschner, 1998; Ling et al., 2009). Chemicals in OSPW have properties of surfactants (Clemente and Fedorak, 2005; Frank et al., 2008; Schramm et al., 1984, 1985), and surfactants enhance the bioavailability of hydrophobic organic compounds in water (Edwards et al., 1992; Jafvert et al., 1994; Volkering et al., 1995; Badr et al., 2004; Subramaniam et al., 2004). For example, synthetic non-ionic surfactants increased apparent solubility and rates

of dissolution of naphthalene and phenanthrene (Volkering et al., 1995). In the current study, regardless of the aqueous phase, retene desorbed from the PDMS to the aqueous phase, but, as indicated by results of the trial in the absence of embryos, the concentration of retene was greater when the aqueous phase was OSPW, and was greatest when the aqueous phase was 5× of BML-OSPW. The lesser concentrations of retene observed in the presence of 1× of BML-OSPW or 5× P9-OSPW likely was because the concentration of dissolved organics was smaller in these samples than in 5× of BML-OSPW. Several studies have shown that concentrations of dissolved organic chemicals, including carboxylic acids such as NAs, are approximately 50% lesser in OSPW from Pond 9 than in OSPW from the west-in-pit (Han et al., 2009; Anderson et al., 2012a).

In addition to greater exposure resulting from enhanced desorption of retene from PDMS caused by OSPW, greater abundance of transcripts of *cyp1a* and greater incidences of pericardial edema might be caused by greater uptake of retene in larvae co-exposed to 5× of BML-OSPW compared to embryos exposed to retene alone or co-exposed to retene and 1× of BML-OSPW or 5× of P9-OSPW. Greater uptake of retene might result from alterations of cell membranes caused by dissolved organic chemicals in OSPW. Changes in permeabilities of cell membranes was proposed as a mechanism by which activation of AhR by TCDD was greater in the presence of humic acids (Glover and Wood, 2005; Bittner et al., 2009). Studies have shown that surfactants increase permeabilities of cell membranes (Xia and Onyuksel, 2000). For example, bioaccumulation of BaP was greater in catfish exposed to the surfactant C-12 linear alkyl-benzene-sulfonate compared to freshwater (Tan et al., 2010). It is not known whether dissolved organic chemicals in OSPW alter cell membranes, but such an effect would enhance bioavailability of retene and therefore might result in greater toxicity.

It has been suggested that blue-sac disease in early-life stages of fishes exposed to retene might be caused by oxidative stress resulting from generation of reactive oxygen species (ROS) produced during metabolism of retene by CYP1A enzymes (Billiard et al., 1999; Bauder et al., 2005). Also, there is an abundance of evidence that dissolved organic chemicals in OSPW cause oxidative stress. Concentrations of ROS were greater in embryos of fathead minnows exposed to OSPW from the west-in-pit settling basin (He et al., 2012b). Concentrations of lipid hydro peroxides were greater in larvae of *Chironomus dilutus* exposed to OSPW from the west-in-pit settling basin and greater abundances of transcript of several genes important for the response to oxidative stress, including *gst*, *sod*, and *cat* were greater in in fathead minnows and *Chironomuis dilutus* exposed to OSPW from the west-in-pit settling basin (Wiseman et al., 2013a, b; He et al., 2013). Therefore, it was hypothesized that co-exposure to OSPW and retene would cause a greater effect than exposure to retene alone. However, expressions of genes important for the response to oxidative stress do not support this mechanism of toxicity. Abundances of transcripts of *gst*, *sod* and *cat*, were not significantly greater in fry exposed only to retene or co-exposed to retene and 5× of BML-OSPW, both of which caused pericardial edema. However, abundance of transcripts of *cat* was greater in fry exposed to 5× of BML-OSPW, which did not cause pericardial edema. These results suggest that oxidative stress does not play a significant role in the incidence of pericardial edema observed in this study.

Inability to metabolise the greater amounts of retene that larvae co-exposed to retene and 5× of BML-OSPW were exposed to might have contributed to the greater effects of retene on these fish. The ABC-transporter proteins, ABCB2 and ABCG2, eliminate glutathione-, glucuronide-, and sulfate-conjugated metabolites of xenobiotic, including glutathione-conjugated metabolites of PAHs, from cells (Konig et al., 1999; Ebert et al., 2005; Hessel et al., 2013). Therefore, greater

incidences of pericardial edema in larvae co-exposed to 5× of BML-OSPW and retene compared to larvae exposed only to retene might have been a result of inhibition of activity of ABC-transporter proteins, which has been reported previously (Alharbi et al., 2016a). In addition to this potential mechanism of toxicity, greater toxicity might have been caused by effects on expression of genes important for metabolism of retene. Previously, it has been demonstrated that abundances of transcripts of *abcc2* and *abcg2* are greater in fathead minnows exposed to OSPW (Wiseman et al., 2013a). Results of the current study support this effect because abundances of transcripts of *abcc2* and *abcg2* was greater in larvae exposed to 5× of BML-OSPW. Also, the lack of any effect of exposure only to retene on expression of *abcc2* and *abcg2* is consistent with effects of retene on expression of these genes in embryos of rainbow trout (*Oncorhynchus mykiss*) (Vehniäinen et al.,2016). However, expression of *abcc2* and *abcg2* in larvae co-exposed to retene and 5 × equivalent of BML-OSPW was significantly lesser compared to larvae exposed only to 5× of BML-OSPW. The mechanism of this response is not known, but the same response occurred for expression of *gst*, which is important for phase II metabolism of PAHs. If lesser abundances of transcripts of *abcc2*, *abcg2* and *gst* in larvae co-exposed to retene and 5 × equivalent of BML-OSPW compared to larvae exposed only to 5 × equivalent of BML-OSPW resulted in lesser abundances of these proteins it might have compromised the ability to detoxify the greater amounts of retene that these fish were exposed to and might have contributed to the toxic effects that were observed.

Results of the current study demonstrate that dissolved organic compounds in OSPW have potential to alter partitioning and effects of PAHs on aquatic organisms. While mechanisms by which these effects occur are not known at this time, it appears that dissolved organic compounds in OSPW might increase exposure and uptake of PAHs by aquatic organisms. However, it does

not appear that acute effects related to activation of the AhR occur at environmentally relevant concentrations. Also, results of the study provide additional evidence that aging of OSPW in reclamation ponds is an effective method to attenuate adverse effects of OSPW. The study emphasises the importance of future research to investigate interactions among chemicals that co-exist in tailings ponds rather than focusing on one specific group of chemicals.

5 CHAPTER 5: TOXICOKINETICS AND TOXICODYNAMICS OF CHLORPYRIFOS IS ALTERED IN EMBRYOS OF JAPANESE MEDAKA EXPOSED TO OIL SANDS PROCESS-AFFECTED WATER: EVIDENCE FOR INHIBITION OF P-GLYCOPROTEIN

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Author Contributions:

Hattan A. Alharbi (University of Saskatchewan) conceived, designed, managed and did the experiment, generated and analyzed the data, wrote, and drafted the manuscript.

Ahmed Al-Mousa (University of Saskatchewan) provided laboratory assistance with the multi drug resistance assay systems.

Jane Alcorn (University of Saskatchewan) provided scientific input on multi drug resistance assay systems data, guidance, commented on and edited the manuscript.

Drs. Steve Wiseman and John P. Giesy (all at University of Saskatchewan) provided inspiration, scientific input, and guidance, commented on and edited the manuscript, and provided funding for the research.

5.1 ABSTRACT

Oil sands process-affected water (OSPW) is generated during extraction of bitumen in the surface mining oil sands industry in Alberta, Canada. Studies were performed *in vitro* by use of Caco-2 cells, and *in vivo* with larvae of Japanese medaka (*Oryzias latipes*) to determine if organic compounds from the aqueous phase of OSPW inhibit ATP binding cassette protein ABCB1 (permeability-glycoprotein, P-gp). Neutral and basic fractions of OSPW inhibited activity of P-gp in Caco-2 cells by 1.9- and 2.0-fold, respectively, while the acidic fraction had the least effect. The organophosphate pesticides chlorpyrifos (a substrate of P-gp) and malathion (not a substrate of P-gp), were used as model chemicals to investigate inhibition of P-gp in larvae. Co-exposure to chlorpyrifos and an extract of OSPW containing basic and neutral compounds reduced survival of larvae to 26.5% compared to survival of larvae exposed only to chlorpyrifos, which was 93.7%. However, co-exposure to malathion and the extract of OSPW did not cause acute lethality compared to exposure only to malathion. Accumulation and bioconcentration of chlorpyrifos, but not malathion, was greater in larvae co-exposed with the extract of OSPW. The terminal elimination half-life of chlorpyrifos in larvae exposed to chlorpyrifos in freshwater was 5 days compared with 11.3 days in larvae exposed to chlorpyrifos in OSPW. Results suggest that in non-acute exposures, basic and neutral organic compounds in the water-soluble fraction of OSPW inhibit activity of P-gp, which suggests that OSPW has the potential to cause adverse effects by chemosensitization.

5.2 Introduction

Extraction of bitumen in the surface mining oil sands industry in northern Alberta, Canada, generates liquid fine tailings that are held in tailings ponds and recycled for use in the extraction of bitumen. As fine tailings settle a layer of mature fine tailings is formed at the base of tailings ponds, leaving behind an aqueous layer of oil sands process-affected water (OSPW) (Allen, 2008). Because companies do not discharge the contents of tailings ponds to the ambient environment there is more than 1 billion m3 of OSPW currently being held in tailings ponds, and this volume will increase as surface mining continues (Government of Alberta, 2011). Chemical compositions in tailings ponds are complex. In addition to a variety of metals and salts, more than 3000 elemental compositions containing oxygen (O_x), sulfur (SO_x) and nitrogen (NO_x) have been detected in the water-soluble organic fraction of OSPW by use of ultrahigh resolution mass spectrometry (Barrow et al., 2010; Grewer et al., 2010; Pereira et al., 2013a). In addition, polycyclic aromatic hydrocarbons (PAHs) and dibenzothiophenes are constituents of tailings ponds and are associated primarily with fine tailings (Galarneau et al., 2014; Madill et al., 1999; Rogers et al., 2002).

Exposure to OSPW causes a variety of toxicities. Disruption of sex steroid synthesis and signaling (He et al., 2010, 2011, 2012a; Leclair et al., 2015), impaired reproduction of fish (Kavanagh et al., 2012), immunotoxicity (MacDonald et al., 2013) and impairment of growth and development of invertebrates (Anderson et al., 2012a, b) and fish (He et al., 2012b) have been reported. Recently, a novel mechanism by which OSPW might cause adverse effects was reported. Specifically, dissolved organic compounds in OSPW inhibit activity of the ATP-binding cassette (ABC) superfamily proteins (Alharbi et al., 2016a). Members of the ABC superfamily of transporters are involved in a variety of processes, including transport of endogenous and exogenous chemicals across biological membranes (Epel et al., 1998; 2008; Kurelec, 1992; Leslie

et al., 2005; Luckenbach et al., 2014; Schinkel and Jonker, 2003). ABC transporters are important in the defense against xenobiotic compounds by mediating the active efflux of parent compounds or products of their phase II biotransformation (Luckenbach et al., 2014). For example, multidrug resistance proteins (MRPs), which are members of the ABC superfamily of proteins, remove glutathione-conjugated metabolites of PAHs from cells (Hessel et al., 2013; Kranz et al., 2014). However, numerous chemicals that are structurally diverse can competitively or non-competitively inhibit activity of ABC transporters, causing greater bioaccumulation of substrates of ABC transporters and greater sensitivity to these substrates, a phenomenon known as chemosensitization (Kurelec, 1997; Kurth et al., 2015; Luckenbach et al., 2014). Therefore, there is the potential for compounds that might otherwise be considered as not posing a threat to the health of organisms to cause adverse effects, particularly when they occur as part of complex mixtures with toxic compounds that are substrates of ABC proteins (Epel et al., 2008; Kurelec, 1997). Although numerous chemicals are chemosensitizers, whether chemosensitization is a mechanism of toxicity at environmentally relevant concentrations of chemicals is not known (reviewed in Kurth et al., 2015).

The permeability-glycoprotein (P-gp or ABCB1) is the most studied ABC transporter and understanding of chemosensitization is based mostly on results of studies of P-gp (Luckenbach et al., 2014). Efflux activity of P-gp protects cells from the effects of a variety of natural and anthropogenic xenobiotic that are diverse in their physiochemical properties (Leslie et al., 2005). Inhibition of this transporter is of toxicological importance as any disruption of its functioning can enhance toxicities of compounds that are substrates of P-gp. For example, mortality of zebrafish (*Danio rerio*) embryos increased when they were co-exposed to inhibitors of P-gp and the environmentally relevant PAH, phenanthrene or the anticancer drug vinblastine (Fischer et al.,

2013). Similarly, the mortality of zebrafish embryos was greater when they were exposed to vinblastine and perfluorooctane sulfonate, which inhibits P-gp (Keiter et al., 2016).

It has been proposed that chemicals in the water-soluble organic fraction of OSPW might act as chemosensitizers by inhibiting activity of ABC transporters but it was not known if these chemicals inhibit P-gp (Alharbi et al., 2016a). Therefore, the goal of this study was to use *in vitro* and *in vivo* methods to determine if compounds in the water-soluble organic fraction of OSPW inhibit P-gp and cause chemosensitization to substrates of P-gp. First, the effects of organic compounds extracted from the aqueous phase of OSPW on accumulation of the fluorescent dye calcein-AM, which is a substrate of P-gp, was quantified in Caco-2 cells. Second, the effects of organic compounds extracted from the aqueous phase of OSPW on uptake, depuration and toxicity of the organophosphate pesticides, chlorpyrifos and malathion, to larvae of Japanese medaka was determined. Although chlorpyrifos or malathion are not of toxicological relevance in the context of oil sands mining or tailings ponds used to hold OSPW, these model chemicals were used to investigate P-gp inhibition and chemosensitization by OSPW. Cytochrome P450 enzymes convert chlorpyrifos to a toxic metabolite, chlorpyrifos oxon, which causes acute lethality by inhibition of acetylcholine esterase (Fukuto, 1990). P-gp affords protection from acute lethality by active efflux of chlorpyrifos (Zaja et al., 2011) and chlorpyrifos oxon (Lanning, 1996). Therefore, it was hypothesized that inhibition of P-gp by OSPW would cause an increase in concentrations of chlorpyrifos and therefore an increase in chlorpyrifos oxon in cells, which would result in greater incidence of acute lethality. Malathion causes acute lethality by the same mechanism, but it is not a substrate of P-gp (Zaja et al., 2011). Therefore, inhibition of P-gp by OSPW would not be expected to cause an increase in concentrations of malathion in cells and therefore would not cause an increase in acute lethality.

5.3 Materials and methods

5.3.1 Chemicals and oil sands process-affected water samples

Verapamil was purchased from the Cayman Chemical Company (Ann Arbor, MI, USA) and calcein-AM was from AAT Bioquest (Sunnyvale, CA, USA). Dimethyl sulfoxide (DMSO) and trypan blue were from the Sigma Chemical Company (Oakville, ON, Canada). Caco-2 cells (human colon adenocarcinoma cells) were purchased from the American Type Culture Collection (Rockville, MD, USA). Fetal bovine serum (product no. F6178) that was sterile filtered was purchased from the Sigma Chemical Company. Penicillin/streptomycin, 10 mm minimal essential medium non-essential amino acids, 100 mm (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, penicillin streptomycin, phosphate-buffered saline, Hanks' balanced salt solution, trypsin-EDTA (0.25% trypsin with 1 mm EDTA.4Na) and Dulbecco's modified Eagle's medium with high glucose, l-glutamine, pyruvate and phenol red (product no. 11995-065) were purchased from ThermoFisher Scientific (Burlington, ON, Canada). Chlorpyrifos, chlorpyrifos oxon, malathion and malathion-*d10* standards, each of which was >98% purity, were purchased from AccuaStandard (New Haven, CT, USA). Acetone, hexane, dichloromethane and ethyl-acetate, each of high-performance liquid chromatography grade, were purchased from Fisher Scientific (Ottawa, ON, Canada). Anhydrous ethanol was obtained from GreenField Ethanol Inc. (Brampton, ON, Canada). OSPW was collected from Base Mine Lake, which is an end-pit-lake constructed from the West-In-Pit settling basin that received input of tailings from the main extraction facility until December 2012 after which all new inflow of fresh OSPW from the extraction plant was ceased (Syncrude Canada, Ltd., Fort McMurray, AB, Canada). Samples were collected during September 2012 and shipped to the University of Saskatchewan (Saskatoon, SK, Canada), where, upon arrival, they were inspected visually and observed to be free of any residual bitumen or

mature fine tailings. OSPW was stored in the dark until extraction to prevent any potential photomodification of dissolved organic compounds.

