Responses of Fishes Following Developmental Exposure to Complex Hydrocarbon Mixtures

by

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Abstract

The continuing use of crude oil and development of the oil industry have resulted in complex hydrocarbon mixtures becoming common environmental contaminants. These contaminants, such as oil sands process affected water (OSPW) and crude oil, are especially of concern in aquatic ecosystems. OSPW is a complex alkaline and saline mixture with both organic and inorganic components. Naphthenic acids (NAs) are currently considered to be the primary toxic component, though other components of OSPW such as metals and salts likely contribute to its toxicity as well. Polycyclic aromatic hydrocarbons (PAHs) are considered the main source of toxicity in crude oil, though other aromatic hydrocarbons may also be responsible for some of its toxicity. Both of these complex hydrocarbon mixtures have been shown to cause many adverse effects in fishes. My thesis focuses on the effects of developmental exposures to OSPW in zebrafish and crude oil in both zebrafish and sheepshead minnow.

Zebrafish were developmentally exposed to either raw or ozonated OSPW from 0-7 days post fertilization (dpf). Survival from 1-7dpf was not affected by exposure to either type of OSPW. However, otolith area was slightly smaller in exposed zebrafish at 7dpf, potentially indicating a slight physiological change due to exposure. The expression of cardiac development genes was also affected by exposure but heart rate and cardiac area were not adversely affected. The expression of biotransformation enzymes cytochrome P450 1A (*cyp1a*) and *cyp1b* were induced by exposure to raw OSPW, with *cyp1b* more highly expressed than *cyp1a*. The expression of CYP enzymes returned to levels similar to that of the control within 2 days after exposure ended, indicating the fish were likely capable of excreting or metabolizing compounds present in OSPW relatively quickly. Exposure to neither raw nor ozonated OSPW affected overall fish length, yolk sac absorption, jaw morphology, apoptosis, or the expression of neurodevelopment genes and vitellogenin, a marker of estrogenicity.

Overall, the OSPW exposures were not overtly toxic to embryonic zebrafish and very few endpoints were affected.

For the crude oil studies, zebrafish and sheepshead minnow were exposed to water accommodated fractions (WAFs) of three types of crude oil (source, moderately weathered, and heavily weathered) plus dispersant. The chemical composition of these WAFs was compared and contrasted between freshwater and saltwater and responses to these WAFs were compared between zebrafish, a model freshwater species, and sheepshead minnow, an ecologically relevant saltwater species. WAFs varied little between freshwater and saltwater except for the source oil plus dispersant treatment group. Dispersant increased amount of PAHs in both the saltwater and freshwater source oil WAFs but lead to a much greater amount in saltwater compared to freshwater (~10 fold). The source oil plus dispersant WAF was the only treatment group to significantly decrease survival in both zebrafish and sheepshead minnow. Species-species differences in responses were found in cardiotoxicity and estrogenicity endpoints. Overall, saltwater and freshwater WAFs were considerably different when dispersant was present with source oil; furthermore, their impacts differed between species, indicating that freshwater systems and species may not be reliable for studying marine oil spills. The study of complex hydrocarbon mixtures can be challenging due to the large variability in environmental samples and the differences in species' sensitivities.

Preface

This thesis is an original work by Danielle Lyons. Research ethics approval from the University of Alberta Research Ethic Board was given for this research project under the animal use protocol 052 – Chemicals, effluent, and fishes.

Chapter two of my thesis is an accepted journal article published in the journal Environmental Pollution. The citation is:

Lyons, D.D., Philibert, D.A., Zablocki, T., Qin, R., Huang, R., Gamal El-Din, M., and Tierney, K.B. (2018). Assessment of raw and ozonated oil sands process-affected water exposure in developing zebrafish: Associating morphological changes with gene expression. Environ. Pollut. *Accepted February 2018.*

I was responsible for writing this journal article as well as collecting embryos, extracting RNA, and the completing the whole process leading to qPCR and the TUNEL assay and analyzing that data. The work of breeding fish, embryo exposure and care, and counting embryo survival was shared between Danielle Philibert and me. Danielle Philibert also collected and analyzed the heart rate and jaw morphology data included in this article. Taylor Zablocki analyzed heart rate videos to collect and analyze the heart arrhythmia data. Rui Qin and Rongfu Huang measured the chemical composition of both types of OSPW and made the chemistry figures.

