University of Alberta

Analysis of goldfish innate immunity following exposure to oil sands process affected water

by

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A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of
Master of Science
in
Physiology, Cell and Developmental Biology

Biological Sciences

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Fall 2013

Edmonton, Alberta

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Abstract

In this thesis I examined the acute and sub chronic effects of complete oil sands process affected water (OSPW) and its toxic component, naphthenic acids (NAs). I exposed goldfish for one (acute), or 12 (sub chronic) weeks and examined immune gene expression, macrophage function, the ability of exposed fish to control the infection with *Trypanosoma carassii*, and endocrine disruption. Acute exposure to 20 mg/L commercial naphthenic acids (C-NAs) or OSPW, induced up-regulation of the expression of immune genes (IFNγ, IL1-β1, TNFα-2). This up-regulation in gene expression was related to observed increase in macrophage functions in fish exposed to C-NAs, but not in in fish exposed to 25% OSPW (NAs content = 10 mg/L). Interestingly, acute exposure of fish to C-NAs or OSPW induced increased resistance to *T. carassii*. After sub chronic exposures of goldfish to C-NAs or OSPW, a significant decrease in the expression of majority of immune genes was observed, suggesting that the fish were immunosuppressed. This was confirmed by decreased ability of primary macrophages from exposed fish to generate antimicrobial responses. Furthermore, sub chronic exposure of fish to C-NAs caused increased susceptibility to *T. carassii*, and eventual parasite-induced host mortality. Acute exposure to fresh OSPW induced up-regulation in genes encoding endocrine receptors and higher vitellogenin levels in male fish; while exposure to aged or ozonated OSPW led to a sub chronic alteration in endocrine gene profiles, suggesting a possible long-term endocrine disruption following exposure to ozonated OSPW. Treatment with ozone ameliorated the immunotoxic effects of OSPW, because immune gene expression, macrophage functions and control of *T. carassii* infecton were similar between ozonated OSPW-exposed and non-exposed goldfish. The results in this thesis represent the first report of immunotoxicity caused by C-NAs or OSPW exposure in aquatic vertebrates.

Acknowlegements

First and foremost thank you to my supervisor Dr. Mike Belosevic for his advice and guidance throughout the years and for giving me this opportunity. He has given me advice and suggestions and his dedication, generosity, and kindness has made my time in the lab memorable and treasurable. Mikes enthusiasm for science is contagious and it has been an absolute pleasure working with him. Words cannot express how thankful I am.

I would like to thank my committee members Dr. Greg Goss and Dr. Keith Tierney, who have been a wealth of knowledge and support. They have provided mentorship and guidance throughout my thesis and without them my science experience would have been incomplete. I would also like to thank my defense chair, Dr. Julia Foght, as well as my arms length, Dr. Kinga Kowalewska-Grochowska, thank you for aiding me in this endeavor. I would also like to thank Dr. Al Shostak who helped me with stats, which would have been impossible otherwise, as well as for the guidance and helpful comments and constructive criticism he has given me throughout my science career.

Thank you to all the lab mates past and present: Jordan Hodgkinson, Leon Grayfer, Barb Katzenback, Ayo Oladiran, Dave Beauparlant, Scott Mitchell, Matt Karpman, Steve Hitchens, Jon Pun, Fumi and Akie Katakura, Jiasong Xie, Chao Li, Nikolina Kovacevic, you all kept me sane and provided friendship and support. The lab would not have been as fun or exciting without all of you.

Lastly, thank you to my family and friends. My mother, Lee, father, Roy, and Peter, my brother Martin, as well as grandma Mary and Odette and grandpa Eldon and Basil, as well as Auntie Lizzy and Grace and Uncle John and Max thank you all for the support you have given and enthusiasm for my work, even though many of you admit to not understanding any of it. Thank you to my dearest girlfriends, Zofia, Jyening, Colleen, and Cienya, without the lunches, beers, and vodka sessions I would not have been able to do this without you!

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List of Abbreviations

ANOVA – analysis of variance

BTEX – benzene, toluene, ethylbenzene, xylene

CYP1a – cytochrome P450 1A

DNA – deoxyribonucleic acid

E2 – 17β -estradiol

EDC – endocrine disrupting compound

ELISA – enzyme linked immunosorbent assay

ER – estrogen receptor

EROD – 7- ethoxyresorufin-O-deethylase

IFNγ – interferon gamma

IL1-β1 – interleukin one-beta one

iNOS – inducible nitric oxide synthase

NAs – naphthenic acids

C-NAs – commercial naphthenic acids

OSPW – oil sands processed water

PAH – polyaromatic hydrocarbon

PKM – primary kidney macrophages

qPCR – quantitative polymerase chain reaction

ROI – reactive oxygen intermediates

RNA – ribonucleic acid

RNI – reactive nitrogen intermediates

T – testosterone

TNFα-2 – tumor necrosis factor two

Vtg - vitellogenin

Chapter I

1.0. Introduction

The oil sands in northern Alberta are the third largest oil deposit in the world. During the process of oil sand mining, hot water (79ºC - 93ºC) and caustic soda (sodium hydroxide) are used to leech bitumen from the sands, resulting in the generation of oil sands process-affected water (OSPW) (Del Rio et al., 2006). The sand and larger particles are removed from this mixture and the resulting slurry is placed in a primary separation vessel. In this vessel, the top and middle layers containing finer particles (major fine tailings) and bitumen froth are further processed, eventually decreasing froth viscosity via naphtha dilutions and upgrading of the froth into synthetic crude oil. The remaining sand and water are confined to cement-lined basins termed tailings ponds. This water is then re-used by oil companies to repeat the extraction process but, in doing so, contaminants in wastewater are further concentrated. Because Alberta currently has a zero-discharge policy, OSPW is destined to remain in these tailings ponds until toxicity of the water is ameliorated, by reducing the concentration of salts, heavy metals, naphthenic acids (NAs), and poly-aromatic hydrocarbons (PAHs). To date, there are no feasible remediation techniques, and aging of the ponds is currently the only remediation approach used for decreasing the toxicity of this highly complex wastewater matrix.

The major toxic component in OSPW are naphthenic acids (NAs) (Dokholyan and Magomedov, 1983; MacKinnon and Boerger, 1986; Madill et al., 2001). NAs are found naturally in the environment at low concentrations, however, due to re-use of water during bitumen extraction processes, NAs are much more concentrated in OSPW. Due to the high concentration of NAs and other contaminants found in OSPW, it is crucial that we examine the toxic effects of OSPW in aquatic and terrestrial organisms in order to gain insight into how animals, ecosystems, and humans, may be affected by exposure to OSPW. The tailing ponds are continuously being produced by the oil mining industry and their

number and size are going to significantly increase in the future. Therefore, it is essential that we develop strategies to remediate these industrial wastewaters prior to their release into the environment.

The toxic effects of xenobiotics on living organisms are commonly studied in environmental sciences. A number of different endpoints have been used in toxicological studies to examine adverse effects of pollutants and industrial-derived wastewaters on aquatic vertebrates. Among them are those that deal with the effects of chemical pollutants on the immune responses of the host. Many toxins have been shown to disrupt homeostatic mechanisms, including the endocrine pathways, and given that endocrine hormones have been implicated in properly functioning immune responses, a comprehensive analysis of the endocrine-immune axis is required for appropriate analysis of toxic effects of xenobiotics.

Aquatic organisms make good sentinels to assess water toxicity. In my studies I used goldfish (*Carassius auratus* L.) as the model organism to assess toxicity of OSPW or commercial naphthenic acids (C-NAs) preparations, using immune gene expression and immune function as endpoints. It has been proposed that due to similar pathogenic indices between yellow perch (native species) and goldfish, goldfish are an appropriate laboratory model species to assess OSPW toxicity (Nero et al., 2006b), although they differ at the level of Order.

Much is known about goldfish innate immunity, which makes this fish species an excellent model organism for immunotoxicology. In addition to the available immunological reagents for goldfish, their temperature tolerance and large size allow for the functional assessment of immunotoxicity, as well as their mid-range sensitivity to NAs.

Previous studies have been done on histopathology and phenotypic indicators of disease following chronic and acute exposure to OSPW or C-NAs, but immunotoxicity of C-NAs and OSPW to fish has not been examined. Immunotoxic effects of potentially remediated ozonated OSPW on aquatic vertebrates has also yet to be examined. In my studies I used a real-time flow through exposure systems to assess the toxic effects after exposure of fish to

varying dilutions of fresh and aged OSPW. This is therefore the first study to use a flow-through exposure system to assess OSPW toxicity.

1.1 Objectives of the Thesis

The central objectives of my thesis were to: (1) assess whether the acute and sub chronic exposures of goldfish to C-NAs, aged OSPW, or fresh OSPW cause immunotoxicity, and whether the immunotoxic effects can be altered by treatment of OSPW with ozone; (2) examine the effects of OSPW and C-NAs *in vitro* using different bioassays; (3) examine whether exposure to OSPW altered the ability of goldfish to control a parasitic (*Trypanosoma* carassii) infection; (4) assess the possible estrogenic effects of OSPW by measuring hormone gene expression and vitellogenin levels in male fish.

1.2 Outline of thesis

Following the general introduction (chapter 1) is the comprehensive literature review (chapter 2) which summarizes current knowledge of OSPW toxicity. Chapter 3 contains the detailed materials and methods I used throughout my research. In chapter 4, I present results of fish exposed to C-NAs. Chapter 5 contains findings of fish exposed to aged OSPW, fresh OSPW, or ozonated OSPW. In chapter 6, I present evidence on potential endocrine disrupting properties of OSPW. Chapter 7 is a general discussion of results and future directions that I believe should be undertaken to further assess OSPW toxicity.

Chapter II: Literature Review

2.0 Industrial wastewater

The rapid increase in human population in the world parallels the exponential expansion in agricultural and industrial practices that require significant amounts of potable water. Industrial wastewaters are an undesirable by-product of human advancement and cause pollution of potable water. This pollution may come from industrial runoff containing effluents such as chemicals, fabricated metals, endocrine disrupting compounds (EDCs), personal care products (PPCPs), and by-products of petroleum refineries (USEPA, 1988). The exposure to industrial wastewaters may lead to many physiological changes affecting homeostasis of animals. These include neurological effects, developmental delays, reproduction, inflammation, suppression of immune responses, higher prevalence of neoplasias, and increased susceptibility to infectious diseases. It is, therefore, not surprising that significant efforts have been made to re-claim polluted industrial wastewaters. Reclaimed wastewaters may be used for non-potable applications such as agriculture, industrial activities, environmental and recreational use, as well as potable reuse. However, if xenobiotics are not properly removed from these waters, they may accumulate in the environment and cause adverse effects in animals including humans. It is important, therefore, to understand the impact of industrial wastewaters on the environment and its inhabitants.

2.1 Oil sands process-affected water (OSPW)

In processing the Athabasca oil sands, bitumen (a form of crude oil) is extracted from the oil sands by the Clark hot water extraction process (Schramm and Smith, 1989; Schramm et al., 2000). This process utilizes hot water (79ºC - 93ºC) and caustic soda (sodium hydroxide), which results in water and unrecovered hydrocarbons present in bitumen froth (Nero et al., 2006). Ultimately, this process requires the use of 3 parts of water to generate 1 part of crude oil (v/v) (Martin et al., 2008). The excess water containing organic and

inorganic contaminants is termed oil sands process-affected water (OSPW) stored in tailings ponds, that are currently accumulating at a rate of ~10^5 m^3/ day (Madill et al., 2001). Due to legislation, this water cannot be released into the environment until toxicity is decreased (Alberta Environmental Protection and Enhancement Act, Section 23, 1993; Headley and McMartin, 2004; Madill et al., 2001), and as a result, the OSPW is stored in tailing ponds and it is estimated that by 2025 over 1 billion m^3 of OSPW will be generated in northern Alberta (Del Rio et al., 2006).

Storage of immense amounts of OSPW in tailing ponds causes concern regarding contamination of groundwater, riverbank erosion, and accidental direct effluent release (Headley et al., 2011; Headley and McMartin, 2004) that may negatively impact wildlife living in the Athabasca region as well as public health. It is, therefore, imperative to develop methods for remediation of OSPW (Clemente and Fedorak, 2005; Headley and McMartin, 2004; MacKinnon and Boerger, 1986; Rogers, *et al*., 2002).

2.2 Composition and toxicity of OSPW

OSPW composition can serve as a "fingerprint" to the oil company that created it (Headley et al., 2012). There are differences in composition between OSPW generated by different companies and mining sites. OSPW contains a complex mixture of pollutants, with more than 400 organic and inorganic contaminants. The organic fraction of OSPW is a complex mixture of naphthenic acids (NAs), BTEX (benzene, toluene, ethylbenzene, and xylene), phenols, and polycyclic aromatic hydrocarbons (PAHs) (Gosselin et al., 2010; Sadek, 2008). The inorganic fraction of OSPW contains heavy metals and salts (Puttaswamy and Liber, 2012; Colavecchia et al., 2004; Gosselin et al., 2010; van den Heuvel et al., 2012). Because oil companies reuse OSPW during the Clark water extraction process, these contaminants are concentrated further, leading to generation of highly toxic industrial wastewater (Leung, 2003).

OSPW was found to be toxic to both eukaryotes and prokaryotes. Current research shows that NAs and cyclic and acyclic alkyl-substituted aliphatic

carboxylic acids are the major toxic components in OSPW (Dokholyan and Magomedov, 1983; MacKinnon and Boerger, 1986; Madill et al., 2001).

2.2.1 Naphthenic acids

Approximatelly 40% of the organic fraction of OSPW are NAs. NAs are a large group of compounds that include all carboxylic acid containing groups in crude oil (Brient et al., 1995). They are commonly found in liquid form, are acidic, and have sulfur or phenolic impurities (Brient et al., 1995). NAs are low molecular weight alkyl-substituted aliphatic acyclic, cyclic, or polycyclic carboxylic acids, accounting for 4% of petroleum by weight (Rogers et al., 2002). They have a general formula of $C_mH_{2m+Z}O_2$, where *m* is the number of carbon atoms, and *Z* is zero or a negative integer representing the number of hydrogen atoms lost during the formation of cyclic structures (Headley and McMartin, 2004).

Although the NAs are available commercially, it is currently very difficult to separate different NA species from OSPW. As a result, commercial preparations vary in their purity levels and composition depending on the petroleum source and the refining techniques used (Brient et al., 1995). In order to make commercial preparations, the sodium naphthenate phase of the slurry of bitumen hydrocarbons and MFT recovered from the Clark process are treated with sulfuric acid (Brient et al., 1995). This allows further separation of sodium naphthenate, ultimately resulting in the recovery of naphthenic acids that still retain some phenolic and sulfuric contamination (Brient et al., 1995).

In tailing ponds, NA levels may range from 20 mg/L (Quagraine et al., 2005) to 120 mg/L (Holowenko et al., 2002) depending on the age of the pond, with concentrations of less than 5 mg/L found in ponds aged for more than 15 years. However for complete degradation of larger NAs species, >100 years is required. Due to natural leeching of oil sands into the Athabasca River, NA concentrations in the environment, are found to be ~1 mg/L (Schramm et al., 2000) As a result, we must focus on reclaiming the oil sands process-affected water and reducing its toxicity.

2.2.1.1 Toxicity of naphthenic acids

It is speculated that NAs behave in a similar fashion as surfactants due to their chemical composition and hydrophilic head and hydrophobic tail (Ivankovic and Hrenovic, 2010). They act mechanistically as narcosis agents by disrupting membranes via insertion into the lipid bilayer, or effecting transmembrane proteins, causing increased membrane permeability and cell death ((Frank *et al*., 2008; Klopman *et al*., 1999; Konemann, 1981). Due to their chemical makeup, they are insoluble in water, but soluble in organic solvents including oil (Brient et al., 1995).

NAs have been shown to be toxic to a variety of aquatic species. Zooplankton was found to be intolerant to sodium naphthenates at concentrations above 0.15 mg/L (Dokholyan and Megomedov, 1983). A study done on phytoplankton communities in 10 water bodies near Fort McMurray, showed no alterations in community biomass in ponds with higher NAs concentrations, however the presence of napthenates and inorganic ions ecologically affected the phytoplankton, suggesting possible synergistic or additive effects (Leung et al., 2003). Similarly, phytoplankton mesocosms from a reference lake that was less than 5 years old (NAs content > 20 mg/L) exhibited significant community effects (Leung et al., 2001). However, how invertebrates are affected by NAs may not be similar to the effects of these compounds in higher vertebrates. In a study comparing *Vibrio fischeri, Daphni magna* and rainbow trout, it was found that OSPW was most toxic to *D. magna* followed by trout, and least toxic to *V. fischeri* using the Microtox assay (MacKinnon and Boerger, 1986). Therefore, the Microtox assay may not be sufficiently sensitive to assess toxicity of OSPW *in vitro*, and the assessment of the effects of OSPW exposure in multicellular organisms would be more representative of toxic effects of OSPW in vertebrates (Garcia-Garcia et al., 2011b). As a result, the number of studies on aquatic organisms has steadily increased over the past decade, and new physiological endpoints such as immunotoxicity are being documented.

Toxicity of NAs has been shown to vary depending on the fish species examined. For example, Dokholyan and Megomedov (1983) looked at acute toxicity of a variety of fish species exposed to 12 -100 mg/L NAs over a 10 day period. It was found that 50% mortality of 2 month old salmon chum occurred after exposure to 25 mg/L NAs. However, different species appeared to be less sensitive to OSPW: 2- month old kutum and 2 year-old sturgeon were able to withstand double that amount (Conversely, 2 year-old Caspian gobys were exposed to 75 mg/L NAs before 50% mortality occurred (Dokholyan and Megomedov, 1983). Rainbow trout appear to be especially sensitive to NAs since 2.5-5 mg/L NAs was acutely toxic to this fish species (Dorn, 1992). Nero et al. (2006a) found that C-NAs were less toxic than OSPW-extracted NAs (E-NAs), due to 100% mortality of yellow perch fingerlings at 3.6 mg/L, and 6.8 mg/L, respectively. They also observed that when fingerlings were exposed to 25% of the lethal NAs levels, the fish exhibited increase in gill cell alterations and proliferation, leading to reduced gill surface area and anoxia (Nero et al., 2006a). Variability in NAs toxicity levels gives insight into acceptable target concentrations of NAs prior to OSPW being released. NAs are a complex group of compounds (more than 100,000 different forms in OSPW), all of which may affect aquatic organisms differently to cause toxicity.

2.2.2 Endocrine disrupting compounds (EDCs)

EDCs are capable of disrupting synthesis, secretion, transport, binding, or elimination of steroids in multicellular organisms. The hormones affected, such as estrogen or testosterone and/or their receptors, are involved in homeostasis, reproduction, development and behavior (CEPA, 1999). Estrogenic compounds are a class of EDCs that interfere with estrogen homeostasis and reproductive physiology, usually resulting in feminization of fish. These compounds may mimic, agonize, or antagonize estrogen and its receptors.

2.2.2.1 Toxicity of EDCs

Studies have shown that some compound(s) in OSPW act as estrogenic agonists due to binding of said compounds to estrogen receptors, as well as causing anti-androgenic effects through decreases in testosterone levels (He et al., 2011). To date, few studies have been done examining endocrine disruption in fish exposed to OSPW, and results are somewhat conflicting. It has been found that ovarian and testicular tissues extracted from goldfish and exposed to OSPW for 19 days have decreased ability to synthesize testosterone and E2 (Lister et al., 2008). In contrast, fathead minnow gonads exposed to OSPW exhibited increased mRNA levels of gonadotropin receptors and enzymes responsible for sex hormone steroidogenesis (He et al., 2012b). There is also evidence of significant changes in testosterone and 17β-estradiol levels in yellow perch and goldfish exposed to OSPW (Lister et al., 2008; van den Heuvel, 1999) as well as in cell lines, H295R (He et al., 2010), T47D-kbluc and MDA-kb2 (He et al., 2011). The estrogen-responsive genes were also found to increase in the liver of male fathead minnows after exposure to fresh OSPW (He et al., 2012b). In the same study, He et al. (2012b) reported that exposure of fish to OSPW induced increased expression of genes that encode molecules involved in synthesis of gonadotropins in male and female fish. Other studies using fathead minnows reported that exposure for 21 days to 10 mg/L E-NAs resulted in decreased egg clutch sizes and reduced secondary sexual characteristics in male fish. Male fish exposed to 10 mg/L also had lower plasma sex hormone levels, namely 11-ketotestosterone (Kavanagh et al., 2012). Exposure to OSPW was shown to decrease reproductive capacity, alter synthesis of sex hormones, and fish were found to have less pronounced secondary sex characteristics (Kavanagh, 2011). The exposed fish also exhibited premature hatching, increases in embryo hemorrhaging, spine malformation, pericardial edema, and increases in *cyp1a* (cytochrome P450) transcripts, which is involved in drug metabolism and breakdown of testosterone (T) and 11-ketotestosterone during E2 formation (He et al. 2012b).

Related to E2 and *cyp1a* levels, vitellogenin, an egg yolk precursor protein, has also been found to increase in Japanese medaka (Yamaguchi et al., 2005), zebrafish (Martyniuk et al., 2007), and fathead minnows (Filby et al.,

2007) exposed to estrogen-like compounds. In a recent study by Reinardy et al. (2013), zebrafish larvae exposed to low levels of E-NAs (alicyclic) did not have altered Vtg levels; when the NAs content was raised to 1 mg/L and contained only aromatic NAs a small increase in Vtg gene expression was observed, suggesting that that estrogenic effects associated with OSPW may be due to specific species of NAs present in OSPW.

He et al. (2011) also reported that anti-androgenic components of OSPW alter cellular membrane and increase androgen receptor prevalence. However, unlike the estrogenicty data, exposure of cells to C-NAs show anti-androgenic properties, suggesting that smaller species of C-NAs may be responsible for changes in testosterone levels (He et al., 2011). However, it has been found that exposure of fathead minnow to C-NAs induced only slight changes to sex hormones (He et al., 2011), even though NAs have been found to be structurally similar to sex steroids, particularly estrone and estradiol (Rowland et al., 2011). Therefore, because the majority of the literature supports endocrine disruption following exposure to OSPW, it seems likely that there is an EDC activity present in OSPW, however, it may not necessarily be due solely to NAs.

2.2.3 Polyaromatic Hydrocarbons (PAHs)

PAHs are a large family of aromatic compounds that are known to be carcinogenic and immunotoxic as well as capable of disrupting the endocrine system (Thurmond et al., 1987; White, 1986; White et al., 1985). They consist of fused aromatic rings and the simplest example of a PAH is naphthalene, a by product of coking of coal. PAHs occur naturally in oil and coal deposits and are water insoluble. They are also formed through combustion of carbon-containing fuels. PAHs are found to be the most toxic of the hydrocarbon families and 16 of them are recognized as priority pollutants.