5.3.2 In vitro assays

5.3.2.1 Extraction of dissolved organic chemicals from oil sands process-affected water

Acidic, basic and neutral fractions of OSPW were generated by use of a method that has been described previously (Alharbi et al., 2016a). Because the goal of the study was to investigate inhibition of P-gp by chemicals in the water soluble organic fraction of OSPW, a 500 mL sample of OSPW was passed through a glass microfiber filter (GF/D 0.47 mm, Whatman) to remove particulates. Next, the sample was acidified to pH 2 by use of concentrated HCl (37%). To isolate the basic fraction, cartridges containing 500 mg of mixed-mode Strata®-X Polymeric-C solid-phase sorbent (Phenomenex, Milford, MA, USA) were conditioned with 6 ml of methanol and 6 ml of acidified water, and OSPW that had been acidified to pH 2 was passed through cartridges under vacuum. Cartridges then were washed with 2% of formic acid and dried under vacuum for 30 min. Acidic and neutral compounds were isolated in the first elution with methanol. The second elution was performed with 5% (v/v) of NH_4OH in methanol and this fraction contained basic compounds. Acidic and neutral compounds from the first elution were separated by use of 500 mg of Strata®-X-A solid-phase matrix in plastic cartridges (Phenomenex). Prior to use cartridges were conditioned by washing with 100% methanol followed by 5% (v/v) of NH_4OH (aq). Eluant I from the Strata®-X Polymeric-C sorbent (acidic and neutral compounds) was evaporated to approximately 0.5 mL, adjusted to a pH of 10-11 with NaOH, and then passed through the Strata®-X-A solid-phase matrix without vacuum. The cartridge was washed with 5% (v/v) of NH_4OH (aq) and left to dry under vacuum for 30 min. A fraction containing neutral

compounds was eluted with 100% of methanol and a fraction containing acidic compounds was eluted with 2% (v/v) of formic acid in methanol. Each fraction was dried under a gentle stream of nitrogen and reconstituted in 500 µL of absolute ethanol to generate a sample in which the concentration of organics was enriched to an equivalent of 1000× greater than in the original sample of OSPW. A pooled sample representative of the organic fraction was generated by pooling equal volumes of the acidic, neutral, and basic fractions. The profile of heteroatom classes in these fractions has been described previously (Alharbi et al., 2016a). These samples were used in the calcein-Am assay that was performed in Caco-2 cells.

5.3.2.2 Cytotoxicity

The human colon adenocarcinoma cell line (Caco-2) is the most common in vitro model used to investigate substrates and inhibitors of P-gp (Balimane and Chong, 2005; Elsby et al., 2008; Siissalo et al., 2007). Effects of OSPW on viability of Caco-2 cells were quantified to identify sub lethal concentrations that could be used to determine inhibition of P-gp. Cell viability was determined by use of the CellTiter 96® AQueous One Cell Proliferation Assay (MTS) according to the protocol provided by the manufacturer (Promega Corporation, Madison, WI, USA). Caco-2 cells (5000 cells per well) were seeded into each well of a 96-well plate and placed in a humidified atmosphere of 5% CO_2 at 37 °C. After 24 h the medium, which was Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, was replaced with medium containing 0.5, 1, 2.5 or 5× equivalent of the pooled sample of OSPW or 5× equivalent of the acidic, basic or neutral fraction. Cells were incubated in a humidified atmosphere of 5% CO_2 at 37 °C for 1 or 24 h. The concentration of ethanol in exposure solutions was 0.1% (v/v) and controls were performed to ensure this concentration was not cytotoxic. At the

end of the exposure period, cells were washed with fresh media and incubated with the MTS solution in a humidified atmosphere of 5% CO_2 for 4 h at 37 °C. Viability of cells was quantified using a spectrophotometric Elx800TM microplate reader (BioTek Instruments, Winooski, VT, USA). Three independent experiments were conducted and there were eight replicates (n = 8) per experiment.

5.3.2.3 Effect of oil sands process-affected water on accumulation of calcein in Caco-2 cells to assess inhibition of the permeability-glycoprotein

An assay with the fluorescent dye, calcein-AM, was used to determine the effects of the acidic, basic and neutral fractions on activities of P-gp in Caco-2 cells, based on a method described elsewhere (Volpe, 2011). Caco-2 cells overexpress P-gp and other membrane transporters comparable to the human small intestine (Englund et al., 2006). Calcein-AM, which is the acetoxymethyl ester of calcein, enters cells by passive diffusion and is hydrolyzed enzymatically into the fluorescent dye calcein by esterases. Because calcein is transported from cells by ABC transporters, including inhibition of activity of P-gp, for example by verapamil, this results in accumulation of calcein that can be measured as an increase of intracellular fluorescence (Bansal et al., 2009; Eneroth et al., 2001; Glavinas et al., 2011). Briefly, 5000 cells were seeded in each well of a 96-well black plate with clear bottoms (Thermo Scientific). Cells were incubated for 48 h in a humidified atmosphere of 5% CO_2 at 37 °C to maintain 80–90% confluence. The medium was replaced after 24 and 48 h. Medium was removed and cells were washed with phosphate-buffered saline warmed to 37 °C, then cells were exposed for 15 min in a humidified atmosphere of 5% CO_2 at 37 °C to either 10 μm of verapamil dissolved in DMSO (positive control), 0.5, 1, 2.5 or 5× equivalent of the pooled organic fraction, or 5× equivalent of fractions of OSPW. Cells were exposed to either DMSO or ethanol at a final concentration of 0.1% to

control for solvent effects. Next, cells were washed twice with media warmed to 37 °C, then 200 μL of media containing samples of OSPW or verapamil and calcein-AM was added and plates were incubated for 60 min in a humidified atmosphere of 5% CO_2 at 37 °C. Fluorescence of calcein (excitation 485 nm, emission 538 nm) was recorded immediately by use of a microtiter plate reader (Elx800TM microplate reader; BioTek Instruments). The accumulation of calcein in cells was calculated by subtracting the background fluorescence measured in a blank well and the fold-change in fluorescence caused by exposure to fractions was calculated compared to the solvent control.

5.3.3 In vivo assays

5.3.3.1 Isolation of basic and neutral compounds from oil sands process-affected water

The method used to prepare OSPW for *in vivo* assays was different from the method used to generate fractions of OSPW for use in Caco-2 cells. Because results of the *in vitro* assay indicated that basic and neutral compounds, but not acidic compounds, in the water soluble organic fraction of OSPW inhibit P-gp, a method that isolates basic and neutral compounds into one fraction and contains smaller amounts of acidic compounds was used to prepare samples for assays with embryos (Vieno et al., 2006). Details of this method have been described previously (Alharbi et al., 2016b). Briefly, 1000 mL of OSPW was passed through a glass microfiber filter (GF/D 0.47 mm, Whatman) to remove any particulate matter and the pH of the OSPW adjusted to pH 9 by use of NH_4OH. Pre-concentration of samples was performed in one generic step by use of EVOLUTE®ABN sorbent (Biotage, Charlotte, NC, USA). Before addition of OSPW, two cartridges were conditioned with 6 mL of methanol followed by 6 mL of ultrapure Milli-Q water (Millipore, Mississauga, Canada). Next, 500 mL of OSPW was passed through each cartridge under vacuum at a flow rate of 10–15 $mL.min^{-1}$. At a pH 9, acidic compounds will not bind the

ABN sorbent. Subsequently, cartridges were washed with Milli-Q water and allowed to dry under vacuum for 30 min. Basic and neutral compounds were isolated in one step in 6 mL of methanol without use of vacuum. Samples were pooled, evaporated to dryness under a gentle stream of nitrogen, and reconstituted in 500 µL of absolute ethanol. Therefore, the concentration of dissolved organic compounds in the final sample was 2000× greater than in the original sample of OSPW. The profile of heteroatom classes in these fractions has been described previously (Alharbi et al., 2016b).

5.3.3.2 Acute lethality

Embryos of Japanese medaka (orange-red strain) were collected from a culture maintained in the Aquatic Toxicology Research Facility at the University of Saskatchewan (Saskatoon, SK, Canada). Culturing of adult fish and rearing of embryos until they were required for exposures was conducted in dechlorinated City of Saskatoon municipal tap water at a temperature of 28 °C and a photoperiod of 16 h:8 h (light: dark). Protocols for culturing of fish were approved by the University of Saskatchewan's Animal Research Ethics Board, and adhered to the Canadian Council on Animal Care guidelines for humane animal use (UCACS-AREB; # 20090108).

Effects of OSPW on acute lethality of chlorpyrifos and malathion were quantified. Larvae at 8 ± 1 dpf were exposed for 24 or 48 h to 100 or 250 µg L^{-1} chlorpyrifos or 15 mg L^{-1} malathion, either in freshwater or in 1 or 2.5× equivalent of OSPW. Acute lethality of larvae exposed only to 2.5× equivalent of OSPW also was quantified. Exposures were performed in 6-well tissue culture plates with 5 ml of solution in each well. Each exposure consisted of 10-12 larvae and all exposures were conducted 5 times (n=5) each with a new batch of larvae.

5.3.3.3 Body burden of chlorpyrifos and malathion

Concentrations of chlorpyrifos, chlorpyrifos oxon, and malathion were quantified in larvae to assess effect of OSPW on body burden. Larvae at 8±1 dpf were exposed to 100 µg L^{-1} of chlorpyrifos or malathion either in freshwater or in 1× equivalent of OSPW because these exposure conditions did not cause acute lethality. Exposures of larvae to chemicals was performed in 6-well tissue culture plates with 5 ml of solution per well. Each exposure consisted of 30 larvae and all exposures were conducted in triplicate (n=3), each with a new batch of larvae. Ten larvae and 1 ml of solution were sampled at 24 h of exposure. Next, larvae exposed only to chlorpyrifos or malathion were transferred to a solution of freshwater and larvae exposed to chlorpyrifos or malathion in 1× equivalent of OSPW were transferred to a solution of 1× equivalent of OSPW for 24 h to assess depuration. Ten larvae and 1 ml of solution was sampled at the end of the depuration period. Samples were stored at -20 ºC until required for analysis.

5.3.3.4 Kinetics of bioaccumulation and depuration of chlorpyrifos

Effects of OSPW on kinetics of uptake and depuration of chlorpyrifos were determined. Protocols used in this assay have been described previously (El-Amrani et al., 2012). The concentration of chlorpyrifos was 10 µg l^{-1}, the same concentration used previously to quantify bioaccumulation of chlorpyrifos in larvae of zebrafish (El-Amrani et al., 2012). This concentration is less than 1% of the LC_{50} of chlorpyrifos and therefore meets recommendations for assessing bioaccumulation in fish (OECD, 2012). Approximately 100 larvae at 8 ± 1 dpf were placed in glass petri dishes (100 × 15 mm) containing 50 ml of chlorpyrifos either in freshwater or 1× equivalent of OSPW. Larvae were collected at 0, 2, 6, 12, 21, 29, 45 and 48 h of exposure during the uptake phase. For the depuration phase, larvae were transferred to petri dishes containing 50 ml of

freshwater or 1× equivalent of OSPW and larvae were sampled at 50, 54 and 72 h. Larvae were killed by use of hypothermic shock in ice water (Strykowski and Schech, 2015). Ten larvae and 1 ml of solution were collected at each time point and larvae were stored at –20 °C until required for analysis. No mortality was observed during the uptake or depuration phases of the study. Bioaccumulation and depuration of chlorpyrifos was calculated using a model that uses first-order kinetics to describe uptake and depuration (El-Amrani et al., 2012; Gobas and Zhang, 1992; Mackay and Fraser, 2000; Sanz-Landaluze et al., 2015; Tu et al., 2014). Rate of uptake Eqn (1) and depuration Eqn (2) of chlorpyrifos were calculated.

$$\frac{dC_B}{dt} = k_1 \,.\, C_w - k_2 . C_B \qquad (1)$$

$$\frac{dC_B}{dt} = -k_2 \,.\, C_B \qquad (2)$$

where C_B is concentration of chlorpyrifos in larvae (expressed in ng g^{-1} w/w), t is the exposure time (h), k_1 is the first-order uptake constant (l kg^{-1} wet mass h^{-1}), C_w is concentration in solution (expressed in ng ml^{-1}) and k_2 is the first-order elimination rate constant (per h).

The bioconcentration factor (BCF) of chlorpyrifos was calculated (Equation 3).

$$BCF_k = k_1/k_2 \qquad (3)$$

The terminal elimination half-life ($t_{1/2}$) of chlorpyrifos in larvae was calculated (Equation 4).

$$t_{1/2} = 0.693/K_2 \qquad (4)$$

5.3.3.5 Quantification of chlorpyrifos and malathion by liquid chromatography-tandem mass spectrometry

Concentrations of chlorpyrifos, chlorpyrifos axon and malathion in solutions and larvae were determined by use of a method described previously (El-Amrani et al., 2012). One ml of each exposure solution was extracted twice with 500 µL of a 1: 1 mixture of hexane/ethyl acetate before analysis. Pools of larvae were rinsed several times with nano-pure water to remove any chemicals adsorbed to the body surface. Next, larvae were homogenized in 1 ml of a 1: 1 mixture of hexane/ethyl acetate, followed by ultra sonication for 1 min. After extraction, samples of larvae and exposure solutions were centrifuged at 9000 g for 5 min, and the organic phase was removed and concentrated under a gentle stream of nitrogen until dry. Next, samples were reconstituted in 195 µL of 100% of methanol and transferred to an autosampler vial fitted with a glass insert and 5 µL of a solution containing 1 µg ml^{-1} malathion-*d10* was added to samples as an internal standard for analysis. After extraction, samples were filtered through a 13 mm × 0.2 µm nylon syringe filter (Whatman) and into a 1.5 ml amber LC vial. Separation of chlorpyrifos, chlorpyrifos oxon and malathion was performed by use of high-performance liquid chromatography (Agilent Technologies, Santa Clara, CA, USA) on a Kinetex C18 100 A column (Phenomenex, 100 mm × 4.6 mm, 5 µm particle size), using water (A) and methanol (B) as solvents at a flow rate of 0.250 ml min^{-1}. The solvent gradient was 10% B increasing to 85% B over 15 min, then increasing to 95% B at 25 min, before returning to 10% B and holding for 5 min. Mass spectra were collected by use of an ABI SCIEX (Milford, MA, USA) 3000 triple quadrupole tandem mass spectrometer fitted with an electrospray ionization source operated in positive ionization mode (multiple reaction monitoring), by using the following operation parameters: temperature 500 °C; capillary voltage, 5.5 kV; collision gas, nebulizer gas, curtain gas were 4, 8 and 10 respectively.

Data were analyzed was by use of Analyst 1.4.1 software (Applied Biosciences, Foster City, CA, USA). Multiple reaction monitoring, recovery, limit of detection and limit of quantification are presented in Table 5.1.

Table 5.1. Multiple reaction monitoring (MRM), Recoveries, Relative Standard Deviations (RSDs), Limits of Detections (LODs), and Limits of Quantifications (LOQs) of chlorpyrifos, chlorpyrifos oxon and malathion analyzed by use of LC-MS.MS.

Pesticide	**LOD (ng/g)**	**LOQ (ng/g)**	**Concentration in exposure solutions**		**Concentration in Larvae**		**MRM**	
			Spiked (ng/mL)	Recovery ±RSD%	Spiked (ng/g)	Recovery ±RSD%	Quantification ion	Confirmation ion
Chlorpyrifos	0.19	0.73	50	98 ±5.4%	500	97±2.8%	349.9>197.9	349.9>97
Chlorpyrifos oxon	0.11	0.45	10	96.4±3.7%	250	94±3.3%	336>280	336>308
Malathion	1.67	6.2	50	95.7±7.8%	500	97.4±1.4%	331>127	331>99
Malathion d_{10} (IS)	ND	ND	ND	ND	ND	ND	343.3>132	343.3>100

5.4 Statistical Analysis

Effects of treatments on cell viability, accumulation of calcein in Caco-2 cells, survival of larvae, and concentrations of chlorpyrifos, chlorpyrifos oxon and malathion in larvae or solutions was determined by use of GraphPad Prism 5 software (San Diego, CA, USA). Normality of data was assessed using the Kolmogorov–Smirnov one-sample test and homogeneity of variance was determined using Levene's test. If necessary, data were log transformed to ensure normality and homogeneity of variance. Significant differences among treatments were evaluated by use of an unpaired t-test (for internal concentration of chlorpyrifos and malathion and accumulation of calcein-AM in caco-2 exposed to verapamil) or one-way ANOVA followed by Tukey's post-hoc

test (for cell viability, accumulation of calcein-AM in Caco-2 cells exposed to OSPW and survival of larvae of Japanese medaka). Differences were considered significant at $P \leq 0.05$. Concentrations of chlorpyrifos in larvae and in solutions were used for calculations of BCF by use of Origin 9.1 software (OriginLab Corporation, Northampton, MA, USA).

