THE ENDOCRINE DISRUPTING AND EMBRYOTOXIC EFFECTS OF UNTREATED AND OZONE-TREATED OIL SANDS PROCESS-AFFECTED WATER

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THE ENDOCRINE DISRUPTING AND EMBRYOTOXIC EFFECTS OF UNTREATED AND OZONE-TREATED OIL SANDS PROCESS-AFFECTED WATER

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ABSTRACT

Due to a policy of no release, oil sands process-affected water (OSPW) produced by the surface-mining oil sands industry in North Eastern Alberta, Canada, is stored on-site in tailings ponds. There is concern regarding the toxic effects of OSPW on aquatic organisms. Knowledge of the chemical composition and toxicity of OSPW is limited. Research is necessary for potential remediation and release of OSPW back into the environment. Due to the large volume and persistency of OSPW, active efforts are necessary for the remediation of OSPW before release and habitat reclamation. Currently, ozonation is considered one possible method for remediation of OSPW by reducing the concentrations of dissolved organic compounds, including naphthenic acids (NAs), which are considered among the primary toxic constituents. However, further work is needed to evaluate the effectiveness of ozonation in reducing the toxicity of OSPW and to ensure that ozonation does not increase the toxicity of OSPW. The overall objective of this work was to determine the toxic effects of OSPW on endocrine disruption and embryo development, using both *in vitro* and *in vivo* models, and the effectiveness of ozone treatment for reducing the toxicity of OSPW.

In the first study, untreated and ozone-treated OSPW were examined for effects on sex steroid production using the H295R cell line steroidogenesis Assay. The results indicate that exposure to untreated OSPW can significantly decrease synthesis of testosterone (T) and increase synthesis of 17β-estradiol (E2) by 0.55 ± 0.06 and 2.0 ± 0.13-fold, respectively, compared to that of control groups ($\rho < 0.05$). These effects were due to increased aromatase enzyme activity and

decreased E2 metabolism. The results also suggest that ozonation is an effective treatment to reduce concentrations of NAs in OSPW without altering steroidogenesis.

In the second study, the T47D-kbluc (estrogen responsive) and MDA-kb2 (androgen responsive) cell assays were used to determine whether OSPW might act as either agonists or antagonists of the estrogen receptor (ER) or androgen receptor (AR), respectively. The estrogenic responses to untreated OSPW were significantly greater by 2.6±0.22-fold compared to control group ($\rho < 0.05$). Exposure to untreated OSPW produced significant antiandrogenic response in the presence of 0.01, 0.05 and 0.1 nM T by 16±6.5%, 47±7.6% and 75±9.7%, respectively, of that of the corresponding concentrations of T alone ($\rho < 0.05$). The results suggest that compounds in the dissolved organic fraction of OSPW have estrogenic and anti-androgenic properties, acting as ER agonists and/or AR antagonists. Ozonation of the OSPW partially mitigated the antiandrogenicity but had no effect on the estrogenicity of OSPW.

In the third study, the endocrine-disrupting effects of OSPW and ozone-treated OSPW were determined by quantifying relative changes in the abundances of transcripts of genes along the brain-gonad-liver (BGL) axis in male and female fathead minnows (*Pimephales promelas*). The results indicate that OSPW has endocrine-disrupting effects at all levels of BGL axis and these effects of impaired expression of genes along the BGL axis are sex specific. For example, exposure to OSPW resulted in significantly greater abundances of transcripts of *vtg* (Vitellogenin), *chg-l* (Choriogenin L) and *chg-h* (Choriogenin H minor) by 4.9±1.2, 5.4±1.5 and 3.4±0.78-fold, respectively, compared to those of control groups ($\rho < 0.05$) in livers from male fathead minnow. However, in livers from female fathead minnows, exposure to OSPW resulted

in significantly lesser abundances of transcripts of *vtg*, *chg-l* and *chg-h* by 0.002±0.0011, 0.022±0.007 and 0.036±0.024-fold, respectively, compared to those of control fish ($\rho < 0.05$). Ozonation of OSPW attenuated the effects on abundances of transcripts of some genes, and the attenuation was more prominent in males than in females. However, impact of ozonation on endocrine-disrupting effects of OSPW was less evident than in the *in vitro* studies described in Chapter 2 and 3. The results also provide a mechanistic basis for the endocrine-disrupting effects of OSPW from other studies, including impaired reproduction of fathead minnows exposed to OSPW.

In the final study the effects of untreated, ozone-treated, and activated charcoal-treated OSPW (OSPW, O3-OSPW, and AC-OSPW) on the survival, growth, and development of embryos of fathead minnows were determined. Compared to the control group, which had an embryo survival rate of 98±2.1%, survival was significantly less when exposed to OSPW (44±7.1%; $\rho < 0.05$). Eggs exposed to untreated OSPW exhibited a significantly greater rate of premature hatching, and embryos exhibited more frequent spontaneous movements. Incidences of hemorrhage (50±3.4%), pericardial edema (56±7.1%), and malformation of the spine (38±5.4%) were significantly greater in embryos exposed to OSPW compared to control group ($\rho < 0.05$). Significantly greater concentrations of ROS (1.7±0.11-fold), and greater abundances of transcripts *cyp3a*, *gst*, *sod*, *casp9*, and *apopen* (2.4±0.34, 2.2±0.26, 3.1±0.74, 3.3±0.57 and 2.4±0.25-fold, respectively) compared to control groups ($\rho < 0.05$), indicated that exposure to OSPW caused oxidative stress, which can result in damage to mitochondria and promote activation of caspase enzymes and apoptotic cell death. Removal of dissolved organic constituents in OSPW by ozone treatment, or by activated charcoal, significantly attenuated all

of the adverse effects associated with untreated OSPW. The results suggest that the organic fraction of OSPW can negatively impact the development of fathead minnow embryos through oxidative stress and apoptosis, and that ozonation attenuates this developmental toxicity.

Overall, the findings from the research described in this thesis provide novel and important insights into the toxicity and mechanisms of the toxicity of OSPW with respect to endocrine disruption and development of embryos of fish. In addition, the research provides compelling evidence that ozonation might be an effective method for accelerating the remediation of OSPW. The results of the research might help regulators develop effective strategies for reclamation, remediation and potential release of OSPW back to the environment.

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LIST OF ABBREVIATIONS

℃ = degree Celsius

µL = microliter

µm = micrometer

µM = micromolar concentration

ΔO3 = amount of utilized ozone (mg/L)

3βHSD = 3β-hydroxysteroid delta dehydrogenase

17βHSD = 17β-hydroxysteroid delta dehydrogenase

18S = ribosomal RNA 18S

Abb. = abbreviation

AC = activated charcoal

ANOVA = analysis of variance

APOPIN5 = apoptosis inhibitor 5

APOPEN = apoptosis enhancing nuclease

AR = androgen receptor

ATCC = American Type Culture Collection

atm = atmosphere

ATRF = Aquatic Toxicology Research Facility, Toxicology Centre, University of Saskatchewan

BAX = B-cell lymphoma 2-associated X

BGL = brain-gonad-liver

BMDM = mouse bone marrow-derived macrophages

CAN$ = Canadian dollar

CAPP = Canadian Association of Petroleum Producers

CASP-3 = caspase-3

CASP-9 = caspase-9

CAT = catalase

cDNA = complimentary deoxyribonucleic acid

$C_{G,in}$ = ozone concentration in the feed gas

$C_{G,out}$ = ozone concentration in the off gas

CHG-H = choriogenin H minor

CHG-L = choriogenin L

C_L = residue ozone concentration in the liquid phase (mg/L)

Conc. = concentration

CYP1A = cytochrome P450, family 1, subfamily a

CYP3A = cytochrome P450, family 3, subfamily a

CYP11A = cytochrome P450, family 11, subfamily a

CYP11B = cytochrome P450, family 11, subfamily b

CYP17 = cytochrome P450, family 17

CYP19a = cytochrome P450, family 19, subfamily a

CYP19b = cytochrome P450, family 19, subfamily b

CYP21 = cytochrome P450, family 21

d = day

DCM = dichloromethane

DEX = dexamathasone

DNA = deoxyribonucleic acid

dT = deoxythimidine

E2 = 17β-estradiol

Eff. = efficiency of polymerase chain reaction

ELISA = enzyme-linked immunosorbent assay

ELS = early life stage

ER = estrogen receptor

ERE = estrogen-responsive element

FBS = fetal bovine serum

FSHβ = follicle-stimulating hormone beta subunit

FSHR = follicle-stimulating hormone receptor

FTFC = Fine Tailings Fundamentals Consortium

g = gram

g/L = gram per liter

GC x GC/ToF-MS = comprehensive two dimensional gas chromatography / time-off light- mass spectrometry

GC-MS = gas chromatography- mass spectrometry

GDP = gross domestic product

GHG = greenhouse gas

GnRH = gonadotropin releasing hormone

GnRHR = gonadotropin releasing hormone receptor

GR = glucocorticoid receptor

GST = glutathione-S-transferase

GTH = gonadotropin hormone

h = hour

HEPES = 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

hpf = hours post-fertilization

HPG = hypothalamus-pituitary-gonad

HPGL = hypothalamus-pituitary-gonad-liver

HRMS = high-resolution mass spectrometry

IC_{20} = inhibitory concentration of chemicals causing 20% decreases in methanogenic activity

ICI 182,780 = Faslodex

IHS CERA = Information handling service, Cambridge Energy Research Associates

KI = potassium iodide

KISS1R = kisspeptin 1 receptor

km^2 = square kilometer

L = liter

LHβ = luteinizing hormone beta subunit

LHR = luteinizing hormone receptor

m^3 = cubic meter

MFT = mature fine tailings

mg/L = milligram per liter

min = minitue

mL = milliliter

MMTV = mouse mammary tumor virus

mRNA = messenger ribonucleic acid

MS-222 = tricaine methanesulfonate

MTT = (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

$NaHCO_3$ = sodium bicarbonate

NAs = naphthenic acids

NASA = National Aeronautics and Space Administration (USA)

N.D. = not detected

nM = nanomolar concentration

No. = number

O3 = ozone

O3-OSPW = ozone-treated oil sands process-affected water

OECD = Organization for Economic Cooperation and Development

OSPW = oil sands process-affected water

PAH = polycyclic aromatic hydrocarbon

PBDG = porphobilinogen deaminase

PBS = phosphate buffered saline

PCR = polymerase chain reaction

$Q_{G,in}$ = feed-gas flow rate (L/min)

$Q_{G,out}$ = off-gas flow rate (L/min)

qPCR = quantitative polymerase chain reaction

RNA = ribonucleic acid

ROS = reactive oxygen species

rRNA = ribosomal ribonucleic acid

s = second

SEP = south east pond

SOD = superoxide dismutase

Sp. movement = spontaneous movement

StAR = steroidogenic acute regulatory protein

t = ozone contact time (min)

T = testosterone

UPLC = ultra-pressure liquid chromatography

USEIA = United States Energy Information Administration

USEPA = United States Environmental Protection Agency

VL = effective reactor volume (L)

VTG = vitellogenin

v/v = volume per volume

WIP = west-in-pit

w/v = weight per volume

yr = year

CHAPTER 1: General introduction

1.1. Oil sands as a source of unconventional oil

The International Energy Agency reports that global energy consumption will increase by 53% from 2008 to 2035 (USEIA, 2011). Most of the growth in energy consumption will occur in countries outside the Organization for Economic Cooperation and Development (OECD). Energy consumption in non-OECD countries will increase by 85%, compared with an increase of 18% in OECD countries, from 2008 to 2035 (USEIA, 2011). Currently, approximately 85% of global energy consumption is supplied by fossil fuels (USEIA, 2011). However, with the decline of reserves of conventional crude oil, the development and production of unconventional oil is increasing rapidly.

Deposits of oil sands are an important source of unconventional oil. Oil sands are loose or partially consolidated sandstone containing sands, clay, water, and a viscous form of petroleum referred to as bitumen. The volume of oil contained within oil sands worldwide are estimated at 250 billion barrels, which is approximately 10% of the volume of oil contained within discovered deposits of conventional oil. Most of the oil sands deposits are in Canada (177 billion barrels), Kazakhstan (42 billion barrels), and Russia (28 billion barrels) (World Energy Council, 2010).

1.2. The oil sands industry in Alberta, Canada

In Canada, most of the oil sands deposits are located in northern Alberta. The oil sands deposits in the Athabasca Basin of northeastern Alberta and Northwestern Saskatchewan, which are regarded as a secure and safe source of petroleum for North America, are one of the largest reserves of oil in the world (Hunt, 1979). The total recoverable oil from these deposits is estimated to be 173.2 billion barrels (Government of Alberta, 2008). In 2000, Alberta's oil sands industry produced approximately 0.6 million barrels of marketable bitumen and crude oil per day (Alberta Energy and Utilities Board, 2004-2005), and production increased to an average of 1.2 million barrels per day in 2008 (Alberta Energy and Utilities Board, 2007-2008). Despite the downturn in world economy that began in 2008, development of oil sands continues to grow at a great pace due to the addition of several new projects, reflecting growing producer confidence. The Canadian Association of Petroleum Producers (CAPP) predicted that the production of oil from the Canadian oil sands would grow to 5 million barrels per day in 2030, and would account for over 80% of total crude oil production in Canada (Figure 1.1) (CAPP, 2012).

The oil sands industry has significant economic benefits for the province of Alberta, the entire country of Canada, as well as other countries. From 2000 to 2020, development of the oil sands and related activities are expected to contribute a total gross domestic product (GDP) of approximately CAN$885 billion to Canada and other countries. Of this total GDP impact, $789 billion (89%) will occur in Canada, which is 61% of Canadian GDP in 2004, and $634 billion (72%) would occur in Alberta, which is 339% of Alberta's GDP in 2004 (Timilsina et al., 2005). The oil sands industry generates great profits. In 2007, Royal Dutch Shell announced that in

2006 its Canadian oil sands unit made an after tax profit of CAN$21.75 per barrel, which was almost double of its worldwide profit per barrel based on conventional oil (Mortished, 2007). With the high price of crude oil, as well as the decline in global conventional oil supplies, expansion of production of oil from bitumen in the major reserves in the Alberta Basin will continue (Williams, 2003, Alberta Energy and Utilities Board, 2006-2007).

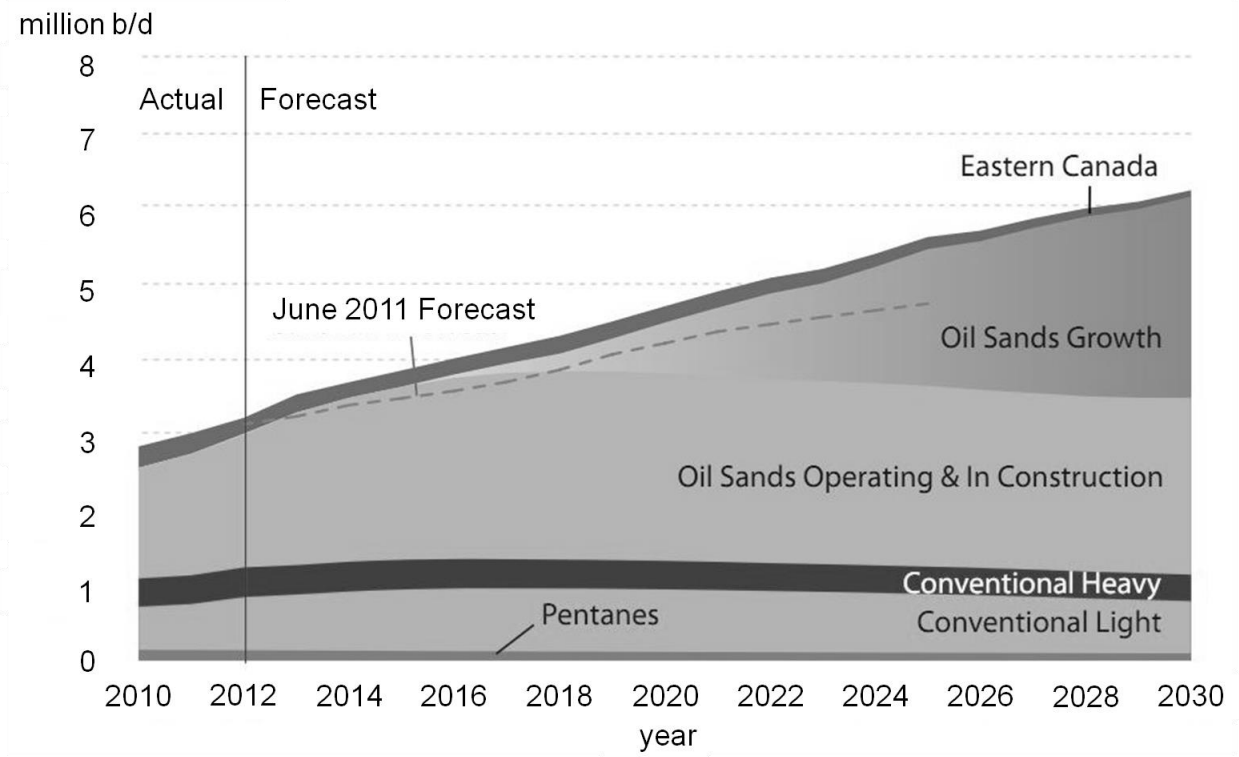


Figure 1.1. Projected crude oil production in Canada. Despites the resurgence of conventional oil production, supplies from the oil sands will continue to comprise the bulk of the predicted future increases in overall crude oil production. The oil sands projects that are currently in operation or under construction account for the growth until 2015 or 2016. Figure adapted from "Crude oil, forecast, market and pipelines" prepared by Canadian Association of Petroleum Producers (CAPP), June 2012. With permission. b/d = barrels per day.

1.3. Environmental issues - land use and emissions of greenhouse gases

Despite the great economic benefits, development and expansion of the oil sands industry is controversial. Due to the high viscosity of bitumen, oil sands must be extracted by surface mining, or the oil made to flow into wells by in situ techniques, depending on the proximity of the resource to the surface. Surface mining extracts bitumen from deposits that are less than 75 m from the ground, while *in situ* extraction is used for deposits that are greater than 75 m below the surface. About 20% of the deposits of oil sands in Alberta, covering 3% of total oil sands surface area, are suitable for surface mining (Government of Alberta, 2012). Advances in extraction using *in situ* techniques such as steam injection processes have started to replace the traditional surface mining process. However, the majority of bitumen is still recovered by surface mining practices that require the clearing of forest and muskeg as well as removing the overburden including topsoil, sand, clay and gravel from the oil sands deposit, resulting in the destruction of habitat for aquatic and terrestrial organisms. The surface mining area is estimated to be 4,800 km^2, 715 km^2 of which has been disturbed by current operations (Government of Alberta, 2011). The government of Alberta requires the oil sands companies to implement a land reclamation plan. However, as of 2011, only 1.04 km^2 of disturbed land is certified reclaimed, and 71 km^2 of disturbed land is in the process of being reclamation. At the current pace, reclamation will take decades to complete (Government of Alberta, 2011).

A major concern associated with the development of the deposits of oil sands is the emission of great quantities of greenhouse gasses (GHG). The production of bitumen and synthetic crude oil from oil sands emits more greenhouse gas compared to the production of

conventional oil. Alberta's oil sands account for about 6.5% of Canada's GHG emission, and this number will increase to 8% in 2015 (CAPP, 2008, Government of Alberta, 2011). A report from Cambridge Energy Research Associates found that the average well-to-wheels life-cycle greenhouse gas emissions from oil sands products is about 6% higher than the average of other crude consumed in the United States (IHS CERA, 2010, Government of Alberta, 2011).

1.4. Effects on quantity and quality of freshwater - Oil sands process-affected water (OSPW)

Mining of deposits of oil sands and the upgrading of bitumen negatively affect both the quantity and quality of freshwater. In the surface mining oil sands industry, the extraction of bitumen from oil sands involves the Clark hot water extraction method. Currently, this method requires 2 to 4 volume units of water to produce each volume unit of recoverable oil from oil sands (Holowenko et al., 2002). The freshwater used in the extraction of bitumen is supplied from the Athabasca River (Figure 1.2). The oil sands industry is allowed to allocate about 1.8% of the annual flow volume of the Athabasca River, which equates to 359 million m^3 of water per year. This is greater than twice the annual requirement for water of the city of Calgary (Griffiths et al., 2006). The actual volume of water withdrawn in 2002 was 114 million m^3, and this volume decreased to 73 million m^3 in 2007, despite a 50% increase in the production of bitumen (Government of Alberta, 2011). The majority of the growth in oil sands mining and decrease in freshwater usage during this time period were due to the establishment of *in situ* recovery projects. Since *in situ* projects recycle as much as 95% of the water, and use deep underground

saline water instead of fresh groundwater wherever possible, with the advances in extraction technologies, the amount of water used by oil sands industry continues to decrease (Government of Alberta, 2011).

The extraction of bitumen from oil sands using the Clarke hot water extraction process results in the production of process waters that contain sand, clay, unrecoverable bitumen, hydrocarbons, and a water soluble organic acid fraction containing a group of organic compounds generally known as naphthenic acids (NAs). This water is commonly referred to as oil sands process-affected water (OSPW). OSPW is not intentionally released in accordance with a zero discharge policy (MacKinnon, 1989, FTFC, 1995). Instead, OSPW is stored on-site in active settling basins, or tailing ponds, and the clarified fraction can eventually be recycled for use in the extraction process. Despite reducing the amount of freshwater required for extraction, this recycling process affects the quality of water stored in tailings ponds by concentrating the organic and inorganic components in OSPW. As of 2006, greater than one billion m^3 OSPW was being stored in on site settling basins (Del Rio et al., 2006) and this number continues to grow as the surface mining industry expands. Ultimately, this OSPW needs to be remediated and reclaimed either as viable aquatic habitats or released to the receiving environment. This presents a major challenge for both industrial and academic communities.

Figure 1.2. An aerial view of Athabasca oil sands mining and extraction facilities with adjacent oil sands process-affected water (OSPW) tailing pond and the Athabasca River. Image by NASA Earth observatory. Jesse Allen and Robert Simmon using E0-1 ALI data courtesy of the NASA E0-1 team. Caption by Holli Riebeek. Available at (http://earthobservatory.nasa.gov/IOTD/view.php?id=40997) (access September 20, 2012).

1.5. Chemistry of oil sands process-affected water (OSPW)

It has been reported that oil sands operation releases polycyclic aromatic hydrocarbons (PAHs) into aquatic and terrestrial environments (Kelly et al., 2009). Although PAHs are natural components and derived from the erosion of natural bitumen on the river banks and bed in the Athabasca River area, it is suggested that fishes and other aquatic wildlife may also be exposed to additional PAHs resulting from the liberation of oil sands mining practices or emissions from refining facilities (Kelly et al., 2009). In addition, the oil sands practices also release heavy metals, via air and water, to the Athabasca River and its watershed. Seven heavy metals including cadmium, copper, lead, mercury, nickel, silver, and zinc were detected in excess of Canada's guidelines for the protection of aquatic life in melted snow and/or collected near or downstream of the development facilities (Kelly et al., 2010).

However, it has been suggested, but not unequivocally validated, that naphthenic acids, or their naphthenate salts, are the primary toxic constituents of OSPW (Dokholyan and Magomedov, 1984, MacKinnon and Boerger, 1986, Holowenko et al. 2002, Frank et al., 2008). The classical definition of NAs is "a group of naturally-occurring acyclic, monocyclic, and polycyclic carboxylic acids with the general formula of $C_nH_{2n+Z}O_2$, where n represents the number of carbons and Z is zero or a negative even integer related to the number of rings in the molecule" (Brient et al. 1995, Grewer et al., 2010). Greater than one isomer can exist with a given "Z" series. Characterization of the spectrum of classical NAs based on the above formula has been intensively studied for many years (Holowenko et al., 2002, Del Rio et al. 2006, Martin et al., 2008, Headley et al., 2009a, b).

Recently, it has been suggested that the classical definition of naphthenic acids is not appropriate to describe the NAs in OSPW. The classical formula $C_nH_{2n+Z}O_2$ only accounts for a limited proportion of the polar fraction of extractable organic compounds in OSPW. In addition to NAs that can be described by the formula of $C_nH_{2n+Z}O_2$, "oxy-naphthenic acids" with the formula of $C_nH_{2n+Z}O_x$ (x from 3 to 7) have been detected in various samples of OSPW (Han et al., 2009, Headley et al., 2009b, Grewer et al., 2010). Heteratomic species containing SO(2), SO(3), SO(4), SO(5), SO(6), and NO(4) have also been reported to be present in organic acid extracts from the Athabasca oil sands (Headley et al., 2009b). Recently, identification of numerous individual diamondoid acids, which refers to the organic acids with 3-dimension structures resembling diamonds, including tri-, tetra- and pentacyclic NAs as well as aromatic carboxylic acids in OSPW has been accomplished using comprehensive two dimensional gas chromatography / time-off light- mass spectrometry (GC x GC/ToF-MS) (Rowland et al., 2011a, b, c, d).

A new definition of NAs that more accurately describes the acid extractable fraction of OSPW has been proposed. The new definition encompasses both classical and oxy-naphthenic acids with formula $C_nH_{2n+Z}O_x$, along with the unsaturated groups, the nitrogen and sulphur containing species $C_nH_{2n+Z}SO_x$ and $C_nH_{2n+Z}NO_x$, and the aromatic carboxylic acids especially the steroidal naphthenic acids. Such extensions expand the scope (almost double in abundance) of the extractable organic acids compared to classical naphthenic acids that need to be considered in the identification, characterization, measurement, biodegradation, and toxicity of OSPW, as well as the individual species and/or fractions of extractable organic acids from OSPW. In addition, NAs with steroid-like structures might be particularly important since these species might be the

part of the origin of the estrogenic / endocrine disruptive properties of OSPW. Moreover, the profile of classical/oxy-naphthenic acids ratio, as well as the degree of hydrogen deficiency among the classical, oxy-, nitrogen-, and sulphur- containing naphthenic acids might provide useful information on routine monitoring, reclamation, regulation, and management of OSPW that might be released to the receiving environment in the future (Han et al., 2009, Headley et al., 2009b, Grewer et al., 2010, Rowland et al., 2011a, b, c, d).

1.6. Toxicity of oil sands process-affected water (OSPW)

Fresh OSPW is acutely toxic to a range of aquatic organisms, including rainbow trout (*Oncorhynchus mykiss*) and green algae (*Pesudokirchneriella subcapitata*) (MacKinnon and Boerger, 1986, Warith and Yong, 1994, Clemente and Fedorak, 2005). However, the mechanisms of subchronic toxicity of OSPW are not known. It has been suggested that the toxicity of OSPW is mainly due to the dissolved organic fraction, in particular the NAs, among which the lower molecular weight NAs are suggested to be most toxic (Holowenko et al., 2002, Frank et al., 2008, 2009). Because NAs are surfactants - owing to the presence of hydrophobic alkyl groups and a hydrophilic carboxylic moiety - this had lead to speculation that NAs do not bind to cellular receptors but exert their mode of acute toxicity via narcosis (Lipnick, 1993, Roberts, 1991). The results of several recent studies indicate that the acute toxicity is related to the molecular weight and structure of NAs. The acute toxicity of NAs decreased with increased carboxylic acid content within NA structures decreases hydrophobicity (Frank et al., 2008, 2009).

Oil sands process-affected water has many non-lethal effects on aquatic organisms. Several studies demonstrated effects of OSPW or oil sands sediments on embryos of various species of fish, including fathead minnows (*Pimephales promelas*), white sucker (*Catostomus commersoni*), yellow perch (*Perca flavescens*), or Japanese medaka (*Oryzias laptipes*) (He et al., 2012a, Colavecchia, et al., 2004, 2006, 2007, Peters et al., 2007). Embryos exposed to these treatments typically displayed greater mortality, premature or delayed hatch, greater spontaneous movement, greater incidences of malformation of the spine, pericardial edema, and hemorrhaging. Exposure to OSPW might also have effects on the immune system. Exposure to organic fraction of OSPW resulted in decreased pro-inflammatory gene expression and macrophage antimicrobial responses in mouse bone marrow-derived macrophages (BMDM) and mice (Garcia-Garcia et al., 2011a, b).

Several studies have demonstrated that OSPW has endocrine disruptive properties (van den Heuvel et al., 1999, Lister et al., 2008, Kavanagh et al., 2011). Exposure to OSPW caused endocrine disrupting effects *in vitro* on sex hormones synthesis and receptor signaling (He et al., 2010, 2011). The abundances of transcripts of regulatory genes in all tissues of the hypothalamus-pituitary-gonad-liver axis were also significantly affected in fathead minnows (*P. promelas*) exposed to OSPW (He et al., 2012b). Exposure to OSPW also adversely affected the reproductive capacity of fathead minnows. Fecundity was less, synthesis of sex steroids was altered, and less pronounced secondary sex characteristics of male and female minnows were observed in fathead minnows exposed to OSPW (Kavanagh et al., 2011, 2012). It is unknown which components of OSPW are responsible for these adverse effects; however, some NAs that are structurally similar to sex steroid hormones have been identified as potential candidate

contaminants for these effects in OSPW (Rowland et al., 2011a, Scarlett et al., 2012). Furthermore, a study using hepatocytes isolated from livers from rainbow trout revealed that exposure to OSPW resulted in greater expression of genes related to estrogenicity, including the induction of estrogen receptor (ER) and vitellogenin (VTG) (Gagné et al., 2012).

The endocrine disruptive properties of OSPW are a source of great concern. The OSPW that is currently stored in tailings ponds ultimately needs to be released back to the environment or reclaimed as viable habitat, such as lake ecosystems. However endocrine disrupting chemicals in OSPW will definitely affect the water quality, not only threatening the fishery downstream, but also the safety of municipal water as well. The creation of healthy lake systems from OSPW tailing ponds is also unlikely, because the organisms such as fish and benthos will be exposed to endocrine disruptive chemicals, resulting in impaired growth, development, and reproduction. Therefore, a healthy food chain is unlikely to exist in this environment.