Chapter three of my thesis is a journal article submitted to the journal Chemosphere. For this journal article, I collected and analyzed the growth, yolk sac absorption, otolith size at 7 days post fertilization, and qPCR data. I was responsible for writing the majority of the article, however, Christie Morrison wrote the methods and results for the otolith back-calculation analysis. She also collected and analyzed the otolith data and performed the growth back-calculations. The embryo exposures and care was a shared responsibility between Danielle Philibert and me.

Chapter four of my thesis is a journal article that has been submitted to Environmental Science and Technology. Danielle Philibert is the lead author on this article. I completed all the steps in collecting gene expression data in this article (embryo collection, RNA extraction, cDNA synthesis, primer selection/design, and qPCR). I also wrote all the information on gene expression within the article. Danielle Philibert was responsible for the collection of all other data in this article. The embryo exposures and care were shared between Danielle Philibert and me, though Danielle made all of the water-accommodated fractions of oil that the embryos were exposed to.

Dedication

“Relax! It’s not that toxic.”

-D^2

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Chapter 1: General Introduction

Background information

Aquatic toxicology has been defined as the study of effects caused by anthropogenic and natural materials (collectively referred to as toxic compounds) in aquatic organisms on many levels of organization – from subcellular and whole organisms to communities and ecosystems (Rand, 1995). The focus of aquatic toxicology is on adverse effects, or endpoint measurements that are divergent from normal for a healthy individual. These effects can be caused by acute or chronic exposures and may be lethal or sublethal. Exposure can be through many different routes including via water, sediment, or food.

More than half the earth's surface is covered in water, most of which contains life in some form. Aquatic environments are complex and varying and include rivers, lakes, coastal marine habitats, and deep oceans to name a few. Fishes are often studied in aquatic toxicology and are good indicators of contamination and water quality (Karr and Dudley, 1981). Fish are the largest group of vertebrates with over 33,000 species identified (FishBase, 2017). They are incredibly diverse and live in many different aquatic environments and are, therefore, exposed to a myriad of contaminants.

Anthropogenic sources of toxicity in the environment are diverse and may include pollutants such as metals, effluent, pesticides, herbicides, and petroleum hydrocarbons. The increase in oil production and, therefore, spillage and release over the past many years has spurred research in petroleum hydrocarbon contamination of aquatic systems. The diversity, usefulness, and value of the vast variety of species within aquatic ecosystems make them important to preserve. Consequently, a great deal of importance has been placed on the study of hydrocarbon contamination.

Oil production and transportation are an important part of Canada's economy (Ganesh Doluweera et al., 2017). In 2016 the oil and gas extraction industry employed over 57,000 employees in Canada (Statistics Canada, 2017). Crude oil is conventionally drilled in Canada, but 97% of Canada's oil reserves lie in the oil sands (Natural Resources Canada, 2016). The Canadian Oil Sands are the third largest oil reserve in the world (BP, 2017). Approximately 4460 thousand barrels of oil per day were produced in Canada in 2016, 4.8% of the world's production of oil (BP, 2017). Not only does Canada produce a relatively large amount of the world's supply of oil, the country trades over 3900 thousand barrels of oil per day. This amount of oil transport in Canada alone inevitably leads to hydrocarbon release into the environment via both seepage and leakage.

Currently, complex hydrocarbon mixtures such as oil sands process affected water (OSPW) and crude oil are of major concern in aquatic environments. Both OSPW and crude oil have been, currently are, or will be either unintentionally or intentionally released into the environment. OSPW is currently stored in on-site tailings ponds due to a no-release policy in the Canadian oil sands industry. Due to this policy, the amount of OSPW stored on-site is continuously increasing. Currently, approximately 1.18 trillion liters of OSPW are stored in tailings ponds in Alberta, Canada (McNeill and Lothian, 2017). With this much contaminated water being stored, seepage into the environment is a possibility.

Thousands of barrels of crude oil are also spilled into the environment every year, though the amount has continuously decreased over the past 40 years of oil transportation (Etkin, 2009). These spills occur in both freshwater and marine environments and vary vastly in amount of oil spilled. Large oil spills such as Exxon Valdez off the coast of Alaska in 1989 and Deepwater Horizon in the Gulf of Mexico in 2010, which released hundreds of thousands of barrels of crude oil, have major impacts on the environment and the health of aquatic species and ecosystems.