2.2.3.1 Toxicity of PAHs

PAHs are found to be toxic to aquatic organisms at 0.2-10 ppm (Neff, 1985) and are known to be bio-transformed by *cyp1a* oxidation, producing very

reactive intermediates that bind to proteins and DNA causing enhanced gene transcription (Peltonen and Dipple, 1995).

Many studies have assessed the toxicity of PAHs during fish development. Zebrafish embryos exposed to non-alkylated PAHs have many developmental defects including: changes in kidney development, neural tube structure, as well as heart formation (Incardona, et al., 2004). These observations were related to phenanthrene (a PAH with 3 rings) concentrations, whose increased levels were linked to defects in cardiac and kidney development, suggesting that > 3 ringed PAHs are more toxic to embryos (Incardona, et al., 2004). Sudenberg et al. (2006) injected toluene extracts (i.e. PAHs, PCBs), into rainbow trout eggs to examine their toxicity. The endpoints measured were increases in egg mortality and larval malformations such as edema, hemorrhages, and cellular necrosis (Sudenberg et al., 2006). Heintz et al. (1999) exposed salmon eggs in a chamber that simulated hydrocarbon leeching from crude oil. They recorded mortality and developmental defects that were most likely due to alkyl- phenanthrenes (Heintz et al., 1999). Taken together these results indicate that PAHs are acutely toxic to fish embryos and cause developmental defects.

Adult fish exposed to PAHs exhibit immunosuppression as well as histopathology manifested as lesions in gills, skin, and fins from opportunistic infections (Seeley and Week-Perkins, 1991). A study examining the sub chronic effects of PAHs in sediments on winter flounder found that very low levels of hydrocarbons produce a biological response, such as fatty livers as well as increased mixed function oxidase (MFO) activity. MFO catalyzes a reaction such that two oxygen molecules may be used for separate reactions (Payne et al., 1988). Payne and Fancey (1989) showed that sub chronic exposure to PAH contaminated sediment induced immunosuppression in flounder by decreasing their liver melano-macrophage centres (MMC). Faisal et al. (1993) also found the spot croaker (*Leiostomus xanthrurs)* and mummichog (*Fundulus heteroclitus)* (1991) were immunosuppressed after exposure to PAHs, manifested by decreased T lymphocyte numbers and their tumor lytic capacity.

In vitro studies have shown that carp macrophages exposed to 3-methylcholanthrene have increased respiratory burst activity (Reynaud et al., 2001). When carp were injected with 3-methylcholanthrene a significant increase in lymphocyte proliferation was also observed. This immune response parameter was the first immune biomarker developed for PAH exposure in fish (Reynaud et al., 2005). However, the exact mechanisms by which PAHs act to induce immunotoxcity are not known (Reynaud and Deschaux, 2005; Reynaud et al., 2001; Reynaud et al., 2003).

Due to the fact that PAHs constitute a broad spectrum of molecular species, different physiological effects have been attributed to PAHs (Reynaud and Deschaux, 2006). PAHs affect both adaptive and innate immunity and because of the large group of toxins that PAHs contain, more research must be done of the effects on fish immunity, in order to determine if they contribute significantly to the overall OSPW toxicity or act synergistically with other compounds in OSPW to cause toxic effects.

2.2.4 Chemical composition of BTEX

BTEX (benzene, toluene, ethylbenzene, xylene) are volatile, monoaromatic, organic compounds found in petroleum and oil sands. BTEX are usually released as a mixture and not as single compound, thereby increasing its toxicity. BTEX are known toxins that are capable of bio-accumulating in the food chain and are relatively soluble in water. However, little is known about BTEX mechanism of action in different organisms, and additive, synergistic, or antagonistic effects of these compounds are possible.

2.2.4.1 Toxicity of BTEX

It has been shown that exposure of rainbow trout to aromatic hydrocarbons (AH) resulted in 100% mortality after one hour of exposure, causing liver damage and adverse effects on blood-clotting enzymes and depletion of red blood cells (Zbanyszek and Smith, 1983). Another study reported that fish are severely stressed following exposure to AHs, with toluene being the major toxic

component responsible for this effect (Casillas and Smith, 1977). It has also been found that exposure of fish to BTEX induced cellular narcosis, likely due to the alteration in the fatty acids of the lipid bilayer (Di Marzio et al., 2001). Another study has shown a reduction in hematopoietic tissues in juvenile striped bass exposed to benzene for 30 days (Taberski, 1983). Sakanari and colleagues (Sakanari et al., 1983) examined the effect of a nematode infection on its striped bass host following exposure to sub lethal concentrations of both zinc and benzene and found that the number of lymphocytes decreased significantly in the fish, which in turn altered immunoglobin production (Sakanari et al., 1983). Not only have immunotoxic effects been found following exposure to BTEX and its derivatives, but also endocrine disrupting effects as well. In a study examining estrogenic effects of xylene on fish cells *cyp1a* gene expression and EROD activity were reduced after acute exposure (Delle Torre et al., 2011).

The exposure of *Drospophila melanogaster* to mixtures of benzene, toluene and xylene was found to be less toxic than benzene alone, as indicated by the expression of genes that encode stress related proteins as well as reactive oxygen species (ROS) generation (Singh et al., 2010). This suggests an antagonistc effect of xylene and toluene on benzene. However, toxicity was found to increase when xylene and toluene without benzene were used, suggesting an additive/synergistic effect of xylene on toluene toxicity (Singh et al., 2010). Similarly, a mixture containing xylene and toluene was found to be more toxic to *Chironomus plumosus* larvae than ethylbenzene alone (Li et al., 2012). However, due to the complexity of the BTEX mixture and the large number of chemical species in that family, it is often difficult to narrow down which compounds are responsible for toxic effects.

2.2.5 Heavy metals

Metals are common contaminants in aquatic environments and are hazardous due to high toxicity, diverse anthropogenic sources of contamination, as well as their ability to accumulate in the ecosystem (Atchison, et al., 1987). Water pH and hardness affect toxicity of metals in an aquatic setting (Borgmann,

1983) because it is possible for metal ions to bind to other components and form insoluble precipitates that accumulate in sediment and remain relatively harmless to aquatic life. However, if metal ions remain free in water their toxicity increases by inhibiting enzymes and impairment of osmotic control, respiration, and reproduction (Atchison, et al., 1987). Heavy metals have a specific gravity of 4 or 5 and contain elements between 22-34, 40-51, 57-83, 89-103. The most toxic metals are known to readily bind to sulphur and nitrogen, often irreversibly, while less toxic metals bind to oxygen (Nieboer and Richardson, 1980). Heavy metals include zinc, mercury, lead, cadmium, magnesium, aluminum, copper, thallium, as well as 13 elements considered to be primary pollutants (PPE) by the US Environmental Protection Agency's Clean Water Act, have been found to increase in concentration at sites situated close to mining areas (Alberta and Badillo, 1991; Debenest et al., 2012; Eisler, 2004; Kelly et al., 2010; Sharma et al., 2004; Wang et al., 2004).

One method of examining metal toxicity is via the biotic ligand model (BLM), which utilizes the basic principles of metal toxicity: metal toxicity is due to free ions, the binding of these ions to ligands will affect toxicity, many factors in wastewater alter metal toxicity, and cations associated with water hardness (i.e. calcium, magnesium) compete with metal ions for binding sites in organisms and aid in protection against metals (Niyogi and Wood, 2004). The BLM predicts metal toxicity based on water quality and the assumption that more toxic ions bind to ligands with stronger affinity than less toxic ions. (Niyogi and Wood, 2004). However, this methodology is relatively new and although it appears to be a good model for assessing metal toxicity, more research must be done on this model before it can be applied for prediction of OSPW toxicity.

2.3.5.1 Heavy metal toxicity

The adverse effects of metal presence in industrial wastewater has been studied since 1960, showing that metal drainage affects primary and secondary nutrient production and cycles, energy flows, as well as decomposition, while reduction in abundance, taxa, and biodiversity have been associated with metal-

mining (Byrne et al., 2012). In terms of physiology, acute metal toxicity has been linked to cranial deformities and mutations of feeding structures in macro-invertebrates, which are more sensitive to xenobiotics, compared to predators or herbivores (Byrne et al., 2012). Metal toxicity is also known to bio-accumulate in the food chain, particularly in benthic organisms that are susceptible to sediment-bound metals such as iron, lead, and aluminum (Byrne et al., 2012). However, the metabolism and accumulation of metals in the environment has been linked to many factors, and depending on the source of the contamination, different metals will be found at different concentrations.

During the process of bitumen coking and purification, Environment Canada's National Pollutant Release Inventory has shown heavy metals are aerosolized, causing concern among inhabitants located near oil sand mining sites (Allen, 2008; Jack et al., 1979; Jang and Etsell, 2006; Kelly et al., 2010). Heavy metal contamination of sites along the Athabasca river may also affect fish reproduction and population dynamics, especially high level of copper, which has been shown to decrease fathead minnow embryos tolerance to other toxins (Kelly et al., 2010; Welsh et al., 1996). The presence of zinc (Abou-Mohamed et al., 1995; Fosmire, 1990) and copper (Sokolik et al., 2002, 2006) has also been linked to decreased inflammatory responses and chronic toxicity in mammals and fish (Handy, 1996; Montaser et al., 2010). OSPW toxicity to green algae has also been linked to presence of heavy metals and metalloids (Debenest et al., 2012). The mechanism by which heavy metals are thought to act on fish is by disruption of osmolality, and alterations of enzyme synthesis (Jezierska and Lugowska, 2009).

Heavy metals have also been shown to affect embryonic development and reproduction of fish. For example, cadmium was shown to inhibit estrogen receptors (Le Guevel et al., 2000), disrupt growth hormone secretion (Jones et al., 2005) and inhibit thyroid hormone synthesis by altering iodine metabolism (Chaurasia et al., 1996). Studies examining fish egg swelling due to excessive water influx, found that copper, cadmium, and lead cause this effect (Jezierska and Lugowska, 2009). Heavy metal exposure decreases the rate of embryonic development and hatching success of fish (Jezierska and Lugowska, 2009).

Because of immunotoxic and developmental effects of heavy metals, it is important to document the effect of these elements on organisms before release of remediated OSPW.

2.3 Toxicity of OSPW in aquatic organisms

2.3.1. Toxicity of OSPW in vitro

Little research has been done regarding toxicity of NAs and OSPW *in vitro*. Studies using mouse primary cells have shown that exposing bone-marrow derived macrophages (BMDM) cultures to C-NAs and OSPW organic fraction (OSPW-OF) caused decreased macrophage antimicrobial responses and decreased ability of cells to proliferate (Garcia-Garcia et al., 2011a). Since OSPW contains a variety of other potentially toxic chemicals in addition to NAs (i.e. PAH, etc.), it is not known whether the toxic effects of OSPW are due solely to NAs or a combination of NAs and other toxic compounds in the organic fraction of OSPW (Nero et al., 2006; van den Heuvel et al., 2000; Garcia-Garcia et al., 2011a).

Toxicity of OSPW *in vitro* may be assessed a variety of ways. One such way is examining functional behavior of cell lines after exposure. A study done using 6 fish cell lines: WF-2, GFSk-S1, RTL-W1, RTgill-W1, FHML, and FHMT exposed to 49 different samples of OSPW and NAs concluded that acute toxicity was caused by NAs present in the OSPW-OF (Sansom et al., 2012). A different study examining four rainbow trout cell lines found C-NAs to be toxic, and that toxicity was enhanced in the presence of high salinity, while low doses of NAs induced enhanced cell proliferation (Lee et al., 2000). Rainbow trout hepatocytes exposed to OSPW, exhibited alterations in the mRNA levels of genes related to glycolysis, oxidative stress and DNA repair (Gagne et al., 2011, 2012, 2103). Exposure of trout cells was also found to lead to changes in cell function, particularly due to an increase in membrane permeability (Gagne et al., 2013).

Studies using H295R human cell lines focused on estrogenic effects of OSPW and have also found the induction of *cyp1a* and increase in E2 production, decrease in testosterone levels, and increases in the expression of genes that encode proteins involved in oxidative burst, apoptosis, and immune function (He

et al., 2010; Knag et al., 2013). Another method of assessing toxicity is by using the microbial genome wide live cell reporter array system that utilizes real time gene profiling (Zhang et al., 2011). These NAs exposed *E.coli* were found to have alterations in genes related to NADH and NADPH binding, as well as alterations in genes related to transcription factors, ultimately signifying DNA damage (Zhang et al., 2011). Several new approaches are being developed for inexpensive assessment of OSPW toxicity. However, it should be noted that *in vitro* assessment of OSPW effects may not necessarily reflect how a whole organism will react to a toxic substance(s), and therefore it is important to establish a causal relationship between the *in vitro* and *in vivo* assessments of toxic effects.

2.3.2. *Toxicity of OSPW in vivo*

OSPW has been shown to be toxic to several aquatic species including *Vibrio fischeri* (Jones et al., 2011; Scott et al., 2008; Martin et al., 2010), green algae (*Pseudokirchneriella subcapitata*) (Debenest et al., 2012; Warith and Yong, 1994) and aquatic invertebrates such as *Chironomus dilutes* (Anderson et al., 2011, 2012) and *Daphnia magna* (MacKinnon and Boerger, 1986). However, few studies have examined how benthic invertebrates react to NAs and OSPW (Anderson et al., 2012).

Yellow perch (*Perca flavescens*) exposed to reclaimed-simulated waters were shown to have decreased disease resistance as a result of increased cortisol levels associated with stress (van den Heuvel et al., 2000). Pathological manifestations such as fin erosion, virally induced tumors, gill abnormalities (aneurisms), and increased epithelial cell proliferation were also reported (van den Heuvel et al., 2000). Chronic exposure of these fish to OSPW resulted in increased prevalence of tumors (van den Heuvel et al., 2000), presumably due to a decrease in immune responsiveness. Other studies have found that yellow perch and goldfish exposed for 3 weeks to OSPW (NAs concentrations of 24 mg/L), exhibited increased mucous cell proliferation and inflammation, histological alterations such as increase in inflammatory cell influx in the liver, and degenerative changes such as gill epithelial cell necrosis; with similar

pathological responses appearing in yellow perch and goldfish (Nero et al., 2006b). Lister et al. (2008) found that goldfish exposed to OSPW for 19 days exhibited increases in plasma cortisol levels, and were therefore stressed.

Other fish species have also been examined. Rainbow trout were affected by OSPW containing NA concentrations similar to those found in tailings ponds (MacKinnon and Boerger, 1986), and appear to be more susceptible to the toxic effects caused by OSPW and NAs. Immunotoxic effects of OSPW on rainbow trout immune systems include increased granulocytes but decreased lymphocytes in the blood, as well as increased susceptibility to opportunistic infections (McNeill et al., 2012), suggesting that exposed fish were immunosuppressed. However, a recent study done by MacDonald et al. (2011) has shown that injecting rainbow trout with NAs isolated from OSPW did not increase bile phenanthrene or EROD activity, thereby questioning whether NAs are the major toxic component in OSPW (MacDonald et al., 2011; McNeill et al., 2012).

Fathead minnows are another fish species that have been used to assess OSPW toxicity. Kavanaugh et al. (2013) found that fathead minnows, sub chronically exposed to OSPW in demonstration ponds, had higher incidence of degenerative changes in the gills. It has also been found that fathead minnows acutely exposed to OSPW exhibited increased expression genes in the liver that encode proteins involved in apoptosis and oxidative stress (Wiseman et al., 2013).

OSPW has been shown to alter development of embryos. Exposure to the wetland reclaimed waters of Mildred Lake has been linked to larval developmental defects in both yellow perch and Japanese medaka fish eggs, resulting in decreased length at hatch (Peters et al., 2007). Similarly, studies examining development of fathead minnow embryos exposed to OSPW observed mortality, malformations, reduced size, as well as larval edema and hemorrhages (Colaveccia et al., 2004; He et al., 2012a; Kavanagh et al., 2011). *In vivo* studies are costly and time consuming, and as a result faster and cheaper methods that maintain accuracy and are representative of how whole organisms respond must be developed.

2.4 Remediation of OSPW

To date, there are no appropriate remediation techniques for OSPW to reduce toxicity to natural environmental levels. Aging of tailing ponds is the most common practice but, although the total NAs decrease, toxicity remains (Leung et al., 2001). Microbial remediation is also possible but, due to the complexity of NAs present in OSPW, and the poorly understood mechanism by which microbial communities are capable of breaking down these compounds, as well as inability to completely remediate toxicity, it is not a popular choice for remediation (Martin et al., 2010; Whitby, 2010).

Advanced oxidation of OSPW is on the forefront of the possible remediation techniques that could be used to decrease OSPW toxicity. This process utilizes ozone to create hydroxyl radicals that are found to break down NAs either partially (He et al., 2011), or totally (Scott et al., 2008). Ozone treatment of OSPW has been shown effective in reducing OSPW toxicity by a number of studies including decrease in OSPW-OF toxicity in mice (Garcia-Garcia et al., 2011a,b), effects on population dynamics of *Chironimus dilutes* (Anderson et al., 2011), and *Vibrio fischeri in vitro* (Martin et al., 2010; Scott et al., 2007), and in human cell lines where apoptosis and endocrine disruption were measured pre- and post-ozonation (He et al., 2010; Knag et al., 2013). Other studies using fathead minnows have shown reduction of EDCs in OSPW after ozonation, as indicated by transcriptional analysis of brain-gonad-liver axis (He et al., 2012b), however there have been many conflicting results as to whether ozonation completely ameliorates EDCs effects (He et al., 2012a; Knag et al., 2013). Transcriptional studies using fathead minnows exposed to OSPW and OSPW-O_3 have found amelioration of gene expression of *cyp1a* and other genes associated with endocrine disruption (He et al., 2011, 2012b, Wiseman et al., 2013). He et al. (2012a) examined the effects of OSPW and OSPW-O_3 on development of fathead minnows, and found that ozonation attenuated survival rate, malformation of the spine, and apoptosis triggered by non-treated OSPW exposure.

To date, studies assessing toxicity of ozonated OSPW focused on the acute time point, and there are conflicting results as to whether full amelioration of OSPW is possible. However, there is evidence that by-products generated during the ozonation treatment may themselves be toxic (Garcia- Garcia et al., 2011a) and, as a result, immunotoxicity of aquatic vertebrates exposed to ozone-treated OSPW must be assessed before the technique can be adopted for remediation.

2.5 Summary

The cause and effect examinations of industrial wastewater exposure on the physiology of an organism are complex. Aquatic organisms may act as bio-sentinels for xenobiotic contamination and because chemical analysis alone does not give insight into toxic physiological effects whole organism studies are needed. Many aspects of fish physiology are shared with higher-level vertebrates, including humans. Therefore, by learning how fish respond to a xenobiotic may give insight into how humans may be affected as well. However, care must be taken when extrapolating the findings across taxa. More research is needed to better understand the effects of environmental pollutants on the physiology of both invertebrates and vertebrates. This is particularly true for OSPW, because of its complex chemical makeup.

CHAPTER III: Materials and Methods

3.1 Fish

Goldfish (*Carassius auratus* L.) ~ 3-6 cm in length, were purchased from Aquatic Imports (Calgary, Alberta) and allowed to acclimate for a minimum of three weeks at 17ºC in the Aquatic Facility of the Department of Biological Sciences at the University of Alberta before use. Fish were acclimated and simulated with a 14h light/10 hour dark photoperiod and fed trout pellets until satiated. Fish were cared for according to the Canadian Council of Animal Care (CCAC-Canada) standards. All fish were anesthetized with tricaine methane sulfonate (TMS; 40 mg/L water, buffered with equal volume of sodium bicarbonate) prior to manipulation (infection, tissues harvest, clipping, bleeding).

3.2 Goldfish exposure to commercial naphthenic acids

NAs were purchased from Merichem Chemicals and Refinery Services LLC (Houston, TX). NAs were solubilized by mixing in 3 mL of 1M sodium hydroxide (NaOH) and diluted and mixed with 1L of water prior to adding to 40L static system fish tanks, yielding concentrations of 5 mg/L, or 20 mg/L NAs. Each tanks housed 25 fish. Each week the tanks were cleaned and the water and NAs were refreshed in each system (i.e. static renewal exposure). The concentrations of NAs were not monitored weekly, thus NAs concentrations of should be considered approximate. Fish exposed to each treatment were kept in tanks for 1, 8, or 12 weeks, and a de-chlorinated city water control treatment was included for every time point analyzed.

3.3 OSPW exposure apparatus

Fish were exposed to OSPW via a real-time flow through exposure system, the only one we are aware of to date that assesses OSPW toxicity (Fig. 3.1 & Fig. 3.2). The apparatus was built and housed at the Oil Sands Research Innovation Facility (Devon, AB). This system involved frequent water shipments from Syncrude Ltd. (Fort McMurray, AB) where water was stored in a ~10,000 L

barrel until use. Water from the barrel was pumped to header tanks on the second floor of the facility and peristaltic pumps then transferred water continuously to fish tanks. The peristaltic pumps were set to varying dilutions depending on the content in the header tank (i.e. aged or fresh OSPW) and a total of 75 L/day cycled through each 150 L fish tank over a 24-hour period. Dilutions and control treatments were made with de-chlorinated city water by injection with sodium thiosulfate such that the total chlorine concentration was less than 0.03 mg/L. Each day, half the water would be drained from each tank to ensure fresh water circulation. Once used, the wastewater would drain to a small barrel and be sump-pumped into 2 large wastewater tanks (~ 5,000 L each) where they would be re-toted and sent back to Syncrude Ltd. site in Fort McMurray, AB.

Basic water chemistry was conducted daily for each experimental period and temperature, chlorine, and pH levels were measured (Table 2)

3.4 Goldfish exposure to OSPW

Fish were exposed at the Oil Sands Research Innovation Facility (Devon, AB) via a real-time flow through apparatus. Fish were exposed for either 1 or 12 weeks to (1) undiluted aged OSPW with a final NAs content of 5 mg/L (Syncrude Ltd. Pond 9, Fort McMurray, Alberta), or fresh tailings with an original NAs concentration of 41 mg/L (Syncrude Ltd. West-in-Pit, Fort McMurray, Alberta) diluted to a final NAs content of (2) 20 mg/L (50% OSPW/ 50% de-chlorinated city water), (3) 10 mg/L (25% OSPW/ 75% de-chlorinated city water), (4) ozonated undiluted fresh tailings (final NAs = 2 mg/L), or (5) de-chlorinated city water (controls). For assessing aged OSPW toxicity a total of 20 fish were randomly assigned to each of four tanks, for assessing endocrine response a total of 20 fish were assigned to each of four tanks. For assessing fresh OSPW immunotoxicity a total of 50 fish were randomly assigned to each of eight tanks, and 20 fish were assigned to each of eight tanks to assess endocrine disruption. Each exposure experimental group was made up of 6 fish per time point and each group was run in time-matched duplicates (n = 12 fish/ experimental group).