1.7. Remediation of oil sands process-affected water (OSPW)

Companies operating in the mining and upgrading of bitumen are required, under provincial legislation, to remediate and reclaim the great volumes of OSPW that are stored in tailings ponds. However, currently there are no proven methods to accomplish remediation and reclamation. Field studies have demonstrated that OSPW degraded in tailings ponds for as little as one month were less lethal to fathead minnows (MacKinnon et al., 1986). However, while removal of acute toxicity is achievable, chronic toxicity is not completely eliminated (Leung et al.

2001). A fraction of NAs in OSPW can be partially removed through aerobic microbial degradation under laboratory conditions (Herman et al. 1993, Scott et al. 2005, Han et al. 2008), but the extent of degradation of NAs in the field is slower. At best, the disappearance half-life for NAs in OSPW is 12.8-13.6 yr (Han et al. 2009). Consequently, more aggressive efforts are being tested for the remediation of OSPW.

A laboratory scale microwave system was developed for rapid degradation of NAs. Using this system the half-life for degradation of NAs in OSPW and commercial NAs were 3.32 and 3.61 h, respectively. In a Microtox® assay, the microwave system reduced toxicity of water containing commercial NAs, but a slight increase in toxicity of NAs in OSPW was observed (Mishra et al., 2010). The presence of species of NAs with relatively high O(x) content in ultraviolet- and microwave-treated samples indicates the resistance of the relatively high O(x) content against oxidation of the parent acids in the treated samples (Headley et al., 2010). A combination treatment of ultraviolet with further ozonation by adding hydrogen peroxide was also in development for degradation of NAs present in OSPW (Drzewicz et al., 2010).

1.7.1. Remediation of oil sands process-affected water (OSPW) using ozonation

One method that has shown promise for attenuating the toxicity of OSPW is ozonation. Ozonation is commonly used as a process to decontaminate water. It is increasingly used in the treatment of municipal wastewater effluents to improve quality and greater compliance with microbiological and physiochemical standards. The ozonation of municipal wastewater is likely

to produce low molecular weight organic intermediate compounds as byproducts, which may either decrease or increase the toxicity. A review of the effects of ozonation on toxicity of municipal effluent was presented by Paraskeva and Graham (2002).

Ozonation is a promising method for remediation of OSPW as it significantly reduces the concentration of NAs (Scott et al., 2008, Martin et al., 2010, Gamal El-Din et al., 2011). Analysis using GC-MS showed that ozonation mainly degraded the proportion of NAs with relatively high molecular weight. After 50 min of ozonation the concentrations of NAs in OSPW was decreased by 70% and after 130 min of ozonation concentrations of NAs was decreased by 95%. The resulting samples were not toxic in the Microtox® bioassay. It has to be mentioned that oxidized NAs, which are naturally present in untreated OSPW, are both degraded and formed during the ozonation process such that there is little net change in their total concentration (Han et al., 2008, Martin et al., 2010). Therefore, it is possible that ozonation may not attenuate the toxic effects related to oxidized NAs. In general, ozonation preferentially degrades the proportion of NAs in OSPW that are most persistent to biodegradation (Martin et al., 2010), suggesting that ozonation is complementary to the biodegradation and might be useful to accelerate the microbial degradation of OSPW.

Work is needed to fully evaluate the effectiveness of ozonation in reducing the toxicity of OSPW and to ensure that ozonation does not cause the formation of byproducts that might impart greater toxicity to the OSPW. In the studies to date, OSPW that has been treated with ozone is generally less toxic *in vivo* and *in vitro*. Ozonation attenuates all the adverse endocrine disrupting effects in the H295R cells (He et al., 2010). Ozone treatment can ameliorate the toxic

effects of the organic fraction of OSPW on the immune system of mice (Garcia-Garcia et al., 2011c). Ozonation is also reported to attenuate effects of OSPW on the growth and development of the non biting midge, *Chironomus dilutus*, (Anderson et al., 2012b) and has lesser effects on the development of embryos of fathead minnows (He et al., 2012a). However, there is concern regarding the possibility of formation of estrogenic oxidized NAs during ozonation of OSPW based on the findings that ozonation partially mitigates the potential anti-androgenic effect in MDA-kb2 cells, but not the estrogenic effect in the T47D-kbluc cells (He et al., 2011).

1.8. Use of immortal cell lines in toxicity testing

Immortal cell lines are commonly used as simple systems in toxicology. The results from studies using these assay systems might be extrapolated to whole organisms and can inform the design of studies with whole organisms. The main advantage of using an immortal cell line for scientific research is that the cells can be grown indefinitely in culture. Compared to the exposure experiment designed based on animal models, which have a limited lifetime, cell culture assays developed based on immortal cell lines are relatively rapid, accurate, high throughput, mechanistically based and cost effective (Zhang et al. 2010). Immortal cell lines were used in two of the studies presented in this thesis to investigate the potential endocrine disruptive properties and the mechanisms of the potential effects of OSPW and ozone-treated OSPW.

1.8.1. The H295R cell line

The H295R cell line was derived from a human female adrenocortical carcinoma. It produces steroid hormones including estrogens, androgens, progestins, glucocorticoids, and mineralocorticoids, and as such, expresses the full suite of enzymes responsible for the synthesis of these hormones. Bioassays used to evaluate endocrine disruptive effects of chemicals on a number of endpoints including gene expression, catalytic enzyme activities, and steroid hormone production have been established for this cell line (Hilscherova et al., 2004, Zhang et al., 2005, Hecker et al., 2006, 2007, Villeneuve et al., 2007, He et al., 2008).

1.8.2. The T47D-kbluc cell line

The T47D-kbluc cell line was derived from human breast cancer cells, which naturally express estrogen receptor (ER) alpha and beta. The cell line was established by stable transfection with a triplet estrogen-responsive element (ERE)-promoter-luciferase reporter gene construct. The T47D-kbluc cells are very sensitive to estrogens, and the antiestrogen, ICI 182,780 is able to completely inhibit expression of luciferase. The T47D-kbluc cell line has been used to screen large numbers of chemicals for estrogenic and antiestrogenic activity (Wilson et al., 2004).

1.8.3. The MDA-kb2 cell line

The MDA-kb2 cell line was derived from the breast cancer cell line MDA-MB-453, which was stably transformed with a mouse mammary tumor virus (MMTV) luciferase reporter gene construct. Since MDA-kb2 cells express both androgen receptor (AR) and glucocorticoid receptor (GR), and both receptors can act through the MMTV promoter, chemicals that bind to

either AR or GR can activate the MMTV luciferase reporter. Therefore, the MDA-kb2 cell line can be used to screen agonists and antagonists and to determine the specificity and sensitivity of the endocrine disruptive properties of chemicals for both AR- and GR-mediated activities (Wilson, et al., 2002).

1.9. Fathead minnow (*Pimephales promelas*) is an ideal small fish model to investigate the toxicity of oil sands process-affected water (OSPW)

The fathead minnow is a member of the ecologically important cyprinidae family, that is broadly distributed in both lotic and lentic environments throughout North America. This species is an opportunistic omnivore, and tolerant to a wide range of basic water quality characteristics including pH, alkalinity/hardness, turbidity, and temperature. The adult male fathead minnow is larger than the female (3~5 g versus 2~3 g, respectively) and, when reproductively active, males exhibit several secondary sex characteristics (e.g., dark banding, dorsal pad, and nuptial tubercles) not normally seen in females (USEPA, 1987). The development of fathead minnow embryos has been characterized. Embryos that have been fertilized proceed through several well-defined developmental stages, and are easily viewed through the transparent egg. Embryos hatch within 4-5 d after fertilization (at 25 ℃). The embryo assay is easy to perform and is ideal for high through-put and measuring multiple endpoints. This is an ecologically relevant assay since the development of embryos is important for the establishment of fish populations. In addition, through high throughput sequencing of the transcriptome of the fathead minnow, as well as sequences available in NCBI, it is easy to

perform quantification of gene expression in this species. In the *in vivo* part of the research in this thesis, the fathead minnow was used as animal model for testing the toxicity of OSPW on endocrine disruption (Chapter 4). Potential toxicity of OSPW to embryos of fathead minnow was also determined (Chapter 5). In addition, the efficacy of ozonation to attenuate the toxicity of OSPW was also assessed.

1.10. Objectives

The overall objective of the research in this PhD thesis was to identify the toxicity of OSPW using *in vitro* and *in vivo* assays and to determine whether ozonation of OSPW attenuates the toxicity. Several approaches were utilized to achieve these goals. The endocrine disrupting effects of fresh OSPW and ozone-treated OSPW were determined *in vitro* and *in vivo* using the H295R, MDA-kb3, and T47D-kbluc cells lines, as well as fathead minnows (*Pimephales promelas*). In addition, the embryotoxic effects of untreated and ozone-treated OSPW were determined in embryos of fathead minnows. Overall, the null hypotheses that were tested were: (1) OSPW does not have endocrine disrupting effect either *in vitro* or *in vivo*, and is not toxic to embryos of fathead minnows; and (2) there is no difference in the endocrine disrupting and embryotoxic effects of untreated OSPW and ozone-treated OSPW. The specific research objectives and the experimental approaches employed are outlined below:

1. **Ozonation attenuates the steroidogenic disruptive effects of sediment free oil sands process-affected water in the H295R cell line (Chapter 2)**. There is concern regarding

the endocrine disruptive properties of OSPW. One of the most important endpoints for endocrine disruption is the production of sex steroid hormones. Although ozonation significantly reduces concentrations of NAs in OSPW, and the acute toxicity of OSPW, it was hypothesized that oxidation of OSPW might produce hydroxylated products with steroidogenic activity. Therefore, untreated and ozone-treated OSPW were examined for effects on sex steroid production using the H295R Steroidogenesis Assay. The H295R cells were exposed to untreated and two different degree of ozone-treated OSPWs (24% O3-OSPW and 85% O3-OSPW). The analyzed endpoints included fold change of concentrations of testosterone (T) and 17β-estradiol (E2), CYP19a (aromatase) mRNA abundance and enzyme activity, and the rate of E2 metabolism. Therefore, objectives of this study were to: (1) assess the effects of untreated sediment free OSPW on sex hormone production using the H295R cell line; and (2) to determine if these baseline effects are modulated by ozone treatment of OSPW.

2. **Effect of ozonation on the estrogenicity and androgenicity of oil sands process-affected water (Chapter 3)**. The specific mechanism(s) of action of OSPW and the component NAs, in particular on the endocrine axis, are not well understood to date. Receptor-mediated estrogenicity and (anti)androgenicity have been reported for some crude oil and offshore platform effluent samples. It is necessary to study the potential receptor-mediated estrogenicity and androgenicity of OSPW and ozone-treated OSPW. In this study, the estrogen receptor- (ER) and androgen receptor- (AR) mediated effects of OSPW and ozone-treated OSPW were investigated *in vitro* using T47D-kbluc and

MDA-kb2 reporter cell assays, respectively. The objectives of this study were to determine: (1) the estrogenicity OSPW via the receptor mediated signaling pathway by use of the T47D-kbluc (estrogen responsive) cell line; (2) the *in vitro* androgenicity of OSPW via the receptor mediated signaling pathway by use of the MDA-kb2 (androgen responsive) cell line; and (3) whether baseline effects of OSPW are modulated by ozonation of OSPW.

3. **Transcriptional responses of the brain−gonad−liver axis of fathead minnows exposed to untreated and ozone-treated oil sands process-affected water (Chapter 4)**. Altered concentrations of sex steroid hormones, impaired reproductive performance, and less prominent secondary sexual characteristics have been reported for fish exposed to OSPW. However, the mechanism(s) by which these effects occur and whether ozonation can attenuate these effects in fish was unknown. The objectives of this *in vivo* study were to investigate the endocrine-disrupting effects of OSPW and ozone-treated OSPW by quantifying abundances of transcripts of genes in the brain-gonad-liver (BGL) axis in male and female fathead minnows (*P. promelas*). To this end it was determined: (1) whether OSPW has endocrine-disrupting effects at all levels of the BGL axis; (2) whether OSPW has the same or different effects in male and female fathead minnows; (3) whether ozonation attenuates the effects of OSPW on abundances of transcripts; and (4) whether ozonation has the same or different attenuating effects in male and female fathead minnows.

4. **Toxicity of untreated and ozone-treated oil sands process-affected water (OSPW) to early life stages of the fathead minnow (*Pimephales promelas*) (Chapter 5)**. Several studies demonstrated effects of OSPW or oil sands sediments on embryos of various species of fish. However, the effects of OSPW on the survival, growth, and development of embryos from fish were not well understood. Moreover, there is no information regarding the effects of ozone-treated OSPW on these endpoints. Success of hatching of eggs, spontaneous movement, and incidences of hemorrhage, pericardial edema, and malformations of the spine were examined. The concentrations of reactive oxygen species (ROS) in embryos exposed to OSPWs were also measured, and the abundances of transcripts of genes involved in biotransformation of xenobiotics, response to oxidative stress, and apoptosis were quantified by real-time PCR. The objectives of this study were to: (1) examine the effects of untreated, ozone-treated, and activated charcoal-treated OSPW (OSPW, O3-OSPW, and AC-OSPW) on the early life stage (ELS) of fathead minnow (*P. promelas*); (2) to elucidate the mechanism of the embryotoxic effects caused by OSPWs; and (3) to determine whether the ozonation attenuate the embryotoxic effects.

CHAPTER 2: Ozonation attenuates the steroidogenic disruptive effects of sediment free oil sands process-affected water in the H295R cell line [1]

[1] This chapter has been published in Chemosphere (2010) Volume 80, Issue 5, Pages 578-584, with minor modification. To investigate the endocrine disrupting effects of untreated and ozone-treated oil sands process-affected water (OSPW and O3-OSPW) on sex steroids production, the objectives of this study were to: (1) assess the effects of untreated sediment free OSPW on sex hormone production using the H295R cell line; and (2) to determine if these baseline effects are modulated by ozone treatment of OSPW. The entire study, including ethics approval security, experimental design, data collection and analyses, and manuscript writing, was conducted by Yuhe He supervised by co-authors Steve B. Wiseman (University of Saskatchewan) and John P. Giesy (University of Saskatchewan). Co-authors Xiaowei Zhang (University of Saskatchewan), Markus Hecker (University of Saskatchewan), and Paul D. Jones (University of Saskatchewan) made valuable comments on manuscript writing. Co-author Mohamed Gamal El-Din (University of Alberta) and Jonathan W. Martin (University of Alberta) cooperated on providing analytical chemistry information related to this study and made valuable comments on manuscript writing.

2.1. Introduction

The oil sands deposits in the Athabasca Basin, located in northeastern Alberta, Canada, are one of the largest reserves of petroleum in the world (Hunt, 1979), and are increasingly being developed by a rapidly growing oil sands industry. In 2000, Alberta's oil sands industry produced approximately 604,700 barrels of marketable bitumen and crude oil per day (Alberta Energy and Utilities Board, 2004-2005), and production increased to an average of 1,184,000 barrels per day in 2008 (Alberta Energy and Utilities Board, 2007-2008). With the costs associated with bitumen recovery from oil sands decreasing due to technological advances, and a decline in global conventional oil supplies, development of the major reserves in the Alberta Basin, which are estimated to be at least 173.2 billion barrels, is expected to continue (Williams, 2003, Alberta Energy and Utilities Board, 2006-2007).

In the surface mining oil sands industry, extraction of bitumen from oil sands involves the Clarke hot water extraction method. This process results in the production of large volumes of process-affected waters that contain sand, clay, unrecoverable bitumen, hydrocarbons, and a water soluble organic acid fraction known commonly as naphthenic acids (NAs). This wastewater is commonly referred to oil sands process-affected water (OSPW). Oil sands process-affected water is stored on-site in active settling basins, or tailing ponds, where the clarified fraction can eventually be recycled back into the extraction plant for further use. In accordance with a zero discharge policy OSPW is not intentionally released (MacKinnon, 1989, FTFC, 1995) and in 2006 it was estimated that greater than 10^9 m^3 OSPW were currently being stored on-site in various settling basins (Del Rio et al., 2006).

Naphthenic acids, or their naphthenate salts, have been identified as the primary toxic constituents of OSPW (Dokholyan and Magomedov, 1984, MacKinnon and Boerger, 1986, Holowenko et al., 2002). Naphthenic acids are a group of naturally-occurring acyclic, monocyclic, and polycyclic carboxylic acids with the general formula of $C_nH_{2n+Z}O_2$, where n represents the number of carbon atoms and Z is zero or a negative even integer related to the number of rings (or double bond equivalents) in the molecule (Brient et al., 1995). Oil sands process-affected water has been found to be toxic to a range of aquatic organisms, including rainbow trout (*Oncorhynchus mykiss*), *Daphnia magna*, and *Vibrio fischeri* (reviewed in Clemente and Fedorak, 2005) as well as the green alga (*Pesudokirchneriella subcapitata*) (Warith and Yong, 1994). The toxicity of OSPW is mainly associated with lower molecular weight NAs (Holowenko et al., 2002, Frank et al., 2008). However, a recent study suggested that increased carboxylic acid content within NAs structures decreases hydrophobicity and, consequently, the toxicity of higher molecular weights NAs (Frank et al., 2008). The mechanism of toxicity of NAs from various sources is the subject of some debate and has received little attention. However, NAs are surfactants - owing to the presence of hydrophobic alkyl groups and a hydrophilic carboxylic moiety. This had lead to speculation, and some experimental evidence, that NAs do not bind to cellular receptors but rather exert their mode of acute toxicity via physical disruption of membranes, or narcosis (Lipnick, 1993, Frank et al., 2008).

Ultimately, there is a long-term need to reclaim the large volumes of toxic OSPW, but currently there are no proven methods to do so. Field studies have demonstrated that OSPW confined to tailings ponds for as little as one month was less lethal to fathead minnows (MacKinnon and Boerger, 1986). However, while removal of acute toxicity is achievable,

chronic toxicity is not completely removed (Leung et al., 2001). Previous studies have also demonstrated that a certain fraction of OSPW NAs can be partially removed through aerobic microbial degradation under laboratory conditions (Herman et al., 1993, Scott et al., 2005, Han et al., 2009), but the extent of NAs degradation in the field is less. At best, the disappearance half-lives for NAs in OSPW are 12.8-13.6 years (Han et al., 2009). Consequently, more aggressive efforts are being tested for the remediation of OSPW. One method that has already shown promise is ozonation (Scott et al., 2008). Ozonation of OSPW decreases NAs concentrations and results in an effluent that is effectively non-toxic in the acute Microtox® microbial toxicity assay. However, little is known about the effectiveness of ozone treatment with respect to toxicity towards other organisms. Ozonation of other matrixes has been shown to change the activity of treated molecules, and in some cases results in compounds that can modulate the endocrine system (Esplugas et al., 2007, Petala et al., 2008).

Little is known about the impact of OSPW and NAs in particular on the endocrine axis, and specifically the process of sex hormone production known as steroidogenesis. Significant decreases in plasma T and E2 have been reported in goldfish (*Carassius auratus*) (Lister et al., 2008) and yellow perch (*Perca flavescens*) (van den Heuvel et al., 1999) exposed to OSPW. Reduced *in vitro* production of T and E2 by ovarian and testicular tissues was also reported in goldfish exposed to OSPW (Lister et al., 2008). Therefore, the objectives of this study were to: (1) assess the cellular toxicity and steroidogenic effects of untreated sediment free OSPW on sex hormone production using the H295R cell line and (2) to determine if these baseline effects are modulated by ozone treatment.

The H295R cell line expresses all key enzymes involved in steroidogenic pathways, and bioassays used to evaluate effects of chemicals on gene expression, enzyme catalytic activities, and steroid hormone production have been established (Hilscherova et al., 2004, Zhang et al., 2005, Hecker et al., 2006, 2007, Villeneuve et al., 2007, He et al., 2008). In the present study, T and E2 production, E2 metabolism, and aromatase (CYP19a) gene expression and enzyme activity were also measured to investigate the effect(s) that untreated and ozonated OSPW had on steroidogenesis in the H295R cell line.

2.2. Materials and methods

2.2.1. Oil sands process-affected water (OSPW) collection and treatment

Oil sands process-affected water was collected in December 2007 from the West-in-Pit, an active settling basin on the site of Syncrude Canada Ltd. (Fort McMurray, AB, Canada). Three 1-L batches of OSPW were filtered through a 0.45 μm cellulose membrane filter (Osmonics, Inc., Minnetonka, MN). Filtering of OSPW does not remove NAs. One batch was left untreated (control) while the two other batches were each placed in 2-L glass bottles for treatment by ozonation. Ozone was bubbled through the OSPW at a rate of 2 L/min and stopped occasionally to take samples for ultra-pressure liquid chromatography (UPLC) high-resolution mass spectrometry (HRMS) analysis of NAs (Bataineh et al., 2006). The ozonation treatment was continued until the total degradation of parent NAs in these two batches reached 24% and 85%, as determined by the remaining sum response of all UPLC-HRMS peak area corresponding to NAs. These ozonated samples will be referred to hereafter as 24% and 85% ozonated OSPW, respectively. An AGSO 30 Effizon® ozone generator (WEDECO AG Water Technology, Herford, Germany) was used to produce ozone gas from extra dry, high purity oxygen. All glassware was cleaned and pre-treated with ozone before experimentation. Ozonation was performed at room temperature in open glass bottles on a stir plate.

It has been previously suggested that NAs have relatively low potential to partition from water to air, with Henry's Law Constants estimated to be $<10^{-6}$ atmosphere $\times$ m^3 /mole, and bubbling of air through OSPW for 9 days resulted in only 7% reduction in naphthenic acid concentration (Han et al., 2009). Thus, physical loss of naphthenic acids during ozonation was

assumed to be negligible in the current work. The pH of the initial untreated OSPW was 8.1 and this value was not affected by the ozonation.

2.2.2. Oil sands process-affected water (OSPW) medium preparation

Exposure media were prepared according to standard cell culture medium recommendations, except that the nano-pure water was replaced with either untreated OSPW (OSPW), 24% ozonated OSPW (24% O3-OSPW) or 85% ozonated OSPW (85% O3-OSPW). Dilutions of exposure medium were prepared by diluting with medium prepared with nano-pure water. All exposure media was adjusted to pH 7.4 as this is optimal for hormone production (Hecker et al., 2006).

2.2.3. Experimental design

H295R cells were obtained from the American Type Culture Collection (ATCC CRL-2128, ATCC, Manassas, VA, USA), and cultured at 37 ℃ in a 5% CO_2 atmosphere (atm) according to previously described methods (Hecker et al., 2006). Briefly, the cell culture medium was a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 Nutrient mixture (SIGMA Chemical Co., St. Louis, MO, USA), supplemented with 2.5% Nu-Serum (BD Biosciences, San Jose, CA, USA), 1% ITS + Premix (BD Biosciences), and 1.2 g/L $NaHCO_3$. Prior to initiation of exposure experiments cells were plated in 24-well culture plates at a density of 3×10^5 cells/mL, and cells were allowed to attach for 24 h. After this pre-incubation period medium was replaced with test solutions. Cells were exposed to either 10,000-, 1000-, 100-, and

10-fold diluted sediment free OSPW or full-strength untreated sediment free OSPW, 24% O3-OSPW, or 85% O3-OSPW.

2.2.4. Cytotoxicity assay

Cytotoxicity of untreated and ozonated OSPW was assessed in 96-well plates using the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Mosman, 1983).

2.2.5. Hormone measurement

Quantification of T and E2 was conducted according to Hecker et al. (2006). After a 48 h exposure period, 500 mL of medium was extracted twice with 2.5 mL diethyl ether. The solvent phase containing target hormones was evaporated under a stream of nitrogen, and then reconstituted in 250 μL ELISA assay buffer (Cayman Chemical Company). Hormone concentrations were measured by competitive ELISA following the manufacturer's recommendations on a Dynex DSX® four-plate automated ELISA processing station (Dynex Technologies, Chantilly, VA, USA). Neither the untreated nor ozone-treated OSPW cross reacted with the ELISA assay.

2.2.6. Estradiol metabolism assay

A method was developed to measure the rate of E2 metabolism using the H295R cell line. Briefly, 1 mL of cell suspension with a density of 1×10^6 cells/mL was plated into 24-well culture plates and cells were allowed to attach for 24 h. After this pre-incubation period medium was replaced with 250 μL of OSPW medium spiked with 1 nM 6, 7 [3H]-E2 (PerkinElmer, Boston, MA, USA) was added and cells were incubated at 37 ℃ and 5% CO_2 for 1 h. At the end

of the exposure, 200 μL medium was collected and extracted with 500 μL DCM, and 100 μL supernatant was collected for liquid scintillation measurement using a Beckman LS6500 scintillation counter (Beckman Coulter, Inc., CA, USA). The rate of E2 metabolism was calculated by the fold change of degraded E2 in the spiked media compared with control groups.

2.2.7. Aromatase assay

The activity of CYP19a (aromatase) was determined based on the method described by Lephart and Simpson (1991). Briefly, to measure the indirect effects on aromatase activity 1 mL of cells were plated in 24-well culture plates at a density of 1×10^6 cells/mL for 24 h, and then the medium was replaced with media prepared with either full-strength or diluted untreated OSPW, or 24% ozonated OSPW, or 85% ozonated OSPW. After 24 h exposure, the OSPW medium was removed and cells were exposed to 54 nM [1β-^{3}H] androstenedione (PerkinElmer) in serum-free medium according to Sanderson et al. (2002). After 1 h exposure at 37 ℃ and 5% CO_2, 200 μL of culture medium was extracted and used for measuring the relative fold change in radioactivity.

2.2.8. Quantitative real-time polymerase chain reaction (qPCR)

Expression of *cyp19a* and the housekeeping gene porphobilinogen deaminase (*pbgd*) was measured by quantitative real-time PCR (qPCR) using the SYBR® green methods of Hilscherova et al. (2004) and Zhang et al. (2005). Briefly, cells were plated at a density of 1×10^6 cells/mL in 1 mL cell suspension per well of a 24-well culture plate. After 24 h, the medium was decanted and replaced with fresh media prepared with full-strength untreated OSPW, 24% O3-

OSPW, or 85% O3-OSPW for 1, 2, 4, 8, and 24 h. Following the exposure period cells were harvested and total RNA was isolated from cells harvested from each well using the Agilent Total RNA Isolation Mini Kit (Agilent, Santa Clara, CA, USA) following the manufacturers recommendations. RNA concentrations were determined at A260 using a ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The first strand cDNA was synthesized from 2 μg of total RNA using an iScript™ cDNA Synthesis kit (BioRad, Mississauga, ON, Canada) with an oligo dT primer according to the manufacturer's instructions. The ABI 7300 fast real-time PCR system (Applied Biosystems, Foster City, CA, USA) was used to perform quantitative real-time PCR. The primer sequences for *cyp19a* are

5'-AGGTGCTATTGGTCATCTGCTC-3' (sense) and

5'-TGGTGGAATCGGGTCTTTATGG-3' (antisense),

and the primer sequences for *pbdg* are

5'-CTGGAGGAGTCTGGAGTCTAG-3' (sense) and

5'-TGGAATGTTACGAGCAGT GATG-3' (antisense).

PCR reaction mixtures (20 μL) contained 1 μL of forward and reverse primers, 5 μL of cDNA sample, and 10μL of 2 × SYBR® green PCR Master Mix (Applied Biosystems). The thermal cycle profile was as follows: denaturation at 95 ℃ for 10 min, followed by 40 cycles of denaturation for 15 s at 95 ℃, annealing with extension for 1 min at 60 ℃; and a final cycle of 95 ℃ for 15 s, 60 ℃ for 1 min, and 95 ℃ for 15 s. Melting curve analyses were performed during the 60 ℃ stage of the final cycle to differentiate between desired PCR products and primer-dimers or DNA contaminants. The delta-delta Ct method (Livak and Schmittgen, 2001) was used to calculate the fold change in *cyp19a* expression relative to *pbdg*.

2.2.9. Statistical analyses

All experiments were conducted in duplicate, and triplicate measurements were used for each individual exposure. Statistical analyses were conducted using SPSS 16 (SPSS Inc., Chicago, IL, USA). The normality of each data set was assessed using the Kolomogrov-Smirnov one-sample test and homogeneity of variance was determined using the Levene's test. Significant differences were evaluated by one-way ANOVA followed by *post-hoc* Dunnett's tests. Linear regression was used to test the relationship between degree of ozonation of OSPW and either the decrease in T production or increase in E2 metabolism in cells exposed to full-strength untreated and ozonated OSPW. Differences with $\rho < 0.05$ were considered significant. All data are presented as mean $\pm$ standard deviation.

2.3. Results and discussion

2.3.1. Cytotoxicity of oil sands process-affected water (OSPW)

Exposure of H295R cells to either untreated OSPW, or 24% or 85% O3-OSPWdid not produce significant cytotoxicity at any of the dilutions used. The acute toxicity of OSPW has been demonstrated in a range of aquatic species (reviewed in Clemente and Fedorak, 2005). Using the Microtox® assay to assess acute toxicity, IC_{20} values of approximately 10% (v/v) OSPW have been reported by Mackinnon and Boerger (1986) and Scott et al. (2008). In the current study no significant acute cytotoxicity of sediment free fresh OSPW towards H295R cells was observed using the MTT assay. This result suggests that H295R cells are less sensitive to the cytotoxic effects of OSPW than *Vibrio fischeri*. Ozonation of OSPW did not increase the cytotoxicity of OSPW. This is consistent with results reported by Scott et al. (2008) that ozonation decreased the cytotoxicity of OSPW in the Microtox® assay.