Though both OSPW and crude oil are petroleum hydrocarbon contaminants, they differ in their composition. OSPW is a complex saline and alkaline mixture composed of water, silt and clay, residual bitumen, and other organic and inorganic material and is both acutely and chronically toxic to aquatic organisms (Allen, 2008; Anderson et al., 2012; Clemente and Fedorak, 2005; He et al., 2012a; Wiseman et al., 2013b, 2013a). Organic compounds originating from bitumen, the heavy oil produced in the Canadian oil sands, such as naphthenic acids (NAs), are the main contaminants in OSPW (Laurier L. Schramm et al., 2000). NAs are thought to be the main toxic component in regards to the lethality of OSPW towards aquatic organisms (Brown and Ulrich, 2015; Hughes et al., 2017; Morandi et al., 2015). Other compounds present in OSPW, such as polycyclic aromatic hydrocarbons (PAHs), metals, and salts, also likely contribute to the toxicity of OSPW (Allen, 2008; Anderson et al., 2012; Li et al., 2017).

PAHs are thought to be the main source of toxicity in crude oil. These compounds are naturally occurring but the vast majority of the PAHs contaminating the environment are present due to human release (ex. oil spills, combustion of fossil fuels) (Rand, 1995). The main toxic components of the water accommodated fraction (WAF) of crude oil are thought to not only include PAHs, but also benzene, toluene, ethyl benzene, and xylene (also known as BTEX) (Philibert et al., 2016).

Many studies have been performed on the effects of OSPW, NAs, crude oil, and PAH exposure on the development of fishes. It is critical to understand the impact of both OSPW and crude oil on fish species for risk assessment and management purposes as well as for understanding the implications of

future OSPW release and crude oil spills. Among other effects, OSPW and crude oil exposure may cause a reduction in survival (Heintz et al., 2000; Hughes et al., 2017), endocrine disruption (Arukwe et al., 2008; He et al., 2012b; Lister et al., 2008; Martin-Skilton et al., 2006; Wiseman et al., 2013b), impaired immune function (Leclair et al., 2013; Nakayama et al., 2008), impaired olfaction (Lari and Pyle, 2017; Lari et al., 2015; Reichert et al., 2017), and spinal curvature (Incardona et al., 2004; Peters et al., 2007) in exposed fishes.

Exposure to environmental toxicants is particularly harmful during the embryonic stage, as this is when aquatic species are often the most susceptible (Rand, 1995). Unfortunately, marine oil spills often occur in critical habitats for aquatic species. The Exxon Valdez spill exposed pink salmon and Pacific Herring to crude oil in their spawning habitats (Ward et al., 2017), while the Deepwater Horizon spill affected bluefin tuna and blue marlin populations (Frias-Torres and Bostater, 2011; Ward et al., 2017). It was reported that 33% of the Bluefin tuna spawning area and 38% of the blue marlin larval area in the Gulf of Mexico were covered by the Deepwater Horizon spill (Frias-Torres and Bostater, 2011).

Because of the toxic effects of OSPW and crude oil exposures, remediation and clean up processes are crucial for decreasing the effects of these contaminants. To enable future release of OSPW into the environment and to reduce the effects of seepage and oil spills, reclamation and cleanup techniques must be studied, developed, and applied properly. Currently, the most commonly used and studied process of detoxification for OSPW is ozone treatment, as it has been shown to reduce toxicity induced by OSPW (Garcia-Garcia et al., 2011; Hughes et al., 2017; Wiseman et al., 2013b), although, the findings on the ability of ozonation to decrease the toxicity of OSPW exposure are varying. Some have speculated that ozonation does not decrease all sublethal effects of OSPW exposure (Garcia-Garcia et al., 2011; Wiseman et al., 2013a).

For marine oil spills, chemical dispersants are one of the most commonly used cleanup methods. This cleanup technique is used to protect diverse and sensitive shorelines from exposure to crude oil and increase the rate of biodegradation. However, it has been shown that chemical dispersants, when applied to marine oil spills, may increase the bioavailability and toxicity of crude oil towards fishes (Ramachandran et al., 2004; Wu et al., 2012). Other methods of crude oil cleanup in aquatic environments include skimming, pumping, and burning (Fingas, 2013). The effectiveness of these current remediation/cleanup processes is not completely clear. Much debate still remains over the consequences of using dispersant as a clean up practice for crude oil spills and over whether ozonation is an adequate remediation process for OSPW or not.

Although biomarkers have been well established for crude oil exposure (i.e. cytochrome p450) due to extensive research instigated by large oil spills, no biomarkers specific to OSPW exposure are currently known. Biomarkers are key factors for risk assessments and management since they are easy and quick to measure. Therefore, it is important to characterize the expression of genes induced by OSPW exposure in order to determine whether biomarkers of exposure can be identified (rather than biomarkers of effects caused by exposure).