3.5 Analysis of NAs in OSPW

Chromatographic separations were done using Waters UPLC Phenyl BEH column (1.7 µm, 150 mm x 1 mm). Final injection was 1 mL with 500 µL sample, 100 µL of 4 mg/L myristic acid $-^{13}C$ in methanol and 400 µL methanol (Fisher Scientific, ON). Mobile phase through the column consisted of a 10 mM ammonium acetate solution in Optima-grade water (A), and 10 mM ammonium acetate in 50% methanol, 50% acetonitrile (B). The gradient elution was: 1% B passed for 2 minutes, ramped to 60% B for 3 min, 70% B 7 min, and 95% B for 13 min, hold for 14 min, 1% B for 5.8 min until equilibrium. Flow rate was 100 µL/min and column temperature was 50ºC, samples were maintained at 4ºC. UPLC was connected to high resolution Synapt G2 high definition (HR)- time of flight (TOF)- mass spectrometer (MS) with electrospray ionization source in the negative ion mode. To analyze data the TargetLynx ver 4.1 was used, and in order to control the system the MassLynx ver 4.1 was used. Tuning was done with leucine encephalin standard solution and sodium formate was used for calibration. This procedure is similar to the one outlined by Wang et al. (2013).

3.6 Ozonation of fresh OSPW

An ozone generator (C, GSO-40, Herford, Germany) was used to ozonate undiluted fresh tailings (35 mg/L). Extra dry high-purity oxygen was used to generate ozone and a 200L plastic barrel with a ceramic fine bubble gas diffuser was used to sparge gas into a liquid phase. Throughout the procedure, ozone concentrations were monitored in the feed and off-gas lines via two ozone monitors (model HC-500, PCI-WEDECO) and the ozone dose was calculated using the following equation (Gamal El-Din et al., 2011):

$$\Delta O_3 = \int_0^t \frac{(Q_{G,in}C_{G,in} - Q_{G,out}C_{G,out})}{V_L} dt - C_L \,,$$

ΔO_3 is the ozone concentration (mg/L) in the ozonated product; $C_{G,in}$ is the ozone concentration in the feed gas calculated by the reading from the first monitor; $C_{G,out}$ is ozone concentration in the off gas, also calculated by the first monitor; C_L

is the residue ozone concentration (mg/L) in the liquid phase; $Q_{G,in}$ is the feed-gas flow rate (L/min); V_L is the effective reactor volume (L); t is the ozone contact time (min); and $Q_{G,out}$ is the off-gas flow rate (L/min). After ozone treatment, the OSPW was purged with nitrogen for 20 minutes to rid the residual ozone.

3.7 Analysis of gene expression with quantitative PCR

Gills, kidney, and spleen were harvested from goldfish following 1, 8, or 12 weeks of exposure. Fish were anesthetized and cervically dislocated prior to organ removal, and tissues were flash frozen in liquid nitrogen and stored at -80ºC until TRIzol (Invitrogen) RNA extraction could be performed according to the manufacturers instructions. In order to preserve RNA quality and quantity, tissues were not perfused (blood remained). Once isolated, 2.5 μg of total RNA was reverse transcribed using Superscript II cDNA synthesis kit (Invitrogen, 11904-018) according to manufacturers directions. cDNA was used for qPCR via Dynamite SYBR Green qPCR fluorescence. All primers were obtained from DNA Technologies and validated for real-time PCR with primer sequences published (Chen et al., 2010; Choi and Habibi, 2003; Grayfer and Belosevic, 2009 a,b,c; Tchoudakova et al., 1999) and are located in Table 1. The cycling parameters were as follows: 95◦C, 10min; 95◦C, 15s; 60◦C, 1min; 95◦C, 15s; 60◦C, 1min; 95◦C, 30 s; 60 ° C, 15 s (melting curve); for 40 cycles. The RQ (fold difference) was found as instructed by the qPCR instruction manual (Applied Biosystems, part number 4347852). All samples were run in triplicate and results are presented as the mean ± SEM of 12 fish per experimental group per time point.

3.8 Isolation of goldfish primary kidney macrophage (PKM)

Following one week or 12 weeks exposure to respective treatments, goldfish macrophage antimicrobial functions were analyzed and PKMs isolated as described (Neumann and Belosevic, 1996), with the following modifications: PKMs were re-suspended at 2 x 10^7 viable cells/mL (trypan blue exclusion) in complete medium (medium containing 10% new-born calf serum and 5% carp serum). One hundred microliters of this suspension was added to each well of 48

well-plates and incubated at 22ºC for 1.5 hours. Following incubation, non-adherent cells were washed away with phosphate buffered saline (PBS). Two hundred microliters of complete medium was added to each well, now containing adherent primary macrophages. These cells were then used to measure reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI).

3.9 Reactive oxygen intermediates (ROI) production assay

PKMs were isolated as described in section 3.7. ROI production was measured via the nitrobluetetrazolium (NBT) reduction assay (Grayfer and Belosevic, 2009b) with minor modifications. PKMs were activated with 3.6 x 10^7 CFU heat-killed *Aeromonas salmonicida* for 4 hours. Two hundred microliters of NBT (2 mg/mL, Sigma) with or without 300 ng/ mL phorbolmyristate acetate (PMA) was added to each well. NBT reduction was measured after 30 minutes. The plates were then centrifuged at 300 x g for 10 minutes, the supernatants removed, and the cells fixed and washed with 70% methanol to remove unreduced NBT. Assays were done in 48 well plates and all samples were run in triplicate. Reduced NBT was then dissolved by addition of 120 μL 2M KOH and 140 μL of DMSO was added to each well to induce a calorimetric response. The supernatants were transferred to a 96 well plate and were read at 630 nm using a microplate reader (Softmax Pro 5). Data is expressed as optical density A_{630} and are mean ± SEM of 8 fish per experimental group.

3.10 Nitric oxide production assay

PKMs were isolated as described in section 3.7. NO production was measured by the Griess reaction as described by Haddad and Belosevic (2009). PKMs were stimulated for 48 hours with 3.6 x 10^7 CFU heat-killed *A. salmonicida* (as described in section 3.8). Following incubation, plates were centrifuged at 300 x g for 10 minutes and then their supernatants collected. One hundred microliters each of Griess reagent A (sulfanilamide, 2.5% phosphoric acid) and Griess reagent B (N-ethyl-enediamine, 2.5% phosphoric acid) was added to the supernatant in a 96 well plate. All samples were run in triplicate and

data are expressed as nitrite concentration as read by a microplate reader (Softmax Pro 5 and calculated via a standard curve) and are mean ± SEM of 8 fish per group.

3.11 *T.carassii* infection

T.carassii strain TrCa (*T.danilewskyi* Laveran and Mesnil 1904) was originally isolated from a wild crucian carp in 1977 by J.Lom. Parasites were shipped from P.T.K. Woo, University of Guelph, ON, and maintained *in vivo* via infection of naïve goldfish until development of and *in vitro* culturing system. Parasites were maintained using trypanosome growth media enriched with 10% heat-inactivated goldfish serum and passaged every 5-7 days as described (Bienek and Belosevic, 1997; Wang and Belosevic, 1994).

Goldfish were exposed for one week to (1) control (de-chlorinated city water), or 20 mg/L C-NAs or (2) control (de-chlorinated city water), 50% fresh OSPW/ 50% water, 25% OSPW/ 75% water, or ozonated fresh undiluted OSPW. Fish were exposed sub-chronically (8 weeks) to (3) control, 5 mg/L, or 20 mg/L C-NAs prior to being injected intraperitoneally with 6.26×10^6 parasites suspended in 100 μL of trypanosome growth medium (TDL-15) using a 26G needle and syringe. Fish were fin clipped in order to follow the infection over a 30-day period, during this time the fish remained in their respective exposure treatments. Parasitemia was determined on days 5, 10, 15, 20, and 30 post-infection by collecting 50 μL of blood from anesthetized fish and diluting it 25 fold in tri-sodium citrate anticoagulant (100 mM tri-sodium citrate, 40 mM glucose, pH 7.3). Parasites were enumerated using a hemocytometer and bright-field microscopy. If fish exhibited low parasite titre, capillary tubes containing undiluted blood were centrifuged 200 x g for 5 minutes in a hemocrit centrifuge and parasitemia was determined by enumerating parasites at the cell-plasma interface of spun, heparinized capillary tubes. If parasite numbers were too high, further dilutions of blood were made in anti-coagulant. Numbers of parasites per mL were log transformed and data are mean ± SEM $\log_{10}$ parasite number per mL of blood of 11 fish per experimental group.

3.12 Collection of plasma

Plasma samples were collected from male goldfish following exposure to either C-NAs, aged, fresh, or ozonated OSPW after week1 and week 12. Following fish anesthetization, blood was collected from their caudal vein using a $27G^{1/2}$ needle coated with cold heparin and aprotinin (2 trypsin inhibitory units (TIU)/mL; Sigma). The blood was then transferred to an equal volume of ice-cold phosphate buffered saline and aprotinin solution (PBS, 2 TIU/mL), and centrifuged at 200 x g for 10 minutes. The plasma was collected, aliquoted, flash frozen with liquid nitrogen, and stored at -80ºC until vitellogenin analysis.

3.13 Vitellogenin ELISA assay

Vtg was quantified using the pre-coated carp Vtg ELISA plates (Carp Vitellogenin ELISA kit, Biosense). Plasma samples were diluted 1:50 in dilution buffer (phosphate buffered saline, 1% bovine serum albumin) and 100 μL of this solution was added to the sample wells in the pre-coated plates. One hundred microliters of dilution buffer was added to each non-specific binding well as a reference. The plate was incubated at 37ºC for 1 hour and washed 3 times with 300 μL of washing buffer (phosphate buffered saline, 0.05% Tween 20). One hundred microliters of diluting detecting antibody (1:500 dilution of stock in dilution buffer) was added to each well and the plate was once again incubated for 1 hour at 37ºC. Following this, plates were washed 3 times with washing buffer and the secondary antibody was added to each well (1:2000 dilution of stock in dilution buffer). The plate was incubated again for another hour at 37ºC and then washed 5 times with washing buffer (300 μL/well). One hundred microliters of substrate solution (urea hydrogen peroxide and O-phenylenediamine dissolved in distilled water) was added to each well and the plate was incubated in the dark, at room temperature, for 30 minutes prior to stopping the reaction with 50 μL of 2M H_2SO4. All samples were run in duplicate and absorbance was read at 492 nm using a microplate reader (Bio-Tek Instruments).

3.14 Statistical Analysis

Vtg data were analyzed using a non-linear 4 parameter regression analysis on SAS in order to derive vitellogenin concentrations (ng/mL blood) using a carp Vtg standard curve according to manufacturers instructions. Prior to statistical analysis all data were square-root transformed to ensure a similar degree of variation between groups and to satisfy statistical test requirements. All data was analyzed using either a one-way factoral analysis of variance (ANOVA) followed by a Dunnet's multiple comparison test, or when only 2 groups were analyzed, an unpaired t-test was used. These tests were used to determine significance between control and experimental groups. With exception of Vtg data (done using SAS), all statistical analyses were done using Prism Graphpad (6.0) software. Probability level of $P < 0.05$ was used to assign significant differences.

Table 1: List of primer sequences for qPCR

Primer Name	**5'-3' Sequence**
IFNγ sense	GAA ACC CTA TGG GCG ATC AA
IFNγ antisense	GTA GAC ACG CTT CAG CTC AAA CA
IL-1β1 sense	GCG CTG CTC AAC TTC ATC TTG
IL-1β1 antisense	GTG ACA CAT TAA GCG GCT TCA C
TNFα-2 sense	TCA TTC CTT ACG ACG GCA TTT
TNFα-2 antisense	CAG TCA CGT CAG CCT TGC AG
IFNR1 sense	TTT TAC GAC TGC CCA CAT GCT
IFNR1 antisense	GGG TCC GTA ACT ATC TAC CGT ATC T
IFNR2 sense	CAG TAA CCC AAC TGA ACA GAC GAA
IFNR2 antisense	CAC TGT TTG GGA AGG ACT TTC A
TNFR1 sense	GCC CCC TGA CTC AAA AGA AAT
TNFR1 antisense	GCC AGC AAC GTC AGG AAA
TNFR2 sense	CCA AAA CAA CCG CGT GAA T
TNFR2 antisense	CAA GAT GTG GTG AAG GTC GTA TC
ERα1 sense	GCA CCC CCT GCT GAA GAA
ERα1 antisense	GCT GGA GGG TGC AAA CAT G
ERα2 sense	ATC TGC AGT CGT TAG GCA GCC CT
ERα2 antisense	CCA GGA TGG CTC AGG TAT GG
ERβ1 sense	CCT TCG CCA TGT CCG AGT AT
ERβ1 antisense	CCA TCC TGC TGG AGT CAA CTT C
ERβ2 sense	CGA AAG CCT GGT CCC TTT AAA
ERβ2 antisense	CAG TGC AGA GAC GGC GTA GA
Q-NPR sense	TTT GCA CAT CAA TGA CCA GAT G
Q-NPR antisense	CTC CAA CCC AGC GAG AAC AA
EF-1α sense	CCGTTGAGATGCACCATGAGT
EF-1α antisense	TTGACAGACACGTTCTTCACGTT

Table 2: Basic chemistry analysis of the water

Parameters	C-NAs	Aged OSPW	Fresh OSPW	Ozonated fresh OSPW
Temperature(ºC)	17.20 ± 0.5	20.30 ± 1.5	23.30 ± 3.0	23.30 ± 3.0
pH	7.40 ± 0.15	7.80 ± 0.20	8.40 ± 0.50	7.80 ± 0.20
Chlorine (Cl_2)	0.01± 0.01	0.02 ± 0.02	0.02 ± 0.02	0.01± 0.01
Flow rate (L/min)	0	0.05 ± 0.02	0.05 ± 0.02	0.05 ± 0.02

Figure 3.1: The real time exposure apparatus system located at the Oil Sands Research Innovation facility in Devon, AB. OSPW is pumped to the 300 L header tanks (white). From there the water is either pumped straight into the green 150L tanks containing goldfish, or diluted with peristaltic pumps (black). Water is renewed at a flow rate of 75L/ day.

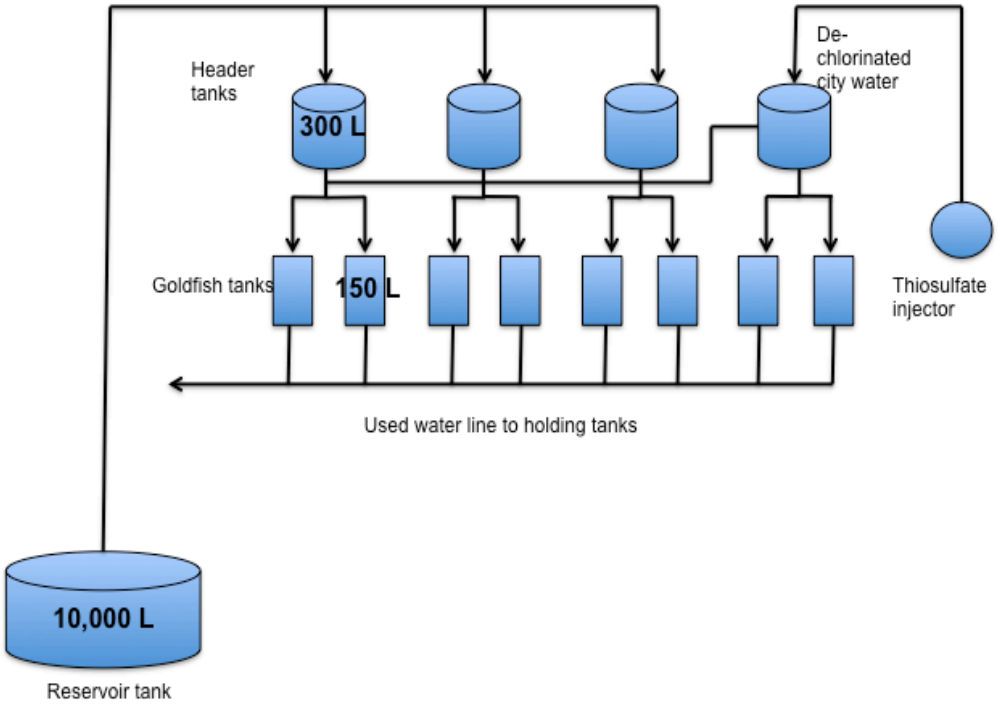

Figure 3.2: A schematic drawing of the real time flow through experimental setup at the Oil Sands Research Innovation facility in Devon, AB. OSPW was pumped from the 10,000L reservoir tank to 300L header tanks. The water was then either pumped directly, or diluted with thiosulfate injected city water, to 150L goldfish holding tanks. Each tank had a flow-through rate of 75L/ day and the used OSPW was stored in two 5000 L holding tanks (not shown).

Chapter IV: Analysis of the innate immunity of goldfish after acute and sub chronic exposure to commercial naphthenic acids[1]

4.1 Introduction

The immune system of all organisms is essential to fighting off invading pathogens as well as being involved in many homeostatic mechanisms. The first cell types to encounter pathogens and react are monocytes/macrophages, granulocytes, and lymphocytes. Upon activation, these cells initiate a cellular cascade to activate other leukocytes and elicit an immune response to neutralize the antigen. Any one of these cells or their secretions may be used as sentinels of specific exposure to a pathogen. Studies involving the infection of a host to a pathogen *in vivo* are capable of directly measuring the host's ability to control and resist the pathogen, whether it is bacterial, or parasitic. This method of assessing host immunocompetence offers a much more complete view of how a pollutant is effecting the hosts ability to combat an infection than studies done *in vitro*. *In vitro* studies generally look at single cell populations and may not necessarily predict how a complete organism will react to a xenobiotic. Fish in aquatic settings are consistently exposed to pathogens, and as a result it is not enough to only examine the toxicity of pollutants, but also how they are able to manage a primary infection in order to determine physiological relevance of a xenobiotic.

The exposure of an organism to particular toxins or xenobiotics may be assessed by certain physiological biomarkers usually associated with shifts in genetic flags relating to metabolism, stress, growth, reproduction, or immune response. Recognizing genetic biomarkers as traits of wastewater pollution is advantageous in that only small amounts of tissue are needed to isolate vast quantities of mRNA; which can be amplified by quantitative polymerase chain reaction (qPCR) to quantify, based on endogenous controls, the relative expression of a biomarker. In doing this it is possible to test for contamination of a particular xenobiotic and understand how it will affect exposed organisms. Control of the immune system and various facets of homeostasis such as cell

[1] A version of this chapter has been published: Hagen et al., 2012; *Aquatic Toxicology* **109**:143-149.

proliferation and inflammation, are controlled by soluble, secretable molecules such as chemokines and cytokines. These molecules are produced not only by immune cells but also by endothelial and epithelial cells as well. In this chapter we examined the changes of three pro-inflammatory cytokines:

Interferon gamma (IFNγ) is an antiviral cytokine produced mainly by activated TH1 cells (Mosmann and Coffman, 1989) and natural killer cells (Perussia, 1991). It has also been shown that induction of IFNγ in trout cell lines leads to an increase of reactive oxygen species (Zou et al., 2005) and may synergistically act with TNFα to induce nitric oxide synthase (iNOS) (Grayfer and Belosevic, 2009c).

Interleukin-one beta (IL-1β1) is produced by macrophages/monocytes as well as neutrophilic granulocytes and is a potent T-cell stimulator (Verburg-van Kemenade et al., 1995); it is also known to mediate communication among immune cells (Blalock, 1994).

Tumor necrosis factor alpha (TNFα) is secreted by macrophages as well as granulocytes and other immune cells to induce an inflammatory response. This molecule is important for initiating a respiratory burst response (Garcia-Castillo et al., 2004) as well as iNOS (Ding et al., 1988). It has been demonstrated to promote chemotaxis of macrophages and neutrophils (Ming et al., 1987) as well as enhancing phagocytic ability (van Strijp et al., 1991). It is also important for up-regulating IL-1β1 levels as well as working synergistically with IFNγ (Gupta et al., 1992)

It is known that pro-inflammatory cytokines such as IL-1β1, IFNγ, and TNFα-2 activate macrophages and initiate induction of ROI as well as iNOS (Grayfer and Belosevic, 2009a,b,c; Grayfer et al., 2008, 2010), which is a method of measuring immune response is *in vitro*. Macrophages produce reactive oxygen and nitrogen species following exposure to pathogens. Respiratory burst is one of the main microbicidal responses of these cells, activating NADPH oxidase to reduce oxygen to generate a superoxide anion (Forman and Torres, 2001). Nitrogen intermediates on the other hand are transcriptionally activated and create NO radicals to act as an antimicrobial molecule (Nathan, 1992). Therefore, we

chose to examine the effects of C-NAs on macrophage antimicrobial functions to determine if gene expression data (*in vivo*) is related to *in vitro* macrophage functions.

In this study, I chose the previously mentioned cytokines as sentinel biomarkers for C-NAs pollution because they are well characterized and involved in inflammation: which has previously been shown to affect fish exposed to OSPW (Nero et al., 2006b; van den Heuvel, 2000). To date, NAs are thought to be the major toxic component in OSPW (Madill et al., 2001). They occur naturally in petroleum and are highly concentrated in tailings ponds with concentrations that range from 20-120 mg/L (Quagraine et al., 2005). NAs have been shown to be toxic to fish at as low as 2.5 mg/L, with 50% mortality in some species exposed to concentrations of 25-75 mg/L (Clemente and Fedorak, 2005). NAs have also been associated with gill inflammation (Nero et al., 2006a) as well as liver histopathology (Nero et al., 2006b).

In order to determine physiological relevance of C-NAs on goldfish, I infected fish with *T. carassii*, an extracellular, blood dwelling flagellated protozoan parasite of cyprinid fish, including goldfish. In aquaculture settings, the prevalence of *T. carassi* infections can be as high as 100% (Lom and Dykova, 1992). The parasite is transmitted from one fish to another by way of blood-feeding leeches (*Hemiclepsis marginata* and *Pisciola*) (Quadri, 1962). Upon ingestion of parasites by the leech, dividing epimastigotes mature and migrate to the crop; becoming non-dividing trypomastigotes. After a short maturation phase these trypomastigotes became metacyclic and are transferred to a new fish host when the leach takes the next blood meal (Lom, 1979; Woo 1987; Lom and Dykova, 1992). Within the vascular system of fish, the parasites develop further, acquire the capacity to divide rapidly, and within two to three weeks the parasite load in fish blood reaches a maximum (~50 x 10^7 trypanosmes/mL of blood) (Lom, 1979; Woo 1987; Lom and Dykova, 1992; Katzenback et al., 2008).

In this chapter, I report on the effects of varying concentrations of C-NAs on goldfish (*Carassius auratus* L.) pro-inflammatory cytokine gene expression in the gill, kidney, and spleen of exposed and non-exposed fish. The gill was chosen

because it is in direct contact with the environment and would be the main point of entry for water-borne pathogens, the kidney because it is a hematopoietic organ, and the spleen because it is a major immune organ. I will also report on the effects of macrophage antimicrobial functions, and physiological relevance of the study and its relation to gene expression.

