The evaluation of toxic effects induced by exposure of mammals to oil sands process-affected water and its organic fraction

By

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Abstract

Oil sands process-affected water (OSPW) produced by the surface-mining oil sands industry in Alberta, has been shown to be toxic to a variety of organisms. Much of this toxicity has been attributed to the dissolved organic compounds, primarily naphthenic acids (NAs). Under a no-release practice, OSPW is stored on-site in tailings ponds with the understanding that eventual reclamation of this water must be undertaken. Successful remediation of OSPW is expected to require a reduction in total dissolved organics (including NAs) and the removal of other compounds that may contribute to OSPW toxicity. Ozonation is currently identified as one of the promising methods for reduction of OSPW toxicity. However, there is concern regarding the possible hazardous byproducts that may be created by ozonation. To date, most toxicological data of OSPW were generated using aquatic organisms, while information on the effects of OSPW exposure in mammals is limited. The overall objective of my research was to determine possible adverse effects of whole OSPW and its organic fraction (OSPW-OF) in a representative mammal, using *in vitro* and *in vivo* assessments, and evaluate the effectiveness of ozone treatment in reducing OSPW toxicity.

The raw OSPW and OSPW-OF were chemically analyzed, revealing that classical NAs ($C_nH_{2n+z}O_2$) and oxy-NAs ($C_nH_{2n+z}O_x$, x=3-6) accounted for 37.9% of the total mass of OSPW-OF. *In vitro* assays using RAW 264.7 mouse macrophage-like cell line demonstrated that whole OSPW, and not OSPW-OF, reduced cell viability at NAs ≥ 14 mg/L that was related to the cell membrane damage, and suppressed cellular proliferation. Whole OSPW (10 mg/L NAs content) also modulated the antimicrobial function of RAW 264.7 cells by altering NO production and subsequent release of pro-

inflammatory mediators. Furthermore, this dose of OSPW induced higher mRNA levels of heme oxygenase 1 (*hmox1*) in RAW 264.7 cells, suggesting that oxidative stress were induced by the constituents in tailing ponds water. Despite ~ 90% reduction of NAs after ozonation, this treatment failed to ameliorate the toxicity of OSPW. These results suggest that in addition to NAs, other components in OSPW (other organic substances or the constituent(s) of the inorganic fraction) or the additive and/or synergistic effects between the constituents present in whole OSPW are responsible for the observed toxic effects.

In vivo toxicity studies using BALB/c mice revealed that acute exposure during the gestation and sub-chronic exposure throughout gestation and lactation had minimal effects on the reproduction of female mice. Mating behavior, pregnancy success, embryonic implantation, gestation length, and litter size were not affected by NA doses up to 100 mg/kg body weight/week. The growth and development of the offspring was similar between pups from OSPW-OF orally exposed and sham-treated non-exposed mothers. Changes in pregnancy-associated hormones (progesterone, 17β-estradiol, prolactin and aldosterone) and immune response (T helper 1 (Th1)/Th2 cytokines shift) were observed in non-exposed pregnant mice, which promoted maternal tolerance of the fetus. OSPW-OF exposure did not bring about significant changes in plasma hormone levels and cytokine protein secretion, consistent with normal reproductive performance. The gene expression analysis suggested that liver was the main target organ for OSPW-OF. Acute exposure to OSPW-OF at 55 mg/L NAs down-regulated the expression of glutathione-S-transferase genes (*gstp1* and *gstm1*) in the liver, suggesting that cellular detoxification functions may be impaired. The expression of *ogg1* (8-oxoguanine DNA glycosylase), a gene involved in DNA repair, was also suppressed in mice after acute

exposure to OSPW-OF (55 mg/L NAs). However, these inhibitory effects were transitory and not observed after sub chronic (6 week) exposure of mice to OSPW-OF. Following sub-chronic exposure to OSPW-OF at high dose, an elevated mRNA level of uracil-DNA glycosylase (*ung*) was observed, indicating a possible increase in OSPW-OF induced mutagenesis.

The results of my thesis research present the first comprehensive analysis of mammalian toxicity associated with OSPW and OSPW-OF exposure, using a series of *in vitro* and *in vivo* bioassays. Additionally, it provides important information regarding the feasibility of ozonation as an option to reduce OSPW contaminants and associated toxicity.

Preface

All of the research in this thesis was designed and conducted by myself and supervised by Professors Mohamed Gamal El-Din and Miodrag (Mike) Belosevic at the University of Alberta.

Portions of Chapter 2 and 3 of this thesis have been published. The published manuscripts are: (1) Li, C., Fu, L., Stafford, J., Belosevic, M., and Gamal El-Din, M., 2017. The toxicity of oil sands process-affected water (OSPW): A critical review. Science of The Total Environment, 601-601: 1785-1802; (2) Fu, L., Li, C., Lillico, D., Phillips, N., Gamal El-Din, M., Belosevic, M., and Stafford, J., 2017. Comparison of the Acute Immunotoxicity of Nonfractionated and Fractionated Oil Sands Process-Affected Water Using Mammalian Macrophages. Environmental Sciences & Technology, 51 (15): 8624-8634. The manuscript in preparation is: Assessment of acute and sub-chronic toxicity of OSPW organic fraction in mice (Chapter 4 and 5).

The research presented in this thesis represents the first comprehensive analysis of the mammalian toxicity of oil sands process-affected water and its organic fraction. I was responsible for extraction of the organic fraction from OSPW, treatment of mice, tissue collection, data collection, analysis and writing of the manuscript (1) and thesis. Fu, L. assisted in mouse exposure, tissue collection, data analysis and writing of the manuscript (2). Singh, A. assisted in the OSPW organic fraction preparation, mouse administration and data collection. Lillico, D. contributed to RNA isolation and phagocytosis assays. Stafford, J., contributed to the critical discussion of the methods and preparation of manuscripts. Gamal El-Din, M., and Belosevic, M. were supervisory authors and involved in discussion of concepts and writing of the manuscripts.

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List of Abbreviations

ABC	adenosine triphosphate (ATP)-binding cassette
AhR	aryl hydrocarbon receptor
AOPs	advanced oxidation processes
BALB/c	Bagg albino laboratory-bred strain of the house mouse
BMDM	bone marrow-derived macrophages
BrdU	bromodeoxyuridine
BTEX	benzene, toluene, ethyl benzene, and xylenes
CF	coagulation/flocculation
CFS	coagulation/flocculation/sedimentation
-COOH	carboxyl group
CT	consolidated tailings
CYP 450	cytochrome P450
DMEM	dulbecco's modified eagle's medium
E2	17β-estradiol
EC_{50}	half maximal effective concentration
EDCs	endocrine disrupting compounds
ERα	estrogen receptor alpha
EROD	ethoxyresorufin-o-deethylase
ESCs	embryonic stem cells
FBS	fetal bovine serum
H_2O_2	hydrogen peroxide
$HO^{\cdot}$	hydroxyl radical
IC_{20}	concentration that induces an inhibitory effect of 20%
IC_{50}	the half maximal inhibitory concentration
LC_{50}	median lethal concentration
LDH	lactate dehydrogenase
LPS	lipopolysaccharide
MF	microfiltration
MFT	mature fine tailings
MLSB	Mildred Lake Settling Basin
MTT	3-(4,5-dimethylthiazolyl-2)-2,5 diphenyltetrazolium bromide

MW	molecular weight
NAs	naphthenic acids
NF	nanofiltration
NO	nitric oxide
O_3	ozone
OD	optical density
OSPW	oil sands process-affected water
OSPW-AEOs	OSPW acid extractable organics
OSPW-NAEs	OSPW naphthenic acids extracts
OSPW-OF	OSPW organic fraction
PAHs	polycyclic aromatic hydrocarbons
PC	petroleum coke
qPCR	quantitative real time polymerase chain reaction
RO	reverse osmosis
T	testosterone
TDS	total dissolved solids
UF	ultrafiltration
UV	ultraviolet light
VTG	vitellogenin
WIP	West In Pit
ZVI	zero valent iron
11-KT	11-ketotestosterone

Chapter 1: General introduction

1.1 Background

The oil sands in Northern Alberta are the third largest oil reservoir in the world, containing approximately 2.5 trillion barrels of recoverable bitumen (Penner and Foght, 2010; Jiang et al., 2016). The bitumen is extracted using a caustic hot water process, which requires about 3 barrels of fresh water for each barrel of bitumen produced (Allen, 2008a; Panikulam et al., 2015). This process leads to the generation of oil sands process-affected water (OSPW), which has been widely reported to be toxic to microorganisms (Jones et al., 2011; Wang et al., 2013), aquatic invertebrates (Anderson et al., 2012a, 2012b), fish (Hagen et al., 2013; van den Heuvel et al., 1999a, 2000, 2014), amphibians (Hersikorn and Smits, 2011; Melvin and Trudeau, 2012; Pollet and Bendell-Young, 2000), birds (Gentes et al., 2006), and mammals (Garcia-Garcia et al., 2011a, 2011b; Rogers et al., 2002). Under a no-release practice, OSPW is retained on-site in tailings ponds, with more than 10^9 m^3 of OSPW accumulated up in the Athabasca region (Del Rio et al., 2006). Ultimately, this accumulation of OSPW needs to be reduced in toxicity for future reincorporation into the ecosystem. Currently ozonation is considered a promising method for remediation of OSPW by reducing the concentration of dissolved organic constituents. However, it is also suggested that the byproduct generated during ozonation might increase the OSPW toxicity, thus it is imperative to evaluate toxicological properties of OSPW after ozonation.

Naphthenic acids (NAs) are a diverse group of naturally occurring petrogenic carboxylic acids that are released from bitumen during the extraction process (Allen, 2008a; Melvin et al., 2013). The toxicological aspects of NAs have been investigated,

and several lines of evidence point to NAs as the primary toxic constituent (Dokholyan and Magomedov, 1983; Frank et al., 2009a; MacKinnon and Boerger, 1986). Recent research showed that NAs account for less than 50% of the organic fraction in OSPW, and other dissolved organics may also contribute to the OSPW toxicity (Garcia-Garcia et al., 2011a, 2011b). Ambient levels of NAs in the Athabasca river and regional lakes in oil sands mining sites are commonly below 1-2 mg/L (Allen, 2008a; Headley and McMartin, 2004). However, NAs in tailings ponds are concentrated, ranging from ~20 to 80 mg/L in fresh settling basins, and ~5 to 40 mg/L in reclamation ponds or experimental wetlands (Li et al., 2017). NAs and other pollutants can enter the surface water systems through OSPW leaching from the tailings ponds, which may pose serious threats to the aquatic biota (Frank et al., 2014; Grewer et al., 2010; Headley and McMartin, 2004). Due to the high complexity of OSPW dissolved organics (including NAs), the identification of all the compounds responsible for the water toxicity is an impossible task. Therefore, the assessment of toxicity in complex OSPW organic fraction (OSPW-OF) requires integrative procedures combining chemical analysis and a set of suitable bioassays (Garcia-Garcia et al., 2011a; Petrovic et al., 2003).

Information about the mammalian toxicity of OSPW organics is sparse, being restricted to the toxic effects of OSPW-OF in murine immune systems (Garcia-Garcia et al., 2011a, 2011b), and behavioural and histopathological effects in rats exposed to OSPW NAs (Rogers et al., 2002). To the author's knowledge, only one study has demonstrated the reproductive toxicity of OSPW NAs in mammals (Rogers, 2003). Female rats after exposure to OSPW-derived NAs during pre-breeding, breeding and gestation, exhibited lower plasma progesterone levels, resulting in notable pregnancy loss

(Rogers, 2003). However, the dose of NAs used in this study were around 10 times the "worst-case single-day acute exposure scenario for wild animals" and these concentrations of NAs are unlikely to be found in the environment (Rogers, 2003). The research presented in this thesis focuses on the possible toxicological aspects of OSPW organics at naturally occurring levels of NAs in tailings ponds. The results from this study will help complete the assessment of the risk associated with OSPW exposures to wildlife and humans.

1.2 Research objectives

As a classical mammalian model organism, the biology of mice is considered representative of terrestrial mammals, including humans. The overall goal of this research is to evaluate the possible toxic effects of OSPW-OF and whole OSPW in mice using both *in vitro* and *in vivo* model systems.

The specific objectives were to:

1) Isolate organic fraction (including NAs) from OSPW so that these compounds could be used in toxicity testing.
2) Develop multiple *in vitro* tools for assessment of OSPW and OSPW-OF toxicity using RAW 264.7 mouse macrophage-like cell line.
3) Determine the acute and sub-chronic toxic effects of OSPW-OF, particularly on development and reproduction of mice, as well as possible immunomodulatory effects of OSPW-OF.
4) Assess whether the effects caused by OSPW and OSPW-OF could be ameliorated by ozone treatment.

Chapter 2: Literature review[1]

2.1 Introduction

The oil sands in northern Alberta, have the third largest oil reserves in the world (after Venezuela and Saudi Arabia), containing an estimated 2.5 trillion barrels of recoverable bitumen (a heavy and viscous form of crude oil) (Penner and Foght, 2010; Jiang et al., 2016). There are three major oil sands deposits in northern Alberta including Athabasca, Cold Lake and Peace River, covering a surface area of more than 140,000 km^2 (Figure 1) (Allen, 2008; Honarvar et al., 2011). The bitumen production by the Alberta's oil sands industry has reached 2.15 million barrels/day (bpd) in 2014, and is estimated to grow to 3.95 million bpd by 2030 (CAPP, 2015). The modified Clark water extraction process is commonly used to separate bitumen from oil sands in surface mining (Allen, 2008a; Gao et al., 2010). The detailed description of the Clark extraction process, and the resulting tailings streams (a mixture of water, solids and unrecovered bitumen) can be found in previous publications (Allen, 2008a; Mahaffey and Dube, 2016).

Any water that has been in contact with oil sands is referred to as oil sands process-affected water (OSPW) (Natural Resources Canada-Canmet ENERGY, 2010). This broad definition encompasses a variety of water types, including fresh OSPW that is retained in active settling basins or tailings ponds, consolidated tailings (CT) released water that is released after treatment of fine tailings with chemical/physical separation techniques, seepage or dyke drainage water collected from areas surrounding the active settling basins, and aged or treated OSPW from wetlands and reclamation ponds (Mahaffey and Dube, 2016). Consequently, there is significant variability in the chemical

[1] A version of this chapter has been published in *Science of the Total Environment* (2017), 601-602, 1785-1822.

composition of different OSPWs. Despite the differences in water chemistry, OSPW always contains several major classes of contaminants including: naphthenic acids (NAs), polycyclic aromatic hydrocarbons (PAHs), BTEX (benzene, toluene, ethyl benzene, and xylenes), phenols, heavy metals and ions (Allen, 2008a; Puttaswamy and Liber, 2012; van den Heuvel et al., 2012; Mahaffey and Dube, 2016). NAs are postulated to be the primary source of OSPW toxicity in the early stages of investigating the OSPW toxicity (Madill et al., 2001; MacKinnon and Boerger, 1986; Allen, 2008a; Clemente and Fedorak, 2005). Recent studies demonstrate that NAs account for less than 50% of all the compounds in the OSPW organic fraction (OSPW-OF) (Headley et al., 2009; Grewer et al., 2010; Garcia-Garcia et al., 2011a,b; Garcia-Garcia et al., 2012; Hagen et al., 2012; Hagen et al., 2013). In addition to NAs, PAHs and other organic species, dissolved ions, and heavy metals may also contribute to the overall OSPW toxicity (Alharbi et al., 2016b; Garcia-Garcia et al., 2011b, 2012; Kavanagh et al., 2012a; Leclair et al., 2013; Morandi et al., 2015; Sansom et al., 2013).

Due to the complexity of OSPW, it is difficult to specifically assess the toxic effects of individual constituent(s). Consequently, most of the studies to date have examined the toxic effects using whole OSPW or its complex fractions (i.e., organic fraction, NAs fraction). However, in the majority of studies, the OSPW types and sources are not often detailed. The inconsistent terminology and variance in different OSPW types have confounded comparisons of toxic effects induced by OSPW. In this chapter, a detailed summary of the exposures and endpoints for various organisms (i.e., microorganisms, invertebrates, fish, amphibians, birds, and mammals) exposed to different OSPW types is provided.

2.2 Toxicity of OSPW

OSPW has been shown to be toxic to both prokaryotes and eukaryotes. The main contributors of this toxicity appear to be the acid-extractable organic compounds, known as NAs (Madill et al., 2001; MacKinnon and Boerger, 1986; Allen, 2008a; Clemente and Fedorak, 2005). This organic fraction is comprised of NAs with an empirical formula $C_nH_{2n+z}O_2$, oxy-NAs ($C_nH_{2n+z}O_x$, x = 3-5), nitrogen and sulfur-containing species, and other organic acids (Kannel and Gan, 2012). The identification and synthesis of individual, structurally representative NAs in OSPW has enabled research on the relationship between structure and toxicity (Jones et al., 2011; Scarlett et al., 2013). NAs concentrations vary between ponds, ranging from ~20 to 80 mg/L in fresh settling basins (as reviewed by Mahaffey and Dube, 2016), and ~5 to 40 mg/L in reclamation ponds or experimental wetlands (Anderson et al., 2012a; Hagen et al., 2013; Hersikorn et al., 2010; Kavanagh et al., 2013, 2011). Significant research efforts have focused on evaluation of NAs toxicity using OSPW NAs extracts (NAEs), although other organics are also present in this complex mixture. Multiple physiological changes have been caused by exposure to NAEs, including impaired reproduction, developmental delays, suppressed immune response, and histological alterations in aquatic species and mammals (Garcia-Garcia et al., 2012, 2011a; Kavanagh et al., 2012a; Marentette et al., 2015b, 2015a; Nero et al., 2006a; Rogers, 2003; Rogers et al., 2002), suggesting that removal of NAs from OSPW, would result in decreased toxicity.

The potential toxic effects caused by other constituents in OSPW (e.g., PAHs, dissolved ions, heavy metals, etc.) have not received much attention. While specific information on the toxicity of these compounds present in OSPW is limited, they have

been associated with a wide range of biological dysfunctions in exposed organisms such as mutagenicity, carcinogenicity, immunotoxicity, and endocrine disruption (as reviewed by Li et al., 2014). It is probable that given the extreme complexity of OSPW composition, possible synergistic or antagonistic chemical interactions may be responsible for the observed toxicity. For example, studies have demonstrated that the addition of salts decreased NAEs toxicity in fish (Nero et al., 2006a; Kavanagh et al., 2012a). However, this reduction in toxicity was likely achieved by decreasing toxicant entry (i.e., NAs) through a reduced gill surface area caused by salts addition, which could also impact the efficiency of gas exchange and long-term issues in terms of fish health (Nero et al., 2006a).

2.2.1 Assessment of OSPW toxicity using *in vitro* assays

Various *in vitro* model systems (e.g., bacteria, immortalized cell lines, primary isolated cells, etc.) have been used to investigate the potential adverse effects of OSPW (Appendix A). The *in vitro* toxicity is commonly demonstrated based on IC_{50} or IC_{20} values; concentrations causing 50% or 20% inhibition of a specific biological or biochemical function. IC_{50} (24% - 67%) and IC_{20} (9%- 33%) values (v/v) of fresh OSPW towards the marine bacterium *Vibrio fischeri* have been reported (Gamal El-Din et al., 2011; Holowenko et al., 2002; Scott et al., 2008; Wang et al., 2013; Zubot et al., 2012). The toxic effects were reduced following *in-situ* biodegradation, as shown by increased IC_{50} (64%-100%) and IC_{20} (11%-100%) values (v/v) of aged OSPW obtained from remediation ponds and wetlands (Holowenko et al., 2002). The reported amelioration of OSPW acute toxicity might be due to the altered structure and composition of NAs over

time. Indigenous microbial populations seem to preferentially degrade NAs with < 22 carbons, resulting in increased proportions of C_{22+} with OSPW aging (Biryukova et al., 2007; Clemente et al., 2004; Quagraine et al., 2005). Similarly, NAs with higher molecular weight (MW) were shown to be less toxic to *V. fischeri* (Frank et al., 2008). These observations seemed to contradict the concept that, for typical NAs structures, the larger MW compounds have a greater hydrophobicity, are more likely to bioaccumulate, and thus should exhibit stronger narcotic potency (Frank et al., 2009a). However, Frank et al. (2009a; 2009b) hypothesized that the reduced toxicity of higher MW NAs was due to the presence of increased NAs -COOH content which decreased hydrophobicity and, consequently, reduced toxic effects.

Continuous or permanent cell lines have been also used for the evaluation of toxic effects induced by OSPW. Research that employed fish cell lines has demonstrated a good correlation between acute cellular toxicity and NAs or total acid-extractable organics in OSPW (Lee et al., 2008; Sansom et al., 2013). Endocrine disruptive properties of fresh OSPW have been reported in human cell lines, shown by estrogenic and antiandrogenic effects observed in T47D-kbluc and MDA-kb2 cells, respectively (He et al., 2011), as well as elevated 17β-estradiol (E2) and decreased testosterone (T) concentrations produced by H295R cells (He et al., 2010). OSPW has also been shown to induce immunotoxicity in primary cells. The OSPW organic fraction (OSPW-OF) was reported to cause a dose-dependent decrease in nitric oxide and respiratory burst responses of mouse bone marrow-derived macrophages (BMDM), as well as suppressed phagocytosis (Garcia-Garcia et al., 2011b). Recently, *in vitro* exposures of fish hepatocytes to OSPW and OSPW acid-extractable organics (OSPW-AEOs) were

reported to induce genotoxic and mutagenic changes in the cells (Lacaze et al., 2014; Zetouni et al., 2016). It has also been reported that *in vivo* exposure to OSPW constituents, such as diamondoid naphthenic acids, can cause genetic damage in gills and haemocytes of marine mussels (Dissanayake et al., 2016).

In vitro tests have become a common approach for evaluating OSPW toxicity due to their low cost, simplicity, high capacity, and reproducibility. These characteristics make them effective screening tools to quickly identify priority pollutants in OSPW, and to evaluate the effectiveness of wastewater treatment methods. Bioluminescence is the endpoint of the Microtox assay using *V. fischeri*; it is a direct measure of narcosis (membrane disruption) which is hypothesized to be the primary mode of action for acute toxicity of NAs (Frank et al., 2008, 2009b). However, *V. fischeri* is a marine organism and therefore not an appropriate model to predict effects on freshwater species. The use of hormone-responsive cell lines and primary cells isolated from animal tissues have revealed more specific mechanisms (endocrine disrupting effects and immunotoxicity) affected by OSPW; however, the abnormal functions in cancerous cell lines, and the absence of biokinetics in *in vitro* models might lead to a misinterpretation of the data when extrapolated to the potential effects at the organismal level (Saeidnia et al., 2015). Therefore, interpretation and reporting of the effects from *in vitro* experiments requires careful consideration, and appropriate follow-up *in vivo* studies to assess whether the toxic effects observed *in vitro* are reproducible *in vivo* are needed.

2.2.2 Assessment of OSPW toxicity using animal models

2.2.2.1 Toxicity of OSPW in invertebrates

The lethal effects of fresh OSPW exposure of invertebrates are shown in Appendix B. Early research demonstrated acute toxicity of fresh OSPW from the Mildred Lake Settling Basin (MLSB, Syncrude Canada Ltd.) towards *Daphnia magna* and *Daphnia pulex*, with low 96h-LC_{50} (the median lethal concentration) values of 10% (v/v) and 2%-10% (v/v), respectively (MacKinnon, 1986; MacKinnon and Boerger, 1986; MacKinnon and Retallack, 1982). A recent report indicated 48h-LC_{50} value of >100% (v/v) for fresh OSPW from the West In Pit (WIP) settling basin (Syncrude Canada Ltd.) for *D. magna* (Zubot et al., 2012). This low acute lethality of *D. magna* was also reported by Lari and colleagues (Lari et al., 2016), however, exposure to sub-lethal concentrations of OSPW (1%) impaired feeding, growth, and reproduction of *D. magna*, which may threaten their survival (Lari et al., 2016). Results of studies using *Ceriodaphnia* bioassays indicated both acute (survival, 6-d LC_{50} of 65%), and chronic (fecundity, 6-d IC_{50} of > 39%) toxicity for fresh OSPW (Zubot et al., 2012). It was suggested that OSPW salinity affected the ability of *Ceriodaphnia* to reproduce (Zubot et al., 2012). Multiple toxic effects of fresh OSPW were observed using *Chironomus dilutus* larvae, including reduced body mass, lower pupation levels, decreased rates of emergence, abnormal behavior, oxidative stress responses, and altered endocrine signaling (Anderson et al., 2012a; Wiseman et al., 2013a). The suppressed larval growth might be due to oxidative stress and disruption of endocrine processes, as suggested by the changes in relevant gene expression (Wiseman et al., 2013a). While NAs were correlated strongly with toxic endpoints, it was suggested that some metals (e.g., nickel, manganese, and uranium)

present in OSPW may also contribute to the reported toxic effects (Anderson et al., 2012a).

The wetland reclamation represents a passive treatment method under the assumption that NAs concentrations and OSPW toxicity will eventually diminish through *in-situ* biodegradation (Kavanagh et al., 2011). The characteristics of OSPW-impacted wetlands have been studied. They appeared to display a lower overall benthic invertebrate diversity (Bendell-Young et al., 2000; Whelly, 2000), a greater chironomid diversity (Bendell-Young et al., 2000), and decreased growth of *Chironomus riparius* larvae (Kennedy, 2012). The mouthpart deformities of chironomids were also examined as evidence of teratogenic effects, and no significant difference was found between wetlands (Bendell-Young et al., 2000). The toxicity of OSPW may decline by aging, as indicated by less diverse invertebrate populations in young (< 7-year old) OSPW-impacted wetlands than older ones (Leonhardt, 2003). This reduction in toxicity might be explained by NAs biodegradation in aged wetlands, but may also be due to the colonization of more tolerant species over time (Kennedy, 2012). A recent study using invertebrates showed that NAs from fresh OSPW were less toxic than those from aged OSPW (Bartlett et al., 2017). These results were contradictory from previous research reporting a decrease in OSPW toxicity occurring with degradation processes associated with aging (Anderson et al., 2012a; Holowenko et al., 2002; MacKinnon and Boerger, 1986). These different observations raised the possibility that the reduction in OSPW toxicity with age was the result of a difference in whole OSPW composition, including potential interactions with organic and inorganic compounds, other than NAs degradation. In fact, laboratory experiments have indicated the antagonistic interaction between NAs

and salts, when *C. riparius* was exposed to water mimicking combinations of these two constituents (Kennedy, 2012).

Benthic invertebrates are commonly used as indicators of water quality, as they spend all or most of their life cycle in water, and many of them are sensitive to the pollutants. The toxicity of OSPW in the invertebrates has been demonstrated in terms of effects on the ecologically relevant endpoints of survival, growth, pupation, and emergence, as well as community characteristic such as abundance and diversity.

2.2.2.2 Toxicity of OSPW in fish

Fish serve as valuable aquatic vertebrate models for toxicological research, owing to their relatively small size, rapid growth and development, short generation time, and externally developing embryos that facilitate experiments in developmental toxicology (Hinton et al., 2009). OSPW is widely reported to be toxic to fish by affecting a variety of endpoints (Appendix C). Early studies reported the 96h-LC_{50} values of $< 35\%$ (v/v) for fresh OSPW collected from various tailings ponds in rainbow trout and fathead minnows, and in some cases, low to ~ 3% v/v caused mortality (MacKinnon, 1981, 1986; MacKinnon and Retallack, 1982; Nix and Martin, 1992). The mortality of rainbow trout exposed to fresh OSPW (100% mortality of rainbow trout after exposure to 50% of MLSB-OSPW for 96 hours) was also reported by Rogers and colleagues (Rogers et al., 2007). Fathead minnow survived a 96-h exposure with altered hematology in CT water and dyke seepage water, but all died in a prolonged period (28 days) (Farrell et al., 2004). The different responses to OSPW exposures could be associated with the different sensitivities of fish species to pollutants, and the variance in composition of OSPW

tested. Research on OSPW organic fractions demonstrated that the fraction containing alicyclic, 'classical' NAs were acutely toxic to larval zebrafish, with 96h-LC_{50} of 13.1 mg/L (Scarlett et al., 2013). Interestingly, an aromatic NAs fraction, containing compounds like dehydroabietic acid, was more toxic (96h-LC_{50} 8.1 mg/L) (Scarlett et al., 2013). These observations suggest that NAs are at least partially responsible for the lethal effects of OSPW on fish, and that these effects are composition- and structure- dependent.

The non-lethal effects of fresh OSPW have also been extensively studied in fish, including reduced fertilization success, premature hatching, increased embryo deformities, and elevated transcript of genes associated with xenobiotics metabolism, oxidative stress and apoptosis (He et al., 2012a; Peters et al., 2007). Oxidative stress could result in damage to mitochondria and promote activation of caspase enzymes and apoptotic cell death (He et al., 2012a). Some of these toxic effects in fish embryos were also caused by OSPW-NAEs (Marentette et al., 2015a,b; Wang et al., 2015). Studies at molecular levels indicate that OSPW-NAEs negatively impacted the development and endocrine function of fish, likely via altering the expression of endocrine-disrupting biomarker genes (Wang et al., 2015). Hagen et al. (2013) reported on the immunotoxic effects of both acute and sub-chronic exposures of goldfish to fresh OSPW. Fish acutely exposed to OSPW had higher transcripts of pro-inflammatory cytokine genes, and enhanced ability to control parasites infection (Hagen et al., 2013). However, this does not necessarily mean that fish are healthy, and in fact, prolonged exposure resulted in significant down-regulations of pro-inflammatory cytokine genes that may influence the susceptibility of fish to infectious diseases (Hagen et al., 2013).

Similar to the results obtained using microorganisms and invertebrates, the acute

toxicity of OSPW appears to decline in aged OSPW ponds (Nero et al., 2006b; Hagen et al., 2013). However, aged OSPW still induced toxic effects, manifested by histological changes in liver and gill tissue (Nero et al., 2006b), dysregulation of immune genes expression (Hagen et al., 2013), decreased plasma levels of steroid hormones (Kavanagh et al., 2011; Lister et al., 2008), as well as impaired growth and reproduction performance (Kavanagh et al., 2011) in different fish species. Although the exact causative pollutants in OSPW are not fully known, there is evidence that these toxic effects are due to the NAs. For example, a 21-day exposure of yellow perch to OSPW-NAEs caused histopathological changes in liver and gill (Nero et al., 2006a), and lower fecundity, spawning and plasma steroid concentrations in fathead minnows (Kavanagh et al., 2012a).

Recently, increasing efforts have been made to discover the endocrine disruptive properties of OSPW. An *in vivo* investigation on fathead minnow indicated that fresh OSPW had endocrine-disrupting effects at all levels of brain-gonad-liver axis (He et al., 2012b). The compounds responsible for the activities were not identified, but some NAs have been implicated as the candidate endocrine disrupting compounds (EDCs). For instance, the transcription of estrogen receptor (ERα), and vitellogenin were significantly induced by OSPW-NAEs on the early life stage of zebrafish that might negatively impact the development and endocrine functions (Wang et al., 2015). The endocrine disruptive effects seem to be structure-dependent. For example, studies have demonstrated that some aromatic NAs in OSPW are structurally similar to estrogens (Rowland et al., 2011b), and some polycyclic NAs with a single aromatic ring may possess human estrogenic and androgenic activity (Scarlett et al., 2012). Reinardy and colleagues

(Reinardy et al., 2013) reported the vitellogenin-inducing effects of esterifiable OSPW NAs, particularly the aromatic NAs in zebrafish larvae. These results suggest that some NAs, especially aromatic NAs, might account for some endocrine disrupting activities reported in OSPW and OSPW-NAEs. EDCs could disrupt synthesis, secretion, transport, binding, or elimination of hormones and steroids in organisms. The hormones and steroids or their receptors are often involved in homeostasis, reproductive capacity, development, or behavior (CEPA, 1999; Hagen, 2013). Interestingly, the reduced plasma levels of E2 witnessed in fish (Lister et al., 2008; van den Heuvel et al., 1999) were in contrast to the increased E2 production by H295R cells exposed to OSPW (He et al., 2010). These variable results may be due to the different chemistry of waters tested (i.e., fresh OSPW vs. aged OSPW), and the difference of specificity and sensitivity of endocrine disruptive properties between whole fish and human cell lines.

Given the high complexity of OSPW, other contaminants in OSPW (PAHs, salts, etc.) likely contribute to the overall toxicity in aquatic organisms. While PAHs can be reduced by volatilization, degradation, and sediment absorption, an average concentration of 0.01 mg/L in OSPW substantially exceeds environmental guidelines of 0.00001-0.00006 mg/L (Allen, 2008a; Beck et al., 2015). There is significant evidence that PAHs from different oil sources cause toxicity in organisms. Many PAHs have been shown to induce teratogenic, mutagenic, carcinogenic, endocrine disruptive, and immunotoxic properties (reviewed by Collier et al., 2013), however, the information on OSPW-derived PAHs is limited. Alharbi et al. (2016b) reported the inhibition of ATP-binding cassette (ABC transport proteins) in Japanese medaka exposed to the water-soluble organic fraction isolated from synthetic OSPW (prepared by extraction of bitumen from oil sands

in the laboratory). ABC transporters are important for excretion of PAHs and their metabolites. Inhibition of the protein activity might exacerbate accumulation and effects of PAHs and/or their bio-activated metabolites in cells, resulting in greater toxicity (Alharbi et al., 2016b). Further research on the effects of OSPW on toxicity of retene (the model alkyl-PAH) provided additional evidence that dissolved organic pollutants in OSPW might increase exposure and uptake of PAHs by fish (Alharbi et al., 2016a).

During the bitumen extraction process, salts leaches from oil sands leading to elevated total dissolved solids (TDS) concentrations (~1200-2500 mg/L) and high conductivity of OSPW (~1000 to 4000 μS/cm) (Mahaffey and Dube, 2016), which may induce ionic imbalances in fish and cause osmotic stress and mortality. While the sensitivity is organism-specific, conductivity above 2,000 μS/cm or TDS above 1,340 mg/L represent concentrations sufficient to cause toxicity in fish (Goodfellow et al., 2000; Leah, 2012).

The heavy metals present in OSPW may also contribute to the overall toxicity of this complex chemical matrix, since they are recalcitrant and are known to bioaccumulate. The concentrations and toxicity risk of heavy metals in OSPW have been described previously, and the target heavy metals that exceeded the Canadian Council of Ministers of the Environment (CCME) water quality guidelines include arsenic, copper, cadmium, lead, and chromium (Li et al., 2014; Zhang, 2016). Information on toxicity of OSPW-derived heavy metals is scarce, however, research on heavy metals present in other industrial wastewaters has demonstrated their toxic effects on cardiovascular, respiratory, gastrointestinal, nervous, hepatic, hematopoietic, immunological, endocrine, and reproductive systems in fish and mammals (Li et al., 2014; Hagen, 2013). All of

these results emphasize the importance of continued research to investigate interactions among chemicals co-existing in OSPW rather than focusing on one specific group of pollutants.

2.2.2.3 Toxicity of OSPW in amphibians

Amphibian larvae are very sensitive to contaminants in the aquatic environments, and it is not surprising that the OSPW exposures could affect their health. Both laboratory and field studies have examined the toxicity of OSPW-impacted wetlands towards amphibians, and the findings of these studies are summarized in Appendix D. Higher mortality, stunted growth and development, elevated ethoxyresorufin-o-deethylase (EROD) activity, as well as alterations in hormone production were observed in tadpoles of *Bufo boreas* or *Lithobates sylvaticus* after exposure to OSPW-impacted wetlands waters (Pollet and Bendell-Young, 2000; Hersikorn et al., 2010; Hersikorn and Smits, 2011). EROD measures the activity of the cytochrome CYP 450 enzyme family, and has been well established as a biomarker of contaminant exposure (Whyte et al., 2000; Havelková et al., 2007; Hersikorn and Smits, 2011). Elevated EROD activity in tadpoles from OSPW-impacted wetlands indicates increased detoxification efforts by the animals, reflecting greater concentrations of pollutants (Hersikorn and Smits, 2011). Hersikorn and colleagues have also provided evidence that detoxification occurs in OSPW-impacted wetlands through aging, since “old” OSPW-impacted wetlands showed markedly lower toxicity than “young” wetlands (Hersikorn et al., 2010; Hersikorn and Smits, 2011).

2.2.2.4 Toxicity of OSPW in birds

Reclaimed wetlands receiving oil sands tailings have been constructed by some companies on their mining leases, with the aim to eventually return the lands disturbed by oil sands mining to self-sustaining ecosystems (Gentes et al., 2007a). Tree swallows (*Tachycineta bicolor*) inhabiting the reclaimed sites are good indicator species for evaluating the potential toxicity of OSPW and the sustainability of wet landscape reclamation strategy. They become exposed to OSPW compounds through food-web transfer, because 80% of their diets are the aquatic insects whose larvae develop in OSPW and sediments (Smits et al., 2000; Gentes et al., 2007a). The toxic effects of OSPW-impacted wetlands on tree swallows are summarized in Appendix E. It was found that tree swallows from OSPW-impacted wetlands had reduced reproductive performance and increased mortality of nestlings, though data were obtained during harsh weather (Gentes et al., 2006). When the weather was less challenging, the mortality rates were low, but less weight and higher hepatic EROD activity of nestlings were recorded (Gentes et al., 2006). More recently, there was evidence of endocrine disrupting effects and immunotoxicity resulting from OSPW exposures, including elevated thyroid hormones (Gentes et al., 2007a) and heavy blow fly infestation (Gentes et al., 2007c). The altered thyroid function might compromise the post-fledging survival by negatively impacting the metabolism, behavior, feather development, and molting (Gentes et al., 2007a), and higher blow fly burdens indicated impaired host resistance to parasites (Gentes et al., 2007c). Another study by Gentes and colleagues (Gentes et al., 2007b) reported minimal sub-acute toxicity of commercial NAs mixtures that had comparable chemistry to NAs extracted from OSPW. However, it was also suggested that though

nestling tree swallows might tolerate short-term exposures to environmentally realistic concentrations of NAs, chronic toxicity of NAs still needs to be determined, because birds breed on these reclaimed sites lengthening their exposure to pollutants (Gentes et al., 2007b). A experiment with mallard (*Anas platyrhynchos*) ducklings reared on reclaimed wetlands suggested that such water is not acutely toxic, though the observed differences (lower body mass and skeletal size) might be related to the decreased survival of juvenile waterfowl (Gurney et al., 2005). The negligible adverse physiological effects were also documented in domestic mallards (*Anas platyrhynchos domestica*) after repeated, short-term exposures to OSPW from a recycled water pond (Beck et al., 2014).

So far, the direct assessments of toxicity in birds from OSPW exposures used those either held on reclaimed wetlands (Gentes et al., 2006, 2007b, 2007a, 2007c) or subjected to OSPW from a recycle water pond (Beck et al., 2014). When evaluating the OSPW toxicity from reclaimed wetlands, the interaction of birds and their environment should be considered, given the potential cumulative effects of other stressors such as harsh weather and additional routes of contaminant exposures (e.g., toxins in sediments, plants, and invertebrates). The relatively low toxicity documented in current literature could not rule out the possibility of adverse health effects on birds exposed to other OSPW sources, especially fresh OSPW; therefore, further studies are required.

2.2.2.5 Toxicity of OSPW in mammals

There are limited toxicity data on mammals after exposure to OSPW, which have focused primarily on rodents (Appendix F). An earlier report by Rogers et al. (2002) demonstrated the toxicity of OSPW-NAEs in rats. It was shown that exposure to a high

dose (300 mg/kg bw) of NAEs caused liver and heart damage and heavier ovaries and spleens in female rats, and brain hemorrhage and heavier testes in male rats (Rogers et al., 2002). It should be noted that animals were exposed to an OSPW dose that was 50 times higher than the "worst-case single-day exposure for wild animals" (Rogers et al., 2002). However, in a sub-chronic (90-day) toxicity test, lower dose (60 mg/kg bw/d NAEs) exposure caused adverse health effects in rats (Rogers et al., 2002). Although this dose was 10 times the estimated worst-case daily exposure, there exists the possibility that indigenous mammals might be more sensitive to NAs than rats used in laboratory assessment, since some uncertainty (e.g., age, season, diet, health status, contaminant interaction, etc.) in field toxicity testing could influence the response to NAs (Rogers et al., 2002). Recently, research using mice reported the immunotoxic effects of OSPW-OF manifested by alterations in various macrophage microbicidal functions, and immune gene expression in different organs (Garcia-Garcia et al., 2012, 2011a,b). The doses that induced toxicity in these studies reflected environmentally realistic concentrations of NAs in OSPW. It is also quite possible that other organic compounds present in OSPW-OF, may also contribute to the observed immunotoxicity.