Though OSPW and crude oil exposures have been quite well studied, it is challenging to compare between studies due to the variability of samples, limited chemistry, as well as the use of different species and life-stages (Frank et al., 2016; Hughes et al., 2017). Direct comparisons between species within a study are not common in toxicology, especially with complex mixtures such as OSPW and crude oil. Though it is not commonly done, direct comparisons between species are necessary in order to compare the effects of exposure between multiple species, since exposures occur to various aquatic species and they may have different sensitivities to the contaminants. This is especially important if model species (i.e. zebrafish) are being used since they are known to be hardy and may potentially be more tolerant to exposure than endogenous species (Fogels and Sprague, 1977).

Thesis Goals

The overall goal of my thesis was to determine the effects of complex hydrocarbon mixtures at the subcellular and whole organism levels in embryonic fishes. I aimed to determine how/if fish developmental is impacted by exposure to our OSPW sample and determine whether ozonation decreased the toxicity of exposure, therefore determining whether the organic fraction of OSPW was responsible for toxicity. This was accomplished by comparing gene expression, fish growth, yolk sack absorption, apoptosis, recovery after exposure, and survival between exposure groups.

I also aimed to determine the developmental effects of crude oil exposure along with chemical dispersant in two fish species, sheepshead minnow (*Cyprinidon variegatus variegatus*) a saltwater species, which is endemic to the Gulf of Mexico, and zebrafish (*Danio rerio*) a freshwater species. I accomplished this mainly by measuring survival and the expression of genes related to neurodevelopment, endocrine disruption, and biotransformation in embryos exposed to water accommodated fractions of crude oil. One critical aim of the crude oil exposures was to compare and contrast the chemistry and the effects of exposure between the oil treatments in freshwater and saltwater. Additionally, I sought to determine how dispersant differentially interacts with each type of water in order to shed light on the potential implications of using dispersants in each of these differing aquatic

environments. This study included three oil samples of different weathering amounts collected from the Gulf of Mexico after the Deepwater Horizon spill.

Studying the effects of both of these complex hydrocarbon mixtures on zebrafish allowed a comparison in changes in transcript abundance between OSPW and crude oil exposures. This provided me with a comparison when attempting to identify potential biomarkers for OSPW exposure in fish.

My thesis contains three data chapters of submitted/accepted publications. The first two data chapters assess the developmental effects of raw and ozonated OSPW exposure on zebrafish embryos. The second chapter (first data chapter) of my thesis looks specifically at survival, apoptosis, and heart and jaw development, while the third chapter looks further into developmental effects of OSPW exposure by measuring growth and yolk sac absorption. The third chapter also explores the effects of OSPW exposure on the expression of genes involved in neurodevelopment and biotransformation and tracks the return of these biotransformation genes back to control levels. The fourth chapter in my thesis compares the effects of weathered crude oil, moderately weathered crude oil, and source oil along with dispersant in sheepshead minnow and zebrafish. This chapter specifically focuses on the developmental effects of crude oil exposure and the differential expression of biomarkers between fish species.

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Chapter 2: Assessment of raw and ozonated oil sands process-affected water exposure in developing zebrafish: Associating morphological changes with gene expression

Abstract

With the ever-increasing amounts of oil sands process-affected water (OSPW) accumulating from Canada's oil sands operations, its eventual release must be considered. As OSPW has been found to be both acutely and chronically toxic to aquatic organisms, remediation processes must be developed to lower its toxicity. Ozone treatment is currently being studied as a tool to facilitate the removal of organic constituents associated with toxicity. Biomarkers (e.g. gene expression) are commonly used when studying the effects of environmental contaminants, however, they are not always indicative of adverse effects at the whole organism level. In this study, we assessed the effects of OSPW exposure on developing zebrafish by linking gene expression to relevant cellular and whole organism level endpoints. We also investigated whether or not ozone treatment decreased biomarkers and any associated toxicity observed from OSPW exposure. The concentrations of classical naphthenic acids in the raw and ozonated OSPW used in this study were 16.9mg/L and 0.6mg/L, respectively. Ozone treatment reduced the total amount of naphthenic acids (NAs) in the OSPW sample by 92%. We found that exposure to both raw and ozonated OSPW had no effect on the survival of zebrafish embryos. The expression levels of biotransformation genes *cyp1a* and *cyp1b* were induced by raw OSPW exposure, with *cyp1b* being more highly expressed than *cyp1a*. In contrast, ozonated OSPW exposure did not increase the expression of *cyp1a* and only slightly induced *cyp1b*. A decrease in cardiac development and function genes (*nkx2.5* and *atp2a2a*) was not associates with large changes in heart rate, arrhythmia or heart size. We did not find any indications of craniofacial abnormalities or of increased occurrence of apoptotic cells. Overall, our study found that OSPW was not overtly toxic to zebrafish embryos.