4.2 Experimental Design

4.2.1 Fish and exposure setup

Goldfish (6-9 cm in length) were exposed in a static system at the University of Alberta, Biological Sciences Aquatic Facility to NAs obtained from Merichem Chemicals and Refinery Services LLC (Houston, TX). Eight hundred microliters of C-NAs were solubilized in 3 mL of 1M NaOH and added to 40L tanks to yield 5 mg/L or 20 mg/L NAs concentrations. All tanks were cleaned once every week and freshly prepared NAs solutions were added after.

4.3 Results

4.3.1 Immune gene expression of goldfish exposed to commercial naphthenic acids

Fish exposed to 20 mg/L C-NAs for 1 week exhibited an acute response in the gill, kidney, and spleen, as seen by significant increases in IFNγ, IL-1β1, and TNFα-2 expression (Fig 4.1). Conversely, fish exposed to 5 mg/L NAs showed no significant changes in pro-inflammatory gene expression after 1 week (Fig 4.1).

Fish exposed to 20 mg/L NAs for 8 weeks continued to have augmented expression of IFNγ in the gill, kidney, and spleen (Fig 4.4). By week 8, TNFα-2 mRNA expression was down-regulated in all organs in fish exposed to 20 mg/L NAs (Fig 4.4). However, down-regulation of cytokine expression was more pronounced following 12 weeks of exposure (Fig 4.5). Fish exposed to 20 mg/L NAs were observed to have decreased levels of IL-1β1 and TNFα-2 in the immune organs (Fig 4.5B, C) and gill (Fig 4.5A). Conversely, fish exposed to 5 mg/L NAs had increased expression of IFNγ and IL-1β1 in all organs (Fig 4.5).

4.3.2 Assessment of primary kidney macrophage (PKM) antimicrobial functions

4.3.2.1 PKM oxidative burst response following exposure of goldfish to C-NAs

We examined the acute effects of C-NAs on primary kidney macrophages (PKMs) after an acute period. It was observed that ROI production stimulated by PMA, a mitogen commonly used for oxidative burst assay (Myers et al., 1985) was higher than non stimulated controls (Fig 4.2 B). It was also found that fish exposed to C-NAs exhibited significantly higher levels of ROI than non-exposed fish (Fig 4.2B). C-NAs did not alter ROI production in non-stimulated macrophages (Fig 4.2B).

4.3.2.2 Macrophage nitric oxide response in goldfish exposed to C-NAs

The acute nitric oxide response of macrophages stimulated with *A.salmonicida*, a common fish pathogen found in warm water (Wiklund and Dalsgaard, 1988) was greater than in non-stimulated macrophages (Fig 4.2A). It was found that fish exposed to 20 mg/L NAs induced the greatest amount of nitrites (Fig 4.2A). However, exposure to lower concentrations of NAs (5 mg/L) had no effect on RNI production (Fig 4.2A). Resting, non-stimulated macrophages did not appear to be effected by exposure to NAs (Fig 4.2A).

4.3.3 Experimental infection with Trypanosma carassii

4.3.3.1 Acute exposure to C-NAs increases resistance to T.carassii

It was found that fish exposed to 20 mg/L C-NAs exhibited a consistently lower parasitemia than control fish, with significantly lower parasitemia on days 10 and 15 (Fig 4.3B). No significant differences in mortality were found (Fig 4.3A).

4.3.3.2 Sub-chronic exposure to C-NAs augments goldfish ability to control parasitic infections

Fish exposed for 8 weeks to 20 mg/L C-NAs subsequently followed by *T.carassii* infection exhibited increased mortality compared to control fish, or fish

exposed to 5 mg/L C-NAs (Fig 4.6A). By day 30 only 10% of fish had survived (Fig 4.6A). Throughout the experiment, fish exposed to high concentrations (20 mg/L) of NAs exhibited consistently higher parasitemia than controls, while fish exposed to lower concentrations (5 mg/L) exhibited decreased parasitemia (Fig 4.6B). Fish exposed to 20 mg/L C-NAs were observed to have high numbers of parasites prior to their death (Fig 4.6C). Fish that died prior to experiment completion were found to have much higher parasitemia than the average parasite number of either dead or live unexposed control group (Fig 4.6C).

4.4 Discussion

In this study, I report on the acute and sub chronic immunomodulatory effects of C-NAs. It is known that the immune system involves proper expression of pro and anti-inflammatory cytokines in order to maintain homeostasis. Deviation from these norms or abnormal inflammatory processes may result in autoimmune diseases or chronic inflammation (Bendtzen, 1989; Miossec, 1993; Prud'homme, 2000; Weckmann and Alococer-Varela, 1996). I postulate that alterations in antimicrobial activity observed were due to changes in immune gene expression during acute and sub chronic exposure of goldfish, which also affected the ability of fish to control a primary infection. These results indicate that both acute and sub chronic exposure of fish to C-NAs induce immunotoxicity and altered ability to control a parasitic infection.

Following exposure of fish to 20 mg/L C-NAs for an acute time period, IFNγ, IL-1β1, and TNFα-2 mRNA levels were up-regulated in all organs, however the kidney and spleen appeared to be the most altered. These are major immune organs and the kidney in particular would be the first place that mediation of xenobiotic secretion would begin (Pritchard and Miller, 1980), and as a result disruption in normal kidney function may lead to homeostatic impairment in other organs. Therefore, NAs may induce a systemic inflammatory alteration that involves multiple organs, and the immune organs in particular. However, increased pro-inflammatory cytokine expression in the gills was also observed. Nero et al. (2006) found that exposure of goldfish to C-NAs reduced

gill surface area and gill mucous and epithelial cell alterations in proliferation, this observation may be a result of alterations in pro-inflammatory cytokine profiles, as seen in this study. It was also found that goldfish did not exhibit mortality at NAs concentrations of 20 mg/L, similar to the concentrations that salmon chum can withstand (Dokholyan and Megamedov, 1983), but higher than that of rainbow trout (Dorn, 1992), showcasing the diverse tolerance of various fish species to NAs.

Alterations in cytokine mRNA levels are in turn linked to macrophage antimicrobial functions. However, the mechanism in which NAs confer toxicity and alter the immune system is not known, but it is believed that they act similarly as surfactants by altering the lipid bilayer and increasing membrane fluidity, acting as a narcosis agent (Frank et al., 2008). NAs are found to be toxic to mammalian leukocytes *in vitro*, and exposure of mouse macrophages to NAs decreases macrophage ability to produce ROI and RNI molecules (Garcia-Garcia et al., 2011a,b). Conversely, an increase in macrophage antimicrobial molecules after acute exposure was witnessed. However, similar findings and increases in gene expression of molecules associated with oxidative stress have also been reported in fathead minnows (He et al., 2012a; Wiseman et al., 2013); as a result NAs appear to effect mammals differently than aquatic vertebrates. Alterations in macrophage functionality may be due to NAs directly interacting with cells of the immune system to alter their functions, but the observed responses of exposed fish are most likely not solely due to NAs-leukocyte interactions directly, and instead evidence is suggesting that alterations of genes may be at the cause, for example cyp1α has been found to alter in fish exposed to both OSPW and NAs (He et al., 2012a, Knag et al., 2013; Wiseman et al., 2013) and these alterations have been found to lead to oxidative stress and ultimately result in cell death through apoptosis (He et al., 2012a). In a study done by Young et al. (2011) it was found using gas chromatography mass spectrometry that liver and gills had the highest concentrations of NAs in rainbow trout in comparison to other organs. As a result, it may be novel biotransformants of NAs generated in the liver that may confer

toxicity, similar to other xenobiotics and drugs (DiGiovanni and Juchau, 1980; Lewis et al., 1998; Kulkarni, 2000).

Following 1 week of exposure to 20 mg/L C-NAs an increase in pro-inflammatory cytokine mRNA levels was found, leading also to an increase in macrophage microbicidal functions, and increased resistance to infection by *T.carassii*. However, no research has been done on the ability of fish exposed to NAs to control a primary infection, so little is known as to whether NAs are able to impact the parasite itself or indirectly affect it through alteration of the host immune response. Sub chronic exposure, on the other hand, resulted in decreased expression of IL-1β1 and TNFα-2, resulting in an inability of fish to control the infection and 90% mortality. All fish appeared sluggish and had a loss of appetite. Similarly, tree swallows exposed to OSPW wetland reclaimed areas have been found to be more susceptible to parasitic blow fly infections (Gentes et al., 2007). In this study, the decreased expression of pro-inflammatory cytokines is linked to a TH1 response, and *T.carassii* clearance is antibody-mediated and thus requires a TH2 response. Therefore, it is possible that the shift in TH responses is an underlying cause in increased susceptibility of these fish to infection.

In conclusion, IFNγ, TNFα-2, and IL-1β1 gene expression appear to be useful biomarkers for evaluating fish immunotoxicity to NAs. However, more reagents and antibodies must be made available for goldfish in order to determine transcribed protein levels. I have shown that exposure to NAs induces acute and sub chronic immunotoxicity, and therefore the need for remediation of NAs-contaminated waters and OSPW is of utmost importance.

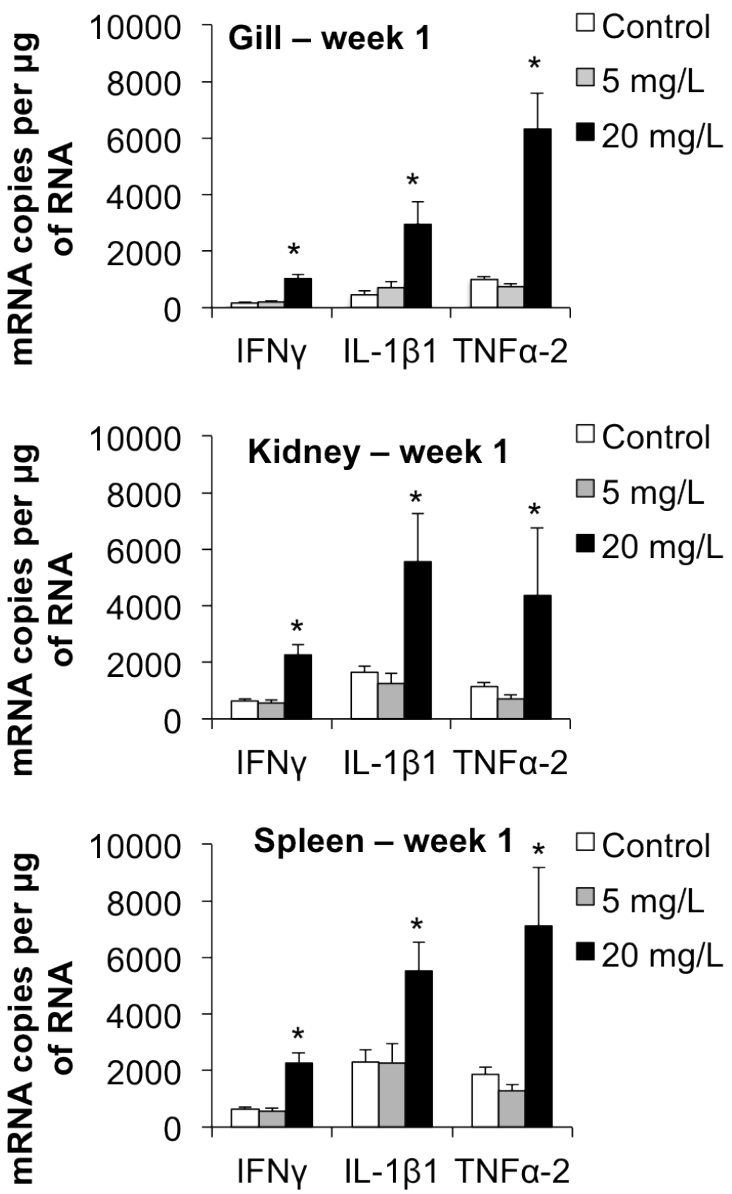

Figure 4.1: Goldfish were acutely (1 week) exposed to dechlorinated city water, 5 mg/L NAs, or 20 mg/L NAs, and gene expression of interferon gamma (IFNγ), interleukin-1 beta 1 (IL-1β1), and tumor necrosis factor alpha-2 (TNFα-2) was assayed in the gill, kidney, and spleen via real time PCR. Data are expressed as mean mRNA copy number and are ± SEM of 6 fish. Asterisks (*) denote statistical significance between exposed and control fish.

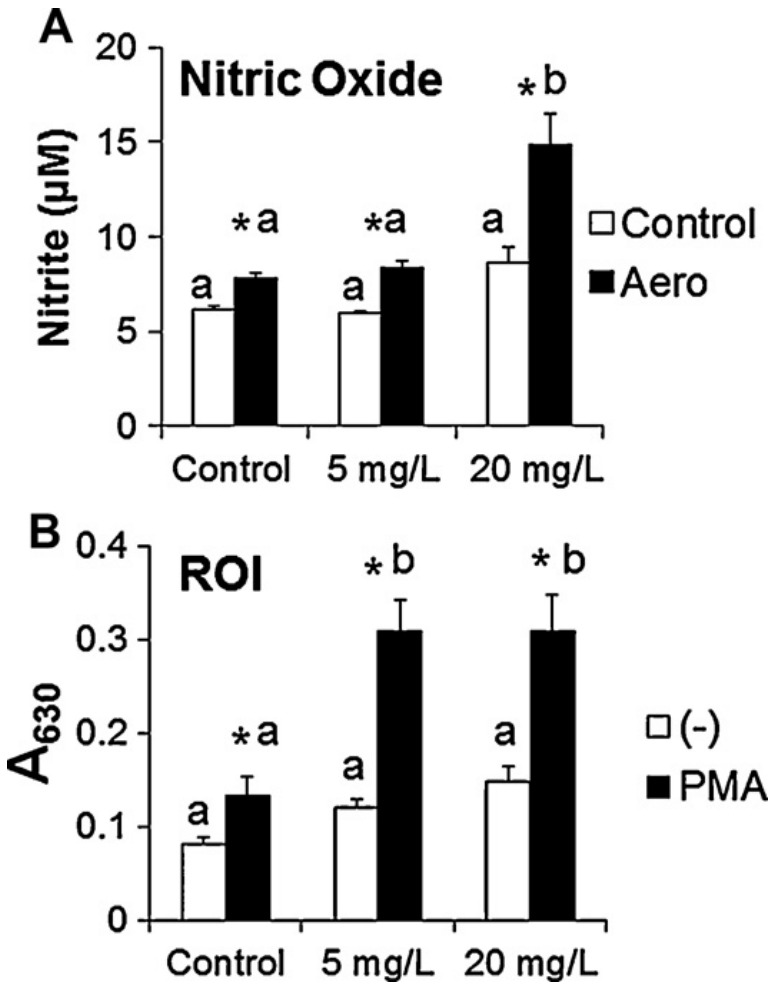

Figure 4.2: Macrophage antimicrobial molecules following 1 week of exposure of goldfish to dechlorinated city water, or C-NAs. Primary kidney macrophages (PKMs) (2x 10^6 cells/ well) were isolated and (A) nitric oxide production was measured by stimulation with *Aeromonas salmoicida* (Aero) and measured by the Griess reaction. Asterisks (*) denote statistical significance from non stimulated negative control (-) and letters denote statistical significance between experimental groups. Data are expressed as nitrite concentration and are mean ±SEM of 6 fish per group. (B) PKMs were activated with *A.salmonicida* for 4 hours and the production of reactive oxygen intermediates (ROI) was stimulated with phorbylmyristate acetate (PMA). Oxygen species were measure after 30 minutes via NBT reduction assay. Asterisks (*) denote statistical significance from unstimulated controls (-) and letters denote significant differences between experimental groups. Data are expressed as optical density A_{630} and are mean ± SEM of 6 fish per group.

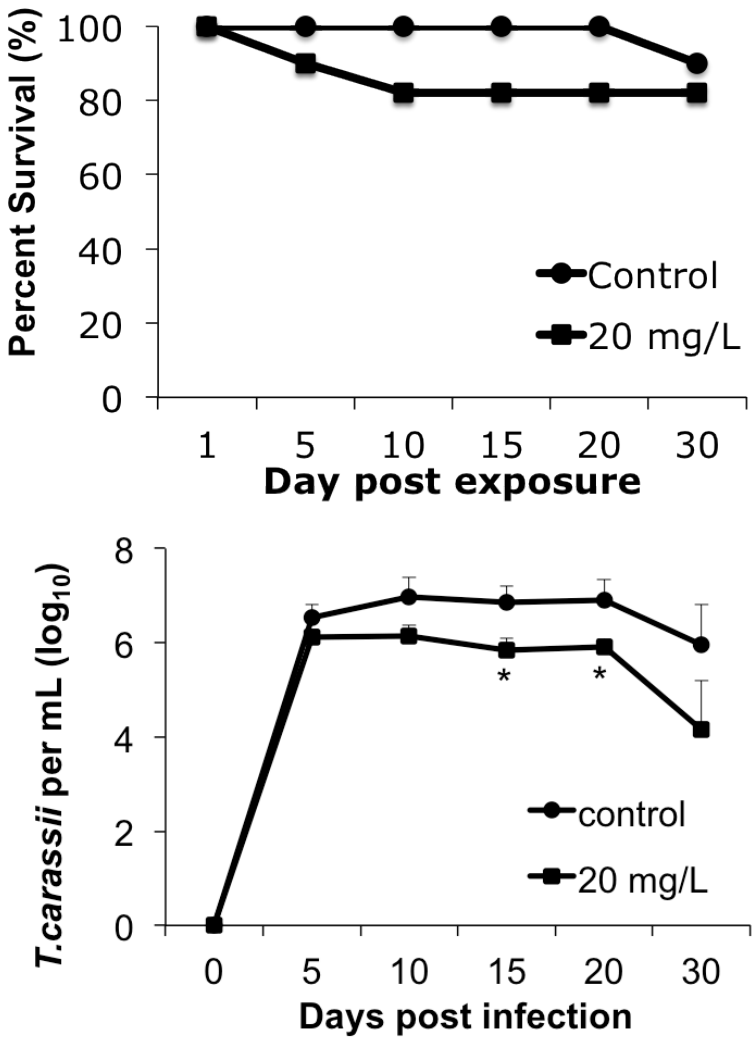

Figure 4.3: Fish were exposed for 1 week to dechlorinated city water, or 20 mg/L C-NAs and infected with *Trypanosoma carassii* and their (A) mortality recorded for 30 days after infection. Data are expressed as % survival. (B) mean parasitemia was calculated over a 30 day period. During this time, fish remained in their respective treatments. Data are expressed as number (log_{10}) parasites per mL of blood and ± SEM of 6 fish.

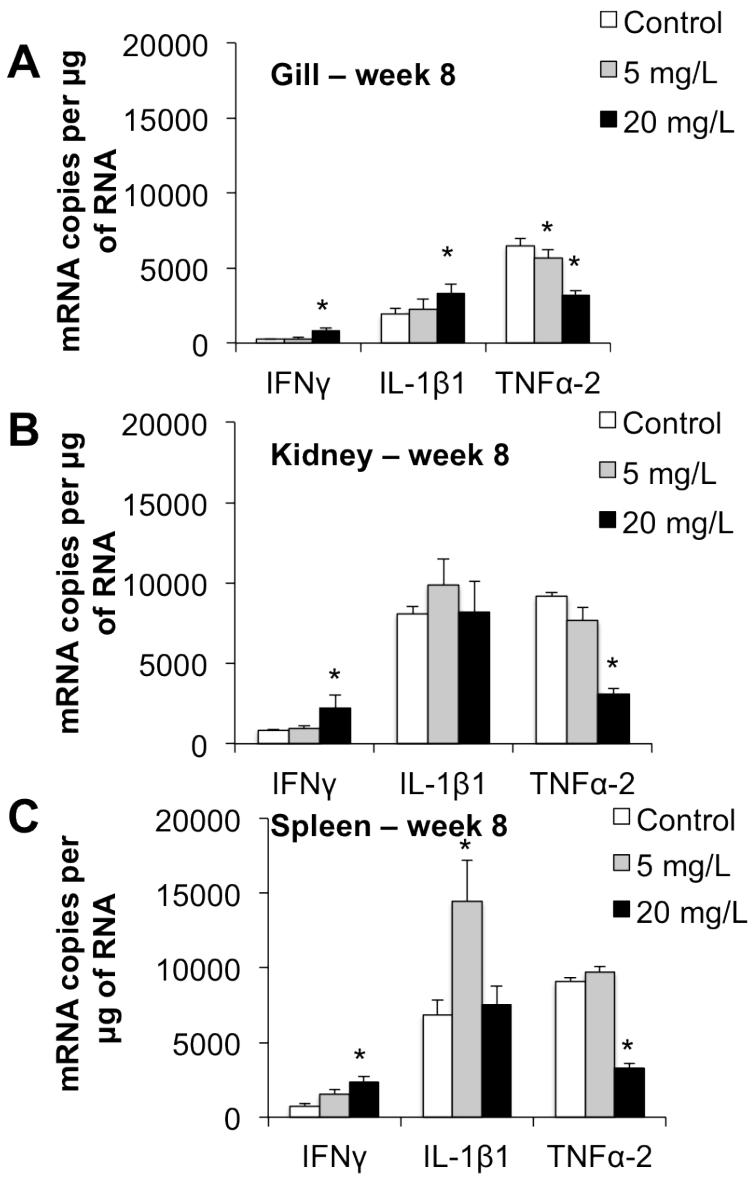

Figure 4.4: Goldfish were sub chronically (8 weeks) exposed to dechlorinated city water, 5 mg/L NAs, or 20 mg/L NAs, and gene expression of interferon gamma (IFNγ), interleukin-1 beta 1 (IL-1β1), and tumor necrosis factor alpha-2 (TNFα-2) was assayed in the gill, kidney, and spleen via real time PCR. Data are expressed as mean mRNA copy number and are ± SEM of 6 fish. Asterisks (*) denote statistical significance between exposed and control fish.