5.5 Results

5.5.1 In Vitro Assays

5.5.1.1 Cytotoxicity of oil sands process-affected water

Organic chemicals extracted from the water-soluble phase of OSPW were acutely toxic to Caco-2 cells, but only at concentrations greater than 1× equivalent in the original sample of this OSPW, and only after 24 h of exposure. Cytotoxicity was not observed after 1 h of exposure (data not shown). Viability of Caco-2 cells exposed to a 5× equivalent of the pooled sample of OSPW was significantly less ($79.6 \pm 1.1\%$) than the solvent control (Figure. 5.1A). At equivalents of 0.5, 1 or 2.5× the pooled sample of OSPW was not cytotoxic. Neither the neutral nor the basic fraction of OSPW was cytotoxic at concentrations tested. Viability of cells exposed to 5× equivalent of the acidic fraction of OSPW was significantly lesser ($74.3 \pm 1.0\%$) compared to the solvent control (Figure. 5.1B).

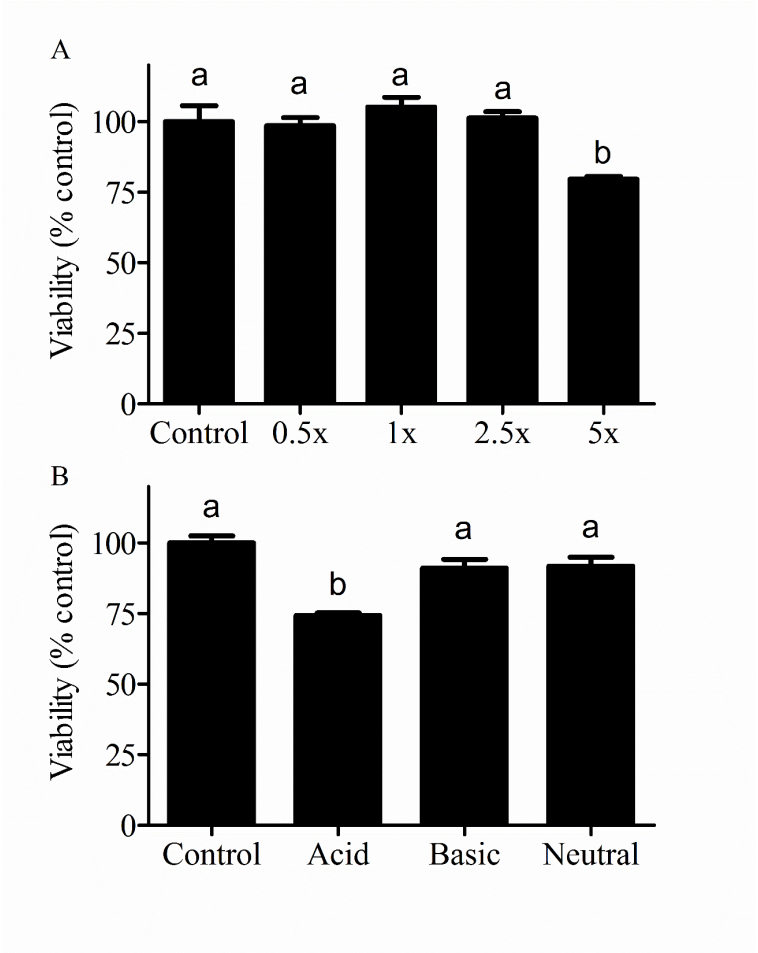

Figure 5.1: Viability of Caco-2 cells exposed to (A) four concentrations of pooled sample of oil sands process-affected water, and (B) a 5× equivalent of the acidic, basic or neutral fraction of oil sands process-affected water. Viability is expressed as a percentage of control. Cells were exposed for 24 h. Data are expressed as mean ± standard deviation of three independent experiments in which the number of replicates per experiment was eight. Significant differences in viability were determined by use of one-way ANOVA followed by a Tukey's post-hoc test ($n = 8$, $P \leq 0.05$) and are designated by different letters. Error bar represents the standard deviation.

5.5.1.2 Effects of oil sands process-affected water on accumulation of calcein in Caco-2 cells

Efflux of calcein from Caco-2 cells was inhibited by the water soluble organic fraction of OSPW. Retention of calcein in cells exposed to verapamil, which is an inhibitor of P-gp, was 1.5 ± 0.1-fold greater compared to cells exposed to the solvent control (Figure. 5.2A). Exposure to 1, 2.5, or 5× equivalent of the pooled fraction of OSPW increased cellular retention of calcein by 1.2 ± 0.03-, 1.2 ± 0.04-, and 1.5 ± 0.05-fold, respectively, compared to cells exposed to the solvent control (Figure. 5.2B). Retention of calcein in cells exposed to 5× equivalent of acidic, basic, or neutral fractions of OSPW was significantly greater by 1.3 ± 0.02-, 1.9 ± 0.01-, and 2.0 ± 0.01-fold, respectively, compared to cells exposed to the solvent control, and the amount in cells exposed to neutral and basic fractions was greater than in cells exposed to the acidic fraction (Figure.5.2C).

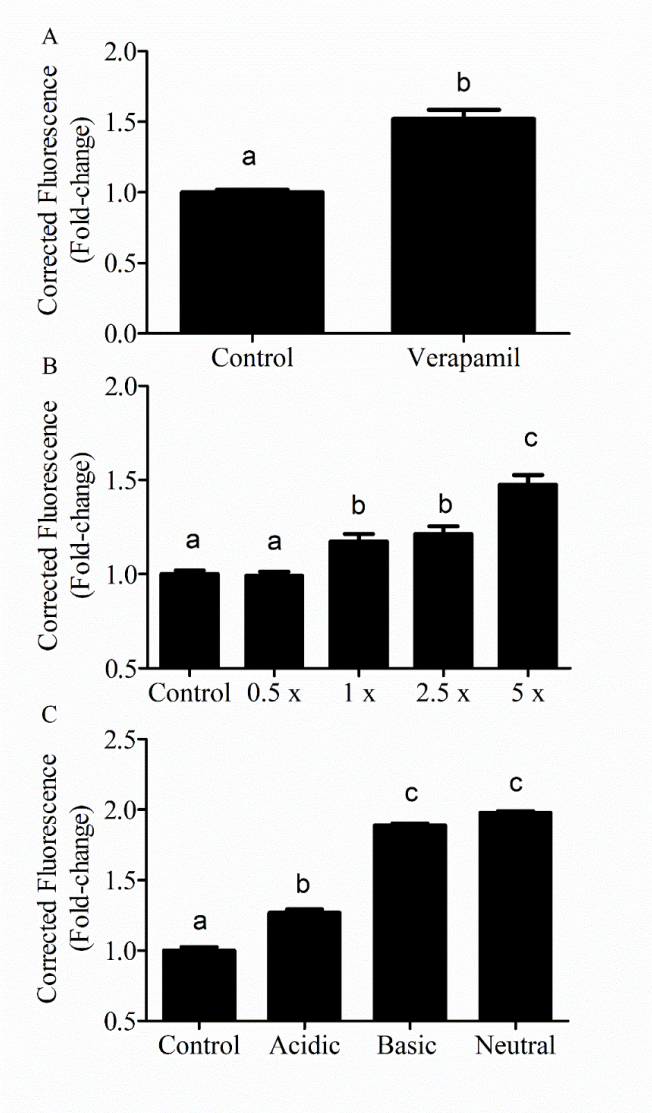

Figure 5.2: Accumulation of calcein in Caco-2 cells exposed to (A) 10 µm verapamil, (B) pooled sample of OSPW and (C) acidic, basic and neutral fractions of OSPW. Cells were exposed to calcein-AM for 15 min before co-exposure to either (B) 5×, 2.5×, 1× and 0.5× equivalents of the pooled organic fraction of OSPW for 1 h or (C) 5× equivalents of the acid, neutral and basic fraction of OSPW for 1 h. Control cells were exposed to cell culture medium containing 0.1% v/v DMSO. Accumulation of calcein was measured as fluorescence and data are expressed as mean ± standard deviation of three independent experiments in which the number of replicates per experiment was eight. Effect of verapamil was assessed by use of an unpaired t-test. Effects of OSPW were assessed by use of a one-way ANOVA followed by Tukey's post-hoc test. Significant differences among treatments are indicated by different letters ($n = 8$, $P \leq 0.05$). Error bar represents the standard deviation. OSPW, oil sands process-affected water.

5.5.2 In Vivo Assays

5.5.2.1 Acute lethality

Co-exposure to chlorpyrifos and OSPW had a significant effect on the survival of larvae (Figure. 5.3A, B). After 24 h of exposure, survival of larvae exposed to the solvent control, 2.5× equivalent of OSPW, or 100 or 250 μg l^{-1} of chlorpyrifos was 97.5 ± 2.0%, 91.6 ± 8.3%, 93.7 ± 6.3% and 88 ± 6.3%, respectively, and no differences in survival was observed among these groups. However, survival of larvae co-exposed to 2.5× equivalent of OSPW and 100 or 250 μg l^{-1} of chlorpyrifos was 26.5 ± 3.0%, and 15.8 ± 2.2%, respectively, which was significantly lesser compared to the effect of the solvent control, 2.5× equivalent of OSPW and chlorpyrifos alone. Survival of larvae co-exposed to 1× equivalent of OSPW, which is an environmentally relevant concentration, and 100 or 250 μg l^{-1} of chlorpyrifos was 94 ± 6.0% and 85 ± 9.0%, respectively, which is not significantly different from the effect of exposure to the solvent control or 100 or 250 μg l^{-1} of chlorpyrifos alone.

Co-exposure to OSPW and malathion did not affect survival of larvae (Figure. 5.3C). After 24 h of exposure, no differences in the survival of larvae exposed to the solvent control, 2.5× equivalent of OSPW, 15 mg l^{-1} of malathion, or co-exposed to 2.5× equivalent of OSPW and 15 mg l^{-1} of malathion were observed. Because there were no effects after 24 h, the exposure was extended to 48 h. After 48 h of exposure, survival of larvae exposed to 15 mg l^{-1} of malathion or co-exposed to 2.5× equivalent of OSPW and 15 mg l^{-1} malathion was 67.9 ± 6.5% and 69.3 ± 3.6%, respectively, which is not different from each other but significantly less than survival of larvae exposed to the solvent control or 2.5× equivalent of OSPW.

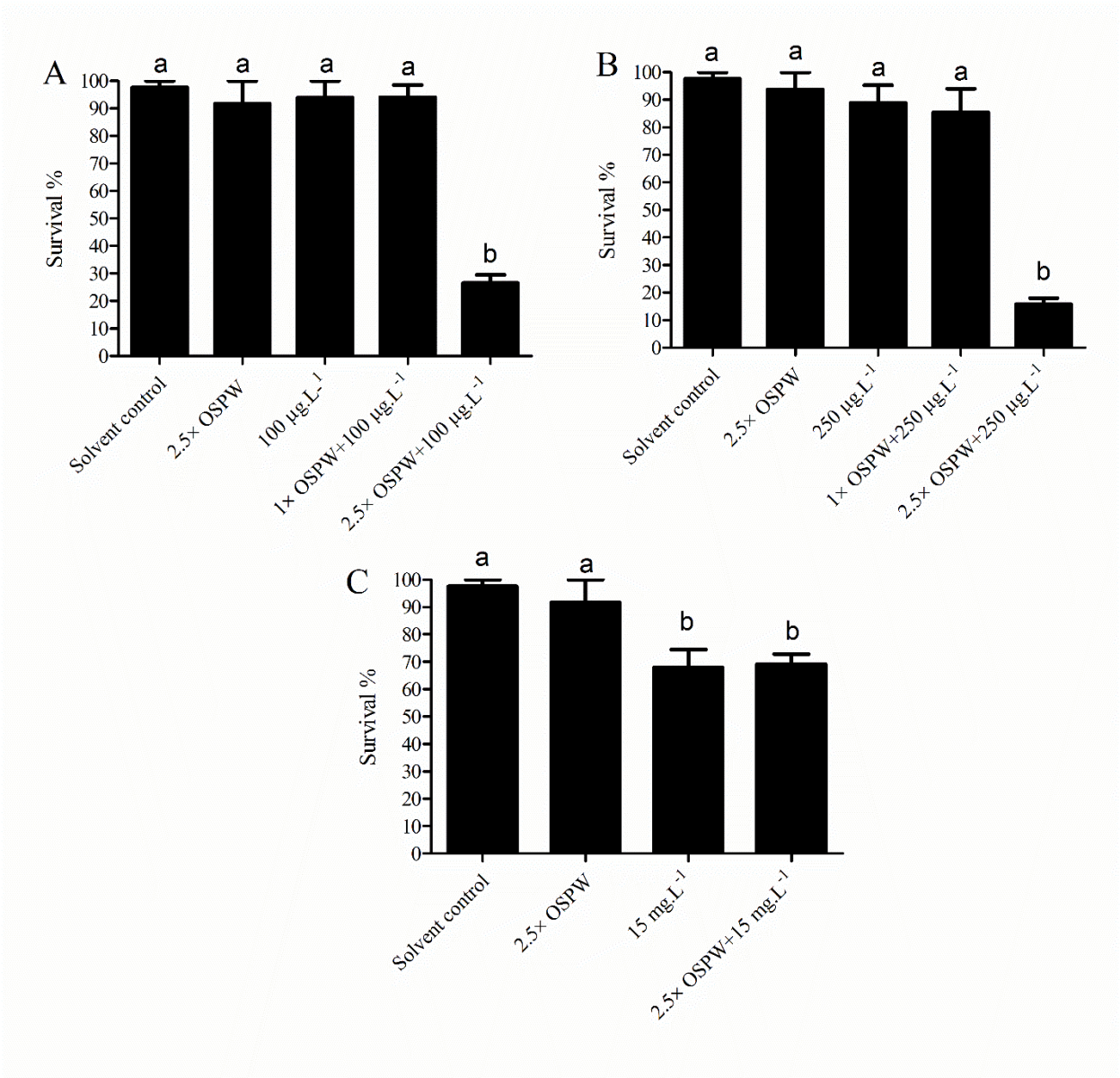

Figure 5.3: Survival of larvae of Japanese medaka exposed to (A) 100 µg l^{-1} of chlorpyrifos, (B) 250 µg l^{-1} of chlorpyrifos or (C) 15 mg l^{-1} of malathion. Approximately 12 larvae at 8 ± 1 dpf were exposed to chlorpyrifos or malathion alone or co-exposed with 1× or 2.5× equivalent of OSPW. Effects of chlorpyrifos were determined after 24 h of exposure. Because malathion did not cause effects after 24 h of exposure the effects were evaluated after 48 h of exposure. Significant differences from control were determined by use of one-way ANOVA followed by Tukey's post-hoc test and different letters indicate significant differences ($n = 5$, $P \leq 0.05$). Error bar represents the standard deviation. OSPW, oil sands process-affected water.

5.5.2.2 Effects of oil sands process-affected water on body burden of chlorpyrifos or malathion

Chlorpyrifos, chlorpyrifos oxon and malathion were quantified in larvae exposed to a nominal concentration of 100 μg l^{-1} of either chemical, either alone or with 1× equivalent of OSPW, because these exposures did not cause acute lethality (Table 5.2). Before the initiation of exposures, concentrations of chlorpyrifos and malathion in freshwater and 1× equivalent of OSPW were less than the limit of detection but concentrations in freshwater and 1× equivalent of OSPW that were spiked with 100 μg l^{-1} chlorpyrifos were 75.1 ± 2.9 and 76.2 ± 5.8 μg l^{-1}, respectively, which are not significantly different. Concentrations of chlorpyrifos in larvae exposed to these solutions for 24 and 48 h were significantly different. Concentrations of chlorpyrifos in samples of solutions taken at 24 h of exposure also were significantly different. After 24 h of depuration, concentrations of chlorpyrifos in freshwater were less than the limit of quantification but mean concentration in the 1× equivalent of OSPW was 16.9 ± 2.9 μg l^{-1}. In addition, concentrations of chlorpyrifos in larvae that had been exposed to chlorpyrifos in 1× equivalent of OSPW were significantly greater than concentrations in larvae exposed to chlorpyrifos in freshwater.

Concentrations of chlorpyrifos oxon in larvae exhibited a similar trend as the parent compound (Table 5.2). Before exposures, concentrations were less than the limit of detection in all solutions, regardless of whether the solution was spiked with chlorpyrifos. After 24 h of exposure, concentrations of chlorpyrifos oxon were less than the limit of quantification in the solution of chlorpyrifos in freshwater but were 0.6 ± 0.1 μg l^{-1} in the solution of chlorpyrifos in 1× equivalent of OSPW. In larvae sampled at 24 h of exposure, concentrations of chlorpyrifos oxon

were significantly lesser in larvae exposed to chlorpyrifos in freshwater compared to chlorpyrifos in 1× equivalent of OSPW. Similar to this observation, after 24 h of depuration, concentrations of chlorpyrifos oxon in larvae that had been exposed to chlorpyrifos in freshwater were significantly lesser than concentrations in larvae that had been exposed to chlorpyrifos in 1× equivalent of OSPW.