2.3.2. Effects of oil sands process-affected water (OSPW) on sex hormone production

Generally, untreated OSPW decreased production of T and increased production of E2 after 48 h exposure (Figure. 2.1). T production was significantly decreased by 0.45-, 0.42-, 0.32-, and 0.26-fold with exposure to full-strength untreated, 24% and 85% ozonated OSPW, and 10-fold diluted untreated OSPW, respectively (Figure. 2.1a). Significantly greater E2 production was detected when cells were exposed to full-strength untreated (2.0-fold) and 24% O3-OSPW (1.7-fold), whereas exposure to full-strength 85% O3-OSPW did not affect E2 production at any dose (Figure. 2.1b). E2 production was also significantly increased in cells exposed to 24% O3-

OSPW diluted 10,000-fold, however, this result is likely an experimental anomaly as the effect was small and no significant effects were observed at dilutions of 1000-, 100-, and 10-fold (Figure. 2.1b). Linear regression analysis of hormone concentrations released by cells exposed to full-strength untreated, 24% ozonated or 85% O3-OSPW indicated a significant relationship between degree of ozonation and the decrease in T production (r^2 = 0.578). A significant relationship between degree of ozonation and the increase in E2 production (r^2 = 0.964) was also observed.

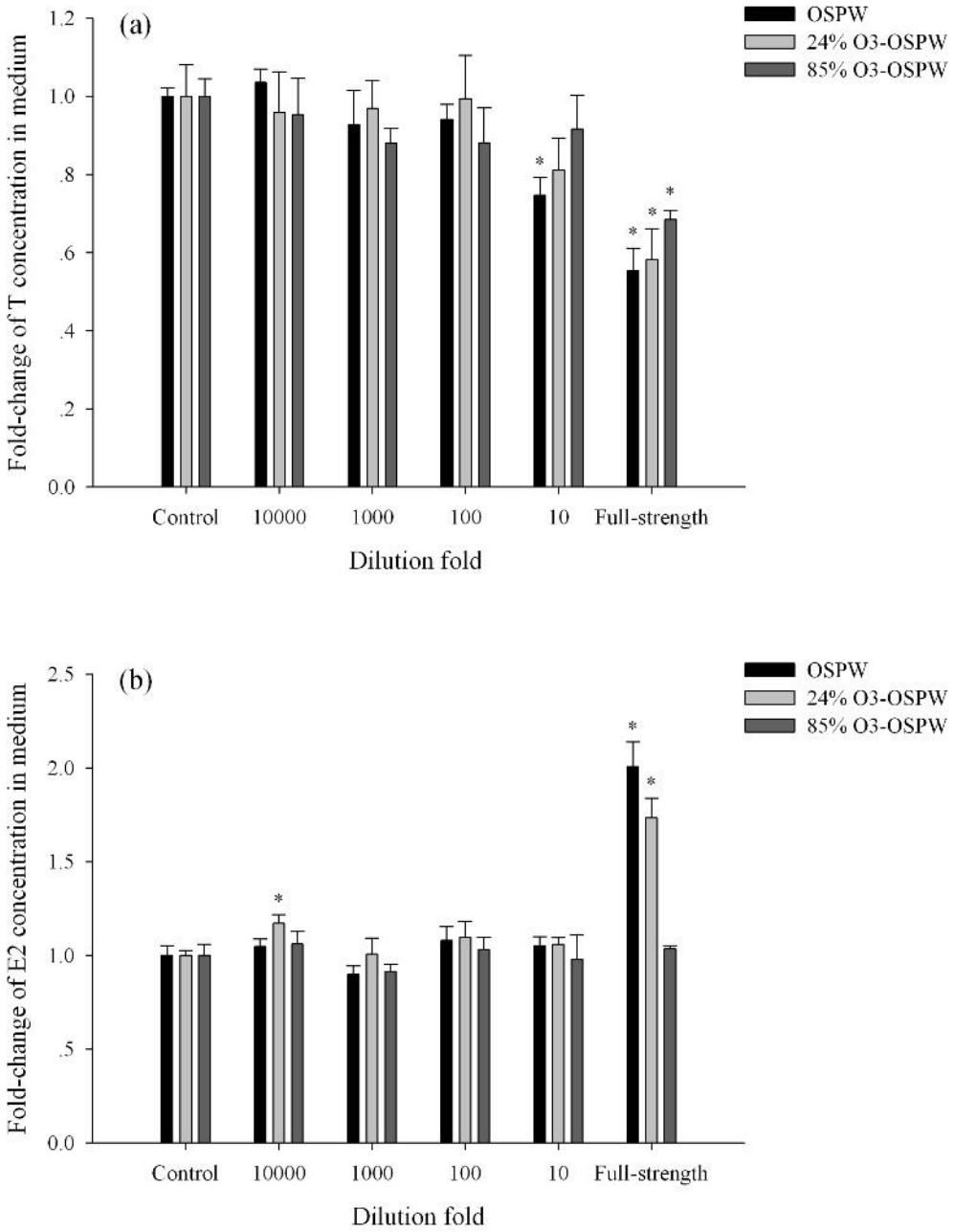


Figure 2.1. Concentrations of (a) testosterone (T) and (b) 17β-Estradiol (E2) in the culture medium of H295R cells exposed to untreated, 24% ozonated, and 85% ozonated oil sands

process-affected water (OSPW, 24% O3-OSPW, and 85% O3-OSPW) for 48 h. Fold changes are relative to the medium control group. Significant differences were evaluated with one-way ANOVA followed by *post-hoc* Dunnett's test. An asterisk indicates a significant change in response compared to the control group ($\rho < 0.05$).

2.3.3. Effects of oil sands process-affected water (OSPW) on E2 metabolism

For untreated OSPW, exposure to 100- and 10-fold diluted and full-strength significantly reduced the metabolism of E2 by 1.2, 1.4, and 2.3-fold, respectively. For 24% ozonated OSPW, exposure to 10-fold diluted and full-strength significantly reduced E2 metabolism by 1.2 and 1.6-fold, respectively. For 85% ozonated OSPW, exposure to full-strength did not impact E2 metabolism (Figure. 2.2).

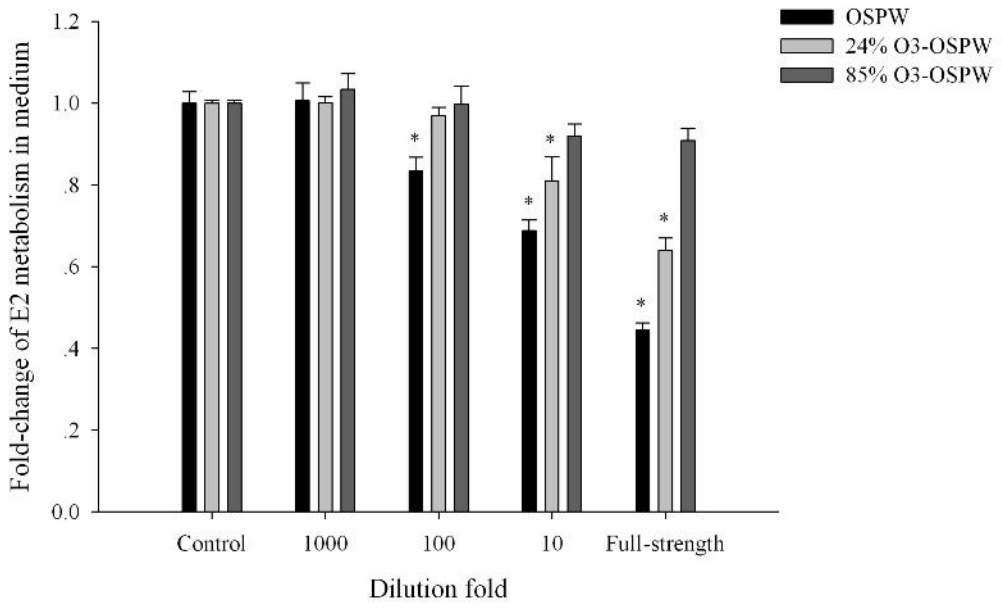


Figure 2.2. Effect of oil sands process-affected water (OSPW) on 17β-Estradiol (E2) metabolism in the H295R cell line exposed to untreated, 24% ozonated, and 85% ozonated oil sands process-affected water (OSPW, 24% O3-OSPW, and 85% O3-OSPW). Fold changes are relative to the medium control group. Significant differences were evaluated with one-way ANOVA followed by *post-hoc* Dunnett's test. An asterisk indicates a significant change in response compared to the control group ($\rho < 0.05$).

2.3.4. Effects of oil sands process-affected water (OSPW) on *cyp19a* mRNA expression and aromatase enzyme activity

Exposure to full-strength untreated OSPW significantly increased *cyp19a* mRNA expression after 2 h, 4 h, and 8 h by 1.8-, 2.0-, and 3.0-fold, respectively, post-exposure. Exposure to either full-strength 24% O3-OSPW for 4 h or 85% O3-OSPW for 8 h also resulted in a significant increase in *cyp19a* mRNA expression by 1.9- and 1.5-fold, respectively. In all exposure groups mRNA abundance returned to control levels by 24 h post-exposure (Figure. 2.3). Exposure to 10-fold diluted and full-strength untreated OSPW, full-strength 24% ozonated OSPW, and full-strength 85% O3-OSPW increased aromatase activity 1.9-, 2.5-, 2.5-, and 2.2-fold, respectively, at 24 h post-exposure (Figure. 2.4).

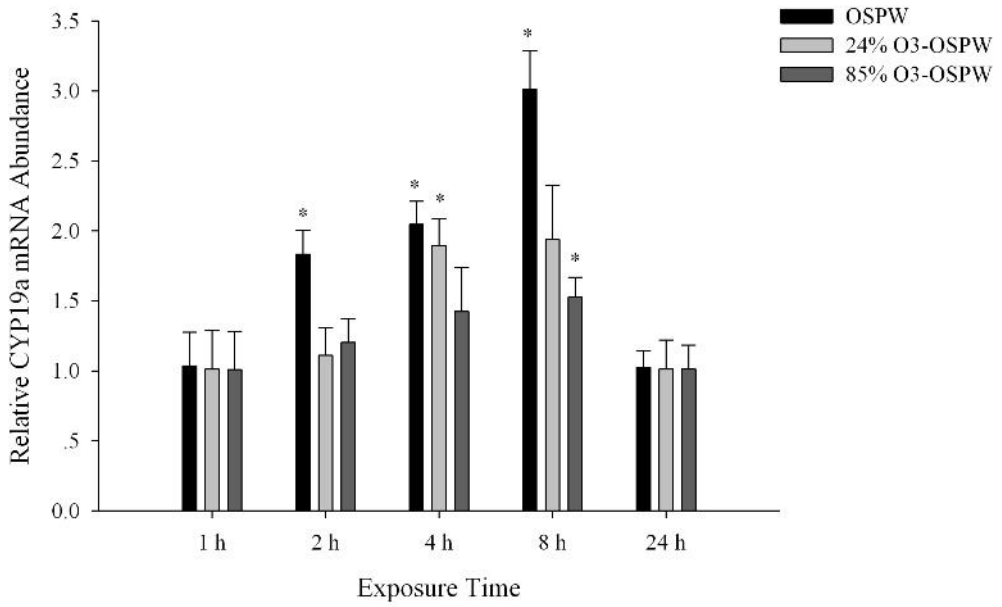


Figure 2.3. Time course analysis of the effect of oil sands process-affected water (OSPW) on *cyp19* mRNA expression in the H295R cell line exposed to untreated, 24% ozonated, and 85% ozonated oil sands process-affected water (OSPW, 24% O3-OSPW, and 85% O3-OSPW). Fold changes are relative to the medium control group. Significant differences were evaluated with one-way ANOVA followed by *post-hoc* Dunnett's test. An asterisk indicates a significant change in response compared to the control group ($\rho < 0.05$).

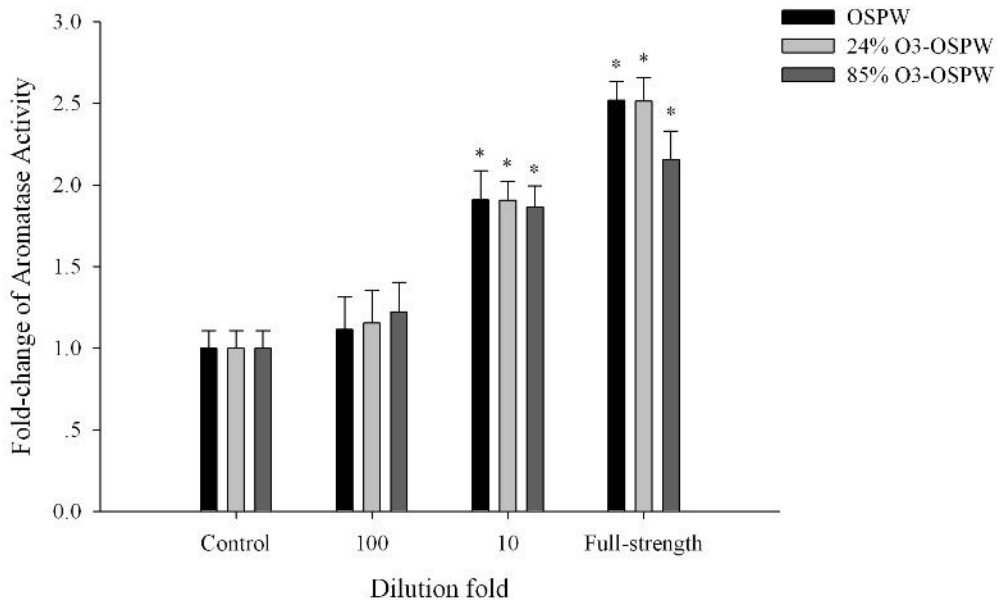


Figure 2.4. Effect of oil sands process-affected water (OSPW) on aromatase activity in the H295R cell line exposed to untreated, 24% ozonated, and 85% ozonated oil sands process-affected water (OSPW, 24% O3-OSPW, and 85% O3-OSPW). Fold changes are relative to the medium control group. Significant differences were evaluated with one-way ANOVA followed by *post-hoc* Dunnett's test. An asterisk indicates a significant change in response compared to the control group ($\rho < 0.05$).

2.3.5. Effects of oil sands-process-affected water (OSPW) on steroidogenesis

Although little is known about the effects of OSPW or NAs on the endocrine axis, there is information suggesting that OSPW and NAs can impact sex hormone production. Significantly less concentrations of T and E2 have been reported in blood plasma of goldfish (Lister et al., 2008) and yellow perch (Van den Heuvel et al., 1999) exposed to OSPW. While the steroidogenic pathway remained intact in goldfish exposed to OSPW, reduced *in vitro* production of T and E2 by ovarian and testicular tissues has been reported (Lister et al., 2008). The decrease in T production in H295R cells is consistent with lesser concentrations of T in blood plasma *in vivo* (Van den Heuvel et al., 1999, Lister et al., 2008) and lesser *in vitro* T production reported in fish (Tetrault et al., 2003). However, this is the first study to demonstrate an increase in E2 production in response to OSPW exposure. Decreased T production was observed in cells exposed to both 10-fold diluted and full-strength OSPW. In contrast, increased E2 production was observed only in cells exposed to full-strength OSPW. These results suggest that the threshold for effects on E2 production may be greater than the threshold for effects on T production.

Aromatase (CYP19a) aromatizes T to convert it to E2. Exposure to 10-fold diluted and full-strength OSPW increased both *cyp19a* expression and aromatase enzyme activity and decreased E2 metabolism. The combined effect of an increase in aromatase activity and decreased E2 metabolism likely explains the observed increase in E2 due to exposure to full-strength OSPW. It is unclear why no significant increase in concentrations of E2 was observed in cells exposed to 10-fold diluted OSPW. In other studies (van den Heuvel et al., 1999, Lister et

al., 2008) that have investigated the effect of OSPW on sex hormone levels no effects on either aromatase gene expression or activity, or E2 metabolism, were investigated.

The difference in E2 concentrations observed in this study, versus the results of *in vivo* studies, in which fish were exposed to OSPW (van den Heuvel et al., 1999, Lister et al., 2008) may be due to several factors. It is unknown whether the results from H295R cells exposed to OSPW are representative of effects on exposed fish since a complementary study was not performed. It has been demonstrated that some chemicals exert similar effects on E2 and T production in both H295R cells and in fathead minnow (*Pimephales promelas*) ovarian explants, while others perform differently in these two systems (Villeneuve et al., 2007). Although the basis for differences in responses observed in the two systems are unclear, it has been suggested that differences in the complexity of the two systems (e.g., cells versus tissues) and species-related differences should be considered when comparing results from the H295R assay with results from fish (Villeneuve et al., 2007). The dominant steroidogenic pathways in adrenal tissue differ from those in gonad tissue (Norris, 1997). Additionally, fish and mammalian steroidogenic enzymes may differ in specificity for some chemicals (Baker, 2001). Consequently, differential regulation of sex hormone steroidogenesis in fish and mammals could result in different sensitivities and/or responses to OSPW. The results of this study also raise an interesting question regarding mammalian health. Although the effect of OSPW on hormone production in mammals is unknown, the results of this study suggest the potential for disruption of steroidogenesis. Indeed, for model chemicals with different effects on steroidogenesis, the H295R assay has been shown to be a relatively sensitive predictor of effects (Hecker et al., 2006).

One difference between this study and previous *in vivo* studies of the effects of OSPW on fish is the nature of the OSPW. The OSPW used in this study was collected from West-in-Pit (WIP), an active settling basin that was established in 1995 and receives fresh OSPW from both the Southwest Settling Basin and South East Pond (SEP). The NAs concentration in the OSPW used in this study was approximately 77 mg/L. In the study by Lister et al. (2008) sexually mature goldfish were caged in an experimental reclamation pond (termed P5) that was constructed from mature fine tailings (MFT) capped with uncontaminated water. The NAs concentration in P5 was reported as approximately 24.1 mg/L. Although the NAs concentrations in the study by van den Heuvel et al. (1999) was not reported, the effects on T and E2 were observed in fish from ponds constructed in 1993, which had lesser NAs concentrations than OSPW (Han et al., 2009). However, van den Heuvel et al. (1999) observed no consistent relationship between exposure to OSPW or NAs and steroid hormones. In addition to the differences in NAs concentration between OSPW and water from these other studies the NAs profile is likely to be different since aging of OSPW has been shown to decrease NAs concentration, while increasing the relative importance of the hydroxylated NAs fraction (Han et al., 2009). Consequently, it is possible that the differences in effects on T and E2 levels between the current study and those in fish may be due to the differences in NAs concentration, and NAs profile including hydroxylated NAs.

2.3.6. Effects of ozone treatment

If the goal of reclaiming OSPW as functional lakes and wetlands, or releasing OSPW back into the environment, is to be realized then effective methods of reducing the toxicity of

OSPW must be developed and validated. Ozonation of OSPW holds promise because it has been shown to rapidly decrease NAs concentrations and their toxicity to bacteria as determined in the Microtox® assay (Scott et al., 2008). However, different NAs profiles with a greater proportion of lower molecular weight NAs was found in the treated OSPW (Scott et al., 2008). Consequently, a major concern is that low molecular weight NAs formed during ozonation, or hydroxylated NAs resembling steroids, can become more bioavailable and consequently adversely affect aquatic organisms. Indeed, ozone treatment is effective in removing both synthetic and natural estrogenic compounds and their associated activity (Alum et al., 2004, Bila et al., 2007, Maniero et al., 2008). However, questions have been raised about potential hazardous substances produced during the ozonation process (Esplugas et al., 2007, Petala et al., 2008). As such we were interested in determining whether ozonation as a treatment method impacted the effect of OSPW on steroidogenesis in the H295R cell line.

Generally, ozonation attenuated the adverse effects of exposure to OSPW on T and E2 production. The significant decrease in T production in cells exposed to 10-fold diluted OSPW was abolished by ozonation, since no significant decrease in T production was detected in either the 24% or 85% O3-OSPW group relative to the control. In contrast, in cells exposed to full-strength OSPW T production remained significantly decreased in both the 24% and 85% O3-OSPW groups, albeit the extent of the decrease appeared to be diminished with increasing ozonation. The increase in E2 concentrations observed in cells exposed to full-strength untreated OSPW was only detected in cells exposed to full-strength 24% O3-OSPW as exposure to 85% O3-OSPW did not affect E2 concentrations.

As was observed in the untreated OSPW exposure groups, aromatase mRNA abundance and enzyme activity remained significantly elevated, though to a lesser extent in cells exposed to ozone-treated OSPW. Although *cyp19a* mRNA expression remained induced by each of the OSPW treatments, ozonation delayed maximal expression of CYP19a gene expression, and increased ozone treatment further increased the time required for maximal induction. Ozonation also impacted the effect of OSPW on the metabolism of E2. This attenuation was most pronounced in cells exposed to 85% ozonated OSPW, since no significant inhibition of E2 metabolism was detected in exposure groups. In addition, when diluted sufficiently, 24% O3-OSPW abolished the effects on E2 metabolism. Overall, these results suggest that inhibition of E2 breakdown is the major reason for increased E2 in the 24% O3-OSPW exposure groups. Furthermore, these results suggest that ozonation may abolish the attenuating effects of OSPW on E2 production. No changes to aromatase mRNA expression or activity, or E2 metabolism, were observed in cells, where OSPW did not affect hormone production. These results suggest that ozonation of OSPW does not impart new or enhanced steroidogenic altering properties on OSPW.

Exposure to ozone-treated OSPW resulted in lesser effects on E2 and T production than did exposure to full-strength OSPW. There was an inverse relationship between degree of ozonation and related decreases in NAs and effects on both T and E2 production. Together, these results suggest that greater ozonation decreases the impact of OSPW on T and E2 production. Again, these results support the hypothesis that ozonation of OSPW does not impart new or enhanced steroidogenic altering properties on OSPW.

2.4. Conclusions

In summary, exposure to untreated OSPW from an active settling basin can alter the steroidogenic pathway in the H295R cell line, and all the adverse effects are mitigated or even recovered by ozonation treatment, suggesting that ozonation may be an effective treatment method to reduce some impacts of OSWP on steroidogenesis. Further study is needed to more fully determine the *in vivo* effects of OSPW and ozonation of OSPW on steroidogenesis.

CHAPTER 3: Effect of ozonation on the estrogenicity and androgenicity of oil sands process-affected water [2]

[2] This chapter has been published in Environmental Science and Technology (2011) Volume 45, Issue 15, Pages 6268-6274, with minor modification. To investigate the endocrine disrupting effects of untreated and ozone-treated oil sands process-affected water (OSPW and O3-OSPW) on estrogen and androgen receptors-mediated signaling, the objectives of this study were to determine: (1) the estrogenicity OSPW via the receptor mediated signaling pathway by use of the T47D-kbluc (estrogen responsive) cell line; (2) the *in vitro* androgenicity of OSPW via the receptor mediated signaling pathway by use of the MDA-kb2 (androgen responsive) cell line; and (3) whether baseline effects of OSPW are modulated by ozonation of OSPW. The entire study, including ethics approval security, experimental design, data collection and analyses, and manuscript writing, was conducted by Yuhe He supervised by co-authors Steve B. Wiseman (University of Saskatchewan) and John P. Giesy (University of Saskatchewan). Co-authors Markus Hecker (University of Saskatchewan), Xiaowei Zhang (University of Saskatchewan), and Paul D. Jones (University of Saskatchewan) made valuable comments on manuscript writing. Co-author Nang Wang (University of Alberta), Leonidas A. Perez (University of Alberta), Mohamed Gamal El-Din (University of Alberta) and Jonathan W. Martin (University of Alberta) cooperated on providing analytical chemistry information related to this study and made valuable comments on manuscript writing.

3.1. Introduction

The oil sands in northeastern Alberta, Canada, are one of the largest proven oil deposit on Earth (Williams, 2003, Alberta Energy and Utilities Board, 2006-2007). In current surface mining operations, extraction of bitumen from oil sands involves the Clarke hot water extraction method. This results in production of oil sands process-affected water (OSPW). In accordance with current zero-discharge policies, OSPW is stored on-site in active settling basins, otherwise known as tailings ponds. It is estimated that greater than 1 billion m^3 of OSPW are currently stored on-site in the various settling basins (Del Rio et al., 2006). The volume of OSPW stored in tailing ponds will continue to increase as mining activities expand. Consequently, there is a need to pursue effective means of remediating or reclaiming OSPW.

Oil sands process-affected water is acutely and chronically toxic to aquatic organisms, including fish (Clemente and Fedorak, 2005). The toxicity of OSPW has been attributed in part to the persistent class of organic acids know as naphthenic acids (NAs) (MacKinnon and Boerger, 1986, Holowenko et al., 2002). NAs are a group of acyclic, monocyclic, and polycyclic carboxylic acids with the general formula of $C_nH_{2n+Z}O_2$, where n represents the number of carbon atoms and Z is zero or a negative even integer related to the number of rings (or double bond equivalents) in the molecule (Brient et al., 1995). However, NAs account for < 50% of all compounds in the organic fraction of OSPW (Headley et al., 2009b, Grewer et al., 2010). Because toxicity of OSPW has been attributed primarily to NAs, these compounds have often been the target of remediation efforts. In-situ remediation relying on natural microbial degradation can reduce a portion of NAs in OSPW, but chronic toxicity still remains (Leung et

al., 2001, Scott et al., 2005, Han et al., 2009). Natural half-lives for NAs in OSPW are 12.8 to 13.6 years (Han et al., 2009). Consequently, more rapid methods of OSPW remediation are being tested. Treatment with ozone is capable of decreasing concentrations of NAs, thereby decreasing the toxicity of OSPW (Scott et al., 2008). Moreover, treatment with ozone accelerates microbial degradation of residual NAs (Martin et al., 2010). While little is known about the biological properties of ozone-treated NAs, studies have warned that ozonation of wastewater could result in the production of endocrine disrupting compounds (Esplugas et al., 2007, Ning et al., 2007, Petala et al., 2008).

The specific mechanism(s) of toxicity of OSPW and the component NAs are unknown, although acute toxicity via physical disruption of membranes, or narcosis, has been suggested (Frank et al., 2008, 2009). Little is known about the potential effects of OSPW, and NAs in particular, on the endocrine axis. Significant alterations in concentrations of testosterone (T) and 17β-estradiol (E2) have been reported in goldfish (*Carassius auratus*) and yellow perch (*Perca flavescens*) that had been exposed to OSPW (Van den Heuvel et al., 1999, Lister et al., 2008). Previous research has also demonstrated altered T and E2 steroidogenesis in H295R cells exposed to OSPW (He et al., 2010).

Endocrine disruption by environmental contaminants has been identified as a potential threat to humans and wildlife. The endocrine disrupting capabilities of some chemicals result from their interaction with nuclear receptors, including the estrogen receptor (ER) and androgen receptor (AR). Disruption of ER and AR signaling pathways can result in alterations of physiological processes, including early embryonic development, growth, and reproductive

success or fecundity (Colborn et al., 1993, Janosek et al., 2006). Several *in vitro* assays have been developed to screen for potential ER and AR agonists or antagonists. Among those are assays using the T47D-kbluc and MDA-kb2 cell lines that screen for direct-acting agonists and antagonists of the ER and AR, respectively (Wilson et al., 2002, 2004). Here results of a study of the endocrine disrupting potency of OSPW and ozone-treated OSPW by use of these reporter assays are reported. The objectives of this study were to determine the following: (1) *in vitro* estrogenic effect of OSPW via the receptor mediated signaling pathway by use of the T47D-kbluc cell line; (2) *in vitro* androgenic effect of OSPW via the receptor mediated signaling pathway by use of the MDA-kb2 cell line; and (3) whether these baseline effects are modulated by ozonation of OSPW.

3.2. Materials and methods

3.2.1. Oil sands process-affected water (OSPW) and commercial Naphthenic acids (NAs)

OSPW was collected in February 2010, from the West-In-Pit (WIP), an active settling basin on the Syncrude Canada, Ltd. site (Fort McMurray, AB, Canada). The OSPW in the WIP is received from two other settling ponds that receive input directly from the main bitumen extraction plant. A schematic illustration is provided by Han et al., 2009. Concentration of NAs in the OSPW sample, determined by ultra pressure liquid chromatography high resolution mass spectrometry (UPLC-HRMS), was 19.7 mg/L (Wang, 2011). Since previous studies have reported activation of *in vitro* AR and ER signaling by NAs, a commercial mixture of high purity NAs provided by Merichem Chemicals and Refinery Services LLC (Houston, TX, USA) was used as a reference in this study so that comparisons of the results of this study could be made to previous studies (Tollefsen et al., 2006, Vrabie et al., 2010). There are similarities and differences between the NAs in OSPW and the Merichem NAs mixture (Bataineh et al., 2006, Martin et al., 2010). The NAs distribution of both mixtures is similar by carbon number, with the major clusters in the range of C12 to C15 (Bataineh et al., 2006). However, in OSPW the most intense ions by high pressure liquid chromatography/ quantitative time-of-flight mass spectrometry (HPLC/ QTOF-MS) are for Z = −4 (36%) and Z = −6 (36%), followed by Z = −12 and −10 (7.8%) > Z = −2 (6.7%) > Z = −8 (5.6%) > Z = 0 (0.4%). In the Merichem NA mixture the most intense ions were for the Z = −2 (41%), followed by Z = −4 (32%) > Z = 0 (20%) > Z = −6 (5.8%) > Z = −8 (1.7%) > Z = −10 (0.3%) (Bataineh et al., 2006). In addition, OSPW contains a significant oxidized NA fraction that is barely detectable in the Merichem NAs

mixture (Martin et al., 2010). Moreover, although NAs are considered one of the major toxic constituents, other organic compounds in OSPW may contribute to toxic effects.