While there is a significant dataset on the effects of OSPW exposure on reproduction and development in fish, there is very little information on the acute and sub-chronic effects of OSPW exposure on development and reproduction of mammals. The exposure of mammals (rats) to OSPW-NAEs caused impaired embryonic implantation, which was likely associated with the changes in cholesterol availability and a parallel decrease in progesterone levels (Rogers, 2003). Recently, research using mouse embryonic stem cells (ESCs) showed that the OSPW-NAE affected the expression of

cardiac specific markers in differentiating mouse ESCs, which may potentially cause developmental abnormalities (Mohseni et al., 2015). These findings suggest that OSPW organic compounds (including NAs) may affect mammalian reproduction and development, and emphasize the importance of testing organisms during sensitive developmental stages when establishing an environmental risk assessment of OSPW exposures.

2.3 Remediation of OSPW

OSPW remediation for greater recycle and reuse, or potential release, is important in optimizing water management in oil sands industry. Many processes have been studied to treat OSPW, though there are no proven methods to accomplish complete remediation and reclamation. Biodegradation, also known as OSPW aging, allows the microbial populations indigenous to the tailings ponds to degrade NAs and other constituents over many years. This method is the most cost-effective way to mitigate OSPW toxicity; however, field studies have indicated that the detoxification process by biodegradation is slow and incomplete, which was likely due to the presence of recalcitrant and toxic NAs and/or other toxic constituents in OSPW (Kavanagh et al., 2011; Scott et al., 2008). Ozone and advanced oxidation processes (AOPs) have received much attention for OSPW treatment due to their high oxidizing ability to degrade recalcitrant NAs, and have been proposed as a complementary technology to biodegradation for remediation of OSPW. Ozone and ozone-based AOPs are discussed in section 2.3.1, below.

Currently, many other OSPW treatment methods are under investigation. Coagulation/flocculation/sedimentation (CFS) is common pre-treatment process for

removing the suspended solids, colloidal particles, as well as NAs with higher molecular weight from OSPW (Pourrezaei, 2013). Adsorption using activated carbon alone, or combined with other adsorbents, has shown effectiveness in the removal of organic compounds (e.g., NAs and BTEX) from wastewaters including OSPW (Adhoum and Monser, 2004; Allen, 2008b; Petrova et al., 2010). Recently, the performance of synthetic copolymers and petroleum coke (PC) in reducing organics via adsorption has also been studied. Copolymer adsorbents containing β-cyclodextrin have displayed favorable sequestration of OSPW-derived NAs, although their adsorption capacity was found to be lower than that of granular activated carbon (Mohamed et al., 2011). PC is a waste by-product generated during bitumen processing that contains an intrinsic adsorptive capacity. PC alone or combined with zero valent iron (ZVI) has been found to effectively remove dissolved organic carbon, the acid-extractable fraction, and NAs, with the resulting effluent non-toxic to *V. fischeri* and rainbow trout (Pourrezaei et al., 2014; Zubot et al., 2012). Membrane-based treatments are applied for removal of ions and organics from water. Four commonly used membrane technologies are microfiltration (MF), ultrafiltration (UF), nanofiltration (NF), and reverse osmosis (RO). NF has shown great capacity of OSPW softening and NAs removal (Peng et al., 2004); however, their applications for OSPW treatment are hindered by membrane fouling caused by high content of suspended solids. Recently, combination of pretreatment methods (CFS or CF) and filtration (NF and RO) has shown effectiveness to reduce both OSPW salinity and membrane fouling (Kim et al., 2011). Heavy metals found in OSPW are of a concern due to their recalcitrance and persistence in the environment. Recently, an indigenous green micro-alga (*Parachlorella kessleri*) in OSPW that is responsible for the removal of

various metals (i.e., nickel, copper, arsenic, strontium, molybdenum, barium, zinc, manganese, chromium, and cobalt), while the removal rate was dependent on water sources and nutrient concentrations (Mahdavi et al., 2013). Pourrezaei et al. (2014) used ZVI alone or combined with PC, demonstrating an effective removal of vanadium, manganese, nickel, molybdenum, arsenic, cadmium, cobalt, and strontium. Although the removal mechanisms of heavy metals from OSPW by ZVI have not been elucidated, research on groundwater suggested that the reduction of metals might be based on their adsorption onto the surface of ZVI and co-precipitation with the iron oxy/hydroxides during the oxidation of ZVI (Abedin et al., 2011; Mak and Lo, 2011).

Few of the OSPW remediation technologies discussed above have been tested at bench- or pilot- scales. The challenge for scale-up is to meet the industrial needs in terms of volumetric flow rates, treatment efficiencies, and the operating and maintenance costs. To date, aging (*in-situ* remediation) of OSPW is the only method for OSPW treatment, and has been ongoing for decades. Remediation using dry and wet landscapes has been proposed as an option for handling the large volumes of OSPW. In these two processes, mature fine tailings and associated OSPW are used. The wet landscape consists of interconnected wetlands that ultimately drain into the end-pit lakes that are created by filling the mined pit with OSPW and freshwater (Allen, 2008b; Kennedy, 2012).

The aim of constructing wetlands is to produce aquatic ecosystems with functionality equivalent to natural water bodies (Han et al., 2009; Leung et al., 2003). While reduced NAs levels and toxicity in OSPW collected from various wetlands have been reported, the detoxification process is incomplete and slow. However, in the absence of adequate

development and scale-up of other technologies, wet landscapes are reasonable options due to their economic feasibility and minimal impacts on the environment (Allen, 2008b).

2.3.1 Ozonation of OSPW

Ozone (O_3) is a strong oxidant that has been found to be an effective way for oxidation of organic pollutants in water and wastewater (Ikehata and Gamal El-Din, 2004, 2005, 2007; Ikehata et al., 2006). Ozonation can proceed through two pathways: direct reaction by molecular ozone and indirect reaction by highly active hydroxyl radical ($HO^{\cdot}$) that is produced via ozone self-decomposition. Ozone can also be used in combination with other chemicals or processes, known as advanced oxidation processes (AOPs), such as O_3/H_2O_2, UV/O_3 and $O_3/H_2O_2/UV$. AOPs have better generation of $HO^{\cdot}$ which is a stronger oxidant than ozone. Ozonation at high pH (pH>8) is also considered as an AOP because basic condition favors the $HO^{\cdot}$ production (Rivas et al., 2003).

Recent studies have shown that ozonation and ozone-based AOPs (i.e. O_3/H_2O_2) can be an effective way to degrade NAs and/or reduce OSPW toxicity. Ozonation has been shown to ameliorate nearly all adverse effects caused by OSPW, including acute toxicity to *V. fischeri*, endocrine disruptive effects in cell lines and fish (He et al., 2010, 2011), impairment in the growth and development of invertebrates and fish (Anderson et al., 2012b; He et al., 2012a), and immunotoxicity in mice (Garcia-Garcia et al., 2011a). However, it should be noted that in most cases ozonation cannot achieve a complete degradation of organic compounds and may generate toxic intermediates. He et al. (2011) found ozonation did not reduce the estrogenic effect of OSPW, using a human cell line. They suggested that the failure of ozonation to reduce estrogenic effects of OSPW was

due to preferential degradation of NAs with higher carbon and ring numbers and inability to degrade the less cyclic and branched NAs could that may be ER agonists (He et al., 2011). The selective degradation of NA fractions by ozonation (preferentially $n \geq 22$) and microorganisms (preferentially ≤ 21) is well established. For example, ozone pre-treatment of OSPW has been shown to result in accelerated microbial degradation of the residual NAs (Hwang et al., 2013; Kannel and Gan, 2012; Martin et al., 2010).

2.4 Discussion and Conclusions

Although the term 'OSPW' is commonly used in toxicology studies, the water chemistry of different OSPW-types varies considerably, depending on the ore sources, extraction and processing methods, and tailings pond characteristics. Different OSPW-types induce different toxic effects both *in vivo* and *in vitro*. A lack of information on water sources, inconsistent analytical methods for water chemistry, and different procedures for OSPW fraction preparations likely accounts in part for the difficulties in the interpretation of toxicological data between taxonomic groups. Studies on whole OSPW and OSPW-derived fractions have identified the constituents with potential toxicity including NAs, PAHs, metals, salts, and other organic or inorganic compounds. NAs are the most widely reported contributors to OSPW toxicity that may induce toxic effects via multiple modes of action such as nearcosis, endocrine disruption, immunotoxicity and carcinogenicity. NAs extracts from different water sources elicit different responses, and are dependent on both concentrations and composition. The great advances in analytical methodology for NAs have improved our understanding of individual NA compounds, and enabled the studies of structure-toxicity relationships.

Future research on NAs-induced toxicity should focus on the compounds that potentially may be more harmful to exposed organisms, such as the more hydrophobic molecules with greater narcotic potency, the diamondoid NAs that causes genotoxicity, and some aromatic NAs that exhibits endocrine disruptive properties. The overall toxicity of OSPW is due to complex interactions between compounds present in the water. While the contribution of other OSPW-derived compounds (e.g., PAHs, dissolved ions, and heavy metals) has not received much attention, studies on other industrial wastewaters have shown a range of biological dysfunctions caused by these compounds. Therefore, elucidation of the biological effects of individual compounds and/or additive or synergistic effects of a group of compounds should be a high priority for future research on OSPW toxicity.

In vitro studies have provided information regarding the mechanisms of OSPW toxicity. OSPW has inhibitory effects on bioluminescence production by marine bacteria *V. fischeri*, estrogenic and antiandrogenic effects on hormone-responsive cells lines, immunotoxicity in mouse primary immune cells, as well as impacts on mammalian development using mouse embryonic stem cells. However, *in vitro* tests only expose single cell types to the contaminants. The results are not necessarily related to the outcomes in complex biological systems (living tissue), due to tissue-specific differences in mechanisms of action, biotransformation, or tissue-specific bioaccumulation (Garcia-Garcia et al., 2011a; Rosengren et al., 2005; Schlenk, 2008). Therefore, the risk assessment for OSPW exposure cannot be easily made without further knowledge of its effects under more physiologically relevant conditions (integrated system of an intact organism), and the combination of biological data generated in both *in vitro* and *in vivo*

assays are required to better understand OSPW toxicity. A variety of organisms have been used for OSPW toxicology research, and they have exhibited different sensitivities; for instance, OSPW was more toxic to *D. magna,* than fish (trout), and least toxic to *V. fischeri* (MacKinnon and Boerger, 1986). These findings suggest that the toxic effects of OSPW are species-specific, and that the responses observed in prokaryotic organisms may not be applicable to eukayotic organisms. To date, the majority of *in vivo* toxicological studies on OSPW have used aquatic organisms. In general, OSPW exposure has induced multiple toxic effects including compromised immunological function, impaired reproduction and development, disrupted endocrine system, and pathological changes in fish, that appear to be dose-, species-, life stage-, and duration of exposure-dependent. However, the extrapolation of data from fish to higher organisms requires careful consideration. For example, differences in routes of OSPW exposure (e.g., skin for fish vs. oral administration for rodents), and potential differences in kinetics and dynamics of xenobiotics between fish and rodents or other mammals could affect the their biological responses. So far, results of mammalian toxicity of OSPW have only been reported using rodents. The adverse effects observed were following assessment of the organic fraction of OSPW (the NAs containing fraction) that may not represent the full toxic effects induced by the whole OSPW (organic and inorganic fractions). Additionally, the laboratory-derived toxicity thresholds for NAs could differ from the possible toxic effects on animals in the wild, due to the uncertainty factors such as weather, diet, health status of wild animals and possible exposure to other contaminants and pathogens.

In summary, the results of the present review suggest that future studies should

provide detailed information on OSPW sources and types (i.e., fresh OSPW, CT water, seepage, and water from reclaimed wetlands, etc.), and conduct the chemical analysis using standard analytical methods. It is also likely that, in addition to NAs, other organic and inorganic compounds present in OSPW may contribute to the water toxicity. We suggest that the research examining the effects of OSPW exposure on aquatic and terrestrial organisms should continue in three main directions: (1) identification of the most toxic components (priority pollutants) of OSPW to enable targeted treatment regimens; (2) investigation of the overall contribution to toxicity of OSPW using parallel assessments of organic and inorganic fractions and whole OSPW to determine potential additive and/or synergistic effects of different toxic components present in OSPW; and (3) comprehensive side-by-side comparisons of the toxic effects induced by different OSPW waters. Furthermore, the cross-species interpretation and extrapolation of the toxicological effects will be required, to enable appropriate risk assessment of OSPW exposure on living organisms.

To date, the aging of tailings ponds and wet landscape remediation relying on natural biodegradation are the only economically-viable methods capable of handling the extremely large volumes of OSPW. However, these options are slow and lead to the incomplete detoxification of OSPW. Some alternative technologies for OSPW treatment have been shown to be effective for the rapid removal of target contaminants from OSPW; however, they have only been tested at a small scale. Further work is required to demonstrate whether they will be effective in full-scale applications and how their resulting by-products will affect the receiving environments.

Chapter 3: The exposure to oil sands process-affected water and its organic fraction changes the physiology of RAW 264.7 mammalian cells[2]

3.1 Introduction

The oil sands surface mining in northern Alberta employs a caustic hot water extraction method to recover bitumen, which results in significant volumes of toxic oil sands process-affected water (OSPW) (Zhang et al., 2016; Shu et al., 2014). OSPW is a complex mixture of water, organic and inorganic compounds. The majority of the OSPW toxicity has been attributed to the water-soluble organic fraction, of which naphthenic acids (NAs) are one of the primary persistent and toxic constituents (Anderson et al., 2012a; Garcia-Garcia et al., 2011a,b). NAs are a group of carboxylic acids with a general formula of $C_nH_{2n+z}O_2$ (classical NAs) (Pérez-Estrada et al., 2011). Recent advancement in high-resolution mass spectrometry has allowed for the identification of various other types of NAs in OSPW, including oxidized, aromatic, and sulphur-/nitrogen-containing NAs (Grewer et al., 2010; Kannel and Gan, 2012; Rowland et al., 2011a).

Substantial studies have shown that OSPW exposures cause lethality and affect the physiological homeostasis in aquatic biota. The adverse effects include impaired immunological function, reproductive and developmental toxicity, as well as disruption in endocrine system in aquatic organisms, mostly fish (as reviewed by Li et al., 2017). However, limited research has been done regarding toxicity of OSPW *in vitro*. Some bioassays using cell lines have been developed to assess OSPW toxicity due to their low cost and high capacity. One study examined the cellular integrity and functionality of

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multifish cell lines after exposure to 49 different OSPW samples and concluded that NAs or total acid-extractable organics were responsible for the acute toxicity (Sansom et al., 2013). Other studies using human cell lines demonstrated that OSPW has endocrine disrupting effects and also altered the expression of genes involved in oxidative burst, apoptosis and immune function (Knag et al., 2013). Primary isolated cells are also important *in vitro* model systems used for OSPW toxicity tests. Rainbow trout hepatocytes exposed to OSPW, exhibited alterations in the mRNA levels of genes related to xenobiotic biotransformation, estrogenicity, oxidative stress and DNA repair activity (Gagné et al., 2013, 2012, 2011). Cytotoxicity and immunological impacts were observed in mouse bone-marrow derived macrophages (BMDM) exposed to OSPW organic fraction (OSPW-OF), including disrupted cellular proliferation, and reduced antimicrobial responses (Garcia-Garcia et al., 2011b). The impairment of BMDM activity was likely associated with a reduction in the expression of pro-inflammatory cytokines gene (Garcia-Garcia et al., 2011b). Parallel *in vivo* experiments further support that OSPW-OF altered immune gene expression profiling in mouse immune organs (Garcia-Garcia et al., 2011b). It should be noted that in addition to NAs, which account for less than 50% of all the organics in OSPW, other compounds such as BTEXs, phenols, polycyclic aromatics, dissolved ions and heavy metals might also contribute to OSPW toxicity (Alharbi et al., 2016b; Garcia-Garcia et al., 2012, 2011b; Kavanagh et al., 2012a; Leclair et al., 2013; Morandi et al., 2015; Sansom et al., 2013).

Under a zero-discharge practice, OSPW is accumulating in on-site tailings ponds. In order for OSPW to be reclaimed, it is essential that the toxic constituents (e.g., NAs) be reduced. This is attempted by aging OSPW to decrease the concentrations of NAs

through natural *in situ* microbial biodegradation. However, higher molecular weight NAs are resistant to biodegradation; as a result, the detoxification of OSPW is incomplete (Martin et al., 2010; Wang et al., 2013). Currently, ozonation has been reported to be a promising method for reduction of NAs content of OSPW-OF and hence the overall OSPW toxicity. It has been shown to ameliorate nearly all adverse effects caused by OSPW, including acute toxicity to *Vibrio fischeri* (Scott et al., 2008; Wang et al., 2013), endocrine disruptive effects in cell lines and fish (He et al., 2011, 2010), impairment in the growth and development of invertebrates and fish (Anderson et al., 2012b; He et al., 2012a), and immunotoxicity in mice (Garcia-Garcia et al., 2011a). However, there is concern regarding the possible hazardous by-products that may be generated during ozonation of complex industrial wastewater such as OSPW.

The purpose of this study was to use an *in vitro* mammalian cell-based assay system to investigate and compare the acute toxicological properties of whole OSPW and OSPW-OF, and also to evaluate the detoxification efficiency of ozone treatment. Specifically, RAW 264.7 cell line (mouse leukaemic monocyte macrophage cell line) was used for OSPW and OSPW-OF exposures at selected doses based on NAs concentrations. The toxicity was examined by measuring changes in cellular viability and proliferation, immune functions in response to bacterial/endotoxin stimulation, and stress responsive gene expression in treated cells. This multiparametric bioassay system allows for rapid screening of the toxic fraction in OSPW, and also for quick evaluation of OSPW remediation effectiveness.

3.2 Materials and Methods

3.2.1 Oil sands process-affected water (OSPW)

Whole OSPW was collected from Aurora (Syncrude), Fort McMurray, Alberta in 2012, and stored in 200-L polyethylene barrels in dark at 4°C prior to use. A detailed composition of this water is shown in Table 3.1.

3.2.2 Ozonation of OSPW

Forty five liters of OSPW was ozonated in a 50-L reactor. Ozone gas was generated from extra-dry high purity oxygen using the ozone generator (WEDECO, GSO-40, Herford, Germany). Ozone was delivered into OSPW via a ceramic fine bubble gas diffuser. Throughout the procedure, ozone concentrations in feed gas and effluent gas (off-gas) were monitored using two ozone monitors (model HC500, PCI-WEDECO). The utilized ozone dose was calculated according to the equation as described previously (Gamal El-Din and Smith, 2002). The ozone dose used in this study was 80 mg/L. This dose was selected based on the preliminary experiments in our research group that it could achieve approximately 90% reduction of NAs. After ozonation, all OSPW samples were stored at 4 °C prior to use.

3.2.3 Extraction of the organic fractions of OSPW and ozonated OSPW

The organic compounds were isolated from 40 L of OSPW and ozonated OSPW (OSPW+O_3), using a liquid-liquid organic extraction protocol developed in our group (Garcia-Garcia et al., 2011b). This method allowed for the isolation of both neutral and acid organic components from OSPW. The step-by-step procedure for extraction of the

organic fraction of OSPW is shown in Figure 3.1. The pH of OSPW or OSPW+O_3 was adjusted to 10.5 with NaOH to ensure that weak organic acids (including NAs) were fully dissolved. OSPW was clarified by centrifugation for 20 min at 15000 x *g*, followed by three rounds of liquid-liquid organic extraction, using 100 mL of dichloromethane (DCM) per liter of OSPW in each round. DCM (organic phase) was collected after each round and combined. The water phase was then adjusted to pH 2 using hydrochloric acid, followed by three additional rounds of DCM extraction. The DCM phase from each round was collected and pooled, followed by the removal of DCM using a rotary evaporator. The resulting organic fraction is known as OSPW-OF or OSPW+O_3-OF. Once the amount of NAs in the organic fractions was determined by UPLC/HRMS (604.66 mg and 56.33 mg in OSPW-OF and OSPW+O_3-OF, respectively), the organic fractions were dissolved in same amount of distilled water (pH ~10) containing final NAs concentrations of 23.8 mg/mL for OSPW-OF NAs, and 2.2 mg/mL for OSPW+O_3-OF NAs.

3.2.4 NAs analysis

Ultra-performance liquid chromatography/high-resolution mass spectrometry (UPLC/HRMS) was used to measure the NAs and oxy-NAs in OSPW and OSPW-OF. Chromatographic separations were performed using a Waters UPLC Phenyl BEH column (1.7 μm, 150 mm × 1 mm) with a gradient mobile phase of (A) 10 mM ammonium acetate solution prepared in Optima-grade water and (B) 10 mM ammonium acetate in 50% methanol and 50% acetonitrile. The gradient elution throughout the column was as follows: 1% B for 2 min, ramped to 60% B for 3 min, to 70% B for 7 min, to 95% B for

13 min, held for another 14 min followed by 1% B for 5.8 min to reach the equilibrium. Constant flow rate was maintained at 100 μL/min and column temperature was 50°C. A high resolution Synapt G2 HDMS mass spectrometer was used for detection of the NAs using an electrospray ionization source operating in negative ion mode. The detection system was controlled with MassLynx® (ver. 4.1) software. Standard solutions, lucine enkaphenlin and sodium formeate, from Waters Corporation (Milford, MA, USA) were used for tuning and calibration, respectively. TargetLynx® (ver. 4.1) was used for data analysis of target compounds with the relative ratio of each analyte chromatographic peak area to the internal standard used for subsequent analysis. In this study, raw OSPW and OSPW-OF stock solutions contained 18.38 mg/L and 23.8 mg/mL of NAs respectively.

3.2.5 Exposure of RAW 264.7 cells to OSPW, OSPW-OF, ozonated OSPW and ozonated OSPW-OF

The RAW 264.7 cell line was purchased from the American Type Culture Collection (ATCC). Cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 in complete medium: Dulbecco's modified Eagle's medium (DMEM; Thermofisher Scientific, ON, Canada) supplemented with 10% fetal bovine serum (FBS; Thermofisher Scientific) and 1% penicillin/streptomycin (Invitrogen). The cells were exposed to whole OSPW (18.38 mg/L NAs in stock sample) or OSPW-OF (23.8 mg/mL NAs in stock sample) by diluting the complete cell culture media with different volumes of OSPW or OSPW-OF to achieve various NAs concentrations (1, 2, 4, 6, 8, 10, 12, 14, 16 and 18 mg/L NAs) in complete medium. The 18 mg/L NAs was selected as our highest dose for OSPW, to reflect the possibility that organisms might be directly exposed to undiluted

OSPW in a specific pond (i.e., Aurora tailings pond, Syncrude). The same dilutions used for OSPW and OSPW-OF samples were done for OSPW+O_3 and OSPW+O_3-OF, respectively. Phosphate buffered saline (PBS) was diluted as the same manner as OSPW, and used as the dilution control for whole OSPW exposure to match the media displacement that occurred when the cells were exposed to the whole OSPW samples. For example, to achieve a 1 mg/L final dose of whole OSPW NAs, 5.4% of the media was displaced by the whole OSPW stock sample, and in the matching dilution control 5.4% of the media was also displaced by PBS. The remaining mg/L and % media displacements used in our study were as follows: 2 mg/L (10.9%), 4 mg/L (21.8%), 6 mg/L (32.6%), 8 mg/L (43.5%), 10 mg/L (54.4%), 12 mg/L (65.3%), 14 mg/L (76.2%), 16 mg/L (87.1%), and 18 mg/L (97.9%).

3.2.6 Gene expression

The expression of representative stress and detoxification genes was determined after exposure of RAW 264.7 cells to OSPW and OSPW-OF. Briefly, 1×10^6 cells were seeded in individual wells of a 6-well plate, and exposed to OSPW, matched PBS controls, and OSPW-OF at 10 mg/L NAs for 18 h. Total RNA was extracted from the cells using the RNA isolation kit (Thermofisher Scientific), and reverse transcribed into cDNA using the Superscript III cDNA synthesis kit (Thermofisher Scientific) according to manufacturer's instructions. The cDNA was used for quantitative PCR (qPCR) using 2× Dynamite SYBR Green PCR master mix (developed by Molecular Biology Service Unit in Biological Sciences at the University of Alberta, Canada). Primers (Integrated DNA Technologies, IDT) used in this study are listed in Table 3.4 and were validated in

the lab as below: standard curve for each primer was generated from qPCR performed on cDNA dilutions. Primers were deemed acceptable for use if the R^2 values from standard curves were greater than 0.980. The qPCR product was subjected to agarose gel electrophoresis and the resulting product was sequenced (done by Molecular Biology Service Unit in Biological Sciences at the University of Alberta, Canada) to confirm the primer specificity. Hypoxanthine-guanine phosphoribosyltransferase (*hprt1*) was used as the endogenous control. The qPCR was performed using a 7500 Fast Real-Time PCR apparatus (Applied Biosystems) according to the cycling conditions: 95°C, 2 minutes; 95°C 15 seconds; 60°C, 1 minute; 95°C 15 seconds; 60°C 1 minute; 95°C, 30 seconds; 60°C, 15 seconds (melting curve); for 40 cycles. Gene expression was analyzed using the ddCt (Delta-Delta-Ct) method, normalizing relative quantitation (RQ) values for OSPW, dilution control, and OSPW-OF to the non-exposed control.

3.2.7 The 3-(4,5-dimethylthiazolyl-2)-2,5 diphenyltetrazolium bromide (MTT) assay

The Vybrant® MTT cell proliferation assay kit (Thermofisher Scientific) was used to measure the cell viability after OSPW and OSPW-OF exposures. It was performed in 96-well plates according to the manufacturer's instructions, using 1×10^4 cells per well. Cells were incubated at 37°C with 5% CO_2 overnight to allow the macrophages to adhere to the plates. The medium was aspirated, and cells were exposed to OSPW (and matched PBS controls), OSPW+O_3, OSPW-OF or OSPW+O_3-OF at indicated NAs concentrations for 18 h. After exposure, the medium was removed, and the cells were incubated with 12 mM MTT reagent for 4 h, followed by the addition of dimethyl sulfoxide. The optical density (OD) was measured at 540 nm using SpectraMax

M_2 microplate reader (Molecular Devices, CA, USA). % Viability = [(mean OD of experimental samples) / (mean OD of non-exposed control) × 100].

3.2.8 Bromodeoxyuridine (BrdU) proliferation assay

The BrdU proliferation assay kit (EMD Millipore, ON, Canada) was used to measure relative cell proliferation activity after OSPW and OSPW-OF exposures. It was performed in 96-well plates, using 1×10^4 cells per well. The cells were exposed to OSPW (and matched PBS controls), OSPW+O_3, OSPW-OF or OSPW+O_3-OF for 18 h as described above. After exposure, cells were labelled with BrdU for additional 2 h, and then fixed with a fixing solution provided in the kit. Fixed cells were stained with anti-BrdU peroxidase conjugated antibody for 30 minutes, and then washed. The bound peroxidase activity was detected using tetramethy benzidine substrate. The OD values were measured at 450 nm using SpectraMax M_2 microplate reader (Molecular Devices, CA, USA). % BrdU incorporation = [(mean OD of experimental samples) / (mean OD of non-exposed control) ×100].

3.2.9 Lactate dehydrogenase (LDH) release assay

The Pierce™ LDH cytotoxicity assay kit (Catalog No. 88954, Themrofisher scientific) was used to measure the cell lysis after OSPW and OSPW-OF exposures. Briefly, the RAW 264.7 cells (1×10^4 cells per well) were seeded in 96-well plates and exposed to OSPW (and matched PBS controls), OSPW+O_3, OSPW-OF or OSPW+O_3-OF for 18 h as described above. The culture supernatants were collected and incubated with LDH reaction mixture provided in the kit at room temperature for 30 minutes in the dark.

Stop solution was then added to stop color formation. LDH release was assessed by measuring absorbance at a wavelength of 490 nm and 680 nm, respectively, using SpectraMax M_2 microplate reader (Molecular Devices, CA, USA). % Cytotoxicity = [(Experimental release – Spontaneous release) / (Maximum release – Spontaneous release) ×100]

3.2.10 Nitric oxide assay

Nitric oxide production, in response to lipopolysaccharide (LPS) stimulation, was measured using the Griess reaction. A total of 4×10^4 cells were seeded in individual wells of a 96-well plate, and exposed to OSPW (and matched PBS controls), OSPW+O_3, OSPW-OF or OSPW+O_3-OF for 18 h. After exposure, cells were stimulated with 1 μg/mL LPS for additional 24 h. Seventy five μL of culture supernatant was recovered, mixed with the Griess reagents (100 μL of 0.1% N-[1-naphthyl]-ethylenediamine in 2.5% phosphoric acid, and 100 μL of 1% sulphanilamide in 2.5% phosphoric acid, sequentially). The absorbance at 540 nm was determined using SpectraMax M_2 microplate reader (Molecular Devices, CA, USA), and the amount of nitrite in supernatants was calculated using a nitrite standard curve.

3.2.11 Cytokine production assay

Upon stimulation, RAW 264.7 cells are capable of producing high levels of cytokines that participate in the regulation of immune responses. A total of 1×10^5 cells were seeded in individual wells of a 24-well plate, and exposed to OSPW (and matched PBS controls), OSPW+O_3, OSPW-OF or OSPW+O_3-OF for 18 h. The cells were then

exposed to 3×10^7 fluorescein-labelled *Escherichia coli* particles (Thermofisher Scientific) for 2 h. The excess *E. coli* was washed and the cells cultured in complete medium overnight to allow the cells to secrete cytokines in the supernatants. The supernatants were collected and analyzed using the Mouse Cytokine/Chemokine Array 31-Plex (Eve Technologies, Calgary, AB, Canada). This assay permitted simultaneous quantification of multiple cytokines by capturing the cytokines to differently colored/fluorescent beads that were detected by the bead analyzer (Bio-Plex 200, BIo-Rad). Only those with significant changes after exposure were presented.

3.2.12 Statistical analysis

Data are presented as mean ± standard error of the mean (SEM). Statistical analysis of data was performed using one-way ANOVA, followed by the Tukey's *post hoc* test, using Prism 6.0 (Graphpad software, CA, USA). Differences between treatment groups were considered statistically significant when $P < 0.05$.

3.3. Results

3.3.1 NAs analysis

The concentrations of NAs in OSPW and OSPW+O_3 are shown in Table 3.2, and extraction efficiency of the organic fraction and ozonated organic fraction in Table 3.3. As shown in Table 3.2, ozone treatment degraded approximately 90% of classical NAs, resulting in OSPW containing lower concentration (1.61 mg/L) of classical NAs than oxy- NAs ($O_{1\text{-}3}$-NAs, 1.81-4.5 mg/L). The reduction of oxy-NAs was also caused by ozonation, while their degradation rates (2.58-57.32%) were much lower than that of

classical NAs. Table 3.3 demonstrates that the NAs were effectively extracted from OSPW before and after ozonation, with the extraction rates > 80%.

3.3.2 Expression of stress responsive and detoxification genes

After exposure to whole OSPW or OSPW-OF, at 10 mg/L NAs for 18 h, cells were analyzed for expression of oxidative stress responsive gene (*hmox1*), cell growth arrest and DNA damage-inducible gene (*gadd45*), and genes involved in xenobiotic biotransformation (*gstp1*) and DNA repair activity (*ogg1*). As shown in Figure 3.2, mRNA level of *hmox1* in OSPW-treated cells was significantly up-regulated, in comparison with the non-exposed control and dilution control. In contrast, the OSPW exposure did not alter the expression of *gstp1*, *gadd45*, or *ogg1*. Similar to other parameters measured, OSPW-OF exposure had no effects on expression of selected genes.

3.3.3 Cytotoxicity

3.3.3.1 Cell viability

I analyzed cell viability using the MTT assay after exposing RAW 264.7 cells to OSPW, OSPW+O_3, or their organic fractions. MTT is a tetrazolium dye that can be reduced by active cellular oxidoreductase enzymes into its insoluble purple colored formazan. This colorimetric assay is used for assessing the cell metabolic activity that may reflect the relative number of viable cells present. The MTT values of the non-exposed control (0 mg/L) were normalized to 100% cell viability, and the MTT values from treatment groups were expressed as the percentage of cell viability relative to non-exposed control. As shown in Figure 3.3A, cells exposed to dilution control (corresponding to ≤ 16 mg/L NAs in OSPW) showed no significant loss of cell viability

as that of the non-exposed cells. The treatment of cells with OSPW and OSPW+O_3 led to decreased cell viability in a concentration-dependent manner (Figure 3.3A). The cell viability was reduced by ~80 - ~95% when the cells were cultured in the highest doses of whole OSPW (i.e., 16 and 18 mg/L NAs), and their corresponding ozonated OSPW (i.e., 1.4 and 1.58 mg/L NAs) for 18 hours. In contrast, OSPW-OF treatment had no effects on cellular viability at indicated NAs concentrations, while OSPW+O_3-OF containing NAs of 1.58 mg/L exhibited cytotoxicity (Figure 3.3B).

3.3.3.2 Cell proliferation

The cellular proliferation was assessed using BrdU assay that is based on measuring the fluorescent thymidine analog BrdU incorporated into the DNA of dividing cells. The incorporation of this compound into the cellular nucleus also indirectly reflects the cell viability as only live proliferating cells can be labeled with BrdU (Ansar Ahmed et al., 1994). As illustrated in Figure 3.4A, a significant reduction of DNA labeling was observed in cells exposed to OSPW at NAs concentration of 10 mg/L and above, relative to non-exposed cells. In contrast, the organic fractions isolated from OSPW before and after ozonation had no effects at up to 18 mg/L NAs (Figure 3.4B). Similar to MTT results, although ozonation effectively reduced around 90% of NAs, an inhibitory effect on BrdU incorporation levels was still observed in the cells exposed to OSPW+O_3 containing low NAs concentration ($\geq$ 0.88 mg/L). Data from the dilution control groups indicated that the media loss caused significant decreases when the percentage of culture medium was 13% or less (corresponding to $\geq$ 16 mg/L NAs in OSPW).

3.3.3.3 Cell membrane integrity

To further investigate potential cytotoxicity of samples tested, the LDH leakage assay was performed which was based on the measurement of lactate dehydrogenase activity in the culture medium. The loss of intracellular LDH and its release into the extracellular medium is an index of irreversible cell death due to cell membrane damage (Fotakis and Timbrell, 2006). As shown in Figure 3.5A, apparent cytotoxicity was observed in cells after treatment with OSPW (14 - 18 mg/L NAs) and OSPW+O_3 (1.23 - 1.58 mg/L NAs) for 18 h. By contrast, neither OSPW-OF nor OSPW+O_3-OF at indicated NAs concentrations had effects on cell membrane integrity (Figure 3.5B). The results from dilution control indicated that medium loss occurred in dilutions did not induce cell lysis of RAW 264.7 macrophages.

3.3.4 Nitric oxide production

Macrophages play an important role in the development of innate immune response against pathogens (Kim et al., 2015). They can be activated by bacterial endotoxins such as LPS, and produce pro-inflammatory molecules like cytokines and reactive nitrogen intermediates (Kim et al., 2015). To investigate the effect of OSPW or its organic fraction on nitric oxide (NO) production, I measured the accumulation of nitrite in the culture media using the Griess reagent. The stimulation of RAW 264.7 cells to LPS for 24 h resulted in an increased nitric oxide response (Figure 3.6A). However, as shown in Figure 3.6B, this NO production by LPS-activated cells was significantly reduced after exposure to OSPW (8 - 10 mg/L NAs) and OSPW+O_3 (0.53 - 0.88 mg/L NAs) without significant cytotoxic effects as measured by MTT and LDH assays. In

contrast, exposure to OSPW-OF or OSPW+O_3-OF had no effect on cellular NO response (Figure 3.6C).

3.3.5 Cytokine production

OSPW and OSPW+O_3 modulated immune response by reducing NO production of LPS-stimulated macrophages. Based on these results, I hypothesized that macrophages exposed to water samples might not properly generate cytokines in response to inflammatory stimuli. I examined several cytokine secretion levels by *E. coli*-stimulated cells. As shown in Figure 3.7A, bacteria-stimulated cells incubated in PBS dilution control secreted comparable levels of all proteins, in comparison with those incubated in complete media. However, pre-treatment with OSPW (10 mg/L) and OSPW+O_3 (0.88 mg/L) for 18 h significantly increased the production of pro-inflammatory mediators including interleukin 1 alpha (IL-1α), interleukin 1 beta (IL-1β), granulocyte-macrophage colony-stimulating factor (GM-CSF), and vascular endothelial growth factor (VEGF) by *E. coli*-activated RAW 264.7 cells. The exposure to organic fractions from OSPW or ozonated OSPW at indicated NAs concentrations had no effects on production of all cytokines tested (Figure 3.7B).

3.4 Discussion

Naphthenic acids have been postulated to be the primary source of OSPW toxicity in multiple early studies (Madill et al., 2001; MacKinnon and Boerger, 1986; Allen, 2008a; Clemente and Fedorak, 2005). However, increasing research has indicated that, in addition to NAs, PAHs and other organic species, dissolved ions, and heavy metals may also contribute to the overall OSPW toxicity (Alharbi et al., 2016b; Garcia-Garcia et al.,

2011a,b; Garcia-Garcia et al., 2012; Kavanagh et al., 2012; Sansom et al., 2013; Leclair et al., 2013; Morandi et al., 2015). Substantial efforts have been made to develop strategies for the removal of the toxic pollutants from OSPW. Ozonation is one promising method that has been shown to cause effective degradation of organic constituents, resulting in the amelioration of OSPW toxicity (Anderson et al., 2012b; Garcia-Garcia et al., 2011a; He et al., 2010, 2011). However, there is also concern regarding the possible hazardous by-products that may be created in ozonation. Our lab has previously established sensitive *in vitro* assays using primary cell cultures from fish and mice, for evaluation of OSPW and OSPW-OF toxicity (Garcia-Garcia et al., 2011a; Hagen et al., 2013). In the present study, we used the immortalized mammalian cell line RAW 264.7 cells to further explore OSPW toxicity, to directly compare the toxic effects between OSPW and OSPW-OF, as well as to evaluate the detoxification effectiveness of ozone treatment. The immortalized cell lines are homogenous and can be grown indefinitely, which allows for rapid, accurate and cost-effective screening system.

I examined the effects of OSPW, OSPW-OF, ozonated OSPW and ozonated OSPW-OF exposure by measuring the proliferation, viability, cytotoxicity and production of inflammatory mediators in LPS- or *E. coli*- stimulated RAW 264.7 cells. We observed that exposure of the cells to OSPW caused significant reduction in their viability and proliferation ability, that were related to the cell membrane damage after exposure to OSPW containing NAs concentrations > 10 mg/L. In contrast, the exposure of cells to OSPW-OF had no impacts on cellular viability at NAs concentrations up to 18 mg/L. This is not surprising given the previous results that mouse primary bone marrow-derived macrophages (BMDM) exposed to OSPW-OF containing NAs as high as 50

mg/L had normal cell viability (Garcia-Garcia et al., 2011b). The cell proliferation in BMDM was increased after exposure to OSPW-OF at low NAs concentrations (6.25 and 12.5 mg/L) (Garcia-Garcia et al., 2011b). In contrast, proliferation of RAW 264.7 cells treated with OSPW-OF up to 18 mg/L NAs were not affected. This different toxic effect could be due to the deviations in composition of NAs and other uncharacterized organic contaminants in organic fractions from different OSPW sources, as well as different sensitivity between cell lines and primary cells in response to pollutants.

Although ozone treatment effectively reduced ~90% of NAs, and approximately 70% of total NAs (NAs and oxy-NAs) in OSPW, the cytotoxicity of OSPW was not ameliorated, and even increased for some endpoints. For example, RAW 264.7 cells exposed to OSPW+O_3-OF, but not those exposed to OSPW-OF, were significantly less viable as indicated by the results of the MTT reduction assay. This observation suggests that high dose ozone treatment of the organic fraction of OSPW may result in generation of by-products that may be more toxic than parent compounds in OSPW.

Macrophages play a crucial role in both the specific and non-specific immune responses, through release of various inflammatory mediators such as NO, cytokines, chemokines, and growth factors (Lee et al., 2012; Park et al., 2013). The production of nitric oxide and other substances such as reactive oxygen intermediates in response to stimuli, is responsible for significant antimicrobial activity of macrophages against pathogens (Nathan and Hibbs, 1991). In the present study, upon exposure to OSPW dilutions containing NAs concentrations of 8 - 10 mg/L, the production of NO was reduced significantly. This inhibited NO response was not ameliorated by ozonation. The down-regulation in NO response indicated impaired macrophage antimicrobial functions

that might produce a profound effect on the ability of hosts to resist infectious diseases. In contrast, exposure to OSPW-OF (≤ 10 mg/L NAs) did not alter NO production by RAW cells, which was in agreement with previous results that BMDM treated with OSPW-OF containing NAs ≤ 12.5 exerted no impacts on NO response (Garcia-Garcia et al., 2011b). It should be noted that, the DCM extraction process did not achieve 100% organic fraction extracted from OSPW. Approximately 82% NAs, or less than 60% total NAs were isolated. Thus, the deviation in immnotoxicity between OSPW and OSPW-OF at the same NAs concentrations may be due to the presence of residual NAs and other organic compounds, as well as inorganic constituents in OSPW. The down-regulation of cellular viability and proliferation caused by OSPW exposure could also result in the reduced NO production.