Exposure to OSPW did not affect concentrations of malathion in larvae (Table 5.2). Before initiation of exposures, concentrations of malathion in freshwater or 1× equivalent of OSPW were less than the limit of detection but concentrations in freshwater or 1× equivalent of OSPW that were spiked with 100 μg l^{-1} of malathion were 63.4 ± 3.6 and 66.8 ± 2.8 μg l^{-1}, respectively, which were not significantly different. After 24 h of exposure of larvae to these solutions, concentrations of malathion in freshwater or 1× equivalent of OSPW were not different. In addition, concentrations of malathion were not different in larvae exposed to malathion in freshwater or 1× equivalent of OSPW for 24 or 48 h. After 24 h of depuration the concentration of malathion in freshwater and in 1× equivalent of OSPW was less than the limit of quantification. There was no difference in concentrations of malathion in larvae exposed to malathion in freshwater or 1× equivalent of OSPW.

Table 5.2: Concentrations of chlrorpyrifos, chlorpyrifos oxon, and malathion in solutions and in larvae of Japanese medaka during assessment of body burden of these chemicals.

		Exposure					Depuration	
Chemical Measured	**Exposure Scenario**	**Solution (0 hr)**	**Solution (24 hr)**	**Solution (48 hr)**	**Larvae (24 hr)**	**Larvae (48 hr)**	**Solution (24 hr)**	**Larvae (24 hr)**
CPF	CPF	75.1 ± 2.9[a]	34.2 ± 0.9[a]	ND	5625 ± 534[a]	5747 ± 495[a]	< LOQ	2541 ± 206[a]
	CPF + OSPW	76.2 ± 5.8[a]	19.5 ± 1.7[b]	ND	8693 ± 774[b]	8811 ± 460[b]	16.9 ± 2.9	5119 ± 210[b]
CPF Oxon	CPF	< LOQ	< LOQ	ND	25.4 ± 2.0[a]	ND	< LOQ	13.1 ± 0.8[a]
	CPF + OSPW	< LOQ	0.6 ± 0.1	ND	56.1 ± 5.0[b]	ND	0.74 ± 0.1	67.8 ± 8.0[b]
MA	MA	63.4 ± 3.6[a]	29.6 ± 0.7 [a]	ND	3698.8 ± 141[a]	4063.5 ± 101[a]	< LOQ	85.2 ± 15[a]
	MA + OSPW	66.8 ± 2.8[a]	30.8 ± 1.8 [a]	ND	3617.2 ± 150[a]	4016.5 ± 93[a]	< LOQ	83.7 ± 18[a]

CPF, chlorpyrifos; CPF oxon, chlorpyrifos oxon; <LOQ, concentration was less than the limit of quantification; MAL, malathion; ND, concentration was not determined.
Statistical analysis compared concentrations of chlorpyrifos, malathion or chlorpyrifos oxon at each time point and comparisons only were made for different exposures to the same chemical. Different letters indicate a statistical difference.

5.5.2.3 Effects of oil sands process-affected water on the kinetics of uptake and depuration of chlorpyrifos

Differences in body burdens of chlorpyrifos after exposure and depuration in freshwater and OSPW suggested that OSPW affects bioaccumulation of chlorpyrifos. Therefore, effects of OSPW on kinetics of bioaccumulation and depuration of chlorpyrifos were assessed. In general, the profile of uptake and depuration of chlorpyrifos was similar in larvae exposed to chlorpyrifos in freshwater or the 1× equivalent of OSPW (Figure. 5.4). In both exposures, concentrations of chlorpyrifos in larvae increased rapidly during the first 2 h of exposure and were greatest at 24 h, but concentrations of chlorpyrifos did not reach steady state by 48 h in either exposure. However,

concentrations of chlorpyrifos were greater by 2-fold in larvae after 24 h of co-exposure to chlorpyrifos in 1× equivalent of OSPW compared to freshwater. At the end of the depuration phase, concentrations of chlorpyrifos were 8-fold greater in larvae exposed in OSPW compared to freshwater.

The BCF of chlorpyrifos was different in larvae co-exposed with 1× equivalent of OSPW compared to freshwater. At the end of the uptake phase, which was 48 h, the mean concentration of chlorpyrifos in freshwater was 4.3 ± 0.5 μg l^{-1} but was 1.8 ± 0.3 μg l^{-1} in 1× equivalent of OSPW. The log BCF in larvae exposed to chlorpyrifos in freshwater was 3.43 but was 3.95 in larvae exposed to chlorpyrifos in 1× equivalent of OSPW. The rate of uptake of chlorpyrifos (k_1) was 374 μg l^{-1} h^{-1} in larvae exposed to chlorpyrifos in freshwater but was 543.4 μg l^{-1} h^{-1} in larvae exposed to chlorpyrifos in 1× equivalent of OSPW. The terminal elimination half-life of chlorpyrifos in larvae exposed to chlorpyrifos in freshwater was 5 days compared to 11.3 days in larvae exposed to chlorpyrifos in 1× equivalent of OSPW. Finally, the rate of depuration of chlorpyrifos (k_2) was 0.14 μg l^{-1} h^{-1} in larvae exposed in freshwater but was 0.06 μg l^{-1} h^{-1} in larvae exposed in 1× equivalent of OSPW. At the end of the depuration phase, the concentration of chlorpyrifos in solution was less than the limit of quantification in all exposures.

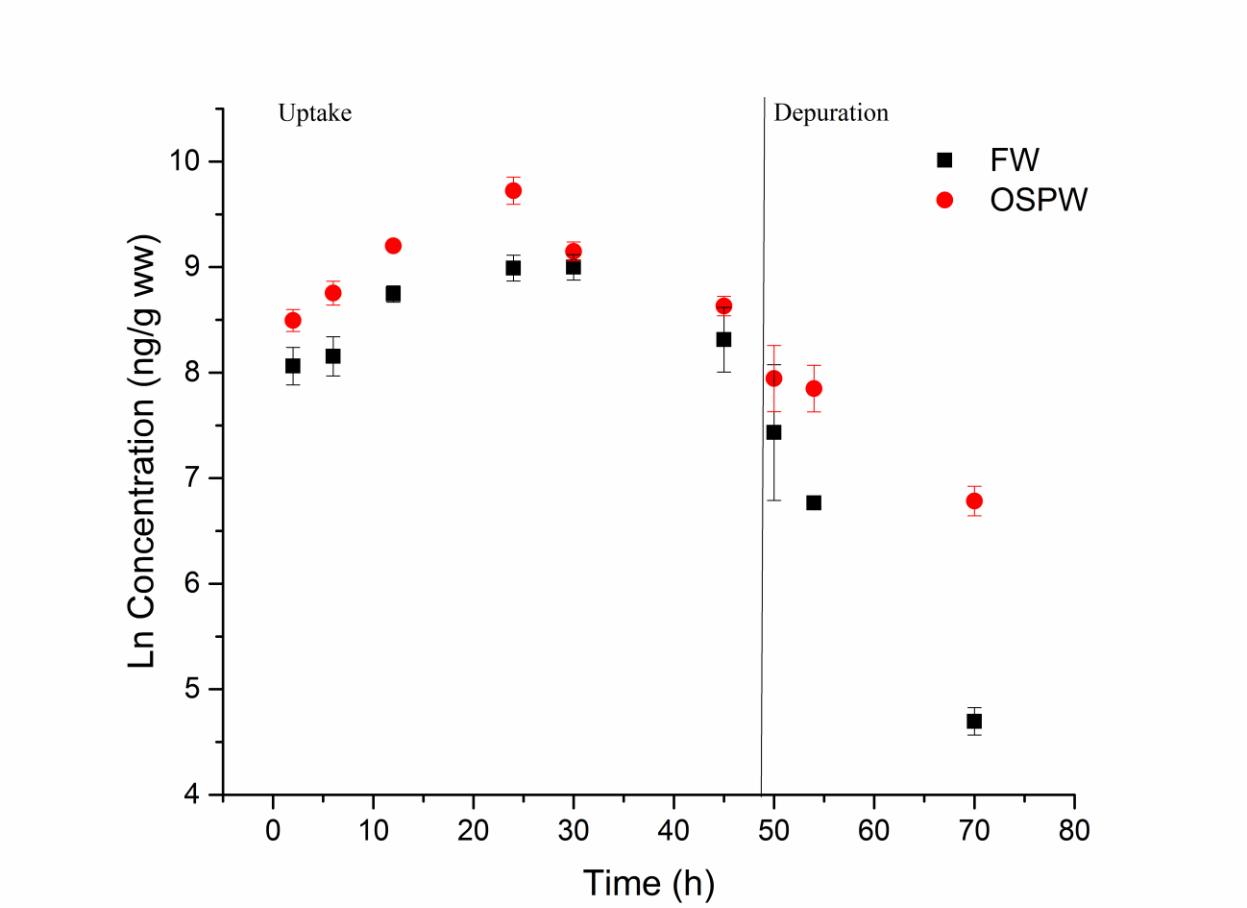

Figure 5.4. Effect of OSPW on kinetics of uptake and depuration of chlorpyrifos. Concentrations of chlorpyrifos in larvae of Japanese medaka were determined at during 48 h of exposure and 24 h of depuration. Approximately 100 larvae at 8 ± 1 dpf were exposed to 10 µg l^{-1} of chlorpyrifos either alone or in co-exposure with 1× equivalent of OSPW. Depuration was performed in solutions without chlorpyrifos. Concentrations are presented as natural-log (Ln) of the arithmetic mean concentration. Three exposures (n = 3) were performed with separate batches of larvae. Error bar represents the standard deviation. FW, freshwater; OSPW, oil sands process affected water.

5.6 Discussion

Results of this study suggest that basic and neutral chemicals in the aqueous phase of OSPW inhibit P-gp, a member of the ABC superfamily of proteins. Previously, inhibition of MRP, which also is a member of the ABC superfamily of proteins, by basic and neutral chemicals in the aqueous phase of OSPW was demonstrated (Alharbi et al., 2016a). Together, these studies suggest that chemosensitization might be a mechanism of toxicity of OSPW.

Greater mortality of larvae co-exposed to chlorpyrifos and the extract of basic and neutral chemicals from the aqueous phase of OSPW are evidence of a chemosensitizing effect caused by inhibition of P-gp. Chlorpyrifos (log K_{ow} 4.7) and malathion (log K_{ow} 2.7) are lipophilic compounds that enter cells by diffusion and are bioactivated via oxidative desulfuration to their oxon metabolite that causes acute lethality by inhibiting acetylcholinesterases (Fukuto, 1990; Ecobichon, 2001). However, transport of chlorpyrifos, but not malathion, from cells by P-gp protects against acute toxicity (Zaja et al., 2011). Therefore, greater lethality caused by co-exposure to chlorpyrifos and the extract of neutral and basic chemicals from the aqueous phase of OSPW likely was a result of inhibition of P-gp resulting in accumulation of chlorpyrifos in larvae. Results of this study support this conclusion because at concentrations of chlorpyrifos that were sub lethal, concentrations of chlorpyrifos and chlorpyrifos oxon were greater in larvae when they were co-exposed to basic and neutral compounds from OSPW, including a 1× equivalent of these chemicals, which is the environmentally relevant concentration. Greater concentrations of chlorpyrifos in larvae were matched by lesser concentrations of chlorpyrifos in the extract of OSPW compared to freshwater after 24 h of co-exposure. The greater concentration of chlorpyrifos in OSPW compared to freshwater after 24 h of depuration likely was because the greater amount of chlorpyrifos that accumulated during the co-exposure with OSPW was being depurated.

Malathion is not transported from cells by P-gp (Zaja et al., 2011), so inhibition of this protein by OSPW would not increase concentrations of malathion in larvae and therefore would not result in greater acute lethality. Results of the current study support this mechanism of toxicity because survival of larvae exposed to malathion was almost the same compared to larvae co-exposed to malathion and the extract of basic and neutral chemicals from OSPW. These results are consistent with those from other studies where concentrations of substrates of P-gp are greater in tissues exposed to inhibitors of P-pg. For example, inhibitors of P-gp increased accumulation of anticancer drugs within cells, and this resulted in greater cytotoxicity (Callaghan et al., 2014). In addition, the sensitivity of zebrafish (*D. rerio*) embryos toward phenanthrene was greater when they were exposed to inhibitors of P-gp (Fischer et al., 2013).

Kinetics of uptake and depuration of chlorpyrifos were altered in larvae co-exposed to the extract of OSPW, which is evidence that the basic and neutral chemicals from OSPW inhibit P-gp. The BCF and half-life of chlorpyrifos were greater in larvae that were co-exposed to 1× equivalent of basic and neutral chemicals from the aqueous phase of OSPW compared to the solvent control. Although BCFs can be predicted from the log *Kow* by use of quantitative structure–activity relationships (Van der Oost et al., 2003), factors affecting the toxicokinetics of compounds can affect BCFs. For chemicals that are eliminated from cells by ABC proteins, inhibitors of these proteins would increase internal concentrations of toxicants and thus toxicity (Choi et al., 2011; Fischer et al., 2013; Kasinathan et al., 2014). The rate of efflux of chlorpyrifos is expected to be slower when the activity of P-gp is inhibited. The greater half-life of chlorpyrifos in larvae that were co-exposed to 1× equivalent of basic and neutral chemicals from OSPW compared to freshwater supports this. In addition, concentrations of chlorpyrifos and chlorpyrifos oxon were greater in larvae during the depuration phase, which can be explained not only because

concentrations of chlorpyrifos were greater in larvae during uptake phases, but also because efflux of chlorpyrifos by P-gp was inhibited by basic and neutral chemicals from OSPW. The implication of this result is that internal concentrations of the substrate of P-gp (chlorpyrifos) are maintained relative to a given external concentration in freshwater while in the presence of environmentally relevant concentrations of OSPW, the equilibrium is affected by inhibition of P-gp.

Results of this study suggest that basic and neutral chemicals in the water-soluble fraction of OSPW have properties similar to those of inhibitors of P-gp. However, identities of these chemicals currently are not known. There is evidence that inhibitors of P-gp have unique physiochemical properties such as presence of two planar aromatic domains, high lipophilicity at a physiological pH with a log K_{ow} > 2.92 and moderate to high molecular mass (Wang et al., 2003; Zamora et al., 1988). Another characteristic of inhibitors of P-gp is the presence of a cationic charge usually in a nitrogen-containing cyclic ring (Ecker et al., 1999; Wang et al., 2003; Zamora et al., 1988). Chemicals containing nitrogen have been detected in the aqueous phase OSPW (Barrow et al., 2010; Grewer et al., 2010; Pereira et al., 2013a). Abundances of these chemicals are greatest in neutral and basic fractions of OSPW and are detected by use of Orbitrap mass spectrometry in positive electrospray ionization (Alharbi et al., 2016a). It has been suggested that chemicals containing nitrogen that are present in the basic and neutral fractions of OSPW from Base Mine Lake might inhibit activity of MRPs in early life stages of Japanese medaka (Alharbi et al., 2016a). Accumulation of calcein in Caco-2 cells and chlorpyrifos in larvae of Japanese medaka exposed to neutral and basic fractions of OSPW compared to the acidic fraction of OSPW supports this finding. In addition, activities of ABC proteins, including P-gp, is inhibited by surfactants (Rege et al., 2002). Anionic surfactants or acid extractable compounds are major constituents of OSPW (Clemente and Fedorak, 2005; Frank et al., 2008), but non-ionic and

cationic surfactants are yet to be characterized. Although dissolved organic compounds in OSPW have values of log K_{ow} ranging from −2 to 3, most acidic compounds, including naphthenic acids (O_2 chemicals that are detected by use of negative electrospray ionization) have log K_{ow} values less than 0. However, polar basic and neutral compounds had values of log K_{ow} greater than 0 at physiologically relevant pH (Wang et al., 2003; Zamora et al., 1988; Zhang et al., 2015). These results support the conclusion that basic and neutral chemicals in OSPW might inhibit the activity of P-gp. Lesser effects of the acid fraction on accumulation of calcein-AM in Caco-2 cells are probably because chemicals in this fraction are acidic and lacking some of the important features of inhibitors of ABC transporters.

In conclusion, the current study provides evidence that dissolved organic compounds in the aqueous phase of OSPW inhibit the activity of P-gp and therefore have the potential to act as chemosensitizers. However, several uncertainties about the environmental relevance of these findings must be addressed in future studies. For example, because the composition of chemicals in OSPW and fine tailings has not been characterized fully, it is not known if substrates of P-gp exist in either of these media. Therefore, studies aimed at identifying substrates of P-gp in tailings ponds are warranted. In addition, the effects of polar basic and neutral compounds from the aqueous phase of OSPW that has been aged on activity of P-gp should be assessed to identify if this potential mechanism of toxicity is attenuated by aging of OSPW in reclamation ponds and end pit lakes.