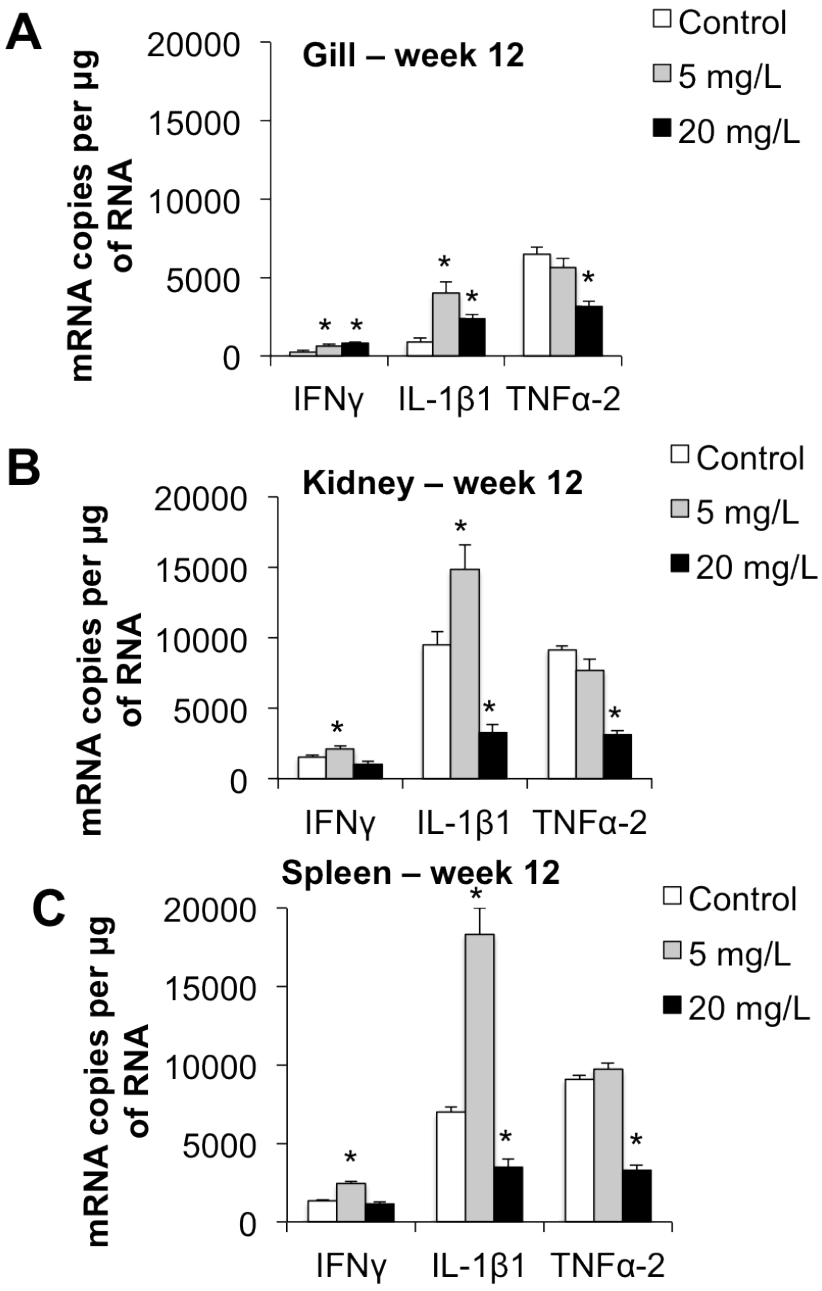

Figure 4.5: Goldfish were sub chronically (12 weeks) exposed to dechlorinated city water, 5 mg/L NAs, or 20 mg/L NAs, and gene expression of interferon gamma (IFNγ), interleukin-1 beta 1 (IL-1β1), and tumor necrosis factor alpha-2 (TNFα-2) was assayed in the gill, kidney, and spleen via real time PCR. Data are expressed as mean mRNA copy number and are ± SEM of 6 fish. Asterisks (*) denote statistical significance between exposed and control fish.

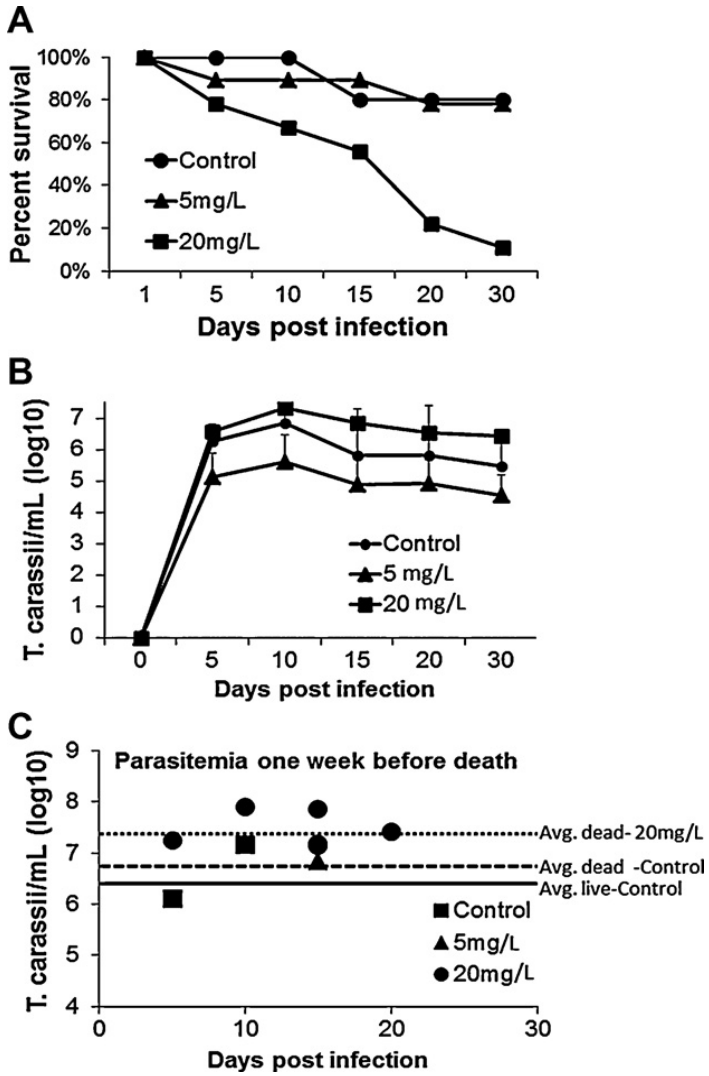

Figure 4.6: (A) fish were exposed to either dechlorinated city water, 5 mg/L C-NAs, or 20 mg/L C-NAs, for 8 weeks followed by *T.carassii* infection. Fish remained in their respective treatments for 30 days and mortality was recorded. Results are presented as % survival (B) Blood parasite numbers were recorded every 5 days post-infection and data are expressed as number (log10) parasites per mL of blood and are ± SEM of 6 fish per group. (C) Fish were exposed for 8 weeks to C-NAs and infected with parasites. Blood parasite number (log 10) are shown for fish that died in each experimental group. The dotted line across the graph is representative of the average (Avg) parasitemia in the dead fish from the 20 mg/L NAs group. The dashed line represents the average parasitemia in dead non-exposed (control) fish. The solid line represents the average parasitemia of non-exposed (control) fish.

Chapter V: Analysis of goldfish innate immunity after acute and sub chronic exposures to oil sands process-affected water[2]

5.1 Introduction

During bitumen extraction, 3 cubic meters of water are used to produce 1 cubic meter of crude oil. This leftover water, termed oil sand process water (OSPW), in addition to bitumen, contains many other toxic compounds including naphthenic acids, BTEX (benzene, toluene, ethylbenzene, and xylene), polycyclic aromatic hydrocarbons, and phenols (Gosselin et al., 2010), while in the inorganic fraction resides heavy metals, chloride, sulfates, sodium, and bicarbonate (Gamal El-Din et al., 2011; Wang et al., 2013). Due to the many toxins in OSPW, this water is confined to cement-lined tailings ponds and legislation prevents its release into the environment (Del Rio et al., 2006). Currently, there are over 10^9 cubic meters of OSPW in tailings ponds and that number is continually growing due to lack of appropriate remediation techniques.

The toxicity of OSPW on aquatic organisms has been widely researched, with yellow perch and goldfish exhibiting liver histopathology (Nero et al., 2006) as well as fin erosion and other physiological alterations (van den Heuvel, 2000). OSPW has also been shown to be acutely and sub chronically toxic to other aquatic organisms such as green algae (Warith and Yong, 1994), and invertebrates (Anderson et al., 2012). This toxicity is thought to be due to the presence of NAs, and in particular, the low molecular weight NA species (Holowenko et al., 2002).

Although there are no appropriate remediation techniques, microbial remediation has been studied, however microbial degradation does not allow for full amelioration because high molecular weight NAs appear to be resistant to this method of remediation (Martin et al., 2010; Wang et al., 2013). A promising technique is ozonation of OSPW and has been shown to ameliorate toxicity in mammals (Garcia-Garcia et al., 2011a, b) as well as in bacteria (*Vibrio fischeri*) and other aquatic organisms (Anderson et al., 2012; He et al., 2010; Martin et al., 2010; Scott et al., 2008; Wang et al., 2013). During the ozonation process,

[2] A version of this chapter has been submitted for publication and is currently in review: Hagen et al., 2013 *Toxicological Sciences* (in review)

hydroxyl radicals are created that breakdown large NAs species into smaller ones, thereby decreasing toxicity of the water (Scott et al., 2008; Wang et al., 2013). However, by products created from the ozonation procedure have been speculated to confer toxicity themselves (Garcia-Garcia et al., 2011a).

To date, there have been few studies examining the effects of OSPW or ozonated OSPW on the immune system. In this chapter of my thesis I will discuss acute and sub chronic immunotoxic effects of goldfish exposed to aged (>15 years), fresh, and ozonated OSPW. I measured immune gene expression, macrophage antimicrobial functions, as well as the ability of exposed fish to control a protozoan parasite infection, *T.carassii*.

5.2. Experimental design

5.2.1 Fish and flow through exposure apparatus

Goldfish (6-9 cm length) were exposed at the Oil sands Research Innovation Facility in Devon, Alberta, Canada in a real-time flow through exposure apparatus. For assessment of aged OSPW toxic effects 20 fish were randomly assigned to four 150L tanks. Fifty fish were used in the fresh OSPW toxicity assessment and were randomly assigned to either 150L tanks. Each tanks had a flow through rate of 75L/ day and were exposed for 1 or 12 weeks to (1) non-diluted aged OSPW (Syncrude Ltd. Pond 9, Fort McMurray, Alberta), or fresh OSPW with a NAs content of 41 mg/L (Syncrude Ltd. West-in Pit) (2) diluted fresh OSPW with a NAs concentrations of 20 mg/L (50% OSPW/ 50% de-chlorinated city water), (3) diluted fresh OSPW to a final NAs content of 10 mg/L (25% OSPW/ 75% de-chlorinated city water), (4) non-diluted ozonated fresh OSPW with a final NAs content of 2 mg/L or (5) de-chlorinated city water. At each observation point 6 fish per tank were collected and each experimental group was run in time matched duplicates (n= 12 fish/ experimental group).

5.3. Results

5.3.1 Gene expression in goldfish after acute and sub chronic exposure to OSPW

5.3.1.1 Immune gene expression of goldfish exposed to aged OSPW

Following acute (one week) of exposure to aged OSPW (NAs content was 5 mg/L); no change in gill cytokine mRNA expression was found (Fig 5.1A). Conversely, mRNA levels of IFNγ and TNFα-2 were found to be significantly elevated in the kidney and spleen in comparison to controls (Fig 5.1C, E), particularily in the spleen where IFNγ and TNFα-2 increased by 16-fold (Fig 5.1E).

After 12 weeks (sub chronic) exposure, all three pro-inflammatory cytokine mRNA levels increased significantly in the gill (Fig. 5.1B), and IL-1β1 and TNFα-2 expression was also found to increase in the kidney (Fig. 5.1D). A 7-fold increase in IL-1β1 gene expression was observed in the spleen (Fig 5.1F). In general, there were minor alterations in mRNA levels of pro-inflammatory cytokine receptor expression between experimental groups (Fig. 5.2).

5.3.1.2 Pro-inflammatory gene expression of fish exposed to fresh OSPW

In general, the two concentrations of fresh OSPW (50% OSPW, and 25% OSPW) produced similar effects on immune gene expression in all tissues after both acute and sub chronic time points; there did not appear to be a dose-dependent effect (Fig. 5.3). The mRNA levels of IL-1β1 were up-regulated throughout the experiment in all tissues, with the exception of OSPW (50%) after sub chronic exposure in the gill (Fig. 5.3B). Changes in IFNγ and TNFα-2 were found to be variable, as increases in IFNγ expression was seen following 1 week exposure to diluted fresh OSPW in the gill and kidney but was not observed in the spleen (Fig. 5.3A, B, C). TNFα-2 expression was down-regulated in the gill after 1 week of exposure, but was up-regulated in the kidney and spleen (Fig. 5.3A, B, C). In contrast, following sub chronic exposure to diluted OSPW it was found that TNFα-2 mRNA expression was significantly lower than controls in all tissues examined (Fig. 5.3B, D, F). Similarly, IFNγ gene expression was found to be significantly decreased in the kidney and spleen of exposed fish (Fig 5.3D, F). Similar to aged OSPW, mRNA levels of pro-inflammatory cytokine gene receptors were not found to have major differences between exposed fish and controls (Fig. 5.4).

5.3.1.3 Immune gene expression of fish exposed to ozonated fresh OSPW

Following acute (1 week) exposure to ozonated OSPW (NAs content = 2 mg/L), it was found that there were no changes in pro-inflammatory gene expression in the gill (Fig. 5.5A) or spleen (Fig. 5.5E), however IL-1β1 was found to be significantly down-regulated in the kidney (Fig. 5.5C), although the biological relevance of this is unknown.

Conversely to this, it was found that fish exposed for a sub-chronic period (12 weeks) to ozonated OSPW exhibited an up-regulation in IL-1β1 in all tissues (Fig. 5.5B, D, F).

Some changes in immune gene receptor expression were seen, however biological relevance of these changes is questionable (Fig. 5.6).

5.3.2 Macrophage antimicrobial responses of exposed goldfish

5.3.2.1 Acute and sub chronic exposure of goldfish to OSPW alters macrophage responses

When PKMs were stimulated with PMA, a known macrophage stimulator, a significant increase in production of ROI was witnessed (Fig. 5.7). However, fish that were exposed to diluted fresh OSPW had PKMs that had significantly impaired ability to produce ROI when compared to the PMA stimulated, non-OSPW treated controls (Fig. 5.7A). Ozonation of fresh OSPW altered ROI production in fish in comparison to fresh OSPW (Fig. 5.7A), and was found to have significantly higher ROI production when compared to controls following acute exposure. However, following sub chronic exposure to ozonated OSPW, PKMs exhibited significantly lower ROI production when compared to controls (Fig. 5.7B), similar to that of PKMs isolated from fresh OSPW (Fig. 5.7B).

5.3.2.2 PKMs isolated from goldfish exposed to OSPW had augmented nitric oxide response

When control fish PKMs were stimulated with heat-killed *A. salmonicida*, the production of nitrates increased significantly (Fig. 5.8). When PKMs were isolated from diluted fresh OSPW-exposed fish it was found that they had impeded ability to produce RNI in comparison to control PKMs (Fig. 5.8). This was found for both acute and sub chronic time points, similar to what we observed for the ROI response (Fig. 5.7). On the other hand, stimulated PKMs isolated from ozonated OSPW exposed fish exhibited a significant increase in RNI from controls (Fig. 5.8A). However, it was found that the levels of nitrites in exposed fish decreased following sub-chronic exposure (Fig. 5.8B), similar to our oxidative burst data (Fig. 5.7).

5.3.3 Control of parasitic infection by goldfish exposed to OSPW

Following exposure of goldfish to OSPW for 1 week subsequently followed by a *T. carassii* infection, fish were observed to have significantly lower parasitemia than non-exposed fish throughout the 30-day period (Fig. 5.9). In contrast, fish exposed to ozonated OSPW exhibited similar parasite burden from controls throughout the experiment (Fig. 5.9).

5.4 Discussion

In this chapter, I examined acute and sub chronic immuntoxicity of goldfish exposed to aged (>15 years), fresh, or ozonated OSPW. I provide data that alterations in pro-inflammatory cytokine expression throughout the experiment may be the cause of altered antimicrobial functions of macrophages as well as increased ability of fish to resist the protozoan parasite, *T. carassii*. Based on these data, I believe that ozonation partially ameliorated the inflammatory response observed in fish exposed to fresh OSPW, and as a result may be an appropriate remediation technique for OSPW.

5.4.1 Acute and sub chronic exposure to OSPW

Acute exposure to xenobiotics are often reversible. On the other hand, sub chronic exposures are long-term, and may be detrimental by morphing into a more serious, chronic disease such as autoimmunity. In this chapter, it was found that fish exposed acutely (1 week) to aged or fresh OSPW exhibited an increase in pro-inflammatory mRNA levels in the immune organs. Cytokines are crucial for homeostasis and inflammation, as well as enhancing antimicrobial functions of immune cells, and high levels of pro-inflammatory cytokines have been linked to macrophage activation and increases in ROI and RNI production (Grayfer and Belosevic, 2009a,c). The increase in acute cytokine expression in fish exposed to fresh OSPW enhanced the ability of fish to control *T.carassii* infection. However, fish exhibited a decrease in IFNγ and TNFα-2 following sub chronic exposure, as well as macrophage functions, and as a result these exposed fish may have become immunosuppressed. Therefore, these data show that separate examination of acute and sub chronic effects is crucial, and the effects of acute exposure may not be extrapolated to predict sub chronic effects.

5.4.2 Aged versus fresh OSPW

The term aged OSPW refers to ponds that have been allowed to sit for more than 10 years as a natural remediation strategy, particularily through the breakdown of NAs through microbial degradation. However, this process does not completely remediate toxicity (Leung et al., 2011) and chronic affects of aged OSPW on aquatic organisms has not been well studied. In this study, aged OSPW refers to >15 years old and had a NAs concentration of 5 mg/L, fresh OSPW was diluted to final NAs concentration of 20 mg/L and 10 mg/L. Following acute exposure to both aged and fresh OSPW we found an immunostimulatory response, most likely due to NAs (Dokholuan and Magomedov, 1984), this was supported by acute increases in pro-inflammatory cytokines levels in the immune organs. However, aged and fresh OSPW contained different NAs concentrations, leading to the belief that other compounds present in the organic fraction such as PAHs may also alter immune response (Thurmond et al., 1987). In contrast to

acute exposure, after 12 weeks fish exposed to aged OSPW had continued up-regulation of pro-inflammatory cytokine expression, while fish exposed to diluted fresh OSPW had decreased expression of IFNγ and TNFα-2, while IL-1β1 levels remained up-regulated. There are a variety of other immune-altering substances in OSPW, such as heavy metals, that are more prevalent in fresh OSPW and have been known to down-regulate inflammation (Abou-Mohamed, 1995), and as a result, gene expression.

5.4.3 Non-ozonated versus ozonated OSPW

Ozonation has been shown to partially (He et al., 2010; Martin et al., 2010) or completely (Scott et al., 2008) break down NAs species. This breakdown has been linked to amelioraton of toxic effects in mice (Garcia-Garcia et al., 2011a,b). Similar to mice, we found amelioration of IFNγ and TNFα-2 levels after both acute and sub chronic exposures. After ozonation, we observed the restoration of fish to control parasitic infection similar to that of non-exposed controls. I also witnessed amelioration of macrophage function following acute exposure to ozonated OSPW: fresh OSPW exposed fish were found to have impeded ability to produce ROI and RNI, while an ameliorated and enhanced function of PKMs isolated from fish exposed to ozonated OSPW was observed. However, exposure of fish to ozonated OSPW for a sub chronic period resulted in impeded ability to produce antimicrobial molecules. Similarily, Wiseman et al. (2013) found that ozonation of OSPW up-regulated genes in fathead minnows that were associated with oxidative burst, although sub chronic effects were not examined. Although ozonation ameliorated IFNγ and TNFα-2 effects in fish, IL-1β1 mRNA levels were found to increase significantly in all tissues following sub chronic exposure. It is well documented that IL-1β1 mediates communication among immune cells and aids in antigen presentation (Bird et al., 2002), suggesting that chronic alterations in immune responsiveness of fish may not be remediated by ozonation. Persistent up-regulation of genes may lead to autoimmune diseases and ongoing inflammation. Due to the ozonation process not being selective strictly for NAs, generated by products from this process may

confer toxicity as well and aid to induce/repress antigen presentation and inflammation in fish (Petala et al., 2008). Although ozonation appears to be a promising remediation technique, physiological relevance must be established in order to determine how an exposed organism will respond to a pathogen.

5.4.4 Parasite challenge and physiological relevance

Proper macrophage function and expression of pro-inflammatory cytokines have been linked to resistance and elimination of pathogens and parasites. Following exposure of fish to fresh OSPW followed by infection with *T.carassii*, an enhanced ability to resist infection was observed, as seen by lower parasitemia in exposed fish. This observation paralleled with increased expression of pro-inflammatory cytokines as well as antimicrobial properties of macrophages. Fish exposed to ozonated OSPW, on the other hand, exhibited a parasite load similar to that of controls, thereby remediating the fresh OSPW exposed fish responses to *T.carassii*. However, we did not examine sub chronic effects of a parasite challenge, and due to decreasing levels of IFNγ and TNFα-2 in fresh OSPW, increased parasitemia would have been expected. However, because the fish are able to better control an infection after acute exposure does not necessarily mean the fish are healthy and prolonged exposure may increase fish susceptibility to disease and infection. Therefore, future studies assessing physiological relevance of OSPW exposure, particularly sub chronic exposure, need to be examined prior to release of OSPW.

These results show that there is an alteration of immune response following fish exposure to OSPW for both acute and sub chronic periods. These data shed light on how higher aquatic organisms may react to OSPW as well as confirming that ozonation may be an adequate remediation technique for OSPW.