3.2.2. Ozonation of oil sands process-affected water (OSPW)

Ozonation of OSPW was conducted at the University of Alberta (Edmonton, AB, Canada). An AGSO 30 Effizon® ozone generator (WEDECO AG Water Technology, Herford, Germany) was used to produce ozone gas from extra dry, high purity oxygen. To obtain a stable ozone concentration in the feed-gas, the ozone generator was allowed to stabilize for 10 min. The feed gas was sparged into the OSPW through a ceramic fine bubble gas diffuser located at the bottom of a PVC plastic reactor. During the ozonation process, ozone concentrations in the feed and off-gas lines were continuously monitored by two identical ozone monitors (model HC-500, PCI-WEDECO). The ozone monitors were calibrated by use of the potassium iodide (KI) method periodically according to Standard Methods for the Examination of Water and Wastewater. Ozone treatment was continued until the total degradation of parent NAs reached approximately 90%, as determined by the remaining sum response of all UPLC-HRMS peak area corresponding to NAs. After treatment with ozone, the OSPW was purged for 10 minutes with a purified nitrogen gas to strip residual ozone and oxygen from the reactor. Ozonation of the OSPW reduced the NA concentration from 19.7 mg/L to 1.9 mg/L (Wang, 2011). The ozone residual in the reactor was measured using the Indigo method (APHA, 2005). The ozone dose for this system can be calculated according to equation 1 (Gamal El-Din and Smith, 2002).

$$\Delta O3 = \int_0^t \frac{(Q_{G,in}C_{G,in} - Q_{G,out}C_{G,out})}{V_L} dt - C_L \quad (1)$$

where ΔO3 is the amount of utilized ozone (mg/L), $C_{G,in}$ is the ozone concentration in the feed gas, which was calculated from reading the first ozone monitor (mg/L), $C_{G,out}$ is the ozone concentration in the off gas, which was calculated from reading the first ozone monitor (mg/L), C_L is residue ozone concentration in the liquid phase (mg/L), VL is effective reactor volume (L), $Q_{G,in}$ is feed-gas flow rate (L/min), $Q_{G,out}$ is off-gas flow rate (L/min), and t is ozone contact time (min).

3.2.3. T47D-kbluc and MDA-kb2 cell culture

The estrogenicity and androgenicity of OSPW was tested in T47D-kbluc and MDAkb2 cells, respectively, based on methods described by Wilson et al., 2002, 2004. T47D-kbluc cells (ATCC CRL-2865) and MDA-kb2 cells (ATCC CRL-2713) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). T47D-kbluc cells were maintained in RPMI culture media (Sigma) supplemented with 2.5 g/L glucose, 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 1 mM sodium pyruvate, 1.5 g/L $NaHCO_3$, 0.2 U/mL insulin, and 10% normal fetal bovine serum (FBS), at 37 ℃ in a 5% CO_2 atm. MDA-kb2 cells were cultured in L15 media (Sigma, Mississauga, ON, Canada) supplemented with 10% FBS (Sigma) at 37 ℃, without CO_2. For T47D-kbluc and MDA-kb2 assays, additional withdrawal and assay/exposure media were also prepared by supplementation with 10% and 5%

dextran-charcoal treated FBS (Sigma), respectively, rather than 10% normal FBS. All the media were filtered through a 0.22 μM bottle top filter (Corning) to avoid microbial contamination.

3.2.4. T47D-kbluc and MDA-kb2 cell assay

To minimize the baseline reporter gene activity, prior to experimental exposures, both cell types were subcultured in their respective withdrawal medium for 24 hr, and then experimental exposures were conducted by use of the corresponding assay/exposure medium. For exposure experiments, T47D-kbluc cells were plated at 4×10^4 cells/mL, and MDA-kb2 cells were plated at 8×10^4 cells/mL in a 96-well luminometer plate (Perkin-Elmer, Woodbridge, ON, Canada) with 250 μL of assay medium per well. Next, the assay medium was replaced with either fresh assay medium as control or exposure medium containing full-strength and different dilutions of untreated OSPW, ozone-treated OSPW, or the Merichem NAs mixture. Exposure media were prepared the same as standard assay medium, except that the nanopure water was replaced with untreated OSPW, ozone-treated OSPW, or the Merichem NAs mixture. Dilutions of exposure medium were prepared with assay medium. All exposure media containing untreated OSPW, ozone-treated OSPW, or the Merichem NAs mixture were prepared fresh on the day of exposure, and the pH was adjusted to 7.6. E2 or T standard curves were also included on each plate. Stock standard solutions of E2 and T were prepared in 100% ethanol, and 10-fold serial dilutions were prepared in assay medium, with final concentrations ranging from 0.0001 to 10 nM and 0.001 to 10000 nM for E2 and T, respectively. The final concentration of ethanol in all exposures was 0.1%. Control experiments demonstrated that this concentration did not affect cell viability or the reporter gene assays. The estrogen receptor antagonist ICI 182,780

(Faslodex) (Sigma, Mississauga, ON, Canada) was used to confirm activation of ER signaling in T47D-kbluc cells. The MDA-kb2 cell assay was performed with the glucocorticoid receptor (GR) agonist dexamethasone (DEX) to determine any GR activity of untreated OSPW, ozone-treated OSPW, or the Merichem NAs mixture. No significant cytotoxicity was observed in any of the treatments.

After dosing with standards or exposure medium the cells were incubated for 24 h, and luciferase activity was determined as a measure of estrogenicity or androgenicity by use of the Steady-Glo® luciferase assay system (Promega, Sunnyvale, CA, USA) according to the manufactures recommendations. Cells were washed once with phosphate buffered saline (PBS) at room temperature. Twenty-five μL of lysis buffer was added per well, and plates were incubated for 15 min at room temperature. After cell lysis, 25 μL of the luciferase reagent was added to each well, and luminescence was read by use of a POLARStar OPTIMA microplate reader (BMG Labtech, Offenburg, Germany).

3.2.5. Statistical analyses

All experiments were performed on two separate occasions, with triplicate exposures performed for each individual experiment (n = 6). There was no significant difference between the results obtained from the two experimental dates. Statistical analyses were conducted by use of SPSS 16 (SPSS Inc., Chicago, IL, USA). The normality of each data set was assessed by the Kolomogrov-Smirnov one-sample test, and homogeneity of variance was determined by the Levene's test. Significant differences were evaluated with a one way ANOVA followed by *post-*

hoc Tukey's test. Differences with $\rho < 0.05$ were considered significant. All data are presented as mean ± standard deviations fold change from control groups.

3.3. Results and discussion

3.3.1. Effects on estrogenicity

Estrogenic responses were proportional to concentrations of untreated OSPW, ozone-treated OSPW, and the Merichem NAs mixture (Figure 3.1). At full strength (100%) concentrations, the estrogenic responses to untreated OSPW and ozone-treated OSPW were 2.58 ± 0.22-fold and 2.48 ± 0.13-fold compared to the control group, respectively. Thus, ozonation neither attenuated nor intensified the estrogenicity of OSPW. Exposure to the Merichem NAs mixture stimulated a nonsignificant induction (2.18 ± 0.40-fold at 100%) in the estrogenic response compared to control levels. In coexposure experiments, the estrogenic response in the cells coexposed to untreated OSPW or ozone-treated OSPW, and E2, was significantly greater than the response to E2 alone at concentrations between 0 and 0.01 nM in both coexposure scenarios (Figure 3.2a and 3.2b). These results suggest that neither untreated OSPW nor ozone-treated OSPW has significant ER antagonist properties, but rather, there is an additive estrogenic effect of OSPW or O3-OSPW with E2. The estrogenic response in cells coexposed to the Merichem NAs mixture and E2 (Figure 3.2c) resembled the response of cells coexposed to E2 with untreated OSPW or ozone-treated OSPW. At lesser concentrations of E2 (for untreated and ozone-treated OSPW: E2 $\leq$ 0.01 nM; for the NAs mixture: E2 $\leq$ 0.001 nM), significant additive estrogenicity was observed. While the results suggest that ER agonists are present, given that the concentration of NAs in the Merichem mixture was 60 mg/L while the concentration of NAs in the untreated OSPW was 19.7 mg/L, the coexposure experiment indicated that the estrogenicity of the Merichem NAs mixture is weak compared to OSPW (no significantly greater response at

0.01 nM E2). However, at greater concentrations of E2 the additive estrogenic effect of untreated and ozone-treated OSPW and the Merichem NAs mixture was not statistically significant, probably due to saturation of the ER.

To confirm whether the estrogenic effects of OSPW and ozone-treated OSPW were due to binding to the ER, T47D-kbluc cells were coexposed, with the potent ER antagonist ICI 182,780, to untreated OSPW, ozone-treated OSPW, or the Merichem NAs mixture, along (Figure 3.3). Co-exposure to ICI 182,780 attenuated the estrogenicity observed in cells exposed to untreated OSPW or ozone-treated OSPW, such that levels were not significantly different from control levels (Figure 3.3a and 3.3b). These results are consistent with estrogenicity of both untreated OSPW and ozone-treated OSPW being due to binding of some component of the OSPW mixture to the ER. Also, the estrogenic response observed for the Merichem NAs mixture was abolished by coexposure with ICI 182,180 (Figure 3.3).

There are several reports related to binding and activation of hormone receptors by offshore effluent from oil production platforms, including crude oil and refined products (Gamal El-Din and Smith, 2002, APHA, 2005, Tollefsen et al., 2006, Thomas et al., 2009, Vrabie et al., 2010). Naphthenic acids present in North Sea offshore produced water were ER agonists and AR antagonists (Thomas et al., 2004). Oil sands process-affected water is a mixture of inorganic and organic compounds, with NAs being the predominant organic compound (Tollefsen et al., 2007). Due to the complex chemistry of OSPW it is difficult to identify specific ER agonist(s). However, the results from the exposures to the Merichem NAs mixture suggest that NAs present in OSPW are probably responsible for the observed effects. The possibility that other

unidentified compounds present in OSPW, and those are not affected by ozonation, are ER agonists cannot be eliminated. Because the T47D-kbluc cells contain both ERα and ERβ, it is unknown whether the agonist(s) preferentially bind to one of these receptors or have equal affinity for both (Wilson et al., 2002).

If the NAs present in OSPW are responsible for the estrogenic effects and ozonation removes NAs, it is inconsistent that ozonation did not attenuate estrogenicity. Several studies have demonstrated that ozonation of OSPW decreases NA levels, thereby attenuating the acute toxicity (Scott et al., 2008). In a previous study, ozonation of OSPW attenuated effects on steroidogenesis in the H295R cell line (He et al., 2010). Ozonation primarily targeted the OSPW NAs with more rings and was hypothesized to preferentially degrade NAs with greater extents of alkyl branching (Martin et al., 2010). One explanation of the observed results is that less cyclic and less branched NAs present in OSPW that are unaffected by ozonation act as the ER agonists. Alternatively, oxidized NAs (i.e., $C_nH_{2n+Z}O_3$) might be responsible for ER agonism of OSPW. Oxidized NAs are naturally present in untreated OSPW and are both degraded and formed during the ozonation process such that there is little net change in their total concentration (Han et al., 2008, Martin et al., 2010). Therefore, if the oxidized NAs are estrogenic, then ozonation may not attenuate observed effects.

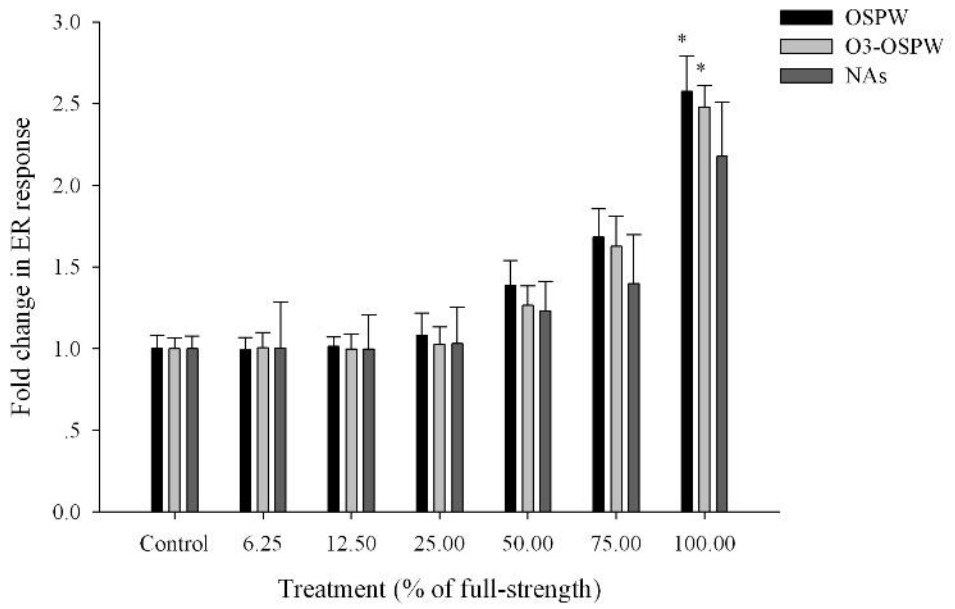
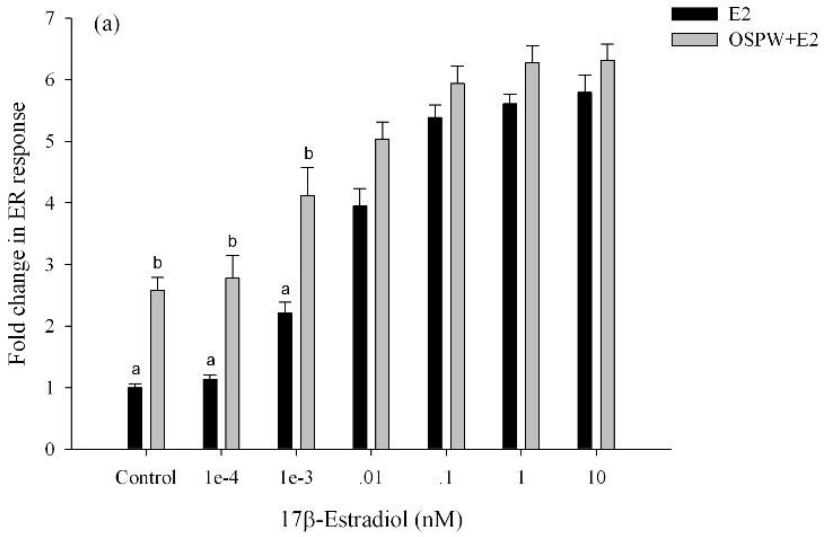
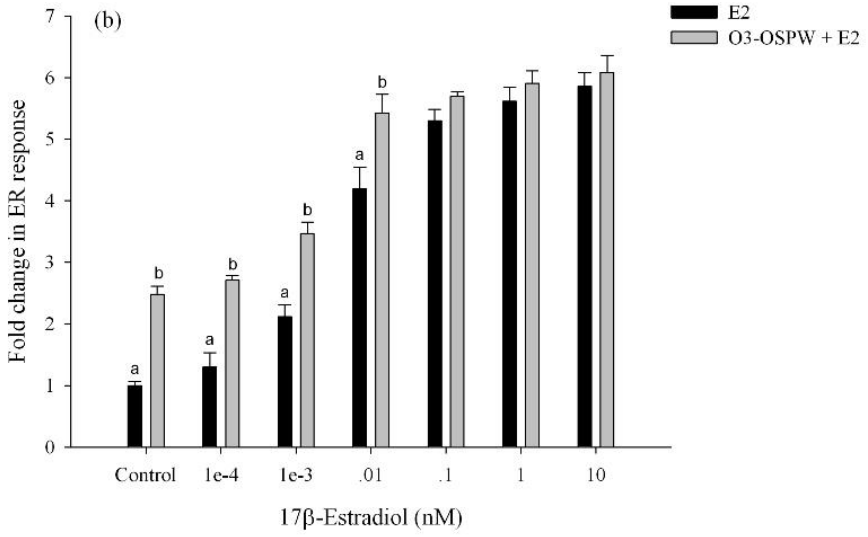


Figure 3.1. Concentration-response relationship for estrogenic response in T47D-kbluc cells exposed to untreated oil sands process-affected water (OSPW), ozone-treated oil sands process-affected water (O3-OSPW), or a commercial naphthenic acids mixture (NAs) (Merichem, Houston, Texas, USA). Fold changes are relative to the medium control group. Significant differences were evaluated with one-way ANOVA followed by *post-hoc* Tukey's test. An asterisk indicates a significant change in response compared to the control group ($\rho < 0.05$).


(a)
E2
OSPW+E2
Fold change in ER response
0
1
2
3
4
5
6
7
a
b
a
b
a
b
Control
1e-4
1e-3
.01
.1
1
10
17β-Estradiol (nM)


(b)
E2
O3-OSPW + E2
Fold change in ER response
0
1
2
3
4
5
6
7
a
b
a
b
a
b
Control
1e-4
1e-3
.01
.1
1
10
17β-Estradiol (nM)

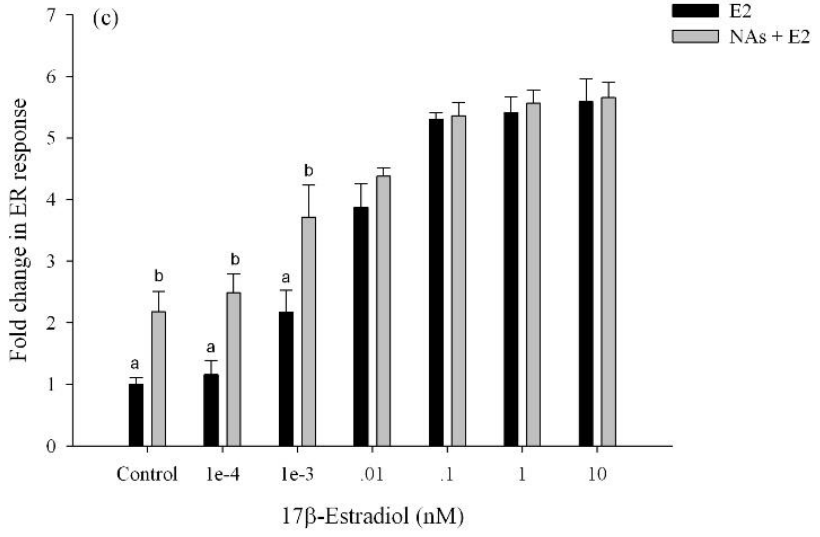

(c)
E2
NAs + E2
Fold change in ER response
0
1
2
3
4
5
6
7
a
b
a
b
a
b
Control
1e-4
1e-3
.01
.1
1
10
17β-Estradiol (nM)

Figure 3.2. Concentration-response relationship for estrogenic response in T47D-kbluc cells co-exposed to different concentrations of 17β-Estradiol (E2) and 100% (a) untreated oil sands process-affected water (OSPW), (b) ozone-treated oil sands process-affected water (O3-OSPW), or (c) Merichem naphthenic acids (NAs). Fold changes are relative to the medium control group. Significant differences were evaluated with one-way ANOVA followed by *post-hoc* Tukey's test. An asterisk indicates a significant change between treatment groups at the same concentration of 17β-Estradiol (E2) ($\rho < 0.05$).

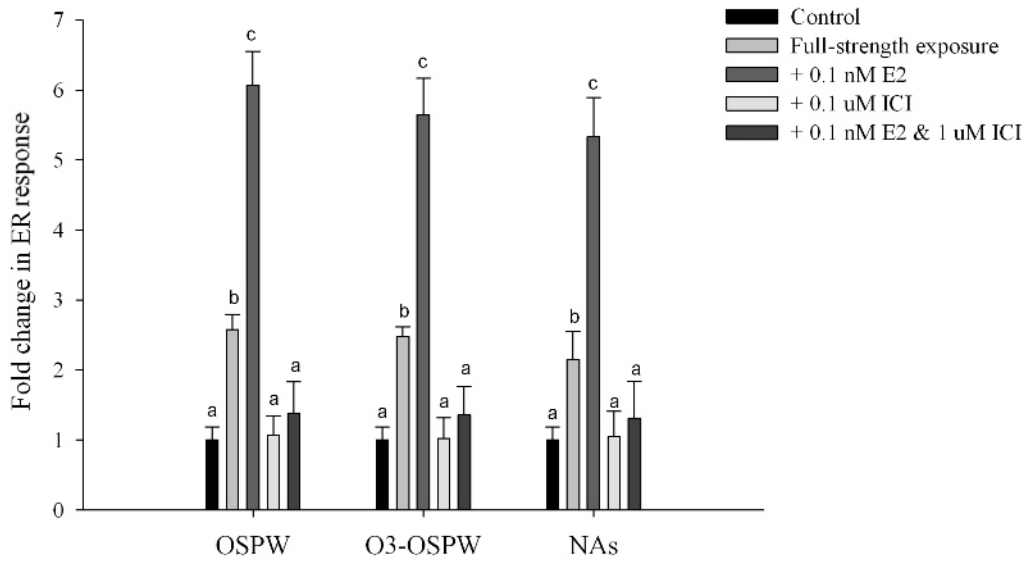


Figure 3.3. Effect of the ER antagonist ICI 182,780 (Faslodex) on the estrogenicity of 100% (a) untreated oil sands process-affected water (OSPW), (b) ozone-treated oil sands process-affected water (O3-OSPW), or (c) Merichem naphthenic acids (NAs), alone or as a co-exposure with 0.1 nM 17β-Estradiol (E2) and/or 1 μM ICI 182,780 in T47D-kbluc cells. Fold changes are relative to the medium control group. Significant differences were evaluated with one-way ANOVA followed by *post-hoc* Tukey's test. Different letters represent significant differences among treatment groups ($\rho < 0.05$).

3.3.2. Effects on androgenicity

Significant antiandrogenic response was observed in cells coexposed to different concentrations of T with full-strength untreated OSPW, ozone-treated OSPW, and the Merichem NAs mixture, compared to the same concentrations of T alone (Figure 3.4). The magnitude (M) of the anti-AR response in coexposed cells versus cells exposed to the corresponding concentrations of T alone is shown in Figure 5 and was determined by equation 2.

$$\mathrm{M} = \frac{Fold\ induction\ of\ AR\ response\ in\ coexposure\ treatement - 1}{Fold\ induction\ of\ AR\ response\ in\ coresponding\ conc.\ of\ Talone - 1} \quad (2)$$

Co-exposure with OSPW resulted in a significant antiandrogenic effect at T concentrations of 0.01, 0.05, and 0.1 nM (Figure 3.4), in which the magnitude of the anti-AR response was 16±6.5%, 47±7.6%, and 75±9.7%, respectively (Figure 3.5). Co-exposure with ozone-treated OSPW resulted in a significantly antiandrogenic effect at T concentrations of 0.01 and 0.05 nM (Figure 3.4b), in which the magnitude of anti-AR response was 64±12% and 70±11%, respectively (Figure 3.5). Compared with untreated OSPW, significantly less antiandrogenic capability was observed in cells coexposed to ozone-treated OSPW and 0.01, 0.05, and 0.1 nM of T (Figure 3.5). Ozone-treated OSPW was less potent as an antiandrogen than untreated OSPW. Similar to the OSPW and ozone-treated OSPW, coexposure with NAs resulted in a significant antiandrogenic effect at T concentrations of 0.01 (49±13%) and 0.05 nM

(64±13%) (Figure 3.4c). The antiandrogenic response in cells exposed to the Merichem NAs mixture is weaker than that of untreated OSPW but is greater than that of the ozone-treated OSPW.

MDA-kb2 cells also contain glucocorticoid receptor (GR) (Vrabie et al., 2010). To confirm the antiandrogenic response rather than an antiglucocorticoid response, a coexposure experiment with the GR agonist, dexamathasone (DEX), was conducted (Figure 3.6). Co-exposure to different concentrations of DEX with untreated OSPW, ozone-treated OSPW, or the Merichem NAs mixture did not result in significantly less response compared to the corresponding concentrations of DEX alone, which suggests that neither the untreated OSPW, ozone-treated OSPW, or the Merichem NAs mixture possess an anti GR effect. Crude oil has been reported to have antiandrogenic effects. There are several reports of steroid hormone receptor binding and activation by offshore effluent from North Sea oil production platforms, crude oil, and refined products in recombinant yeast assays (Gamal El-Din and Smith, 2002, APHA, 2005, Tollefsen et al., 2006, Thomas et al., 2009, Vrabie et al., 2010). Significant AR antagonist potency has been demonstrated in at least one of these effluents fractions containing mixtures of NAs. In those reports the observed antiandrogenic effects were attributed to an enriched NA fraction and not hydroxylated polycyclic aromatic hydrocarbons (PAHs) as was shown in a mammalian and human cell line study (Kizu et al., 2000).

Given the apparent antiandrogenic properties of untreated OSPW and ozone-treated OSPW, it was hypothesized that coexposure with T and OSPW would have resulted in attenuation of the androgenic response. However, there was no significant difference in the

response to T alone and in combination with 100% untreated OSPW at the 0.05 nM T (Figure 3.3a). In fact, coexposure with OSPW significantly increased the androgenic response compared to that for 1, 10, and 100 nM of T alone (Figure 3.3a). The magnitude of this response was 121 ± 8.5%, 140 ±10.7%, and 118 ±5.3%, respectively (Figure 3.4). At concentrations of T greater than 100 nM there was no statistical difference in the androgenic response between cells exposed to T alone or in combination with 100% OSPW (Figure 3.3a). Similar responses were observed in cells coexposed to ozone-treated OSPW, resulting in a greater AR response compared to that for 1 or 10 nM of T alone (Figure 3.3b), and the Merichem NAs mixture, which resulted in a significantly greater AR response compared to that for 1 nM of T alone (Figure 3.3c). Compared with untreated OSPW, the magnitude of the androgenic effect caused by ozone-treated OSPW was significantly weaker in coexposure with 100 nM T, while for the Merichem NAs mixture it was significantly weaker in coexposure with 10 and 100 nM T (Figure 3.4).

The mechanism of the increased androgenic response in cells coexposed to moderate concentrations of T and untreated OSPW, ozone-treated OSPW, or Merichem NAs is unknown. One explanation is that untreated OSPW, ozone-treated OSPW, and the NAs mixture might have altered the physiological properties of the cell membrane, which resulted in greater bioavailability of T and a greater AR response compared to cells exposed to the same concentration of T alone. This type of effect has been reported for other hydrophobic organic compounds (Glover and Wood, 2005, Mayer et al., 2007). Under such a scenario the antiandrogenic effects of untreated OSPW, ozone-treated OSPW, and the Merichem NAs mixture would be considered weak since T is apparently able to compete with the antagonist for

the ligand binding domain of the AR. Further work is needed to fully elucidate the mechanism of antiandrogenicity observed here.

Regardless of the mechanism(s) of action, ozonation attenuated the antiandrogenic effects of OSPW on the AR. The compounds in OSPW and ozonated OSPW responsible for the antiandrogenic effect are unknown, but to some extent NAs likely play a role, as evidenced by the experiments with the NAs. Considering the antiandrogenicity was still evident in cells exposed to full-strength ozone-treated OSPW compared to that for 0.01 and 0.05 nM T alone, in which 90% of NAs had been degraded by ozone, ozonation was not able to completely abolish the effects. This suggests that other unidentified compounds present in OSPW might possess antiandrogenic activity. Given that the same antiandrogenic effect was observed in cells exposed to the NAs, which is composed entirely of lesser molecular weight NAs, these results suggest that the lesser molecular weight NAs might be responsible for some of the antiandrogenicity of OSPW. Whereas attenuation of antiandrogenicity was observed in cells exposed to ozone-treated OSPW alone, under a coexposure scenario both untreated OSPW and ozone-treated OSPW potentiated the effect of T, and ozonation only attenuated the potentiating effect compared to that for 100 nM T alone. This result suggests that the component of untreated OSPW responsible for this potentiating effect might be partially affected by ozonation. Because the same effect was also found in the NAs mixture, then that component of OSPW may share properties with the Merichem NAs mixture.