Introduction

The oil sands deposits in northern Alberta, Canada, are the third largest oil reserve in the world with up to approximately 50 billion cubic meters of recoverable of bitumen (National Energy Board, 2006). The extraction of bitumen from the oil sands area is based on a hot water alkaline extraction process that separates bitumen from sand, silt and clay. The process water is commonly referred to as oil sands process-affected water (OSPW), and is stored on-location in tailings containment structures due to a no-release practice due to concerns regarding its quality. This storage enables the water to be recycled for

production uses including bitumen extraction, material hydro transport and process cooling. For every barrel of bitumen extracted from the oil sands, approximately 1.67 barrels of fresh water is used in the extraction process (Shell Canada Ltd., 2016). Though 85-90% of the water used in bitumen extraction is recycled back into the extraction process from tailing ponds, water is still continuously accumulating (National Energy Board, 2006; Shell Canada Ltd., 2016).

Organic compounds originating from bitumen, such as naphthenic acids (NAs), are the main contaminants of OSPW (Laurier L. Schramm et al., 2000). NAs are believed to be the major source of OSPW's lethality to aquatic organisms (Brown and Ulrich, 2015; Hughes et al., 2017; Morandi et al., 2015). NAs are carboxylic acids that are classically defined with a formula of $C_nH_{2n+z}O_2$ (where n=carbon number, z=number of hydrogen atoms lost due to the amount of rings in the compound) (Headley et al., 2009a, 2009b). The acid extractable fraction of OSPW also contains oxidized, aromatic, and heteroatom NAs, which are NAs with 3 or more oxygen atoms, aromatic rings, and nitrogen or sulfur atoms, respectively (Headley et al., 2009a). The oil sands extraction process solubilizes these complex carboxylic acids into the OSPW. Other organic and inorganic compounds may also contribute to the toxicity of OSPW, however, NAs have the greatest potency in regards to lethality (Hughes et al., 2017). Fewer studies have focused on determining the toxicity of other compounds in OSPW such as polycyclic aromatic hydrocarbons (PAHs), metals, and salts, though they likely contribute to the toxicity of OSPW (Allen, 2008; Anderson et al., 2012; Li et al., 2017). Not all compounds present in OSPW have been identified and associated with their potential toxicity (Klamerth et al., 2015; Leclair et al., 2013; Li et al., 2014; Morandi et al., 2015).

The complexity of OSPW and its constituents is thought to contribute to various toxicological effects observed in aquatic species. Previous studies have shown multiple effects of OSPW and NA exposure in fishes such as reduced survival (Scarlett et al., 2013; Zubot et al., 2012), increased incidence of deformities (He et al., 2012a; Wang et al., 2015), endocrine disruption (He et al., 2012b; Reinardy et al., 2013; Wiseman et al., 2013a), impaired olfaction (Lari and Pyle, 2017; Reichert et al., 2017), and induction of apoptosis (He et al., 2012a, 2012b). Therefore to return OSPW to the environment, reclamation efforts will require decreasing OSPW toxicity. Recent studies have focused on the use of ozonation as a tool to expedite remediation efforts, as it has potential to minimize effects such as endocrine disruption and immunotoxicity induced by OSPW exposure (Garcia-Garcia et al., 2011; Wiseman et al., 2013a). Ozonation breaks down organic compounds and, therefore, reduces the amount of NAs in OSPW (Wang et al., 2013). However, it is still unclear whether or not ozonation completely

attenuates the adverse effects of OSPW exposure, as degradation by-products of ozonation (e.g. O_x-NAs) may also be toxic or more bioavailable to organisms (Klamerth et al., 2015).

Impacts of exposure to xenobiotic compounds at the cellular and tissue level are often linked to alterations in gene expression (Incardona, 2017; Wiseman et al., 2013b). Since OSPW is a complex mixture, it likely has multiple mechanisms of action. Understanding the mechanisms by which OSPW affects aquatic organisms is important for characterizing the toxicity of OSPW and increasing the understanding of how toxicity may be attenuated, potentially leading to future release of the water. The principal aim of this study was to characterize the effects of raw OSPW exposure on embryonic zebrafish and determine the role of ozone treatment on OSPW toxicity. A secondary goal was to link gene expression to a suite of whole organism responses after OSPW exposure in order to establish whether changes at the transcript level lead to changes at higher levels of organization. The expression levels of genes involved in heart development and function, jaw development and apoptosis were measured and linked to heart rate, jaw morphology and occurrence of apoptotic cells. Survival was also measured alongside the expression of cytochrome P4501A and 1B, which are common biomarkers of exposure to organic contaminants. Zebrafish were used as the model organism in this study because their genome is sequenced and they are transparent as embryos. This enabled us to link gene expression to whole organism endpoints, which is not as easily accomplished with species whose genomes are less well known. Their transparency throughout development also permitted the measurement of endpoints such as heart rate and jaw morphology.