In addition to the NO response, I evaluated whether OSPW or its organic fraction could modulate the cytokine production by RAW 264.7 cells. Exposure to OSPW at 10 mg/L NAs significantly elevated production of pro-inflammatory mediators (i.e. IL-1α, IL-1β, GM-CSF, and VEGF) in *E. coli*-stimulated RAW 264.7 cells. Cytokines and growth factors are involved in responses to endogenous and exogenous insults, repairs, and homeostasis restoration (Lee et al., 2012). However, the excessive production of cytokines may also result in pathogenesis (Lee et al., 2012; Ritchlin et al., 2003). Research has reported that some chronic inflammatory diseases, such as rheumatoid arthritis and asthma, might be induced by overexpression of cytokines like IL-1β and GM-CSF (Lee et al., 2012; Lopalco et al., 2015). VEGF, as a critical pro-angiogenic factor, is also involved in the initiation and progression of rheumatoid arthritis (Lee et al., 2012).

In the present study, ozonation of OSPW failed to abolish its ability to alter the cytokine resonse by RAW 264.7 cells. Similar to other endpoints, OSPW-OF had no effects on production of all cytokines tested. These results further confirm that some constituents other than organic fraction extracted, are likely to be responsible for the altered cytokine profiling of RAW 264.7 cells. Previous findings have shown that exposure of mouse BMDM to OSPW-OF with 50 mg/L NAs resulted in reduction of both pro-inflammatory (IL-1β) and anti-inflammatory (IL-10) cytokines at the genetic levels (Garcia-Garcia et al., 2011b). However, our results showed up-regulated cytokine production by RAW cells after OSPW exposure to lower NAs concentration (10 mg/L). These contradictory observations might be due to the different composition of samples tested (OSPW-OF and OSPW) and the cells used (primary cells versus cell line). In should be noted that although transcriptional activation of cytokine genes is a major regulatory step in cytokine production by macrophages, the mRNA levels do not necessary reflect the protein abundances. Research has demonstrated that some regulatory processes occurring after mRNA (i.e. post-transcriptional, translational and protein degradation regulation) can affect the protein synthesis (Vogel and Marcotte, 2012).

Whole OSPW containing 10 mg/L NAs did not affect the cellular viability (by MTT assay) and cytotoxicity (by LDH assay), but significantly reduced DNA labeling. This dose also caused a dramatic alteration in the cell morphologies that featured a ruffling of their surfaces and formation of multiple spiky projects of the plasma membrane, suggesting the cells were stressed (data unpublished). Therefore, I measured the mRNA levels of several selected stress response genes, to further investigate the toxic

mechanisms caused by OSPW. After exposure to 10 mg/L NAs of whole OSPW for 18 h, mRNA expression of *hmox1* was significantly increased, compared to both non-exposed control and dilution control, but not an equivalent dose of OSPW-OF. Cells activate antioxidant responses to mitigate cellular stress and damage in the presence of increased reactive oxygen species (Birben et al., 2012). However, the excessive accumulation of these species might result in oxidative stress, leading to altered expression of genes encoding antioxidant enzymes such as *hmox1* whose induction is associated with cellular protection against injury induced by reactive oxygen species. In this study, elevated mRNA level of *gadd45* was also observed in cells exposed to OSPW at 10 mg/L, though no significant difference was found in comparison with control. Research has demonstrated that oxidative stress and other toxicant-induced mechanisms of cellular injury may cause accumulated DNA damage that can result in mutations leading to cell cytotoxicity (Yakes and Van Houten, 1997). Cells use an integrated DNA damage repair response to mitigate the DNA damage, by the induction of various DNA repair enzymes (Jackson and Bartek, 2009), such as the one encoded by the *gadd45* gene that contributes to cellular protection from physiological stressors (Schäfer, 2013).

3.5 Summary

Using *in vitro* cellular assays in the present study, I assessed the cytotoxicity and immunotoxicity of OSPW and its fractions, using RAW 264.7 macrophage-like cell line. I demonstrated that whole OSPW, rather than OSPW-OF at indicated NAs concentrations reduced cell viability and proliferation in a dose-dependent manner. OSPW also modulated the antimicrobial function of RAW 264.7 cells by altering the NO production

and subsequent release of pro-inflammatory mediators. Despite significant reduction of NAs after ozonation of OSPW (~90% of NAs in OSPW), no amelioration of the toxicity was achieved. These results suggest that in addition to NAs other components in OSPW (other organic substances, inorganic fraction) or the additive and/or synergistic effects among the constituents present in whole OSPW are responsible for the observed effects.

Table 3.1 General parameters of raw OSPW

General parameter	OSPW
pH	8.41
Total dissolved solids (TDS) (mg/L)	2050.00
Total alkalinity (mg/L $CaCO_3$)	644.15
Hardness (mg/L $CaCO_3$)	182.50
Chemical oxygen demand (COD) (mg/L)	224.79
Biochemical oxygen demand (BOD) (mg/L)	13.61
Total organic carbon (TOC) (mg C/L)	55.70
Acid-extractable fraction (mg/L)	79.53
Naphthenic acids (NAs) (mg/L)	18.38
Chloride (Cl) (mg/L)	457.25
Sulfate (SO_4-S) (mg/L)	131.80

Table 3.2 NAs analysis for OSPW and OSPW+O_3

[a]Parameters, mg/L	OSPW	OSPW+O_3	Reduction[b] (%)
NAs	18.38	1.61	91.25
O-NAs	8.75	4.5	48.53
O_2-NAs	9.57	4.08	57.32
O_3-NAs	3.98	1.81	54.39
O_4-NAs	0.62	0.6	2.58
Sum	41.3	12.61	69.46

[a] NAs: $C_nH_{2n+z}O_2$; O-NAs: $C_nH_{2n+z}O_3$; O_2-NAs: $C_nH_{2n+z}O_4$; O_3-NAs: $C_nH_{2n+z}O_5$; O_4-NAs: $C_nH_{2n+z}O_6$;

[b] (Conc. of NAs in OSPW – Conc. of NAs in OSPW+O_3) / Conc. of NAs in OSPW × 100.

Table 3.3 Extraction rate of NAs from OSPW and OSPW+O_3

Parameter (mg)	OSPW[a]	OSPW-OF[b]	Extraction rate[c], %
NAs	735.37	604.66	82.23
O-NAs	349.86	130.89	37.41
O_2-NAs	382.71	167.42	43.75
O_3-NAs	159.16	36.13	22.70
O_4-NAs	24.84	9.35	37.65
Sum	1651.93	948.45	57.41
Parameter (mg)	OSPW+O_3[a]	OSPW-OF+O_3[b]	Extraction rate[c], %
NAs	64.33	56.33	87.55
O-NAs	180.07	69.85	38.79
O_2-NAs	163.32	49.54	30.33
O_3-NAs	72.59	15.23	20.98
O_4-NAs	24.20	4.87	20.11
Sum	504.52	195.81	38.81

[a] NAs mass calculated by multiplying the NAs concentration in OSPW or OSPW+O_3 by the volume of 40 L;

[b] NAs mass calculated by multiplying the NAs concentration in OSPW-OF or OSPW+O_3-OF dissolved in acetonitrile (550 and 500 mL for OSPW-OF and OSPW+O_3-OF, respectively) by the sample volumes;

[c] mass of NAs in OSPW-OF / mass of NAs in OSPW × 100.

Table 3.4 Sequence of the investigated genes in a RT-PCR analysis

Gene	Primer sequence (5' → 3')	
	Sense	**Anti-sense**
gstp1	CAAATATGTCACCCTCATCTACACCA	CAAAGGAGATCTGGTCACCCAC
hmox1	CCTGGTGCAAGATACTGCCC	GAAGCTGAGAGTGAGGACCCA
ogg1	CTGCCTAGCAGCATGAGACAT	CAGTGTCCATACTTGATCTGCC
gadd45	AGTCAGCGCACCATTACGGT	GGATGAGGGTGAAATGGATCTG
hprt1	GTTAAGCAGTACAGCCCCAAAATG	AAATCCAACAAAGTCTGGCCTGTA

gstp1 = Glutathione S-transferase pi 1; *hmox1* = Heme oxygenase 1; *ogg1* = 8-oxoguanine DNA glycosylase; *gadd45* = Growth arrest and DNA damage-inducible protein 45; *hprt1* = Hypoxanthine-guanine phosphoribosyltransferase

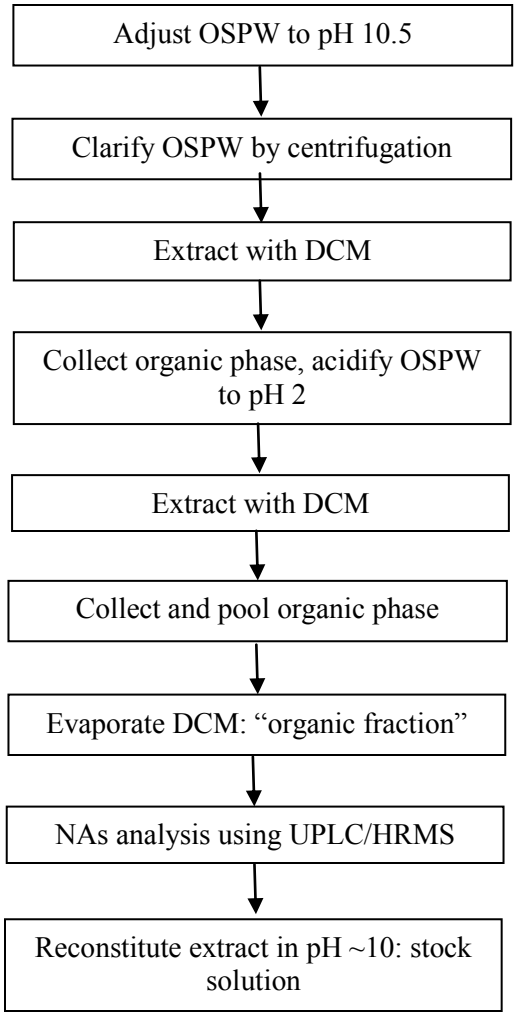

Figure 3.1 Procedure for extraction of organic fraction from OSPW

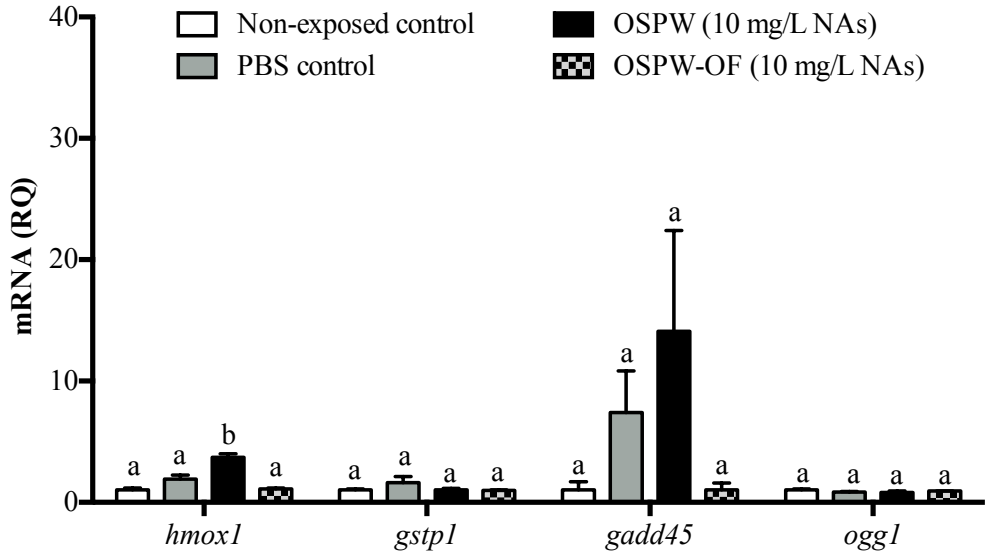

Figure 3.2 Gene expression of RAW cells. RAW cells were exposed to dilution control, OSPW or OSPW-OF at 10 mg/L NAs for 18 hours. mRNA levels of stress responsive and detoxification genes (*hmox1*, *gstp1, gadd45* and *ogg1*) were analyzed by qPCR using the ddCT method. Data are expressed as relative quantification (RQ) values. RQ values for the dilution control, OSPW, or OSPW-OF groups were normalized against the RQ values of non-exposed (i.e. [NAs] = 0) cells for each gene. Each value represents mean ± SEM of three independent experiments. For each gene, statistical analysis was performed using one-way ANOVA with Tukey's *post hoc* test. Different letters above each bar denote statistical difference ($p < 0.05$), and the same letter indicates no statistical differences between groups.

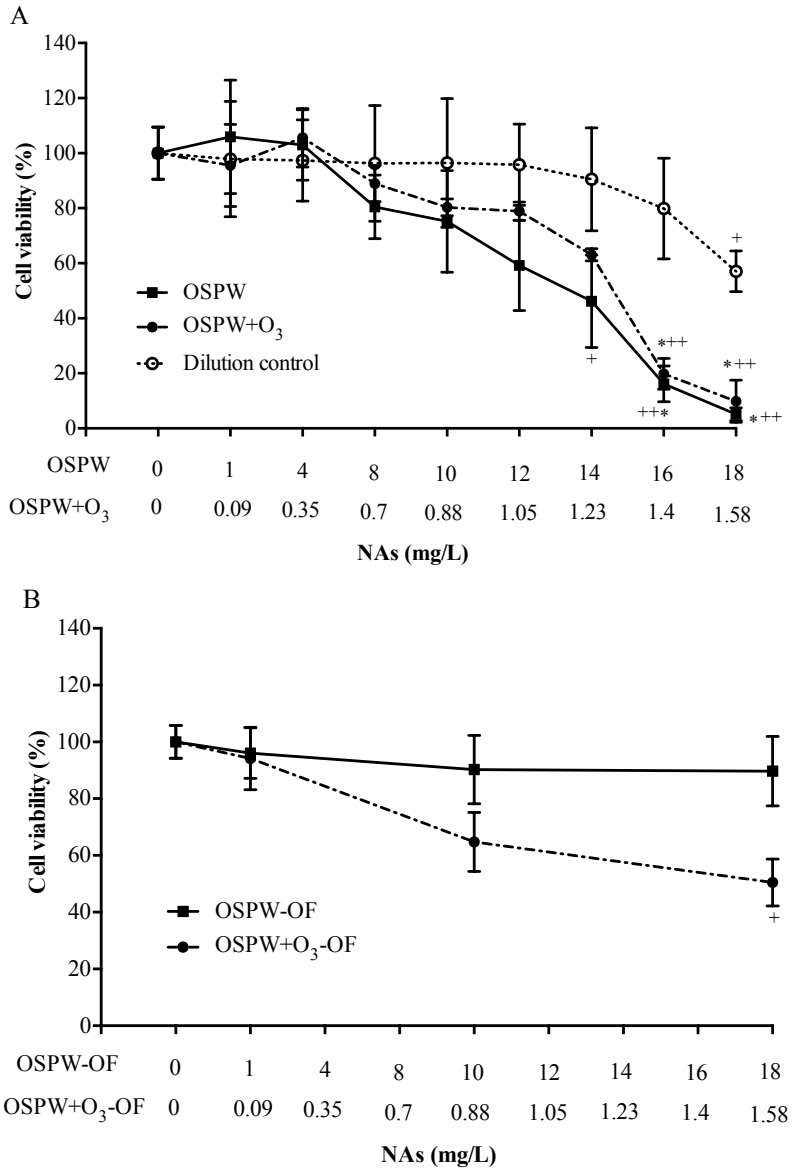

Figure 3.3 Viability of RAW cells. RAW cells were exposed to OSPW, OSPW+O_3, and their dilution control (A), or OSPW-OF and OSPW+O_3-OF (B) for 18 hours. Cell viability was determined using the MTT assay. The data are expressed as the percentage of cell viability relative to non-exposed control (i.e. [NAs] = 0) cells. Each value represents mean ± SEM of triplicate experiments (n = 3). For each NAs dilution, statistical analysis was performed using one-way ANOVA with Tukey's *post hoc* test. * p <0.05 compared to dilution control; + p <0.05 compared to non-exposed control; ++ p <0.01 compared to non-exposed control.

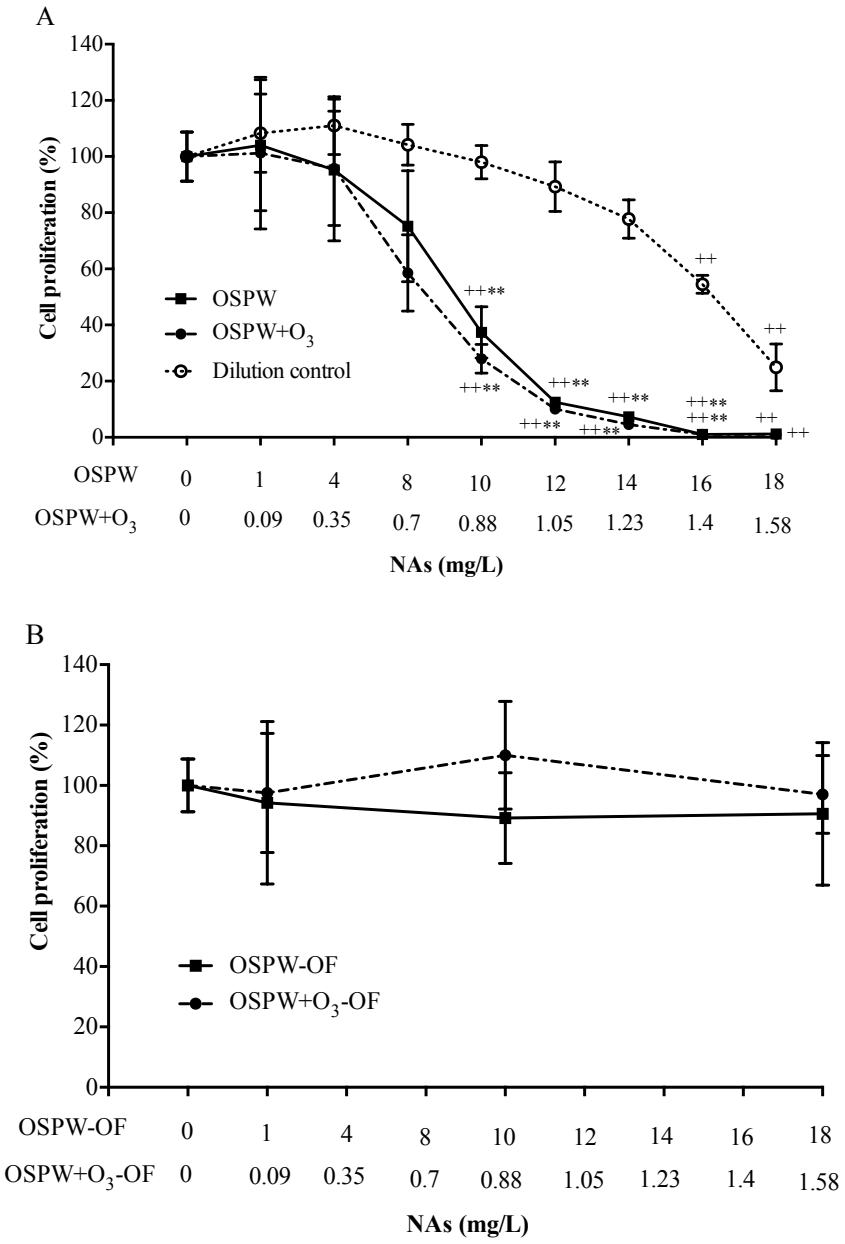

Figure 3.4 Proliferation analysis of RAW cells. RAW cells were exposed to OSPW, OSPW+O_3, and their dilution control (A), or OSPW-OF and OSPW+O_3-OF (B) for 18 hours. The Cell proliferation was determined using the BrdU incorporation assay. The data are expressed as the percentage of cell proliferation relative to non-exposed control (i.e. [NAs] = 0) cells. Each value represents mean ± SEM of triplicate experiments (n = 3). For each NAs dilution, statistical analysis was performed using one-way ANOVA with Tukey's *post hoc* test. ** $p < 0.01$ compared to dilution control; ++ $p < 0.01$ compared to non-exposed control.

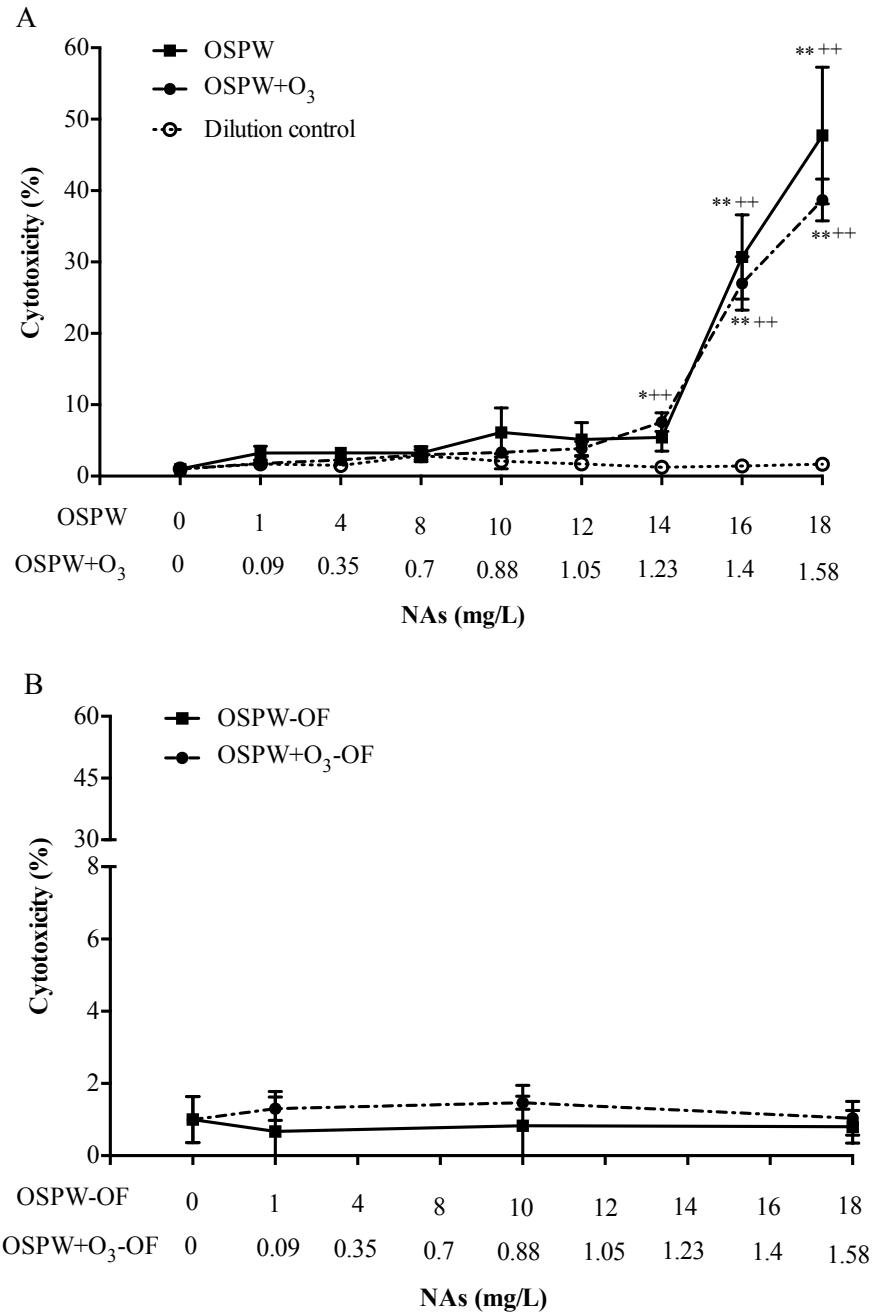

Figure 3.5 Cytotoxicity of RAW cells. RAW cells were exposed to OSPW, OSPW+O_3, and their dilution control (A), or OSPW-OF and OSPW+O_3-OF (B) for 18 hours. The cell cytotoxicity was measured using LDH release assay. Percentage cytotoxicity was calculated using the following equation: Cytotoxicity (%) = (experimental release – spontaneous release) / (maximum release – spontaneous release) × 100. Each value represents mean ± SEM of triplicate experiments (n = 3). For each NAs dilution, statistical analysis was performed using one-way ANOVA with Tukey's *post hoc* test. * p <0.05 compared to dilution control; ** p <0.01 compared to dilution control; ++ p <0.01 compared to non-exposed control.

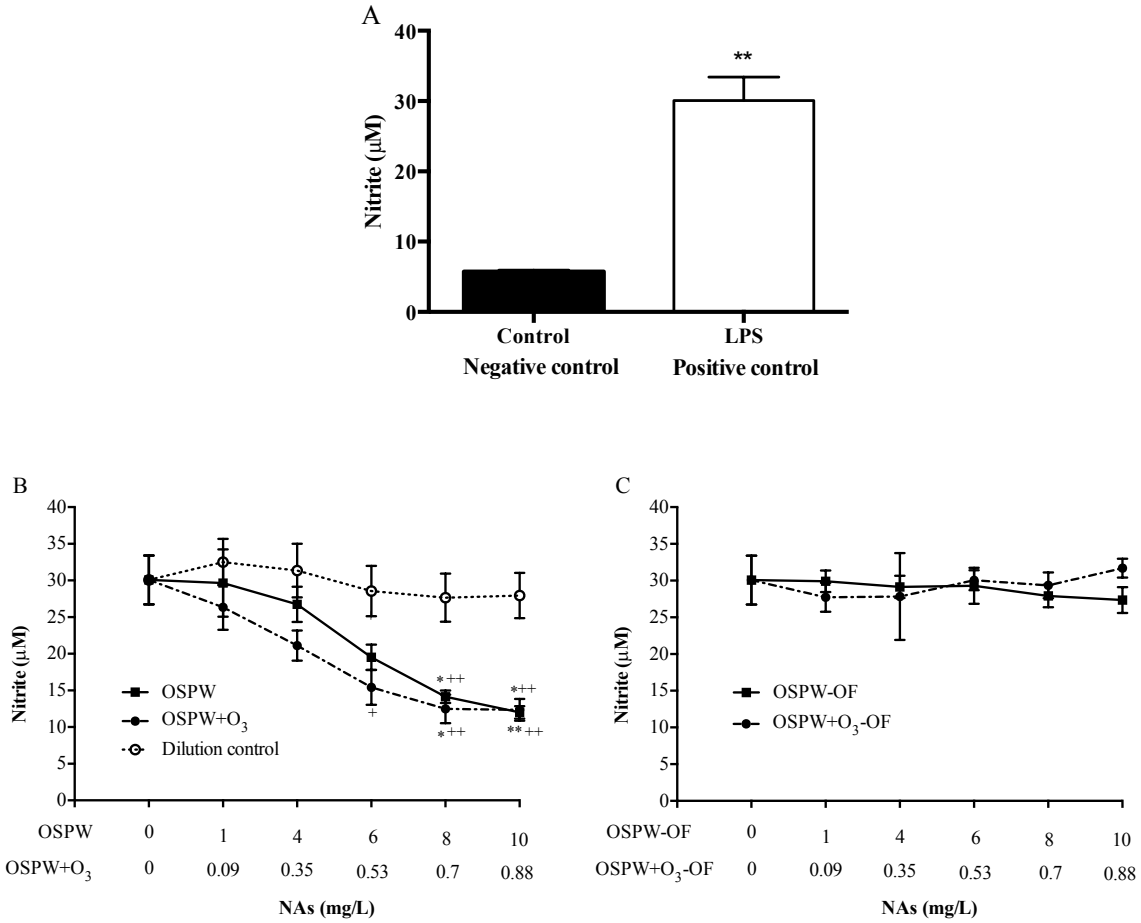

Figure 3.6 NO production of RAW cells. (A) RAW cells were cultured in complete medium for 18 hours, and stimulated (positive control), or not (negative control), by LPS for 24 hours. RAW cells were exposed to OSPW, OSPW+O_3, and their dilution control (B), or OSPW-OF and OSPW+O_3-OF (C) for 18 hours, followed by LPS stimulation for 24 hours. Nitric oxide response was analyzed using the Griess reaction. Results are expressed as nitrite production by cells. Each value represents mean ± SEM of at least three independent experiments. For each NAs dilution, statistical analysis was performed using one-way ANOVA with Tukey's *post hoc* test. * p <0.05 compared to dilution control; ** p <0.01 compared to dilution control; ++ p <0.01 compared to positive control.

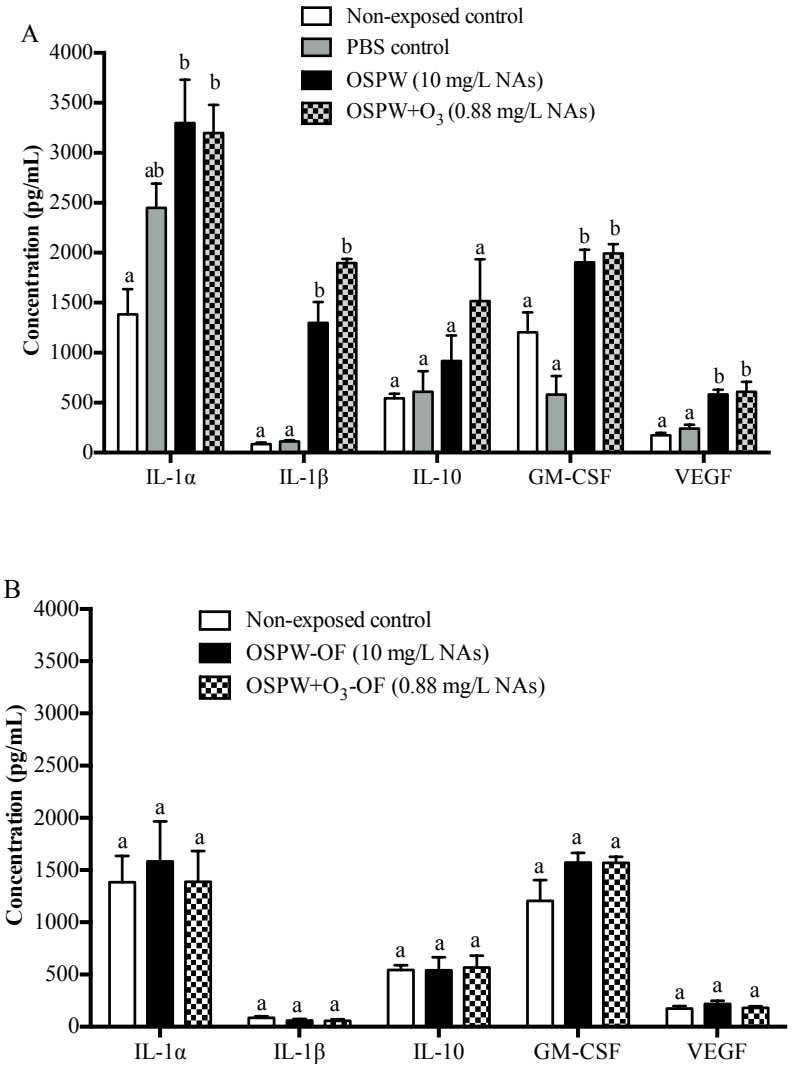

Figure 3.7 Cytokine production of RAW cells. RAW cells were exposed to OSPW, OSPW+O_3, and their dilution control (A), or OSPW-OF and OSPW+O_3-OF (B) for 18 hours. Cells were then stimulated with *E. coli* for 18 hours. The cytokines in the medium supernatants were measured using a bead-based antibody capture assay. Each value represents mean ± SEM of three independent experiments. For each cytokine, statistical analysis was performed using one-way ANOVA with Tukey's *post hoc* test. Different letters above each bar denote statistically different ($p < 0.05$), and the same letter indicates no statistical differences between groups.

Chapter 4: Toxic effects in mice during pregnancy after acute oral exposures to the organic fraction of oil sands process water

4.1 Introduction

The Alberta's oil sands industry has generated large volumes of OSPW during bitumen extraction. OSPW has shown toxic to varieties of organisms, likely due to the presence of the acid-extractable organic compounds. NAs ($C_nH_{2n+z}O_2$) have been used to describe this group of organics over time. However, Grewer and colleagues (Grewer et al., 2010) demonstrated that, less than 50% of the compounds in the OSPW extracts could be assigned to classical NAs and oxy-NAs (Grewer et al., 2010). Similarly, according to the UPLC/HRMS analysis in my previous study (as shown in Chapter 3), only 37.9% of the total mass of OSPW organic fraction (OSPW-OF) was NAs (24.2% for classical NAs, and 13.7% for oxy-NAs), and the remaining 62.1% of the mass remained uncharacterized. The identification of all the compounds and their possible toxic effects, constitutes a major analytical challenge (Grewer et al., 2010), not given that the composition of OSPW and NAs in it varies with many factors such as ore sources, extraction process, tailings age and remediation strategies.

The adverse effects of OSPW-OF have been increasingly reported in aquatic species, including developmental delays, impaired reproduction, and histological changes (Garcia-Garcia et al., 2011a, 2012; Kavanagh et al., 2012a; Marentette et al., 2015b, 2015a; Nero et al., 2006a; Rogers et al., 2002; Rogers, 2003). The causative agents and the mechanisms how they exert the toxic effects are not fully understood. However, some organic compounds (e.g., the surfactant-like and estrogen-like structures of NAs) may be candidate chemicals for narcotic and endocrine disruptive effects of OSPW (He et al.,

2010, 2011; Scarlett et al., 2013; Wiseman et al., 2013a). Additionally, OSPW-OF may also induce oxidative stress, as evidenced by the findings that reduction of organic compounds in OSPW attenuated its effects on expression of oxidative stress responsive genes in fish (Wiseman et al., 2013b). It is suggested that the oxidative stress or disruption of endocrine processes caused by OSPW might be associated with impaired growth, development, and reproductive capacity (He et al., 2012b; Wiseman et al., 2013a).

Limited toxicity studies have been conducted using mammals. Rogers et al. (2002) were first to examine the toxicity of OSPW-OF in rats. Acute exposure to high dose (300 mg/kg body weight/d NAs) caused behavioral and histopathological effects in rats including temporary appetite suppression and pericholangitis (a biliary inflammatory response) (Rogers et al., 2002). Sub-chronic toxicity testing using a smaller dose (60 mg/kg body weight/d NAs) also resulted in toxicity such as suppressed body weight, hepatotoxicity, and depressed plasma cholesterol (Rogers et al., 2002). Rogers (2003) also demonstrated that oral exposure to OSPW-OF (60 mg/kg body weight/d NAs) during pre-breeding, breeding and gestation caused impaired embryonic implantation in rats, which was likely associated with the changes in cholesterol availability and a parallel decrease in plasma progesterone (a downstream molecule of cholesterol metabolic pathway). However, it should be noted that Rogers and colleagues (Rogers et al., 2002; Rogers, 2003) used OSPW-OF doses for rat exposures that were 10 to 50 times higher than the estimated worst-case daily intake, which are unlikely to be found in the environment. In reality, NAs concentrations range from ~20 to 80 mg/L in fresh settling basins, and ~5 to 40 mg/L in reclamation ponds or experimental wetlands (Li et al.,

2017). NAs levels in Athabasca River adjacent to the lease sites are typically less than 1 mg/L (Allen, 2008a). For my research project, NAs at environmentally relevant concentrations (1, 10, and 55 mg/L) were selected to investigate the possible toxicity of OSPW-OF in mammals.

The objective of this study was to determine whether OSPW-OF posed a health risk to mammals (mice used as the model organism) following oral exposure to OSPW-OF, during early pregnancy (early- to mid-gestation). Pregnancy is a comprehensive process that requires the fine-tuned endocrine (hormonal changes) and immune (T helper 1 (Th1)/Th2 cytokines shift) adaptation of the mother to the semi-allogeneic fetus. The OSPW-OF induced changes in hormone levels have been reported both *in vitro* and *in vivo* (Rogers, 2003; Wang et al., 2015b). Our lab previously reported that OSPW-OF could act as immunomodulatory agent by altering the cytokines/chemokines genes involved in the regulation of important immune responses (Garcia-Garcia et al., 2011a,b; 2012). The results of these studies suggested that the OSPW-OF may impair pregnancy by perturbing endocrine and immune systems. To explore this possibility, the reproductive performance combined with the production of pregnancy-associated hormones, and cytokines/chemokines were assessed following acute exposure of mice to environmentally relevant concentrations of OSPW-OF. Besides, the expression of stress responsive genes and genes involved in detoxification process in liver and spleen, as well as several pregnancy-associated genes in placenta were analyzed, to investigate the potential toxic effects at molecular levels induced by OSPW-OF exposures.

4.2 Materials and Methods

4.2.1 Oil sands process-affected water (OSPW)

The OSPW sample was collected from Aurora pond (Syncrude Canada Inc.), Fort McMurray, Alberta in 2012, and stored in 200-L polyethylene barrels in dark at 4°C prior to use. A detailed composition of this water is shown in Table 3.1 (Chapter 3).

4.2.2 Ozonation of OSPW

The ozone treatment of OSPW was carried out according to the protocol described in Section 3.2.2 (Chapter 3).

4.2.3 Extraction of the organic fractions from raw OSPW and ozonated raw OSPW

The organic compounds were isolated from 40 L of raw OSPW, ozonated raw OSPW (OSPW+O_3), or distilled water using a liquid-liquid organic extraction protocol described in Section 3.2.3 (Chapter 3). The total mass of the organic fraction of OSPW was 2.50 g, and the total mass of the organic fraction of OSPW+O_3 was 1.11 g. Once the amount of NAs in the organic fractions was determined by UPLC/HRMS (604.66 mg and 56.33 mg in OSPW-OF and OSPW+O_3-OF, respectively), the organic fractions were dissolved in distilled water (pH ~10) containing final NAs concentrations of 23.8 mg/mL for OSPW-OF NAs, and 2.2 mg/mL for OSPW+O_3-OF NAs. The same extraction protocol was performed using 40 L of distilled water, and the material obtained was also dissolved in same volume of distilled water, which was used as a sham-control (pregnant control) solution. These stock solutions were diluted in distilled water to prepare working solution (100 µL) for subsequent mouse gavage using 22G stainless steel bulb tipped

gavage needles. Gavaging was selected over dissolving the organic fractions in the drinking water, so that each mouse would receive specific NAs doses based on their body weight. For the first set of exposure, the doses adopted were 1.82 and 18.2 mg/kg body weight/week NAs for OSPW-OF, and 1.69 mg/kg body weight/week NAs for OSPW+O_3-OF. These doses reflect the amount of NAs that an animal would consume in a week, if its drinking waters were OSPW (1 or 10 mg/L NAs) or ozonated OSPW (0.93 mg/L NAs). This estimate was based on a daily water consumption of 7.8 mL per 30 g of mouse body weight (United States Environmental Protection Agency, 1998). For the follow-up experiments, OSPW-OF at the dose of 100 mg/kg body weight/week NAs was used for treatment, to investigate the risk when mice were exposed to a high level of NAs (55 mg/L).

4.2.4 NAs analysis

Ultra-performance liquid chromatography/high-resolution mass spectrometry (UPLC/HRMS) was used to measure the NAs and oxy-NAs in organic fractions according to the procedures described in Section 3.2.4 (Chapter 3).

4.2.5 Exposure of mice to OSPW-OF and OSPW+O_3-OF

Six-to-eight week old female BALB/c mice were purchased from Charles River Laboratories. Mice were given *ad libitum* access to food (PicoLab mouse diet 20-5058) and tap water. They were kept at 20-22°C with a 12 h light cycle. After two weeks of acclimatization, adult BALB/c male mice were randomly assigned to breed with females in a 1 male: 2 female ratio overnight. The presence of a vaginal plug, indicating that

mating had occurred, was determined the following morning. The day of appearance of the plug was designated gestation day 0 (GD 0), and the mating day was referred as GD-1. Females showing a vaginal plug were separated from the males prior to exposure to OSPW-OF. Beginning on GD 0, vaginal plug-positive animals were dosed weekly for two weeks (early- to mid-gestation). The gavaging was selected for mice exposure over dissolving the contaminants in their drinking water, so that each mouse would receive exactly the same NAs dose based on their weight. The non-mated and non-exposed female mice were housed in separate cages and used as the non-pregnant control.