6 CHAPTER 6: DIFFERENTIAL EXPRESSION OF GENES ENCODING BIOTRANSFORMATION ENZYMES IN JAPANESE MEDAKA (*Oryzias latipes*) EXPOSED TO ACIDIC, BASIC AND NEUTRAL COMPOUNDS FROM THE AQUEOUS PHASE OF OIL SANDS PROCESS AFFETED WATER

This chapter is under preparation of submission to science of the total environment under joint authorship with David Saunders, John P. Giesy, and Steve B. Wiseman (University of Saskatchewan). The tables, figures and references cited in this article have been re-formatted here to the thesis style. References cited in this chapter are listed in the reference section of this thesis.

Author Contributions:

Hattan A. Alharbi (University of Saskatchewan) conceived, designed, managed and did the experiment, generated and analyzed the data, wrote, and drafted the manuscript.

David Saunders (University of Saskatchewan) provided laboratory assistance with designing the primers.

Drs. Steve Wiseman and John P. Giesy (all at University of Saskatchewan) provided inspiration, scientific input, and guidance, commented on and edited the manuscript, and provided funding for the research.

6.1 ABSTRACT

Understanding of effects of oil sands process affected water (OSPW) on aquatic organisms has come either from studies that expose animals to intact OSPW or to the total acid extractable fraction of organic chemicals in OSPW. However, because of the complexity of OSPW, toxicological profiling of either intact OSPW or total acid extractable fraction might not be an effective approach. A greater understanding of the effects of OSPW on aquatic organisms is likely to be generated if the organic compounds in the aqueous phase are separated based on their chemical properties. Thus, in the current study, dissolved organic chemicals in OSPW were separated into acidic, basic, and neutral fractions and expression of phase I and phase II drug-metabolizing enzymes and phase III ABC-efflux transport proteins were quantified in larvae of Japanese medaka exposed to these fractions. This approach was used to test the hypothesis that expression of genes encoding for xenobiotic metabolizing proteins is different in organisms exposed to the most acutely toxic fraction (i.e. acidic) of organic compounds from the aqueous phase of OSPW compared with fractions that are not acutely toxic (i.e. basic and neutral). Abundances of transcripts of the phase I enzyme *cyp3a* and the phase III proteins *abcb4* and *abcg2a* were significantly less in larvae exposed to the basic and neutral fractions but not the acidic fraction or pooled sample. Abundances of transcripts of *cyp1a and cyp3a,* were greater in larvae exposed to the acidic fraction or the pooled sample. Abundances of transcripts of the phase II enzymes *ugt1a* and *sult1a* were greater in larvae exposed to the acidic fraction and the pooled sample but were not affected in larvae exposed to either the basic neutral fractions. Results indicated that separation of organic compounds in OSPW is an effective approach to understand their effects on aquatic organisms. Using the differential gene expression information form this

study is a useful approach for future development of biomarkers of exposure to class of compounds in OSPW.

6.2 Introduction

Extraction of bitumen from oil sands requires hot caustic water to separate bitumen from oil. The process results in large volumes of oil sands process-affected water (OSPW) that is contained in tailings ponds (Grewer et al., 2010). OSPW is acutely and chronically toxic to aquatic organisms. Toxicity is caused primarily by organic chemicals in the aqueous phase (Anderson et al., 2012a, b; He et al., 2012 Morandi et al., 2015). A variety of acidic, basic and neutral organic compounds are present in the aqueous phase of OSPW and have been identified as oxygen-, sulphur- and nitrogen-containing compounds, including naphthenic acids (NAs) (Barrow et al., 2010; Grewer et al., 2010; Pereira et al., 2013a; Morandi et al., 2015; Alharbi et al., 2016a, b, c).

Aquatic organisms utilize a variety of mechanisms to cope with exposure to xenobiotic. Metabolism of some xenobiotic depends on biotransformation enzymes, including members of the cytochrome P450 (CYP) superfamily that catalyse phase I biotransformation reactions, phase II enzymes such as Sulfotransferases (SULT), Glutathione-s-transferase (GST), and Uridine diphosphate glucuronsyltransferase (UGT) that transform xenobiotic into more hydrophilic, excretable forms (Shimada et al., 2006), and phase III ATP-binding cassette transporters (ABC-transporters), which eliminate conjugated chemicals from cells (Ebert et al., 2005). Genes encoding for phase I, II or III-proteins are differentially expressed in fathead minnows (*Pimephales promelas*; He et al., 2012; Wiseman, et al., 2013b) and in primary cultures of rainbow trout hepatocytes (Gagné et al., 2012; 2013) exposed to OSPW.

The diverse mixture of chemicals present in OSPW complicates their assessment by routine chemical or biological analyses. One concern when working with complex mixtures is that some chemicals can mask effects of other chemicals in the mixture, including those chemicals that are bioavailable. For example, one approach to assess these bioavailable compounds is to fractionate complex mixtures so these compounds are separated from those that are not bioavailable (Brack et al., 2016). Thus, in our previous studies, the total organic fraction of OSPW was separated into three distinct fractions based on their physicochemical properties in order to decrease the complexity of mixture and therefore increase the information regarding the drivers of toxicity in each fraction compared to the total organic fraction of OSPW (Alharbi et al., 2016a, c). Using this approach, it has been demonstrated that the acidic but not the basic or neutral fractions of OSPW caused acute lethality to early life-stages of Japanese medaka (*Oryzias latipes)*. However, basic and neutral fractions, but not the acidic fraction, inhibited activity of ABC transporters (Alharbi et al., 2016a), which might enhance toxicity of OSPW to aquatic organisms via a chemo-sensitisation effect (Luckenbach et al., 2014; Kurth et al., 2015; Alharbi et al., 2016a, c). Moreover, abundances of transcripts of phase I such as *cyp3a4*, and phase II such as Glutathione-S-transferase (*gst*) or catalase (*cat*), and phase III such as *abcc2* whose enzyme products and proteins are important for clearance of reactive metabolites, were greater in male fathead minnow that was exposed to unfractionated OSPW (Wiseman et al., 2013b). Recently, it was demonstrated several basic and neutral compounds in OSPW are bioaccumulative in tissues of aquatic organisms (Zhang et al., 2015;2016). The goal of the current study was to further understanding of the effects of acidic, basic and neutral compounds from OSPW by testing the hypothesis that genes encoding for phase I, II, and III xenobiotic metabolizing proteins are differentially expressed in organisms exposed to the acidic, basic or neutral fraction of the aqueous phase of OSPW. This work will not only further

understand of responses of aquatic organisms to different classes of compounds in the aqueous phase of OSPW, but also is a useful step forward in the development of biomarkers of exposure to different classes of chemicals that have different toxicological properties.

6.3 Materials and methods

6.3.1 OSPW fractionation

OSPW was collected on the site of Syncrude Canada, Ltd. (Fort McMurray, AB, Canada) from Base Mine Lake (BML-OSPW), which is an end-pit lake constructed from the West-In-Pit settling basin that received input of tailings from the main extraction facility. Samples were collected in September of 2012, shipped to the University of Saskatchewan (Saskatoon, SK, Canada) and fractionated immediately upon arrival, into acidic, basic, and neutral compounds by use of methods described previously (Alharbi et al., 2016a). Fractions were resuspended in 100% ethanol. Equal volumes of each fraction were pooled to create a sample, referred to here as the "pooled", that is representative of the dissolved organic phase of OSPW. The profile of organic chemicals in the acidic, basic, and neutral fractions have been reported previously (Alharbi et al., 2016a).

6.3.2 Exposure of larvae to fractions of OSPW

All work with fish was approved by the University of Saskatchewan's Council on Animal Care and Supply (Protocol 20090108). Japanese medaka (*Oryzias latipes*) were cultured in the Aquatic Toxicology Research Facility at the University of Saskatchewan. Eggs were collected daily and maintained in embryo rearing medium (ERM) (1 g/L NaCl, 0.030 g/L KCl, 0.040 g/L $CaCl_2 \cdot H_2O$, 80 mg/L $MgSO_4$ and 1 mg/L Methylene Blue in distilled water), until hatch. Fish at

the fry stage of development (larvae), which is developmental stage 40 that extends from hatching until appearance of fin rays in the caudal and pectoral fins (Iwamatsu, 2004), were collected immediately upon hatch for use in exposures. All culturing of adult fish and rearing of embryos was conducted at a water temperature of 28 ˚C with a photoperiod of 16 h: 8 h (light: dark).

Fry were exposed to fractions of OSPW in 6-well tissue culture plates. Fry at one-day post-hatch were exposed to 2.5× equivalents and 1× equivalents of the pooled sample or acidic fraction, and 5×, 2.5× and 1× equivalents of the neutral or basic fractions of OSPW for 24 h at 28 ˚C with a photoperiod of 16 h:8 h (light: dark). Fry were exposed to 0.1% (v/v) of ethanol as a control. Exposures were replicated five times (n=5) and each with a separate batch of embryos. There were 10 fry per replicate. Concentrations of fractions were based on a previous study in which acute lethality was quantified (Alharbi et al., 2016a). Animals were not fed during exposures. Fry were euthanized by hypothermic shock in an ice bath until for 10 minutes (Strykowski and Schech, 2015), and were stored at -80℃ until needed for quantification of gene expression.

6.3.3 Primers

Primers to amplify cytochrome P450 1A (*cyp1a*), cytochrome P450 2A (*cyp2a*), cytochrome P450 2C (*cyp2c*), cytochrome P450 3A (*cyp3a*), sulfotransferase (*sult1a*), uridine diphosphate glucuronsyltransferase (*ugt1a*, and *ugt2a*), and pregnane X receptor (*pxr*) were from Zhao et al (2013). Primers to amplify ATP-binding cassette subfamily b member (*abcb4*), ATP-binding cassette subfamily c (*abcc1, abcc2, abcc3, abcc5*), and subfamily G member 2 (*abcg2*), were designed using Primer express 3.0 software (Applied Biosystems, Foster City, CA) and were purchased from Invitrogen (Burlington, ON, Canada). Nucleotide accession numbers of ABC transporters were reported previously (Luckenbach et al., 2014) and sequences were retrieved from

the Ensembl database (http://uswest.ensembl.org/Oryzias_latipes/Info/Index). All primers were synthesized by Invitrogen (Burlington, ON, Canada).

Efficiency of PCR reactions was established by performing qPCR with cDNA that was generated by pooling equal volumes of each sample of cDNA and that was serially diluted by 5-fold, from 1× (undiluted) to 3125×, in water that was free of nucleases. Reactions without cDNA were performed to check for contamination of reagents and to check for primer-dimers. For all reactions, the coefficient of determination (r^2) was at least 0.99 and efficiencies were 84–106%. Target genes, sequences of primers, accession numbers, product length, and efficiencies of reactions are given in Table 6.1.

Table 6.1. Primer sequences, efficiency of reaction, product length and Genbank accession numbers, for target genes of Japanese medaka used in quantitative real-time PCR.

Target Gene	Accession #	Primer Sequence (5'-3')	Efficiency (%)	Product length
cyp3a	AF105018	F: GAGATAGACGCCACCTTCC R: ACCTCCACAGTTGCCTTG	95	179
cyp1a	AY297923	F: GAGCACCTGGTCAAAGAGATA R: AGCACATGCCACAGATAACA	96	181
cyp2a	EF546459	F: ATATGGGATCGGGATCAGCAA R: CCGCAGCGTCGTCAGAGTG	88	70
cyp2c	Zhao et al., 2013	F: AGGAGAAAATGCAGGAGGAGA R: GTGAGGGAGGCTGAAAGGTGT	93	146
Pxr	Yamamuchi et al., 2008	F: GAGGAGCAAGAGCACGAATC R: ATGAAGCACAGAGGCTGGAG	84	136
abcb4	ENSORL T00000011623	F: CTGTTCGCCACCACAATTTC R: GTCATAGGCGTTGGCTTCT	93	99
abcc1	ENSORL T00000021452	F: TCGTGTCGTTTGCTGCTTTG R: GCCTCTTTCTCTGTGTCGCT	103	193
abcc2	ENSORL T00000010370	F: GGCGGTCACATTAGGAGAGG R: ACGTCACACAGAACCAGCAA	91	198
abcc3	ENSORL T00000025691	F:GAGGAACAACAGAGGCTACATC R:TCTCGTGGAAGGAGTAGAAGAG	91	113
abcc5	ENSORL T00000000960	F: CGTCTTCCCTTGGTTCCTAATC R: GATGTTCTCCAGGCGCTTTA	91	109
abcg2	ENSORL T00000008770	F: AGGGTAAGCAGGGGATGACT R: GAGAGCTCCAACGATCAGGG	106	176
sult1a	EF546450	F: AGAGAATCCTCGCCGTGAAGTT R: CCACGATTTGGTTGATGACCTC	96	83
ugt1a	EF546456	F: TGACCTTTTAGCCCATCCCAA R: GCAGATGCCCTCATAGATTCCA	90	76
ugt2a	EF546458	F: AGATCTGCCCGCTGACTTAGCT R: CATTCTGTGGCAGCCAATCAA	99	140
rpl-7	DQ118296	F: GTCGCCTCCCTCCACAAAG R: AACTTCAAGCCTGCCAACAAC	94	99

6.3.4 Relative Quantification of Gene Expression

Total RNA was extracted from was extracted from each of the 5 pools of 10 fry by use of a Qiagen RNeasy Mini Kit (Qiagen, Mississauga, ON, Canada). Quantities of mRNA were determined by use of a ND-1000 Spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA) and samples were stored at -80 °C. cDNA was synthesized from 1 µg of total RNA by use of a QuantiTect Reverse Transcription Kit (Qiagen). Real-time quantitative PCR (qPCR) was performed in 96-well plates by use of an ABI 7300 Real-Time PCR System (Applied Biosystems). A 50 µl reaction mixture of 2× concentrated QuantiFast SYBR Green master mix (Qiagen), an optimized concentration of cDNA, 10 pmol of gene-specific qPCR primers, and nuclease free water was prepared for each cDNA sample and primer combination. Reactions were conducted in duplicate with 20 µl reaction volumes per well. The PCR reaction mixture was denatured at 95 °C for 10 min before the first PCR cycle. The thermal cycle profile consisted of denaturing at 95 °C for 10 s and extension for 1 min at 60 °C for a total of 40 PCR cycles. Amplification of a single PCR product was confirmed by melt curve analysis and target gene transcript abundance was quantified by normalizing to abundance of transcripts of ribosomal protein L7 (*rpl-7)* (Pfaffi, 2001). A control reaction without cDNA was performed for each primer pair. Expressions of genes are presented as fold-change of abundances of transcripts of target genes in fry exposed to samples of OSPW relative to abundances in fry exposed to the solvent control.

6.3.5 Statistical analysis

Statistical analyses were performed by use of GraphPad Prism 5 software (San Diego, CA, USA). Normality of data was assessed by use of the Kolmogorov Smirnov one-sample test and homogeneity of variance was determined by use of Levene's test. If necessary, data were $\log_{10}$

transformed to ensure normality and homogeneity of variance. Changes in abundances of transcripts of target genes in fry of Japanese medaka exposed to different fractions of OSPW relative to abundances in fry exposed to the solvent control were evaluated by use of one-way ANOVA followed by Dunnett's post-hoc test. Differences were considered significant at a ρ-value < 0.05. Correlation of abundances of transcripts with exposure to the pooled, acidic, basic and neutral fraction of OSPW was performed using correlation analysis with a two-tailed test for significance (analyzed with the mean values of fold-change of larvae exposed to 2.5×).

6.4 Results

6.4.1 Effects of fractions of OSPW on expression of genes encoding phase I enzymes

Fractions of OSPW affected expression of phase I biotransformation enzymes. Abundances of transcripts of *cyp1a* in larvae exposed to 2.5× and 1× of the pooled sample were greater by 4.85- and 2.72-fold, respectively, and by 2.59-fold in fry exposed to 5× of neutral fraction. Abundances of transcripts of *cyp1a* were not significantly different in fry exposed to acidic or basic fractions. (Figure. 6.1A). Abundances of transcripts of *cyp2a* were greater by 2.99- and 3.95-fold greater in fry exposed to 2.5× of pooled sample and the acidic fraction, respectively. Abundances of transcripts of *cyp2a* were greater by 2.23-fold in larvae exposed to 5× of the basic fraction and by 5.21- and 2.90-fold in fry exposed to 5× and 2.5×, respectively, of the neutral fraction (Figure. 6.1B). Abundances of transcripts of *cyp2c* were significantly lesser in fry exposed to 5× of the basic fraction (Figure. 6.1C). Abundances of transcripts of *cyp3a* were greater by 3.39- and 4.44-fold in fry exposed to 2.5× of the pooled sample and the acidic fraction, respectively (Figure 6.1D). Abundances of transcripts of *cyp3a* were 18.56-, 13.47-, and 5.87-fold less in fry exposed to 5×, 2.5×, and 1× of the basic fraction, respectively and were 4.75-fold less in larvae

exposed to 5× of the neutral fraction (Figure. 6.1D). Abundances of transcripts of *pxr* were 2.92-fold greater in fry exposed to 2.5× of acidic fraction and 3.12-fold greater in fry exposed to 5× of the neutral fraction, but was not different in fry exposed to other fractions of OSPW (Figure 6.1E).