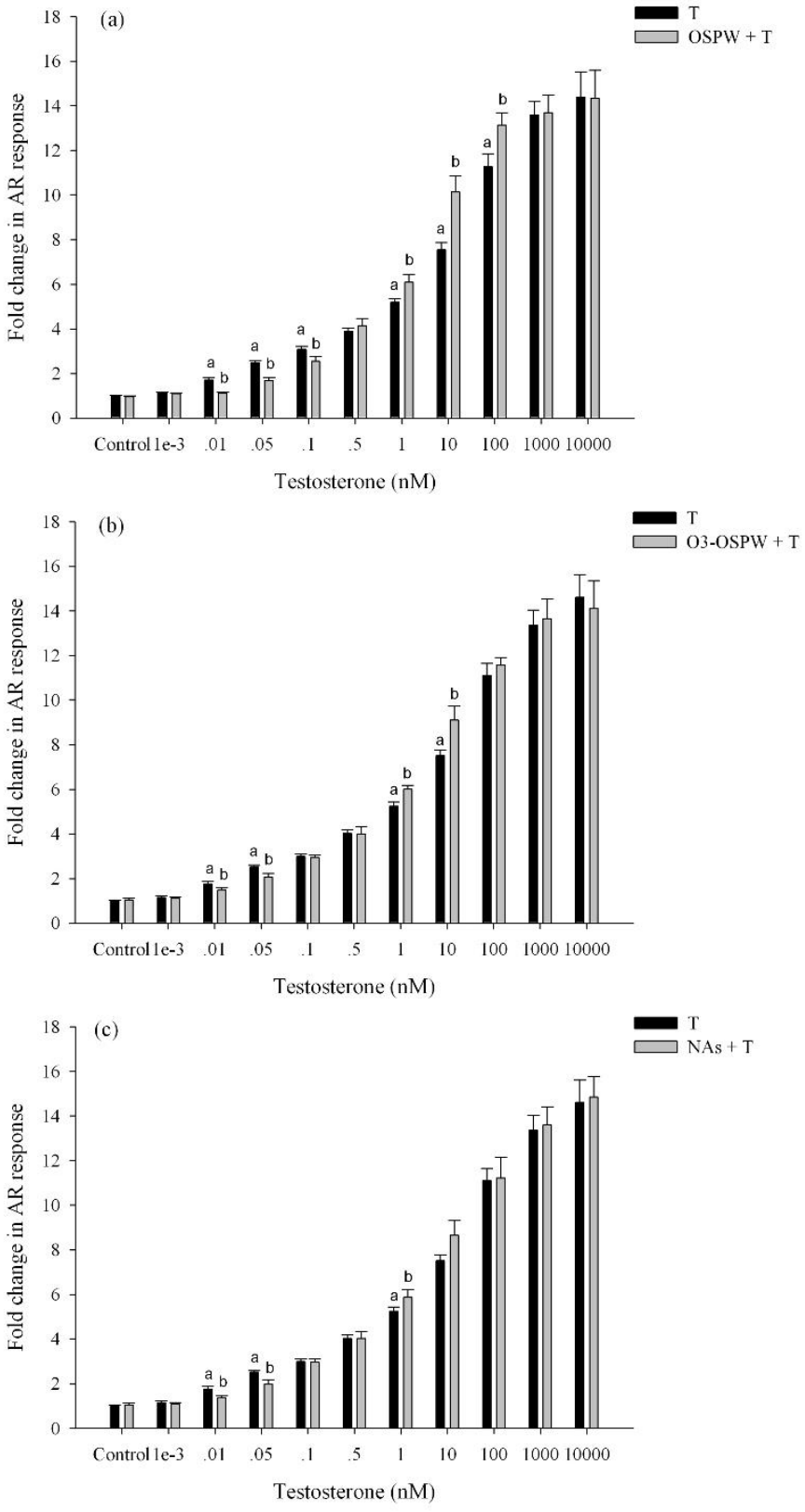

(a)
T
OSPW + T
Fold change in AR response
Testosterone (nM)
Control 1e-3 .01 .05 .1 .5 1 10 100 1000 10000
(b)
T
O3-OSPW + T
Fold change in AR response
Testosterone (nM)
Control 1e-3 .01 .05 .1 .5 1 10 100 1000 10000
(c)
T
NAs + T
Fold change in AR response
Testosterone (nM)
Control 1e-3 .01 .05 .1 .5 1 10 100 1000 10000

Figure 3.4. Concentration-response relationship for androgenic response in MDA-kb2 cells co-exposed to different concentrations of testosterone (T) and 100% (a) untreated oil sands process-affected water (OSPW), b) ozone-treated oil sands process-affected water (O3-OSPW), or c) Merichem naphthenic acids (NAs). Fold changes are relative to the medium control group. Significant differences were evaluated with one-way ANOVA followed by *post-hoc* Tukey's test. An asterisk indicates a significant change between treatment groups at the same concentration of T ($\rho < 0.05$).

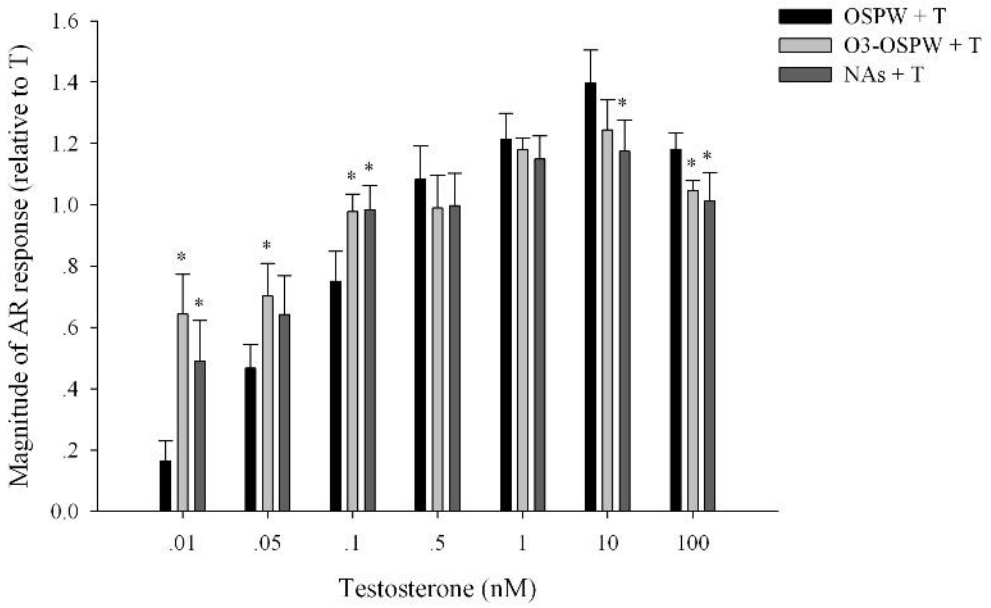


Figure 3.5. Magnitude of androgenic response in MDA-kb2 cells co-exposed to untreated oil sands process-affected water (OSPW), ozone-treated oil sands process-affected water (O3-OSPW), or Merichem naphthenic acids (NAs) with testosterone (T). Fold changes are relative to the same concentrations of testosterone (T). Significant differences were evaluated with one-way ANOVA followed by *post-hoc* Tukey's test. An asterisk indicates a significant change in response from the androgen receptor (AR) response of co-exposure group of untreated oil sands process-affected water (OSPW) and testosterone (T) ($\rho < 0.05$).

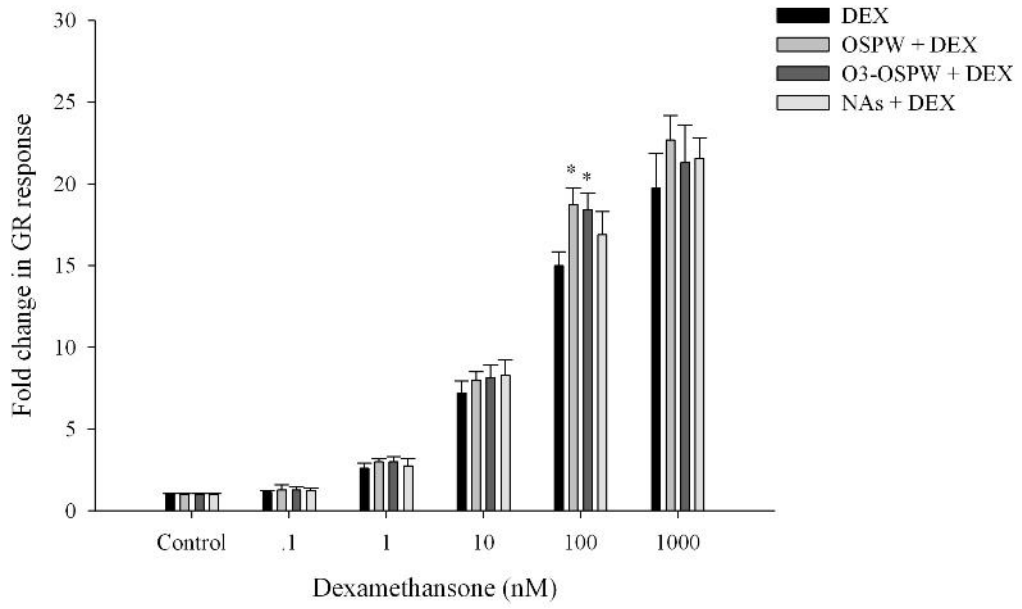


Figure 3.6. Concentration-response relationship for glucocorticoid response in MDA-kb2 cells co-exposed to different concentrations of dexamathasone (DEX) and 100% of untreated oil sands process-affected water (OSPW), ozone-treated oil sands process-affected water (O3-OSPW), and Merichem naphthenic acids (NAs). Fold changes are relative to the media control group. Significant differences were evaluated with one-way ANOVA followed by *post-hoc* Tukey's test. An asterisk indicates a significant change between treatment groups at the same concentration of DEX ($\rho < 0.05$).

3.4. Conclusions

In summary, exposure to OSPW as well as NAs can result in both ER agonist and AR antagonist properties, and ozonation can partially mitigate the potential antiandrogenic and androgen potentiating effects of OSPW. However, the results of the current study do not support the idea that ozonation reduces the estrogenic effect. To some extent, ozonation can be an effective treatment to reduce the effects of OSPW due to endocrine disruption. Further study is needed to clarify the mechanism(s) of action of OSPW dissolved organics as ER/AR agonist/antagonists, including more detailed determination of the ER and AR mediated effects of OSPW and the effect of ozonation on the *in vivo* responses of organisms.

CHAPTER 4: Transcriptional responses of the brain-gonad-liver axis of fathead minnows exposed to untreated and ozone-treated oil sands process-affected water [3]

[3] This chapter has been published in Environmental Science and Technology (2012) Volume 46, Issue 17, Pages 9701-9708, with minor modification. To investigate the endocrine disrupting effects of untreated and ozone-treated oil sands process-affected water (OSPW and O3-OSPW) the specific objectives of this *in vivo* study were to investigate the effects of OSPW and O3-OSPW by quantifying abundances of transcripts of genes in the brain-gonad-liver (BGL) axis in male and female fathead minnows (*P. promelas*). To this end it was determined: (1) whether OSPW has endocrine-disrupting effects at all levels of the BGL axis; (2) whether OSPW has the same or different effects in male and female fathead minnows; (3) whether ozonation attenuates the effects of OSPW on abundances of transcripts; and (4) whether ozonation has the same or different attenuating effects in male and female fathead minnows. The entire study, including ethics approval security, experimental design, data collection and analyses, and manuscript writing, was conducted by Yuhe He supervised by co-authors Steve B. Wiseman (University of Saskatchewan) and John P. Giesy (University of Saskatchewan). Co-author Nang Wang (University of Alberta), Leonidas A. Perez-Estrada (University of Alberta), Mohamed Gamal El-Din (University of Alberta), and Jonathan W. Martin (University of Alberta) cooperated on providing analytical chemistry information related to this study and made valuable comments on manuscript writing.

4.1. Introduction

Oil sands process-affected water (OSPW) is a byproduct of the extraction of bitumen from the oil sands in Alberta, Canada. Oil sands process-affected water is a mixture of water, residual bitumen, silts, clays, and other inorganic and organic compounds. The water-soluble organic fraction of OSPW has been shown to be responsible for the majority of the toxicity of OSPW (Garcia-Garcia et al., 2011a, b, Pourrezaei et al., 2011, Anderson et al., 2012a, b). Naphthenic acids (NAs) are one of the primary persistent organic constituents of OSPW. These are a group of carboxylic acids with the general formula $C_nH_{2n+Z}O_2$, where n indicates the number of carbons and Z relates to the number of rings or double bonds (Holowneko et al., 2002, Headley and McMartin, 2004, Clemente and Fedorak, 2005, Frank et al., 2008, 2009, Rowland et al., 2011a).

Oil sands process-affected water has endocrine-disrupting effects. Exposure to OSPW decreases concentrations of testosterone (T) and estradiol (E2) in plasma of yellow perch (*Perca f lavescens*) (van den Heuvel et al., 1999) and goldfish (*Carassius auratus*) (Lister et al., 2008) and causes less synthesis of T and E2 by explants of ovarian and testicular tissue from goldfish exposed to OSPW. Exposure to OSPW impairs reproductive capacity of fathead minnows (*Pimephales promelas*) as exemplified by decreased fecundity, altered synthesis of sex steroid hormones, and less pronounced secondary sex characteristics of male and female minnows (Kavanagh et al., 2011). *In vitro*, OSPW reduces synthesis of T and increases synthesis of E2 in the H295R cell line (He et al., 2010) and stimulates estrogenic and antiandrogenic effects in T47Dkbluc and MDA-kb2 cell lines, respectively (He et al., 2011). Although the components of

OSPW that are responsible for these effects are unknown, some NAs are structurally similar to sex steroid hormones (Rowland et al., 2011a).

In accordance with a policy of zero-discharge, OSPW is stored on-site in active settling basins, otherwise known as tailings ponds. Currently, there are greater than 1 billion m3 of OSPW stored on-site in tailings ponds of several companies operating in this region (Del Rio et al., 2006). Eventually this OSPW needs to be remediated and reclaimed either as viable aquatic habitats or released to the receiving environment. In order for OSPW to be reclaimed it is essential that toxicity of the water-soluble organic fraction be reduced. Currently, this is attempted by aging OSPW in the tailing ponds, or experimental ponds, to decrease the concentrations of NAs through natural in situ biodegradation (Holowenko et al., 2002, Del Rio et al., 2006, Han et al., 2009). However, some NAs in OSPW are resistant to biodegradation (Han et al., 2008, 2009). Consequently, aging is only moderately effective for removing the toxicity of OSPW. Therefore, to more rapidly remediate OSPW, a treatment approach that targets NAs, and other persistent dissolved organics, is required. Ozonation has been identified as a potentially effective treatment method (Scott et al., 2008, Gamal El-Din et al., 2011, Perez-Estrada et al., 2011). Ozone preferentially degrades NAs with more rings which are most resistant to biodegradation, thereby accelerating subsequent microbial remediation (Martin et al., 2010). Ozonation reduces the acute toxicity of OSPW toward *Vibrio fischeri* as measured by the Microtox® assay (Scott et al., 2008, Gamal El-Din et al., 2011) and attenuates some of the endocrine-disrupting effects on eukaryotic cells *in vitro* (Van den Heuvel et al., 1999, Lister et al., 2008).

The mechanism(s) of endocrine disruption caused by exposure to OSPW are unknown. To develop effective treatment, monitoring, and remediation programs, as well as to be able to conduct risk assessments and set regulatory standards, knowledge of the mechanism of toxic action would be beneficial. Therefore, in the current study, a PCR array was utilized to investigate the mechanistic basis of the endocrine disrupting effects of OSPW by examining transcriptional responses of key genes along the brain-gonad-liver (BGL) axis of male and female fathead minnows. In addition, to further investigate the usefulness of ozonation for reducing the toxicity of OSPW the effects of ozone-treated OSPW on transcriptional responses of these genes were determined.

4.2. Materials and methods

4.2.1. Oil sands process-affected water (OSPW) collection and ozonation

Oil sands process-affected water was collected in February 2010, from the West-In-Pit (WIP), an active settling basin on the site of Syncrude Canada Ltd. (Fort McMurray, AB, Canada). The OSPW is untreated process water from the main bitumen extraction plant as described previously (Han et al., 2009). The total concentration of NAs in OSPW, as determined by ultra pressure liquid chromatography high resolution mass spectrometry (UPLC-HRMS), was 19.7 mg/L (Wang, 2011). Ozonation of OSPW was conducted at the University of Alberta (Edmonton, AB, Canada) using a semibatch ozonation system and following a standard protocol described elsewhere (GamalEl-Din et al., 2011, Wang, 2011). Ozonation was continued until approximately 90% degradation of parent NAs was achieved, as determined by the remaining sum response of all UPLC-HRMS peak area corresponding to NAs. The total concentration of NAs in the ozone-treated OSPW (O3-OSPW) was 1.9 mg/L. A more detailed description of the effects of ozonation on the distribution of the NAs in this sample is given by Wang (2011).

4.2.2. Experimental protocol

This study was approved by the University of Saskatchewan's Animal Research Ethics Board and adhered to the Canadian Council on Animal Care guidelines for humane animal use. The experiment was conducted in the Aquatic Toxicology Research Facility (ATRF) at the University of Saskatchewan's Toxicology Centre. Adult male and female fathead minnows of approximately 6 months of age were randomly selected from a culture that is maintained within

the ATRF. Minnows were maintained in 200-L tanks supplied with running water at approximately 20 ℃, maintained under a 12L:12D photoperiod and fed approximately 2% body weight of frozen bloodworms once daily. Thirty-six fathead minnows (18 male and 18 female) were randomly assigned to one of six 25-L aquaria containing 20 L of either dechlorinated city of Saskatoon tap water as the control group, untreated OSPW at full strength, or O3-OSPW at full strength. All exposures were performed in duplicate aquaria, with six males and six females per treatment. The aquaria were supplied with constant aeration, the water temperature was maintained at 22 ℃, and a 12 h light: 12 h dark photoperiod was used. Approximately 50% of the water volume of each aquarium was replaced daily, and the exposure was maintained for 7 days. Minnows were fed approximately 2% body weight of frozen bloodworms once daily. There were no mortalities in either of the exposure groups. At the end of exposure period, minnows were netted and immediately anesthetized with 150 mg/L MS-222. Brain (including the pituitary gland), liver, and gonad were collected from each fish and frozen at −80 ℃ for analysis of the abundances of transcripts of the genes of interest.

4.2.3. Quantification of abundances of transcripts by quantitative real-time Polymerase chain reaction (qPCR)

Total RNA was extracted from livers by use of the Qiagen RNeasy® Plus Mini Kit according to the manufacturer's protocol (Qiagen, Mississauga, ON, Canada). Total RNA was extracted from brains and gonads by use of a Qiazol RNeasy® lipid tissue mini kit according to the manufacturer's protocol (Qiagen). Purified RNA was quantified by use of a NanoDrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE), and samples were stored at

−80 ℃ until analysis. First strand cDNA was synthesized from 1 μg of total RNA by use of an iScript™ cDNA Synthesis Kit (Bio-Rad, Mississauga, ON, Canada) according to the manufacturer's instructions. The cDNA samples were stored at −80 ℃ until further analysis. Real-time PCR (qPCR) was performed on an ABI 7300 Real-Time PCR System in 96-well PCR plates (Applied Biosystems, Foster City, CA). A PCR reaction mixture for one reaction contained 10 μL of SYBR® green master mix (Applied Biosystems), 2 μL of sense/antisense gene-specific primers (Invitrogen, Carlsbad, CA), and 8 μL of cDNA that was diluted in RNase-free water (Qiagen). The PCR reaction mix was denatured at 95 ℃ for 10 min before the first PCR cycle. The thermal cycle profile was denaturizing for 15 s at 95 ℃ and annealing and extension for 1 min at 60 ℃ for a total of 40 PCR cycles. After amplification reactions were completed, dissociation curves were generated to ensure amplification of a single product. Efficiency, uniformity, and linear dynamic range of each qPCR assay were assessed by construction of standard curves by use of serially diluted cDNA standards. Changes in abundances of transcripts of target genes were quantified by normalizing to 18s rRNA, according to the method of Simon (2003). Amplification of genes of interest and reference genes was performed in separate reactions.

4.2.4. Gene selection and model development

Twenty-three genes representing key signaling pathways and functional process of the BGL axis in fathead minnows were selected based on principles of a previous study using zebrafish (Zhang et al., 2008). All primers were designed based on the sequences available in

the NCBI GeneBank database or from sequences obtained by Illumina RNA sequencing (unpublished data). Sequences of nucleotide primers are given in Table 4.1.

Table 4.1. Abbreviations (Abb.), name of target gene, category, nucleotides sequence, efficiency (Eff.) and GeneBank number of nucleotides sequence used in primer pair design for quantitative real-time PCR (qPCR). * Sequence information obtained by Illumina RNA sequencing (unpublished data).

Abb.	Target gene	Category	Primer pair (5'-3')	Eff.	GenBank
18S	Ribosomal RNA 18S	Reference gene	F:GCCCTGTAATTGGAATGAGC R:TCCCGAGATCCAACTACGAG	1.99	AY855349
AR	Androgen receptor	Steroid receptor	F:CAACGCGTCTAAATCCCATT R:TGTTCGAACTGACACGAAGC	2.04	AY727529
ERα	Estrogen receptor alpha	Steroid receptor	F:CGGTGTGCAGTGACTATGCT R:CTCTTCCTGCGGTTTCTGTC	2.06	AY775183
ERβ	Estrogen receptor beta	Steroid receptor	F:CGTTTTGGCATAACCATGTG R:TGCTGTCAGACTTCCGAATG	1.99	AY566178
KISS1R	Kisspeptin 1 receptor	Peptide receptor	F:GATGAGTGGAGACCGTTGCT R:CCTCAAGCCTCTGGTACAGG	2.02	EF672266
GnRH2	Gonadotropin releasing hormone 2	Hormone	F:TCACTCGAGGGAAAGCAGAC R:AACTGGGCACTTAAACACAGC	2.03	EF672264
GnRH3	Gonadotropin releasing hormone 3	Hormone	F:TCTATTCCTGCGGACACTCC R:CTCCAAGGGTTCAACATCGT	2.04	EF672265
GnRHR	Gonadotropin releasing hormone receptor	Peptide receptor	F:TGCAAAGCCAGTGAAAATTG R:TTGTCAAACTGGGACGTGAG	2.05	*
FSHβ	Follicle-stimulating hormone beta	Hormone	F:AGCTGCATCACAATCGACAC R:AGGGCAGCCTTTAAACTCGT	1.99	DQ242616
FSHR	Follicle-stimulating hormone receptor	Peptide receptor	F:CACGTACTGCTGTCCAGACG R:GTGGCTGGGGTATGTCAGAT	2.01	EF219401
LHβ	Luteinizing hormone beta	Hormone	F:GTCGTTGCTCAAAGCTCCTT R:TGGAGAACGGGCTCTTGTAT	1.85	DQ242617
LHR	Luteinizing hormone receptor	Peptide receptor	F:CTTTCAACCACCTTCCCAAG R:AGCATTTGGTGGGACTGAAC	1.92	DT281016
StAR	Steroidogenic acute regulatory protein	Steroidogenesis	F:ATGCCCGAGAAGAAAGGATT R:CCCGGTTGATGACTGTTTTT	1.98	DQ360497

CYP11A	Cholesterol side-chain cleavage enzyme	Steroidogenesis	F:CACACTGATGTGGACGCTCT R:AGGGCTCCTTTAAGCAGAGG	1.95	DQ360498
CYP17	17α-hydroxylase	Steroidogenesis	F:CTGCCCATCATTGGAAGTCT R:GCATGATGGTGGTTGTTCAC	1.98	AJ277867
CYP19a	Aromatase alpha	Steroidogenesis	F:GCTGCACAAGAAGCACAAG R:CGTGGCTCTGAGCGAATATC	1.93	AF288755
CYP19b	Aromatase beta	Steroidogenesis	F:AGGGTGTATCCTGGCAACTG R:ATCTGCACCCGTTTCATTTC	1.97	AJ277866
3βHSD	3β-hydroxysteroid dehydrogenase	Steroidogenesis	F:TAACTGGAGGATGCGGTTTT R:TGCACCACTACCACCTTCAC	1.99	DT361291
17βHSD	17β-hydroxysteroid dehydrogenase	Steroidogenesis	F:ATCCAGAGTGTGCTGCCTTT R:AGGGAAATAGCCGTTGGTCT	1.98	DT161033
VTG	Vitellogenin	yolk precursor	F:TTGCTCTCCAGACCTTTGCT R:GCAGAGCCTCCACCTTGTAG	1.97	AF130354
CHG-L	Choriogenin L	yolk precursor	F:CAAGCACAATCGCAGAGAAC R:GTCCCTGTTGGGTTTGTGAG	2.08	*
CHG-H	Choriogenin H minor	yolk precursor	F:GCAGCATCAATTGCGTTTAC R:TCTTCTGGGGATCAAACCAT	2.06	*

4.2.5. Statistical analyses

Fathead minnows were exposed to the treatment waters in duplicate tanks. The authors are aware that this is technically pseudoreplicattion. However, there were no differences in the responses to the same treatment between fish in different tanks. Therefore, individual male and female fish were considered the experimental unit. Statistical analyses were conducted by use of SPSS16.0 (SPSS, Chicago, IL). All data are expressed as mean ± SEM. Normality of each data set was assessed by use of Kolomogrov−Smirnov one-sample test, and homogeneity of variance was determined by use of Levene's test. When necessary to meet assumptions of parametric tests, data were log-transformed to ensure homogeneity of variance. Nontransformed data are presented in figures. Where data met the required assumptions, statistical differences were evaluated with one-way ANOVA followed by *post-hoc* Tukey's test. A Kruskal−Wallis test was used when neither the untransformed nor transformed data met the assumptions of parametric statistics. Differences were considered statistically different at $\rho < 0.05$.

4.3. Results and discussion

4.3.1. Effects on males

Exposure to untreated OSPW affected the abundances of transcripts of target genes expressed in brains from male fathead minnows. The abundances of transcripts of *erα*, *kiss1r*, *fshβ*, *lhβ*, and *cyp19b* in brains from male fish exposed to untreated OSPW were greater by 5.14±3.22, 6.11±1.51, 3.96±0.96, 3.04±1.80, and 3.44±1.20-fold, respectively, than in brains from male fish exposed to freshwater. Ozonation of OSPW attenuated these effects. Abundances of transcripts of *erα*, *kiss1r*, *fshβ*, *lhβ*, and *cyp19b* in brains from male fish exposed to O3-OSPW were not different from abundances of these transcripts in brains from male fish exposed to freshwater. The abundance of transcripts of *gnrhr* was less in brains from males exposed to untreated OSPW or O3-OSPW by 0.13±0.05 and 0.11±0.06-fold, respectively, compared to the abundance of transcripts of *gnrhr* in brains from male fish exposed to freshwater. Abundances of transcripts of *erβ*, *ar*, *gnrh2*, and *gnrh3* were not different among brains from male fish exposed to any of the treatments (Figure 4.1a).

Exposure to untreated OSPW significantly affected abundances of transcripts of target genes expressed in gonads from male fathead minnows. Abundances of transcripts of *fshr* and *lhr* were greater in gonads from male fish exposed to OSPW by 3.7±0.43 and 2.5±0.59-fold, respectively, compared to abundances in gonads from male fish exposed to freshwater. Ozonation of OSPW partially attenuated effects on abundances of transcripts of *fshr* and *lhr*. The abundance of transcripts of *lhr* was not different in gonads from male fish exposed to O3-OSPW compared to abundance in gonads from male fish exposed to freshwater and was less

(0.86±0.19-fold relative to freshwater) than in gonads from male fish exposed to freshwater. In contrast, the abundance of transcripts of *fshr* was not different in gonads from male fish exposed to O3-OSPW compared to that for untreated OSPW but was greater, by 3.8±0.77-fold, than that in gonads from male fish exposed to untreated OSPW. Abundances of transcripts of *cyp11a* and *3βhsd* were greater in gonads from male fish exposed to OSPW by 8.0±1.3 and 7.0±1.8-fold, respectively, compared to abundances in gonads from fish exposed to freshwater. Abundances of transcripts *cyp11a* and *3βhsd* in gonads from male fish exposed to O3-OSPW (5.16±0.56 and 3.72±0.78-fold relative to freshwater, respectively) were less than abundances in gonads from male fish exposed to untreated OSPW but were greater than abundances in gonads from male fish exposed to freshwater. The abundance of transcripts of *cyp17* was greater in gonads from male fish exposed to O3-OSPW compared to abundance in gonads from male fish exposed to freshwater and untreated OSPW. Abundances of transcripts of other genes including *star, 17βhsd,* and *cyp19a* were not affected by any of the treatments (Figure 4.1b).

Exposure to untreated OSPW affected abundances of transcripts of target genes in livers from male fish. Abundances of transcripts of *erα*, *vtg*, *chg*-l, and *chg*-h in livers from male fish exposed to untreated OSPW were greater by 4.1±0.85, 4.9±1.2, 5.4±1.5, and 3.4±0.78-fold, respectively, compared to abundances in livers from male fish exposed to freshwater. Exposure to O3-OSPW attenuated these effects, and abundances of transcripts of *erα*, *vtg*, *chg*-l, and *chg-h* in livers from male fish exposed to O3-OSPW were not different from abundances of transcripts in male fish exposed to freshwater. The abundance of transcripts of *ar* and *erβ* were not different in livers from male fish exposed to either untreated OSPW or O3-OSPW compared to the abundance in livers from male fish exposed to freshwater (Figure 4.1c).

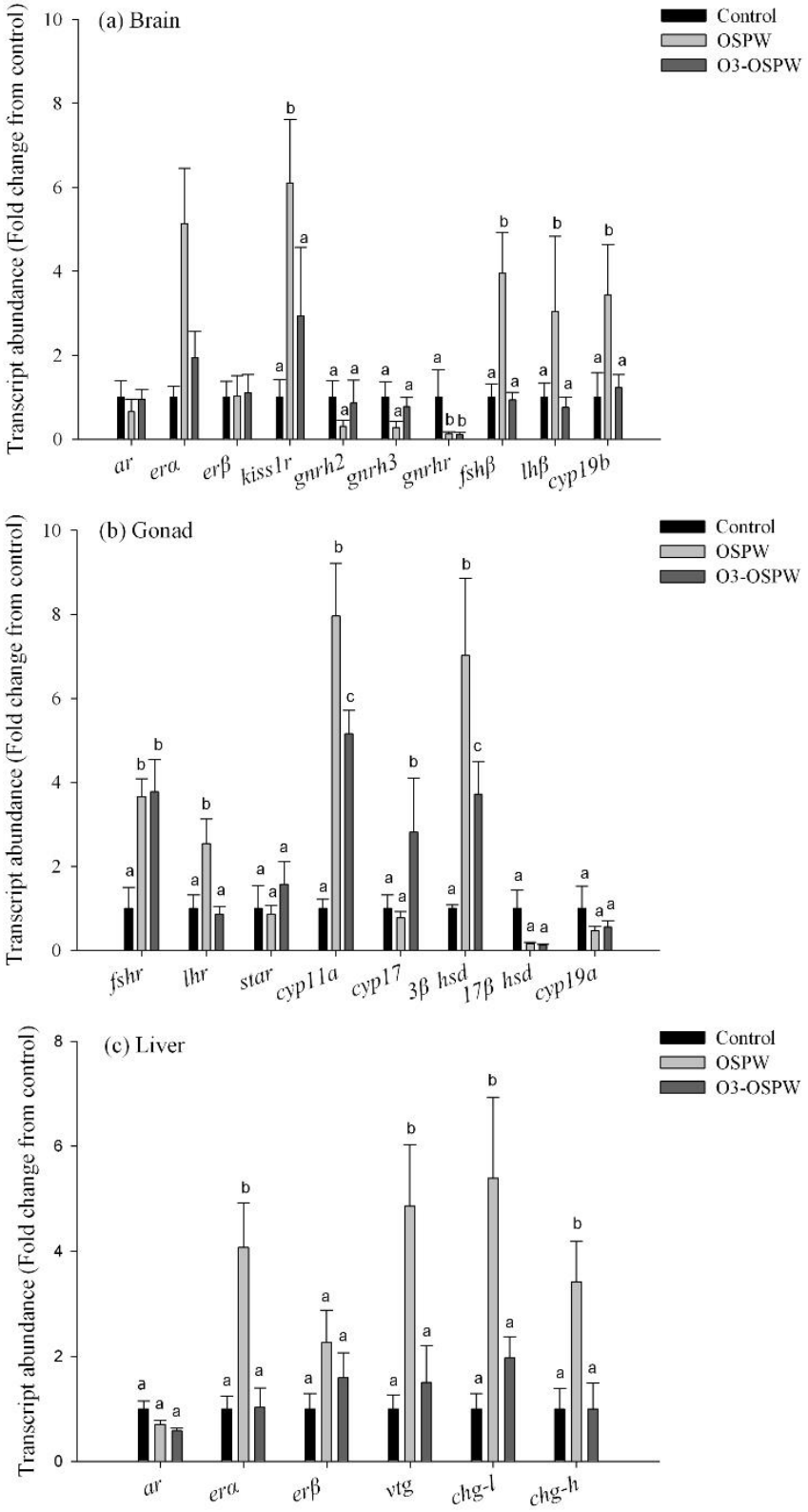

(a) Brain
Control
OSPW
O3-OSPW
Transcript abundance (Fold change from control)
ar
erα
erβ
kiss1r
gnrh2
gnrh3
gnrhr
fshβ
lhβ
cyp19b
(b) Gonad
fshr
lhr
star
cyp11a
cyp17
3β hsd
17β hsd
cyp19a
(c) Liver
ar
erα
erβ
vtg
chg-l
chg-h

Figure 4.1. Abundances of transcripts of genes involved in sex steroid hormone synthesis and signaling in male fathead minnows exposed to freshwater as control, oil sand process-affcted water (OSPW) or ozone-treated oil sand process-affcted water (OSPW) (O3-OSPW). (a) Barin, (b) Gonad, (c) Liver. Significant differences were evaluated with One-way ANOVA followed by *post-hoc* Tukey's test. Different letters represent significant differences in the abundances of transcripts among treatment groups ($\rho < 0.05$).