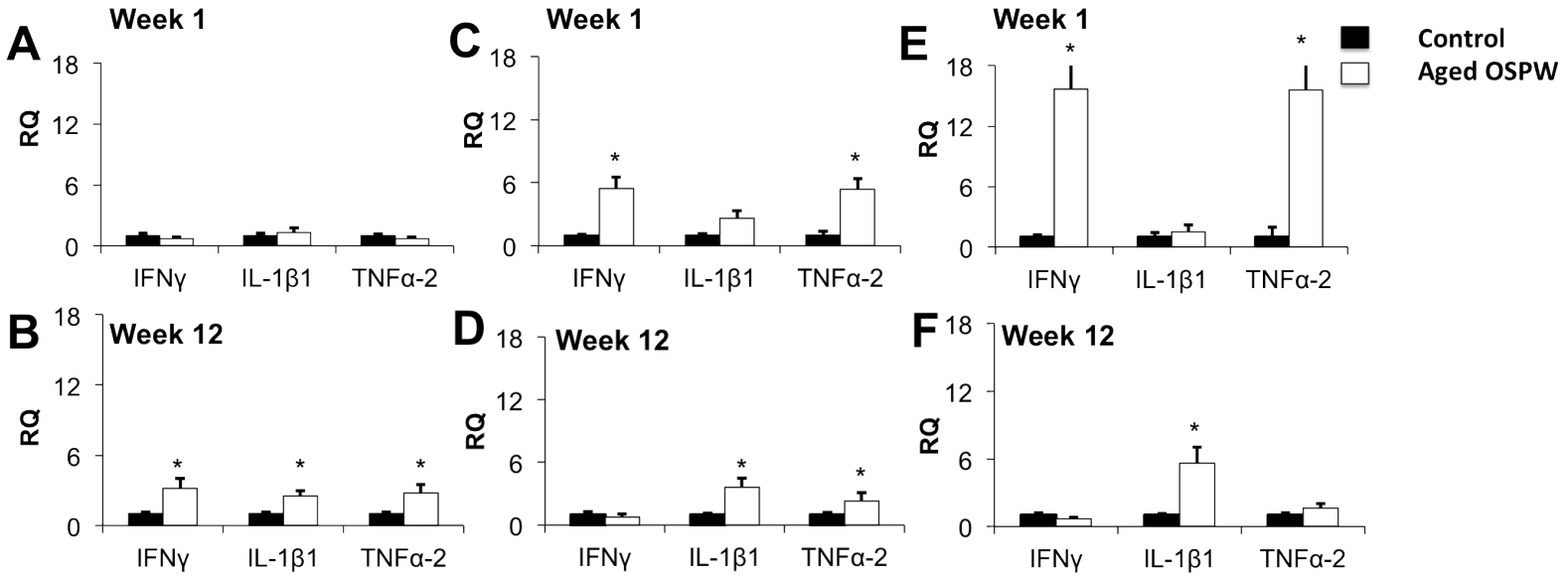

Figure 5.1: Immune gene expression in gill (AB), kidney (CD), and spleen (EF) of goldfish exposed to de-chlorinated city water, or aged OSPW (NA = 5 mg/L). Fish were exposed in a flow through apparatus for 1 or 12 weeks and mRNA levels of gamma interferon (IFNγ), interleukin-1 beta 1 (IL-1β1) and tumor necrosis factor alpha-2 (TNFα-2) were found using quantitative PCR. Data are mean RQ (fold difference compared to the EF-1a normalized control gene expression) ± SEM of 12 fish. Asterisks (*) denote statistical significance between exposed and control fish.

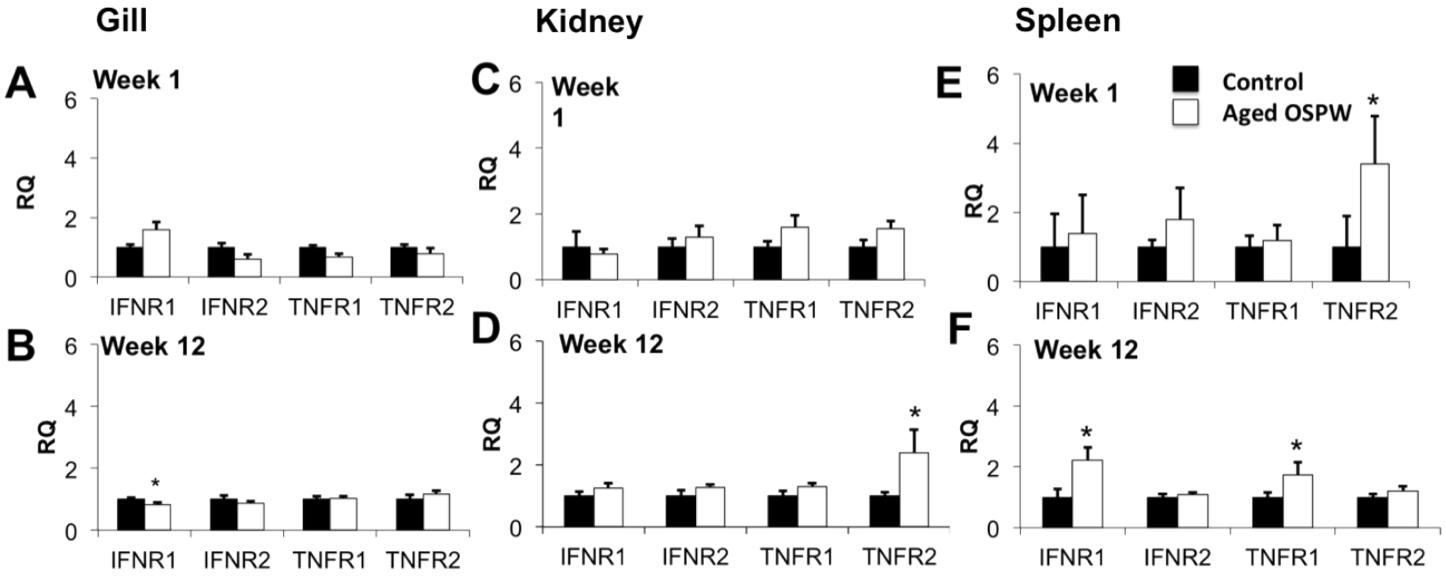

Figure 5.2: Gene expression of immune receptors in the gill (AB), kidney (CD), and spleen (EF) in goldfish exposed to de-chlorinated city water, or aged OSPW (NA = 5 mg/L). mRNA levels of interferon receptor 1 and 2 (IFNR1, IFNR2), and tumor necrosis factor receptor 1 and 2 (TNFR1, TNFR2) gene expression were determined by qPCR. Data are mean RQ (fold difference between EF1-α normalized to control gene expression) ± SEM of 12 fish. Asterisks (*) denote statistical significance between exposed and non-exposed controls.

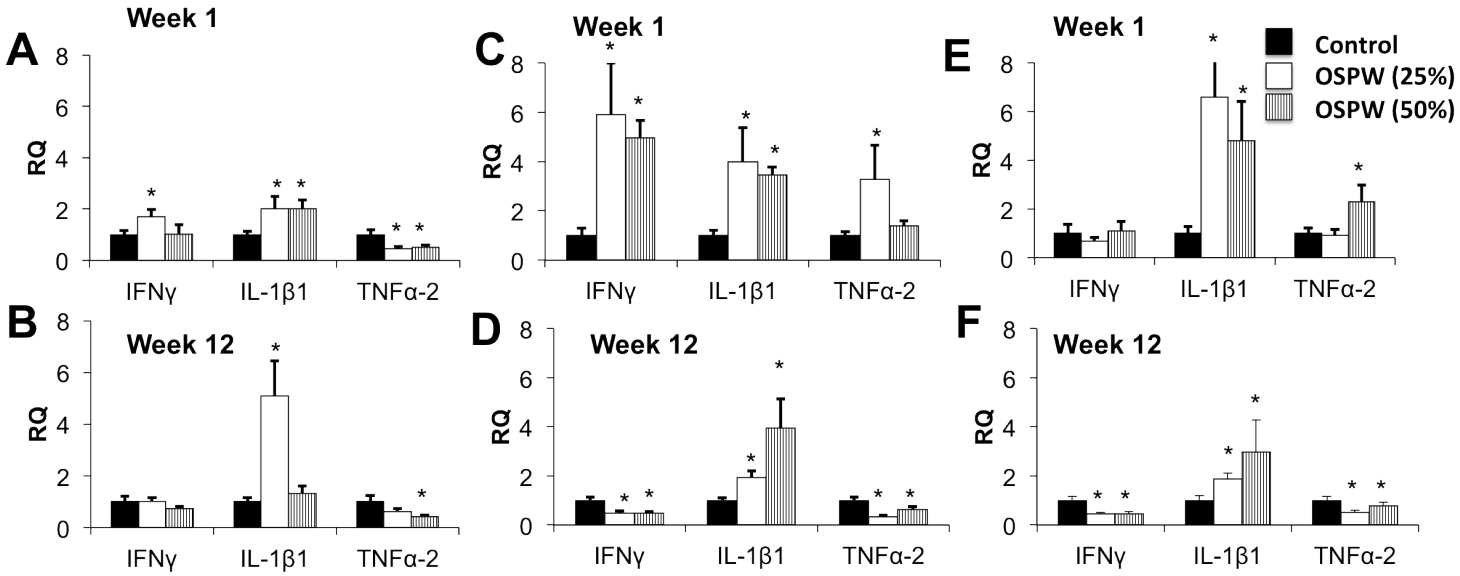

Figure 5.3: Immune gene expression in gill (AB), kidney (CD), and spleen (EF) of goldfish exposed to de-chlorinated city water, or fresh OSPW. Fish were exposed in a flow through apparatus for 1 or 12 weeks to either 50% OSPW/50% dechlorinated city water (NA = 20 mg/L), 25% OSPW/ 75% dechlorinated city water (NA = 10 mg/L). mRNA levels of gamma interferon (IFNγ), interleukin-1 beta 1 (IL-1β1) and tumor necrosis factor alpha-2 (TNFα-2) were found using quantitative PCR. Data are mean RQ (fold difference compared to the EF-1α normalized control gene expression) ± SEM of 12 fish. Asterisks (*) denote statistical significance between exposed and control fish.

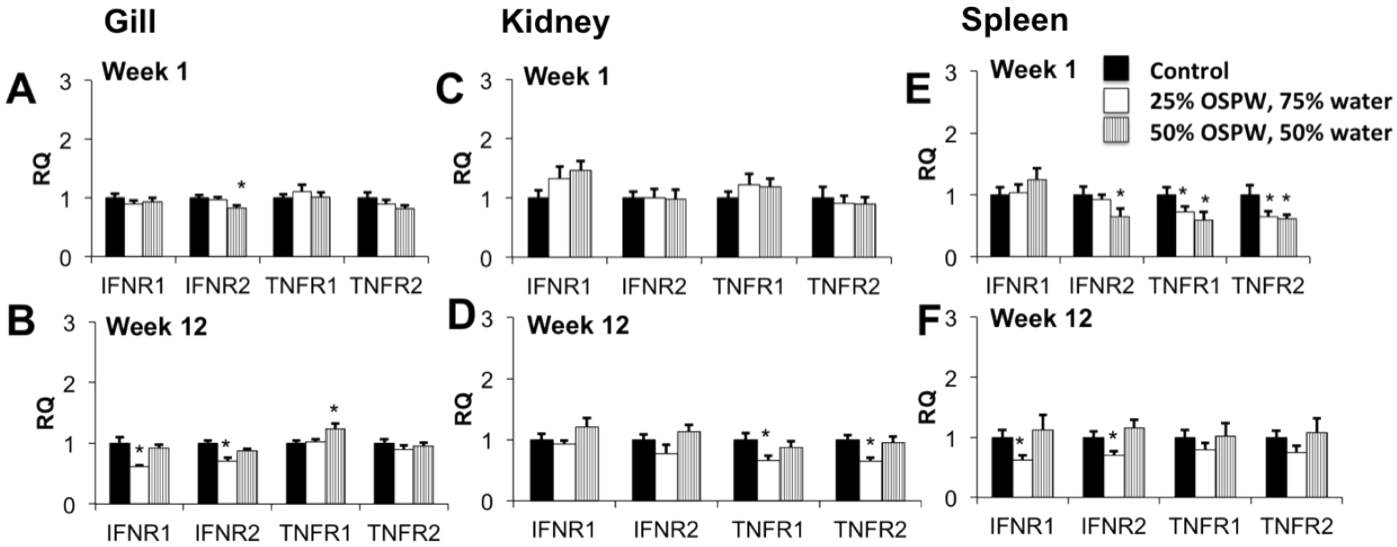

Figure 5.4: Gene expression of immune receptors in the gill (AB), kidney (CD), and spleen (EF) in goldfish exposed to de-chlorinated city water, fresh OSPW: 50% OSPW/50% dechlorinated city water (NA = 20 mg/L), or 25% OSPW/ 75% dechlorinated city water (NA = 10 mg/L). mRNA levels of interferon receptor 1 and 2 (IFNR1, IFNR2), and tumor necrosis factor receptor 1 and 2 (TNFR1, TNFR2) gene expression were determined by qPCR. Data are mean RQ (fold difference between EF1-α normalized to control gene expression) ± SEM of 12 fish. Asterisks (*) denote statistical significance between exposed and non-exposed controls.

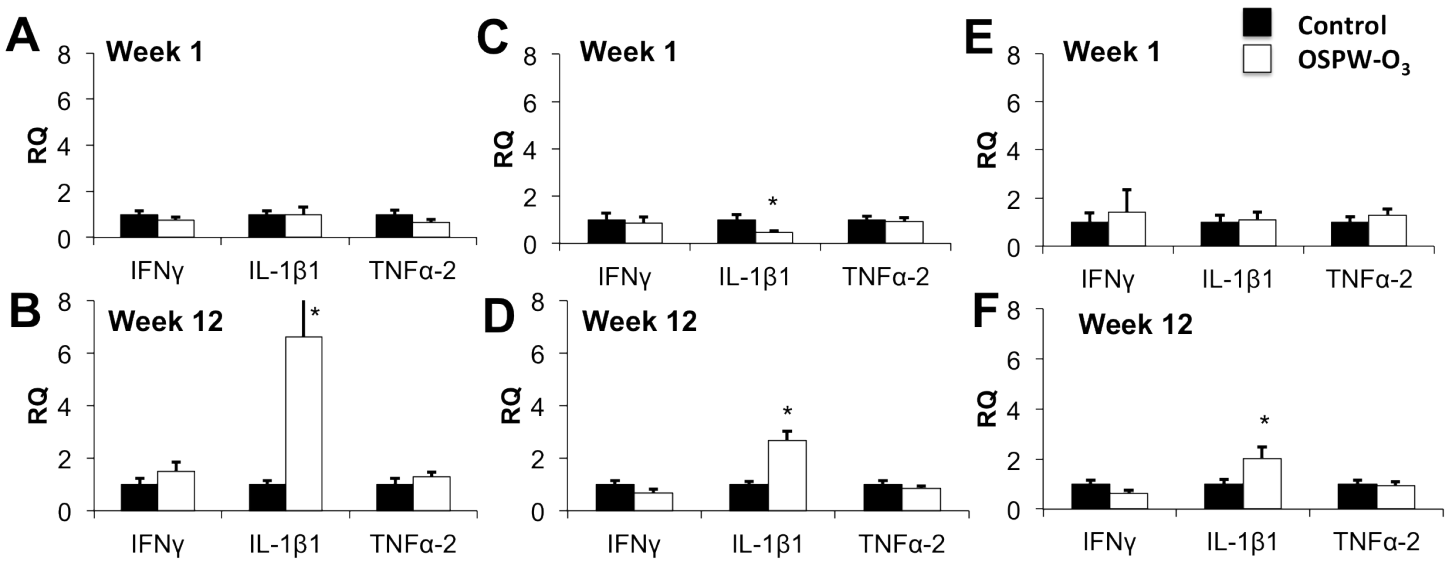

Figure 5.5: Immune gene expression in gill (AB), kidney (CD), and spleen (EF) of goldfish exposed to ozonated fresh OSPW. Fish were exposed in a flow through apparatus for 1 or 12 weeks to de-chlorinated city water, or ozonated undiluted fresh OSPW (NA = 2 mg/L). The mRNA levels of gamma interferon (IFNγ), interleukin-1 beta 1 (IL-1β1) and tumor necrosis factor alpha-2 (TNFα-2) were found using quantitative PCR. Data are mean RQ (fold difference compared to the EF1-α normalized control gene expression) ± SEM of 12 fish. Asterisks (*) denote statistical significance between exposed and control fish.

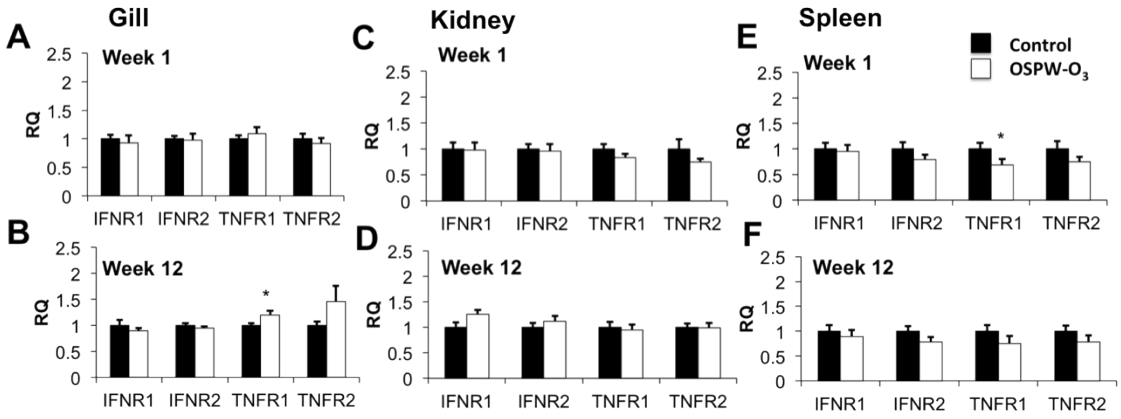

Figure 5.6: Gene expression of immune receptors in the gill (AB), kidney (CD), and spleen (EF) in goldfish exposed to de-chlorinated city water, or ozonated undiluted fresh OSPW (NA = 2 mg/L). The mRNA levels of interferon receptor 1 and 2 (IFNR1, IFNR2), and tumor necrosis factor receptor 1 and 2 (TNFR1, TNFR2) gene expression were determined by qPCR. Data are mean RQ (fold difference between EF1-α normalized to control gene expression) ± SEM of 12 fish. Asterisks (*) denote statistical significance between exposed and non-exposed controls.

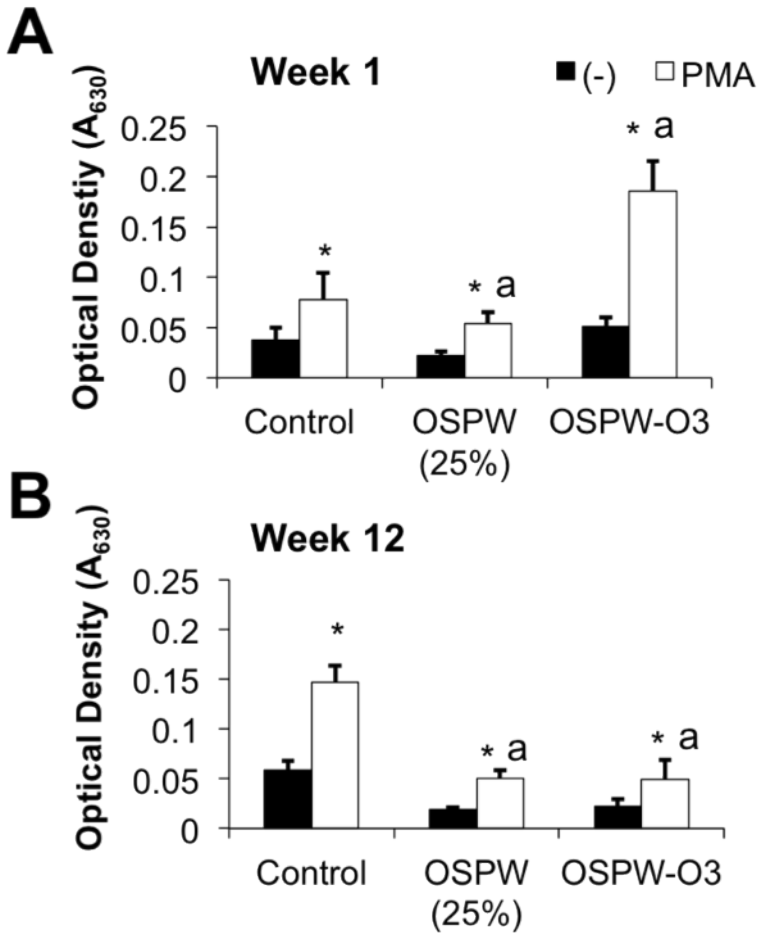

Figure 5.7: Reactive oxygen intermediates production from primary kidney macrophages (2x 10^6 cells/ well) isolated from fish exposed to de-chlorinated city water, diluted fresh OSPW (25% OSPW/ 75% water, NAs = 10 mg/L), or ozonated undiluted fresh OSPW (NAs = 2 mg/L). Fish were exposed for either 1 or 12 weeks and PKMs were activated via *Aeromonas salmonicida* for 4 hours prior to being stimulated with phorbol myristate acetate (PMA). Production of ROI was determined using the NBT reduction assay. Data are presented as optical density A_{630} and asterisks (*) denote statistical significance from non-stimulated controls, and letter denotes statistically different from stimulated controls ± SEM of 8 fish. OSPW- O_3

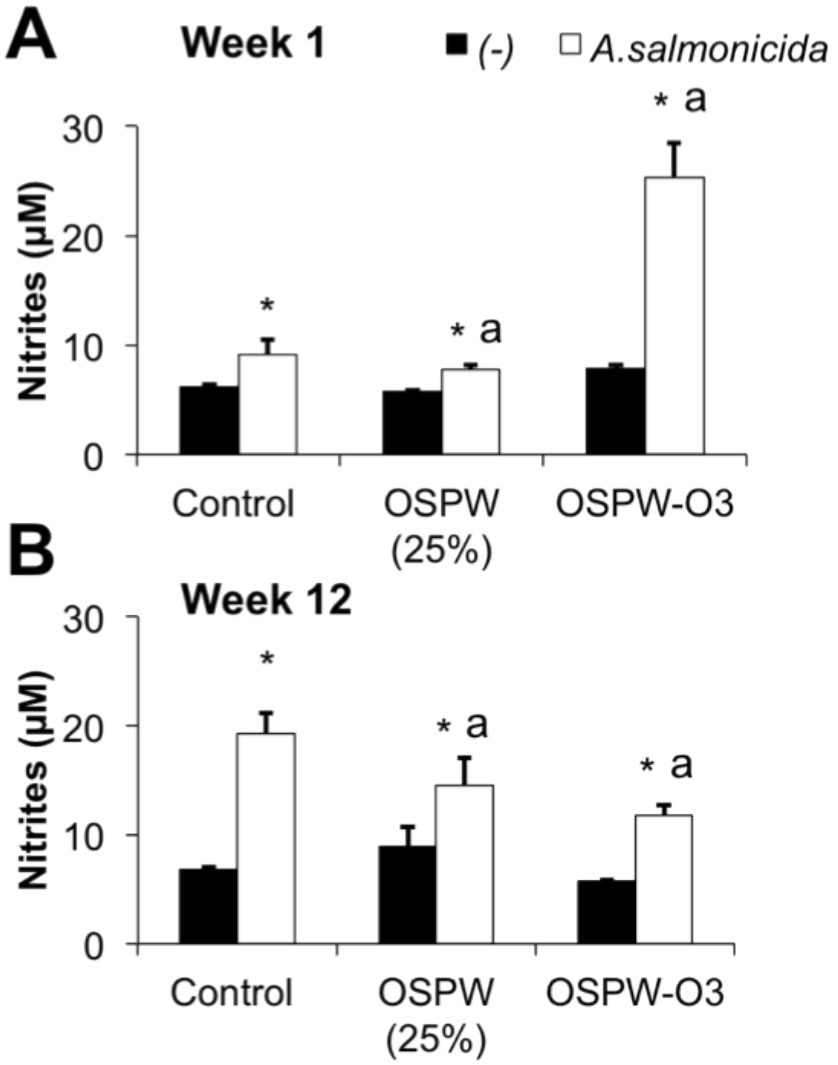

Figure 5.8: Reactive nitrogen intermediates (nitrite) production from primary kidney macrophages (2x 10^6 cells/ well) isolated from fish exposed to de-chlorinated city water, diluted fresh OSPW (25% OSPW/ 75% water, NAs = 10 mg/L), or ozonated undiluted fresh OSPW (NAs = 2 mg/L). Fish were exposed for either 1 or 12 weeks and PKMs were activated via *Aeromonas salmonicida* for 48 hours and nitrite concentration was determined using the Griess reaction. Data are presented as nitrite concentration and asterisks (*) denote statistical significance from non-stimulated controls, and letter denotes statistically different from stimulated controls ± SEM of 8 fish.