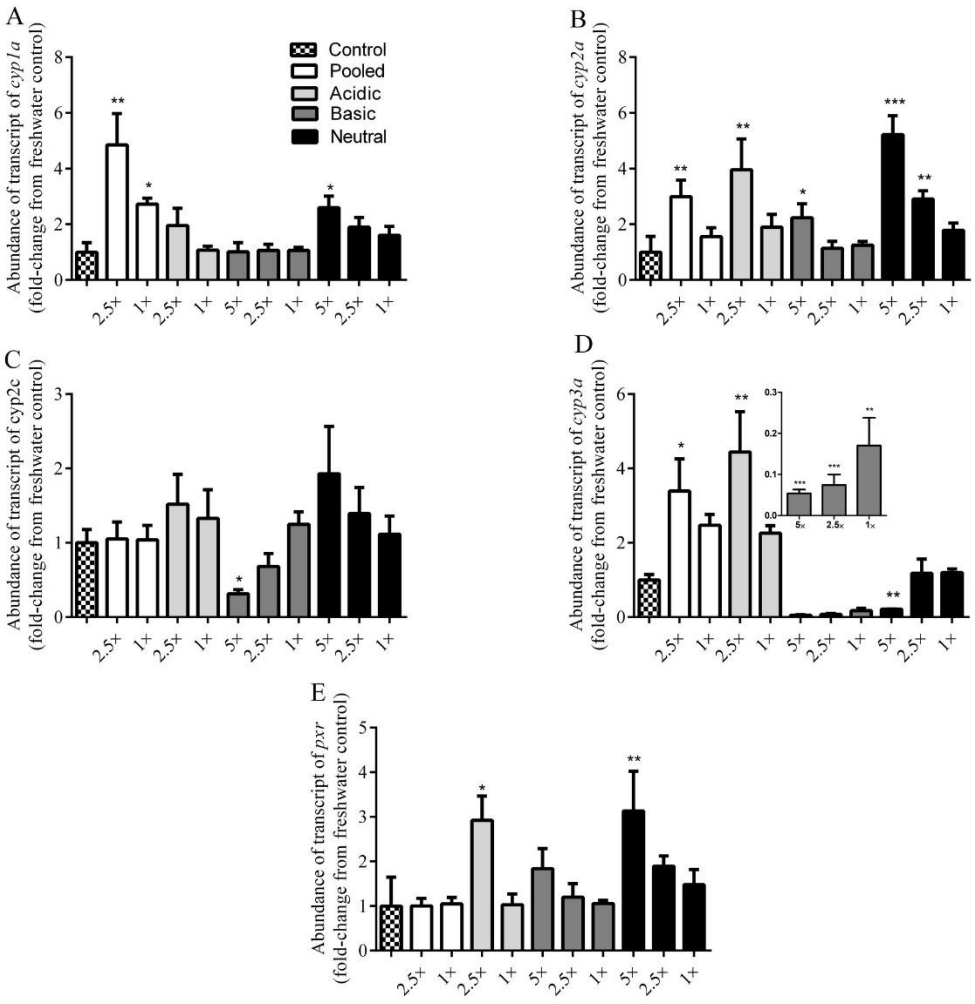

Figure. 6.1: Abundances of transcripts of (A) *cyp1a*, (B) *cyp2a*, (C) *cyp2c*, (D) *cyp3a*, and (E) *pxr* in fry of Japanese medaka after 24 hours of exposure to pooled sample, and acidic, neutral, and basic fractions of OSPW. Bars represent the mean (± SEM) abundance of transcripts relative to the solvent control. Abundance of *rpl-7* was used for normalization. Significant differences were determined by use of a one-way ANOVA followed by Dunnett's post hoc test (*n = 5).* Differences were considered significant at $\rho \leq 0.05$ and are indicated by an asterisk (*).

6.4.2 Effects of fractions of OSPW on expression of genes encoding phase II enzymes

Genes encoding phase II enzymes were differentially expressed in larvae exposed to fractions of OSPW. Abundances of transcripts of *sult1a* were greater by 9.26- and 9.72-fold in fry exposed to 2.5× of the pooled and acidic fractions, respectively. However, there were no effects of other fractions on expression of *sult1a* (Figure. 6.2A). Abundances of transcripts of *ugt1a* were greater by 6.89- and 9.40-fold in fry exposed to 2.5× of the pooled sample and the acidic fraction, respectively, and lesser by 3.90-fold in fry exposed to basic 5× (Figure. 6.2B). Abundance of transcripts of *ugt2a* was 5.07-fold less in fry exposed to 5× of the basic fraction, but was not different in fry exposed to other fractions of OSPW (Figure. 6.2C).

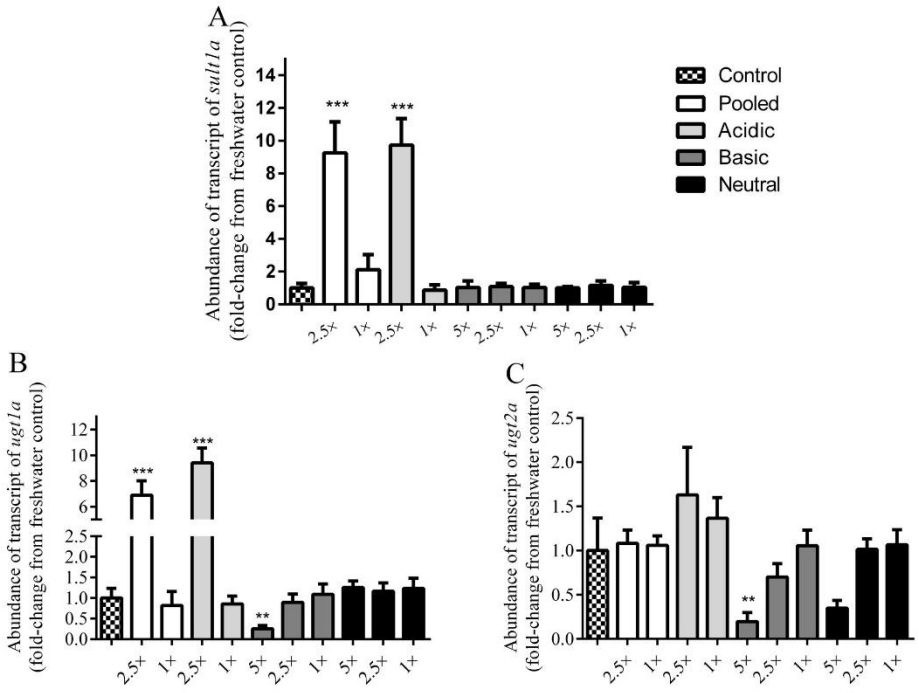

Figure. 6.2: Abundances of transcripts of (A) *sult1a*, (B) *ugt1a*, and (C) *ugt2a* in fry of Japanese medaka after 24 hours of exposure to pooled sample, and acidic, neutral, and basic fractions of OSPW. Bars represent the mean (± SEM) abundance of transcripts relative to the solvent control. Abundance of *rpl-7* was used for normalization. Significant differences were determined by use of a one-way ANOVA followed by Dunnett's post hoc test (*n = 5).* Differences were considered significant at $\rho \leq 0.05$ and are indicated by an asterisk (*).

6.4.3 Effects of fractions of OSPW on expression of ABC-transporters

Genes encoding ABC transporter proteins were differentially expressed in fry exposed to fractions of OSPW. There was a trend of lesser abundances of transcripts of most ABC-transporters. Abundances of transcripts of *abcc1* were lesser by 3.96-fold in fry exposed to 2.5× of the acidic fraction and by 6.92- and 3.69-fold in fry exposed to 5× and 2.5× of the basic fraction, and 2.90-fold in fry exposed to 5× of the neutral fraction (Figure. 6.3A). There were no effects on the abundance of transcripts of *abcc2* in fry exposed to either fraction of OSPW (Figure. 6.3B). Abundance of transcripts of *abcc3* was 3.66-fold lesser in fry exposed to 5× of the basic fraction (Figure. 6.3C). Abundance of transcripts of *abcc5* was greater by 2.84-fold in fry exposed to 2.5× of the pooled sample and by 3.37- and 2.79-fold in fry exposed to 2.5× and 1×, respectively, of the acidic fraction. In contrast, the abundance of transcripts of *abcc5* was 5.93- and 3.29-fold lesser in fry exposed to 5× of the basic and neutral fractions, respectively (Figure. 6.3D). Abundances of transcripts of *abcb4* were lesser by 17.53- and 6.69-fold in fry exposed to 5× and 2.5× of the basic fraction, respectively, but was not different in fry exposed to any other treatment (Figure. 6.3E). Abundances of transcripts of *abcg2* were lesser by 6.12-, 8.48-, and 3.96-fold in fry exposed to 5×, 2.5×, and 1×, respectively, of the basic fraction. The abundance of transcripts of *abcg2* was 5.59-fold less in fry exposed to 5× of the neutral fraction (Figure. 6.3F).

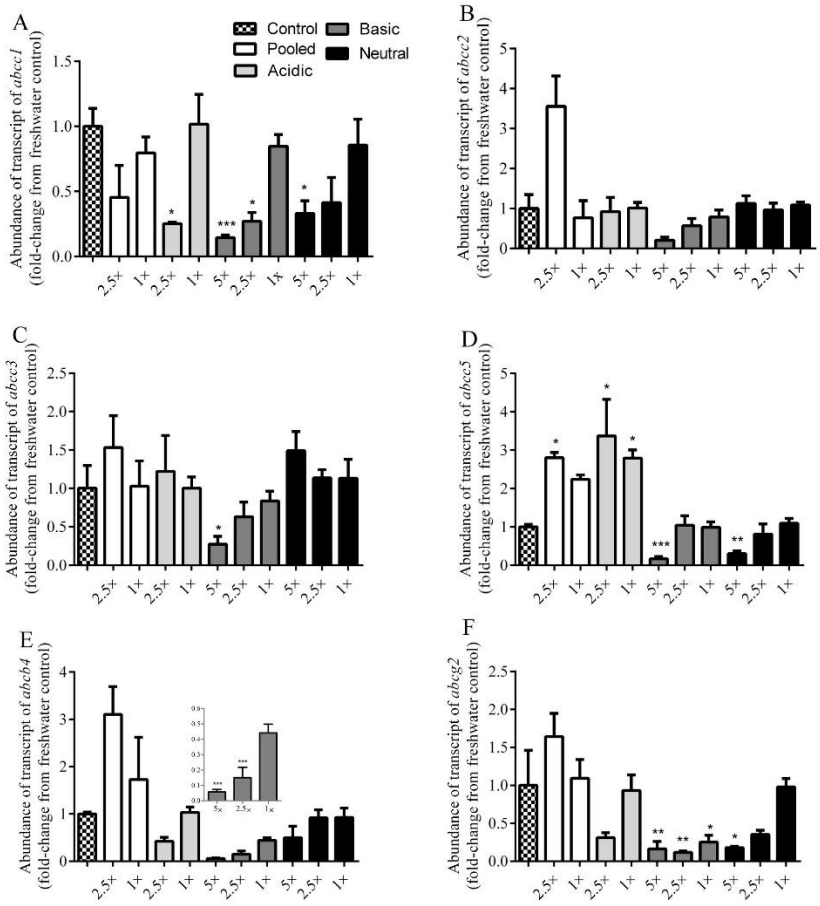

Figure. 6.3: Abundances of transcripts of (A) *abcc1*, (B) *abcc2*, (C) *abcc3*, (D) *abcc5*, (E) *abcb4*, and (F) *abcg2* in fry of Japanese medaka after 24 hours of exposure to pooled sample, and acidic, neutral, and basic fractions of OSPW. Bars represent the mean (± SEM) abundance of transcripts relative to the solvent control. Abundance of *rpl-7* was used for normalization. Significant differences were determined by use of a one-way ANOVA followed by Dunnett's post hoc test (*n = 5).* Differences were considered significant at $\rho \leq 0.05$ and are indicated by an asterisk (*).

6.4.4 Correlations between fractions

Correlation analysis was used to determine which of the three fractions was driving the changes in gene expression in fry exposed to the pooled sample. Results of the analysis demonstrated that changes in abundance of transcripts in response to the pooled sample and the acidic fraction were correlated ($r^2 = 0.604$; $P < 0.001$), and changes in abundance of transcripts in fry exposed to the basic fraction and the neutral fraction were correlated by ($r^2 = 0.689$; $P < 0.001$). Changes in abundance of transcripts in fry exposed to the acidic fraction or pooled sample did not correlate with either the basic or neutral fraction (Table 6.2).

Table 6.2. Linear correlations for effects of acidic, basic, neutral and pooled fractions of OSPW on abundances of transcripts of genes encoding proteins involved in biotransformation reactions. Data represent coefficients of determination (r^2) in the upper diagonal and the probabilities (P) of 2-tailed test in the lower diagonal. Coefficients of determination were calculated using mean abundance of transcripts across all concentrations of samples of OSPW. Correlation is significant at $\rho \leq 0.05$ and is indicated by an asterisk (*).

	Acidic	Basic	Neutral	Pooled
Acidic		0.149	0.226	0.604
Basic	0.172		0.689	0.028
Neutral	0.084	0.000*		0.051
Pooled	0.001*	0.562	0.436	

6.5 Discussion

Organic chemicals in the aqueous phase of OSPW affect expression of genes encoding drug-metabolizing enzymes and ABC transporters in aquatic organisms (Wiseman et al., 2013b; Gagné et al., 2012; 2013). However, little is known about the classes of chemicals that cause these effects. In the present study, expressions of genes encoding phase I, II, and III proteins was investigated in fry (larvae) of Japanese medaka exposed to fractions of organic chemicals from the aqueous phase of OSPW.

Expression of genes encoding phase I enzymes in Japanese medaka were affected differently by fractions of OSPW. Abundances of transcripts of *cyp1a* were greater in fry of Japanese medaka exposed to the pooled sample and the neutral fraction of OSPW. Identities of chemicals in the aqueous phase of OSPW that induce expression of *CYP1A* are not known, but the most likely candidates are PAHs/alkyl-PAHs. Although PAHs/alkyl-PAHs were not determined in fractions used in the current study, studies have reported that they are present in OSPW, albeit concentrations in the aqueous phase of OSPW are small compared to concentrations in the sediment phase of tailings ponds (Madill et al., 1999; Colavecchia et al., 2004, 2006, 2007; Kelly et al., 2009; Korosi et al., 2016). PAHs with greater solubilities in water, such as naphthalene, have lesser molecular mass and lipophilicity, whereas PAHs of greater molecular mass are rarely detected in water (Neff et al., 2011). PAHs are neutral compounds and if they were present in the sample of OSPW they most likely would occur in the neutral fraction, which is supported by the greater abundances of transcripts of *cyp1a* in fry exposed to this fraction.

Several studies have investigated effects of OSPW on expression of *cyp1a* in fish, but less is known about effects on expression of other CYP genes, including *cyp2* and *cyp3*, that are important for the biotransformation of xenobiotic. Both enzymes are important for detoxification

of aromatic hydrocarbons in the liver of fish (Celander et al. 1996; Wassmur et al., 2010). The present paper for the first time provides evidence that expression of *cyp2* genes were affected by exposure to OSPW. However, changes in abundances of transcripts of *cyp2a* were significant in response to each fraction of OSPW, which not only suggests that structurally diverse substrates of *cyp2a* might be present in OSPW, but also that there is little promise for use of this gene as a biomarker of exposure to specific classes of compounds in OSPW. Similar to effects on expression of *cyp2a*, each fraction of OSPW significantly affected abundance of transcripts of *cyp3a*, and the magnitude and direction of these effects was not the same across each fraction. Abundance of transcripts of *cyp3a* was lesser, in a dose-dependent manner, in fry exposed to the basic fraction of OSPW and in fry exposed to the greatest concentration of the neutral fraction. In contrast, the abundance of transcripts of *cyp3a* was greater in fry exposed to the acidic fraction and the pooled sample. Other studies have reported changes in abundances of transcripts of *cyp3a* in response to OSPW. For example, abundance of transcripts of *cyp3a* was greater in embryos of fathead minnow exposed to OSPW that was not fractionated (He et al., 2012), which is consistent with the greater expression of *cyp3a* in medaka exposed to the pooled sample. Differential expression of *cyp3a* in response to the acidic fraction versus the basic and neutral fraction suggest that this gene might be useful as an indicator of exposure to classes of compounds is OSPW that are acutely toxic versus those that do not cause acute toxicity. However, expression of *pxr*, which regulates expression of *cyp3a*, might not be useful as an indicator of exposure to different classes of chemicals in OSPW. Previously it was demonstrated that expression of *pxr* mirrors that of *cyp3a* (Wassmur et al., 2010), but this was not observed in the current study.