4.3.2. Effects on females

Exposure to untreated OSPW significantly affected abundances of transcripts of several target genes expressed in brains from female fathead minnows. The abundance of transcripts of *lhβ* was greater by 5.3±2.2-fold in brains from female fish exposed to OSPW compared to the abundance in brains from female fish exposed to freshwater. However, in brains from female fish exposed to O3-OSPW, the abundance of transcripts of *lhβ* was less than the abundance in brains from female fish exposed to untreated OSPW and not different from the abundance in brains from female fish exposed to freshwater. The abundance of transcripts of *fshβ* in brains from female fish exposed to untreated OSPW was not different from that of female fish exposed to freshwater. However, the abundance of transcripts of *fshβ* in brains from female fish exposed to O3-OSPW was greater by 2.0±0.25-fold compared to the abundance in brains from female fish exposed to freshwater but not different from the abundance in brains from female fish exposed to untreated OSPW. Abundances of transcripts of *gnrh2* or *gnrh3* in brains from female fish exposed to OSPW were not affected. However, the abundance of transcripts of *gnrh2*, but not *gnrh3*, was significantly greater in brains of female fish exposed to O3-OSPW, by 1.6±0.36-fold, compared to the abundance in brains from female fish exposed to freshwater. Abundances of transcripts of *kiss1r*, *cyp19b*, *erα*, *erβ*, or *ar* were not different between brains from female fish exposed to either of the waters (Figure 4.2a).

Exposure to untreated OSPW affected abundances of transcripts of several target genes expressed in gonads from female fathead minnows. Abundances of transcripts of *fshr*, *lhr*, and *cyp19a* were less by 0.02±0.004, 0.33±0.056, and 0.28±0.10-fold in gonads from female fish

exposed to untreated OSPW compared to abundance in gonads from female fish exposed to freshwater. Ozonation did not attenuate these effects, and abundances of these transcripts were not different between fish exposed to untreated OSPW and O3-OSPW. However, abundances of transcripts of *fshr*, *lhr*, and *cyp19a* were less by 0.01 ±0.002, 0.14 ±0.045, and 0.07 ±0.013-fold in gonads from female fish exposed to O3-OSPW compared to abundances in gonads from female fish exposed to freshwater. Abundances of transcripts of *cyp11a* and *cyp17* were not different between gonads from fish exposed to freshwater or those exposed to untreated OSPW. However, abundances of transcripts of *cyp11a* and *cyp17* were less by 0.044 ±0.017 and 0.28 ±0.078-fold in gonads from fish exposed to O3-OSPW compared to gonads from fish exposed to freshwater. Abundances of transcripts of *star*, *3βhsd*, and *17βhsd* were not significantly different in gonads from female fish exposed to either untreated OSPW or O3-OSPW compared to the abundance in gonads from female fish exposed to freshwater (Figure 4.2b).

Exposure to untreated OSPW significantly affected abundances of transcripts of target genes in livers from female fish. In livers from female fish exposed to OSPW, abundances of transcripts of *ar*, *erα*, *erβ*, *vtg*, *chg*-l, and *chg-h* were less by 0.18 ±0.025, 0.14 ±0.075, 0.080 ±0.011, 0.002 ±0.0011, 0.022 ±0.007, and 0.036 ±0.024-fold compared to thosein livers from female fish exposed to freshwater. Ozonation did not attenuate these effects. Abundances of transcripts of *ar*, *erα*, *erβ*, *vtg*, *chg-l*, and *chg-h* were not different between brains from female fish exposed to untreated OSPW and those exposed to O3-OSPW. Abundances of transcripts of *ar*, *erα*, *erβ*, *vtg*, *chg-l*, and *chg-h* were less by 0.26 ±0.015, 0.13 ±0.050, 0.11 ±0.016, 0.0015 ±0.0004, 0.072 ±0.033, and 0.021 ±0.008-fold in livers from female fish exposed to O3-

OSPW compared to abundance of transcripts in livers from female fish exposed to freshwater (Figure 4.2c).

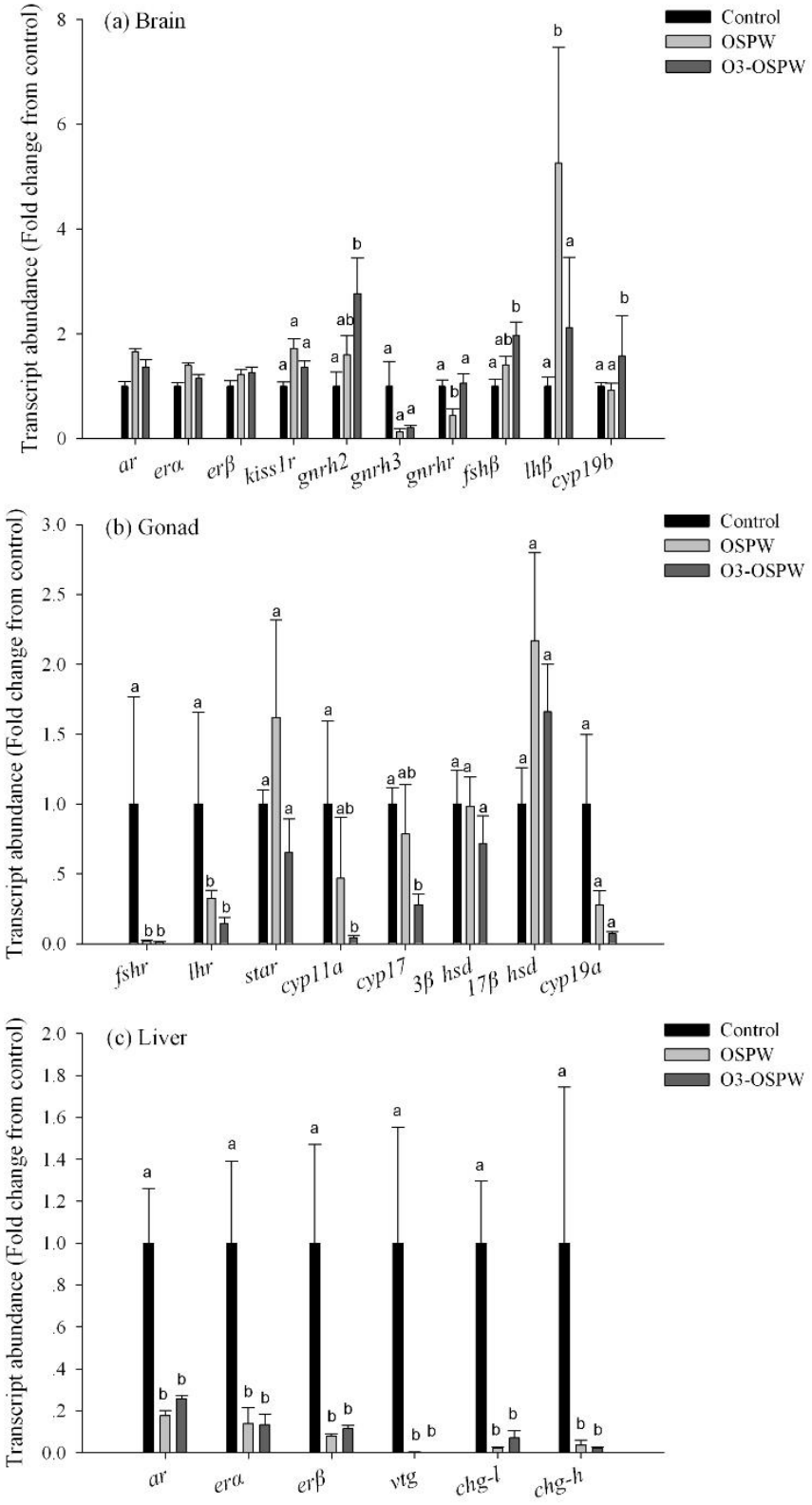

(a) Brain
Control
OSPW
O3-OSPW
Transcript abundance (Fold change from control)
ar
erα
erβ
kiss1r
gnrh2
gnrh3
gnrhr
fshβ
lhβ
cyp19b
(b) Gonad
fshr
lhr
star
cyp11a
cyp17
3β hsd
17β hsd
cyp19a
(c) Liver
ar
erα
erβ
vtg
chg-l
chg-h

Figure 4.2. Abundances of transcripts of genes involved in sex steroid hormone synthesis and signaling in female fathead minnows exposed to freshwater as control, oil sand process-affcted water (OSPW) or ozone-treated oil sand process-affcted water (OSPW) (O3-OSPW). (a) Barin, (b) Gonad, (c) Liver. Significant differences were evaluated with Kruskal-Wallis test. Different letters represent significant differences in the abundances of transcripts among treatment groups ($\rho < 0.05$).

4.3.3. Exposure to oil sands process-affected water (OSPW) and ozone-treated oil sands process-affected water (O3-OSPW) impairing the expression of genes along the brain-gonad-liver axis of fathead minnows

In teleost fishes, regulation of sexual reproduction is dependent upon a complex network of signaling pathways between brain (the hypothalamus and pituitary), gonads, and liver. GnRHs act via a G-protein-coupled receptor, termed the GnRHR, to regulate synthesis and release of FSH and LH from the pituitary (Dufour et al, 2010). Gonadotropins consist of a noncovalently linked glycoprotein−hormone α-subunit (GTHα) and a specific β-subunit (FSHβ and LHβ) (Sower et al., 2009). The gonadotropins stimulate gonadal development by regulating synthesis of the sex steroid hormones T and E2 (Kanda et al., 2008). In teleost fishes, FSH is involved in early gamete maturation, and LH is mainly involved in the final stage of gamete maturation which results in ovulation and/or spermiation (Levavi-Sivan et al., 2010). Estradiol and T synthesized in response to LH and FSH also feedback to the hypothalamus and the pituitary, thereby regulating the synthesis and release of FSH and LH (Kanda et al., 2008). Results of recent studies indicate a important role of Kiss peptides and Kiss peptide receptors, in particular the Kiss1 system, in HPG axis regulation via its action on GnRH1 neurons, thereby regulating synthesis and release of LH and FSH in fishes (Akazome et al., 2010, Kanda et al., 2011).

Genes involved in synthesis and regulation of gonadotropins, which are expressed in the hypothalamus and pituitary, including Kiss1R, FSH, LH, GnRHs, and GnRHR, are critical for control and regulation of sexual maturation and reproduction of male and female fathead

minnows. The greater abundance of transcripts of *kiss1r* in male fathead minnows exposed to OSPW and the attenuation of this effect by ozonation of OSPW suggest that OSPW might stimulate secretion of FSH and LH via the Kiss1 system. Although the abundances of transcripts of *gnrh2* and *gnrh3* were not different in fathead minnows exposed to OSPW, the abundance of transcripts of *gnrhr* was less in males and females exposed to untreated OSPW. The reason for down-regulation of expression of the GnRHR might be due to compensation feedback via activation of GnRH or direct inhibition by dissolved organic compounds in OSPW. Exposure to OSPW caused greater abundances of transcripts of *fshβ* and *lhβ* in brains from male fathead minnows, but in brains from female fish only the abundance of transcripts of *lhβ* was up-regulated. Greater abundances of transcripts of the gonadotropin hormones, in particular in brains from male minnows, might be due to stimulation of ERα signaling. Effects of estrogens on the pituitary are exerted primarily through the ERα isoform (Sánchez-Criado et al., 2004), and exposure to OSPW caused greater abundance of transcripts of *erα*, but not *erβ*, in brains from male fathead minnows exposed to untreated OSPW. These effects on transcription were all attenuated by ozonation. The results suggest that exposure to OSPW might have a greater effect on hypothalamic and pituitary control of sexual maturation in male fathead minnows than in maturing female fathead minnows.

After FSH and LH are released from the pituitary, they are transported in blood to the gonads where they bind to FSHR and LHR and regulate sex hormone steroidogenesis (Levavi-Sivan et al., 2010, Zohar et al., 2010, Luckenbach et al., 2011). In addition to acting via their respective receptors, there is some cross-activation of FSHR by LH (Levavi-Sivan et al., 2010). The greater abundances of transcripts of *fshr* and *lhr* in gonads from male fish exposed to OSPW

compared to gonads from fish exposed to freshwater might be a response to greater FSHβ and LHβ released from the pituitary in response to OSPW as suggested by the greater abundances of transcripts of these gonadotropins. Several studies have demonstrated FSHR- and LHR-activating properties by FSH and LH, respectively (Han et al., 1996, Campbell et al., 1997, Grossmann et al., 1997). In contrast, lesser abundances of transcripts of *fshr* and *lhr* in gonads from female fish exposed to OSPW compared to that for freshwater might be a mechanism of regulating steroidogenesis in response to a greater concentration of LH. Another possible mechanism for less *fshr* and *lhr* in gonads from female fish is that E2-like compounds in OSPW directly interact with the ER and, in turn, feedback to inhibit steroidogenesis and E2 synthesis. The combination of these two effects might explain the conflicting effects between male and female, thus feminization in males and less fecundity in females that was reported by Kavanagh and colleagues (He et al., 2011). The reason ozonation only attenuated effects on *lhr* in male gonad is unknown at this time and requires further study.

If concentrations of FSHR and LHR are greater in gonads from male fish exposed to OSPW, it might increase sensitivity of the gonads to circulating FSH and LH, which would cause greater activity of steroidogenic enzymes (Planas and Swanson, 1995, Kusakabe et al., 2006). Greater abundances of transcripts of *cyp11a*, *3βhsd*, and *11βhsd* observed in male fathead minnows exposed to OSPW suggest that the capacity for steroidogenesis is greater in these fish. Ozonation partially attenuated effects of OSPW on this signaling pathway. In female fathead minnows exposed to untreated OSPW and O3-OSPW, abundances of transcripts of *11βhsd*, *cyp11a*, and *cyp17*, respectively, were less than in gonads from minnows exposed to freshwater. This suggests a down-regulation of steroidogenesis and is consistent with the down-regulation of

fshr and *lhr*. These results suggest that OSPW, possibly due to the "steroid-like" NAs (Rowland et al., 2011a), might decrease steroidogenic activity in female fathead minnows, by directly inhibiting transcription of genes in the gonads. Exposure to untreated OSPW resulted in sex-specific effects on expression of target genes in livers of male and female fathead minnows. Up-regulation of ERα and estrogen-response genes such as VTG, CHG-L, and CHG-H in livers of fish exposed to estrogens has been previously demonstrated in Japanese medaka (*Oryzias latipes*), zebrafish (*Danio rerio*), and fathead minnow (Yamaguchi et al., 2005, Filby et al., 2007, Martyniuk et al., 2007). The fact that the sex steroid receptor ERα, as well as the estrogen-responsive genes VTG, CHG-L, and CHG-H, were up-regulated in livers from male fathead minnows exposed to OSPW suggests that exposure to OSPW resulted in an estrogenic effect. This is consistent with results of a previous *in vitro* study that demonstrated the stimulation of ER signaling by OSPW and identification of estrogen-like compounds in OSPW (Lister et al., 2008, Rowland et al., 2011a). In addition, activation of ER signaling might have been due to greater synthesis of E2 caused by stimulation of steroidogenesis by FSH and LH released from the pituitary. Either way, activation of ER signaling suggests that male fathead minnows exposed to untreated OSPW are exposed to an estrogenic internal environment, and this might explain the less pronounced secondary sex characteristics in male fathead minnows exposed to OSPW (He et al., 2011). Ozonation of OSPW attenuated these effects on male fathead minnows. The presence of estrogen-like compounds in OSPW (Lister et al., 2008, Rowland et al., 2011a) might explain lesser abundances of transcripts of *ar*, *erα*, *erβ*, *vtg*, *chg-l*, and *chg-h* in livers of female fathead minnows exposed to OSPW compared to that in females exposed to freshwater. These results suggest that OSPW might impair HPG signaling and E2 synthesis. Lesser abundances of

transcripts of *fshr*, *lhr*, *cyp11a*, and *cyp17* support this mechanism of action of OSPW. Regardless of the mechanism of action, any impairment of ER signaling and subsequent inhibition of synthesis of egg envelope proteins might explain the lesser fecundity of female fathead minnows exposed to OSPW (He et al., 2011).

4.4. Conclusions

In summary, exposure to OSPW resulted in changes in abundances of transcripts at all levels of the BGL axis in male and female fathead minnows. This is perhaps not surprising for a mixture with the complexity of OSPW. Estrogen-like NAs in OSPW might have caused some of the effects in the liver and might have influenced negative feedback pathways that regulate synthesis and release of gonadotropins and sex steroid hormones. It is also possible that organic compounds in OSPW, including NAs, might have directly affected actions of gonadotropin releasing hormones in the hypothalamus, gonadotropins in the pituitary, and sex steroid hormones in the gonads. The results of this study provide a mechanistic basis for the impaired reproductive performance and less pronounced secondary sexual characteristics of fathead minnows exposed to OSPW. Effects of ozonation on endocrine disrupting effects of OSPW were less clear than previous *in vitro* studies where ozonation of OSPW either attenuated or had no effect on the endocrine-disruptive effects of OSPW. Ozonation attenuated the effects of OSPW on some endocrine end points, and the effects were more prominent in males than in females.

CHAPTER 5: Toxicity of untreated and ozone-treated oil sands process-affected water (OSPW) to early life stages of the fathead minnow (*Pimephales promelas*) [4]

[4] This chapter has been published in Water Research (2012) Volume 46, Issue 19, Pages 6359-6368, with minor modification. To investigate the embryotoxic effects of untreated and ozone-treated oil sands process-affected water (OSPW and O3-OSPW), the specific objectives of this study were to: (1) examine the effects of untreated, ozone-treated, and activated charcoal-treated OSPW (OSPW, O3-OSPW, and AC-OSPW) on the early life stage (ELS) of fathead minnow (*P. promelas*); (2) to elucidate the mechanism of the embryotoxic effects caused by OSPWs; and (3) to determine whether the ozonation attenuate the embryotoxic effects. The entire study, including ethics approval security, experimental design, data collection and analyses, and manuscript writing, was conducted by Yuhe He supervised by co-authors Steve B. Wiseman (University of Saskatchewan) and John P. Giesy (University of Saskatchewan). Co-author Sarah Patterson (University of Saskatchewan) also did a part of the laboratory work. Co-authors Markus Hecker (University of Saskatchewan) made valuable comments on manuscript writing. Co-author Nang Wang (University of Alberta), Mohamed Gamal El-Din (University of Alberta), and Jonathan W. Martin (University of Alberta) cooperated on providing analytical chemistry information related to this study and made valuable comments on manuscript writing.

5.1. Introduction

There is increasing concern about the potential environmental effects of petroleum production from oil sands surface-mining in Alberta, Canada. One of the issues is that oil sands process-affected water (OSPW), which is generated by extraction of bitumen using the "Clark hot water extraction process", is toxic to aquatic organisms. Due to a policy of no-release, more than 10^9 m^3 of OSPW are currently stored in on-site tailing ponds, with volumes continuously increasing (Del Rio et al., 2006). Remediation of this process water is a current priority of industries and government agencies (Del Rio et al., 2006, Government of Alberta, 2006).

The majority of the toxicity of OSPW has been attributed to the water soluble organic fraction, of which naphthenic acids (NAs) are one of the primary persistent constituents (Garcia-Garcia et al., 2011a, b, Anderson et al., 2012a, b). Naphthenic acids exist as a mixture, characterized as a group of cyclic and acyclic alkyl-substituted carboxylic acids with the general formula $C_nH_{2n+Z}O_2$, where n is the number of carbons and Z relates to the number of rings (Holowenko et al., 2002, Headley and McMartin, 2004, Clemente and Fedorak, 2005, Frank et al., 2008, Rowland et al., 2011a, b). Ozonation is a promising method for remediation of OSPW as it has been shown to significantly reduce the concentration of NAs (Scott et al., 2008, Martin et al., 2010, Gamal El-Din et al., 2011). However, further work was needed to fully evaluate the effectiveness of ozonation in reducing the toxicity of OSPW and to ensure that ozonation does not cause the formation of byproducts that might impart greater toxicity to OSPW.

Oil sands process-affected water is both acutely and chronically toxic to aquatic organisms (Clemente and Fedorak, 2005) and caused endocrine disrupting effects *in vitro* on sex

steroids synthesis and receptor signaling (He et al., 2010, 2011). Exposure to OSPW decreased synthesis of and plasma concentrations of testosterone (T) and estradiol (E2) in yellow perch (*Perca flavescens*) and goldfish (*Carassius auratus*) (van den Heuvel et al., 1999, Lister et al., 2008). Abundances of transcripts of regulatory genes in all tissues of the hypothalamus-pituitary-gonad-liver axis were significantly different in fathead minnows (*Pimephales promelas*) exposed to OSPW (He et al., 2012b). Exposure to OSPW also adversely affected reproductive capacity of fathead minnows. Fecundity was less, synthesis of sex steroids was altered, and less pronounced secondary sex characteristics were observed in male and female fathead minnows exposed to OSPW (Kavanagh et al., 2011, 2012). It is unknown which components of OSPW are responsible for these adverse effects; however, some NAs that are structurally similar to sex steroid hormones have been identified as potential candidate contaminants for these effects in OSPW (Rowland et al., 2011a, Scarlett et al., 2012). Furthermore, a study using hepatocytes isolated from livers from rainbow trout revealed that exposure to OSPW resulted in greater expression of genes related to the biotransformation of xenobiotics, estrogenicity, and oxidative stress (Gagné et al., 2012).

Oil sands process-affected water is toxic towards early life stages of several species of fishes. When exposed to OSPW, oil sands sediment, or commercial NAs, a greater rate of premature hatching of eggs, lesser survival of embryos, greater incidence of deformities including hemorrhage, pericardial edema, and malformation of spine were observed in fathead minnow, white sucker (*Catostomus commersoni*), yellow perch (*Perca flavescens*), and Japanese medaka (*Oryzias laptipes*) (Colavecchia, et al., 2004, 2006, 2007, Peters et al., 2007). In this study, potential toxicity of OSPW to embryos of fathead minnow was determined and the

efficacy of ozonation to attenuate toxicity of OSPW was assessed. Assessment endpoints included rates of hatching of eggs, spontaneous movement of embryos, and morphological alterations such as malformations, especially of the spine, hemorrhage, and pericardial edema. Measurements of reactive oxygen species (ROS) and abundances of transcripts of genes related to biotransformation, responses to oxidative stress, and apoptosis were also measured to elucidate the mechanism(s) of toxicity of OSPW, and ozone-treated OSPW on fish embryo development.

5.2. Materials and Methods

5.2.1. Exposure waters

Oil sands process-affected water was collected from the West-In-Pit (WIP), an active settling basin on the Syncrude Canada Ltd. site at Fort McMurray, AB, Canada, in February 2010. The concentration of NAs in the OSPW was determined by use of ultra pressure liquid chromatography high resolution mass spectrometry (UPLC-HRMS) to be 19.7 mg/L (Wang, 2011). Ozonation of OSPW was conducted at the University of Alberta (Edmonton, AB, Canada) following standard procedures (Gamal El-Din and Smith, 2002, Wang, 2011). Ozone gas was generated from extra dry, high purity oxygen using an AGSO 30 Effizon® ozone generator (WEDECO AG Water Technology, Herford, Germany). Prior to operation of the generator a ten min stabilization period was utilized to obtain a stable ozone concentration in the feed-gas. The feed gas was sparged into the OSPW through a ceramic fine bubble gas diffuser located at the bottom of a PVC plastic reactor. During the ozonation process, concentrations of ozone in the feed- and off-gas lines were continuously monitored by two identical ozone monitors (model HC-500, PCI-WEDECO). The potassium iodide (KI) method was used to calibrate the ozone monitors periodically according to Standard Methods for the Examination of Water and Wastewater (APHA, 2005). Treatment of the OSPW with ozone was continued until the total degradation of parent NAs reached approximately 90%, as determined by the remaining sum response of all UPLC-HRMS peak area corresponding to NAs. Residual ozone and oxygen were stripped from the generator by purging for 10 min with purified nitrogen gas. Residual ozone in

the reactor was determined by use of the Indigo method (APHA, 2005). Dose of ozone delivered by this system can be calculated (Equation 1) (Gamal El-Din and Smith, 2002).

$$\Delta O3 = \int_0^t \frac{(Q_{G,in}C_{G,in} - Q_{G,out}C_{G,out})}{V_L} dt - C_L \qquad (1)$$

Where: ΔO3 is the amount of utilized ozone (mg/L), $C_{G,in}$ is the ozone concentration in the feed gas, which was calculated from reading the first ozone monitor (mg/L), $C_{G,out}$ is the ozone concentration in the off gas, which was calculated from reading the first ozone monitor (mg/L), C_L is residue ozone concentration in the liquid phase (mg/L), V_L is effective reactor volume (L), $Q_{G,in}$ is feed-gas flow rate (L/min), $Q_{G,out}$ is off-gas flow rate (L/min), and t is ozone contact time (min).

Efficiency of removal of NAs by ozonation was greater than 90% with the total concentration of NAs having been decreased from 19.7 mg/L to 1.9 mg/L in the ozone-treated OSPW (O3-OSPW), which has been determined in a parallel study (Wang, 2011). The activated charcoal-treated OSPW (AC-OSPW) was prepared by mixing OSPW with 5% (weight/volume, w/v) activated charcoal according to Anderson et al. (2012a). Because the organic compounds in OSPW were efficiently absorbed by activated charcoal, this water sample was used as a control to determine whether toxic effects were due to the organic compounds in OSPW.

5.2.2. Embryo Exposure

The fathead minnow is a small fish species that is native to the oil sands region and is commonly used in aquatic toxicology testing in North America and whose life history is well known (OECD, 1992). Fathead minnows were cultured in 200-L tanks in the Aquatic Toxicology Research Facility (ATRF) at the University of Saskatchewan. Three breeding tanks consisting of one sexually mature male and two sexually mature females were established in order to collect eggs for the exposure studies. Each breeding tank contained 20-L of dechlorinated tap water and half this volume was replaced daily. Tanks were maintained at 25±1 ℃ with a 16/8 h day/night photoperiod. Fish were fed twice daily with frozen blood worms.

A 7-day assay to assess effects on development of embryos was designed based on OECD Guideline 210: Fish, Early Life-stage Toxicity Test (OECD, 1992). Fertilized embryos were collected within 1 h post fertilization (hpf) from the different breeding tanks and were pooled in a petri-dish containing control water. Eggs were rinsed 3 times in dechlorinated tap water and any unfertilized eggs were removed. Equal numbers of eggs were randomly placed into wells of a 6-well plate. Depending on the number of eggs available from each spawn the minimum number of eggs per well was 10 and the maximum number of eggs per well was 15 in an exposure replicate. Each well contained 2 mL of control water, which consisted of dechlorinated municipal tap water, OSPW, O3-OSPW, or AC-OSPW. The pH of the control water, OSPW, O3-OSPW, and AC-OSPW was 8.2, 8.7, 8.8 and 9.9, respectively. Fifty-percent of the volume (1 mL) was replaced daily with fresh test solutions. Exposures were performed at

25±1 ℃ with a 16/8h day/night photoperiod. Any dead eggs or larvae were removed daily. Exposure experiments were replicated 8 times and each experiment was performed with a separate batch of eggs.

Observations of embryos were made daily and values of measurement endpoints were recorded prior to the 50% water renewal. Daily measurements made included the number of live and dead embryos, the number of spontaneous embryo movements/minute (measured only at 26 hpf), rate of premature hatching, and prevalence of hemorrhage, pericardial edema, and malformation of spine. Exposures were terminated 168 hpf. At the end of exposure the cumulative percent occurrence of each endpoint was determined and used for statistical analyses. Percentage survival was calculated as the number of live larvae at the end of the experiment divided by the initial number of embryos.