During the exposure, the behavioral and clinical signs of females were monitored daily. The maternal body weight was measured weekly. Animals were anesthetized on GD 14 with carbon dioxide. The blood was collected by cardiac puncture using a syringe rinsed with sodium heparin (1000 U/mL). Plasma was prepared by centrifuging the blood at 15,000 x *g* at 4°C for 5 minutes. Plasma was flash frozen in liquid nitrogen and stored at -80°C until used for hormones and cytokines/chemokines assays. Mice were killed by cervical dislocation, prior to organ collection. The uteri were examined for implantations and fetal resorptions. The resorbing fetus was identified by its notably smaller size and necrotic or hemorrhagic appearance, as shown in Figure 4.1. Normal fetus and placenta were separated and weighed. A portion of the liver, spleen and placenta samples were fixed in 4% PFA/PBS for the following histology assessment. The remaining liver, spleen and placenta tissues were flash frozen and stored at -80°C for gene expression analysis. Other tissues collected for histology examination included intestine, kidney, brain and lung.

4.2.6 Measurement of hormones in plasma using ELISA assays

Progesterone (P4), 17β-estradiol (E2), prolactin (PRL) and aldosterone (ALDO) in plasma samples were measured using rodent ELISA kits according to the manufacturer's instructions. The limits of sensitivity of the assays were for 0.04 ng/mL for P4 (cat# 55-PROMS-E01, ALPCO), 3 pg/mL for E2 (cat# ES180S-100, Calbiotech), 30 pg/mL for PRL (cat# ab100736, Abcam,), and 4.7 pg/mL for ALDO (cat#ab136933, Abcam).

4.2.7 Plasma cytokine/chemokine analysis

The plasma cytokine/chemokine profile was analyzed using the Mouse Cytokine/Chemokine Array 31-Plex (Cat# MD31, Eve Technologies, Calgary, AB, Canada). This assay permitted simultaneous quantification of multiple cytokines by capturing the cytokines to differently colored/fluorescent beads that were detected by the bead analyzer (Bio-Plex 200, BIo-Rad). The cytokines and chemokines tested were: interleukin (IL)-1α, IL-1β, IL-5, IL-9, IL-13, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), macrophage inflammatory protein (MIP)-1α/CCL3, MIP-1β/CCL4, MIP-2/CXCL2, RANTES/CCL5, keratinocyte chemoattractant (KC/CXCL1), monokine induced by gamma interferon (MIG/CXCL9), and interferon gamma-induced protein 10 (IP-10/CXCL10).

4.2.8 Gene expression analysis

Total RNA was extracted from liver, spleen, and placenta using the RNA isolation kit (Cat# AM1924, Ambion) and reverse transcribed into cDNA using the Superscript III

cDNA synthesis kit (Cat# 18080-051, Life Technologies) according to manufacturer's instructions. The cDNA was used for real-time PCR using 2× Dynamite SYBR Green PCR master mix (developed by Molecular Biology Service Unit in Biological Sciences at the University of Alberta, Canada). The stress responsive and detoxification genes that were examined in liver and spleen included *gstp1*, *gstm1*, *hmox1*, *lig1*, *ogg1*, *gadd45*, *ung* and *pcna*. Four pregnancy-associated genes (*vegfa*, *pgf*, *erα* and *pr*) were monitored in placenta. The primers (Integrated DNA Technologies, IDT) used in this study are listed in Table 4.3. *hprt1* was used as the endogenous control. The real-time PCR was performed using a 7500 Fast Real-Time PCR apparatus (Applied Biosystems) according to the cycling conditions: 95°C, 10 min; 95°C 15 sec; 60°C, 1 min; 95°C 15 sec; 60°C 1 min; 95°C, 30 sec; 60°C, 15 sec (melting curve); for 40 cycles. Gene expression was analyzed using the ddCT method, normalizing relative quantitation (RQ) values for treatment groups to their sham-controls.

4.2.9 Histological assessment

Various tissues (liver, spleen, placenta, kidney, intestine, brain, and lung) were collected from mice exposed to OSPW-OF (1, 10, 55 mg/L NAs), and were fixed in 4% PFA/PBS for 24 hours. Tissues were then processed by dehydration in a series of increasing ethanol solutions and embedded in paraffin. Each liver specimen was sectioned to 5 μm thickness using a microtome. The sections were mounted on glass slides, followed by the deparaffinization and rehydration using toluene and a series of decreasing ethanol solutions. The tissues were then stained with haematoxylin-eosin (H

& E) for light microscopic examination that was done by a certified veterinary pathologist (Dr. Nick Nation, University of Alberta).

4.2.10 Statistical analysis

Data are presented as mean ± SEM. Statistical analysis of data was performed using one-way ANOVA, followed by the Dunnett's's *post hoc* test. All analysis was processed with Prism 6.0 (Graphpad software, CA, USA). Differences between treatment groups were considered statistically significant when $p < 0.05$.

4.3 Results

4.3.1 Behavioral observations and body weight changes

During the exposure, the pregnant mice from all groups had no signs of morbidity, mortality, behavioral changes, or changes in general appearance. The maternal body weight before pregnancy (GD-1) and on mid-gestation (GD14) is illustrated in Figure 4.2. Pregnant mice from OSPW-OF treated groups gained similar body weight (40 and 36% for 10 and 55 mg/L NAs exposures, respectively), in comparison with the weight increase observed in sham-controls (40%) after 2-week exposure ($p > 0.05$).

4.3.2 Pregnancy outcomes of mice after acute exposure to OSPW-OF and OSPW+O_3-OF

Table 4.1 summarizes various reproductive parameters associated with the mice exposed to OSPW-OF (1 and 10 mg/L) and OSPW+O_3-OF. Animals exposed to OSPW-OF (1 and 10 mg/L) and OSPW+O_3-OF had pregnancy rates of 62.5 - 70.8%, which were

comparable to sham-control (62.5%). Also, the treated mice carried a median of 9-10 embryos, which is close to that of control animals (median value = 9). When I examined the placenta for spontaneous fetal resorption, an indicative of a fetus that died in utero, mice gavaged with OSPW-OF (10 mg/L NAs) had a 37% higher resorption rate than sham-controls, but the difference was not statistically significant. The health status of the embryos were assessed by measuring the fetal weight, placental weight, as well as the ratio of a fetal weight to placental weight (F/P), that is an indicator of the balance between fetal and placental growth. It was shown that F/P values did not differ significantly between treatment and control groups. Furthermore, as shown in Table 4.2, when the dose of OSPW-OF was increased to 55 mg/L NAs, none of the parameters associated pregnancy and reproduction were significantly different between exposed and non-exposed pregnant mice.

4.3.3 Hormonal levels in plasma of mice after acute exposure to OSPW-OF and OSPW+O_3-OF

Reproduction in female mammals is dependent on precise production and regulation of various hormones. In this study, I examined and compared the basal levels of four steroid hormones (progesterone, 17β-estradiol, prolactin and aldosterone) in non-pregnant mice and pregnant mice on GD 14. As shown in Figure 4.3, plasma P4 level was 3.6 ng/mL in non-pregnant females, and significantly increased to 16.9 ng/mL at mid-gestation (GD14). The pregnancy led to minor but significant increase in E2 levels, from basal concentration of 4.8 pg/mL to 9.6 pg/mL on GD14. Also, higher circulating level of prolactin was observed in mice on GD14 (36.7 ng/mL) relative to the non-

pregnant control (5.7 ng/mL). By contrast to other results, pregnancy resulted in a significant reduction in aldosterone production, with the plasma concentration of 77% lower than non-mating animals.

The effects of OSPW-OF exposures on hormonal levels in pregnant mice were examined. The results are shown in Figure 4.4 and Figure 4.5. Oral exposure to OSPW-OF (1, 10, and 55 mg/L) or OSPW+O_3-OF for two weeks did not cause significant alterations of these four hormones, when in comparison with the sham-control.

4.3.4 Cytokine/chemokine levels in plasma of mice after acute exposure to OSPW-OF and OSPW+O_3-OF

There is a general agreement that, during the pregnancy, a bias of the immune system towards Th2 occurs to promote fetal-maternal tolerance (Sykes et al., 2012b). Thus, in this research, I monitored the circulating concentrations of multiple cytokines and chemokines in non-pregnant control, pregnant control and treatment groups. As shown in Figure 4.6, several Th2 cytokines were elevated on GD14. For examples, IL-9 and IL-13 increased from 33.6 and 15.9 pg/mL to 57.3 and 33.1 pg/mL, respectively, though the differences were not statistically significant. The growth factor G-CSF exhibited a significant increase from basal level of 89.7 pg/mL to 150 pg/mL on GD14. Regarding to the Th1 cytokines, the plasma levels of IL-1α, IL-1β and GM-CSF in mid-gestation did not differ from non-pregnant control. In the study, several chemokines belonging to the CC and CXC families (i.e., MIP-1α/ CCL3, MIP-1β/CCL4, RANTES/CCL5, KC/CXCL1, MIG/CXCL9 and IP-10/CXCL10) were measured. As illustrated in Figure 4.6, pregnancy did not alter their circulating concentrations.

The production of cytokines and chemokines among the OSPW-OF (1, 10, and 55 mg/L NAs), OSPW+O_3-OF and their control groups was illustrated in Figure 4.7 and Figure 4.8. The Th1/Th2 cytokine profile in dosed pregnant mice was not affected by OSPW-OF treatment at all doses. The chemokine CXCL10/IP-10 was significantly elevated in animals exposed to OSPW-OF (10 and 55 mg/L NAs), compared with the sham-controls. Interestingly, KC/CXCL1 was elevated in mice treated with OSPW-OF at 10 mg/L NAs, but dropped back to the control level when the dose was increased to 55 mg/L NAs.

4.3.5 Expression of stress responsive and detoxification genes in the liver and spleen of mice after acute exposure to OSPW-OF and OSPW+O_3-OF

The hepatotoxicity caused by OSPW and OSPW-OF has been reported both *in vitro* (Gagné et al., 2013, 2011), and *in vivo* (Rogers et al., 2002). In the present study, the toxic effects of OSPW-OF in the mouse liver were examined by measuring the expression of the following genes to track changes in: xenobiotic biotransformation (*gstp1, gstm1*), oxidative stress (*hmox1*), DNA repair activity (*lig1*, *ung*, *ogg1*), cell proliferation and growth arrest (*pcna*, *gadd45*). The transcripts of these genes were also investigated in the spleen, to determine whether acute exposure to OSPW-OF caused stress to this tissue. As shown in Figure 4.9, after 2-week exposure to OSPW-OF (1, 10 mg/L NAs) and OSPW+O_3-OF, all the genes in the liver and spleen were expressed at levels that were not statistically different from those of non-exposed animals. When the dose of OSPW-OF was increased to 55 mg/L NAs, mice exhibited a down-regulation of mRNA levels of *gstp1*, *gstm1,* and *ogg1* in the liver (Figure 4.10).

4.3.6 Expression of pregnancy-associated genes in the placenta of mice after acute exposure to OSPW-OF and OSPW+O_3-OF

Four pregnancy-associated genes (*vegfa*, *pgf*, *era*, *pr*) were examined in placenta to determine if acute OSPW-OF exposure could potentially affect the placental functions at molecular levels. The results are summarized in Figure 4.11. No changes in *vegfa* gene expression were observed between the sham-control and OSPW-OF treatment groups at all doses. Similarly, OSPW-OF and OSPW+O_3-OF exposures did not lead to alterations in *pgf* mRNA levels. In this study, the transcripts of two hormone receptors (*era* and *pr*) were determined in mice after acute exposures to OSPW-OF and OSPW+O_3-OF. No changes were observed in treatment groups at the indicated doses.

4.3.7 Histological assessment of tissues from mice after acute exposure to OSPW-OF and OSPW+O_3-OF

The tissues collected for histological examination included liver, spleen, placenta, intestine, kidney, brain, and lung. Table 4.4 summarizes the histopathological findings in selected tissues. When compared with the sham-control samples, the intestine, kidney, placenta, brain and lungs of mice acutely exposed to different concentrations of OSPW-OF (1 to 55 mg/L NAs) were not histologically different. While there were some minor effects on the liver (mild lipid vacuolation) and spleen (mild hyperplasia of lymphoid germinal centres) in some animals exposed to 55 mg/L NAs, as shown in Figure 4.12, they were suggested to be normal physiologic reactions and not pathologic.

4.4 Discussion

The possibility that NAs extracts or OSPW-OF may affect the endocrine system, immune responses, reproduction and development of both invertebrates and vertebrates, was suggested by the results of previous studies. The results of the experiments presented in this chapter represent a first comprehensive evaluation on the possible effects of environmentally relevant concentrations of OSPW-OF exposures on the pregnancy in mammals. I found that the exposure to OSPW-OF did not cause mortality in pregnant mice during the 2-week duration, and there were no obvious signs of distress measured by behaviour changes, loss of body weight, pregnancy failure, or the development of embryos and placentas (based on the weight of fetus and placenta, F/P weight ratio). However, it is important to know that to evaluate the toxicity of exposures to xenobiotics (i.e., OSPW-OF) more indicators of potential adverse impacts in the absence of the overt toxicity are needed. Thus, some biological responses were examined through proteomic and genomic approaches including circulating levels of hormones and cytokines, expression of stress genes and pregnancy-associated genes.

The 17β-estradiol (E2) and progesterone (P4) are two important hormones that play crucial roles in the control of embryo implantation and maintenance of successful pregnancy. In agreement with previous studies (Chung et al., 2012; Mao et al., 2010), elevated E2 and P4 levels were observed in normal midterm pregnancy (pregnant control) in this research, compared to the non-pregnant animals. Prolactin is another hormone with main function related to the development of mammary glands and milk production (Bole-Feysot et al., 1998; Grattan and Kokay, 2008). The circulating level of prolactin is high during pregnancy (Nagaishi et al., 2014) , which was also observed in the present

study where a significant increase of prolactin was observed in mice on GD14 relative to the non-pregnant control. In contrast, I found a reduced plasma aldosterone concentration in pregnant control mice. This decrease was contradictory to the previous findings that circulating aldosterone levels are persistently high during gestation in humans and mice, which promotes the blood volume expansion to support fetal well-being (Lindheimer and August, 2009; Lumbers and Pringle, 2014). However, fluctuating concentrations of plasma aldosterone were reported in normal pregnant ewes (Boulfekhar and Brudieux, 1980), indicating the aldosterone production might vary between different species and/or strains of the animals. The aldosterone reduction during pregnancy observed in this study may be a true phenomenon occurring at the end of mid-pregnancy in the BALB/c mice. However, the aldosterone profile during mice pregnancy needs to be further investigated in future.

The earlier toxicological assessment using rats reported that reproductive failure was induced after exposure to OSPW-OF (60 mg/kg body weight/d NAs) during pre-breeding, breeding and gestation. This reproductive failure was associated with the reduction in progesterone and estradiol (Rogers, 2003). In contrast, the results of this study show that circulating levels of the four tested hormones in mice on GD 14 were not affected following exposure to OSPW-OF. Differences in the results obtained may be due to different animals used (rats versus mice), short duration (two weeks) of exposure, the method of exposure (gavage once per week), and different chemical composition (source) of OSPW. It is well established that the organic fraction obtained from different sources of OSPW vary in the type and concentration of NAs as well as in the type and concentration of other chemical contaminants including the endocrine disruption

compounds. Previous reports have indicated the presence of aromatic NAs in OSPW that are structurally similar to estrogens (Rowland et al., 2011a), and some polycyclic NAs with a single aromatic ring that may possess estrogenic and androgenic activity (Scarlett et al., 2012). Thus, the OSPW-OF containing greater portions of endocrine disruption compounds may be cause greater effects on the pregnancy outcomes in small mammals.

The previous cytokines/chemokines studies demonstrated that OSPW-OF exposure altered their transcriptional mRNAs (Garcia-Garcia et al., 2012, 2011a,b). In my research, the protein levels of multiple cytokines and chemokines were monitored, because proteins, rather than mRNA, are the actual biological effectors and could be better correlate with the biological outcome. During the pregnancy, a bias of the immune system towards Th2 occurs to promote fetal-maternal tolerance (Sykes et al., 2012b). This Th1/Th2 shift was also observed in this study, demonstrated by the significant increase of the growth factor G-CSF, which has shown a marked anti-abortion activity in normal pregnant mice (Litwin et al., 2005; Saito et al., 1994) compared to the non-pregnant control. Regarding chemokines, pregnancy did not alter their circulating levels. It is not surprising, because many of these chemokines are likely induced in early pregnancy, which contributes to the trophoblast invasion and migration during implantation (Du et al., 2014; Santoni et al., 2008). I also looked into the effects of OSPW-OF exposures on the cytokine/chemokine production during mice gestation. The plasma Th1/Th2 cytokine profile in pregnant mice treated with OSPW-OF was similar to sham-control animals. In contrast, OSPW-OF at 10 and 55 mg/L NAs caused an up-regulation of the chemokine CXCL10/IP-10. CXCL10 is a chemokine with pro-inflammatory and anti-angiogenic properties. It has been shown that humans exhibit

elevated levels of CXCL10 during pre-eclampsia (Gotsch et al., 2007; Szarka et al., 2010), and this was associated with higher risk for spontaneous preterm delivery (Gervasi et al., 2012). However, in my research, the OSPW-OF exposures did not affect time to delivery (data shown in the 6-week exposure study, Chapter 4). Furthermore, there were no statistically significant differences in female fertility, including the pregnancy rate and embryo implantation number.

Early study on rats has suggested liver as a target organ for OSPW NAs toxicity, indicated by increased liver weight that could be because of the induction of hepatic detoxification enzymes (Rogers et al., 2002). The *in vitro* exposure of rainbow trout hepatocytes to OSPW organic fraction or whole OSPW led to changes in expression of important stress responsive and detoxification genes (Gagné et al., 2013, 2012). These results suggest the hepatotoxicity caused by OSPW or its constituents. In this research, a suite of stress genes were examined in the liver and spleen, including *gstp1*, *gstm1*, *hmox1*, *lig1*, *ogg1*, *gadd45*, *ung* and *pcna*. The changes of mRNA levels for selected genes were only observed in the liver, which provided evidence that liver might be the target organ in rodents after exposure to OSPW-OF. Specifically, the abundances of transcripts of two glutathione-S-transferases (GSTs) were down-regulated in livers of mice exposed to high dose of OSPW-OF (55 mg/L NAs) for two weeks including *gstp1* and *gstm1*. GSTs are a family of detoxification enzymes that function to protect cells from damage caused by reactive electrophiles by conjugating a variety of endogenous and exogenous compounds (including carcinogens, environmental pollutants, drugs and other xenobiotics) with reduced glutathione to produce less reactive water-soluble compounds and facilitate their elimination (Cnubben et al., 2001; Sharma et al., 2014).

The results found in the present study were inconsistent with greater abundance of transcripts of GST in livers of fathead minnows (Wiseman et al., 2013b) and rainbow trout hepatocytes (Gagné et al., 2012) exposed to OSPW. However, Gagné and colleagues (Gagné et al., 2011) also found that GST enzyme activity in trout hepatocytes was significantly inhibited by exposure to OSPW extracts. It was suggested that some inhibitors like organometallic complexes might be present in the extracts that could block the activity and perhaps also block expression of *gst* (Byington and Hansbrough, 1979; Gagné et al., 2011). Evidence exists that organic complexes of some elements in group IVA of the periodic table (e.g., germanium, lead and tin) were potent inhibitors of hepatic GST activity (Byington and Hansbrough, 1979). Since OSPW contains a complex mixture of organics and metals (including those from IVA family), it is possible that some organometallic compound(s) extracted by DCM extraction might be responsible for the decrease in *gst* gene expression. The down-regulated *gst* transcription could lead to decreased *gst* translation and a decrease in protein levels which may compromise the ability of an organism to eliminate (detoxify) xenobiotics.

Recently, there are several studies reporting on the genotoxicity of OSPW. Lacaze and colleagues (Lacaze et al., 2014) found primary DNA damage (assessed by comet assay) in rainbow trout hepatocytes caused by structurally different NAs. The DNA damage was also reported by Gagne and colleagues (Gagné et al., 2013, 2012) with alterations in transcripts of genes involved in DNA repair and synthesis during cell maintenance such as *lig*, *ung*, and *ogg*. DNA ligase (LIG) functions in the ligation of newly synthesized DNA strands to the backbone of DNA molecules (Gagné et al., 2013). Uracil-DNA glycosylase (UNG) is an important enzyme that prevents mutagenesis by

capturing and removing uracil, a base normally present in RNA, from DNA (Zharkov et al., 2010), and 8-oxoguanine DNA glycosylase (OGG) is the DNA glycosylase that initiates the base-excision repair pathway to repair non-bulky oxidative DNA lesions (Sampath et al., 2012). In the previous studies, genotoxicity of OSPW was suggested by elevated mRNA levels of *ung* and *ogg* with decreased *lig* (Gagné et al., 2013, 2012, 2011). In the present research, the *lig* and *ung* mRNA levels were not affected following 2-week exposure of pregnant mice to OSPW-OF at all doses tested, although a significant reduction in the mRNA levels of *ogg* occurred after exposure of mice to 55 mg/L NAs. It should be noted that, despite the changes in liver gene expression, no or minimal (exposure to 55mg/L) histopathological alterations were observed after exposure of pregnant mice to OSPW-OF.

The expression of four important pregnancy-associated genes in placenta was also determined, including *vegfa*, *pgf*, *erα* and *pr*. Vascular endothelial growth factor A (VEGFA) is highly expressed in the utero-placental unit, and is a main regulator of decidual angiogenesis (Kim et al., 2013). Multiple studies have reported on the embryonic lethal effects in genetically VEGFA-ablated mice (Carmeliet et al., 2001; Haiko et al., 2008; Karkkainen et al., 2004). In my study, OSPW-OF exposures did not cause changes in *vegfa* gene expression. Similarly, no changes were observed in *pgf* mRNA levels in treated mice. Placental growth factor (PGF) is another member of the VEGF family and is highly expressed in both human and mouse pregnancy (Tayade et al., 2007; Torry et al., 1998). However, although PGF deficiency in human pregnancy was linked with preeclampsia (Levine et al., 2004), the genetically PGF-deleted mice were viable and fertile (Carmeliet et al., 2001). As mentioned above, estrogen and

progesterone are essential for the initiation (embryo implantation) and maintenance of pregnancy in mammals. These hormones need to bind to their receptors, in order to exert the biological functions. In this study, the transcripts of *era* and *pr* were not affected by OSPW-OF exposures at the indicated doses.

In this study, multiple tissue sections were examined microscopically. Overall, no overt pathological changes related to tissue damage, inflammation, or neoplasia were observed in mice after acute exposures to OSPW-OF. Besides, all the toxicological bioassays depicted in this work were also conducted on the organic fraction isolated from ozonated raw OSPW (OSPW+O_3-OF). It was shown that ozone treatment did not generate by-products of increased toxicity *in vivo*. It is not surprising because in the previous study in our lab, ozone treatment ameliorated the mammalian toxicity of OSPW-OF at much higher NAs concentration (Garcia-Garcia et al., 2011a).

4.5 Summary

The results present in this chapter suggest that the risk of acute toxicity to small wild mammals (based on the mouse as the surrogate mammal) exposed to OSPW organic fraction, at indicated NAs concentrations, in contaminated drinking water is low. No significant signs of distress (including death, behaviour changes, loss of body weight, and pregnancy failure) were associated with the oral administration of OSPW-OF to mice acutely (2-week) at the doses up to 55 mg/L NAs. OSPW-OF exposures did not change the Th1/Th2 cytokine profile in pregnant mice, or the expression of pregnancy-associated genes, implying that the immune environment and placental functions in mid-gestation were not disrupted. Several stress responsive or detoxification genes were altered in

livers at high dose of OSPW-OF, indicating that liver might be a target organ. However, no histopathological changes in livers were observed. Besides, OSPW+O_3-OF did not change any biological responses examined in this study, suggesting the safety of ozonation as the OSPW remediation method.

Table 4.1 Reproductive parameters of mice after acute exposure to OSPW-OF (1 and 10 mg/L NAs) or OSPW+O_3-OF

Parameters	Sham-control (n=15)	OSPW-OF		OSPW+O_3-OF (n=17)
		1 mg/L NAs (n=15)	10 mg/L NAs (n=16)	
# of females	24	24	24	24
# of pregnant mice	15	15	16	17
Pregnancy rate[a] (%)	62.5	62.5	66.7	70.8
# of implantations/dam[b]	9	9	9	10
Resorption rate[c] (%)	23.78 ± 4.27	27.43 ± 4.46	32.60 ± 4.53	22.29 ± 4.49
Fetus weight (g)	0.153 ± 0.005	0.162 ± 0.014	0.158 ± 0.005	0.156 ± 0.005
Placental weight (g)	0.099 ± 0.003	0.091 ±0.004	0.097 ±0.002	0.091 ±0.004
Fetus/Placenta weight (F/P)	1.560 ± 0.055	1.769 ± 0.099	1.627 ±0.058	1.736 ±0.052

Note: Females were gavaged weekly with OSPW-OF (1 and 10 mg/L NAs), OSPW+O_3-OF and the sham-control, for two weeks beginning on GD 0. On GD 14, female mice were euthanized, and reproductive parameters were recorded. Values in rows five to eight represent the mean ± SEM. Statistical analysis was performed using one-way ANOVA with Dunnett's *post hoc* test. [a] Number of pregnant mice/number of female mice mated × 100. [b] Median value. [c] Number of resorptions/number of implantations × 100.

Table 4.2 Reproductive parameters of mice after acute exposure to OSPW-OF (55 mg/L NAs)

Parameters	Sham-control (n=5)	OSPW-OF (55 mg/L) (n=8)
# of females	6	8
# of pregnant mice	5	8
Pregnancy rate[a] (%)	83.3	100
# of implantations/dam[b]	9	8
Resorption rate[c] (%)	21.35 ± 12.84	28.03 ± 5.80
Fetus weight (g)	0.166 ±0.004	0.176 ±0.003
Placental weight (g)	0.101 ±0.002	0.100 ±0.002
Fetus/Placenta weight (F/P)	1.657 ±0.067	1.761 ±0.040

Note: Females were gavaged weekly with OSPW-OF (55 mg/L NAs) and the sham-control for two weeks beginning on GD 0. On GD 14, female mice were euthanized, and reproductive parameters were recorded. Values in rows five to eight represent the mean ± SEM. Statistical analysis was performed using one-way ANOVA with Dunnett's *post hoc* test. [a] Number of pregnant mice/number of female mice mated × 100. [b] Median value. [c] Number of resorptions/number of implantations × 100.

Table 4.3 Sequence of the investigated genes in a RT-PCR analysis

Gene	Primer sequence (5' → 3')	
	Sense	**Anti-sense**
gstp1	CAAATATGTCACCCTCATCTACACCA	CAAAGGAGATCTGGTCACCCAC
gstm1	AGCTCATCATGCTCTGTTACAACC	AATCCACATAGGTGACCTTGTCCC
hmox1	CCTGGTGCAAGATACTGCCC	GAAGCTGAGAGTGAGGACCCA
lig1	GGGTGAGTTTGTCTTCACCACCTCTTTGG	AAGGTCTTCACCATCAGGCCCTCACAGG
ogg1	CTGCCTAGCAGCATGAGACAT	CAGTGTCCATACTTGATCTGCC
ung	GGAAGCCGTACTTCGTCAAG	GAGCCCGTGAGCTTGATTAG
gadd45	AGTCAGCGCACCATTACGGT	GGATGAGGGTGAAATGGATCTG
pcna	TTTGAGGCACGCCTGATCC	GGAGACGTGAGACGAGTCCAT
vegfa	GGAGAGCAGAAGTCCCATGAAGTG	AAGATGTCCACCAGGGTCTCAATC
pgf	AAGTGGAAGTGGTGCCTTTCAAC	CAGCAGCCACTACAGCGACT
erα	CCTCCCGCCTTCTACAGG	CACACGGCACAGTAGCGAG
pr	TTCATCCAATCCCGGACACT	AGGATCTTGGGCAACTGGG
hprt1	GTTAAGCAGTACAGCCCCAAAATG	AAATCCAACAAAGTCTGGCCTGTA

gstp1 = Glutathione S-transferase pi 1; *gstm1* = Glutathione S-transferase mu 1; *hmox1* = Heme oxygenase 1; *lig1* = DNA ligase 1; *ogg1* = 8-oxoguanine DNA glycosylase; *ung* = Uracil-DNA glycosylase; *gadd45* = Growth arrest and DNA damage-inducible protein 45; *pcna* = Proliferating cell nuclear antigen; *vegfa* = Vascular endothelial growth factor A; *pgf* = Placental growth factor; *erα* = Estrogen receptor α; *pr* = Progesterone receptor; *hprt1* = Hypoxanthine-guanine phosphoribosyltransferase.

Table 4.4 Histopathology report summary for various tissues from mice after acute exposure to OSPW-OF

Tissue	OSPW-OF		Summary
	1 and 10 mg/L NAs	55 mg/L NAs	
Liver	No microscopic changes relative to sham-control	Mild lipid vacuolation and cytoplasmic stippling (cytoplasmic effects)	The intestine, kidney, placenta, brain and lungs of mice exposed to OSPW-OF had no significant microscopic changes compared to sham-control. There were some mild effects on the livers and spleens of some animals from 55mg/L group, but they were suggested to be normal physiologic reactions, not pathologic.
Spleen	No microscopic changes relative to sham-control	Mild hyperplasia of lymphoid germinal centers (a nonspecific change indicating antigenic stimulation)	
Placenta	No microscopic changes relative to sham-control	No microscopic changes relative to sham-control	
Intestine	No microscopic changes relative to sham-control	No microscopic changes relative to sham-control	
Kidney	No microscopic changes relative to sham-control	No microscopic changes relative to sham-control	
Brain	No microscopic changes relative to sham-control	No microscopic changes relative to sham-control	
Lung	No microscopic changes relative to sham-control	No microscopic changes relative to sham-control	

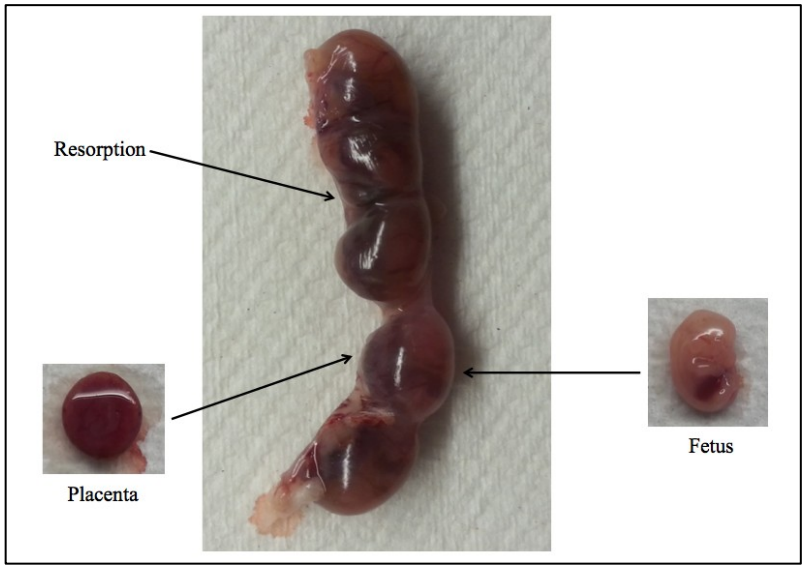

Figure 4.1 Representative picture of the feto-maternal interface in a mouse pregnancy on GD 14

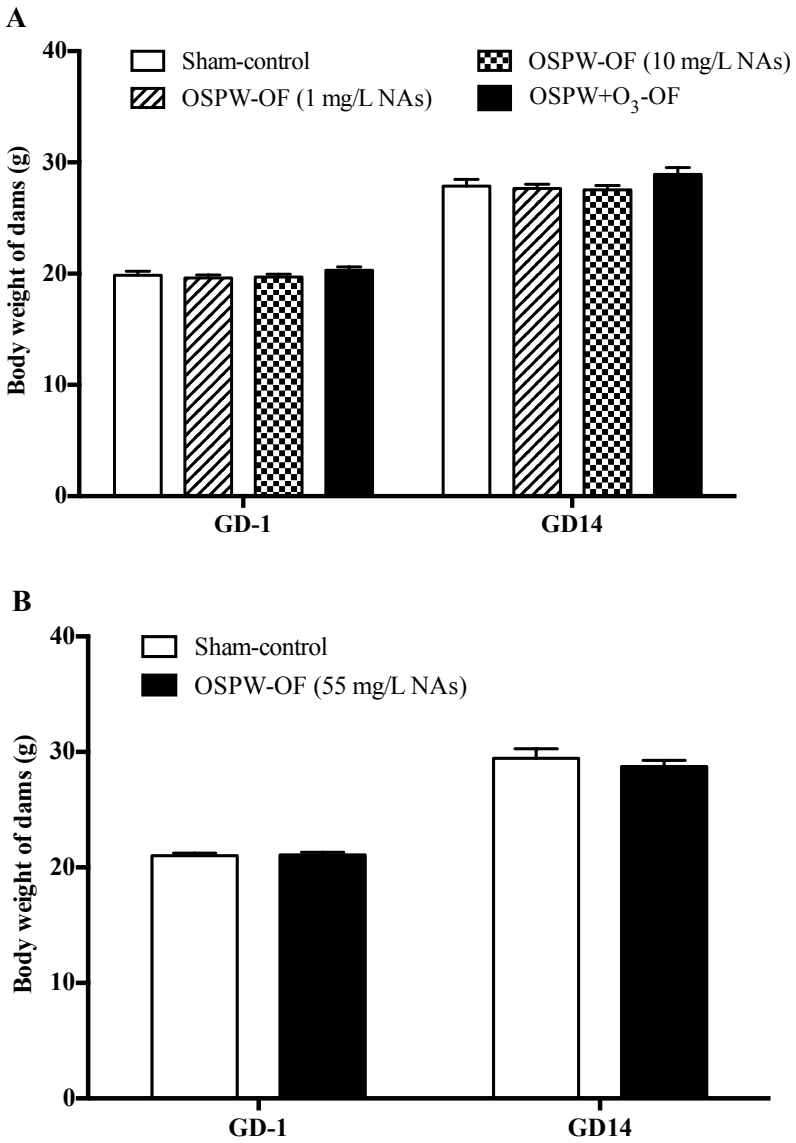

Figure 4.2 Maternal body weight on mating day (GD-1) and at euthanasia (GD14). Animals were gavaged weekly with (A) OSPW-OF (1 mg/L NAs; n=15), OSPW-OF (10 mg/L NAs; n=16), OSPW+O_3-OF (n=16), and their sham-control (n=15), or (B) OSPW-OF (55 mg/L NAs; n=8) and the sham-control (n=5) for two weeks beginning on GD 0. Body weight of each mouse was recorded on GD-1 and GD14. Data represent mean ± SEM. At each time point, statistical analysis was performed using one-way ANOVA with Dunnett's *post hoc* test.

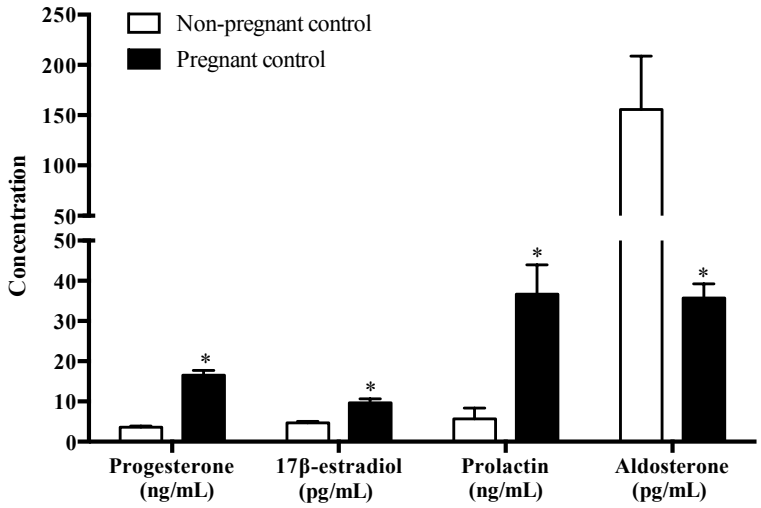

Figure 4.3 Hormonal levels in plasma of non-pregnant and pregnant mice on GD 14. The plasma concentrations of four hormones (progesterone, 17β-estradiol, prolactin and aldosterone) in non-pregnant and pregnant controls were measured on GD14. Data represent the mean ± SEM (n=3 for non-pregnant control and 12 for pregnant control). For each hormone, statistical analysis was performed using one-way ANOVA with Dunnett's *post hoc* test. * denotes statistically significant differences at $p < 0.05$.

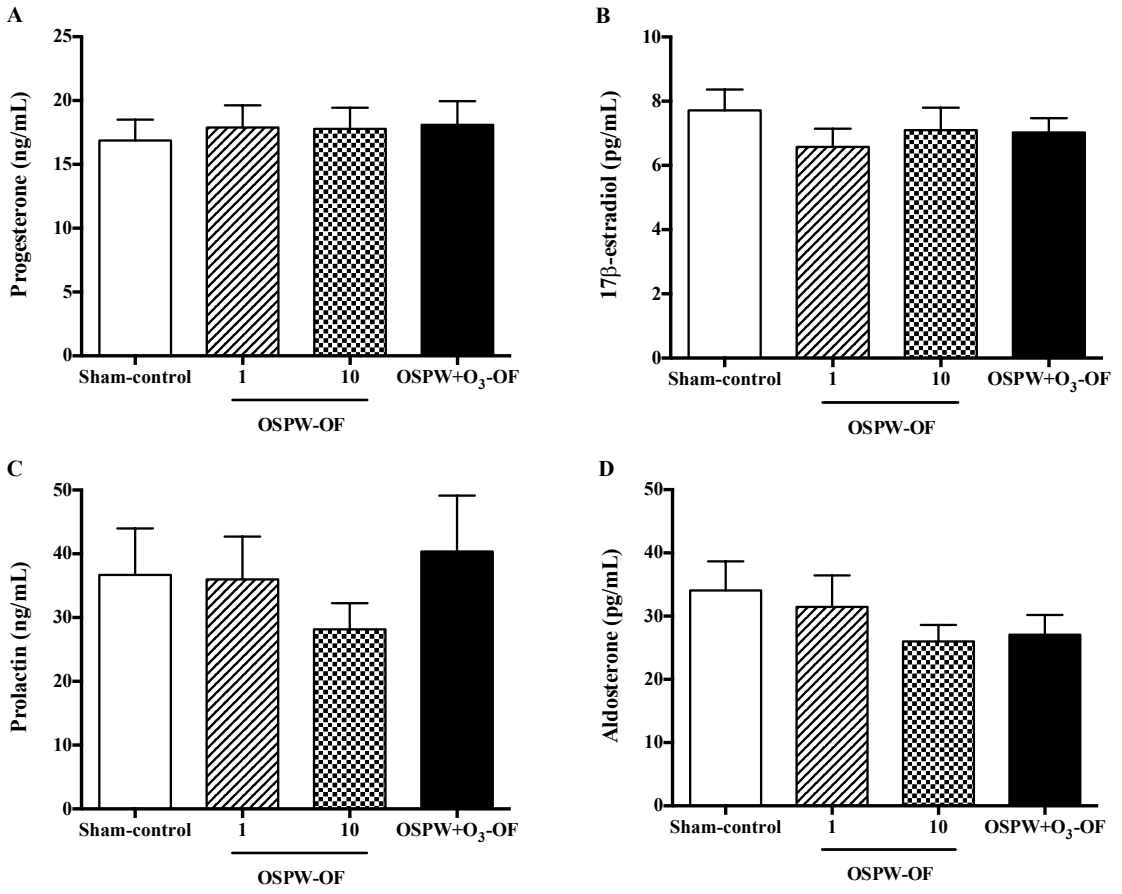

Figure 4.4 Hormonal levels in plasma of pregnant mice after acute exposure to OSPW-OF (1, 10 mg/L NAs) and OSPW+O_3-OF. Animals were gavaged weekly with OSPW-OF (1 and 10 mg/L NAs), OSPW+O_3-OF, and their sham-control for two weeks beginning on GD 0. On GD 14, pregnant mice were euthanized. The plasma concentrations of hormones (progesterone, 17β-estradiol, prolactin and aldosterone) were determined using commercial ELISA kits. Data represent the mean ± SEM (n=11-12 for A, 8-9 for B, 11-12 for C, and 10-12 for D). For each hormone, statistical analysis was performed using one-way ANOVA with Dunnett's *post hoc* test.