Expression of genes encoding UGTs and SULTs were significantly greater in larvae exposed to the acidic fraction and the pooled sample of OSPW indicating that phase II

detoxification reactions might have been initiated in these fish. After phase I biotransformation, reactive electrophiles are eliminated via conjugation by phase II detoxification enzymes, such as GSTs, SULTs, and UGTs (Schlenk et al., 2008). Abundances of transcripts of *ugt1a* and *sult1a* were greater in fry of Japanese medaka exposed to the pooled sample and acidic fraction but did not change in fry exposed to the basic or neutral fractions. Similarly, abundances of transcripts of *sult1, sult2,* and *sult3* were greater in adult fathead minnow exposed to unfractionated OSPW (Wiseman et al., 2013b), which is consistent with the effect of the pooled sample on expression of *sult1a*. Both the pooled sample and acidic fraction, at concentrations of 5×, were acutely toxic to fry of Japanese medaka (Alharbi et al., 2016a). Although the mechanism of this acute lethality is not known, production of reactive metabolites might be greater in fry exposed to the pooled sample and acidic fraction compared to fry exposed to basic and neutral fractions. If these metabolites are formed, it might explain the greater expression of *ugt1a* and *sult1a.* Greater expression of detoxification enzymes, including phase II enzymes, is one response that animals mount to protect against adverse effects of reactive oxygen species and reactive metabolites (McMillian et al., 2004; Xu et al., 2005; Di Bello et al., 2007; Regoli et al., 2011; Zhao et al., 2013). Whether such a response is insufficient to combat oxidative stress and reactive metabolites in organisms exposed to OSPW is not known.

ATP-binding cassette proteins were differentially expressed in Japanese medaka exposed to fractions of OSPW. It has been shown previously that ABC transporter were inhibited by basic and neutral fractions of OSPW in Caco-2 and fry of Japanese medaka (Alharbi et al., 2016a, c), which indicate the potential of chemo-sensitising effects by the complex mixture of chemicals in the basic and neutral fractions derived from OSPW. ABC proteins are efflux pumps that eliminate hydrophobic substrates from cells (Bouige et al., 2002). In Japanese medaka, the *ABCB4* gene, is

the only member of the *ABCB* gene family that has been identified, encodes for p-glycoprotein (P-gp), a multi-xenobiotic efflux pump (Jeong et al., 2015). Abundances of transcripts of *abcb4* were lesser in fry exposed to the basic and neutral fractions but greater in fry exposed to the acidic fraction and the pooled sample. Differential expression of *abcb4* is consistent with previous studies where the expression of multi-drug resistance protein (MDR; P-gp) was greater in rainbow trout hepatocytes exposed to the total organic fraction extracted from OSPW (Gagné et al., 2012), but significantly lesser in rainbow trout hepatocytes exposed to the aqueous phase of an surrogate sample of OSPW generated by mixing bitumen with hot caustic soda under laboratory conditions (Gange et al., 2013). However, the chemicals responsible for these effects were not identified in those studies. In mammals, it has been shown that *ABCB1*, which encodes P-gp, is regulated by PXR (Tolson and Wang., 2010). This also appears to be true in zebrafish (*Danio rerio*) as expression of *PXR* and P-gp are upregulated simultaneously following exposure to the model PXR activator 16α-carbonitril (PCN) (Bresolin et al, 2005).

Other genes coding for ABC transporters are the ABCCs that might be involved in efflux of hydrophobic substrates or chemicals that are conjugated with glutathione, glucuronic acid or sulphate, and are the products of phase II biotransformation reactions (Slot et al., 2011). Abundance of transcripts of *abcc2* did not significantly change following exposure to any fraction but was greater after exposure to 2.5 × of the pooled sample. In another study, abundance of transcripts of *abcc2* was greater in adult fathead minnows exposed to full-strength OSPW for one week (Wiseman et al., 2013b). Abundance of transcripts of *abcc2* also was greater in Japanese medaka exposed to the organic fraction of OSPW (Alharbi et al., 2016b). The third family of ABC transporters quantified were the ABCG. Specifically, abundance of transcripts of *abcg2* was lesser and most responsive to the basic fraction, followed by neutral fraction. While expression of the

ABCG2 has not been characterized previously in organisms exposed to OSPW, in a study of killifish (*Fundulus heteroclitus*) from the Sydney Tar Ponds, Nova Scotia, Canada, which contained high concentration of PAHs, abundance of transcripts of *abcg2* was greater compared to reference site (Paetzold et al., 2009).

Currently, understanding of effects of OSPW on aquatic organisms has come either from studies that expose animals to intact OSPW or to the total acid extractable fraction of organic chemicals in OSPW. However, because of the complexity of OSPW, toxicological profiling of either intact OSPW or total acid extractable fraction might not be an effective approach. Results of the current study suggest that fractionation of the organic phase of OSPW into acidic, basic and neutral compounds might be a more effective approach. Only by separating the mixture into these fractions were effects of neutral and basic compounds on expression of target genes identified. The results of the current study suggest that changes in expression of phase I, II and III drug-metabolizing proteins might be a useful approach for identification of genes as candidates for use as biomarker of exposure to organic acids and non-organic acids (basic and neutral) classes of dissolve organic compounds in OSPW.

7 CHAPTER 7: GENERAL DISCUSSION

7.1 Introduction

Extraction of bitumen during surface mining in the oil sands industry generates large volumes of oil sands process-affected water (OSPW), which is recycled for use in extraction process, and finally stored on site in tailings ponds, in concordance with a policy of zero discharge. OSPW is a complex matrix that contains many classes of chemicals including organics, such as naphthenic acids (NAs), polycyclic aromatic hydrocarbons, and inorganic chemicals such as metals, and ions. A comprehensive understanding of toxic potency of OSPW is needed to understand the risk that any unintentional or intentional release of OSPW might have on aquatic systems. Constituents of OSPW have been shown to be toxic to variety of species of vertebrates and invertebrates (Anderson et al., 2012a, b; He et al., 2012b; Morandi et al., 2015). Investigation of the toxic effects of OSPW is a difficult task because of its complexity, and most studies to date have examined the whole fraction that contains largely organic acids or NAs. Although aquatic species might expose to several organic and inorganic constitutes of OSPW however, the focus in this thesis was on the organic constitutes of OSPW.

The acid extractable fraction of OSPW as well as commercial naphthenic acid (NA) mixtures are the most investigated chemicals in OSPW research, which are used to gather information in support of future ecological risk assessment of OSPW. However, acidic compounds are not representative of all organic chemical species present in OSPW. There is a gap in knowledge in relation to effects of non-acidic compounds in OSPW on aquatic species, which makes the data derived from acid extractable fraction insufficient for future environmental risk assessment to aquatic species.

As indicated above, OSPW is a complex mixture and it is difficult to understand the toxic effects of all organic classes present in this mixture. One strategy to overcome this is to decrease the complexity of the mixture by separating acidic and non acidic species for further toxicity investigation. The objective of this thesis was first to develop a quick, easy and efficient extraction/isolation methodology to comprehensively and simultaneously separate organic constituents in OSPW into fractions based on their physicochemical properties. This extraction methodology decreases the complexity of OSPW components and allows for better determination of toxic potencies of fractions. OSPW was separated into three distinct fractions (acidic, basic and neutral fractions) and the profile of chemicals in each fraction was investigated by use of orbitrap mass spectrometry. These fractions were subsequently investigated for their acute toxicity, effect on the function and expression of ABC transporters proteins, and effects on toxicity of the alkylated PAH, 1-methyl-7-isopropyl phenanthrene (retene) in larvae of Japanese medaka.

In the first study (chapter 2), acute toxicity and profile of organic chemicals in the aqueous phase of fresh and aged OSPW was determined. The acidic fraction of fresh, but not aged, OSPW was acutely toxic to early life stages of Japanese medaka. The profiles of chemicals in fresh and aged OSPW represent a useful tool to evaluate the differences in lethality caused by the two samples. The concentrations of chemicals detected in negative ion mode such as O_2, O_3, and O_4 species were less in aged OSPW than in fresh OSPW

The second study (chapter 3) was designed to investigate the effect of acidic, basic, and neutral fractions of the aqueous phase of fresh and aged OSPW on the activity of ABC transporter proteins in early life-stages of Japanese medaka. A major conclusion of chapter 3 is that organic compounds in the basic and neutral fraction, but not compounds in the acidic fraction, of fresh OSPW but not aged OSPW inhibited the activity of ABC transporters. Based on this finding, basic

and neutral compounds in OSPW might cause adverse effects via chemosensitization. The comparison of the profile of chemicals in fractions of fresh and aged OSPW indicated that basic and neutral showed the concentration of chemical species with nitrogen, and sulfur was less in aged compared to fresh OSPW.

Because results of chapter 3 demonstrated that compounds in the basic and neutral fractions of fresh, but not aged, OSPW inhibited the activity of ABC transporters, the objective of the third study (chapter 4) was to investigate the effect of co-exposure to the model alkylated PAH (retene) and the basic or neutral fraction of OSPW on early life stages of Japanese medaka. Results indicated that fresh but not aged OSPW enhanced the effects of alkylated-PAHs to early life stages of fishes, and enhanced the water solubility of retene. Also, retene suppressed the gene expression induction of gene expression of ABC transporters caused by OSPW.

The fourth study (chapter 5) investigated the effect of basic and neutral compounds of OSPW on the toxicity and bioaccumulation of a model substrate of P-gp (chlorpyrifos), and not P-gp substrate (malathion). The results showed that the toxicity, internal concentration and bioaccumulation of chlorpyrifos against early life stage of Japanese medaka increased in the presence of OSPW, which indicates that OSPW might act as chemosensitizers.

Finally, in the fifth study (Chapter 6), the effect of acidic, basic, and neutral fraction derived from fresh OSPW on expression of genes encoding proteins important for phase I, II, and III detoxification of xenobiotic was investigated in early life-stage of Japanese medaka. Results of this study showed that the genes encoding ABC transporters genes were differentially expressed in larvae exposed to the basic and neutral fractions, whereas genes encoding enzymes of phase II reactions were differentially expressed in response to exposure the acidic.

7.2 Acidic, basic and neutral fractions

7.2.1 Separation of acidic, basic and neutral compounds using MMC

Orbitrap mass spectrometers are one of the most commonly used ultra high-resolution mass spectrometers (HRMS), providing selective detection and accurate measurements. An additional advantage of using Orbitrap instruments is that full scan data is obtained, providing structural information that can be supported by fragmentation of precursor ions (if necessary) (Fedorova et al., 2013). However, preparation of samples for both qualitative and quantitative analysis is as important as instrument analysis. Chemical modification of the analytes of interest is required for isolation, separation, and detection for elucidation of molecular structures. Solid-phase extraction (SPE) is one of the most powerful extraction and separation technique as it efficiently enriches, separates, and purifies analytes from matrices.

The first objective in this thesis was to develop an analytical method capable of extracting a broad range of organic compounds from the aqueous phase of fresh and aged OSPW and to perform detailed analysis of these fractions by use of HRMS. By combining mixed-mode cationic or anionic sorbents with hydrophilic-lipophilic-balance (HLB) sorbent (reversed-phase), it was possible to selectively and simultaneously increase the number of extractable compounds from OSPW and to separate acidic, basic, and neutrals compounds in one extraction step (Chapters 2,3, and 6),

Polar chemicals in petroleum can be classified as acidic, basic or neutral compounds (Stanford et al., 2007). However, simultaneous isolation of acidic, basic and neutral fractions from the aqueous phase of OSPW had not been investigated before. Historically, a generic procedure, such as liquid-liquid extraction, has been used to extract acid extractable organic compounds from

OSPW. Although this method can achieve high recoveries for NAs when the pH of aqueous sample is decreased to approximately 2, it does not support the simultaneous extraction of chemicals with different physicochemical properties. Extraction from OSPW of specific classes of chemicals that have different physicochemical properties can be difficult with the traditional method such as liquid-liquid extraction. An alternative to liquid-liquid extraction is solid phase extraction (SPE), which has been used in chemical extraction of NAs from OSPW. Several methods of solid phase extraction, including Isolute ENV+ (Headley et al., 2013), hydrophilic- lipophilic-balance (HLB) (Bataineh et al., 2006), C18 (Gagné et al., 2013) have been used to isolate organics (mainly NAs) from the aqueous phase of OSPW. SPE is a powerful sample-extraction technique for liquid samples, as it can be employed in the extraction and clean up steps as a pre-concentration as well as for de-salting (Fritz., 1999). Many extraction materials (also known as sorbents) are commercially available, and the extraction can be adjusted depending on the mechanisms of interaction between sorbents and analytes (Fritz., 1999).

Mixed-mode chromatography (MMC), which uses dual cationic and anionic-exchange/reversed-phase is another strategy to selectively isolate acidic, basic and neutral chemicals from environmental samples. A mixed-mode ion-exchange sorbent can be considered as extraction/clean-up/isolation procedure in one step to maximize the extraction efficiency and improve the retention of neutral and ionic analytes. Mixed mode chromatography can also minimize matrix effects and extract complexity by separating acidic, basic and neutral substances into different fractions. The principle of MMC was successfully applied in this thesis, where acidic, basic and neutral fractions were isolated from the aqueous phase of OSPW. However, chemicals in each fraction have similar physicochemical properties which make the isolation of individual compounds within each fraction is difficult if not impossible.

Based on results of this thesis, it might be acceptable that identification of chemicals in OSPW does not provide more information, and it can be suggested that additional research involving extraction and chemical profiling of OSPW should focus only on the acidic, basic and neutral fractions that are representative of water soluble polar chemicals in OSPW. This approach could give a better prediction of the risk of each fraction, if combined with toxicity data. MMC is suggested to reduce the complexity of OSPW and therefore limits the chance of overlooking significant contributors to risks and effects. Combining the MMC approach with characterization of the biological activity of each fraction using responses of (sub) cellular systems or whole organisms, should allow for comprehensive investigation of effects of diverse dissolve organic compounds in OSPW.

There are many ongoing researches towards isolation of distinct NA species such as classical, aromatic, oxidized in small fractions for either acute or chronic toxicity testing. However, the "whole fraction toxicity method" that is similar to "whole effluent toxicity method" (de Vlaming et al., 2000) for the main three fractions of OSPW (i.e. acidic, basic and neutral fractions) is the best approach to assess the toxicity of OSPW based on the physicochemical properties. For example, regarding the acidic fraction of the aqueous phase of OSPW, it is assumed that toxicity of the mixture can be predicted by concentration or dose addition models for chemicals with similar mechanisms of action or response addition for dissimilarly acting chemicals (Morandi et al., 2016).

7.2.2 Toxicity of acidic, basic and neutral fractions

There are many confounding factors in OSPW mixture that might influence toxicity, and therefore might affect the accuracy of predictive methods used in environmental risk assessments.

For example, interactions might occur between acidic, basic and neutral chemicals, which might alter toxicity of each chemical class so that it is more or less than expected from the whole mixture, and therefore the risk posed by each group might be under/over-estimated (Brack et al., 2016). Based on this thesis results, assessment of toxicity of OSPW is suggested to be designed on acidic, basic and neutral fractions, which will provide an accurate estimation of toxicity for future assessment of ecological risk of OSPW.

In acidic, basic and neutral fractions of OSPW, toxicity, either acute or sub lethal, is based on the same mode of action. In other words, the total response in observed toxicity might correspond to the sum of all the individual compounds and their concentrations multiplied with their respective potencies (Brack et al., 2016). The other model that can be applied to OSPW fractions is the response addition, based on independent mode action. However, it is not clear if response addition is applicable to sub lethal effect. For example, acute toxicity of OSPW has been shown to be primarily via narcosis (Morandi et al., 2015), where all chemicals contribute to the overall toxicity (i.e. the toxicity is the sum of concentration of all chemicals in the mixture).

Since OSPW contains neutral and ionizable organic compounds (IOCs), the degree of ionization of IOCs impacts many important molecular properties including but not limited to permeability, lipophilicity, partition or distribution coefficients, and solubility (Avdeef., 2001; Neuhoff et al., 2005; Erickson et al., 2006a, b; Manallack et al., 2013; Stott et al., 2015), which will subsequently impact the persistence, bioaccumulation and either acute or chronic toxicities of IOCs. Toxicokinetics including uptake and bioconcentration, and toxicodynamics (toxicity) of IOCs in OSPW is dependent on both the pH of the surrounding aquatic environment as well as on the acidity constant (p*K*a). The lesser toxicity of charged species might be attributed to its very poor membrane permeability (Krämer et al., 1998; Saparov et al., 2006; Charifson et al., 2014).