5.2.3. Quantification of reactive oxygen species (ROS)

Concentration of ROS in embryos of fathead minnows were measured at 96 hpf by use of 5-(and-6)-chloromethyl -2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA) according to the manufacturers protocol (Invitrogen, Burlington, ON, Canada). Briefly, all live embryos from each exposure group were washed in dechlorinated tap water, homogenized in 120 µL of cold PBS, and centrifuged at 15,000 g at 4 ℃ for 20 min. A volume of 100 µL supernatant was recovered, diluted in pre-warmed PBS, and then added to a 96-well plate. CM-H_2DCFDA was added to a final concentration of 1 µM and the plate was incubated at 37 ℃ for 30 min. Intensity of fluorescence was measured using a POLARStar OPTIMA microplate reader (BMG Labtech) with excitation and emission at 485 nm and 520 nm, respectively. Concentrations of

ROS were normalized to protein content, which was determined by use of the Bio-Rad protein assay (Bio-Rad, Mississauga, ON, Canada).

5.2.4. Quantification of abundances of transcripts

Abundances of transcripts of ten genes related to biotansformation of xenobiotics, response to oxidative stress, and apoptosis were determined. All primers were designed using Primer 3 software and based on sequences obtained by Illumina RNA sequencing (unpublished data). Nucleotide sequences of primers and the biological functions of the target transcripts are given (Table 5.1).

Total RNA was extracted from embryos by use of the Qiagen RNeasy® Plus Mini Kit according to the manufacturer's protocol (Qiagen, Mississauga, ON, Canada) and stored at −80 °C until required. First-strand cDNA was synthesised from 1 μg of total RNA using an iScript™ cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's instructions. The cDNA samples were stored at −20 °C until further analysis.

Quantitative real-time PCR (qPCR) was performed on an ABI 7300 Real-Time PCR System in 96-well PCR plates (Applied Biosystems, Foster City, CA, USA). A PCR reaction mixture for 1 reaction contained 10 μL of SYBR® green master mix (Applied Biosystems), 2 μL of sense/anti-sense gene-specific primers (Invitrogen, Carlsbad, CA, USA), and 8 μL of cDNA that was diluted in RNase free water (Qiagen). The PCR reaction mix was denatured at 95 ℃ for 10 min before the first PCR cycle. The thermal cycle profile was: denaturizing for 15s at 95 ℃ and annealing and extension for 1 min at 60 ℃ for a total of 40 PCR cycles. Efficiency,

uniformity, and linear dynamic range of each qPCR assay were assessed by construction of standard curves by use of serially diluted cDNA standards. Changes in abundances of transcripts of target genes were quantified by normalizing to 18s rRNA according to the method of Simon (2003).

Table 5.1. Abbreviations (Abb.), name of target gene, category, nucleotides sequence, and efficiency (Eff.) used for quantitative real-time PCR (qPCR). Primer pairs are designed based on Illumina RNA sequencing (unpublished data).

Abb.	Target gene	Category	Primer pair (5'-3')	Eff.
18S	Ribosomal RNA 18S	Reference gene	F:GCCCTGTAATTGGAATGAGC R:TCCCGAGATCCAACTACGAG	1.96
CYP1A	Cytochrome p450 1A	Phase I metabolism	F:CCTGCAGGGAGAACTGAG R:TCGACGTACAGTGAGGGA	1.85
CYP3A	Cytochrome p450 3A	Phase I metabolism	F:CGACGAGACCTTCCCAAAT R:GTTTTCTTGCAGACCCGTT	1.89
GST	Glutathione-S-transferase	Phase II metabolism/ Oxidative stress	F:CCGGCAAGAGCTTCACCAT R:GTGAAGTCGTGGGAAATAGGC	1.90
SOD	Superoxide dismutase	Oxidative stress	F:CCAGACATGTCGGAGACCTT R:ATGGAATGTTGCCCTGAGAG	1.96
CAT	Catalase	Oxidative stress	F:TTATCAGGGATGCGCTTCTGT R:TTCACATGAGTCTGCGGATTTC	1.91
CASP3	Caspase-3	Apoptosis	F:GTAACGGGACACACGAGGAT R:GTGATCCTGCCGAGACACTT	1.95
CASP9	Caspase-9	Apoptosis	F:GCAGTTCATGGTGTTGATGG R:CTTCTCACCTCCTCCACAGG	1.93
APOPIN5	Apoptosis Inhibitor 5	Apoptosis	F:GCCACCAGAAGAGGAGAATG R:CCCAGTTGGTGGAAGCTAAA	1.98
APOPEN	Apoptosis Enhancer	Apoptosis	F:AGGATGGGTCACTGCTCTGT R:GTGCTTTAGGGAGCTGTTGG	1.91
BAX	Bax	Apoptosis	F:AGCCTATACGCGAGGTGA R:ACCTTAACCGGAATGCTGT	1.96
P53	P53	Apoptosis	F:GATGGTGAAGGACGAAGG R:AGGCAGTCCAAAAAGAGC	1.95

5.2.5. Statistical analyses

Statistical analyses were conducted using SPSS16.0 (SPSS, Chicago, IL, USA). All data are expressed as mean ± standard error of the mean. Normality of distributions of data was assessed by use of the Kolomogrov-Smirnov, one-sample test and homogeneity of variance was determined by Levene's test. When necessary, datasets were $\log_{10}$-transformed to meet assumptions of parametric tests. Non-transformed data are presented in figures. Statistical differences were evaluated by one-way ANOVA followed by *post-hoc* Tukey's test. Differences were considered statistically significant at $\rho < 0.05$.

5.3. Results and discussion

5.3.1. Developmental toxicity of oil sands process-affected waters (OSPWs)

Effects of OSPW on eggs and embryos of fathead minnows were consistent with the results of other studies. Exposure to OSPW resulted in a significantly greater incidence of premature hatching of eggs (Table 5.2). Cumulative rates of hatching of embryos exposed to OSPW were significantly greater than cumulative rates of hatching of embryos exposed to O3-OSPW or AC-OSPW at 48, 72, 96, 120, and 144 hpf. Embryos exposed to OSPW or O3-OSPW began hatching at 48 hpf and embryos exposed to AC-OSPW began to hatch at 72hpf. No embryos hatched until 120 hpf in the control group. Exposure to OSPW significantly decreased the embryo survival rate (43.8±7.12%) compared to those of control (97.9±2.08%), O3-OSPW (93.8±3.99%), and AC-OSPW (77.1±7.12%) treated fish, respectively (Table 5.2). At 26 hpf, embryos exposed to OSPW exhibited a significantly greater rate of spontaneous movement (48.6±2.74 movement/min) compared to the control (25.3±1.15 movements/min), O3-OSPW (29.25±1.88 movements/min), and AC-OSPW (26.63±1.23 movements/min) treatment groups.

Several studies have reported reduced hatching success and survival of embryos exposed to oil sands sediment, PAHs, petroleum oil, and fractions of crude oil (Middaugh et al., 1998, 2002, Carls et al., 1999, Couillard, 2002, Colavecchia et al., 2004, 2006, 2007, Peters et al., 2007). These studies, together with the results of the present study, indicate that the organic compounds in OSPW are toxic to eggs and embryos, especially during organogenesis. Specifically, a greater incidence of premature hatching of embryos was a significant consequence of exposure to OSPW. Premature hatching has been attributed to rupturing of

hatching glands due to stimulation of respiration or irritation by soluble hydrocarbons (Leung and Bulkley, 1979). The greater rate of spontaneous movement of embryos exposed to OSPW might be due to disruption in neurophysiological function (Drapeau et al., 2002). Increased spontaneous movement of fish embryo has been observed in embryos exposed to chemicals such as polybrominated diphenyl ethers (PBDEs) (Usenko et al., 2011).

Exposure to OSPW resulted in significantly greater incidences of deformities in embryos of fathead minnows (Table 5.2 and Figure 5.1). At the end of exposure (168 hpf) the total incidences of hemorrhage, pericardial edema, and malformation of spine in embryos exposed to OSPW were 50.0±3.40%, 56.3±7.12%, and 37.5±5.38%, respectively. Incidences of deformities of embryos exposed to O3-OSPW or AC-OSPW were significantly less than in embryos exposed to OSPW and were not significantly different from the control groups, except for the significantly greater incidence of hemorrhage at 168 hpf in embryos exposed to O3-OSPW (12.5±2.41%). No deformities were detected in the control group.

Deformities observed during development of embryos exposed to OSPW were consistent with the results of other studies of oil sands sediments and OSPW. Those studies reported hemorrhage, pericardial edema, and malformation of spine in fish embryos after exposure to oil sands sediment or OSPW (Colavecchia et al., 2004, 2006, 2007, Peters et al., 2007). These deformities are similar to symptoms of dioxin-induced "blue sac disease", which is induced when PAHs and other dioxin-like compounds activate the aryl hydrocarbon receptor (AhR) (Fernandez-Salguero et al., 1996). The mechanism of toxicity due to activation of AhR signaling includes induction of CYP1A, oxidative stress, and damage to endothelial cells. Dioxins-

induced expression of CYP1A is correlated with oxidative DNA damage, and is co-localized with damage to tissues and programmed death of cells in both embryos and in visibly healthy post-hatch fry (Cantrell et al., 1996, 1998, Park et al., 1996). Although there is similarity of toxic effects caused by exposure to OSPW and dioxin-like compounds, since the abundance of transcripts of *cyp1a* was not greater in embryos exposed to OSPW compared to the control, the toxic effects observed during development during the present study seem to not be mediated by the AhR. Moreover, no significant AhR-mediated potency was detected when H4IIE-*luc* cells - an *in vitro* assay for the detection AhR agonists and antagonists (Hilscherova et al., 2000) - were exposed to OSPW. These observations are supported by a recent study where the AhR binding potential of individual NAs from OSPW were modeled and it was determined that the compounds tested did not bind to the AhR (Scarlett et al., 2012). A role for sediment bound PAHs in the embryotoxic effects of oil sands sediments has been suggested (Colavecchia et al., 2004, 2006, 2007) but the current study suggests that organic compounds in sediment-free OSPW that do not activate the AhR might cause these embryotoxic effects.

Table 5.2. The survival of embryos, rate of spontaneous embryo movement (Sp. movement), and incidences of premature hatching and deformities including hemorrhage, pericardial edema, and malformation of spine in different time points in the control, untreated oil sands process-affected water (OSPW), ozonated oil sands process-affected water (O3-OSPW), and activated charcoal-treated oil sands process-affected water (AC-OSPW) exposure groups. Numbers represent the mean ± standard error of the mean of 4 independent replicate exposures. Significant differences were evaluated with one-way ANOVA followed by *post-hoc* Tukey's test. Different letters represent significant differences among treatment groups at the same time-point ($\rho < 0.05$). N.D. = Not detected. hpf = Hours post-fertilization.

Endpoint	Time point (hpf)	Control	OSPW	O3-OSPW	AC-OSPW
Survival (% of initial No.)	168	97.92 ± 2.08^{a}	43.75 ± 7.12^{b}	93.75 ± 3.99^{a}	77.08 ± 7.12^{a}
Sp. Movement (No. per min)	26	25.31 ± 1.15^{a}	48.63 ± 2.74^{b}	29.25 ± 1.88^{a}	26.63 ± 1.23^{a}
Hatching (% of initial No.)	48	N.D.^{a}	14.58 ± 2.08^{b}	2.08 ± 2.08^{a}	N.D.^{a}
	72	N.D.^{a}	31.25 ± 3.99^{b}	8.33 ± 5.89^{abc}	12.50 ± 2.41^{c}
	96	N.D.^{a}	68.75 ± 3.99^{b}	29.17 ± 9.92^{c}	22.92 ± 3.99^{c}
	120	18.75 ± 3.99^{a}	87.50 ± 5.38^{b}	60.42 ± 7.12^{c}	52.08 ± 3.99^{c}
	144	97.92 ± 2.08^{a}	89.58 ± 3.99^{a}	95.83 ± 2.41^{a}	83.33 ± 5.89^{a}
Hemorrhage (% of initial No.)	168	N.D.^{a}	50.00 ± 3.40^{b}	12.50 ± 2.41^{c}	10.42 ± 5.24^{ac}
Pericardial edema (% of initial No.)	168	N.D.^{a}	56.25 ± 7.12^{b}	6.25 ± 3.99^{a}	10.42 ± 6.25^{a}

Malformation of spine (% of initial No.)	168	N.D.[a]	37.50 ±5.38[b]	6.25 ±2.08[a]	4.17 ±2.41[a]

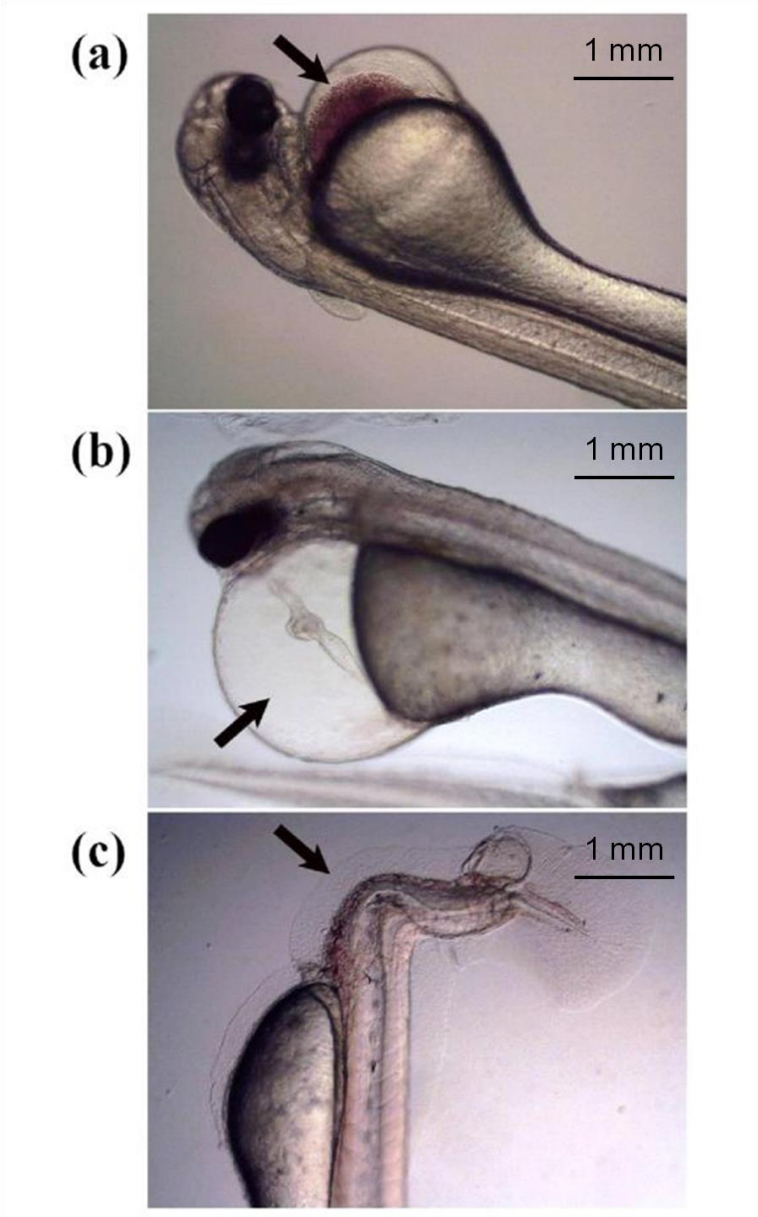


Figure 5.1. Photographs of typical teratogenic responses of fathead minnow embryos: (a) hemorrhage, (b) pericardial edema, and (c) malformation of spine. Images were taken at 20 times magnification.

5.3.2. Abundances of transcripts

Exposure to OSPW affected abundances of transcripts of genes that encode cytochrome P450s (Figure 5.2a). There were no significant differences in the abundance of transcripts of *cyp1a* among exposure groups. However, the abundance of transcripts of *cyp3a* was significantly greater by 2.35±0.34-fold in embryos exposed to OSPW compared to the control group. The abundance of transcripts of *cyp3a* in embryos exposed to O3-OSPW or AC-OSPW were not significantly different from that of the control but were significantly lesser than in embryos exposed to OSPW (Figure 5.2a).

Abundances of transcripts of genes involved in responses to oxidative stress were significantly affected by exposure to OSPW (Figure 5.2b). Abundances of transcripts of *gst* and *sod* were significantly greater by factors of 2.15±0.26 and 3.08±0.74-fold, respectively, in embryos exposed to OSPW compared to the control group. Abundances of transcripts of *gst* and *sod* in embryos exposed to O3-OSPW or AC-OSPW were not significantly different from those of the control, but were significantly lesser than in embryos exposed to OSPW. Abundances of transcripts of *cat* were not significantly different among any of the exposure groups.

Abundances of transcripts of genes that regulate and mediate apoptosis were significantly affected in embryos exposed to OSPW (Figure 5.2c). Abundances of transcripts of *casp9* and *apoen* were significantly greater by a factor of 3.26±0.57 and 2.38±0.25-fold, respectively, in the embryos exposed to OSPW compared to the control group. Abundances of transcripts of *casp9* and *apopen* were not significantly different in embryos exposed to O3-OSPW and AC-OSPW compared to the control groups, but were significantly lesser than in embryos exposed to OSPW.

Abundances of transcripts of *casp3, p53, apopin5,* and *bax* were not significantly different among any of the treatment groups.

Organic compounds in OSPW might act as agonists of the pregnane-x-receptor (PXR). PXR is a nuclear receptor that is activated by endogenous and exogenous chemicals that up-regulate expression of proteins involved in biotransformation of xenobiotics (Kliewer et al., 2002). One of the primary targets of PXR activation is induction of CYP3A, a phase I oxidative enzyme that is responsible for the metabolism of xenobiotics (Bertilsson et al., 1998). In addition, PXR can interact with factors binding to the antioxidant response element to elicit the pregnane induced response and up-regulates expression of phase II conjugating enzymes such as glutathione S-transferase (GST) (Falkner et al., 2001). In the present study, the abundance of transcripts of *cyp3a* was significantly greater in embryos exposed to OSPW compared to embryos exposed to control water, O3-OSPW, or AC-OSPW. This is consistent with a previous study where OSPW caused greater abundance of transcripts of *cyp3a* in hepatocytes isolated from rainbow trout (Gagne et al., 2012). As discussed below, the abundance of transcripts of *gst* was greater in embryos exposed to OSPW. This finding is evidence that compounds in OSPW are agonists of the PXR as this receptor can regulate expression of GST (Higgins and Hayes, 2011). The identities of agonists of the PXR in OSPW are not known. Aromatic acids have been identified in OSPW (Jones et al., 2012) so it will be important to determine whether these compounds activate signaling pathways regulated by the PXR.

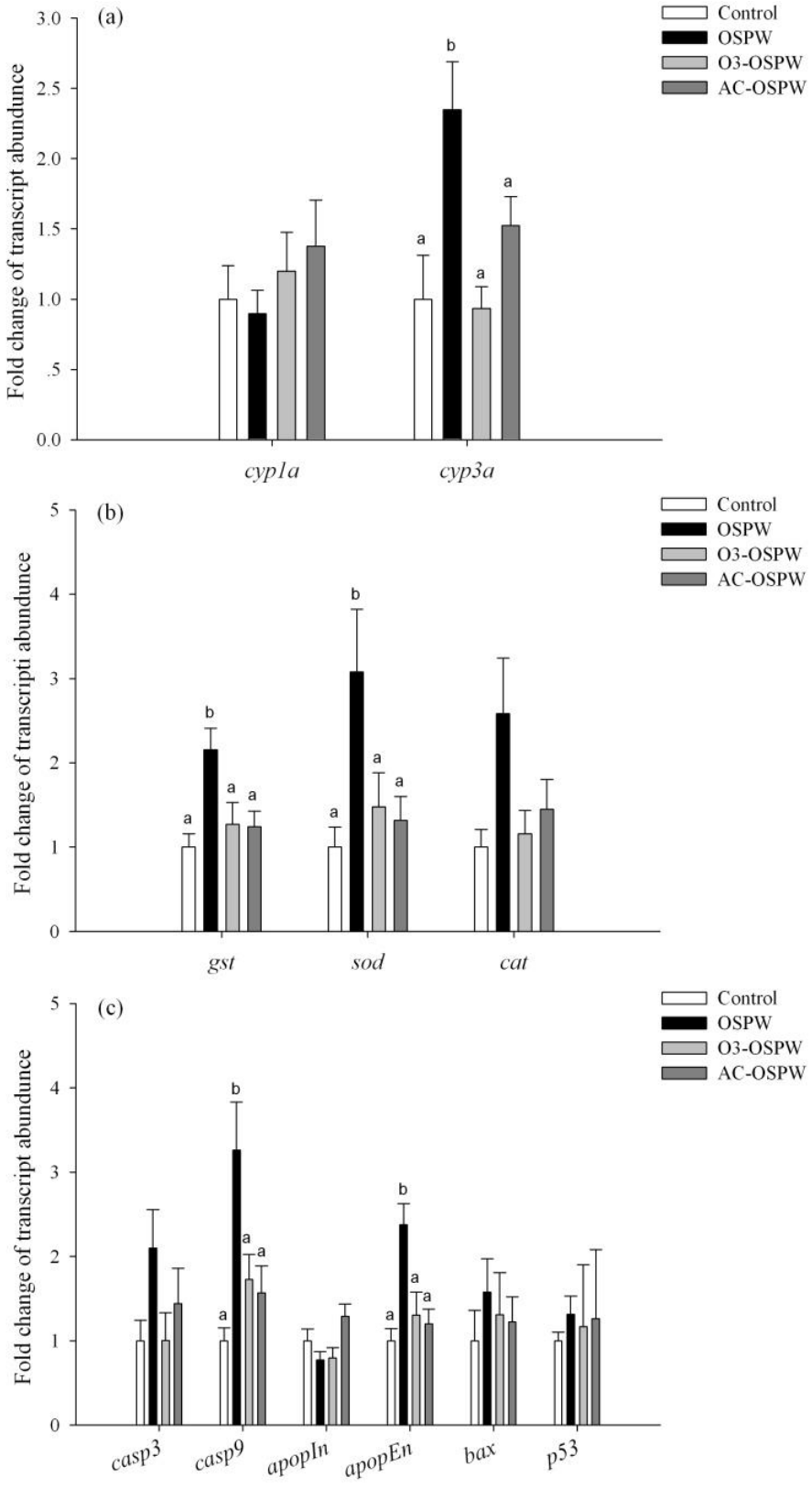

(a)
Control
OSPW
O3-OSPW
AC-OSPW
Fold change of transcript abundunce
3.0
2.5
2.0
1.5
1.0
.5
0.0
a
b
a
a
cyp1a
cyp3a
(b)
Control
OSPW
O3-OSPW
AC-OSPW
Fold change of transcripti abundunce
5
4
3
2
1
0
a
b
a
a
a
b
a
a
gst
sod
cat
(c)
Control
OSPW
O3-OSPW
AC-OSPW
Fold change of transcript abundunce
5
4
3
2
1
0
a
b
a
a
a
b
a
a
casp3
casp9
apopIn
apopEn
bax
p53

Figure 5.2. Fold-change in abundances of transcripts of genes related to (a) the metabolism of xenobiotics (b) oxidative stress, and (c) apoptosis in the control, untreated oil sands process-affected water (OSPW), ozonated oil sands process-affected water (O3-OSPW), and activated charcoal-treated oil sands process-affected water (AC-OSPW) exposure groups. Bars represent the mean ± standard error of the mean of 7 independent replicate exposures. Significant differences were evaluated with one-way ANOVA followed by *post-hoc* Tukey's test. Different letters represent significant differences among treatment groups ($\rho < 0.05$).

5.3.3. Concentrations of reactive oxygen species (ROS)

Concentrations of ROS in embryos exposed to OSPW was significantly 1.68±0.11-fold greater compared to that of the control embryos. Concentrations of ROS in embryos exposed to O3-OSPW or AC-OSPW were not significantly different from concentrations in control embryos but were, nonetheless, significantly lesser than the concentration in embryos exposed to OSPW (Figure 5.3).

Oxidative stress is a plausible explanation for the toxicity of OSPW to developing embryos. The malformations in embryos exposed to OSPW are consistent with those caused by oxidative stress (Deng et al., 2009, Mussi and Calcaterra 2010, Bui et al., 2012). Oxidative stress results when antioxidant defense mechanisms become saturated, and concentrations of ROS exceed the levels produced during normal functioning of cells. This exceedance of the capacity of cells to reduce ROS can then ultimately result in damage to tissues and cells (Zhang et al., 2012). Glutathione-S-transferase (GST), a phase II enzyme that facilitates detoxification of drugs, together with superoxide dismutase (SOD) and catalase (CAT), play key functions in clearance of ROS. Greater abundances of transcripts of these genes suggested that there was greater production of ROS in embryos exposed to OSPW. In a recent study that used hepatocytes from rainbow trout, exposure to extracts of OSPW and water accommodated with oil sands caused significantly greater abundance of transcripts of *gst* and *sod* (Gagné et al., 2012). This observation is consistent with the results of the present study. The source of the ROS is not known, but transformation of substrates by CYP1A and CYP3A results in production of reactive

oxygen species (ROS) (Zangar et al., 2004) and generation of ROS in microsomes has been correlated with total P450 content and CYP3A activity (Shaik and Mehvar, 2010).

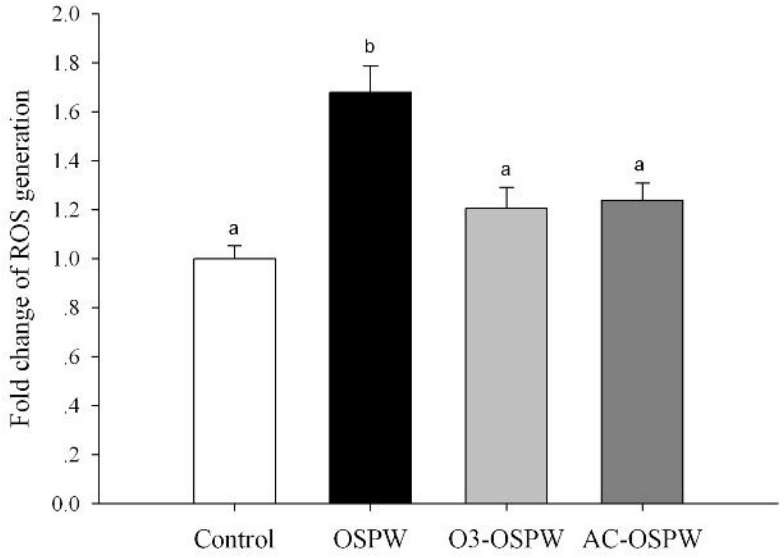


Figure 5.3. Fold change of reactive oxygen species (ROS) generation in the control, untreated oil sands process-affected water (OSPW), ozonated oil sands process-affected water (O3-OSPW), and activated charcoal-treated oil sands process-affected water (AC-OSPW) exposure groups. Numbers represent the mean ± standard error of the mean of 8 independent replicate exposures. Significant differences were evaluated with one-way ANOVA followed by *post-hoc* Tukey's test. Different letters represent significant differences among treatment groups ($\rho < 0.05$).

5.3.4. Apoptosis induced by oxidative stress might be the primary mechanism of embryotoxicity resulted from oil sand process-affected water (OSPW) exposure

Exposure to OSPW might have caused apoptosis. Oxidative stress caused by ROS can induce apoptosis in developing embryos of zebrafish (Yamashita, 2003, Deng et al., 2009). These ROS can damage DNA and when damage to DNA is irreparable apoptosis is initiated by activation of the tumor suppressor protein p53. Once p53 is activated it induces up-regulation of pro-apoptotic proteins, including death receptors and their ligands (Langheinrich et al., 2002). B-cell lymphoma 2-associated X (BAX), which is a member of the BCL-2 family of genes, triggers a mitochondrial pro-apoptotic pathway by inducing mitochondrial outer-membrane permeabilization and promoting the release of cytochrome c, which, in turn, triggers activation of the caspase enzymes cascade (Bernardi et al., 2001, Gottlieb, 2001, Pyati et al., 2007). Caspase-9 (CASP9) is an initiator caspase, which has been linked to the mitochondrial apoptotic pathway. Activated CASP9 initiates cleavage of other inactive pro-caspases such as Caspase-3 (CASP3). CASP3 initiates apoptosis by cleaving cellular substrates, which results in shrinkage of cells and degradation of the contents of cells (Jaeschke et al., 2012). Apoptosis enhancing nuclease (APOPEN) is an exonuclease that is induced by p53 following DNA damage and digests double-stranded DNA to form single-stranded DNA and amplifies DNA signals related to damage to DNA, which results in enhancement of apoptosis (Kawase et al., 2008). Apoptosis inhibitor 5 (APOPIN5) is an inhibitor of apoptosis that prevents fragmentation of DNA after activation by CASP3 (Morris et al., 2006). The significantly greater abundances of transcripts of *casp9* and *apopen*, and the lack of a change in the abundance of transcripts of *apopin5* in embryos exposed

to OSPW suggest that exposure to OSPW resulted in the activation of the oxidative stress-induced apoptotic pathway.

The source of oxidative stress that might have caused the toxic effects of OSPW towards embryos of fathead minnows is not known. Although some of the effects observed in embryos exposed to OSPW are similar to those caused by exposure to AhR agonists it was concluded that the primary mechanism of toxic action during development of embryos was independent of activation of the AhR. Rather, oxidative stress due to greater concentrations of ROS might be due to the metabolism of substrates by the CYP3A that is induced by binding of compounds to the PXR. Further studies are required to identify agonists of the PXR in OSPW.