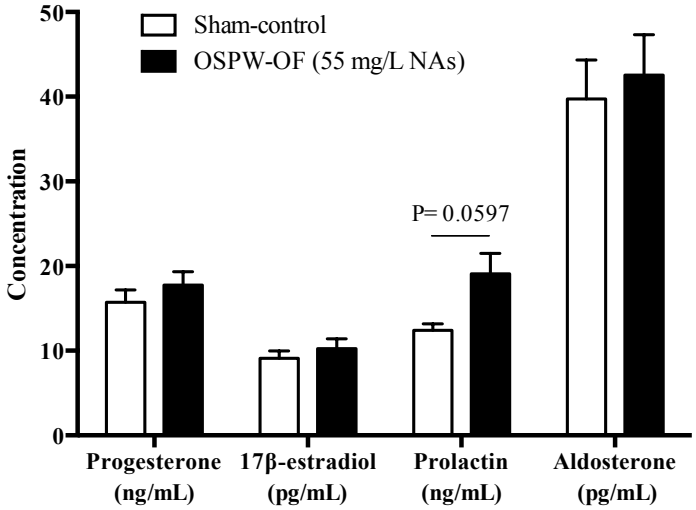

Figure 4.5 Hormonal levels in plasma of pregnant mice after acute exposure to OSPW-OF (55 mg/L). Animals were gavaged weekly with OSPW-OF (55 mg/L NAs) and the sham-control for two weeks beginning on GD 0. On GD 14, pregnant mice were euthanized. The plasma concentrations of four hormones (progesterone, 17β-estradiol, prolactin and aldosterone) were determined using commercial ELISA kits. Data represent the mean ± SEM (n=5 for sham control and 8 for OSPW-OF). For each hormone, statistical analysis was performed using one-way ANOVA with Dunnett's *post hoc* test.

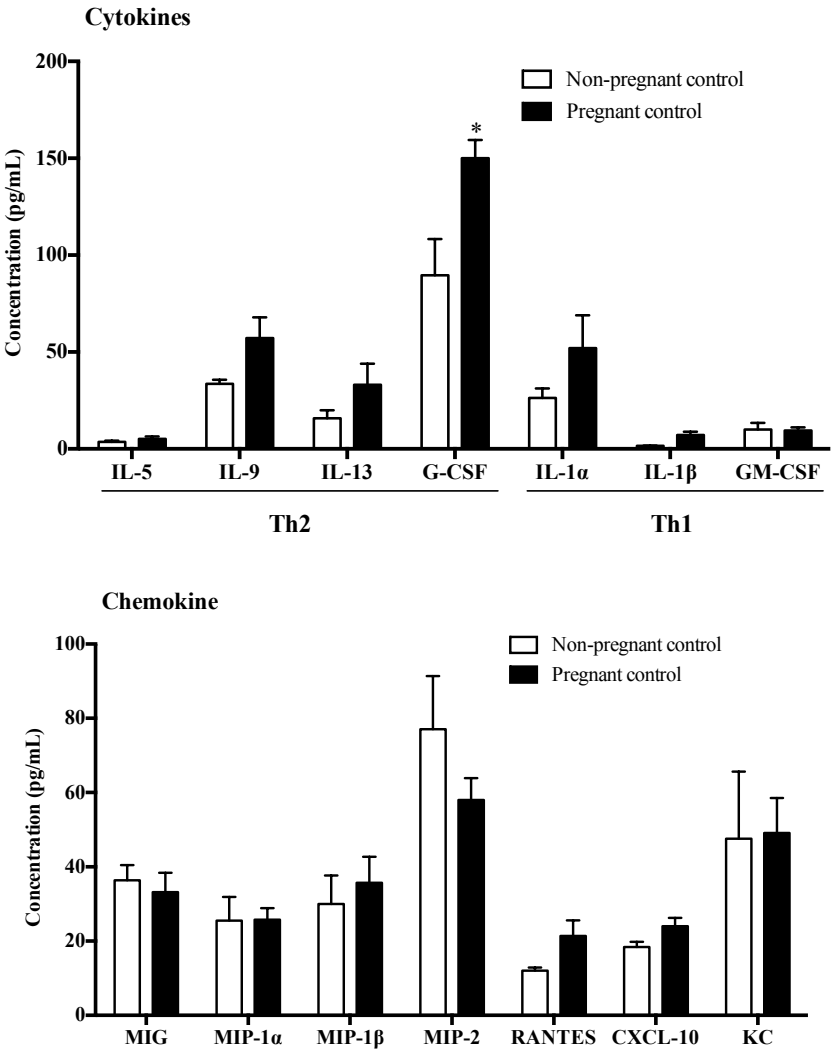

Figure 4.6 Cytokine/chemokine profile in plasma of non-pregnant and pregnant mice on GD 14. The plasma levels of cytokines and chemokines in non-pregnant and pregnant controls were measured on GD14. Data represent the mean ± SEM (n=3 for non-pregnant control and 12 for pregnant control). For each cytokine/chemokine, statistical analysis was performed using one-way ANOVA with Dunnett's *post hoc* test. * denotes statistically significant differences at $p < 0.05$.

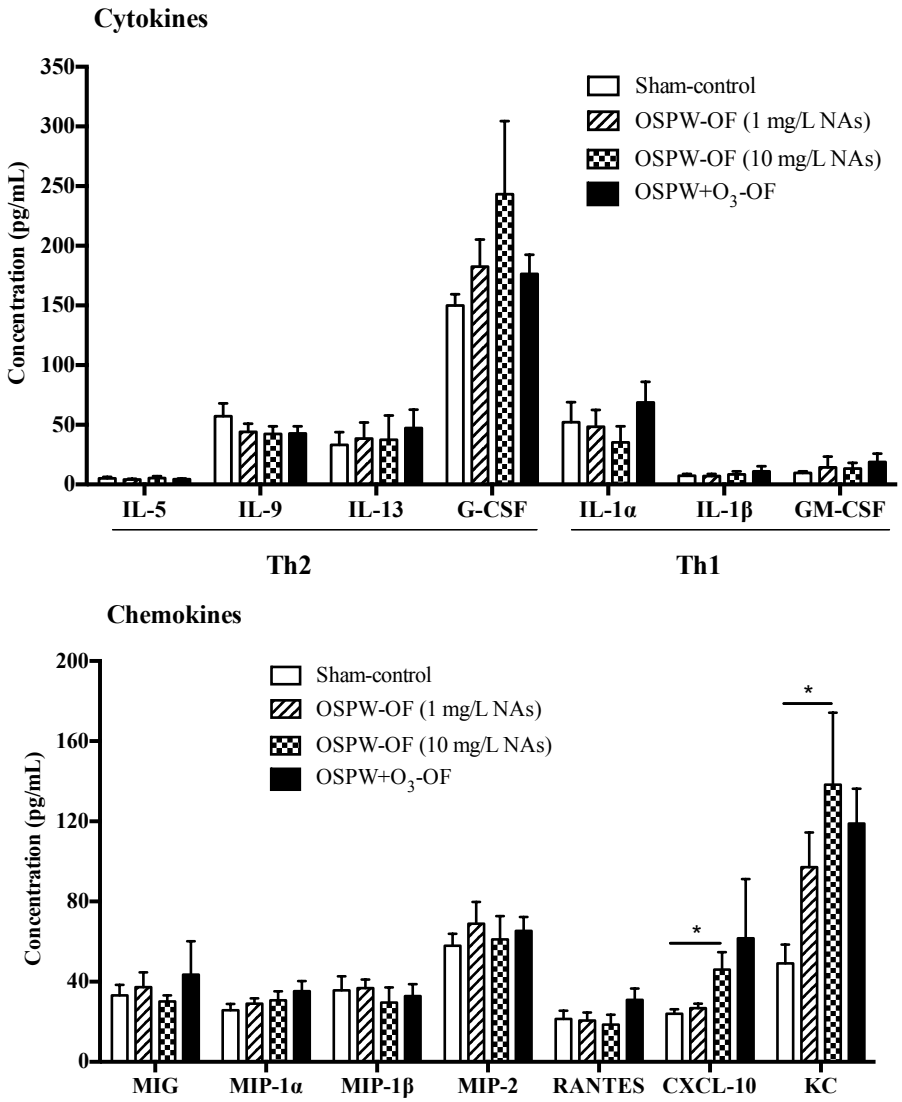

Figure 4.7 Cytokine/chemokine profile in plasma of pregnant mice after acute exposure to OSPW-OF (1, 10 mg/L NAs) and OSPW+O_3-OF. Animals were gavaged weekly with OSPW-OF (1 mg/L NAs; n=12), OSPW-OF (10 mg/L NAs; n=11), OSPW+O_3-OF (n=13), and their sham-control (n=12), for two weeks beginning on GD 0. On GD 14, pregnant mice were euthanized. The plasma levels of cytokines and chemokines were measured. Data represent the mean ± SEM. For each cytokine/chemokine, statistical analysis was performed using one-way ANOVA with Dunnett's *post hoc* test. * denotes statistically significant differences at $p < 0.05$.

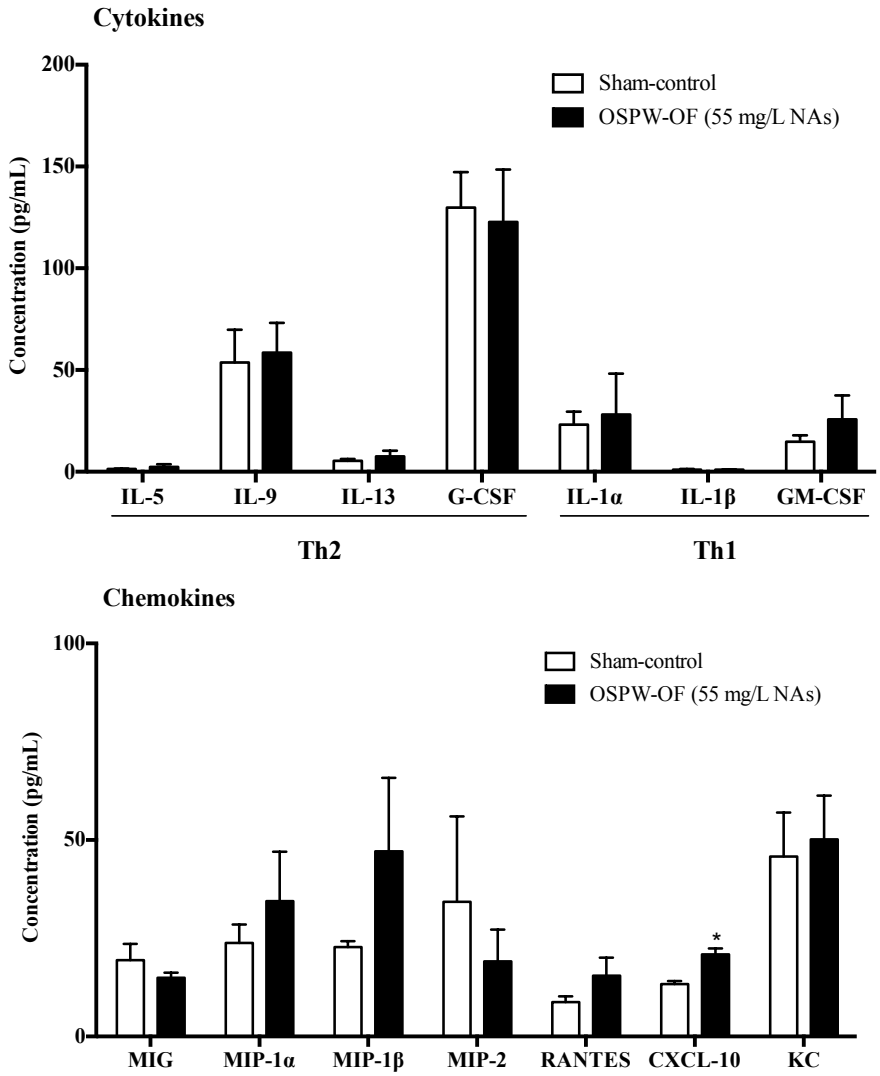

Figure 4.8 Cytokine/chemokine profile in plasma of pregnant mice after acute exposure to OSPW-OF (55 mg/L). Animals were gavaged weekly with OSPW-OF (55 mg/L NAs) and the sham-control for two weeks beginning on GD 0. On GD 14, pregnant mice were euthanized. The plasma levels of cytokines and chemokines were measured. Data represent the mean ± SEM (n=5 for sham control and 8 for OSPW-OF). For each cytokine/chemokine, statistical analysis was performed using one-way ANOVA with Dunnett's *post hoc* test. * denotes statistically significant differences at $p < 0.05$.

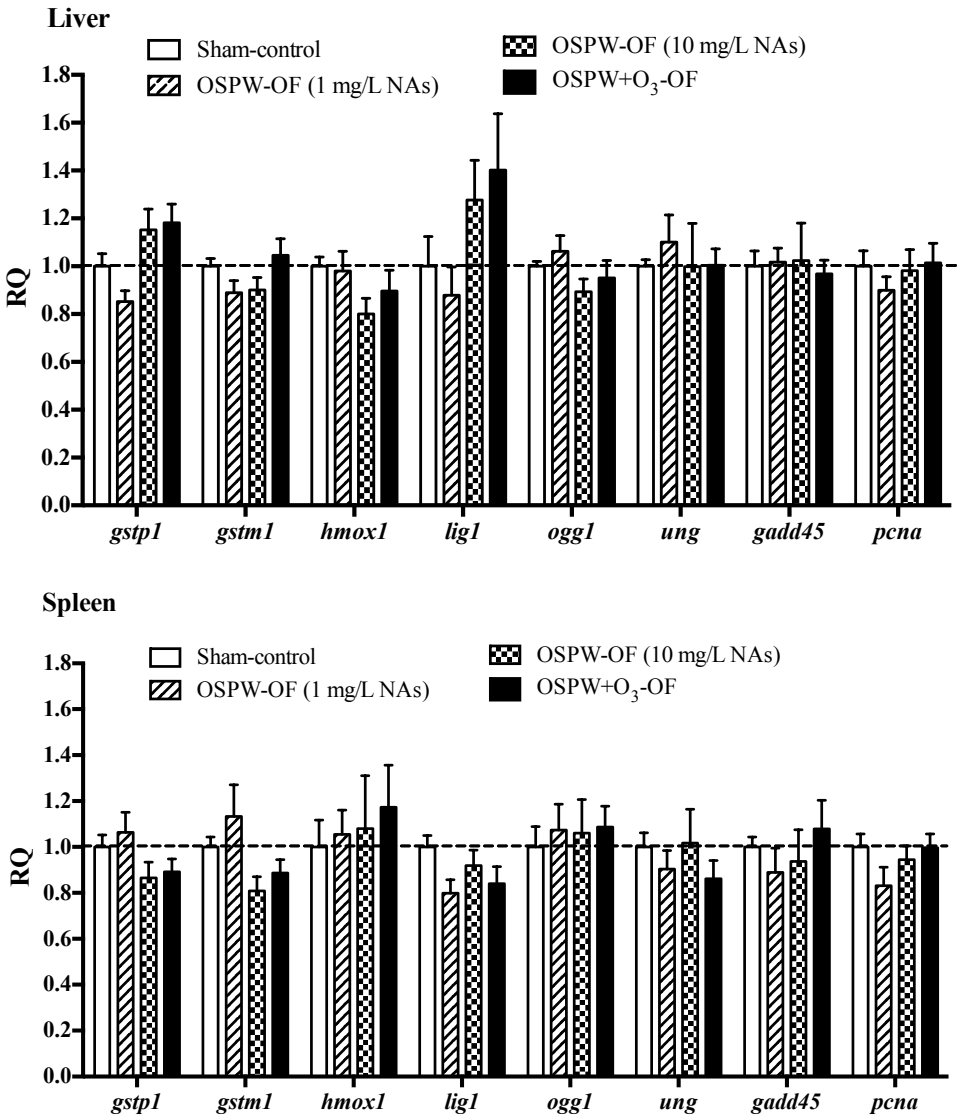

Figure 4.9 Gene expression in the liver and spleen of pregnant mice after acute exposure to OSPW-OF (1, 10 mg/L NAs) and OSPW+O_3-OF. Gene expression was assessed in the liver and spleen of pregnant mice after acute exposure to OSPW-OF (1 mg/L NAs; n=7), OSPW-OF (10 mg/L NAs; n=6), OSPW+O_3-OF (n=8), and their sham-control (n=7) for two weeks. Gene expression was analyzed by qPCR using the ddCT method. Data are expressed as relative quantification (RQ) values. RQ values for the treatment groups were normalized against the RQ values of sham-control. Data represent the mean ± SEM. For each gene, statistical analysis was performed using one-way ANOVA with Dunnett's *post hoc* test.

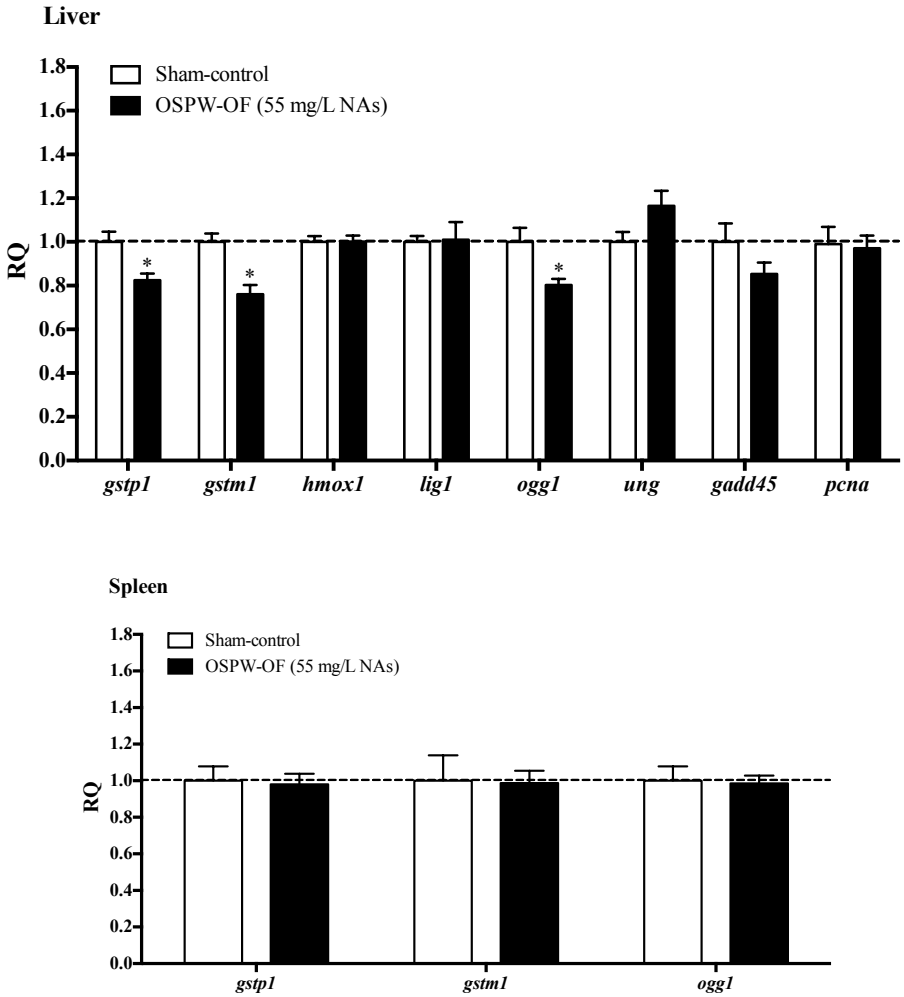

Figure 4.10 Gene expression in the liver and spleen of pregnant mice after acute exposure to OSPW-OF (55 mg/L). Gene expression was analyzed by qPCR using the ddCT method. Data are expressed as relative quantification (RQ) values. RQ values for the treatment group were normalized against the RQ values of sham-control. Data represent the mean ± SEM (n=5 for sham control and 8 for OSPW-OF). For each gene, statistical analysis was performed using one-way ANOVA with Dunnett's *post hoc* test. * denotes statistically significant differences at $p < 0.05$.

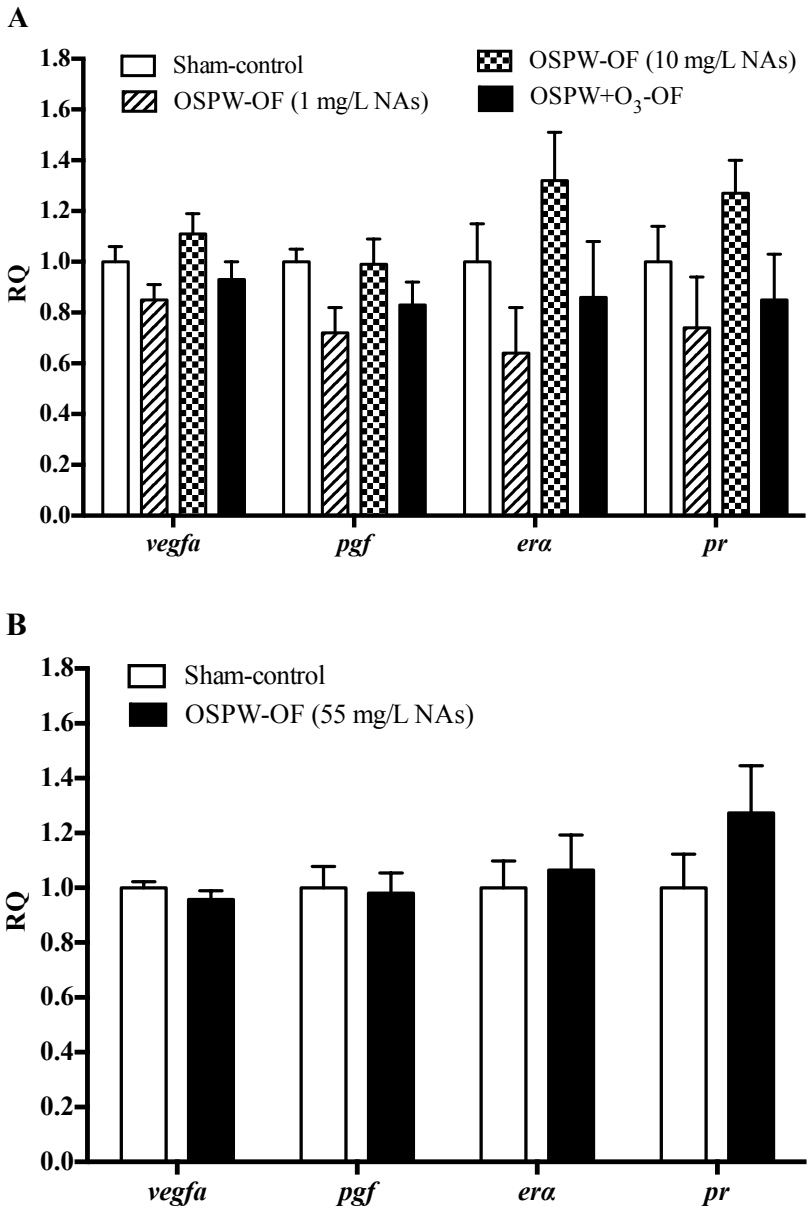

Figure 4.11 Gene expression in the placenta of pregnant mice after acute exposure to OSPW-OF (1, 10, 55 mg/L NAs) and OSPW+O_3-OF. Gene expression was assessed in the placenta of pregnant mice after exposure to (A) OSPW-OF (1 mg/L NAs; n=7), OSPW-OF (10 mg/L NAs; n=6), OSPW+O_3-OF (n=8), and their sham-control (n=7), or (B) OSPW-OF (55 mg/L NAs; n=8) and the sham-control (n=5) for two weeks. Gene expression was analyzed by qPCR using the ddCT method. Data are expressed as relative quantification (RQ) values. RQ values for the treatment groups were normalized against the RQ values of sham-control. Data represent the mean ± SEM. For each gene, statistical analysis was performed using one-way ANOVA with Dunnett's *post hoc* test.

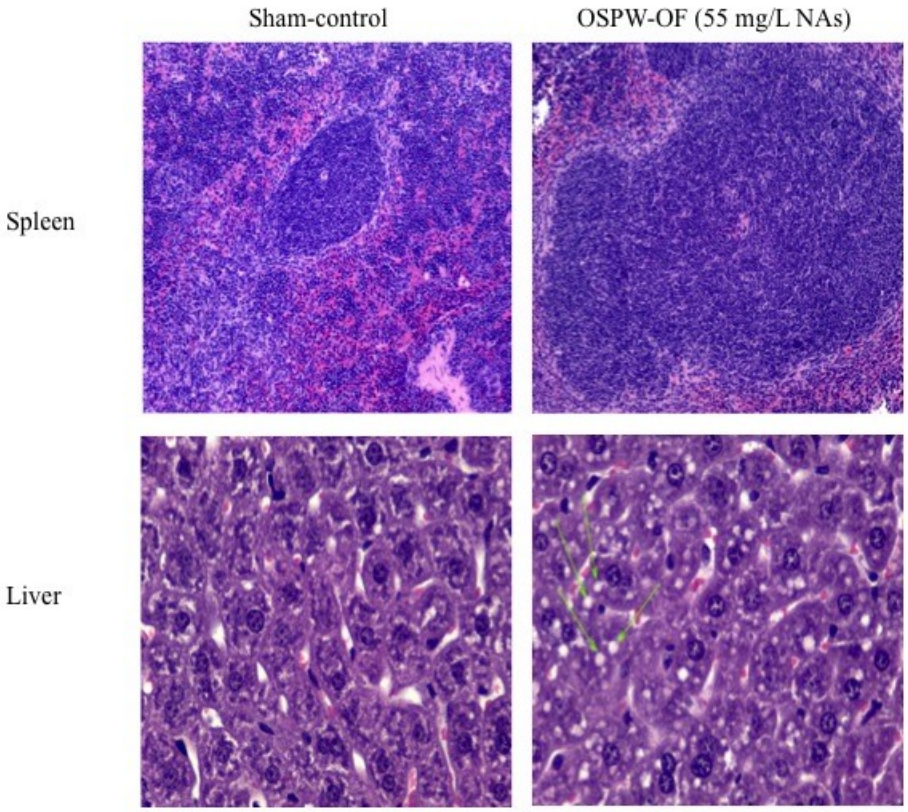

Figure 4.12 Representative histological images of spleen and liver from pregnant mice after acute exposure to OSPW-OF (55 mg/L NAs). Arrows pointing out hepatocyte vacuoles.

Chapter 5: Toxic effects in mice during pregnancy and lactation after sub-chronic oral exposures to the organic fraction of oil sands process water

5.1 Introduction

The complex mixture of oil sands process-affected water (OSPW) has been shown to be toxic to a variety of organisms (Li et al., 2017). The majority of OSPW toxicity has been attributed to the dissolved organic fraction, of which naphthenic acids (NAs) are postulated to be one of the primary toxic constituents (Madill et al., 2001; MacKinnon and Boerger, 1986; Allen, 2008a; Clemente and Fedorak, 2005).

Research has revealed the impact of OSPW on the endocrine axis, and in particular the production of sex hormones. *In vitro*, OSPW decreased testosterone (T) and increased 17β-estradiol (E2) in the H295R cell line (He et al., 2010). *In vivo*, significant reduction in plasma T and E2 were reported in goldfish (Lister et al., 2008) and yellow perch (van den Heuvel et al., 1999a) exposed to OSPW. Exposure to OSPW also impaired the fish reproduction. Less fecundity, altered synthesis of sex hormones and less pronounced secondary characteristics were observed in fathead minnows exposed to OSPW (Kavanagh et al., 2011, 2012a). In addition, transcription of estrogen receptor (ERα) and vitellogenin were significantly induced by OSPW NAs extracts (NAEs) at the early life stage of zebrafish, that negatively impact the development and endocrine functions (Wang et al., 2015).

Due to the complexity of OSPW, it is difficult to identify the individual components that are responsible for the toxicity. However, studies have reported that some aromatic NAs in OSPW are structurally similar to estrogens (Rowland et al., 2011a), and that some polycyclic NAs with a single aromatic ring exhibit estrogenic and

androgenic activity (Scarlett et al., 2012). Therefore, NAs may be responsible for the endocrine disrupting effects caused by OSPW exposure.

In contrast to the numerous studies on the toxicity of OSPW and its organic fraction (OSPW-OF) in aquatic organisms and *in vitro* model systems, there are only a few studies that examined the toxicity of OSPW and OSPW-OF in mammals. An early investigation in toxicity of OSPW-OF (primary NAs) has provided information about non-lethal effects in rats (Rogers et al., 2002). A single, oral administration of 300 mg/kg body weight/d NAs produced signs of acute toxicity including temporary appetite suppression and pericholangitis (a biliary inflammatory response) (Rogers et al., 2002). Repeated exposure at 60 mg/kg body weight/d NAs for 90 days resulted in suppressed growth, hepatotoxicity, and depressed blood cholesterol (Rogers et al., 2002). A subsequent reproductive study by rogers (2003) has shown that exposure to 60 mg/kg body weight/d NAs prior to breeding, throughout breeding and gestation caused poor pregnancy success in rats, which appeared to be associated with the depressed concentration of the sex hormone progesterone (Rogers, 2003). It should be noted that the dose of 60 mg/kg body weight/d NAs reflects 10 times a worst-case exposure scenario for small mammals drinking water containing NAs at concentrations comparable to those in OSPW (Rogers, 2003). The mice exposed to OSPW-OF at environmentally relevant doses that showed that sub-chronic *in vivo* exposure to OSPW-OF down-regulated the expression of genes that encoded pro-inflammatory cytokines (hormones of the immune system) (Garcia-Garcia et al., 2011b). In parallel *in vitro* experiments, the antimicrobial responses of mouse tissue-derived macrophages decreased after treatment with OSPW-OF (Garcia-Garcia et al., 2011b). These results indicate that OSPW-OF has immunotoxic

properties that may impair the ability of exposed hosts to defend against infectious diseases.

In the previous study, we investigated the toxicity of OSPW in mice after short-term exposure (Chapter 4). In the present study, mice were exposed orally to OSPW-OF for six weeks covering the periods of gestation and lactation. The goal of this study was to determine whether sub-chronic exposure of mice to OSPW-OF induced reproductive toxicity, immunotoxic effects and stress in female mice and their offspring. Completion of this objective was important for characterization of the sub-chronic toxic effects of OSPW-OF in mammals.

5.2 Materials and Methods

5.2.1 Oil sands process-affected water (OSPW)

The OSPW sample was collected from Aurora pond (Syncrude Canada Inc.), Fort McMurray, Alberta in 2012. A detailed composition of this water is shown in Table 3.1 (Chapter 3).

5.2.2 Ozonation of OSPW

The ozone treatment of OSPW was carried out according to the protocol described in Section 3.2.2 (Chapter 3).

5.2.3 Extraction of the organic fractions of OSPW and ozonated OSPW

The organic compounds were isolated from OSPW, ozonated OSPW ($OSPW+O_3$), or distilled water using a liquid-liquid organic extraction protocol as

described in Section 3.2.3 (Chapter 3). The stock solutions for OSPW-OF and OSPW+O_3-OF were prepared according to the method exactly as described in Section 4.2.3 (Chapter 4). For the first set of exposures, the doses adopted were 1.82 and 18.2 mg/kg bw/week NAs for OSPW-OF, and 1.69 mg/kg bw/week NAs for OSPW+O_3-OF. These doses reflect the amount of NAs that an animal would consume in a week, if its drinking water was OSPW (1 or 10 mg/L NAs) or ozonated OSPW (0.93 mg/L NAs). For the follow-up experiments, OSPW-OF at the dose of 100 mg/kg bw/week NAs was used for treatment, to investigate the risk when mice were exposed to a high dose of NAs (55 mg/L).

5.2.4 NAs analysis

Ultra-performance liquid chromatography/high-resolution mass spectrometry (UPLC/HRMS) was used to measure the NAs and oxy-NAs in organic fractions according to the procedures described in Section 3.2.4 (Chapter 3).

5.2.5 Exposure of mice to OSPW and OSPW+O_3 organic fractions

Six-to-eight week old female BALB/c mice were purchased from Charles River Laboratories. Mice were given *ad libitum* access to food (PicoLab mouse diet 20-5058) and tap water. They were kept at 20-22°C with a 12 h light cycle. After two weeks of acclimatization, adult BALB/c male mice were randomly assigned to breed with females in a 1 male: 2 female ratio overnight. The presence of a vaginal plug, indicating that mating had occurred, was determined the following morning. The day of appearance of the plug was designated gestation day 0 (GD 0). Females showing a vaginal plug were

separated from the males prior to exposure to OSPW-OF. Beginning on GD 0, vaginal plug-positive animals were dosed weekly for totally six weeks, throughout gestation (3-week) and lactation (3-week). The non-mated and non-exposed female mice were housed in separate cages and used as the non-pregnant control.

During the exposure, the behavioral and clinical signs of females were monitored daily. Mice were allowed to deliver, and the day of parturition was regarded as postnatal day 0 (PND 0). The litter size and offspring-related parameters (viability, sex, and gross external morphological abnormalities) were recorded. The body weight of both dams and pups were measured each week. After 6 weeks, animals were anesthetized with carbon dioxide. The blood was collected from the dams by cardiac puncture using a syringe rinsed with sodium heparin (1000 U/mL). Plasma was prepared by centrifuging the blood at 15,000 x *g* at 4°C for 5 minutes. Plasma was flash frozen in liquid nitrogen and stored at -80°C until used for hormones and cytokine/chemokine assays. The dams and pups were killed by cervical dislocation, prior to organ collection. A portion of the liver and spleen from both dams and pups were fixed in 4% PFA/PBS for histological assessment. The remaining liver and spleen from dams were flash frozen and stored at -80°C for gene expression analysis. Other tissues collected from dams for histological examination included intestine, kidney, brain and lung.

5.2.6 Assessment of pregnancy outcomes

After exposure, the pregnancy-related parameters for each group were calculated: pregnancy rate (number of pregnant mice/number of female mice mated ×100), delivery rate (number of females delivering/number of pregnant females × 100), live-birth index (number of live offspring/number of offspring delivered × 100), viability (number of live

offspring at PND 4/number of live offspring delivered×100), weaning index (number of live offspring at end of lactation/number of live offspring born×100), and offspring sex ratio (number of female pups/litter size × 100).

5.2.7 Measurement of hormones in plasma using ELISA assays

Progesterone (P4), 17β-estradiol (E2), prolactin (PRL) and aldosterone (ALDO) in plasma samples were measured using rodent ELISA kits according to the manufacturer's instructions. The limits of sensitivity of the assays were for 0.04 ng/mL for P4 (cat# 55-PROMS-E01, ALPCO), 3 pg/mL for E2 (cat# ES180S-100, Calbiotech), 30 pg/mL for PRL (cat# ab100736, Abcam,), and 4.7 pg/mL for ALDO (cat#ab136933, Abcam).

5.2.8 Plasma cytokine/chemokine analysis

The plasma cytokine/chemokine profile was analyzed using the Mouse Cytokine/Chemokine Array 31-Plex (Cat# MD31, Eve Technologies, Calgary, AB, Canada). This assay permitted simultaneous quantification of multiple cytokines by capturing the cytokines to differently colored/fluorescent beads that were detected by the bead analyzer (Bio-Plex 200, BIo-Rad). The cytokines and chemokines tested were: interleukin (IL)-1α, IL-1β, IL-5, IL-9, IL-13, granulocyte colony-stimulating factor (G-CSF), macrophage inflammatory protein (MIP)-1α/CCL3, MIP-2/CXCL2, RANTES/CCL5, keratinocyte chemoattractant (KC/CXCL1), monokine induced by gamma interferon (MIG/CXCL9), and interferon gamma-induced protein 10 (IP-10/CXCL10)

5.2.9 Gene expression analysis

Total RNA was extracted from liver and spleen using the RNA isolation kit (Cat# AM1924, Ambion) and reverse transcribed into cDNA using the Superscript III cDNA synthesis kit (Cat# 18080-051, Life Technologies) according to manufacturer's instructions. The cDNA was used for real-time PCR according to the method exactly described in Section 4.2.8 (Chapter 4). The stress responsive and detoxification genes that were examined in liver and spleen included *gstp1*, *gstm1*, *hmox1*, *lig1*, *ogg1*, *gadd45*, *ung* and *pcna*. The primers (Integrated DNA Technologies, IDT) used in this study are shown in Table 4.3 (Chapter 4). *hprt1* was used as the endogenous control. Gene expression was analyzed using the ddCT method, normalizing relative quantitation (RQ) values for treatment groups to their sham-controls.

5.2.10 Histological assessment

Various tissues (liver, spleen, kidney, intestine, brain, and lung), collected from mice exposed to OSPW-OF and OSPW+O_3-OF, were fixed in 4% PFA/PBS for 24 hours. Tissues were then processed by dehydration in a series of increasing ethanol solutions and embedded in paraffin. Each liver specimen was sectioned to 5 µm thickness using a microtome. The sections were mounted on glass slides, followed by the de-paraffinization and rehydration using toluene and a series of decreasing ethanol solutions. The tissues were then stained with haematoxylin-eosin (H & E) for microscopic examination performed by a certified veterinary pathologist (Dr. Nick Nation, University of Alberta).

5.2.11 Statistical analysis

Data are presented as mean ± SEM. Statistical analysis of data was performed using one-way ANOVA, followed by the Dunnett's's *post hoc* test. All analysis was processed using Prism 6.0 (Graphpad software, CA, USA). Differences between treatment groups were considered statistically significant when $p < 0.05$.

5.3 Results

5.3.1 Behavioral observations and body weight trend of both dams and pups

Throughout the gestation and lactation, no behavioral abnormalities were observed for mice from all experimental groups. As illustrated in Figure 5.1, the body weight of dams from the treatment groups did not differ significantly when compared to the sham-control mice. Figure 5.2 shows that exposure of dams to OSPW-OF (1, 10 or 55 mg/L NAs) and OSPW+O_3-OF for six weeks did not influence the weight of pups either at birth or during the lactation.

5.3.2 Pregnancy outcomes of mice after sub-chronic exposure to OSPW-OF and OSPW+O_3-OF

Table 5.1 and Table 5.2 summarize various reproductive parameters determined for the experimental groups of mice. Exposure to OSPW-OF (1, 10 or 55 mg/L NAs equivalent) or OSPW+O_3-OF did not impair the mice fecundity, indicated by the comparable pregnancy rate of treated animals compared to their sham-controls. A reduction of litter size was noted in both OSPW-OF (1, 10 or 55 mg/L NAs) and OSPW+O_3-OF groups, but the differences were not statistically significant in comparison

to that of non-exposed animals. In addition, the live-birth, viability and weaning indexes, as well as the sex ratio of pups delivered were not affected following a 6-week exposure to OSPW-OF at all doses or OSPW+O_3-OF.

5.3.3 Hormonal levels in plasma of mice after sub-chronic exposure to OSPW-OF and OSPW+O_3-OF

In the present study, the basal plasma concentrations of four steroid hormones (progesterone, 17β-estradiol, prolactin and aldosterone) in non-pregnant mice and dams on postnatal day 21 (PND 21) were measured. As shown in Figure 5.3, the plasma aldosterone exhibited a dramatic reduction from ~150 pg/mL in non-pregnant mice to ~50 pg/mL in mice at the end of lactation. By contrast, no significant differences were observed between these two groups for other three hormones.

I also examined the effects of sub-chronic exposures to OSPW-OF on hormonal levels in mice. As shown in Figure 5.4 and Figure 5.5, oral administration with OSPW-OF (1, 10, or 55 mg/L) or OSPW+O_3-OF for six weeks did not cause significant alterations of progesterone, 17β-estradiol, or prolactin when compared with the sham-control. Interestingly, exposure to OSPW-OF at 10 mg/L NAs, rather than 55 mg/L NAs, resulted in notable reduction of plasma aldosterone concentration.

5.3.4 Cytokine/chemokine levels in plasma of mice after sub-chronic exposure to OSPW-OF and OSPW+O_3-OF

In previous study, I assessed Th1/Th2 cytokine profile in mice on GD 14, and observed a significant elevation of Th2 cytokine granulocyte colony-stimulating factor (G-CSF) in comparison with non-pregnant mice. In the present experiments, the protein

level of G-CSF returned to the baseline level at the end of lactation (Figure 5.6). The plasma concentrations of other cytokines and chemokines did not differ between non-pregnant and exposed mice on PND 21.

The production of cytokines and chemokines in mice from OSPW-OF (1, 10, or 55 mg/L NAs) and OSPW+O_3-OF groups is illustrated in Figure 5.7 and Figure 5.8. The Th1/Th2 cytokine profile in exposed mice was not affected by OSPW-OF treatment at all doses. The chemokine MIG/CXCL9 was significantly elevated in animals exposed to OSPW-OF (10 mg/L NAs), compared to that of the sham-control. When the dose was increased to 55 mg/L NAs, MIG/CXCL9 returned to baseline levels and was comparable to the controls.

5.3.5 Expression of stress responsive and detoxification genes in the liver and spleen of mice after sub-chronic exposure to OSPW-OF and OSPW+O_3-OF

The results presented in the previous section (Chapter 4) showed that OSPW-OF acute oral exposure of mice to 1 or 10 mg/L NAs for two weeks, did not affect the expression of stress genes in the liver and spleen. When the exposure was prolonged to six weeks, the mRNA level of *hmox1* was down-regulated (Figure 5.9). I previously also showed that exposing mice acutely (2-week) to OSPW-OF at 55 mg/L NAs resulted in suppressed expression of *gstp1*, *gstm1*, and *ogg1* in the liver. However, following sub-chronic (6-week) exposure at this dose, non-significant changes in the mRNA levels of all genes except *ung* were observed in the liver (Figure 5.10).