Materials and Methods

Ozonation of OSPW

Raw OSPW was collected from Shell Canada Ltd's Muskeg River Mine (located ~60 km north of Fort McMurray, Alberta, Canada) in 2015 and stored in 200 L polyvinyl chloride barrels in a cold room (4°C). The characterization of OSPW is presented in Table 2.1 as well as figures 2.1-2.6. The OSPW ozonation process was carried out in a 200 mL reactor with approximately 80 mg/L utilized ozone dose. Ozone gas was produced by an ozone generator (AGSO 30, Effizon WEDECO AG Water Technology, Herford, Germany). The ozone concentration in feed-gas and off-gas was monitored by two ozone monitors (HC-500, PCI-WEDECO, USA). The ozone feed gas was introduced into the raw OSPW with a flow rate of 10 L/min through a ceramic fine bubble gas diffuser placed at the bottom of the reactor. The flow rate was measured by a calibrated flow meter. The residual ozone concentration in the ozonated OSPW was measured by the Indigo method (American Public Health Association, 2005).

The ozone generator was stabilized for 10 min to obtain a stable ozone concentration before the ozone gas was sparged into OSPW. Firstly, ozone was bubbled to 180 L raw OSPW with a flow rate of 10 L/min for 30 min. Then oxygen was introduced to the ozonated OSPW for 10 min to purge the residual ozone. The utilized ozone dose was calculated by the following equation (Wang et al., 2013):

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

where ΔO_3 is utilized ozone concentration (mg/L), Q is the ozone flow rate (L/min), $C_{G,in}$ and $C_{G,out}$ are the feed-gas and off-gas ozone concentration respectively (mg/L), C_L is the residual ozone concentration in the ozonated OSPW (mg/L).

Analysis of naphthenic acids

Prior to analysis, OSPW was centrifuged at 10,000 RPM for 10 min. The samples were analyzed using ultra performance liquid chromatography time-of-flight mass spectrometry (UPLC-TOF-MS) (Synapt G2, Waters, ON) with the TOF analyzer in high-resolution mode and the investigated mass range of 100-600 (m/z). The electrospray ionization source was operated in the negative-ion mode to measure NAs in the samples (Huang et al., 2016a; Sun et al., 2014). The injection solution was prepared with 500 µL of the supernatant, 100 µL of 4.0 mg L^{-1} internal standard (myristic acid-1-^{13}C) in methanol, and 400 µL methanol to reach a final sample volume of 1 mL. Chromatographic separations were performed using a Waters UPLC Phenyl-BEH column (1.7 µm, 150 mm×1 mm) and a prefilter (0.2 µm), with the mobile phases: (A) 10 mM ammonium acetate in water; and (B) 10 mM ammonium acetate in 50/50 methanol/acetonitrile. The column temperature was 50 °C and sample temperature was 10 °C. The flow rate was 100 µL/min and the elution gradient was 0−2 min, 1%B; 3 min, 60%B; 7 min, 70%B; 13 min, 95%B; 14 min, back to 1%B until 20 min to equilibrate column. Data acquisition was controlled using MassLynx (Waters, ON) and data extraction from spectra was performed using TargetLynx (Waters, ON). One quality control sample was used to ensure the method stability. This method was developed previously for semi-quantification of NAs based on the signal of a compound versus the signal of spiked internal standard (Huang et al., 2015, 2016b).

Fish

The embryos used in this study were produced and collected from a breeding colony of approximately 500 adult AB strain wild type zebrafish. All adults and embryos were housed at 28°C±0.5°C on a 14h:10h light:dark cycle. Adult breeding stock were fed a mixture of TetraMin® flakes (Tetra Holding,

Blacksburg, VA), Cobalt™ Aquatics spirulina flakes (Cobalt, Rock Hill, SC), and Omega One™ freeze dried bloodworms (Omegasea, Sitka, SK).