5.3.5. Effects of ozone treatment

Toxicity of OSPW to developing embryos of fathead minnow is caused by the organic fraction, possibly the NAs. Reducing the concentration of the organic fraction of OSPW by either ozonation or activated charcoal significantly attenuated all of the adverse effects observed in embryos exposed to OSPW. Exposure to O3-OSPW or AC-OSPW did not cause any significant effects on embryo development except for limited incidences of premature hatching. With the exception of some hemorrhaging at 168 hpf in embryos exposed to O3-OSPW there were no cases of deformities in embryos exposed to O3-OSPW or AC-OSPW. Although oxidative stress might have been caused by osmotic stress from salts and metals in OSPW, considering the fact that both ozonation and activated charcoal treatment did not significantly reduced the amount of salts and metals, the attenuation of effects were most likely attributed to the removal of organic content in OSPW. In addition, exposure to O3-OSPW and AC-OSPW

did not cause any significant changes in generation of ROS or changes in abundances of transcripts of genes related to biotransformation, oxidative stress, and apoptosis. The results of the current study, together with those of previous studies (Anderson et al., 2012a, b, He et al., 2010, 2011, Garcia-Garcia et al., 2011c), suggest that ozonation is a promising method for remediation of OSPW.

5.4. Conclusions

In conclusion, the results of the present study demonstrated that exposure to OSPW caused adverse effects in developing fathead minnow embryos. Lesser survival, greater incidences of premature hatching, and greater incidences of deformities such as hemorrhage, pericardial edema, and malformation of spine were caused by exposure to OSPW. The results suggest that caspase-activated apoptotic cell death, induced by oxidative stress resulting from metabolism of substrates by P450 enzymes that are not induced by activation of the AhR, was the primary mechanism of effects on embryos. Ozonation is a promising method for the remediation of OSPW because it significantly attenuated the developmental toxicity towards embryos of fathead minnow.

CHAPTER 6: General discussion

6.1 Introduction

Concerns over the toxicity of oil sands process-affected water (OSPW) generated during the extraction of oil from bitumen in the surface mining oil sands industry are increasing as the volumes of OSPW stored in tailings ponds increases. Oil sands process-affected water is toxic to a range of aquatic organisms (MacKinnon and Boerger, 1986, Holowenko et al., 2002, Clemente and Fedorak, 2005, Frank et al., 2008, 2009, Anderson et al., 2012a, He et al., 2012b). The remediation of OSPW stored in tailings ponds is a major challenge for oil sands industry. Because the disappearance half-lives for NAs in OSPW is 12.8-13.6 yr (Han et al. 2009), more aggressive efforts are necessary for the remediation of OSPW. Ozonation is a promising method for reducing the toxicity and concentration of NAs in OSPW (Martin et al., 2010). However, since the ozonation treatment results in production of new organic chemicals such as oxidized NAs which may present as new toxic components of OSPW, the effectiveness of ozonation treatment on the toxicity of OSPW/NAs needed further investigation.

The objectives of the research conducted in this thesis were to determine the toxicity of OSPW, in particular the endocrine disrupting and embryotoxic effects of OSPW, and to assess the effects of ozonation treatment on the toxicity of OSPW. The first study was designed to investigate the effects of OSPW on synthesis of sex steroid hormones in the H295R cell line. The results of the study indicate that OSPW can disrupt steroidogenesis resulting in impaired

production of T and E2. The second *in vitro* study was designed to determine whether OSPW had estrogenic or androgenic properties. The results of the study indicate the presence of estrogenic and/or antiandrogenic chemicals in OSPW. Because the results from the first two *in vitro* studies suggested that OSPW might have endocrine disrupting effects, a third study was designed to investigate effects of OSPW on the transcription of genes that are important for sexual development and reproduction in small fish model, the fathead minnow (*Pimephales promelas*). The results of this study indicated that exposure to OSPW impairs expression of genes along the brain-gonad-liver axis of the endocrine system, suggesting the possibility of impaired reproduction by fathead minnows. Establishment of healthy and viable populations of fish in an aquatic environment is dependent not only successful reproduction, but also successful growth and development of embryos. Therefore, the fourth study focused on the effects of OSPW on the development of embryos of fathead minnows. The results of the fourth study suggest that OSPW is embryotoxic.

Taken together, the results of the 4 studies suggest that, due to potential effects on reproduction, growth, and development of fathead minnows caused by exposure to OSPW, establishment of healthy and self-sustaining populations of fish in reclaimed tailings ponds is not likely, at least during the initial years when the concentrations of organic compounds is greatest. However, ozonation treatment, which reduced the concentrations of organic compounds in OSPW, especially the NAs, significantly attenuated most of the toxic effects of OSPW both *in vitro* and *in vivo*, suggesting that ozonation is a promising method for remediation of OSPW. Based on the results of studies 1-4, ponds created from OSPW that had been ozonated would

likely be able to sustain viable populations of fish. The null hypotheses, specific project approaches, and conclusions are illustrated in Figure 6.1.

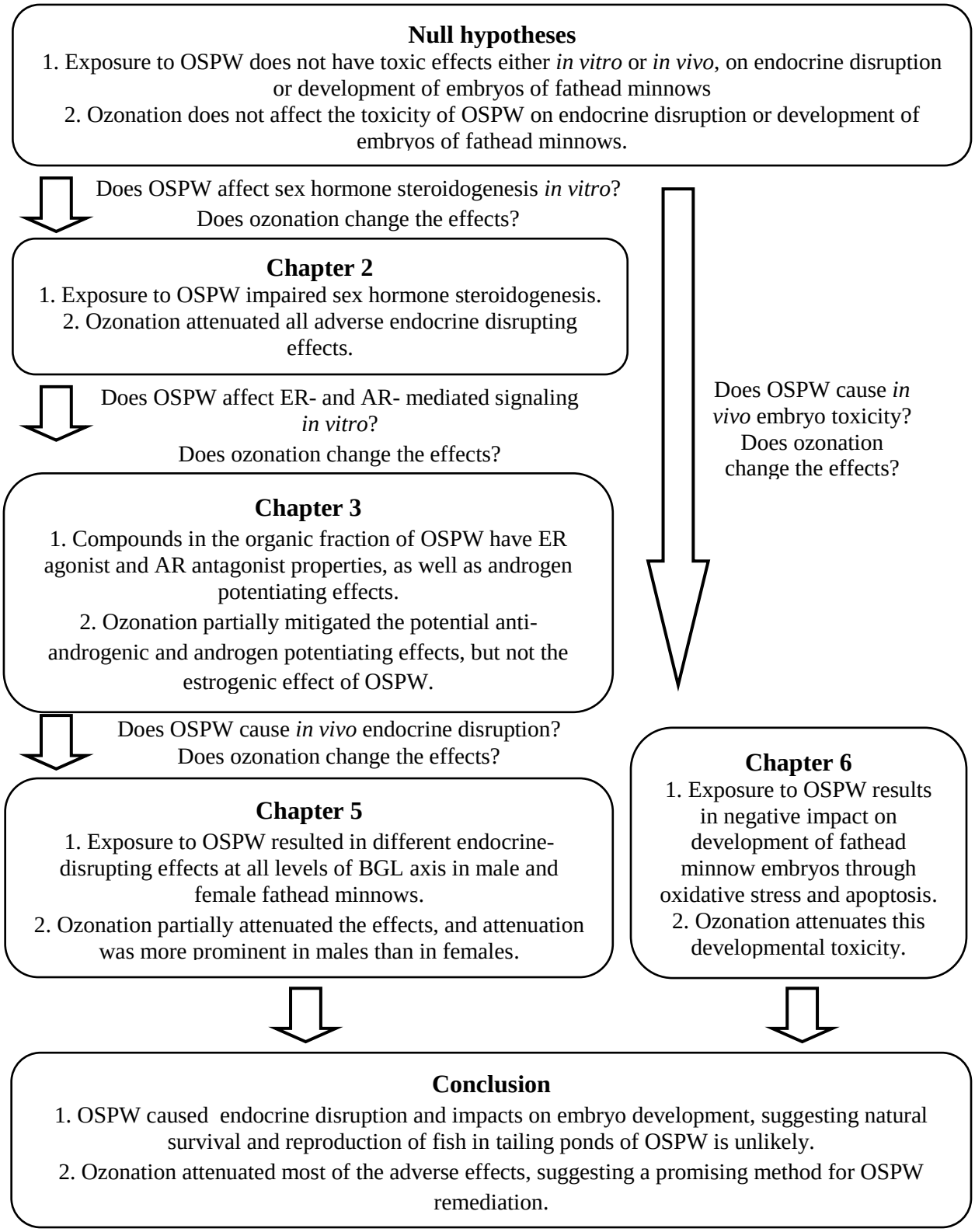

Null hypotheses
1. Exposure to OSPW does not have toxic effects either in vitro or in vivo, on endocrine disruption or development of embryos of fathead minnows
2. Ozonation does not affect the toxicity of OSPW on endocrine disruption or development of embryos of fathead minnows.
Does OSPW affect sex hormone steroidogenesis in vitro?
Does ozonation change the effects?
Chapter 2
1. Exposure to OSPW impaired sex hormone steroidogenesis.
2. Ozonation attenuated all adverse endocrine disrupting effects.
Does OSPW affect ER- and AR- mediated signaling in vitro?
Does ozonation change the effects?
Chapter 3
1. Compounds in the organic fraction of OSPW have ER agonist and AR antagonist properties, as well as androgen potentiating effects.
2. Ozonation partially mitigated the potential anti-androgenic and androgen potentiating effects, but not the estrogenic effect of OSPW.
Does OSPW cause in vivo endocrine disruption?
Does ozonation change the effects?
Chapter 5
1. Exposure to OSPW resulted in different endocrine-disrupting effects at all levels of BGL axis in male and female fathead minnows.
2. Ozonation partially attenuated the effects, and attenuation was more prominent in males than in females.
Does OSPW cause in vivo embryo toxicity?
Does ozonation change the effects?
Chapter 6
1. Exposure to OSPW results in negative impact on development of fathead minnow embryos through oxidative stress and apoptosis.
2. Ozonation attenuates this developmental toxicity.
Conclusion
1. OSPW caused endocrine disruption and impacts on embryo development, suggesting natural survival and reproduction of fish in tailing ponds of OSPW is unlikely.
2. Ozonation attenuated most of the adverse effects, suggesting a promising method for OSPW remediation.

Figure 6.1. An overview of hypotheses, specific project approaches, and conclusions. OSPW = oil sands process-affected water, ER = estrogen receptor, AR = androgen receptor, BGL axis = brain-gonad-liver axis.

6.2 The endocrine disruptive properties of oil sands process-affected water (OSPW)

6.2.1 Effects of oil sands process-affected water (OSPW) and ozone-treated oil sands process-affected water (O3-OSPW) on sex steroid production, *in vitro*

The first two studies (Chapter 2 and 3) focused on endocrine disruptive properties of OSPW determined by *in vitro* assays. In the first study (Chapter 2), exposure to OSPW resulted in decreased production of T and increased production of E2 in the H295R cell line. Lesser concentrations of T and E2 in blood plasma from fish exposed to OSPW (Van den Heuvel et al., 1999, Lister et al., 2008) as well as lesser synthesis of T and E2 by gonads from fish exposed to OSPW (Tetrault et al., 2003) have been reported. The lesser synthesis of T in the H295R cell line is consistent with other studies, while the production of E2 was not, suggesting the difference of specificity and sensitivity of endocrine disruptive properties between human cell lines and fish model. This point must be considered in the future if human cell line assays are applied as rapid screening tools for endocrine disruption in the routine monitoring of OSPW. The first study also gives some mechanistic insight into how OSPW disrupts sex hormone steroidogenesis. Lesser abundance of transcripts and lesser activity of aromatase (CYP19a), which is the enzyme that converts T in E2, as well lesser metabolism of E2 by sulfotransferases, were quantified in cells exposed to OSPW. These effects were not present in cells exposed to ozone-treated OSPW therefore indicating that the endocrine disrupting chemicals in OSPW are present in the dissolved organic fraction.

6.2.2 Effects of oil sands process-affected water (OSPW) and ozone-treated oil sands process-affected water (O3-OSPW) on activation of the estrogen receptor (ER) and androgen receptor (AR), *in vitro*

The second study (Chapter 3) used a series of *in vitro* assays to demonstrate the receptor-mediated estrogenic and anti-androgenic properties of OSPW. It is the first study demonstrating that compounds present in the dissolved organic fraction of OSPW can behave as agonists of the ER and antagonists of the AR. There are several reports of binding and activation of sex steroid hormone receptors by effluent from oil production platforms, including crude oil and refined products (Gamal El-Din and Smith, 2002, APHA, 2005, Tollefsen et al., 2006, Thomas et al., 2009, Vrabie et al., 2010). The NAs present in water from oil platforms in the North Sea were agonists of the ER and antagonists of the AR (Thomas et al., 2004). The compounds in OSPW that are the weak estrogens of the ER are difficult to identify due to the complex chemistry of OSPW. However, a recent study identified steroidal aromatic NAs in OSPW with structural similarities to estradiol (Rowland et al., 2011a). Interestingly, ozonation of the OSPW did not attenuate the estrogenic effects. One explanation is that less cyclic and less branched NAs present in OSPW that are unaffected by ozonation act as the agonists of the ER. Another explanation is that oxidized NAs naturally present in untreated OSPW which are both degraded and formed during the ozonation process act as the agonists of the ER. The second hypothesis is partially supported by Rowland et al. (2011a). According to the authors, ozonation of some of the steroidal aromatic "naphthenic acids" identified in OSPW can result in dehydration, ring-opening, C ring fragmentation and formation of tricyclic acids, keto acids, and hydroxy acids, producing chemicals with less estrogenicity, while some of the steroidal aromatic "naphthenic

acids" become hydroxylated on the aromatic ring, leading to production of estrogen-like compounds.

The data presented in Chapter 3 demonstrates that there are compounds in the dissolved organic fraction of OSPW that are anti-androgenic and compounds that potentiate the effects of T on the AR. The specific mechanism(s) of these effects are not clear. As discussed in Chapter 3, the organic compounds in OSPW might alter the physiological properties of the cell membrane, resulting in greater bioavailability of T compared to cells exposed to the same concentration of T alone, which has been demonstrated with other hydrophobic organic compounds (Glover and Wood, 2005, Mayer et al., 2007). Under such a scenario the antagonists of the AR that are present in OSPW would be considered weak antagonists, since the anti-androgenicity is only evident at the concentrations of T less than 0.1 nM, and concentrations of T greater than 1 nM are able to compete with the androgen potentiating effects of OSPW. Ozonation attenuated the anti-androgenic effect of OSPW, but only partially attenuated the androgen potentiating effect. Because the OSPW and the commercial mixture of NAs both have similar effects on the AR it suggests that some components of OSPW have similar properties with the Merichem NAs mixture, and the components of OSPW responsible for the potentiating effect might be partially affected by ozonation.

6.2.3 Effects of oil sands process-affected water (OSPW) and ozone-treated oil sands process-affected water (O3-OSPW) on expression of genes along the brain-gonad-liver axis of fathead minnows

The third study (Chapter 4) demonstrated that the abundances of transcripts of genes along the brain-gonad-liver (BGL) axis that are important for the regulation of sexual maturation and reproduction are significantly affected in male and female fathead minnows exposed to OSPW. This study provides a mechanistic bridge for filling the gap between the identification of estrogenic organic compounds in OSPW (Rowland et al., 2011a) and the impaired reproductive physiology of fathead minnows exposed to OSPW and extracts of NAs from OSPW (Kavanagh et al., 2011, 2012) (Figure 6.2).

The identification of steroidal aromatic NAs in OSPW provides a mechanistic basis for the estrogenicity of OSPW (Rowland et al., 2011a). It is not surprising that as a complex mixture, OSPW contains numerous estrogen-like NAs which might have effects on the endocrine system. Because compounds in the organic fraction of OSPW are agonists of the ER (Chapter 3), one mechanism by which OSPW might have exerted this affect is via estrogen-like NAs in OSPW altering the abundances of transcripts of estrogen response genes in the liver, including estrogen receptor alpha (*erα*), estrogen receptor beta (*erβ*), vitellogenin (*vtg*), choriogenin-L (*chg-l*) and choriogenin-H (*chg-h*). In turn, negative feedback pathways that regulate synthesis and release of gonadotropins and sex steroid hormones would alter the abundances of transcript in the brain and gonads to compensate for the estrogenic environment in the liver. Alternatively, the organic compounds in OSPW, including NAs, might also have directly affected the synthesis and actions of gonadotropin releasing hormones in the hypothalamus, gonadotropins in the pituitary, and/or sex steroid hormones in the gonads, thereby eliciting a series of effects along the BGL axis. Regardless of the mechanism by which compounds in OSPW affect the expression of genes along the BGL axis, these results provide a mechanistic basis for the lesser concentrations

of plasma sex steroids, and less pronounced secondary sexual characteristics of fathead minnows exposed to OSPW or extract of NA from OSPW (Kavanagh et al., 2011, 2012). For example, the elevated abundance of transcripts of *erα*, *erβ*, *vtg*, *chg-l*, and *chg-h* in the liver of male fathead minnow could explain the less pronounced secondary sexual characteristics in male fathead minnows exposed to OSPW, and the impairment of ER signaling and subsequent inhibition of synthesis of egg envelope proteins might explain the lesser fecundity of female fathead minnows exposed to OSPW.

In contrast to the *in vitro* studies described in Chapter 2 and 3, ozonation of the OSPW was less effective in attenuating the effects of OSPW on the expression of genes along the BLG axis of fathead minnows. This is probably not surprising given that only a single endpoint was measured in the *in vitro* studies. Physiologically, fish are much more complex than immortal cells, and negative or positive feedback pathways both within and across tissues create a much more complex exposure scenario. As discussed in section 6.2.2, the different effects of ozonation on various organic compounds in OSPW might result in production of either less or more estrogenic compounds, which in turn might have effects at different levels of the BGL axis. In general, ozonation of OSPW either attenuated or had no effect on some endocrine-disruptive end points, and the attenuating effects were more prominent in males than in females. Additional studies are required to evaluate the effectiveness of ozonation treatment of OSPW are needed.

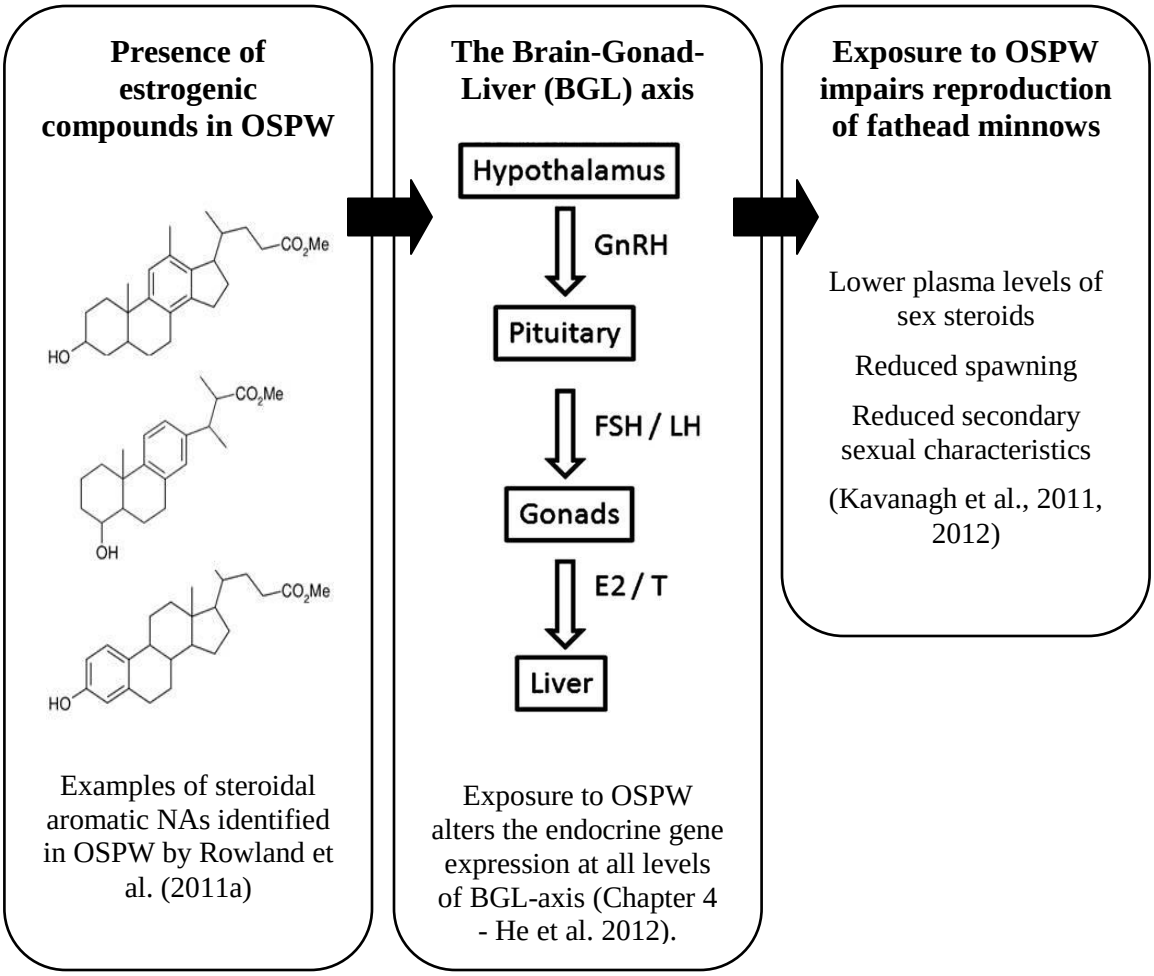


Figure 6.2. Changes in the abundances of transcripts of genes along the brain-gonad-liver (BGL) axis of male and female fathead minnows exposed to oil sands process-affected water (OSPW) provide a mechanistic basis for the effects of OSPW and naphthenic acids (NAs) from OSPW on reproduction by fathead minnows. NAs with structures similar to estrogens have been identified in OSPW (Rowland et al., 2011). Reproduction is impaired in fathead minnows exposed to OSPW and extracts of NAs from OSPW. The results of the studies described in Chapter 4 provide a mechanistic link for the effects.

6.3 The embryo toxicity of oil sands process-affected water (OSPW)

Establishment of healthy populations of fish not only relies on the ability of adults to reproduce, but the successful development of embryos as well. The fourth study (Chapter 5) demonstrated that exposure to OSPW adversely affected development of embryos of fathead minnows, including lesser survival, greater incidences of premature hatching, and greater incidences of deformities such as hemorrhage, pericardial edema, and malformation of the spine.

Deformities during development of embryos exposed to oil sands sediment or OSPW had been reported earlier. These effects were attributed to activation of the aryl-hydrocarbon receptor (AhR) by polycyclic aromatic hydrocarbons (PAHs) (Colavecchia et al., 2004, 2006, 2007, Peters et al., 2007). In the studies described in Chapter 5, the concentrations of PAHs were lesser than the level of detection as there was no activation of the AhR in the H4IIE-luc assay. Together with the fact that the abundance of transcripts of *cyp1a*, a biomarker of exposure to dioxin-like compounds, was not greater in embryos exposed to OSPW compared to the control, the embryo toxic effects of OSPW observed in study 5 seem to not be mediated by the AhR. This finding is also supported by a recent study that reported that individual NAs from OSPW did not bind to the AhR (Scarlett et al., 2012). However, the abundance of transcripts of *cyp3a*, a phase I enzyme regulated by the pregnane-x-receptor (PXR), was significantly greater in embryos exposed to OSPW compared to the control group. Together with the evidence of greater concentrations of reactive oxygen species (ROS) and greater expression of genes related to biotransformation, oxidative stress, and apoptosis, it suggests that agonists of the PXR that are present in OSPW might have caused the expression of CYP3A and other biotransformation

enzymes, which resulted in generations of ROS and subsequent consequences such as death, malformations, and apoptotic cell death. These results are consistent with the results by Gagné et al. (2012) that exposure to OSPW resulted in greater expression of genes related to the biotransformation of xenobiotics and oxidative stress. The removal of the organic fraction of OSPW by ozonation or activated charcoal successfully attenuated these adverse effects. This supports the findings presented in Chapter 2-4 that the organic fraction of OSPW is the main source of the effects of OSPW.

6.4 The potential release of oil sands process-affected water (OSPW)

Ultimately the OSPW currently stored in various tailing ponds needs to be remediated and reclaimed as aquatic habitat or released to the receiving environment. However, remediation of OSPW by natural biodegradation of the organic fraction of OSPW is a slow process, with half-lives for natural degradation of NAs in OSPW estimated at 12 years (Han et al., 2009). In fact, it is estimated that the toxicity of OSPW in tailings ponds will persist for several decades (Royal Society of Canada, 2010). In addition, *in vivo* studies using the benthic invertebrate, *Chironomus dilutus,* demonstrated that exposure to fresh OSPW and OSPW that had been aged for 23 years negatively impacts survival, growth, development, behavior and pupation (Anderson et al., 2012a, b). Benthos in the sediment are critical components of the aquatic food chain, and without the establishment of population of benthos, higher vertebrates like fish are not likely to survive. The data presented in Chapter 2-4 demonstrate that exposure to OSPW can negatively affect process that are crucial for sexual reproduction and the data presented in Chapter 5 suggest

that even if fish are able to reproduce in OSPW, healthy populations of fish might not be expected to be established due to effects on the survival, growth, and development of embryos. Considered together, these studies indicate that establishment of healthy populations of fish in the OSPW is unlikely until concentrations of various toxic compounds are sufficiently decreased to a non-toxic level. Research is needed to establish this concentration.

6.5 Future research

The classical definition of NAs, with the formula $C_nH_{2n+Z}O_2$, only account for a portion of the acid-extractable organic compounds in OSPW (Headley et al., 2009b, Grewer et al., 2010). Some oxidized NAs with formula $C_nH_{2n+Z}O_x$ (x from 3 to 7), together with sulphur- and nitrogen-containing species, have been identified in various oil sands samples (Han et al., 2009, Headley et al., 2009b, Grewer et al., 2010). Recent studies identified some individual NAs with structural similarity to estrogens (Rowland et al., 2011a). However, there is still a large proportion of the extractable organic fraction from OSPW which cannot be identified. It is critically important that the toxic chemicals in OSPW be identified. Some progress has been made using GC/MS by Rowland et al., 2011a, b, c. Compounds in OSPW that are identified can be synthesised, if they are not already commercially available, and used in the *in vitro* and embryo toxicity assays described in this thesis to investigate their toxicities. The major problems with this approach may involve the identification of endocrine disruptive and/or embryotoxic chemicals present in OSPW, as well as the capacity of chemical synthesise. On the other hand, some simple approaches like fractionation of OSPW could also help to determine the toxicity of

OSPW. It is possible that the toxic components of OSPW might be isolated to only one or a few fractions of OSPW. The assays described in this thesis can be used to test the toxicities of fractions of OSPW, and once toxic fractions are identified they can be characterized chemically.

Some mechanisms of action of the organic fraction of OSPW have been demonstrated and discussed in Chapter 2, 3, 4 and 5. However, further studies are needed to elucidate additional mechanisms of toxicity of OSPW. The results from the studies presented in Chapter 5 provide some insight into potential mechanisms of toxicity that should be explored. Exposure to OSPW resulted in greater abundance of transcripts of *cyp3a* rather than *cyp1a*, as well as *gst*, suggesting the main cause of the developmental toxicity of OSPW, including generation of oxidative stress, and apoptotic cell death, was mediated by the PXR. This finding is consistent with a study in which OSPW caused greater abundance of transcripts of *cyp3a* and *gst* in hepatocytes isolated from rainbow trout (Gagne et al., 2012). Although evidence shows that the dioxin-like effects in embryos exposed to OSPW might be PXR- rather than AhR-mediated, the identities of the compound(s) in OSPW that bind to and activate PXR should be determined. Additional studies should be performed to elucidate the exact role(s) of the PXR in mediating the toxic effects of organic compounds in OSPW. Morpholino oligonucleotides are the most widely used anti-sense knockdown tools as a way to inhibit the *in vivo* translation of RNA transcripts. Morpholinos targeting PXR can be designed to identify whether the activation of PXR is involved in the mechanism of toxicity of OSPW on development of embryos. Ultimately, once the chemicals of interest are identified, and successfully synthesized, assessments of toxicity, such as binding affinity, toxic equivalent, and environmental risk assessment can be conducted.

Ozonation can greatly reduce the concentrations of organic content, in particularly NAs, in OSPW (Martin et al., 2010). This study, together with others, support that ozonation might work on reducing the toxicity of OSPW (Anderson et al., 2012b, Garcia-Garcia et al., 2011c, He et al., 2010, 2011, 2012a, 2012b, Martin et al., 2010, Scott et al., 2008). Further studies might be needed to determine the efficacy of ozonation on reducing the toxicity of OSPW, such as what degree of ozonation (eg. ozone concentration, treatment time, presence of catalyst) reduces the toxicity most efficiently. Moreover, special attention should be paid to those byproducts of ozonation, which may enhance the toxicity, or disturb the progress of reducing toxicity. The feasibility of ozonation, from an engineering point of view, is beyond the scope of this thesis, but the results from the studies described in this thesis provide compelling evidence that further investigation of ozonation is warranted.

In conclusion, this thesis provides some novel insights on the toxicity of OSPW which might be helpful for monitoring, regulation and strategy development for OSPW remediation. The results from this thesis demonstrate the endocrine disruptive properties of OSPW *in vitro* and in fathead minnows, as well as the developmental toxicity of OSPW in fathead minnow embryos. Ozonation treatment can significantly attenuate most of the toxic effects of OSPW both *in vitro* and *in vivo*. Taken together, due to the potential toxic effects on growth, development, and reproduction by OSPW exposure, it is not likely for local fish such as fathead minnow to establish a healthy and self-sustaining population in reclaimed tailing ponds, and ozonation is a promising effort for remediation of OSPW to create ponds which would likely be able to sustain populations of fish.

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