5.3.6 Histological assessment of tissues from mice after sub-chronic exposure to OSPW-OF and OSPW+O_3-OF

Liver and spleen were collected from both dams and pups for histological examination. Other tissues obtained and analyzed from dams include intestine, kidney, brain, and lung. Table 5.4 summarizes the histopathological findings in selected tissues, and Figure 5.11 shows representative sub-chronic histological images. No overt pathological changes were observed after sub-chronic exposures of mice to different doses of OSPW-OF that would be indicative of tissue damage, inflammation, or neoplasia for all samples examined.

5.4 Discussion

In the present study, mice were orally exposed to OSPW-OF and OSPW+O_3-OF at the environmentally relevant doses, reflecting the amount of organic compounds a mouse would intake if their water source was OSPW (containing 1, 10 or 55 mg/L NAs), or ozonated OSPW. It was found that repeated dosages of OSPW-OF and OSPW+O_3-OF throughout gestation and lactation did not cause significant effects on the reproductive performance of mice. The body weight and reproductive performance (pregnancy rate, gestation length, litter size, and offspring viability) in the treated animals was comparable to the controls. Also, the growth of pups during lactation was not impacted by OSPW-OF and OSPW+O_3-OF exposures of the dams. These results indicate that sub-chronic oral exposure of mice to OSPW-OF containing NAs of 1 to 55 mg/L, did not affect reproductive performance of dams or the growth rate of pups at parturition or during lactation.

In the acute toxicity experiments (Chapter 4), I reported on the levels of three pregnancy-associated hormones (E2, P4, and prolactin). As expected, these hormone levels decreased at the end of lactation, and the hormone concentrations returned to the basal levels comparable to those in non-pregnant mice. The systemic reduction of these hormones suggests postpartum adaptation of maternal physiology from parturition to the end of lactation. This is not surprising, as parturition is regulated by changes in circulating hormone levels. For example, P4 is normally high during gestation and plays a crucial role in establishment and maintenance of pregnancy. P4 decreases around the time of parturition, which is the trigger that initiates milk production and secretion (Nguyen et al., 2001). In most mammals, including mice and rats, parturition is characterized by a rapid increase of the ratio of estradiol to progesterone concentrations (Knobil, 2006). Although I did not measure the hormonal levels at parturition time, a notable increase of E2: P4 ratio was observed at the end of lactation (0.98) compared to mice in mid-pregnancy (0.58). Also, the plasma concentrations of prolactin in mice at the end of lactation returned to the basal level comparable to that in non-pregnant mice. This was also expected, because the young mice begin to eat solid food at about 16 days post parturition and are weaned during PND 19-21. Less demand of breast milk leads to decreased suckling, which results in diminished prolactin release and the cessation of lactation.

Plasma hormone levels were also measured in mice after sub-chronic exposures to OSPW-OF and OSPW+O_3-OF. The results showed that exposure to 10 mg/L OSPW-OF caused a significant decrease in aldosterone plasma concentrations, whereas the impacts disappeared in mice after exposure to 55 mg/L OSPW-OF. Aldosterone is the main

mineralocorticoid hormone that regulates Na^+ and K^+ homeostasis (Lee et al., 2005). The decrease in aldosterone levels at 10 mg/L NAs might be due to more sodium intake, since OSPW-OF was prepared in NaOH solution. However, it is unknown why the aldosterone levels increased to the control level in mice administered with higher dose of OSPW-OF solution having greater concentrations of Na^+. Because no impacts on the reproductive performance were observed in mice from treatment groups, the significance in the changes of aldosterone production is unclear at present, however, its alteration may affect the renin-angiotensin-aldosterone system that plays a key role in regulating blood pressure and salt balance (Lee et al., 2005).

In the acute exposure experiments, a bias of the immune system towards Th2 was observed in non-exposed animals at mid-gestation, as demonstrated by elevated plasma levels of Th2 cytokine G-CSF in the plasma of mice on GD 14. The Th1/Th2 shift was reported to be associated with a successful pregnancy (Sykes et al., 2012b). As shown in this study, all the cytokines and chemokines in mice on PND 21 were at levels comparable to those in non-pregnant animals, suggesting that the maternal immunity return to homeostasis by the end of lactation.

The cytokine/chemokine production in mice following sub-chronic exposures to OSPW-OF and OSPW+O_3-OF was not influenced by different treatments; however, mice dosed with 10 mg/L NAs of OSPW-OF exhibited up-regulated mRNA levels of MIG/CXCL9 ($p = 0.049$). MIG/CXCL9 belongs to the subfamily of CXC and is a chemokine that attracts and specifically activates Th1 cells (Feng et al., 2015). The increased plasma concentrations of MIG/CXCL9 in OSPW-OF (10 mg/L NAs) exposed mice may be associated with enhanced inflammatory response. However, given that the *p*

value is very close to 0.05, and that MIG/CXCL9 levels at higher dose (55 mg/L) were similar to those in control animals, it is unlikely that sub-chronic oral exposure of mice to OSPW-OF caused a significant inflammatory response.

The sub-chronic oral exposure of mice to OSPW-OF did not cause lethality or obvious distress such as behavioral changes, loss of body weight, or pregnancy failure. Therefore, changes in mRNA levels of stress genes in the liver and spleen might not be indicative of acute toxicity but might be indicators of sub-lethal effects on mice. In the 2-week exposure study (Chapter 4), the mRNA levels of two glutathione-S-transferases (GSTs) were down regulated in liver of mice exposed to high dose of OSPW-OF (55 mg/L NAs), including *gstp1* and *gstm1*. GSTs are enzymes involved in the detoxification of harmful electrophilic endogenous and exogenous compounds including carcinogens, environmental pollutants, drugs and other xenobiotics (Cnubben et al., 2001; Sharma et al., 2014). The down-regulated *gst* transcription may be related to decreased ability of the animals to deal with xenobiotics. Interestingly, down-regulation of *gst* only occurred after the short exposure, while the gene expression returned to the control level or was slightly elevated (i.e., *gstp1*) after prolonged exposure. This indicates a possible shift towards detoxification mechanisms following long duration of the stress conditions generated by the OSPW-OF treatment.

OSPW has been shown to cause oxidative stress, demonstrated by greater concentrations of reactive oxygen species (ROS) and higher expression of genes involved in defending against ROS in fish (Gagné et al., 2012; He et al., 2012a), as well as elevated oxidative stress responsive gene in mammalian cells (i.e., elevated *hmox1* expression in RAW 264.7 cells exposed to OSPW as demonstrated in Chapter 3).

Interestingly, in the present *in vivo* study, 6-week oral exposure of OSPW-OF at 10 mg/L NAs induced a down-regulation of *hmox1* expression in the liver. Similar inhibitory effects have been reported in kidneys of rats (Bolati et al., 2013) and human neutrophils (Alba et al., 2008) under conditions of oxidative stress. These results suggest that *hmox1* expression is not solely dependent on ROS status in the cells. Oxidative stress can damage DNA, and when the DNA damage is irreparable, it can lead to cellular transformation that eventually contributes to the development of tumors (Sampath et al., 2012). Recently, researchers have reported on the genotoxicity of OSPW, as demonstrated by the alterations in transcripts of genes involved in DNA repair and synthesis during cell maintenance such as *lig*, *ung*, and *ogg* (Gagné et al., 2013, 2012). The functions of these genes have been described in Chapter 4. In this sub-chronic exposure study, the expressions of *lig* and *ogg* were not impacted by OSPW-OF treatment. However, an up-regulation in *ung* (involved in DNA repair) transcripts was observed in liver of animals exposed to 55 mg/L OSPW-OF, which was consistent with the previous findings in rainbow trout hepatocytes after exposure to OSPW (Gagné et al., 2013). It should be noted that despite the changes in liver gene expression, there were no major histopathological alterations in all tissues examined after oral exposure of mice to OSPW-OF and OSPW+O_3-OF.

5.5 Summary

The sub-chronic oral exposure to OSPW-OF at indicated NAs concentrations, throughout gestation and lactation, did not lead to obvious distress in dams and their offspring. The circulating concentrations of cytokines and chemokines were not impacted

by OSPW-OF treatment. The alterations of genes involved in oxidative stress response (*hmox1*) and DNA repair (*ung*) were only observed in liver, suggesting that liver might be a target organ. However, no histopathological changes in liver and other tissues were observed. The results suggest that OSPW-OF at the environmentally relevant doses of up to 55 mg/L NAs may be below the threshold required to induce these types of changes. Besides, OSPW+O_3-OF did not change any biological responses examined in this study, indicating the safety of ozonation as the OSPW remediation method.

Table 5.1 Reproductive parameters of mice after sub-chronic exposure to OSPW-OF (1 and 10 mg/L NAs) or OSPW+O_3-OF

Parameters	Sham-control (n=13)	OSPW-OF		OSPW+O_3-OF (n=16)
		1 mg/L NAs (n=15)	10 mg/L NAs (n=13)	
# of females	18	18	18	18
# of pregnant mice	13	15	13	16
Pregnancy rate[a] (%)	72.2	83.3	72.2	88.9
Gestation length[b]	20	20	20	19
Litter size	6.23 ± 0.53	5.73 ± 0.45	5.62 ± 0.53	5.44 ± 0.30
Live-birth index[c] (%)	98.90 ± 1.10	99.26 ± 0.74	100 ± 0.00	100 ± 0.00
Viability[d] (%)	100 ± 0.00	100 ± 0.00	100 ± 0.00	100 ± 0.00
Weaning index[e] (%)	100 ± 0.00	100 ± 0.00	100 ± 0.00	100 ± 0.00
Sex ratio of pups[f] (%)	0.57 ± 0.03	0.64 ± 0.07	0.56 ± 0.04	0.56 ± 0.07

Note: Females were gavaged weekly with OSPW-OF (1 and 10 mg/L NAs), OSPW+O_3-OF and the sham-control, for six weeks beginning on GD 0. On GD 14, Mice were allowed to deliver. After six weeks, dams and pups were euthanized, and reproductive parameters were recorded. Values in rows five to nine represent the mean ± SEM. Statistical analysis was performed using one-way ANOVA with Dunnett's *post hoc* test. [a] Number of pregnant mice/number of female mice mated × 100. [b] Median value.[c] number of live offspring/litter size × 100. [d] number of live offspring at PND 4/number of live offspring delivered×100. [e] number of live offspring at end of lactation/number of live offspring born×100. [f] number of female pups delivered/litter size × 100.

Table 5.2 Reproductive parameters of mice after sub-chronic exposure to OSPW-OF (55 mg/L NAs)

Parameters	Sham-control	OSPW-OF
No. of females	8	6
No. of pregnant mice	7	5
Pregnancy rate[a] (%)	87.5	83.3
Gestation length[b]	19	20
Litter size	6.43 ± 0.78	6.20 ± 0.37
Live-birth index[c] (%)	95.80 ± 2.73	100 ± 0.00
Viability[d] (%)	100 ± 0.00	93.3 ± 6.67
Weaning index[e] (%)	100 ± 0.00	93.3 ± 6.67
Sex ratio of pups[f] (%)	0.58 ± 0.04	0.70 ± 0.07

Note: Note: Females were gavaged weekly with OSPW-OF (55 mg/L NAs) and the sham-control for six weeks beginning on GD 0. After six weeks, dams and pups were euthanized, and reproductive parameters were recorded. Values in rows five to nine represent the mean ± SEM (n=7 for sham control and 5 for OSPW-OF). Statistical analysis was performed using one-way ANOVA with Dunnett's *post hoc* test. [a] Number of pregnant mice/number of female mice mated × 100. [b] Median value. [c] number of live offspring/litter size × 100. [d] number of live offspring at PND 4/number of live offspring delivered×100. [e] number of live offspring at end of lactation/number of live offspring born×100. [f] number of female pups delivered/litter size × 100.

Table 5.3 Histopathology report summary for various tissues from mice after sub-chronic exposure to OSPW-OF

Tissue*	OSPW-OF	Summary
	1, 10 and 55 mg/L NAs	
Liver	No microscopic changes relative to sham-control	No significant difference was found between the treatment and sham-control groups. It was concluded that exposure to OSPW-OF at these doses had no microscopic anatomic effects on mice.
Spleen	No microscopic changes relative to sham-control	
Intestine	No microscopic changes relative to sham-control	
Kidney	No microscopic changes relative to sham-control	
Brain	No microscopic changes relative to sham-control	
Lung	No microscopic changes relative to sham-control	

*Liver and spleen tissues were collected from both dams and pups. Other tissues were only collected from dams.

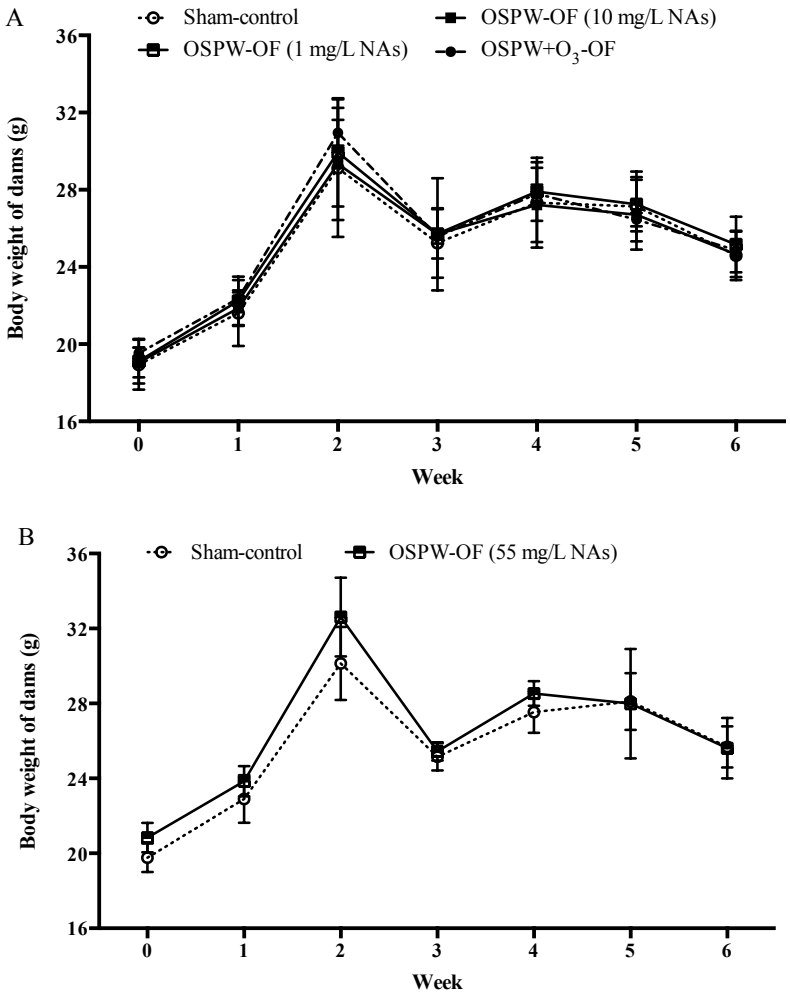

Figure 5.1 Body weight of pregnant mice during sub-chronic exposure. Animals were gavaged weekly with (A) OSPW-OF (1 mg/L NAs; n=15), OSPW-OF (10 mg/L NAs; n=13), OSPW+O_3-OF (n=16), and their sham-control (n=13), or (B) OSPW-OF (55 mg/L NAs; n=7) and the sham-control (n=5) for six weeks beginning on GD 0. Body weight of each mouse was recorded weekly. Data represent mean ± SEM. At each time point, statistical analysis was performed using one-way ANOVA with Dunnett's *post hoc* test.

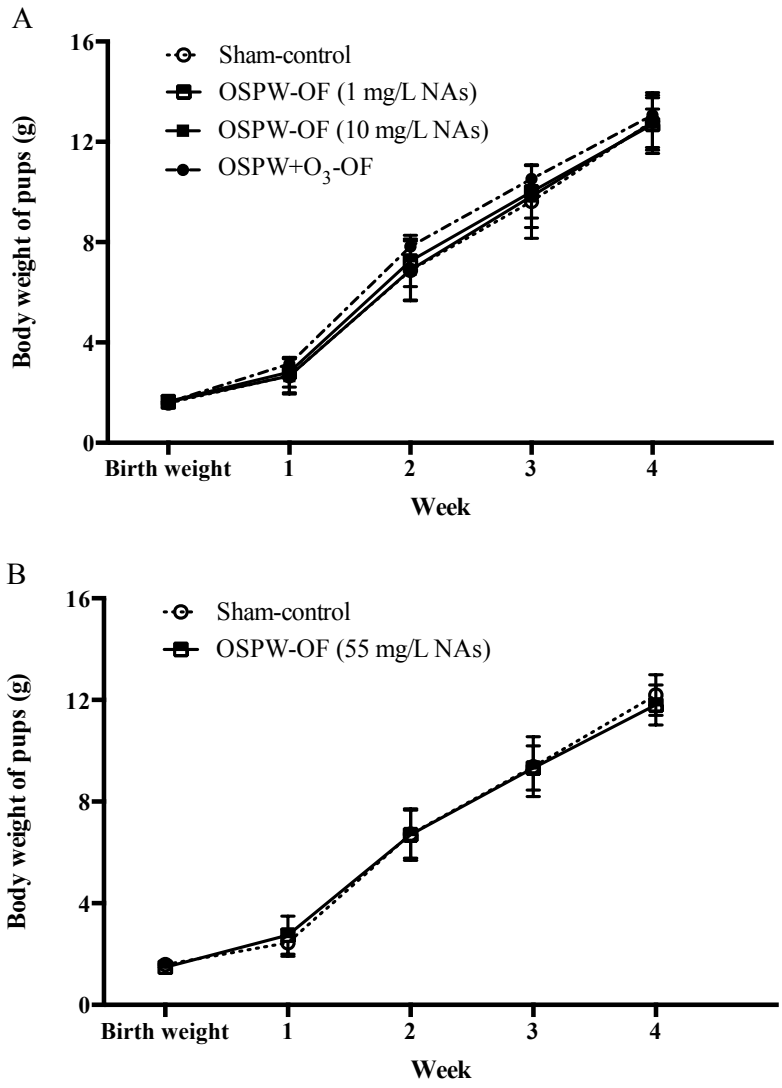

Figure 5.2 Body weight of pups. Pups were delivered by female mice gavaged weekly with (A) OSPW-OF (1 mg/L NAs; n=15; n=number of litters), OSPW-OF (10 mg/L NAs; n=13), OSPW+O_3-OF (n=16), and their sham-control (n=13), or (B) OSPW-OF (55 mg/L NAs; n=7) and the sham-control (n=5) for six weeks beginning on GD 0. Body weight of each pup from each litter was recorded weekly. Data represent mean ± SEM. At each time point, statistical analysis was performed using one-way ANOVA with Dunnett's *post hoc* test.

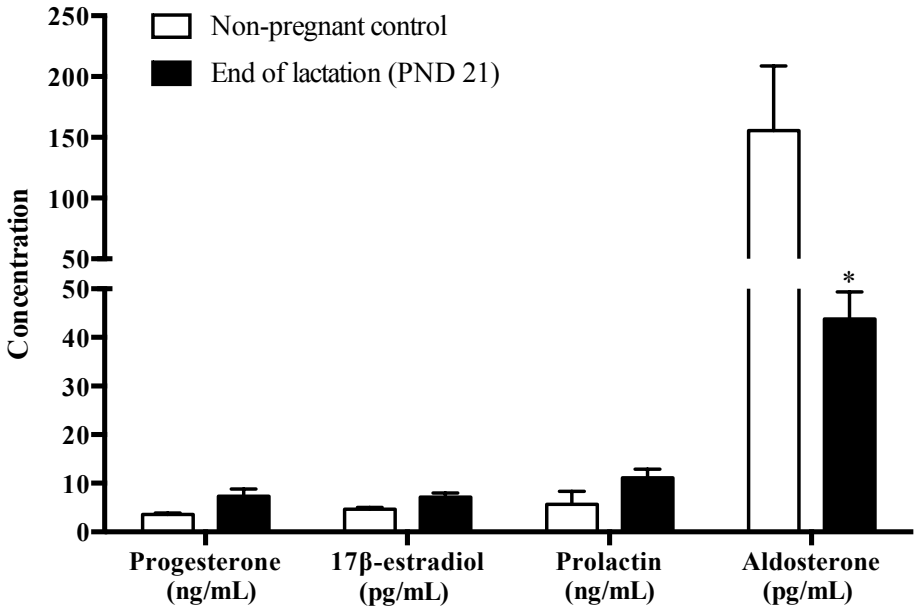

Figure 5.3 Hormone levels in the plasma of non-pregnant and pregnant mice on PND 21. The plasma concentrations of four hormones (progesterone, 17β-estradiol, prolactin and aldosterone) in non-pregnant control and non-treated control on PND 21 were measured. Data represent the mean ± SEM (n = 3 for non-pregnant control, and 9 for end of lactation). For each hormone, statistical analysis was performed using one-way ANOVA with Dunnett's *post hoc* test.

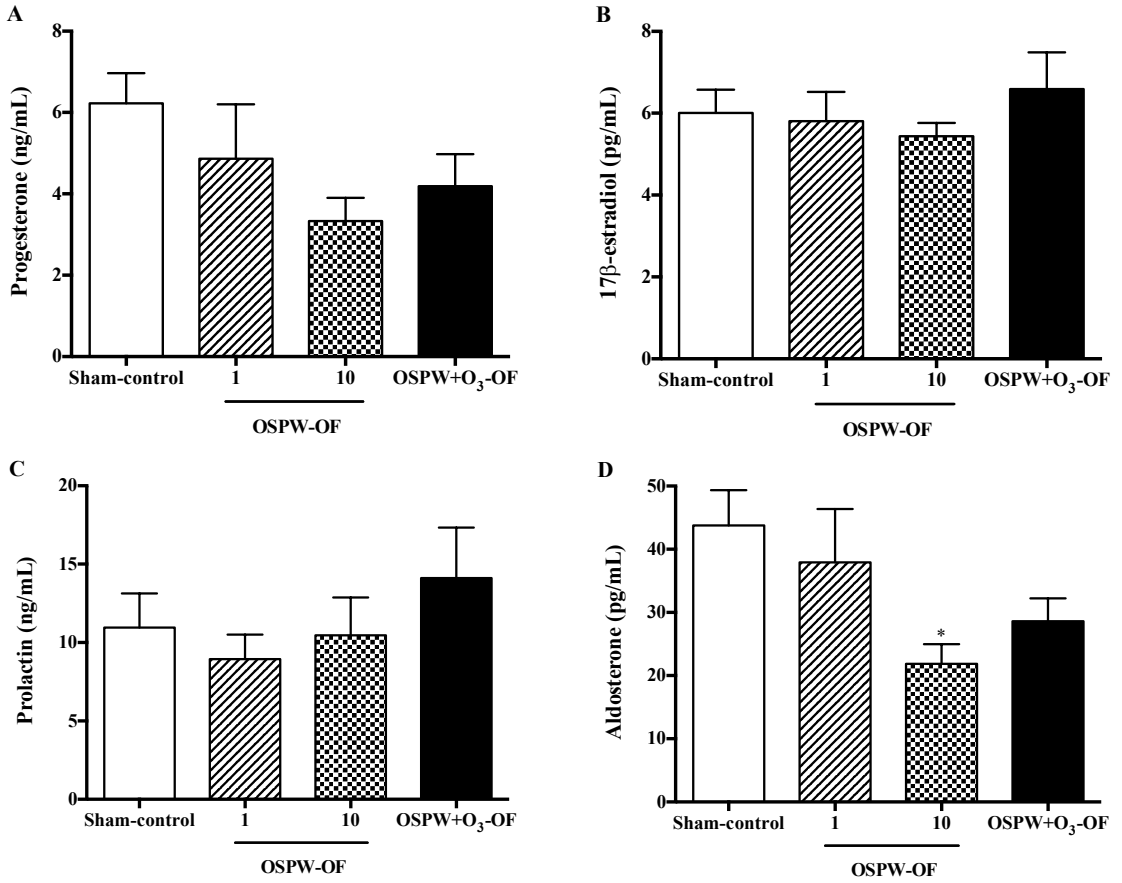

Figure 5.4 Hormone levels in the plasma of mice after sub-chronic exposure to OSPW-OF (1, 10 mg/L NAs) and OSPW+O_3-OF. Animals were gavaged weekly with OSPW-OF (1 and 10 mg/L NAs), OSPW+O_3-OF, and their sham-control for six weeks beginning on GD 0. After six weeks, pregnant mice were euthanized on PND 21. The plasma concentrations of hormones (progesterone, 17β-estradiol, prolactin and aldosterone) were determined using commercial ELISA kits. Data represent the mean ± SEM (n=8-11 for A, 9-11 for B, 9-11 for C, and 9-11 for D). For each hormone, statistical analysis was performed using one-way ANOVA with Dunnett's *post hoc* test.

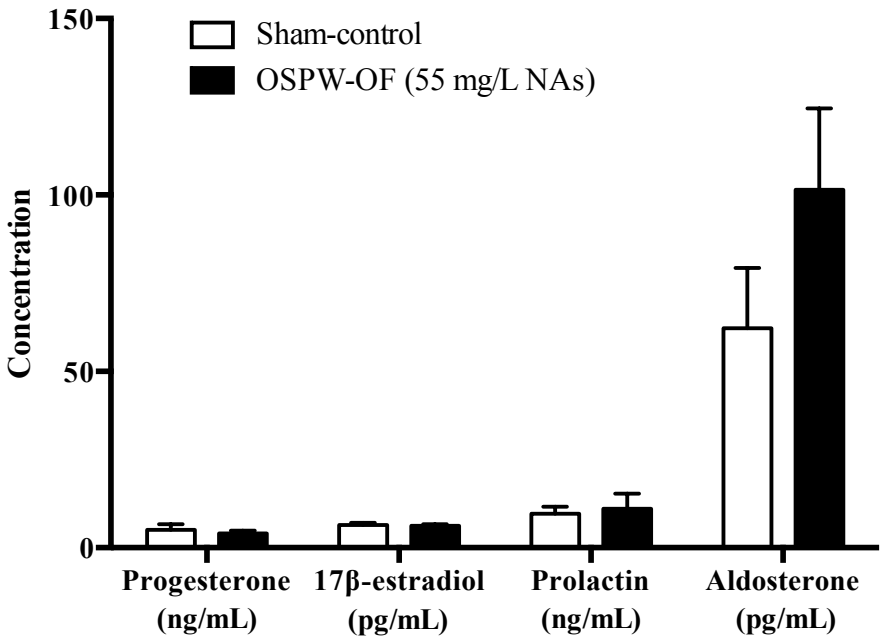

Figure 5.5 Hormone levels in the plasma of mice after sub-chronic exposure to OSPW-OF (55 mg/L NAs). Animals were gavaged weekly with OSPW-OF (55 mg/L NAs) and the sham-control for six weeks beginning on GD 0. On PND 21, pregnant mice were euthanized. The plasma concentrations of four hormones (progesterone, 17β-estradiol, prolactin and aldosterone) were determined using commercial ELISA kits. Data represent the mean ± SEM (n=7 for sham control and 5 for OSPW-OF). For each hormone, statistical analysis was performed using one-way ANOVA with Dunnett's *post hoc* test.

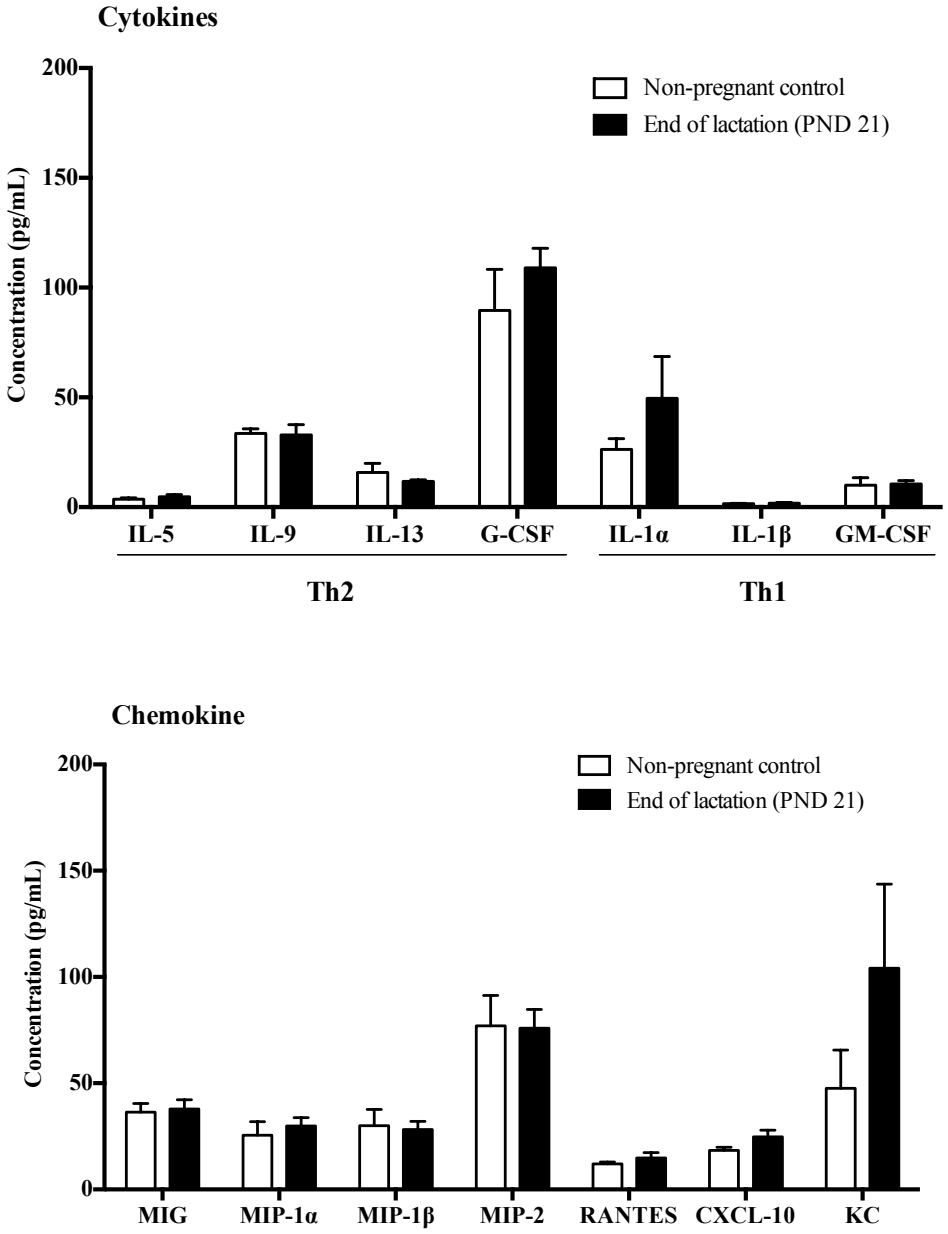

Figure 5.6 Cytokine/chemokine profile in the plasma of non-pregnant and pregnant mice on PND 21. The plasma levels of cytokines and chemokines in non-pregnant control and non-treated control on PND 21 were measured. Data represent the mean ± SEM (n = 3 for non-pregnant control and 9 for end of lactation). For each cytokine/chemokine, statistical analysis was performed using one-way ANOVA with Dunnett's *post hoc* test.

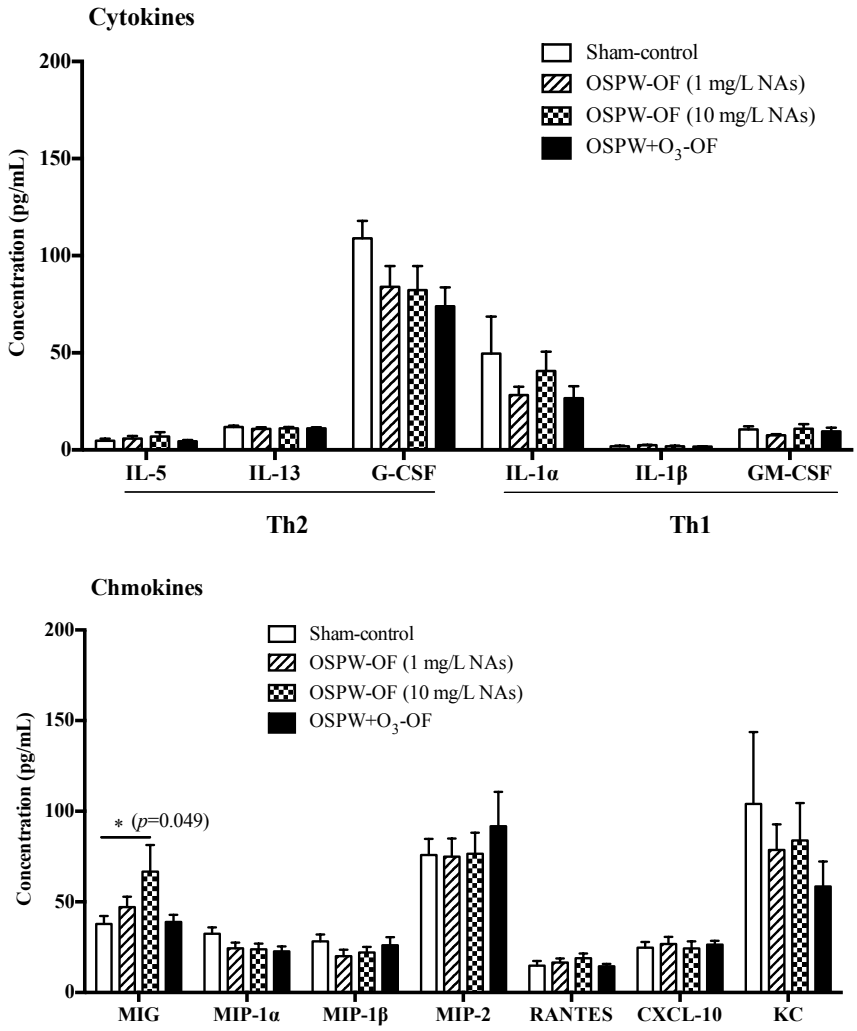

Figure 5.7 Cytokine/chemokine profile in the plasma of mice after sub-chronic exposure to OSPW-OF (1, 10 mg/L NAs) and OSPW+O_3-OF. Animals were gavaged weekly with OSPW-OF (1 mg/L NAs; n=10), OSPW-OF (10 mg/L NAs; n=9), OSPW+O_3-OF (n=10), and their sham-control (n=9), for six weeks beginning on GD 0. On PND 21, dams were euthanized. The plasma levels of cytokines and chemokines were measured. Data represent the mean ± SEM. For each cytokine/chemokine, statistical analysis was performed using one-way ANOVA with Dunnett's *post hoc* test. * denotes statistically significant differences at $p < 0.05$.

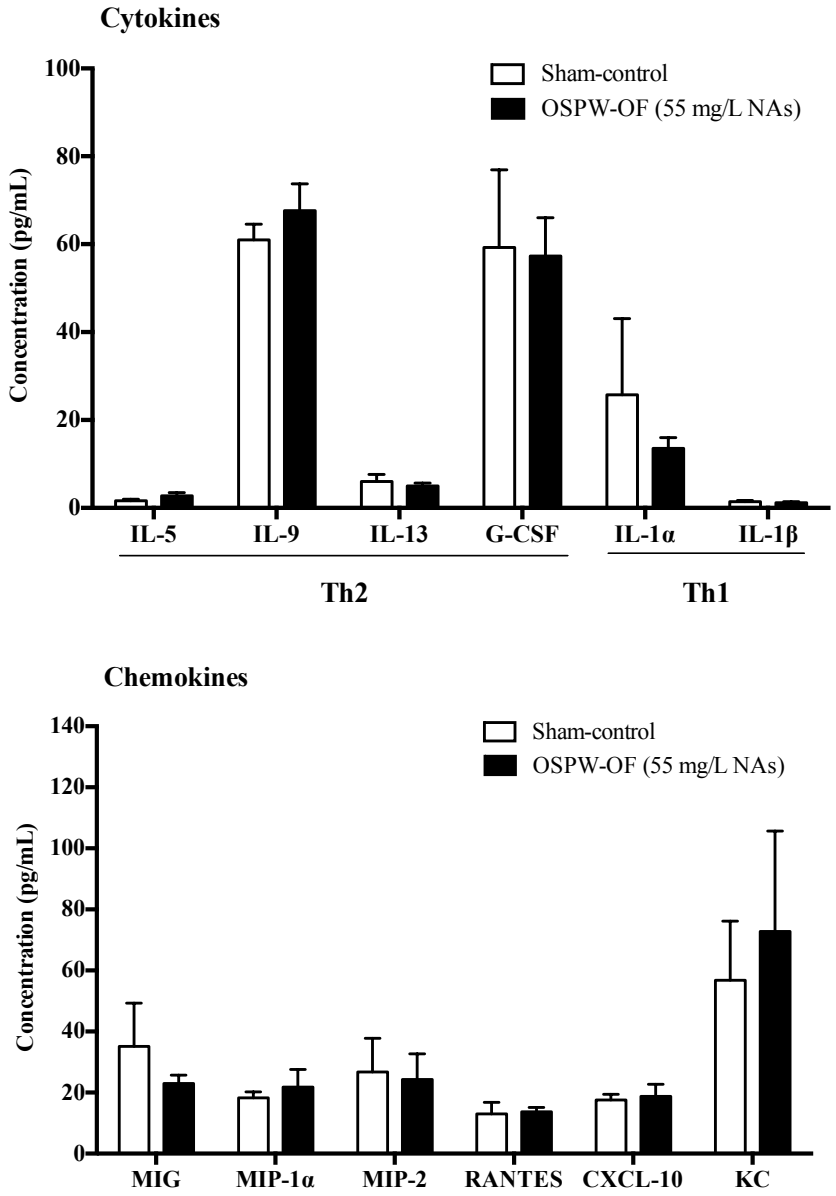

Figure 5.8 Cytokine/chemokine profile in the plasma of mice after sub-chronic exposure to OSPW-OF (55 mg/L NAs). Animals were gavaged weekly with OSPW-OF (55 mg/L NAs) and the sham-control for six weeks beginning on GD 0. On PND 21, dams were euthanized. The plasma levels of cytokines and chemokines were measured. Data represent the mean ± SEM (n=7 for sham control and 5 for OSPW-OF). For each cytokine/chemokine, statistical analysis was performed using one-way ANOVA with Dunnett's *post hoc* test.

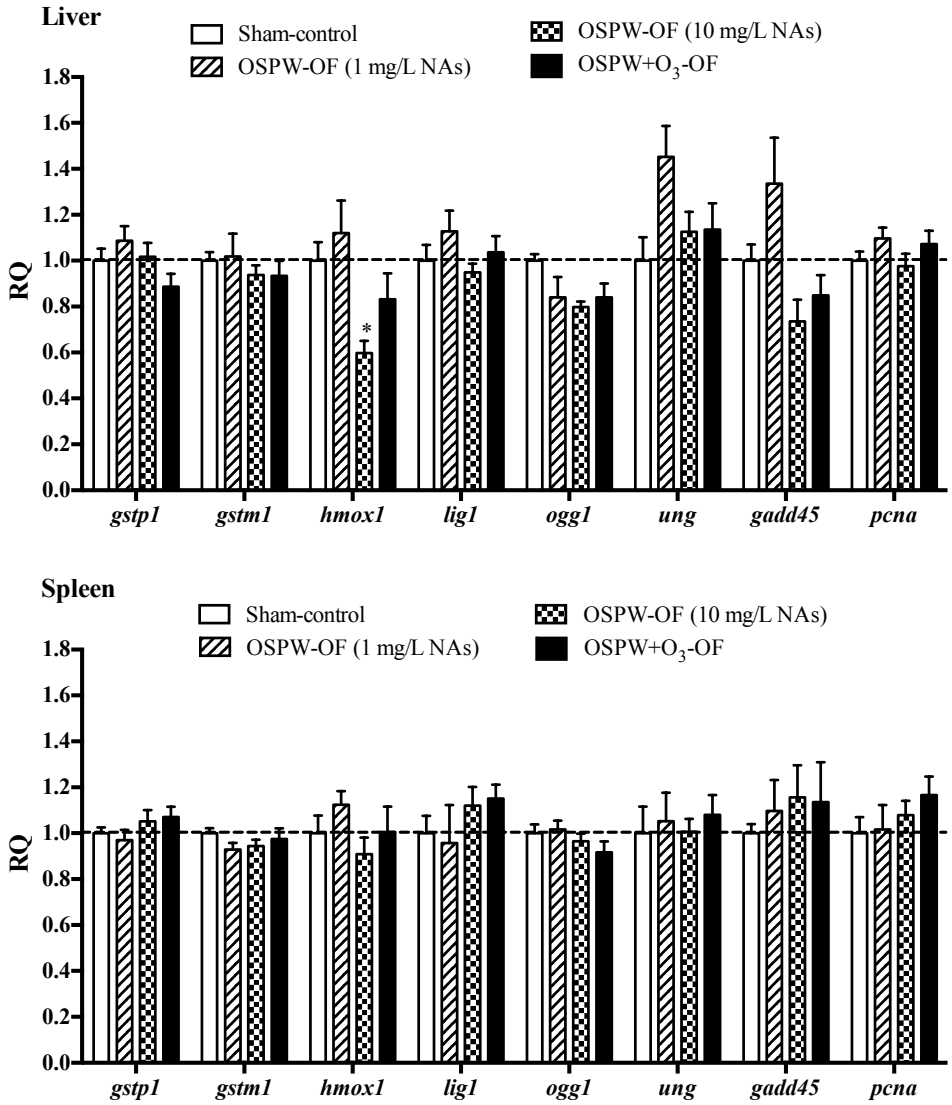

Figure 5.9 Gene expression in the liver and spleen of mice after sub-chronic exposure to OSPW-OF (1, 10 mg/L NAs) and OSPW+O_3-OF. Gene expression was assessed in the liver and spleen of mice after exposure to OSPW-OF (1 mg/L NAs; n=10), OSPW-OF (10 mg/L NAs; n=9), OSPW+O_3-OF (n=11), and their sham-control (n=9) for six weeks. Gene expression was analyzed by qPCR using the ddCT method. Data are expressed as relative quantification (RQ) values. RQ values for the treatment groups were normalized against the RQ values of sham-control. Data represent the mean ± SEM. For each gene, statistical analysis was performed using one-way ANOVA with Dunnett's *post hoc* test. * denotes statistically significant differences at $p < 0.05$.