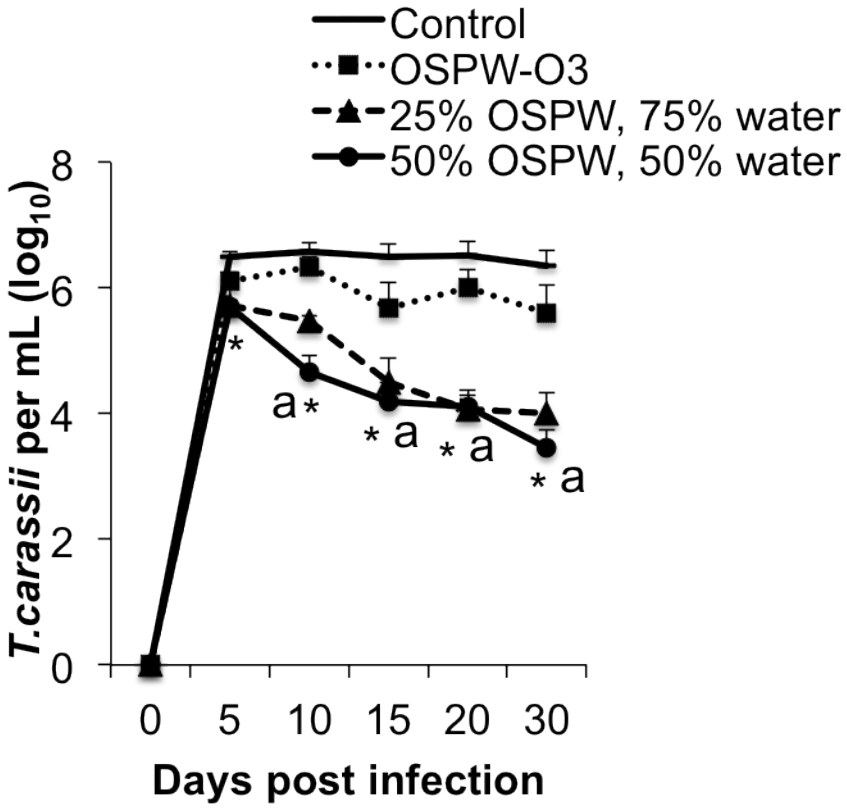

Figure 5.9: *Trypanasoma carassii* course of infection in goldfish exposed to de-chlorinated city water (control), 25% OSPW/ 75% water (NAs = 10 mg/L), 50% OSPW/ 50% water (NA = 20 mg/L), or ozonated undiluted fresh OSPW (NAs = 2 mg/L), for one week followed by subsequent infection with the parasites. Parasite levels were measured every 5 days for a total of 30 days, during this time fish remained in their respective treatments. Asterisks (*) denote statistical significance between exposed and control groups and letter denotes significance between ozone-treated OSPW and fresh OSPW. Data are mean number (log_{10}) parasites per mL of blood ± SEM 11 fish per experimental group.

Chapter VI: Analysis of endocrine receptor expression and vitellogenin levels following acute and sub chronic exposures of male goldfish (*Carrasius auratus* L.) to oil sands process affected water

6.1 Introduction

OSPW exposure has been reported to alter the endocrine-immune axis in fish (He et al., 2012b; Wiseman et al., 2013). In fathead minnows, exposure to OSPW has been shown to impair reproduction, decrease egg clutch size, alter development of secondary sexual characteristics (Kavanagh et al., 2012) as well as induce hemorrhaging and malformation in embryos (He et al., 2012a). These alterations may be due to a decrease in testosterone and 17β estradiol (E2) levels (van den Heuvel et al., 1999; van den Heuvel et al., 2012). Related to E2 levels, vitellogenin, an egg yolk pre-cursor protein, has been found to decrease in Japanese Medaka (Yamaguchi et al., 2005), zebrafish (Martyniuk et al., 2007), and fathead minnows (Filby et al., 2007) when the fish were exposed to estrogen-like compounds. Naphthenic acids (NAs) are structurally similar to sex steroid hormones, particularly estrone and estradiol, with a 4 ring structure (Rowland et al., 2011) and fathead minnows exposed to commercial NAs (C-NAs) exhibited altered sex hormone levels (He et al., 2012a).

One of the remediation strategies for OSPW involves aging of the tailings ponds for indefinite periods. However, advanced oxidation of OSPW is currently being studied as a potential technique to decrease toxicity of these waters (He et al., 2012; Scott et al., 2008). Ozonation involves the break down of large NAs species by hydroxyl radicals (Scott et al., 2008), however during the ozonation process by-products of these compounds are created which may also be toxic (Petala et al., 2008). Despite this, ozonation has been shown to remediate toxicity of OSPW in mice (Garcia-Garcia et al., 2011a,b), cell lines (He et al., 2010), as well as partial remediation of endocrine disruption in fish (He et al., 2012a,b; Wiseman et al., 2013; Knag et al., 2013).

In this chapter I examined the effects of aged (>15 years), fresh, and ozonated OSPW on endocrine disruption in goldfish by measuring endocrine receptor gene expression as well as vitellogenin levels in male fish.

6.2 Experimental design

6.2.1 Goldfish and exposure system

Male goldfish 6-9 cm in length were exposed at the Oil Sands Research Innovation Facility in Devon, Alberta, Canada in a real-time flow through exposure apparatus. For assessment of aged OSPW 15 fish were randomly assigned to each of four 150L tanks, and 15 fish were randomly assigned to each of eight 150L tanks for exposure to fresh OSPW. Each tank had a flow through rate of 75L/ day and were exposed for 1 or 12 weeks to (1) non-diluted aged OSPW (Syncrude Ltd. Pond 9, Fort McMurray, Alberta), or fresh OSPW with a NAs content of 41 mg/L (Syncrude Ltd. West-in Pit) (2) diluted fresh OSPW with a NAs concentrations of 20 mg/L (50% OSPW/ 50% de-chlorinated city water), (3) diluted fresh OSPW to a final NAs content of 10 mg/L (25% OSPW/ 75% de-chlorinated city water), (4) non-diluted ozonated fresh OSPW with a final NAs content of 2 mg/L or (5) de-chlorinated city water. At either 1 or 12 weeks 6 fish per tank were collected for endocrine gene analysis and Vtg and each experimental group was run in time-matched duplicates (n = 12 fish per experimental group).

6.3 Results

6.3.1 Exposure to OSPW alters acute endocrine receptor expression

6.3.1.1 Acute endocrine receptor expression of exposed fish

Following acute (1 week) exposure to aged OSPW, there were no changes in expression of most genes that encode endocrine receptors (Fig. 6.1A) except for an up-regulation of mRNA levels of nuclear progesterone receptor in the spleen (Fig. 6.1B).

In general, gene expression of endocrine receptors in fish exposed to different concentrations of diluted fresh OSPW was similar. After 1 week, fish

exhibited varying changes in gene expression (Fig. 6.1C, D). For example, Erα1 was found to decrease in the kidney (Fig. 6.1C), but not in the spleen (Fig 6.1D). The fish exposed to 25% OSPW exhibited a decrease in ERβ1 mRNA levels, but increases in Erα2 and ERβ2 in the kidney (Fig 6.1C). On the other hand, no major changes in gene expressions for estrogen receptors were observed in the spleen after acute exposure (Fig. 6.1D).

Fish exposed to ozonated OSPW for 1 week exhibited significant down-regulation of ERα1 in the kidney and spleen (Fig. 6.1E, F), similar to the kidney of fish exposed to fresh OSPW (Fig. 6.1C). The endocrine gene receptor expressions were similar to those of non-exposed fish after acute exposure (Fig. 6.1E, F).

6.3.1.2 Exposure of fish to aged OSPW and ozonated fresh OSPW augments endocrine receptors following sub chronic exposure

After sub chronic exposure (week 12) to aged OSPW, Erα2, ERβ1, and Q-NPR mRNA levels were found to increase in the kidney (Fig. 6.2A). Only Erα-2 levels were found to increase in the spleen after sub chronic exposure (Fig 6.2B).

Following sub chronic exposure to fresh OSPW, the mRNA levels of estrogen receptors in both kidney and spleen were similar to those of non-exposed control fish (Fig. 6.2C, D). However, the mRNA levels of Q-NPR were down-regulated in both organs (Fig. 6.2C, D).

After sub chronic exposure to ozonated fresh undiluted OSPW, by week 12 the kidney and spleen were found to have elevated levels of Erα1, Erα2, and ERβ1 receptors (Fig. 6.2E, F).

6.3.2 OSPW exposure altered goldfish plasma vitellogenin levels

6.3.2.1 Vitellogenin levels in male fish after acute exposure to OSPW

Fish exposed for one week to aged OSPW had similar Vtg levels compared to non-exposed fish (Fig. 6.3A).

After 1 week, we observed an increase in Vtg protein levels in the 50% OSPW/50% city water exposed fish (Fig. 6.3B), but saw no significant differences between fish exposed to ozonated OSPW and controls (Fig. 6.3B).

6.3.2.2 Vitellogenin protein levels following 12 weeks of exposure to OSPW

After sub chronic exposure to aged OSPW, Vtg levels decreased significantly (Fig. 6.3C), this was opposite of what was seen after acute exposure (Fig 6.3A).

At week 12 of exposure, I did not observe significant differences between control Vtg levels in fish exposed to 50% OSPW or ozonated fresh OSPW (Fig. 6.3D).

6.4 Discussion

I observed that there were endocrine-disrupting affects of aged and fresh OSPW, and the ability of advanced oxidation to ameliorate these effects was found following acute, but not sub chronic exposure. Following acute exposure to OSPW, little change in endocrine receptor expression was observed, except in the kidneys isolated from fish exposed to fresh OSPW, and alterations of endocrine receptor gene expression following acute and sub chronic exposure of goldfish to OSPW may be related to alterations in vitellogenin (Vtg) levels. Furthermore, I propose that ozonation of fresh OSPW ameliorates, in part, the alterations in acute endocrine disruption in fish exposed to OSPW. However, ozonation may create a different profile of endocrine receptor gene expression disruption that may ultimately alter the endocrine axis in goldfish after sub chronic exposure, as seen by significant increases in three out of four endocrine receptors analyzed.

6.4.1 Acute versus sub chronic exposure to OSPW

Following acute exposure to aged OSPW (> 15 years with a NAs content of 5 mg/L), there was little change in endocrine gene receptor expression or Vtg levels. Endocrine disrupting effects in OSPW have been linked to the organic fraction of OSPW, such as NAs or PAHs (He et al., 2012a,b). Since large

compounds in the organic fraction breakdown during the aging process, endocrine-disrupting effects may be dampened after acute exposure, and these smaller compounds may need to be somewhat broken down or disassembled to act on the fish in order to alter endocrinicity. Fresh OSPW (diluted 50% OSPW/ 50% water, to a NAs content of 20 mg/L, or 25% OSPW/ 75% water, diluted to a final NAs content of 10 mg/L), on the other hand, showed variable alterations in acute endocrine receptor mRNA expression that translated to alterations in Vtg levels compared to controls. It has been shown that fish vitellogenin (Vtg) levels are affected by ERβ1 and estradiol (E2) levels (Soverchia et al., 2005). We saw an acute up-regulation of Vtg protein levels in the 50% OSPW group although we saw a decrease/no change in ERβ1 levels. This may be due to unknown Vtg agonists in OSPW activating the gene, similar to what was found by He et al. (2012). It appears that following acute exposure, fresh OSPW has more of an effect on endocrine receptor gene expression and Vtg than aged OSPW, and therefore may contain more acutely estrogenic compounds at a greater concentration.

After sub chronic exposure, it was found that fish exposed to fresh OSPW had similar estrogen gene receptor profiles as non-exposed fish. In contrast, fish exposed to aged OSPW exhibited siginficant increases in estrogen receptor gene expression. These results suggest a compound in aged OSPW, but lacking in fresh OSPW, induced sub chronic endocrine alteration of fish. To date, it is not known what estrogenic agonists are present in OSPW, although other studies have hypothesized that they may be NAs (Lister et al, 2008; Wiseman et al., 2013) as well as hydroxylated PAHs (Fertuck et al., 2001). It is possible that smaller species of NAs that are created during the aging process (Leung et al., 2001) may also be affecting the endocrine axis.

After sub chronic exposure of goldfish to aged OSPW, we measured an increase in kidney ERβ1 mRNA levels, and a decrease in Vtg levels. This may be due to a negative feedback loop signaling the pituitary to inhibit E2 levels and decrease Vtg activation upon binding of an agonist to ERs (He et al., 2012; Wiseman et al., 2013). Following sub chronic exposure, it appeared that aged

OSPW had more of an effect on endocrine receptor mRNA expression than fresh OSPW, although the opposite was observed after acute exposure. Therefore, effects of aged and fresh OSPW and acute versus sub chronic endocrine responses may not be extrapolated; highlighting the importance of examining short and long term toxic effects caused by OSPW.

6.4.2 Fresh versus ozonated OSPW exposure

The development of time effective remediation techniques for OSPW is an ongoing process and it appears that ozonation is a promising remediation technique. Ozonation of OSPW may result in complete NAs degradation, and has been linked to amelioration of toxic effects (Scott et al., 2008; Garcia-Garcia et al., 2011a). In general, it was observed that endocrine receptor gene expression and Vtg levels in fish exposed to ozonated OSPW for one week were similar to those of non-exposed fish. Similarily, a study on fathead minnows exposed to OSPW found alterations in estrogen receptors after acute exposure, that returned to control levels after ozonation (He et al., 2012b). Although there are conflicting results related to ERβ1 mRNA levels in the experimental groups, we did not examine transcriptional activity of E2, which may be what is ultimately affecting Vtg. The E2 mRNA levels have been found to change in cells exposed to OSPW *in vitro* (He et al., 2010; Knag et al., 2013; van den Heuvel et al., 1999; Lister et al., 2008). He et al. (2012) found an increase in Vtg mRNA levels following acute exposure of undiluted fresh OSPW, but not after exposure to ozonated OSPW, suggesting that exposure to only highly concentrated OSPW affected Vtg mRNA levels.

After sub chronic exposure of fish to fresh OSPW there were no changes in the mRNA levels for estrogen receptors. However, the mRNA levels of three out of 4 estrogen receptors were up-regulated in fish exposed to ozonated OSPW. He et al. (2012a) have found partial amelioration of endocrine disrupting effects following ozonation, and it is possible that smaller NAs created during the advanced oxidation process may be more alkylated and that the oxidation of these species may be directly affecting the endocrine axis. I also observed an increase in

ERβ1 levels in fish exposed to ozonated OSPW after week 12, but no significant differences in Vtg protein levels between exposed and non-exposed fish. However, It has been shown that exposure to OSPW increases cytochrome P450 activity an enzyme involved in E2 production (He et al., 2011; Wiseman et al., 2013), which in turn may affect Vtg protein levels..

Exposure to OSPW changed the expression of Q-NPR expression throughout the experiment, and this effect was remediated by ozonation. It is known that 17α and 17β-hydroxyprogesterone stimulate vitellogenisis in crustaceans (Yano, 1985), and many synthetic molecules in wastewater have been shown to mimic progesterone (Kolpin et al., 2002). Therefore, it is possible that Vtg synthesis may also be affected by the presence of contaminants in wastewater that effect progesterone synthesis such as cadmium, magnesium, and iron (Siah et al., 2003). To date, few studies have been done assessing how OSPW affects progesterone; however, cytochrome P450, involved in drug metabolism and E2 conversion, has also been shown to influence progesterone levels (Spooner et al., 1991) and it has been shown that H295R cell lines exposed to NAs and oil-related hydrocarbons exhibited increased progesterone levels (Knag et al., 2013).

Due to conflicting results in literature of the endocrine disrupting properties of OSPW, appropriate biomarkers must be found to assess disruption of steroidogenesis. My findings show that either acute or sub chronic exposures of fish to OSPW modulate the endocrine receptor gene expression and vitellogenin levels in the male goldfish, and that different OSPW waters (aged versus fresh) induce different effects. The acute modulating effects of OSPW in steroidogenesis of male fish were ameliorated by ozonation. However, persistent exposure to ozonated OSPW may result in endocrine disruption in fish.

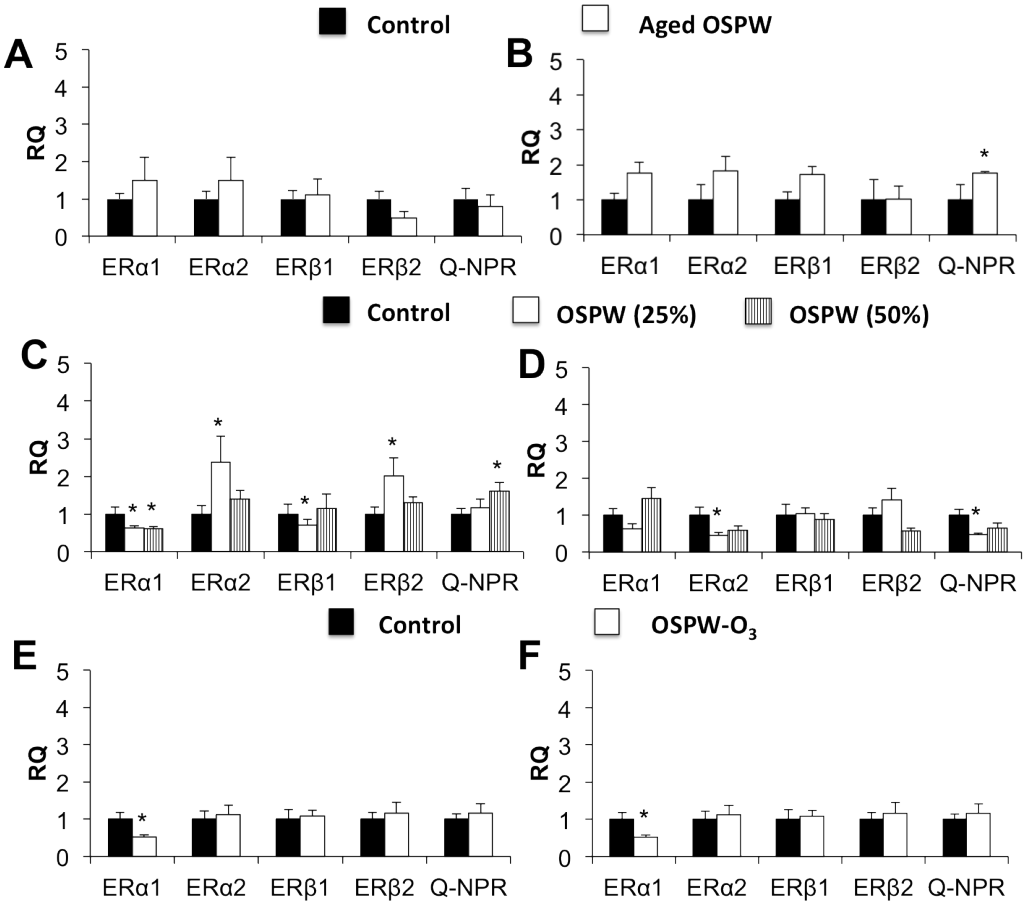

Figure 6.1: Kidney (A, C, E), and spleen (B, C, D) immune gene expression of goldfish exposed to for 1 week to either (1) aged OSPW (NA = 5 mg/L), (2) 25% OSPW, 75% water (NA = 10 mg/L) (3) 50% OSPW, 50% water (NA = 20 mg/L), (4) ozonated fresh OSPW (NA = 2 mg/L), or (5) de-chlorinated city water. Analysis of estrogen receptor alpha 1 or 2, beta 1 or 2 (ERα1, ERα2, ERβ1, ERβ2), or nuclear progesterone receptor (Q-NPR) was performed by real time PCR. Results are mean RQ (fold difference within endogenous control and genes) ± SEM of 8 fish. Asterisks (*) denote statistical significant differences from unexposed fish (control).

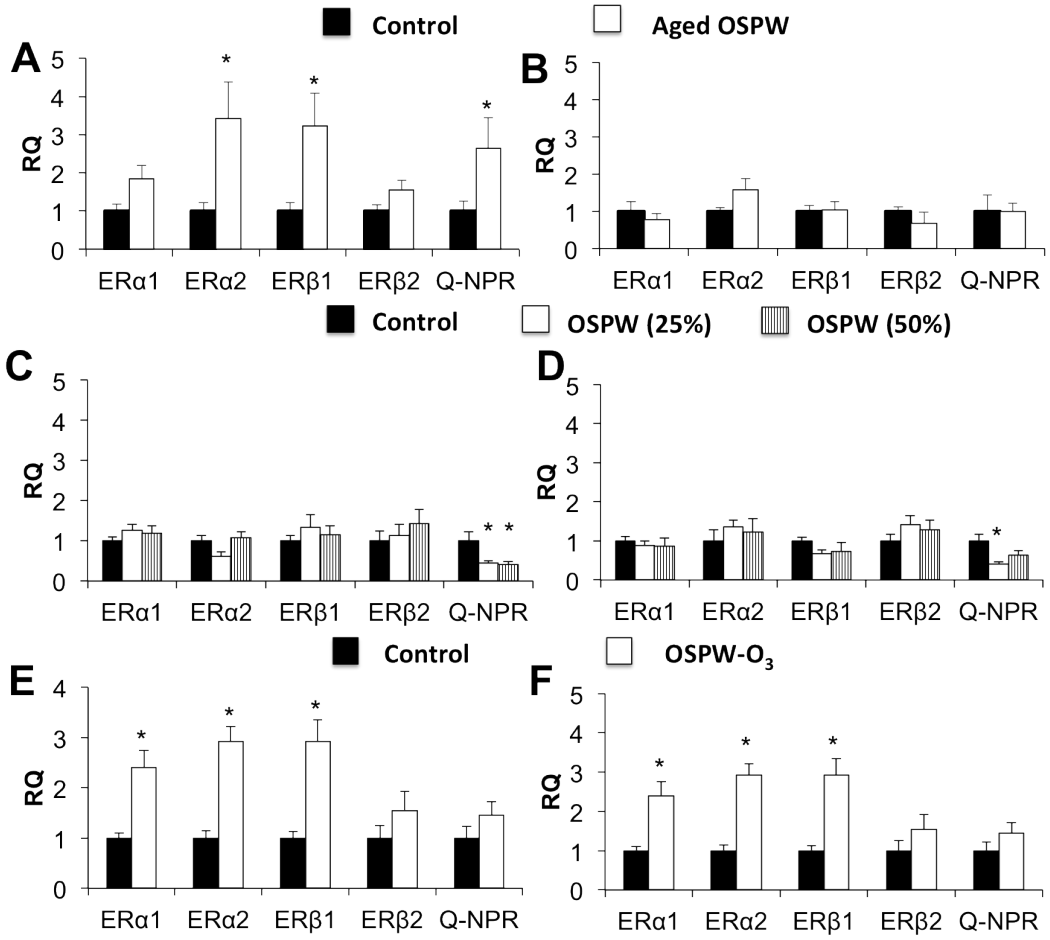

Figure 6.2: Kidney (A, C, E), and spleen (B, C, D) immune gene expression of goldfish exposed to for 12 weeks to either (1) aged OSPW (NA = 5 mg/L), (2) 25% OSPW, 75% water (NA = 10 mg/L) (3) 50% OSPW, 50% water (NA = 20 mg/L), (4) ozonated fresh OSPW (NA = 2 mg/L), or (5) de-chlorinated city water. Analysis of estrogen receptor alpha 1 or 2, beta 1 or 2 (ERα1, ERα2, ERβ1, ERβ2), or nuclear progesterone receptor (Q-NPR) was performed by real time PCR. Results are mean RQ (fold difference within endogenous control and genes) ± SEM of 8 fish. Asterisks (*) denote statistical significant differences from unexposed fish (control).