Embryo Exposures

Embryos were exposed to 100% raw (untreated) and 100% ozonated OSPW within 30 min post-fertilization until 7 days post fertilization (dpf). Embryos from each breeding event were randomized and held in groups of ~70 in glass Petri dishes containing 40mL of exposure water. Approximately 95% of the exposure water was exchanged daily via glass pipette. Control groups of embryos were raised in embryo medium (EM) (M. Westerfield, 2000).

Survival, heart rate, arrhythmia, and cardiac area

Embryos from 4-6 replicates were observed daily throughout their exposure and survival was recorded daily from 1-7 dpf. For heart rate, 2 dpf embryos were recorded for 30s between 14:00-16:00PM and heart rates were determined by manual scoring. Arrhythmia was measured by counting the number of video frames between atrium-to-atrium and ventricle-to-ventricle contractions as well as measuring time spent in both the atrium and ventricle (each frame was $1/29^{th}$ of a second). Cardiac area was measured at 2 dpf in ImageJ using photos of the same embryos used for heart rate and arrhythmia analysis. Videos and images were randomized and scored blind for both the videos and images. For heart rate and arrhythmia, 4-6 replicates were completed with a subset of 10-15 embryos assessed per replicate.

Jaw morphology

Embryos were collected at 7 dpf, fixed overnight in 4% phosphate buffered paraformaldehyde and stored in 100% methanol at -20°C until use. Fish were then rehydrated with phosphate-buffered saline with a 0.1% Tween-20 (PBT). Specimens were bleached in 30% hydrogen peroxide for 2 h or until the eyes became translucent. Embryos were rinsed again with 1 mL of PBT, transferred to an Alcian blue solution (1% HCl, 70% ethanol, 0.1% Alcian blue) and specimens were stained overnight. The following morning the specimens were rinsed 3-4× with 1-1.5 mL of acidic ethanol (5% HCl, 70% ethanol, HCl-EtOH). Embryos were then left in a wash of 1-1.5 ml of HCl-EtOH for 20 min. Embryos were then rehydrated, and stored in glycerol-KOH for imaging on a Leica DMRXA microscope (Meyer; Houston, TX, USA). Three replicates of fish were exposed with 30-40 embryos analyzed per replicate per treatment group. Jaws were analyzed and scored based on presence or absence of gross morphological defects.

TUNEL assay

Cell apoptosis was identified in embryos using whole mount terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay. An *in situ* cell death detection kit (Roche; Mannheim, Germany) was used and the manufacture's instructions were followed. Briefly, embryos (3 dpf) were preserved overnight at 4°C in 4% paraformaldehyde. After preservation, embryos were rinsed twice in PBS tween (1% tween) and incubated in proteinase K (1mg/ml) at 37°C for 30 min. Embryos were then rinsed 2× in PBS tween and the TUNEL reaction mixture was added. Samples were incubated at 37°C for 50 min in a humidified environment away from light. For a positive control, embryos were incubated in DNAse1 (Qiagen) for 10 min at room temperature before the reaction mixture was added. The embryos were then rinsed 3× in PBS tween and photographed under fluorescence with a Leica DMRXA microscope (Meyer; Houston, TX, USA). Since organic pollutants, including OSPW, have been found to cause tail malformations (Incardona et al., 2004; Peters et al., 2007), we focused on the occurrence of apoptosis in the tail region as well as whole embryo. Three replicates were completed, consisting of 6-7 embryos each.

RNA extraction, cDNA synthesis, and qPCR

At 7 dpf, embryos were euthanized on ice, preserved in RNAlater® (Thermo Fisher; Waltham, MA, USA) and stored at -20°C until RNA extraction. Each sample of total RNA was extracted from 20-35 pooled 7dpf whole embryos using TRIzol® Reagent (Ambion; Carlsbad, CA, USA) according to the manufacturer's instructions and 4-6 replicates were completed for each treatment group. Extracted RNA was then purified using an RNeasy® Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's protocol for RNA cleanup with few modifications. Genomic DNA contamination was removed by a 30-minute on-column DNase incubation using RNase Free DNase Set (Qiagen).

Purified RNA was suspended in RNase free water and stored at -80°C until analysis. RNA quality and concentrations were measured using a Nanovue (General Electric, Chicago, IL, USA) and an RNA Nano 6000 Assay Kit for the Agilent 2100 Bioanalyzer (Agilent; Santa Clara, CA, USA). All RNA samples had RNA integrity numbers (RINs) above 9.0. First-strand cDNA was synthesized from 2ug of total RNA for each sample using SuperScript® First-Strand Synthesis System for RT-PCR (Invitrogen™; Carlsbad, CA, USA) as described by the manufacturer on a Mastercycler Pro S (Eppendorf, Hamburg, Germany).