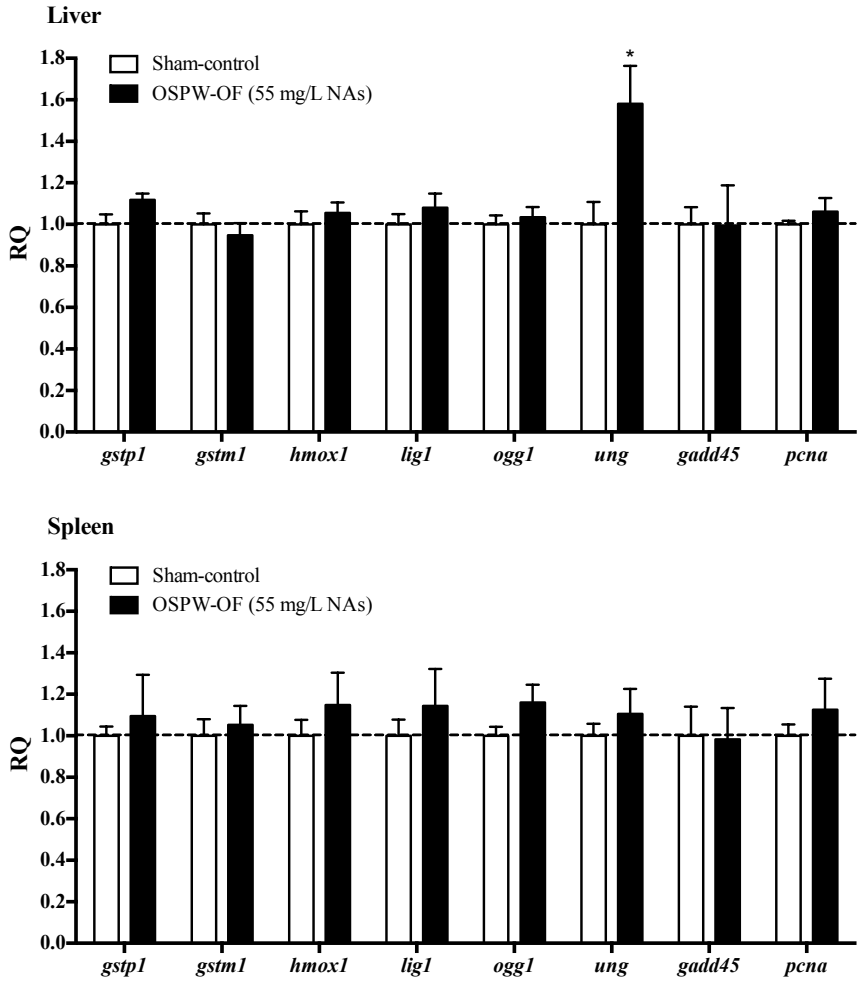

Figure 5.10 Gene expression in the liver and spleen of mice after sub-chronic exposure to OSPW-OF (55 mg/L). Gene expression was analyzed by qPCR using the ddCT method. Data are expressed as relative quantification (RQ) values. RQ values for the treatment group were normalized against the RQ values of sham-control. Data represent the mean ± SEM (n=7 for sham control and 5 for OSPW-OF). For each gene, statistical analysis was performed using one-way ANOVA with Dunnett's *post hoc* test. * denotes statistically significant differences at $p < 0.05$.

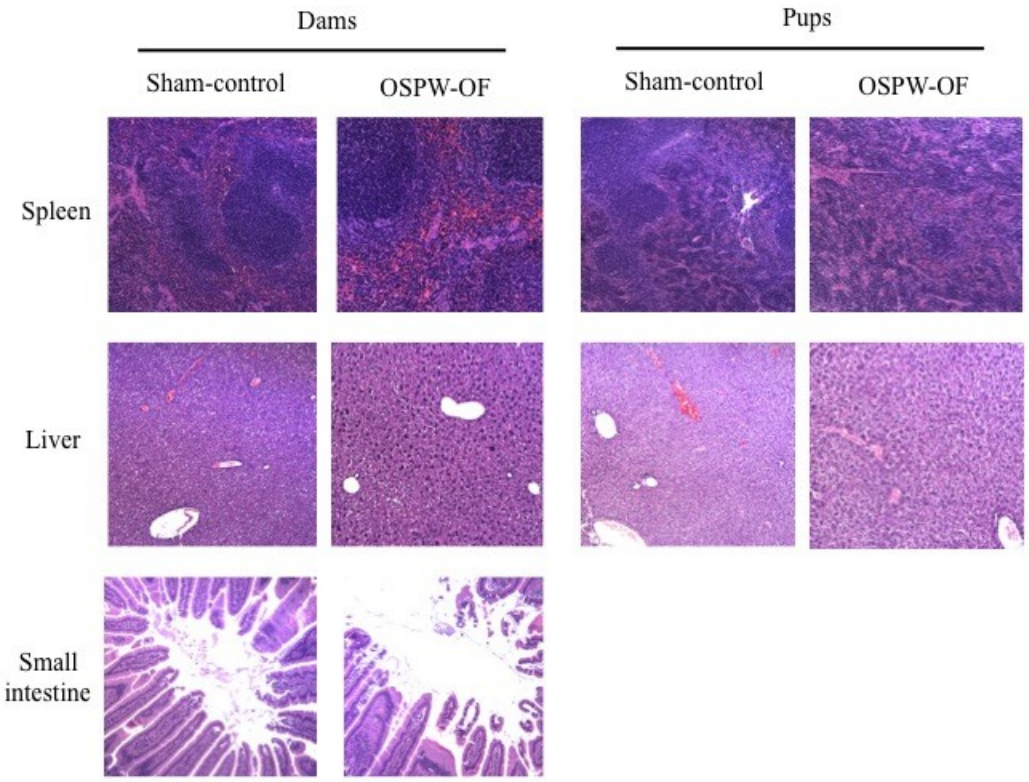

Figure 5.11 Representative histological images of tissues from dams and pups following sub-chronic exposure in pregnant mice.

Chapter 6: General Discussion

6.1 Introduction

The main objectives of the research conducted in this thesis were to (1) compare the toxicity of whole OSPW and OSPW-OF using the RAW 264.7 mouse macrophage cell line; (2) determine the acute and sub-chronic toxicity of OSPW-OF in mice, in particular the effects on reproduction and development; and (3) examine the potential and safety of ozone treatment as the OSPW remediation method. Mouse was chosen as a study species because they represents the classical mammalian model organisms with many similarities to humans in terms of anatomy, physiology, immune responsiveness and genetics.

6.2 Comparison of whole OSPW and OSPW-OF toxicity using RAW 264.7 cell line

There is increasing evidence that a variety of pharmaceuticals and personal care products (PPCPs), pesticides, and by-products of petroleum refineries persist in natural freshwater resources, which may come from the discharges and leaks of municipal, agricultural, and industry wastewaters (Archer et al., 2017; Avci et al., 2005; Bartrons and Peñuelas, 2017; Naidu et al., 2016; Petrie et al., 2015; Stuart et al., 2012). The exposure to these pollutants may lead to many physiological changes, disrupting homeostasis of animals. For example, some PPCPs (e.g., non-steroidal anti-inflammatory drugs like ibuprofen and naproxen) may modulate molecular and/or cellular pathways including those involved in endocrine system function, producing long-term adverse health effects in animals (Archer et al., 2017; Veldhoen et al., 2014). A broad range of pesticides (e.g., organochlorines, organophosphates and carbamates) have been shown to have carcinogenic, immunotoxic and endocrine-disruptive properties, resulting in growth

suppression, organ malformations, delayed hatching, and embryonic mortality (Galloway and Depledge, 2001; Köhler and Triebskorn, 2013; McKinlay et al., 2008; Pašková et al., 2011; Turusov et al., 2002).

Wastewaters from petroleum refineries are a major source of hydrocarbons, PAHs, phenols, metals and other toxic compounds that persist in the environment (De Jonge et al., 2013; Mrayyan and Battikhi, 2005; Wake, 2005). As a result of exposure to petroleum refinery wastewaters (including OSPW) or their polluted water bodies, a wide range of biological dysfunctions have been documented in various animal models both *in vitro* and *in vivo*, including cytotoxicity, mutagenicity, disruption in immune system, impaired reproduction, as well as developmental delays and deformities (Collier et al., 2013; Garcia-Garcia et al., 2012; Hagen et al., 2012; Iqbal et al., 2017; Leclair et al., 2015, 2013). It is often very difficult to identify the individual constituents that are responsible for the specific toxicity of wastewaters, as the contaminants typically occur as complex chemical mixtures in aquatic ecosystems. For example, the possible interactions between contaminants in wastewater may significantly alter the toxicity induced by complex mixtures that is usually different from observed effects of individual pollutants. For example, research has shown that a mixture of environmentally relevant concentrations of PPCPs (i.e., acetaminophen, carbamazepine, gemfibrozil, and venlafaxine) significantly altered embryo production, oocyte development and fecundity in fish (Galus et al., 2013a), while the individual compounds at the same concentrations did not cause the same toxic effects (Galus et al., 2013b). The additive, synergistic or antagonistic effects have also been reported for pollutants present in the wastewaters from petroleum refineries. For example, the results of gene expression of stress related

proteins and reactive oxygen species (ROS) showed that the combination of benzene, toluene and xylene was less toxic to *Drosophila melanogaster* than the effect of benzene alone, while the exposure to toluene and xylene resulted in increased toxicity (Singh et al., 2010). The additive toxic effects of petrochemicals (toluene, ethylbenzene and xylene) on midge larvae (Li et al., 2013) and antagonistic reactions between OSPW NAs extracts and salts in fish reproduction (Kavanagh et al., 2012b) have also been reported.

Interactions between organisms and chemicals are initiated at cellular level, hence, vertebrate cell cultures (*in vitro* models) can be used as valuable tools in predicting toxicity in whole-animals. Application of cell cultures in ecotoxicological assessment offers significant advantages such as ease of culture, availability and reproducibility, and makes them feasible to examine a large variety of environmental pollutants (Bols et al., 2001; Castaño and Gómez-Lechón, 2005; Fu et al., 2017; Leusch et al., 2010; Schirmer, 2006; Song et al., 2011; Wang et al., 2015b; Yang and Chan, 2015). Although existing information on adverse effects induced by OSPW is mainly based on animal experiments, the *in vitro* studies have added to the knowledge on hazardous pollutants present in OSPW. It has been shown that OSPW and its organic fraction (primarily containing NAs) induced cytotoxic, endocrine disruptive, immuntoxic, and oxidative stress responses in bacteria (*Vibrio fischeri*) (Gamal El-Din et al., 2011; Scott et al., 2008; Wang et al., 2013; Zubot et al., 2012), human cell lines (He et al., 2010; He et al., 2011), and primary cell cultures derived from fish and rodents (Gagné et al., 2013; Garcia-Garcia et al., 2012, 2011a,b; Hagen, 2013). However, none of these studies have investigated whole OSPW, either combined extracted fractions (e.g. organic & inorganic fractions) from the same OSPW source, on the same test system, which

makes it difficult to compare the observed toxic effects between different studies. For example, NAs were found to decrease mammalian macrophage ability to produce reactive oxygen and nitrogen intermediates (Garcia-Garcia et al., 2012; Garcia-Garcia et al., 2011b), while an increase in these antimicrobial molecules were reported for goldfish macrophages (Hagen, 2013).

In my thesis, I conducted parallel experiments for organic fraction and the complex mixture of whole OSPW using RAW 264.7 cells, to determine whether they differed in inducing toxic effects. Originally, RAW 264.7 cells were derived from mouse ascites and are neoplastic macrophage-like cells (leukemia) that express inflammatory mediators once activated (Makene and Pool, 2015). This cell line has been widely used for testing of immunomodulatory activities of natural products (Xu et al., 2014; Yang et al., 2014) and environmental pollutants (Jalava et al., 2008; Schneider et al., 2005), including those found in wastewater (Makene and Pool, 2015).

My findings (presented in Chapter 3 of the thesis) demonstrated that the exposure of RAW 264.7 cells to the environmentally relevant OSPW-OF concentrations did not significantly affect cell viability and proliferation compared to those of non-exposed cells. This is similar to our previous report that demonstrated lack of toxicity of OSPW-OF using cellular viability as a readout, in mouse bone marrow-derived macrophages (Garcia-Garcia et al., 2011b).

The reduced viability in OSPW treated cells was related to the impairment of cell membrane integrity, as demonstrated by increased leakage of intracellular enzyme (i.e., lactate dehydrogenase). Other evidence supporting the membrane disruption was the dramatic alteration in cell morphology after exposure of RAW cells to OSPW as reported

recently by our laboratory (Fu et al., 2017). The appearance of a frayed plasma membrane was observed with multiple intrusive-like structures that are indicative of membrane degeneration and/or disruption of membrane architecture (Fu et al., 2017). These results suggest that cells were stressed, which was further confirmed by an elevation in the expression of stress responsive and detoxification genes involved in antioxidant responses (*hmox1*), and DNA repair (*gadd45*). My observations are supported by reports of greater concentrations of reactive oxygen species (ROS) in fish embryos (He et al., 2012a), and higher expression of mRNA levels of genes that encode proteins responsible for biotransformation of xenobiotics, oxidative stress, and DNA repair in fish hepatocytes (Gagné et al., 2013) after exposure to OSPW. Thus, the possible mode of action for OSPW toxicity may include oxidative stress and associated DNA damage. Aerobic organisms have integrated antioxidant systems to mitigate the harmful effects of ROS (Birben et al., 2012); however, the excessive accumulation of ROS may result in oxidative stress, leading to altered expression of genes encoding antioxidant enzymes such as *hmox1* whose induction was associated with cellular protection against injury caused by ROS (Chan et al., 2014; Fu et al., 2017). Oxidative stress may cause DNA damage that, if not repaired, can result in cell mutations (Sampath et al., 2012). To combat the threats posed by DNA damage, cells use an integrated DNA damage and repair response, via complicated signal-transduction cascades (Jackson and Bartek, 2009; Lord and Ashworth, 2012). Among the various stress responsive genes, the growth arrest and DNA damage gene (*gadd45*) encodes an important protein that plays a role in controlling the cell cycle G2-M checkpoint (preventing cells from initiating mitosis when DNA is damaged), providing a chance for repair and arresting the proliferation of

damaged cells (Schäfer, 2013; Stark and Taylor, 2004; Zhan, 2005). The induction of *gadd45* in DNA-damaged cells is a critical signal that limits cell growth and division, resulting in enhanced chemosensitivity toward toxicants and apoptosis (Gagné et al., 2013; Zhang et al., 2012).

Macrophages are important immune cells distributed in tissues throughout the body and contribute to both homeostasis and disease (Epelman et al., 2014). Stimulation of RAW 264.7 macrophage cell line with pathogens such as bacteria and endotoxins produces cytokines, growth factors, and nitric oxide (Fu et al., 2017; Lei et al., 2015; Park et al., 2013). Cytokines and growth factors are important bioactive proteins involved in responses to exogenous and endogenous insults, repairs, and restoration of homeostasis (Ware, 2005). NO serves multiple purposes in the immune processes including: pathogenesis and control of infectious diseases, tumours, autoimmune processes and chronic degenerative diseases (Bogdan, 2001).

I examined the effects of OSPW and OSPW-OF exposure on the production and secretion of cytokines by macrophage-like RAW 264.7 cells in responses to bacterial challenge. I observed that several cytokines which generally function as stimulators of the immune system were elevated following OSPW exposure only, at nonlethal concentrations. The elevated cytokine gene expressions were: (1) IL-1 (IL-1α and IL-1β), which are central mediators of innate immunity and inflammation that regulate cellular proliferation, differentiation, and apoptosis (Garlanda et al., 2013); (2) GM-CSF, an important growth factor that induces activation of monocytes/macrophages and also mediates differentiation of dendritic cells that participate in antigen presentation, a central function of host defense (Ushach and Zlotnik, 2016); and (3) VEGF, a growth factor

essential for angiogenesis during development and in the pathogenesis of cancers and other diseases (Evans et al., 2017). My results also suggest that compounds in whole OSPW, may up-regulate inflammatory responses that may lead to inflammation-related disturbances. The excessive production of the pro-inflammatory mediators may lead to serious systemic complications such as microcirculatory dysfunction, tissue damage, and septic shock, causing high mortality (Lee et al., 2012; Ulevitch and Tobias, 1995). Interestingly, OSPW also caused immunosuppression exemplified by down-regulation of antimicrobial functions of macrophages (i.e. reduced nitric oxide response observed in this study), and an inhibition of phagocytosis (Fu et al., 2017) in RAW 264.7 cells. These observations reflect impairment in antimicrobial activities of macrophages, which could translate to a higher prevalence of bacterial infections in exposed hosts. Given that the whole OSPW is highly complex mixture, the unidentified constituents, in addition to NAs, may act as both immunostimulatory and/or immunosuppressive agents. For example, PAHs that are present in whole OSPW have been shown to up-regulate pro-inflammatory cytokine production (e.g., IL-1β, TNF-α) in human primary macrophages (Lecureur et al., 2005; Sparfel et al., 2010), but also exhibit immunosuppressive effects on immune cells (Grevenynghe et al., 2004, 2003). Other constituents known to be present in whole OSPW such as heavy metals, may also induce immunotoxcity (e.g., cadmium), which has been shown to induce oxidative stress and decreased inflammatory responses in RAW 264.7 cells (Jin et al., 2016).

The exposure of RAW 264.7 cells to OSPW-OF did not induce significant changes in cytokine secretion and nitric oxide production at all environmentally relevant doses tested (<18 mg/L). In previous studies, we reported that exposure of mouse bone

marrow-derived macrophages to OSPW-OF containing higher NAs concentrations (>25 to 50 mg/L) down-regulated their antimicrobial functions (Garcia-Garcia et al., 2011a,b), suggesting a dose-dependent OSPW-OF immunotoxic effect.

Overall, these results agree with the previous data in our lab indicating that immunomodulatory factors are present in OSPW and that they modulate cytokine levels and antimicrobial functions of mammalian macrophages and a macrophage-like cell line *in vitro* (Garcia-Garcia et al., 2012, 2011a,b). At the doses tested in this study, whole OSPW but not OSPW-OF components induced cell cytotoxicity, cell stress, and immunotoxicity, suggesting that inorganic compounds and unidentified organics may be responsible for the observed effects, and/or that the synergistic interactions between organic and inorganic fraction constituents may alter cell functional behavior. It should be noted that *in vitro* toxic effects may not necessarily reflect how a whole organism will react to OSPW and OSPW-OF. Therefore, I conducted a series of *in vivo* experiments using a mouse model, with an aim to determine whether OSPW-OF exposure would induce reproductive toxicity, immunotoxic effects and stress in pregnant female mice and their offspring.

6.3 Comparison of acute and sub-chronic toxicity of OSPW-OF in mice

The ability of OSPW to alter reproductive function has been reported in fish. The sexual maturity and spawning of male fathead minnows inhabiting an OSPW pond that has moderate concentrations of NAs (~10 mg/L) were delayed, which was probably due to the presence of some constituent(s) in OSPW that disrupted the fish endocrine system (Kavanagh et al., 2013). Evidence supporting this is the similar reproductive effects (i.e.,

reduced steroid concentrations, male secondary sexual characteristics, and fecundity) in fathead minnows after exposure to both OSPW and OSPW acid-extractable organic acids (Kavanagh et al., 2012b, 2011). The *in vitro* toxicity results demonstrating the presence of some organic compounds in OSPW that can act as estrogen receptor (ER) agonists and androgen receptor (AR) antagonists (He et al., 2011), and the identification of steroidal aromatic NAs in OSPW with structural similarities to estrogens (Rowland et al., 2011b) suggest that some NAs species may contribute to the estrogenic activity reported in OSPW and its organic fraction. The binding and activation of hormone receptors have also been achieved with effluent from oil production platforms (Gamal El-Din and Smith, 2002; Thomas et al., 2009; Tollefsen et al., 2007, 2006; Vrabie et al., 2010), and petrogenic NAs were found to be important contributors to the estrogenic and anti-androgenic properties of North Sea offshore process-affected water (Thomas et al., 2009). Furthermore, a recent study showed increased mRNA levels of genes along the brain-gonad-liver (BGL) axis that are involved in regulation of synthesis of sex hormones and their receptors, the sex maturation, and reproduction in fathead minnows exposed to OSPW (He et al., 2012b). These results suggest that OSPW and NAs extracts may disrupt the hormonal production and that this may occur through the interference of the estrogen and/or androgen receptor, and possibly other unidentified pathways, causing impaired reproductive physiology of exposed organisms (Kavanagh et al., 2012b, 2011).

There are very few studies on the toxic effects of OSPW and/or its constituents exposure in mammals. An early study demonstrated the reproductive failure in female rats after exposure to OSPW NAs extracts (Rogers, 2003). The mechanisms of this reproductive toxicity involved an indirect effect on sex hormone production that was

secondary to a direct effect that caused hypocholesterolemia (Rogers, 2003). Adequate and accurate production of sex hormones like progesterone and estradiol are required for normal mating behavior, implantation, and maintenance of pregnancy.

In my thesis research I examined the effects of OSPW-OF exposure during the gestation (Chapter 4) and continuous exposure throughout gestation and lactation (Chapter 5). The results showed that at doses up to 100 mg/kg body weight/week NAs (equivalent to 55 mg/L NAs) did not affect the mating behavior, pregnancy success, embryonic implantation, gestation length, and litter size. The results on pregnancy-associated hormones supported this by showing normal circulating concentrations of progesterone and estradiol in OSPW-OF treated mice during mid-pregnancy. The only hormone level that was altered following the OSPW-OF exposure was aldosterone which is the main mineralocorticoid hormone that regulates Na^+ and K^+ homeostasis (Lee et al., 2005). After a six-week exposure to OSPW-OF at 10 mg/L NAs, the mice had significantly lower levels of aldosterone, however, when mice were exposed to a dose of 55mg/L NAs, the aldosterone levels were similar between exposed and non-exposed mice, suggesting that the decrease in aldosterone levels was not a consistent biological effect.

Immune system involves proper production and secretion of pro-inflammatory and anti-inflammatory cytokines in order to maintain homeostasis. The previous studies have reported that OSPW or OSPW-OF affected the immune system of vertebrates by altering the expression of genes encodings cytokines and thereby decreasing the ability of hosts to control infectious diseases (Garcia-Garcia et al., 2012, 2011a, 2011b; Hagen, 2013). Pregnancy creates a unique immune response profile that is normally associated

with a bias from T helper 1 (Th1) cytokine profile (pro-inflammatory cytokines) towards Th2 (anti-inflammatory cytokines) profile (Sykes et al., 2012a). The shift in the panel of cytokines produced during pregnancy decreases the cell-mediated immune response and enhances the humoral (antibody-based) immunity (Mor et al., 2011). This modulation in immune responsiveness during gestation is carefully controlled, leading to different responses depending on the infections and on the stages of the pregnancy (Cardenas et al., 2010; Mor et al., 2011; Mor and Cardenas, 2010). In addition, maternal immunity is also regulated by sex hormones, because progesterone and estradiol have been shown to promote the T helper 2 cell cytokine production profiles and are likely partially responsible for the Th2 bias associated with pregnancy (Huber et al., 1999; Piccinni et al., 1995; Sykes et al., 2012b).

My research results indicate that the Th1/Th2 bias was observed in mid-gestation, as evidenced by a significant increase of Th2 cytokine G-CSF (a growth factor with a marked anti-abortion activity) in non-treated pregnant mice on GD 14 (Chapter 4). By the end of lactation, circulating concentrations of G-CSF returned to basal levels similar to that of non-pregnant mice (Chapter 5). The parallel changes were also observed for concentrations of progesterone and estradiol in the plasma that were elevated in mid-pregnancy (Chapter 4), and returned to baseline levels measured of non-pregnant animals three weeks after parturition (Chapter 5). The Th1/Th2 cytokine modulation during gestation has been shown to exert deleterious effects on pregnancy, including early fetal loss, preeclampsia and preterm labor (Sykes et al., 2012b). Interestingly, the acute exposure to OSPW-OF (1-55 mg/L NAs) did not alter the cytokine profiles in pregnant mice, suggesting that OSPW-OF did not affect the normal reproductive physiology. After

3-week post-parturition adaptation period, the maternal immunity returned to normal, and was similar to that in non-pregnant control mice.

Considering the importance of cytokines in health and disease, especially in host defense during infectious diseases, I examined cytokine plasma concentrations in dams at the end of lactation (at the end of sub-chronic exposure to OSPW-OF). The results indicated that the protein profiles of different cytokines were not affected by exposure to OSPW-OF. Previous research demonstrated that OSPW and OSPW-OF induced immunotoxicity in fish, birds and mammals. For example, chronic exposure to OSPW, at concentrations of NAs within the range used in the present study, caused a higher occurrence of virally induced tumors in fish (van den Heuvel et al., 2000) and greater prevalence of infestation with bird blow flies in tree swallows (Gentes et al., 2007c). In mammals, OSPW-OF exposure at dose equivalent to 55 mg/L NAs resulted in the down-regulation in expression of genes encoding pro-inflammatory cytokines (*in vivo* exposure), which might be associated with impaired ability of an exposed host to defend against infectious disease (Garcia-Garcia et al., 2011b). Similar cytokine protein profiles between treated and non-exposed mice after sub-chronic exposure to OSPW-OF may be due to the lower doses of OSPW-OF used in my experiments or the source of the OSPW-OF that was different from that used in other studies that showed immunotoxic effects caused by OSPW-OF exposure.

Chemokines are also important molecules involved in the regulation of immune system. The majority of chemokines measured in OSPW-OF exposed mice at mid-gestation were at levels comparable to those of non-pregnant animals, with a notable exception of CXCL10/IP-10. This chemokine has both pro-inflammatory and anti-

angiogenic properties. Up-regulation of CXCL10/IP-10 has been associated with preeclampsia in humans (Gotsch et al., 2007; Szarka et al., 2010) that could result in higher risk for spontaneous preterm delivery (Gervasi et al., 2012). However, it appeared that increased levels of CXCL10/IP-10 levels observed after acute exposure to OSPW-OF did not affect the reproduction of mice, since the pregnancy success and gestation length were similar in exposed and non-exposed pregnant mice after exposure of mice to OSPW-OF for 6 weeks.

In female mammals, lactation period is energy-demanding that might have negative impacts on their survival and other physiological functions (Garratt et al., 2010). The limited resources available to animals are delicately balanced and require a trade-off between survival (somatic protection) and reproduction. When animals are under stress conditions, their energy is directed towards processes that will restore homeostasis, and away from reproduction (Fuzzen et al., 2011). Oxidative stress has been suggested as a cost of somatic maintenance that may limit investment of energy resources on reproduction (Xu et al., 2014; Zheng et al., 2015). Oxidative stress occurs when the rate of ROS production exceeds the capacity of the antioxidant defence and repair mechanisms (Metcalfe and Alonso-Alvarez, 2010; Monaghan et al., 2009; Xu et al., 2014), that have been associated with a variety of disease conditions, including reproductive complications (spontaneous abortion, recurrent pregnancy loss and preeclampsia) (Agarwal et al., 2012; Webster et al., 2008) and embryonic development (Dennery, 2007). There have been several studies linking OSPW exposure to oxidative stress (Gagné et al., 2013, 2012; He et al., 2012a), which led to the increased apoptosis of cells and impairment of development of fish embryos (He et al., 2012a). Organisms have

a variety of defense mechanisms that can combat the threat of oxidative stress and associated damage (Paul et al., 2009). Glutathione S-transferase (GST) is an antioxidant family that facilitates detoxification of xenobiotics, together with superoxide dismutase (SOD) and catalase (CAT), play important functions in clearance of ROS (He et al., 2012a; Paul et al., 2009). Greater abundances of transcripts of the genes that encode enzymes of the antioxidant pathway suggested that there was greater production of ROS in animals exposed to OSPW. Gagné and colleagues (Gagné et al., 2012) used hepatocytes from rainbow trout and reported up-regulation of *gst* and *sod* genes after exposure to OSPW. Similar changes in gene expression were reported following exposure of fathead minnow embryos to OSPW (He et al., 2012a). These observations were inconsistent with the results in the present study where acute exposure to OSPW-OF down-regulated the mRNA levels of *gst*. The mechanism for the suppression in *gst* gene expression is unknown, however, the inhibition of GST enzyme activity in trout hepatocytes after exposure to the OSPW extracts (Byington and Hansbrough, 1979; Gagné et al., 2011) suggests that some organometallic compound(s) that are present in OSPW may participate in GST enzyme inhibition. The inhibition of *gst* gene expression and enzyme activity may create an imbalance between the ROS production and the quantities of antioxidants required to restore homeostasis. In this study, after sub-chronic exposure of pregnant mice to OSPW-OF, the expression of the *gst* gene was reversed, since I observed a slight elevation in *gstp1* transcripts, suggesting that a shift towards enhanced detoxification after prolonged period of stress.

ROS could cause oxidative damage to molecules such as lipids, proteins and DNA (Speakman and Garratt, 2014). Animals have protection mechanisms to mitigate

the DNA damage by the induction of various enzymes involved in DNA repair activity (Jackson and Bartek, 2009). In a previous study, rainbow trout hepatocytes exposed to OSPW exhibited genotoxicity as demonstrated by the PAH-DNA adducts, DNA strand breaks, and increased expression of genes encoding enzymes that function to repair DNA damage: *ung* and *ogg* (Gagné et al., 2013, 2012, 2011). In my study, an elevation in *ung* gene expression was also observed in the liver of productive mice after sub-chronic exposure to OSPW-OF at 55 mg/L NAs, which indicated a possible increase in OSPW-OF induced mutagenesis, as the protein UNG functions to eliminate uracil (a base normally present in RNA) from DNA. Interestingly, the acute exposure to OSPW-OF exposure induced a reduction in the expression of *ogg* which encodes DNA glycosylase that initiates the base-excision repair pathway for repair of non-bulky oxidative DNA lesions (Sampath et al., 2012). However, this effect was transitory and not observed after sub-chronic exposure to relatively high dose of OSPW-OF (55 mg/L NAs). These results suggest the time- and dose-dependent effects of OSPW-OF on DNA repair gene expression. It should be noted that despite the changes in liver gene expression that may be indicative of hepatotoxicity, I did observe significant alterations in liver histopathology following exposure to OSPW-OF at all doses tested.

6.4 Comparison of OSPW and OSPW-OF toxicity to their ozonated products

Ozonation is one of the most widely used advanced oxidation processes in full-scale water and wastewater treatment applications, which has been shown to effectively degrade many organic contaminants including various EDCs, pharmaceuticals and personal care by-products, and petrochemicals (Bertanza et al., 2010; Nakada et al., 2007;

Oller et al., 2011; Umar et al., 2013; Gamal El-Din et al., 2011a; Garcia-Garcia et al., 2011a). The reaction of ozone with organic matter proceeds either via direct interaction with molecular ozone or indirect interaction with hydroxyl radicals ($HO^{\cdot}$), resulting in the breakdown of high molecular weight compounds (Wang et al., 2016). In the area of OSPW treatment, ozonation has been shown to greatly reduce the concentrations of organic compounds particularly NAs (Martin et al., 2010), resulting in the effluent being less toxic to *Vibrio fischeri* as measured by the Microtox assay (Gamal El-Din et al., 2011a; Scott et al., 2008). Attenuation of some of the endocrine-disrupting effects on eukaryotic cells *in vitro* (He et al., 2010) and in fish (He et al., 2012b), as well as reducing the adverse impacts on immune responses of mice (Garcia-Garcia et al., 2011a) has been documented following ozonation of OSPW. However, due to the complex nature of the wastewaters, as is the case of OSPW, organic substances are not completely degraded. The formation of organic metabolites during the ozonation process may result in an increased toxicity as documented for some PAHs (e.g., pyrene, fluoranthene, and benzo[a]pyrene) (Petala et al., 2008; Upham et al., 1994), and some NAs that were broken down to hydroxylated NAs resembling steroids with endocrine disrupting potential (He et al., 2011).

The failure of detoxification by ozonation was observed in the present *in vitro* study (Chapter 3) where the results showed that, despite significant reduction of the NAs after ozonation, no amelioration of the OSPW toxicity was observed for all endpoints examined including cellular viability and proliferation, cytotoxicity and immune functions. Furthermore, RAW 264.7 cells exposed to OSPW+O_3-OF, but not those exposed to OSPW-OF, were significantly less viable as indicated by the results of the

MTT reduction assay. These findings are surprising since mechanism of toxic action of NAs is widely accepted to be via narcosis (Frank et al., 2009b), where the OSPW containing NAs with higher carbon number were expected to induce greater cell membrane disruption than the ozone-treated water with smaller molecular NAs.

The results of *in vivo* exposures to ozonated OSPW-OF during pregnancy (Chapter 4 and Chapter 5) indicated that ozonation did not influence the reproduction and immune responses of the animals, although it appears that it decreased the oxidative stress induced by OSPW-OF, as indicated by lower *hmox1* gene expression. These results suggest that ozonation may reduce some toxic properties of organic fraction in OSPW. In addition to the organic fraction, OSPW contains high concentrations of ions such as Na^+, Cl^- , SO_4^{2-}, and HCO_3^- (Allen, 2008). It is possible that these ions may contribute to the toxicity of OSPW by acting alone or in combination with other constituents. For example, it has been reported that gill proliferative changes (epithelial, mucous, and chloride cells) in yellow perch exposed to OSPW NAs extracts increased with the addition of Na_2SO_4. The significance of these changes was a reduced gill surface area, which likely not only decreased the rate of NAs absorption, but also reduced the rate of gas exchange, that decreased the respiratory stress and hypoxia that are known to impair the fish reproductive capacity (Thomas et al., 2007; Thomas and Rahman, 2009).

6.5 Conclusions and Future Directions

The present research investigated the toxic effects of whole OSPW, OSPW-OF and ozonated whole OSPW or OSPW-OF, both *in vitro* and *in vivo* using a number of different end points. The findings of the *in vitro* toxicity studies suggested that at the

doses tested, whole OSPW but not OSPW-OF components reduced cell viability and proliferation in a dose-dependent manner. OSPW exposure also disrupted the cellular antimicrobial responses by inhibiting the NO production and altering the subsequent release of pro-inflammatory cytokines. Gene expression results showed that OSPW treatment resulted in oxidative stress, demonstrated by elevated mRNA levels of *hmox1* whose induction is involved in cellular protection against ROS induced injury. These observations suggest that the inorganic compounds present in the OSPW may contribute to the toxicity of whole OSPW at the concentrations tested. In this research, ozonation failed to ameliorate the adverse effects caused by whole OSPW, which was not surprising since ozone treatment mainly degraded organic compounds that appear not to contribute significantly to the overall toxic effects at concentrations tested. The feasibility of ozonation as a remediation strategy for OSPW, from an engineering point of view, is beyond the scope of this thesis, but the results from this study suggest that further research is needed to assess the efficacy of ozonation for reduction of OSPW induced toxicity, particularly for OSPW containing higher concentrations of NAs, with special attention to the by-products that may be generated during the ozonation process and their possible toxic effects.

The results of the *in vivo* toxicity studies suggest that the risk of acute and sub-chronic toxicity to small wild mammals (based on the mouse as the surrogate mammal) exposed to OSPW-OF in contaminated drinking water is low. No significant signs of distress (including death, behavioral changes, loss of body weight, and pregnancy failure) were associated with the oral administration of OSPW-OF to mice, either acutely (2-week) or sub-chronically (6-week) at the doses up to 55 mg/L NAs. In this study, only

minor changes in plasma hormone levels and cytokine protein secretion were observed, suggesting that exposure to OSPW-OF at indicated doses did not induce endocrine disruption and immunotoxic effects. On the other hand, exposure of OSPW-OF at high dose altered the expression of genes involved in antioxidant pathways (*gst*) and DNA repair processes (*ogg1*, *ugg*), in a dose- and time-dependent manner. The changes were only observed in liver tissue, suggesting that liver was a target organ. However, no histopathological changes in liver and other tissues were observed. In this study, ozonation did not induce toxicity in the reproduction and immune responses of the animals, and it ameliorated the oxidative stress induced by OSPW-OF exposure, suggesting that ozonation may reduce some toxicity of organic fraction of OSPW.

It should be noted that the establishment and maintenance of healthy populations of animals not only relies on the ability of adults to reproduce, but also on normal embryonic development. Exposure to certain chemicals (e.g., EDCs) during development may result in different effects from exposure during adulthood. In my study, the intra-uterine fetal growth and development during gestation (Chapter 4) and the general health of pups during lactation (Chapter 5) were not impacted by exposure to OSPW-OF. In future research, it is suggested that a comprehensive investigation be undertaken to assess whether exposure to OSPW and its organic/inorganic fraction(s) during fetal development or early in childhood have long-lasting adverse health consequences in adulthood and subsequent generations of mammals.

To date, a significant body of literatures have linked the adverse effects of OSPW to its organic fraction mainly composed of NAs, though OSPW-OF at environmentally relevant concentrations of NAs may not be necessarily sufficient to cause effects, as

suggested by the results in the present study. NAs occur together with other compounds such as salts (dominated by sodium, bicarbonate, sulfate and chloride) and trace metals. There is evidence for toxicological effects of salts derived from the bitumen extraction process (Leung et al., 2003; Sansom et al., 2013; Kennedy 2012) and metals (Anderson et al., 2012a). However, these studies focused on the modulatory effects of several major ions (e.g., sodium and bicarbonate) and certain metals present in OSPW. Given the complex nature of OSPW, future investigation of the toxicity of inorganic fraction is suggested, using parallel assessments of toxic effects induced by whole OSPW, OSPW-OF and OSPW-IF, in order to assess the potential additive and/or synergistic effects between different OSPW fractions. In addition, given that OSPW chemistry varies considerably between tailing ponds, it is imperative that a comprehensive side-by-side comparative datasets of the toxic effects induced by different OSPWs are generated. Furthermore, careful cross-species interpretation and extrapolation of the toxicological effects are also required, to enable appropriate risk assessment of OSPW exposure on living organisms including humans.