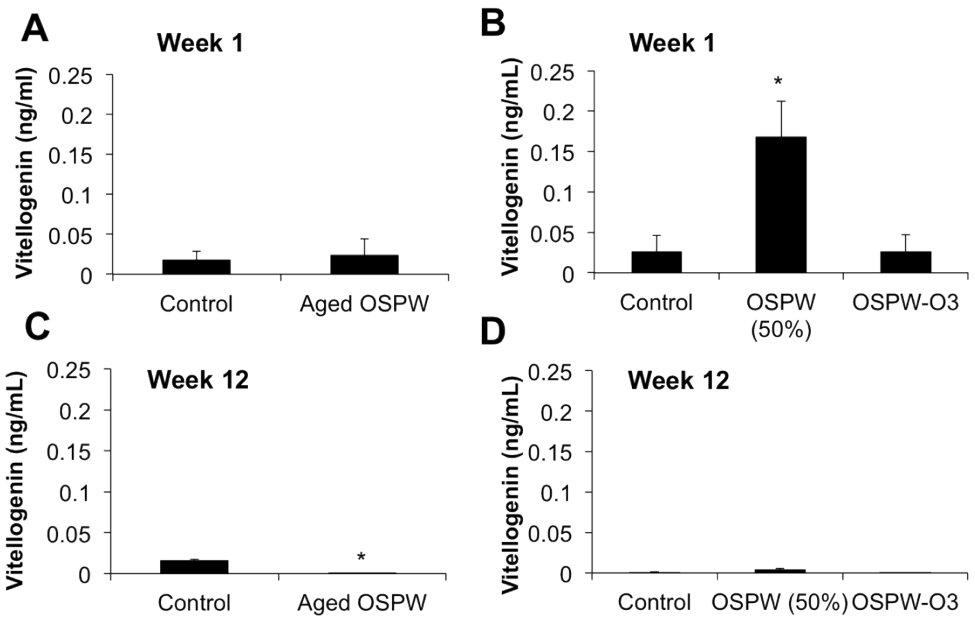

Figure 6.3: Plasma vitellogenin levels of male goldfish exposed for 1 (A, C) or 12 weeks (B, D) to (1) undiluted aged OSPW (NA = 5 mg/L), (2) 50% OSPW, 50% water (NA = 20 mg/L), (3) ozonated fresh OSPW (NA = 2 mg/L), or (4) de-chlorinated city water. Vitellogenin levels were calculated via a standard curve (Biosense Laboratories) and data are represented as mean ± SEM of 8 fish.

Chapter VII: General discussion

7.1 Introduction

The main objectives of my thesis were to: (1) examine the immunotoxic and endocrine disrupting effects of commercial naphthenic acids (C-NAs) and oil sands process affected water (OSPW) using goldfish; and (2), examine potential of ozonation treatment to ameliorate toxic effects of OSPW. The toxic effects of OSPW were studied after acute and sub chronic exposures to goldfish and assessed using the immune gene and endocrine receptor gene expression in different fish organs. Antimicrobial functions of macrophages isolated from kidneys of exposed fish were assessed, as well as vitellogenin levels, (an egg yolk precursor protein), were measured in male fish.

Goldfish were chosen as a study species for the following reasons: (a) they are much larger than zebrafish, and as a result more tissue may be collected from each fish; (b) they are more resistant to temperature fluctuations compared to rainbow trout; and (c) goldfish have been shown to respond to OSPW similar to yellow perch, a species native to the Athabasca River (Nero et al., 2006b).

7.2 Comparison of acute and sub chronic exposure

In chapters IV and V, I examined the effects of C-NAs, OSPW, or ozonated OSPW on goldfish immune response. C-NAs are known to behave differently from NAs found in the OSPW, perhaps due to the fact that OSPW mixture may contain ~100,000 different NAs species. OSPW also contains a variety of compounds other than NAs (heavy metals, BTEX, bicarbonates, salts, PAHs, etc.) that may affect normal immune function. Fresh OSPW is generated during the Clark extraction process used in oil sands mining, and is a highly toxic mixture having containing NAs and other organic and inorganic contaminants. The term “aged OSPW” refers to ponds that have been aged for more than 10 years in order to naturally remediate the waters, through microbial degradation. However, this microbial degradation does not fully remediate toxicity of OSPW (Leung et al., 2011; Martin et al., 2010).

Acute exposure to a xenobiotic is short-term, hours or days, and often has severe initial effects that may result in death, but usually these effects are reversible, provided mortality does not result (Sheline et al., 2003). I found that exposure to 20 mg/L C-NAs, aged, or fresh OSPW induced an acute increase in pro-inflammatory cytokine mRNA levels and a parallel increase in the ability of macrophages to produce antimicrobial molecules, reactive oxygen and nitrogen intermediates. These observations support the results of other studies that the exposure to compound(s) in fresh and aged OSPW, likely NAs (Dokholyan and Magomedov, 1984), induced an acute immunostimulatory response (Knag et al., 2013; Wiseman et al., 2013). As a result, acute exposure to OSPW may initiate inflammation, and acute inflammation has been linked to vascular alterations such as changes in blood flow, and increased permeability of vascular systems, all potentially leading to damage and injury (Ryan and Majno, 1977). Many other physiological functions may also be disrupted such as disorders in leukocyte migration and chemotaxis, or result in systemic effects such as fever (Ryan and Majno, 1977). Therefore, it is possible that these physiological changes may arise in animals following acute exposure to OSPW and disrupt homeostasis.

Sub-chronic exposure, on the other hand, can be more disruptive to an organism, potentially causing more serious diseases due to prolonged exposure to a xenobiotic. (Davies et al., 1976; Sweet and Zelikoff, 2001). In this thesis it was found that sub chronic exposure, defined as 8-12 weeks, to either 5 mg/L C-NAs, aged, or ozonated OSPW induced an increase in pro-inflammatory cytokine expression. Therefore, OSPW that is remediated via aging or ozonation still caused alteration of the immune system of goldfish. These results demonstrate that the exposure of fish to OSPW or C-NAs augments fish immunity, and whether or not this information may be applied to mammals, including humans is not known. However, care must be taken when extrapolating the immune alterations in fish to higher vertebrates, particularly because it has been found that the immune response of teleosts and mammals, particularly goldfish and mice, exposed to OSPW and ozone-treated OSPW (OSPW-O_3) vary considerably; inducing an increase in pro-inflammatory gene expression in fish, but inducing a

decrease in pro-inflammatory cytokine levels in mice (Garcia-Garcia et al, 2011b).

Fish exposed sub chronically to fresh OSPW or 20 mg/L NAs exhibited a decrease in pro-inflammatory cytokine gene expression, the opposite of what was seen following acute exposure, suggesting that high concentrations of NAs present in fresh OSPW and C-NAs caused immunosuppression and down-regulated antimicrobial functions of macrophages. One of the mechanisms responsible for this immunosuppression may be associated with stress (and stress hormones). Chronic immunosuppression caused by xenobiotics may act directly on the immune system by affecting the function of lymphocytes and antimicrobial molecule production, or may affect the immune system indirectly via alterations of the neuroendocrine system and hypothalamus-pituitary axis (Pruett et al., 1993). Other xenobiotics present in OSPW that may induce immunosuppression are high amounts of heavy metals (i.e. Cd) in fresh OSPW, which have been linked to decreased inflammatory responses (Abou-Mohamed et al., 1995; Fosmire, 1990; Sokolik et al., 2002, 2006), and impairment of macrophage functions (Fournier et al., 2000). PAH exposure has also been linked to immunosuppression (Seeley and Week-Perkins, 1991), which may lead to decreased secretion of pro-inflammatory cytokines and negatively impact immune cells (Faisel et al., 2001, 2003). Other corticosteroid agonists present in OSPW have been linked to immunosuppression in rats, such as benzene and toluene (Hsieh et al., 1981). Immunosupression in humans may result in increased susceptibility to pathogens, and bacteria, potentially manifesting as sepsis, and eventual organ failure and death (Schwach and Chaudry, 2002). Thus, exposure to OSPW induced profound and complex change in immune responsiveness of animals that may result in their inability to function properly or survive, which would significantly affect aquatic ecosystems.

A variety of chemicals in OSPW may act to both immunostimulate and/or immunosuppress, depending on the duration of exposure, and act antagonistically or synergistically. As a result, acute effects do not necessarily correlate with sub chronic effects, as showcased in a study done on Atlantic salmon treated with

hydrogen sulphide, where significant gill damage occurred after a single dose of a xenobiotic. However, these fish adaptively responded to the xenobiotic after exposure to multiple doses of hydrogen sulphide (Kiemer et al., 1995). It is, therefore, important to document both acute and sub chronic effects of xeobiotic exposure in animals.

I reported that exposure to C-NAs appeared to largely alter cytokine expression in the spleen compared to the gills or kidney, while gene expression changes in fish exposed to OSPW were most pronounced in the kidney. The kidney is the primary hematopoietic organ in teleosts and along with the liver, would be the first place that mediation and breakdown of xenobiotics would begin (Pritchard and Miller, 1980). However, in the presence of heavy metals such as Cd (present in OSPW), this process of xenobiotic transformation is altered and results in decreased ability of the kidney to biotransform xenobiotics (Masereeuw et al., 2000). As a result, because the kidney is crucial for ion regulation and waste secretion and regulates many processes *in vivo*, disruption in kidney function caused by xenobiotics may lead to homeostatic impairment in other organs, and an altered ability to remove toxins from the system. Spleen tissue, on the other hand, has been found to be less effective in xenobiotic transformation (Taysse, et al., 1998), and in the presence of xenobiotics, spleen function is impeded (Reynaud et al., 2008). Thus, OSPW and C-NAs affected different tissues in the goldfish, and the reasons why this is the case remains to be determined.

7.3 Comparison of C-NAs and OSPW exposure to ozonated fresh OSPW

Ozonation, as a remediation technique, has been reported to either partially (He et al., 2011) or fully (Scott et al., 2008) degrade large NAs species in OSPW. Consequently, ozonation of OSPW decreased toxicity in mice (Garcia-Garcia et al., 2011a,b), cell lines (He et al., 2011), as well as other organisms (He et al., 2010; He et al., 2011; Martin et al., 2010; Scott et al., 2007). I reported that goldfish exposed for one week to ozonated OSPW exhibited no changes in pro-inflammatory cytokine mRNA levels, similar to fish exposed to 5 mg/L C-NAs after one week (Chapter V). Similarly, fish exposed sub chronically to ozonated

OSPW showed no alterations in IFNγ or TNFα-2 gene expression. These data differ from exposure of fish to C-NAs or aged OSPW, where I observed that IFNγ and TNFα-2 expression increased in fish exposed to 5 mg/L NAs or aged OSPW, and decreased in fish exposed to fresh OSPW. IFNγ is a pleiotropic cytokine largely involved in immunomodulation. Impairment in IFNγ has been linked to altered macrophage functions, and reduced expression of major histocompatibility complex class II (MHC II), and in mice has been shown to result in immunosuppression (Kerkvliet et al., 1996). Alteration in TNFα-2 production, which is crucial in tumor regression and septic shock, has also been linked to immunosuppression, particularly following exposure to heavy metal and dioxins (Broeckaert et al., 1997). As a result, high concentrations of metals and dioxins present in fresh OSPW may act as instigators for sub chronic immunosuppression observed in fresh OSPW-exposed goldfish. Although fish exposed for 12 weeks to ozonated OSPW had no changes in IFNγ or TNFα-2 production, increased IL-1β1 mRNA levels were observed, similar to 5 mg/L C-NAs, fresh or aged OSPW, suggesting only acute amelioration of pro-inflammatory cytokine gene expression occurred in the fish exposed to ozonated OSPW.

It is known that IL-1β1 is a major immune cytokine in teleosts and mediates inflammation, communication among immune cells, as well as antigen presentation (Bird et al., 2002). The sub chronic increase IL-1β1 mRNA levels, following exposure to ozonated OSPW, may be caused by other compounds present in OSPW, such as PAHs, or breakdown products generated during ozonation which may themselves be toxic. These by-products of ozonation may have instigated altered antigen presentation and caused changes in the inflammatory response in the exposed fish (Paraskeva and Graham, 2002; Petala et al., 2008; Stalter et al., 2010). Supporting this are the observations that PAHs increase IL-1β1 mRNA levels in human fibroblast-like cells (Tamaki et al., 2004), therefore PAHs not fully broken down by the ozonation process, may cause the observed change in immune responsiveness of cells as well as animals. Thus, the

use of ozone to remediate complex industrial wastewaters, such as OSPW, would require careful assessment if complete reduction of toxicity of OSPW is the goal.

7.4 Parasite challenge and physiological relevance

Cytokine profiles and macrophage functions are related to how an organism responds to a pathogen. However, examining gene expression and cell function alone is not sufficient for proper assessment of possible toxicity of industrial wastewater. An important end point is to assess how an animal exposed to a xenobiotic controls an exposure to an infectious agent. Consequently, I examined the ability of goldfish to control an infection with a protozoan parasite *T. carassii*, after exposure to C-NAs or OSPW. I observed that acute exposure to either C-NAs or fresh OSPW induced resistance of goldfish to *T.carassii*, and following ozonation, exposed fish had similar ability as controls to regulate the infection. My results suggest that alterations in parasite burden caused by fresh OSPW were ameliorated following ozonation. Fish that exhibited increased ability to control parasitic infection were also found to be immunostimulated (enhanced inflammatory response) that correlated with increased resistance to the pathogen. Another study has documented a similar effect: cod exposed to water-soluble oil fraction of oil-polluted water were found to have decreased load of acanthocephalan parasites when compared to non-exposed control fish, and flounders submerged in petroleum contaminated sediment had fewer species of trematodes compared to the non-exposed fish (Khan and Kiceniuk, 1983). It is also possible that toxicants present in OSPW may act directly on the parasite to increase mortality or alter their ability to parasitize the host. However, it is important to note that because the organism is better able to resist infection, does not necessarily mean the fish is healthy, and in fact may fall prey to different pathogens that are controlled by different immune defense mechanisms (e.g. changes in antibody responses).

Following sub chronic exposure to C-NAs, increased mortality was observed in fish exposed to 20 mg/L NAs, suggesting an inability of exposed fish to control heavy parasite loads, resulting in mortality. These data were supported

by the observation that fish exposed to 20 mg/L C-NAs were immunosuppressed. Our findings are similar to what has been reported in other studies. For example, Gentes et al. (2007) reported that tree swallows from OSPW contaminated areas had more parasites. Not only has altered resistance to parasites been found following OSPW exposure, but other pathogens and opportunistic infections have been found to affect exposed fish as well. Fathead minnows exposed to OSPW exhibited increased prevalence of tumors (van den Heuvel et al., 2000), potentially of viral origins, as well as higher incidence of black spot disease following chronic exposure (Kavanagh et al., 2013). Although it is unknown as to how C-NAs and OSPW affect pathogens and hosts, it has been previously shown that pollution affects parasitism. Fish exposed to high levels of PAHs had increased prevalence of gill ectoparasites (Moles and Wade, 2001). Carp infected with a blood fluke and exposed to Cd, were found to be severely immunosuppressed, resulting in increased parasite-induced pathology (Hoole et al., 2003). These findings suggest that although OSPW and C-NAs may act to initially immunostimulate, upon sub chronic exposure they ultimately increase susceptibility of organisms to infectious agents.

7.5 Endocrine disruption

Many chemicals may alter the endocrine axis, by way of mimicking hormones or their antagonists and may affect a variety of targets such as the hypothalamic-pituitary-gonadal axis, as well as affecting hormone secretion, synthesis, and transport. Estrogens and androgens are steroidal molecules with phenanthrene rings, being structurally similar to NAs and PAHs. Estrogen receptors (ERs) are found in all tissues and most cell types and are highly conserved among vertebrates, and their expression is affected by ligand binding. Estrogens are responsible for developing secondary feminine sexual characteristics and are crucial in stimulating the liver to produce Vtg, a non sex-linked egg yolk precursor protein. However, due to different species controlling sexual development in various ways, estrogens and estrogenic agonists and mimics affect different classes of vertebrates differently. For example, in a study

examining fish species downstream from pulp and paper mill effluents it was found that perch, but not white suckers, exhibited decreased viability of developing larvae (Karels et al., 2001); and although zebrafish exposed to oil fractions did not induce increased expression of the Vtg gene (Knag et al., 2013), fathead minnows exposed to OSPW did (He et al. 2012b), similar to what was found in goldfish in this thesis.

In chapter VI, I reported that fresh OSPW induced acute alterations in endocrine receptors and Vtg levels in the kidney but not the spleen, while sub chronic exposure to aged or ozonated OSPW induced increased expression of endocrine receptor mRNA levels in the kidney for both treatments, and both kidney and spleen isolated from fish exposed to ozonated OSPW. As a result, it appears that compound(s) in aged and ozonated OSPW, but lacking in fresh OSPW, induced sub chronic endocrine disruption. This suggests that the products of degradation in OSPW may be more damaging to organisms, as these products may behave as estrogenic agonists.

Research in this area is crucial in determining the effects of OSPW on the endocrine system. Feminization of males may lead to adverse effects such as sterile populations and has also been linked to development of various cancers. Examining the endocrine disrupting effects of OSPW in fish provides a foundation of knowledge, which may be related to other toxicants and other organisms.

The fine balance of sex-hormone regulation is pervasively important for homeostatic regulation, as well as normal sexual function in metazoans (Harukuni et al., 2001; Jobling et al., 2006). Different endocrine disrupting compounds (EDCs) have the ability to bind to ligands with varying affinities. It is also possible for xenobiotics to act in an additive/synergistic manner, or non-additive ways amongst themselves to alter physiological responses of an organism (Li et al., 2012; Singh et al., 2010). However, the concentrations of different EDCs and xenobiotics in OSPW are variable and ascertaining the exact chemical composition is difficult, if not impossible.

It is known that acute exposures to high doses of EDCs can severely disrupt endocrine homeostasis, but this disruption may be ameliorated with time, if there are no further exposures to the toxicant. However, sub chronic exposure to low doses of EDCs has been shown to be harmful, particularly during development of organisms, leading to lifelong homeostatic impairments (Billard et al., 1981; Desbrow et al., 1998). For example, small amounts of estrogens and its mimics can induce homeostatic alterations and it is not surprising that a complex mixture such as OSPW, has been shown to have both estrogenic and antiandrogenic properties (van den Heuvel et al., 2000; He et al., 2011; Kavanagh, 2011). As a result, continuous exposure to OSPW has been found to negatively impact fish as well as their progeny. My results indicate the presence of endocrine disruption following exposure of goldfish to fresh, aged, or ozonated OSPW. This is particularly pronounced after sub chronic exposures of goldfish to OSPW.

7.6 Future Research

Future research on OSPW toxicity in fish and other species may include assessing additional cytokine biomarkers. Continued analysis of the suppression and measurement of a larger panel of genes may help shed light on the effects of OSPW on broader physiological processes that may be influenced by OSPW exposure.. Measuring expression of anti-inflammatory molecules such as IL-10 and TGF-β would be useful to expand this research into alterations of mechanism that effectively "turn off" immune responses. DNA microarrays could also be used to screen expression of a large number of genes simultaneously to determine their relative expression in exposed and non-exposed fish.

The measurement of mRNA levels does not necessarily correlate to protein levels of the molecules that I examined. Developing an ELISA (enzyme-linked immunosorbent assay) to measure cytokines levels would further our understanding of immune disruption caused by OSPW. Additionally, measuring the ability of goldfish to elicit a humoral-based response (TH2 responses; antibody production) during the infection with *T. carassii* would also provide

significant new information about alteration of host defense mechanisms by OSPW exposure.

Analysis of biomarkers for the presence of heavy metals in OSPW would further our knowledge on the toxicity of OSPW. In particular, measuring the levels of molecules such as metallothionein, a cysteine rich intracellular protein that binds heavy metals, may yield interesting results.

A widely accepted method of determining xenobiotic exposure is the 7-ethoxyresorufin-o-deethylase (EROD) assay. This technique involves measuring the catalytic activity of *cyp1a* to convert 7-ethoxyresorufin to a fluorescent product: resorufin. *Cyp1a* is involved in xenobiotic secretion and biotransforms aromatic hydrocarbons into more extractable forms. This assay may also be useful in examining physiological effects in goldfish exposed to OSPW.

Other experiments capable of examining endocrine disruption may include looking at E2 or progesterone protein levels. Competitive estrogen receptor binding assay (ERBA) may be used to measure the binding of estrogenic agonists. This technique may be particularly useful when examining individual NAs species (from OSPW). Finally, radioimmunoassay (RIA) that measure testosterone levels in the plasma could also be employed to assess the effects of EDCs found in OSPW.

Through the use of a real-time flow through exposure apparatus it is possible to get a realistic idea of how an organism will react to OSPW released into the environment. I believe that my work using goldfish as biosentinels for examination of toxicity of OSPW has shed light on the effects of OSPW exposure on the immune responses and host defense of the goldfish. My results indicate that goldfish may be used as an appropriate model organism in assessing industrial wastewater toxicity, as demonstrated by the acute and sub chronic alterations in pro-inflammatory gene expression, macrophage functions, susceptibility to infection, as well as endocrine disruption. Furthermore, I examined whether ozonation would be an appropriate remediation technique for amelioration of OSPW toxicity such that one day we may safely dispose of OSPW and minimize the negative impacts on the environment and public health.

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