Toxicity of acidic fraction of OSPW using a rainbow trout gill cell line was investigated, and showed that toxicity increased with decreasing pH (Brinkman et al., In preparation). Since the pH of OSPW is approximately 8-9, it is expected that there is a decrease in the observed toxicity of acidic species to aquatic species. However, effects of basic and neutral fractions on their target molecules is expected to be great at the ambient pH of OSPW. These results strongly support the importance of testing the pH of the surrounding environment in OSPW toxicity assays to avoid the overestimation of the actual toxicity of acid extractable fraction from OSPW.

Finally, further fractionation of acidic, basic, and neutral fractions is not recommended because it is time consuming and difficult to produce any distinct toxic fractions and identify of individual compounds in OSPW. For example, it has been suggested that the EDA approach is not efficient for non-specific effects, such as baseline toxicity (narcosis) in which all chemicals contribute to the mixture effects based on their concentration and lipophilicity (Brack et al., 2016). Because it has been suggested that acute toxicity of OSPW, more specifically the acidic fraction of OSPW, is caused by a narcosis mode of action, further fractionation would not be informative.

7.3 Chemosensitization potential of OSPW organic constitutes

The role of membrane drug transporters in protection against environmental toxins is well established. Chemicals that can change the function of ABC drug transporters are called "chemosensitizers" (Kurelec, 1997). This thesis successfully established *in vitro* (caco-2 cell line) and *in vivo* (early life-stage of Japanese medaka) assays to test the hypothesis that organic chemicals in the aqueous phase of OSPW cause toxicity via chemosensitization (i.e. via inhibition of ABC transporters). Studies with both assays demonstrated that chemicals in the basic and neutral fractions of the aqueous phase of OSPW, but not in the acidic fraction, decrease the efflux

of substrates of ABC transporters (calcein-am) compared to control. Furthermore, adverse effects, internal concentration, and bioconcentration of a model substrate of P-gp (chlorpyrifos) was increased in early life-stage of Japanese medaka co-exposed with OSPW compared to non-p-gp substrate (malathion). While adverse effects of OSPW on aquatic organisms cannot be explained only by direct inhibition of ABC transporters, these results indicate that chemosensitization might be a mechanism of toxicity of OSPW.

Physicochemical properties of inhibitors of ABC proteins include presence of two planar aromatic domains, lipophilicity at a physiological pH with a log *Kow* > 2.92, and moderate to great molecular mass (Wang et al., 2003; Zamora et al., 1988). Another characteristic is the presence of a cationic charge usually in a nitrogen-containing cyclic ring (Ecker et al., 1999; Wang et al., 2003; Zamora et al., 1988). Most chemicals detected in either basic and/or neutral of OSPW have similar properties to inhibitors of ABC transporters (Alharbi et al., 2016a; Zhang et al., 2015). Thus, these findings suggested the acidic fraction do not have properties of chemosensitizers (i.e. do not act as a chemosensitizers).

Results of this thesis were not able to determine the mechanism(s) of inhibition of ABC transporters in embryos of Japanese medaka or Caco-2 cells. Chemicals in basic and neutral fractions might be substrates of ABC proteins, and displaced calcein from the ABC protein, thus giving the appearance of inhibition. Alternatively, chemicals in the basic and neutral fraction of OSPW might have direct inhibition of functioning of the transporter by competing indirectly with calcein within the substrate binding sites on the protein. Using the ATPase activity assay, Zaja et al (2011) identified three potential mechanisms of chemical interaction with specifically P-gp - substrate, inhibitor, and no interaction. However, it is not possible to distinguish between direct versus indirect inhibition of ABC proteins by OSPW. Because OSPW contains a variety of

surfactant like compounds that might directly or indirectly manipulate the activity of ABC transporters, the uptake and accumulation of chemicals in OSPW with and without a standard inhibitor of specific ABC transporters might not be a reliable approach to investigate whether OSPW constitutes are either substrates or inhibitors.

Effects of acidic, basic and neutral fractions of the aqueous phase of OSPW on mRNA expression of ABC proteins, as well as phase I and phase II biotransformation enzymes were investigated. In general, expression of genes of ABC proteins was inhibited in larvae of Japanese medaka exposed to basic and neutral fractions of OSPW, but not in larvae exposed to the acidic fraction. These results suggested that chemicals in acidic fractions do not regulate gene expression of ABC transporters. It has been shown that areas close to oil sands activities are contaminated with PAHs (Ohiozebau et al., 2016; Korosi et al., 2016), which are inducers of phase I enzymes such as cyp1a. The greater activity of cyp1a after exposure to benzo(*a*)pyrene (B*a*P) generates highly reactive and toxic metabolites that binds to DNA. Thus, inhibition of ABC efflux transporters, that are involved in the elimination of the reactive intermediates of benzo(*a*)pyrene, can increase DNA damage (Myllynen et al., 2007). In this thesis, it was not possible to investigate the effect of OSPW on amounts of protein of any ABC transporters because antibodies against these proteins in fish have not been developed. An anti-p-glycoprotein mouse mAb (C219) has been used to investigate levels of P-gp in fish, but it cross-reacts with other ABC proteins, thus it is not possible to investigate effects of OSPW on concentrations of specific ABC proteins (Childs et al., 1995; Georges et al., 1990; Loncar et al., 2010; Zaja et al., 2008).

Oil sands related matrices contain naturally occurring surfactants in the water column, as well as hydrophobic compounds, such as PAHs in sediment. Results of this thesis suggest that chemosensitization might be a mechanism of toxicity of OSPW. Specifically, exposure to basic

and neutral fractions of OSPW and PAHs might result in greater adverse effects in organisms than the exposure to only PAHs. Future risk assessments should consider this mechanism of toxicity.

7.4 Interaction of water soluble constituents (surfactants) in OSPW with hydrophobic organic contaminants

7.4.1 Relation to solubility, bioavailability

The solubility of hydrophobic chemicals, such as PAHs, decreases linearly with the increase of molecular weight. Solubility is a major factor that influences bioavailability of PAHs. Bioavailability and biodegradability of higher molecular weight PAHs is mainly limited by their low solubility, however, other factors such as strong sorption of PAHs to the adsorbents in soil might limit bioavailability (Schreiber et al., 2008).

Surfactants are chemicals that have both hydrophobic and hydrophilic moieties and can accumulate along air-liquid and liquid-liquid interfaces. It is well-known that solubility and biodegradability of PAHs can be enhanced by surfactants, where the hydrophobic core of the surfactant micelles can accumulate PAHs and increase the aqueous solubility. In general, rates of biodegradation of PAHs depends on desorption kinetics of PAHs from contaminated soil. Desorption of PAHs by surfactants from contaminated soil is controlled by molecular weight and concentrations of PAHs, concentration and type of surfactants, temperature, pH, salinity, dissolved organic matter, and soil type (Lamichhane et al., 2017).

The role of dissolved organic compounds in OSPW in affecting the aqueous solubility of PAHs in OSPW has not been previously addressed. In chapter 4, it was found that the concentration of retene in the aqueous phase increased when the aqueous phase was OSPW compared to freshwater. However, the type of surfactant (i.e. anionic, cationic, and non-ionic) that might enhance solubilities of PAHs could not be identified. These results suggest that traditional

methods and studies for assessing ecological risks of organic compounds in OSPW should be expanded to consider possible enhancement of solubility, biodegradability, and toxicity of other hydrophobic contaminants such as PAHs that might co-exist with OSPW constitutes in the same area. It is expected that the rate and amount of PAHs dissolving from sediment into the aqueous column will be higher in the presence of dissolved organic compounds generated from oil sands, which modulate PAHs solubility, bioavailability, and chemical activity.

7.4.2 Relation to toxicity and risk assessment

The identity and toxicological properties of the majority of organic chemicals in the aqueous phase of OSPW are unknown, and the toxicity data available are based on testing with the whole mixture or fractions, using either *in vivo* or *in vitro* test systems. It has been suggested that effects of mixtures on responses of biomarkers in aquatic organisms are different than effects of single chemicals. The lack of data on mixture toxicity can negatively impact legislation, remediation-plan for contaminated sites, and government's decisions and actions (Celander, 2011). In complex mixture, components can interact and cause substantial changes in toxicity of components from situations where they exist individually.

The principle of toxicity modulation (either enhancement or suppression) of hydrophobic organic contaminants (i.e. PAHs) by the dissolved organic fraction of OSPW has not been previously addressed. In this thesis, the effect of co-exposure to OSPW and the model of alkylated-PAH (retene) was investigated. PAHs are a contaminant of concern in the oil sands mining region, and have been detected in area close to oil sands industry (Korosi et al., 2016). Based on results presented in chapter 4, it can be stated that toxicity of PAHs can be enhanced in the presence of

dissolved organic fraction of OSPW however, it is possible that PAHs and dissolved compounds from OSPW act via a dissimilar mode of action.

7.5 Fresh versus aged OSPW

Based on this work and others (Anderson et al., 2012a, b; Wiseman et al., 2013a), toxicity of aged OSPW is lesser compared to fresh OSPW. There are many explanations for the observed lesser toxicity of aged OSPW, but the small concentration of total organic compounds in all fractions of aged compared to fresh OSPW might be a good indicator of the lesser toxicity of aged OSPW. Among all end points tested in this thesis (i.e. acute toxicity, toxicity enhancement, solubility enhancement, and inhibition of ABC drug transporters), aged OSPW exhibit lesser effect compared to fresh OSPW. Abundances of total organic class of chemicals, such as NAs, SO, NO, detected using HRMS were lesser in aged OSPW compared to fresh OSPW (except for O_x detected in positive ionization). However, the lack of true quantitative analysis of these chemical species make it difficult to fully illustrate the observed less toxicity of aged compared to fresh OSPW. Finally, although aging of OSPW attenuated the acute toxicity, inhibition of ABC transporters, and modulation of retene aqueous solubility; chronic exposure of aquatic organisms to aged OSPW might still negatively impact aquatic organisms, and therefore further research is required.

7.6 Future studies

Several potential research directions could be undertaken to improve understanding of the toxicity of OSPW. Separating OSPW into acidic, basic and neutral fractions is a new strategy to understand the chemical profile and toxicity of this complex mixture. For chemical analysis, the determination of total NAs has been suggested for chemical fingerprinting in OSPW and

environmental samples. However, there is a need (in future studies) to include other non-acidic chemicals such as basic and neutral for comprehensive characterization using HRMS of compounds derived from oil sands industry. It should be noted that one of the limitations of using HRMS is lack of a valid quantification methods of chemicals in OSPW. All the applied protocols in OSPW chemical profile do not consider quality assurance procedure, due to an absence of representative internal standards that can used for analytical method validation.

For toxicity testing, future research could investigate effects of chronic exposure to acidic, basic, and neutral fractions as well as their interactions with PAHs on some apical endpoints (such as reproductive fitness) in native species of fish. Also, effects of OSPW on absorption, metabolism, and elimination of free and conjugated PAHs in adult fish should be investigated to understand how much surfactants influence the toxicity of PAHs. These assays might represent a promising step towards the prediction of the potential toxicity and sensitivity of wild fish species to OSPW dissolved chemicals-PAHs mixture, which is important for future monitoring programs.

Studies of ecotoxicological relevance of ABC transporters has been limited by the absence of relevant techniques, limited availability of environmentally relevant substrates and inhibitors, and antibodies for ABC drug-transporters that can properly confirm their functionality in aquatic organisms. Although results of this thesis have shown modulation of expression of ABC efflux transporters as well as activity during short term exposure of larvae of Japanese medaka, functional studies are needed to demonstrate their activity in transportation of metabolites of PAHs in fish co-exposed to OSPW. This is very important since some ABC transporters such as ABCC2 and ABCG2 might have a role in the efflux of phase II metabolites of PAHs (Kranz et al., 2014). It should be noted that interactions between PAHs and dissolved organic compounds of OSPW is currently not covered under any risk assessment or regulation in environmental and health impact of oil sands industry.

7.7 Conclusion

Knowledge of how aquatic organisms are affected by chemical mixtures of surfactants and PAHs in OSPW is lacking. However, results presented in this thesis provided some novel approaches to address these gaps. This thesis investigated the direct and indirect toxicity of acidic, basic and neutral fractions in fresh and aged OSPW. While the acute toxicity of acidic fraction in OSPW is well-studied, this thesis provides novel insights into indirect effects of basic and neutral fractions. In this thesis, effects on the detoxification pathway and biomarker responses in fish exposed to OSPW fractions, either alone or their mixture are addressed.

In the ABC transporter inhibition assay (Chapter 3, 5, and 6), it was identified that OSPW influenced the efflux of calcein-am dye (substrate of ABC transporters) and also inhibited genes of expression of several ABC transporters. Moreover, concentrations and toxicity of P-gp model (chlorpyrifos) were enhanced in larvae of Japanese medaka when they co-exposed to basic and neutral fractions of OSPW which indicate they are likely the major driver of effects, but due to the complexity of each fraction, it was not possible to identify the components in each fraction. Chemical mixtures in the environment can cause chemical interactions resulting in adverse responses not seen by exposure to chemical alone. Data from this work suggest that chemosensitization in one mechanism by which OSPW can lead to increase in the toxicity of PAHs and other substrates of ABC transporters that might be present in OSPW.

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Appendix

APPENDIX A: Toxicity of oil sands process affected water fractions on early life stage of medaka (*Oryzias latipes*)

Embryos at stage 7–8 in early morula stage (3–4 h post fertilization [hpf]; Iwamatsu, 1994) were used in this test. Embryos were exposed in 6-well plates (10-12 egg per well) and incubated with 5×, 2.5×, 1× and 0.5× of BML-acidic, and only at 5× of BML-basic, BML-neutral, and acidic, basic and neutral -P9 with control and solvent control (see chapter 3 for detailed exposure media and conditions). However, in this test; embryos were exposed for the duration of their complete embryonic development (around 9 days) and the media was renewed daily by 50%.

Observations of embryos were made daily and the measurements made included; rate of premature hatching, pericardial edema, spinal malformation, and the number of live and dead embryos. However, except for lethality; non-of these endpoints showed any abnormal development in embryos exposed to any fractions. The cumulative percent occurrence of death was determined at the end of the exposure and used for statistical analyses as percentage survival of the number of live larvae experiment divided by the initial number of embryos.

The results showed that exposure to acidic fraction of BML at 5 and 2.5× significantly decreased the embryo survival rate (0%), compared to that of control (98.33±2.88%), 0.1% of ethanol (96.67±5.7%), 1× acidic-BML (80.0±26%), 0.5× acidic-BML (90±17%), 5× basic-BML (93.33±6.05%), 5× neutral-BML (88.0±1.9%), 5× acidic-P9 (90.00±4.89%), 5× basic-P9 (97.2±3.33%), and 5× neutral-P9 (94.0±12%) (Figure A1).

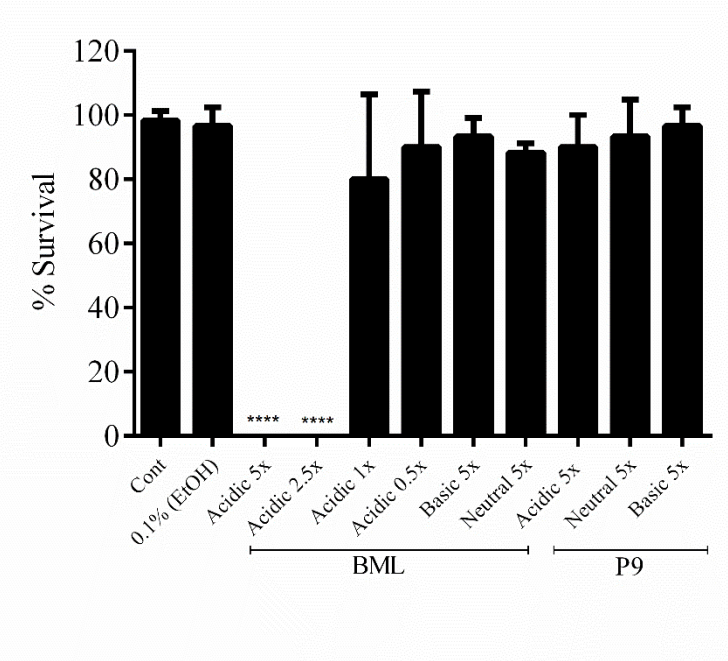

Figure A1: Survival of embryos of Japanese medaka exposed to A) 0.5, 1, 2.5, or 5× equivalent of the acidic fraction of BML-OSPW or a 5× concentration of basic and neutral of BML-OSPW, and 5× equivalent of the acidic, basic and neutral fraction of P9-OSPW. Survival is expressed as mean ± standard deviation of 3 independent studies in which there were 3 replicate exposures with 10-12 eggs per replicate. Significant differences in survival compared to control were determined by use of one-way ANOVA followed by Dunnett's post-hoc test and are designated by an asterisk. (**** = $P \leq 0.0001$).