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Appendices

Appendix A. Studies demonstrating *in vitro* toxicity of OSPW

Sample designation	OSPW type	Test organism	Duration of Exposure	Endpoint & Result	Reference
MLSB	Fresh OSPW	*Vibrio fischeri*	15 min	IC_{50}: 32%	(Holowenko et al., 2002)
Recycle water pond (Syncrude)	Fresh OSPW	*Vibrio fischeri*	15 min	IC_{50}: 100%; IC_{20}: 23%	(Scott et al., 2008)
WIP	Fresh OSPW	*Vibrio fischeri*	5 min	IC_{50}: 24%	(Gamal El-Din et al., 2011)
WIP	Fresh OSPW	*Vibrio fischeri*	15 min	IC_{20}: 9%; IC_{50}: 67%	(Zubot et al., 2012)
WIP	Fresh OSPW	*Vibrio fischeri*	15 min	IC_{20}: 32.6%	(Wang et al., 2013)
WIP	Fresh OSPW	H295R cell line	1hour (Serial dilution)	E2 metabolism (-1.2-fold for 1% OSPW; -1.4-fold for 90% OSPW; -2.3-fold for 100% OSPW)	(He et al., 2010)
WIP	Fresh OSPW	H295R cell line	2, 4, 8 hours (100% OSPW)	cyp19a mRNA expression (+1.8-fold, 2.0-fold, and 3-fold after 2h, 4h, and 8h, respectively)	(He et al., 2010)
WIP	Fresh OSPW	H295R cell line	24 hours (Serial dilution)	Aromatase activity (+1.9-fold for 90% OSPW; +2.5-fold for 100% OSPW)	(He et al., 2010)
WIP	Fresh OSPW	H295R cell line	48 hours (100% OSPW)	T production (-0.45-fold); E2 production (+2-fold)	(He et al., 2010)
WIP	Fresh OSPW	T47D-kbluc cell line	24h (Serial dilution)	Estrogenic response (proportional to concentrations; +2.58 for 100% OSPW)	(He et al., 2011)
WIP	Fresh OSPW	MDA-kb2 cell line	24h (100% OSPW)	Antiandrogenic response coexposed to low T levels; Androgenic response coexposed to moderate T levels	(He et al., 2011)
Experimental ponds (Syncrude)	7-year and 11-year aged OSPW	*Vibrio fischeri*	15 min	IC_{50}: 100% for both ponds	(Holowenko et al., 2002)
OSPW-impacted wetlands (Suncor)	Wetlands composed of CT discharge and/or seepage collection water	*Vibrio fischeri*	15 min	IC_{50}: 100%, 100%, 100%, 100%, 98%, and 64% for increasing NAs concentrations in wetlands; IC_{20}: 100%, 46%, 33%, 52%, 14%, and 11% for increasing NAs concentrations in wetlands	(Holowenko et al., 2002)
OSPW-NAEs (WIP)	NAEs isolated from fresh OSPW	*Vibrio fischeri*	15 min	IC_{50}: 41.9, 58.1, 43.5, 54.7, 64.9, and 52.7 mg/L for five fractionated NAs with increasing MW, and the original NAs mixture	(Frank et al., 2008)

OSPW-NAEs	NAEs isolated from fresh OSPW (source not specified)	Mouse embryonic stem cell	5 days	Up-regulated gene expression of early cardiac markers (*nkx2.5*: 0.0025-2.5 mg/L NAEs; *gata4* and *mef2c*: 0.025-2.5 mg/L NAEs; *nrg1α* and *nrg1β*: 0.25-2.5 mg/L NAEs)	(Mohseni et al., 2015)
OSPW-OF (WIP)	Organic fraction isolated from fresh OSPW; Organic fraction consists of neutral fraction and NAEs	Mouse BMDM	18 hours (Serial dilution)	Viability (NSD); Proliferation (+ for 6.25 and 12.5 mg/L NAs; -40%, for 50 mg/L NAs); RNI (less for 25 and 50 mg/L NAs); iNOS gene expression (-20% for 50 mg/L NAs); ROI (less for 25 and 50 mg/L NAs); NADPH subunit - p91Phox gene expression (-20% for 50 mg/L NAs); Phagocytosis (inhibited for 50 mg/L NAs); Pro-inflammatory cytokines gene expression (less IL-1β for both resting and activated cells at 50 mg/L NAs); Anti-inflammatory cytokines gene expression (less IL-10 for activated cells).	(Garcia-Garcia et al., 2011b)
OSPW-OF (WIP)	Organic fraction isolated from fresh OSPW; Organic fraction consists of neutral fraction and NAEs	Mouse BMDM	18 hours (Serial dilution)	RNI (less for 25 and 50 mg/L NAs); iNOS gene expression (less for 50 mg/L NAs); ROI (less for 50 mg/L NAs); NADPH subunits - p47Phox & p67Phox gene expression (less for 50 mg/L NAs); Phagocytosis (inhibited for 50 mg/L NAs); Cytokines gene expression (less IL-1, IL-6, IL-12, TNF-α for resting cells at 50 mg/L NAs; less IL-1, more IL-12 and TNF-α for activated cells)	(Garcia-Garcia et al., 2011a)
OSPW-OF (Pond 10)	Organic fraction isolated from 17-year aged OSPW; Four fractions tested: NAEs, neutral fraction, C18 MeOH fraction, C18 NaOH fraction;	H4IIE-luc cell line	24 hours for cytotoxicity assay; 24, 48, and 72 hours for AhR transactivation assay	Cytotoxic at 50 mg/L of each fraction; AhR agonist activity (5 mg/L of neutral fraction after 24h, but dissipated at 48 and 72h; NSD for other fractions)	(Leclair et al., 2015)
OSPW-OF (Pond 10)	Organic fraction isolated from 17-	H295R cell line	48 hours	Corticosterone production (+ for 5 mg/L NAEs; NSD for other fractions);	(Leclair et al., 2015)

	year aged OSPW; Four fractions tested: NAEs, neutral fraction, C18 MeOH fraction, C18 NaOH fraction			Progesterone production (+ for 0.05-0.5 mg/L C18 MeOH fraction; NSD for other fractions); Androstenedione production (NSD for all fractions); Testosterone production (NSD for all fractions)	
OSPW-OF (Pond 10)	Organic fraction isolated from 17-year aged OSPW; Four fractions tested: NAEs, neutral fraction, C18 MeOH fraction, C18 NaOH fraction	Yeast (*Saccharomyces cerevisiae*) cells	48 and 72 hours for yeast androgen screen and yeast estrogen screen, respectively	No estrogen or androgen receptor agonists for all fractions; Antiestrogenic potency for neutral fraction, NAEs and C18 MeOH fraction	(Leclair et al., 2015)

Note: (NSD) no significant difference relative to control; (+) significant increase relative to control; (-) significant decrease relative to control; MLSB: Mildred Lake Settling Basin built in 1989, an active settling basin on Syncrude's site; WIP: West-In-Pit, an active settling basin established in 1995, on Syncrude's site; Pond 10: a small tailings storage pond, containing 17-year old OSPW, on Syncrude's site; OSPW-NAEs: OSPW naphthenic acids extracts; OSPW-OF: OSPW organic fraction.

Appendix B. Studies demonstrating OSPW toxicity in invertebrates

Sample designation	OSPW type	Test organism	Duration of Exposure	Endpoint & Result	Reference
MLSB	Fresh OSPW	*Daphnia magna*	96 hours	LC_{50}: 16-27%	(MacKinnon and Retallack, 1982)
MLSB	Fresh OSPW	*Daphnia pulex*	96 hours	LC_{50}: 2%	(MacKinnon, 1986)
MLSB	Fresh OSPW	*Daphnia pulex*	96 hours	LC_{50}: 10%	(MacKinnon and Boerger, 1986)
MLSB	Fresh OSPW	Daphnia	96 hours	LC_{50}: 2%	(Boerger et al., 1986)
WIP	Fresh OSPW	*Daphnia magna*	48 hours	LC_{25}: >100%; LC_{50}: >100%	(Zubot et al., 2012)
OSPW from three major oil sands companies	OSPW source not specified	*Daphnia magna*	24 and 48 hours	Survival (48h-LC_{50}: >100%); Feeding rate (24h-IC_{50}: 5.34%); Chemosensory function (inhibited at ≥ 5% OSPW for 24h); Total activity (inhibited at 10%-20% OSPW)	(Lari et al., 2016)
OSPW from three major oil sands companies	OSPW source not specified	*Daphnia magna*	21 days (1% -10% OSPW)	Feeding rate (-); Reproductive capacity (-); Growth (-)	(Lari et al., 2016)
WIP	Fresh OSPW	*Chironomus dilutus*	10 days (100% OSPW)	Survival (NSD); Growth (-)	(Pourrezaei et al., 2011)
WIP	Fresh OSPW	*Chironomus dilutus*	4 days (100% OSPW)	Survival (NSD); Growth (-49%); Gene expression: Oxidative stress response (*cat*: +1.9-fold; *gpx*: +2.7-fold; *gst* and *aif*: NSD); Endocrine signaling (*err*: -1.8-fold; *esr* and *usp*: NSD)	(Wiseman et al., 2013a)
WIP	Fresh OSPW	*Chironomus dilutus*	7 days (100% OSPW)	Survival (NSD); Growth (-62%); Gene expression: Oxidative stress response (*cat* and *gpx*: NSD; *gst*: -2.4-fold; *aif*: +1.6-fold); Endocrine signaling (*err*: +4.2-fold; *esr*: +4.8-fold; *usp*: +8.9-fold);	(Wiseman et al., 2013a)

				Tissues concentrations of lipid hydroperoxides (+2.9-fold)	
WIP	Fresh OSPW	*Chironomus dilutus*	10 days (100% OSPW)	Survival (WIP-2009: -35%; WIP-2010: NSD); Growth (WIP-2009: -64%; WIP-2010: -79%) Behavior: Case building (smaller, fragile); Case occupation activity (WIP-2009: less active on day 7& 9; WIP-2010: more active on day 3, less active on day 7& 9)	(Anderson et al., 2012a)
WIP	Fresh OSPW	*Ceriodaphnia dubia*	6 days: renewal test	Survival (LC_{25}: 52%, LC_{50}: 65%); Fecundity (IC_{25}: 8%, IC_{50}: >39%)	(Zubot et al., 2012)
WIP	Fresh OSPW	*Chironomus dilutus*	60 days (100% OSPW)	Pupation (WIP-2009: -32%; WIP-2010: -70%); Cumulative emergence (WIP-2009: -84%; WIP-2010: -90%)	(Anderson et al., 2012a)
Recycle water pond (Syncrude)	Fresh OSPW	*Ceriodaphnia dubia*	6-8 days	Survival (LC_{50}: 70.7%); Reproduction (IC_{50}: 49.4%)	(Puttaswamy et al., 2010)
TPW	Aged OSPW	*Chironomus dilutus*	10 days (100% OSPW)	Survival (NSD); Growth (-23%); Behavior: Case building (slightly smaller); Case occupation activity (more active)	(Anderson et al., 2012a)
TPW	Aged OSPW	*Chironomus dilutus*	60 days (100% OSPW)	Pupation (-43%); Cumulative emergence (-72%)	(Anderson et al., 2012a)
FE5	Aged OSPW	*Chironomus dilutus*	10 days (100% OSPW)	Survival (NSD); Growth (NSD); Behavior: Case building (smaller); Case occupation activity (more active)	(Anderson et al., 2012a)
FE5	Aged OSPW	*Chironomus dilutus*	4 days (100% OSPW)	Survival (NSD); Growth (NDS); Gene expression: Oxidative stress response (cat, gpx, gst, and aif: NSD); Endocrine signaling (err, esr, and usp: NSD)	(Wiseman et al., 2013a)
FE5	Aged OSPW	*Chironomus dilutus*	7 days (100% OSPW)	Survival (NSD); Growth (NDS); Gene expression:	(Wiseman et al., 2013a)

				Oxidative stress response (cat, gpx, and aif: NSD; gst: -2-fold); Endocrine signaling (err, esr, and usp: NSD); Tissues concentrations of lipid hydroperoxides (NSD)	
FE5	Aged OSPW	*Chironomus dilutus*	60 days (100% OSPW)	Pupation (NSD); Cumulative emergence (NSD)	(Anderson et al., 2012a)
Big Pit	Aged OSPW	*Chironomus dilutus*	10 days (100% OSPW)	Survival (NSD); Growth (-19%); Behavior: Case building (slightly smaller)； Case occupation activity (more active)	(Anderson et al., 2012a)
Big Pit	Aged OSPW	*Chironomus dilutus*	60 days (100% OSPW)	Pupation (NSD); Cumulative emergence (NSD)	(Anderson et al., 2012a)
OSPW-impacted wetlands (Suncor & Syncrude)	Oil sands process-impacted wetland waters	*Chironomus riparius*	10 days: laboratory bioassays (100% OSPW)	Larvae size (-); When reared in water mimicking combinations of salts and NAs, there was an antagonistic interaction between the two components.	(Kennedy, 2012)
OSPW-impacted wetlands (Suncor)	Oil sands process-impacted wetland waters	chironomids	NA	Density and biomass (+); Incidence of mentum deformities (NSD or +); Mutagenicity (NSD)	(Bendell-Young et al., 2000)

Note: (NSD) no significant difference relative to control; (+) significant increase relative to control; (-) significant decrease relative to control; MLSB: Mildred Lake Settling Basin built in 1989, an active settling basin on Syncrude's site; WIP: West-In-Pit, an active settling basin established in 1995, on Syncrude's site; Big Pit, FE5 and TPW are three on-site experimental reclamation ponds that have been aged by different approaches; Big Pit: have been aging since 1993 and is comprised of fluid fine tailings (FFTs) capped with freshwater; FE5 pond: created in 1989 by capping MFTs with OSPW; TPW: OSPW that has been aging since 1993.

Appendix C. Studies demonstrating OSPW toxicity in fish

Sample designation	OSPW type	Test organism	Duration of Exposure	Endpoint & Result	Reference
MLSB	Fresh OSPW	Rainbow trout	96 hours	LC_{50}: <4%	(MacKinnon, 1981)
MLSB	Fresh OSPW	Rainbow trout	96 hours	LC_{50}: 4-6%	(MacKinnon and Retallack, 1982)
MLSB	Fresh OSPW	Rainbow trout	96 hours	LC_{50}: 7%	(MacKinnon, 1986)
MLSB	Fresh OSPW	Rainbow trout	96 hours	LC_{50}: 8%	(Boerger et al., 1986)
MLSB	Fresh OSPW	Rainbow trout (fingerlings)	96 hours	Survival: 5% OSPW (11%), 10% OSPW (13%), 20% OSPW (5%), 50% OSPW (0%)	(Rogers et al., 2007)
MLSB	Fresh OSPW	Fathead minnow	96 hours	LC_{50}: 6-8.5%	(MacKinnon and Retallack, 1982)
MLSB	Fresh OSPW	Yellow perch	Early life stages (Serial dilution)	Fertilization success (NSD for 0.16%-20% OSPW; 0% for 100% OSPW); Incidence of embryo deformities (+, optic-cephalic & spinal deformities, calculated threshold: 7.52 mg/L OSPW-NAs); Larval hatch length (-, calculated threshold: 1.92 mg/L OSPW-NAs)	(Peters et al., 2007)
MLSB	Fresh OSPW	Japanese medaka	Early life stages (Serial dilution)	Fertilization success (NSD at all OSPW dilutions tested); Incidence of embryo deformities (+, circulatory and head region deformities, calculated threshold: 30 mg/L OSPW-NAs); Larval hatch length (-, calculated threshold: 6.18 mg/L OSPW-NAs)	(Peters et al., 2007)
WIP	Fresh OSPW	Rainbow trout	96 hours	LC_{25}: >25%; LC_{50}: 35%	(Zubot et al., 2012)
WIP	Fresh OSPW	Rainbow trout	End of bioassay	Mortality (100%)	(Zubot et al., 2012)
WIP	Fresh OSPW	Goldfish	1 week	Cytokine gene expression in gill (more IFN γ and IL-1-β1, less TNFα-2 for 25% OSPW; more IL-1-β1, less TNFα-2 for 50% OSPW); Cytokine gene expression in kidney (more IFN γ, IL-1-β1 and TNFα-2 for 25% OSPW; more IFN γ and IL-1-β1 for 50%	(Hagen et al., 2013)

				OSPW); Cytokine gene expression in spleen (more IL-1-β1 for 25% OSPW; more IL-1-β1 and TNFα-2 for 50% OSPW)	
WIP	Fresh OSPW	Goldfish	12 weeks (Serial dilution)	Cytokine gene expression in gill (more IL-1-β1 for 25% OSPW; less TNFα-2 for 50% OSPW); Cytokine gene expression in kidney (less IFN γ and TNFα-2, more IL-1-β1 for 25% OSPW; less IFN γ and TNFα-2, more IL-1-β1 for 50% OSPW); Cytokine gene expression in spleen (less IFN γ and TNFα-2, more IL-1-β1 for 25% OSPW; less IFN γ and TNFα-2, more IL-1-β1 for 50% OSPW)	(Hagen et al., 2013)
WIP	Fresh OSPW	Fathead minnow	Early life stages (100% OSPW)	Larval survival (-55.3%); Spontaneous embryo movement (+92.1%); Premature hatching rate (+); Incidence of deformities (hemorrhage: + 50%; pericardial edema: + 56.2 %; spinal malformations: +37.5%); Transcript of genes related to the metabolism of xenobiotics (cyp1a: NSD; cyp3a: +2.35-fold), oxidative stress (gst: + 2.15-fold; sod: +3.08-fold), and apoptosis (casp9: +3.26-fold; apopen: +2.38-fold); ROI generation (+1.68-fold)	(He et al., 2012a)
WIP	Fresh OSPW	Fathead minnow (males)	7 days (100% OSPW)	Gene expression in brain (erβ, ar, gnrh2, gnrh3: NSD; erα: +5.14-fold; kiss1r: +6.11-fold; fshβ: +3.96-fold; lhβ: +3.04-fold); cyp19b: + 3.44-fold; gnrhr: -0.13-fold); Gene expression in gonads (star, 17βhsd, cyp19a: NSD; fshr: +3.7-fold; lhr: +2.5-fold; cyp11a: +8-fold; 3βhsd: +7-fold); Gene expression in liver (erα: + 4.1-fold; vtg: + 4.9-fold; chg-l: +5.4-fold; chg-h: + 3.4-fold)	(He et al., 2012b)
WIP	Fresh OSPW	Fathead minnow (females)	7 days (100% OSPW)	Gene expression in brain (gnrh2, gnrh3, kiss1r, cyp19b, erα, erβ, ar: NSD; lhβ: +5.3-fold; gnrhr: -); Gene expression in gonads (star, 3βhsd, 17βhsd, cyp11a, cyp17: NSD; fshr: -0.02-fold; lhr: 0.33-fold; cyp19a: -0.28-fold); Gene expression in liver (ar: -0.18-fold); erα: -0.14-fold; erβ: -0.08-fold; vtg: -0.002- fold; chg-l: -0.022-fold; chg-h: -0.036-fold)	(He et al., 2012b)

WIP	Fresh OSPW	Fathead minnow (males)	7 days (100% OSPW)	Gene expression in liver: Phase I biotransformation/detoxification (cyp1a: +2.1-fold; cyp2j28: +2.2-fold; cyp2ad2: +2.7-fold; cyp2k6: +10.1-fold; cyp2k19: +11.7-fold; ao1: +3.1-fold; aldh2: +3.6-fold; moa: +3.2-fold; eh: +2.0-fold); Phase II & III biotransformation/detoxification (gstm: +4.5-fold; gstc: + >23.3-fold; ugt2a3: +6.3-fold; sult1,3: +1.8-fold; ugt5f1: -4.3-fold); Oxidative stress response (gs: +3.1-fold; gr: +3.2-fold; gpx: +1.7-fold; tk: +2.4-fold; 6-pgdh: +10.1-fold; g6pdh: +2.7-fold; trx: +2.5-fold; trxr3: +2.7-fold; pdi p5: +2.2-fold; pdi a3: +1.5-fold; grx5: +1.7-fold); Apoptosis (aif-3: +4.3-fold; aif m2: +4.1-fold; parp: +4.8-fold; pdcd4a: +1.5-fold; dram2: +>23.3-fold; cthpb: +1.5-fold; bnip3: -1.8-fold; foxo3a: -3.3-fold); Immune response (c8β: -2.1-fold; c1q4c: -19.7-fold; c3: -7.6-fold; c3-h1: -2.1-fold; c4-2: -2-fold)	(Wiseman et al., 2013b)
CT water pond (Suncor)	OSPW released through MFT consolidation	Fathead minnows	96 hours (100% OSPW)	Mortality (0%); Hematocrit (+38.8%); Leucocrit (-50.6%); Lymphocrit (-74%); Gill histology (NSD)	(Farrell et al., 2004)
CT water pond (Suncor)	OSPW released through MFT consolidation	Fathead minnows	28 days (100% OSPW)	Mortality (100%)	(Farrell et al., 2004)
DS pond (Suncor)	Dyke seepage water	Fathead minnows	96 hours (100% OSPW)	Mortality (0%); Hematocrit (+36.6%); Leucocrit (-50.6%); Lymphocrit (-80%); Gill histology (less basal epithelial thickening); Critical swimming speed (-)	(Farrell et al., 2004)
DS pond (Suncor)	Dyke seepage water	Fathead minnows	28 days (100% OSPW)	Mortality (100%)	(Farrell et al., 2004)
Suncor Pond 1	Fresh OSPW	Rainbow trout	96 hours	1981 (LC_{50}: 17%), 1982 (LC_{50}: 7.5-10.2%), 1984 (LC_{50}:	(Nix and Martin,

				4.5%), 1989 (LC_{50}: 3.2%)	1992)
Suncor Pond 1A	Fresh OSPW	Rainbow trout	96 hours	1981 (LC_{50}: 27%), 1982 (LC_{50}: 24%), 1984 (LC_{50}: 5.8%), 1989 (LC_{50}: 3.2%)	(Nix and Martin, 1992)
Suncor Pond 2	Fresh OSPW	Rainbow trout	96 hours	1981 (LC_{50}: 16%), 1982 (LC_{50}: 4.2-5.1%), 1984 (LC_{50}: 4.2%), 1989 (LC_{50}: 3.2%)	(Nix and Martin, 1992)
Suncor Pond 3	Fresh OSPW	Rainbow trout	96 hours	1989 (LC_{50}: 3.2%)	(Nix and Martin, 1992)
Syncrude Pond 9	Aged OSPW (>15 years)	Goldfish	1 week (100% OSPW)	Cytokine gene expression in gill (NSD); Cytokine gene expression in kidney (more IFN γ, TNFα-2); Cytokine gene expression in spleen (more IFN γ, TNFα-2)	(Hagen et al., 2013)
Syncrude Pond 9	Aged OSPW (>15 years)	Goldfish	12 weeks (100% OSPW)	Cytokine gene expression in gill (more IFN γ, IL-1-β1, TNFα-2); Cytokine gene expression in kidney (more IL-1-β1, TNFα-2); Cytokines gene expression in spleen (more IL-1-β1)	(Hagen et al., 2013)
Remediation Pond 3 (Syncrude)	Aged OSPW (>12 years); MFT capped with freshwater	Yellow perch	22 days (100% OSPW)	Mortality (0%); Gill pathology (NSD); Liver pathology (NSD)	(Nero et al., 2006b)
Remediation Pond 3 (Syncrude)	Aged OSPW (>12 years); MFT capped with freshwater	Goldfish	19 days (100% OSPW)	Mortality (1 fish dead); Gill pathology (NSD); Liver pathology (NSD)	(Nero et al., 2006b)
Remediation Pond 3 (Syncrude)	Aged OSPW (>12 years); MFT capped with freshwater	Goldfish	19 days (100% OSPW)	Plasma levels of hormones (T: -; E2: -); *In vitro* basal T production by gonadal tissues (NSD); hCG-stimulated T production by gonadal tissues (NSD); Plasma cortisol levels in males (+)	(Lister et al., 2008)
Remediation Pond 5 (Syncrude)	Aged OSPW (>12 years); MFT capped with OSPW	Yellow perch	22 days (100% OSPW)	Mortality (0%); Gill pathology (increased cell proliferation of epithelial and mucous cells); Liver pathology (hepatocellular degeneration, inflammatory cell infiltration)	(Nero et al., 2006b)
Remediation Pond 5 (Syncrude)	Aged OSPW (>12 years); MFT capped with OSPW	Goldfish	19 days (100% OSPW)	Mortality (1 fish dead); Gill pathology (epithelial cell necrosis); Liver pathology (hepatocellular degeneration, hypertrophic hepatocytes)	(Nero et al., 2006b)
Remediation Pond 5 (Syncrude)	Aged OSPW (>12 years); MFT	Goldfish	19 days (100% OSPW)	Plasma levels of hormones (T: -; E2: -); *In vitro* basal T production by gonadal tissues (-);	(Lister et al., 2008)

	capped with OSPW			hCG-stimulated T production by gonadal tissues (NSD); Plasma cortisol levels in males (+)	
Remediation Pond 5 (Syncrude)	Aged OSPW (>15 years); MFT capped with OSPW	Fathead minnows	21 days: laboratory bioassays (100% OSPW)	GSI (NSD); LSI (NSD); Fecundity rate (-21.9%); Mean spawn number (NSD); Plasma steroid concentration in males (T and 11-KT: NSD); Plasma steroid concentration in females (E2 and T: NSD)	(Kavanagh et al., 2011)
Remediation Pond 9 (Syncrude)	Aged OSPW (>15 years)	Fathead minnows	21 days: laboratory bioassays (100% OSPW)	GSI (males: NSD; females: -); LSI (NSD); Fecundity rate (-78.1%-100%); Mean spawn number (-71.7%-100%); Plasma steroid concentration in males (T: -; 11-KT: -); Plasma steroid concentration in females (E2: -; T: NSD)	(Kavanagh et al., 2011)
Demonstration Pond (Syncrude)	Aged OSPW (>15 years); MFT capped with freshwater	Fathead minnows	21 days: laboratory bioassays (100% OSPW)	GSI (NSD); LSI (NSD); Fecundity rate (-18.9%); Mean spawn number (NSD)	(Kavanagh et al., 2011)
Demonstration Pond (Syncrude)	Aged OSPW (>15 years); MFT capped with freshwater	Fathead minnows	Fish collected at various time in 2006-2008 (Jun-06, Jul-07, Aug-07, May-08, Jun-08)	Males: Length (+, Jul-07; -, May-08); Mass (+, Jul-07 and Jun-08; -, May-08); Condition factor (+, Jun-06, Jul-07, and Jun-08; -, Aug-07); GSI (+, 2006-2008); LSI (+, 2007); SSI (-, 2006-2007); Number of tubercles (-, Jun-06 and May-08; +, Aug-07); Plasma steroid concentration in Jun-06 and Jul-07 (T: NSD; 11-KT: -) Females: Length (+, Aug-07; -, Jun-08); Mass (+, Jun-06 and Aug-07) Condition factor (+, Jun-06, Jul-07, Aug-07 and Jun-08; -, May-08); GSI (+, 2006-2007); LSI (+, 2006-2008); SSI (-, 2006-2007);	(Kavanagh et al., 2013)

				Plasma steroid concentration in Jun-06 and Jul-07 (T: NSD; 11-KT: NSD)	
Suncor North MFT Pond	Aged OSPW (>15 years); MFT capped with OSPW	Fathead minnows	21 days: laboratory bioassays (100% OSPW)	GSI (NSD); LSI (NSD); Fecundity rate (-77.5%); Mean spawn number (-68.4%); Plasma steroid concentration in males (T and 11-KT: -); Plasma steroid concentration in females (E2 and T: NSD)	(Kavanagh et al., 2011)
Suncor South MFT Pond	Aged OSPW (>15 years); MFT capped with OSPW	Fathead minnows	21 days: laboratory bioassays (50% and 100% OSPW)	50% OSPW: GSI (NSD); LSI (NSD); Fecundity rate (-14.8%); Mean spawn number (-26.3%); Plasma steroid concentration in males (T and 11-KT: NSD); Plasma steroid concentration in females (E2 and T: NSD); 100% OSPW: GSI (NSD); LSI (NSD); Fecundity rate (-57.4%); Mean spawn number (-50%); Plasma steroid concentration in males (T: NSD; 11-KT: -); Plasma steroid concentration in females (E2 and T: NSD)	(Kavanagh et al., 2011)
OSPW-NAEs (WIP)	Fresh OSPW	Zebrafish	96 hours	Whole acid extract (LC_{50}: 8.4 mg/L); Esterifiable NAs (de-esterified with alkal) (LC_{50}: 5.4 mg/L); De-esterified alicyclic acids/ classical NAs (LC_{50}: 13.1 mg/L); Aromatic NAs (LC_{50}: 8.1 mg/L)	(Scarlett et al., 2013)
OSPW-NAEs (WIP)	Fresh OSPW	Yellow perch	21 days	Mortality: NAEs-6.8 mg/L (100% in ≤ 96h); Gill pathology: NAEs-1.7 mg/L (high levels of gill proliferative changes: epithelial, mucous, and chloride cell); Liver pathology: NAEs -1.7 mg/L (NSD)	(Nero et al., 2006a)
OSPW-NAEs (WIP)	Fresh OSPW	Fathead minnows	21 days (5 and 10 mg/L NAEs)	NAEs-5 mg/L: Fecundity rate (NSD); Mean spawn number (NSD); GSI (males: +; females: NSD);	(Kavanagh et al., 2012a)

				Plasma steroid concentration in males (T: NSD; 11-KT: -); Plasma steroid concentration in females (E2 and T: NSD) NAEs-10 mg/L: Fecundity rate (-68%); Mean spawn number (-68.2%); GSI (males: +; females: NSD); Plasma steroid concentration in males (T and 11-KT: -); Plasma steroid concentration in females (E2 and T: NSD)	
OSPW-NAEs	NAEs isolated from fresh and aged OSPW	Fathead minnow	Early life stages : from <1 dpf to hatch day	Hatch success (EC_{50}: 5-10.6 mg/L for fresh OSPW-NAEs; EC_{50}: 12.4 mg/L for aged OSPW-NAEs); Deformities at hatch (cardiovascular abnormalities)	(Marentette et al., 2015a)
OSPW-NAEs	NAEs isolated from fresh OSPW	Fathead minnow	Early life stages : from <1 dpf to 21 dpf	Hatch success (EC_{50}: 9.5-11 mg/L); Growth (IC_{10}: 24.7-25.8 mg/L for total length; 14.7-15.8 mg/L for total mass); Deformities at hatch (+ at 33.3 mg/L, predominated by cardiovascular abnormalities)	(Marentette et al., 2015b)
OSPW-NAEs	NAEs isolated from fresh OSPW	Walleye	Early life stages : from <1 dpf to 19-21 dpf	Hatch success (EC_{50}: 21.8-24.5-11 mg/L); Deformities at hatch (dose-responsive increase at 0-33 mg/L), predominated by spinal curvature, followed by cardiovascular and craniofacial defects)	(Marentette et al., 2015b)
OSPW-NAEs	NAEs isolated from fresh OSPW	Walleye	Early life stages : from <1 dpf to hatch day	Gene expression: AhR-cytochrome P450 pathway (cyp1a1: +2.11-fold at 4.2 mg/L and +1.95-fold at 8.3 mg/L; arnt: NSD); Oxidative stress response (gpx1b: -1.56-fold at 4.2 mg/L; cat, gst, sod1: NSD); Apoptosis (bax, casp3, p53: NSD); Growth factor signaling (igf1, igf1b, igf1bp: NSD); Tissue differentiation (vim: NSD)	(Marentette et al., 2017)

Note: (NSD) no significant difference relative to control; (+) significant increase relative to control; (-) significant decrease relative to control; MLSB: Mildred Lake Settling Basin built in 1989, an active settling basin on Syncrude's site; WIP: West-In-Pit, an active settling basin established in 1995, on Syncrude's site; OSPW-NAEs: OSPW naphthenic acids extracts.

Appendix D. Studies demonstrating OSPW toxicity in amphibians

Sample designation	OSPW type	Test organism	Duration of Exposure	Endpoint & Result	Reference
Natural wetland (Suncor)	Wetland receiving dyke seepage water	Boreal toad (*Bufo boreas*)	Posthatch to complete metamorphosis (laboratory bioassays)	*B. boreas* tadpoles: Mortality (0%); Delayed metamorphosis (24-d compared to 21-d in reference wetland); Mass change after 96-h exposure (no change)	(Pollet and Bendell-Young, 2000)
Hummock wetland (Suncor)	Wetland receiving CT water (intentional release).	Boreal toad (*Bufo boreas*)	Posthatch to complete metamorphosis (laboratory bioassays)	*B. boreas* tadpoles: Survival (47% died before completing metmorphosis); Delayed metamorphosis (31-d compared to 21-d in reference wetland); Mass change after 96-h exposure (-)	(Pollet and Bendell-Young, 2000)
OSPW-impacted wetlands	Young wetland (≤ 7 years old); old wetlands (> 7 years old)	Wood frog (*Lithobates sylvaticus*)	75 days	Tadpoles in young OSPW-impacted wetlands showed 41.5%, 62.6%, and 54.7% higher mortality than old OSPM-impacted, young reference, and old reference wetlands, respectively. Old OSPW-impacted wetlands had similar effects in tadpoles compared to reference wetlands.	(Hersikorn et al., 2010)
OSPW-impacted wetlands	Young wetland (≤ 7 years old); old wetlands (> 7 years old)	Wood frog (*Lithobates sylvaticus*)	75 days	Tadpoles in young OSPW-impacted wetlands showed delayed metamorphosis (up to 75 d) compared to reference and old OSPM-impacted wetlands (50-60 d). Tadpoles in young OSPW-impacted wetlands had highest T4 concentration, and lowest T3:T4 ratio. Tadpoles in young OSPW-impacted wetlands had highest EROD activity.	(Hersikorn and Smits, 2011)

Note: (NSD) no significant difference relative to control; (+) significant increase relative to control; (-) significant decrease relative to control

Appendix E. Studies demonstrating OSPW toxicity in birds

Sample designation	OSPW type	Test organism	Duration of Exposure	Endpoint & Result	Reference
OSPW-impacted wetland	CT water	Zebra finch (*Taeniopygia guttata*)	4 days: laboratory bioassays (70µL/day orally)	No effects on immunosuppression of T-lymphocyte immune response, or on hematocrit, white blood cell differential and body mass. Had larger bursa of Fabricius.	(Smits and Williams, 1999)
Demonstration Pond (Syncrude)	MFT capped with freshwater	Tree swallows (*Tachycineta bicolor*)	Over two breeding seasons	Clutch size (NSD); Clutch mass (NSD); Hatching success (NSD); Fledging success (NSD); Immune response (+ in 1997; NSD in 1998); Hepatic EROD activity (NSD)	(Smits et al., 2000)
Demonstration Pond (Syncrude)	MFT capped with freshwater	Tree swallows (*Tachycineta bicolor*)	Late May to mid July of 2003 and 2004	In 2003, harsh weather: Mortality (58.8%); Reproductive performance (-) In 2004, less challenging weather: Mortality (0%); Hepatic EROD activity (+1.2-fold); Fledging size (-)	(Gentes et al., 2006)
Demonstration Pond (Syncrude)	MFT capped with freshwater	Tree swallows (*Tachycineta bicolor*)	May 19-July 15, 2014	Plasma hormones (T3 and T4: NSD); Thyroid weight (NSD)	(Gentes et al., 2007a)
Natural wetland (Suncor)	Wetland receiving dyke seepage water	Tree swallows (*Tachycineta bicolor*)	Over two breeding seasons	Clutch size (NSD); Clutch mass (NSD); Hatching success (-); Fledging success (-); Immune response (NSD); Hepatic EROD activity (+)	(Smits et al., 2000)
Natural wetland (Suncor)	Wetland receiving dyke seepage water	Mallard (*Anas platyrhynchos*) ducklings	33 days	Body mass (day 2, 5, 9 and 13: -; after 13 days: NSD); Body size (on day 2, 5, 9 and 13: -; after 13 days: NSD); Plasma triglyceride level (NSD);	(Gurney et al., 2005)

				Plasma glycerol level (day 13: +; day 33: NSD); EROD activity (NSD); PAH metabolite levels in the bile (Pyrene: +; BαP: NSD; Naphthalene: +; Phenanthrene: NSD)	
Natural wetland (Suncor)	Wetland receiving dyke seepage water	Tree swallows (*Tachycineta bicolor*)	Late May to mid July of 2003 and 2004	In 2003, harsh weather: Mortality (89.3%); Reproductive performance (-) In 2004, less challenging weather: Mortality (3.6%); Hepatic EROD activity (+1.9-fold); Fledging size (-)	(Gentes et al., 2006)
Natural wetland (Suncor)	Wetland receiving dyke seepage water	Tree swallows (*Tachycineta bicolor*)	May 19-July 15, 2014	Plasma hormones (T3: +; T4: NSD); Thyroid weight (NSD)	(Gentes et al., 2007a)
CT wetland (Suncor)	Wetland with consolidated tailings	Tree swallows (*Tachycineta bicolor*)	Late May to mid July of 2003 and 2004	In 2003, harsh weather: Mortality (100%) In 2004, less challenging weather: Mortality (0%); Hepatic EROD activity (+2-fold); Fledging size (-)	(Gentes et al., 2006)
CT wetland (Suncor)	Wetland with consolidated tailings	Tree swallows (*Tachycineta bicolor*)	May 19-July 15, 2014	Plasma hormones (T3: +; T4: NSD); Thyroid weight (NSD)	(Gentes et al., 2007a)
Hummock wetland (Suncor)	Wetland receiving CT water (intentional release).	Mallard (*Anas platyrhynchos*) ducklings	33 days	Body mass (on day 2, 5, 9 and 13: -; after 13 days: NSD); Body size (on day 2, 5, 9 and 13: -; after 13 days: NSD); Plasma triglyceride level (NSD); Plasma glycerol level (NSD)	(Gurney et al., 2005)

Note: (NSD) no significant difference relative to control; (+) significant increase relative to control; (-) significant decrease relative to control

Appendix F. Studies demonstrating OSPW toxicity in mammals

Sample designation	OSPW type	Test organism	Duration	Endpoint & Result	Reference
OSPW-NAEs (MLSB)	Fresh OSPW	Wistar rats	14 days	300 mg/kg/d, 5d a week: Brain hemorrhage in males; Cardiac periarteriolar necrosis and fibrosis in females; Significant incidence of pericholangitis in both sexes	(Rogers et al., 2002)
OSPW-NAEs (MLSB)	Fresh OSPW	Wistar rats (females)	90 days	60 mg/kg/d, 5d a week: Increased liver weight; Elevated blood amylase; Reduced hypocholesterolemia; Excessive hepatic glycogen accumulation	(Rogers et al., 2002)
OSPW-NAEs (MLSB)	Fresh OSPW	Wistar rats (females)	Throughout pre-breeding, breeding and gestation	60 mg/kg/d: Hypocholesterolemia; Poor reproductive success; Reduced litter size	(Rogers, 2003)
OSPW-OF (WIP)	Fresh OSPW	Mice	1 week	Cytokine gene expression in liver (less TNF α, IFN γ, IL-1, CSF-1, CCL3, and CCL4 at 100 mg/kg/week); Cytokine gene expression in spleen (NSD at100 mg/kg/week); Cytokine gene expression in MLN (less IL-1 at 100 mg/kg/week)	(Garcia-Garcia et al., 2011a)
OSPW-OF (WIP)	Fresh OSPW	Mice	1 and 2 weeks	Body weight (NSD); Pro-inflammatory cytokines gene expression in liver (less IL-1β at 50 mg/kg/week for 1 week; less IL-1β, CSF-1, and CSFR1 at 100 mg/kg/week for 1 week; less IL-1β at 100 mg/kg/week for 2 weeks); Pro-inflammatory cytokines gene expression in spleen (less IFN γ, IL-1β, at 100 mg/kg/week for 1 week; NSD for 2 weeks); Pro-inflammatory cytokines gene expression in MLN (more CCL3 and CCL4 at 100 mg/kg/week for 1 week; more CCL3 at 50 and 100 mg/kg/week for 2 weeks); Peritoneal macrophage phagocytosis (NSD)	(Garcia-Garcia et al., 2012)
OSPW-OF (WIP)	Fresh OSPW	Mice	4 and 8 weeks	Body weight (NSD);	(Garcia-Garcia et

				Pro-inflammatory cytokines gene expression in liver (NSD for 4 weeks; less TNFRFS1A, IL-1β, and CSF-1 at 100 mg/kg/week for 8 weeks); Pro-inflammatory cytokines gene expression in spleen (less TNF α, TNFRFS1A, IFN γ, IL-1β, CSF-1, CSFR1, CCL3, CCL4, and CCL5 at 50 mg/kg/week for 4 weeks; less CCL4 at 100 mg/kg/week for 4 weeks; less TNF-α, TNFRFS1A, IFN γ, IL-1β, CSF-1, CSFR1, CCL2, CCL3, and CCL4 at 50 mg/kg/week for 8 weeks); Pro-inflammatory cytokines gene expression in MLN (NSD for 4 weeks; more CSF-1 and CSFR1 at 100 mg/kg/week for 8 weeks); Peritoneal macrophage phagocytosis (enhanced at 50 and/or 100 mg/kg/week for 4 weeks; NSD for zymosan phagocytosis at 50 and 100 mg/kg/week for 8 weeks; inhibited phagocytosis for zymosan+complement at 50 and 100 mg/kg/week for 8 weeks)	al., 2012)
OSPW-OF (WIP)	Fresh OSPW	Mice	8 weeks	Pro-inflammatory cytokines gene expression in spleen (less IFN γ, IL-1β and CSF-1 at 100 mg/kg/week)	(Garcia-Garcia et al., 2011b)

Note: (NSD) no significant difference relative to control; MLSB: Mildred Lake Settling Basin built in 1989, an active settling basin on Syncrude's site; WIP: West-In-Pit, an active settling basin established in 1995, on Syncrude's site; OSPW-NAEs: OSPW naphthenic acids extracts; OSPW-OF: OSPW organic fraction.