Primer efficiencies were calculated prior to real-time PCR (qPCR) reactions, with acceptable efficiencies between 90-110%. qPCR was performed in 96-well PCR plates on a 7500 Fast Real-Time

PCR System (Applied Biosystems; Foster city, CA, USA). Each 10 µL qPCR reaction contained 5 µL custom SYBR Green master mix, 2.5 µL of forward/reverse gene specific primers, and 2.5 µL cDNA diluted in nuclease free water (Ambion). Individual target cDNA amplifications were run in triplicates. Transcript levels of target genes were quantified by normalization to the endogenous gene Beta-actin. The threshold cycle (Ct value) was used to determine the amplification levels of target cDNA and the relative fold changes of target genes were quantified using the $2^{-\Delta\Delta Ct}$ method. The qPCR reaction was denatured at 95°C for 2 min then cycled through 95°C for 15 seconds (denature step) and 60°C for 1 min (annealing step) for a total of 40×. After the amplification cycles were complete, dissociation curves were generated to ensure the amplification of a single product. The dissociation steps were 95°C for 15 s, 60°C for 1 min, 95°C for 15 s, and finally 60°C for 15 s.

Targeted genes included two biotransformation genes (*cyp1a* and *cyp1b*), one neurodevelopment gene (*gli2a*), one cardiac development gene (*nkx2.5*), and two markers of apoptosis (*p53* and *casp9*). Specific primer sequences are listed in Table 2.2.

Statistical analyses

Statistical differences between treatments were evaluated using a one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test for gene expression data and Holm-Sidak for all other data. When needed to meet the assumptions of parametric tests, gene expression data was transformed using a $\log_{10}$ transformation. All data are expressed as the mean +/- standard error of the mean (SEM) and a p-value<0.05 was accepted as significant. SigmaPlot 11 (Systat, San Jose, CA) was used for all statistical analyses.

Results

Ozonation

The residual concentration of total organic carbon (TOC) and chemical oxygen demand (COD) revealed that most organics were oxidized to other organic compounds rather than mineralized to CO_2. The NA concentration data (Table 2.1) showed that after approximate 80 mg/L ozonation, the concentration of total NAs (O_2+O_3+O_4 NAs) decreased from 34.6 mg/L in the raw OSPW to 2.9 mg/L in the ozonated OSPW, a decrease of 92%. The degradation efficiency for O_2, O_3 and O_4 NAs were 97% (16.9 mg/L to 0.6 mg/L), 90% (8.6 mg/L to 0.9 mg/L) and 85% (9.1 mg/L to 1.4 mg/L), respectively. This indicates that NAs with more oxygen were less degraded and reflects the formation of O_4 from O_2 species via ozonation. After ozonation, the ratio of O_2 NAs among all the NAs decreased from 49% to 20%, while

O_3-NAs and O_4-NAs ratios increased from 25% to 31% and 26% to 49%. After ozonation, the distribution of NA species shifted to lower carbon number (figures 2.1-2.6).

Survival and cytochrome P450 expression

The overall survival of embryos throughout the exposure period from 1-7 dpf was not significantly affected by exposure to either OSPW type (Figure 2.7a, One Way ANOVA p=0.09). We also found no change in hatch rate, embryo length at hatch, or in the occurrence of spinal curvature or tail malformations due to exposure to either type of OSPW. Although survival was not significantly affected by exposure, an upregulation of the biotransformation genes *cyp1a* and *cyp1b* was observed. Raw OSPW exposure induced the expression of both biotransformation enzyme genes ($p<0.001$) with fold changes of approximately 4 and 8 fold greater than control for *cyp1a* and *cyp1b*, respectively (Figure 2.7b, *cyp1a* $F_{2,20} = 267.87$, *cyp1b* $F_{2,20} = 67.63$). Ozonation treatment of the OSPW completely attenuated the upregulation found in *cyp1a* expression. However, it did not completely eliminate the upregulation found in *cyp1b* expression, as it was slightly upregulated to a fold change of approximately 1.5 by ozonated OSPW (Figure 2.7b, $p<0.05$).

***nkx2.5* expression and heart rate**

Both raw and ozonated OSPW exposure slightly downregulated the expression of genes related to cardiac development and function, but those changes did not translate to effects at the whole organism level. The cardiac development gene *nkx2.5*, which is involved in cardiomyocyte differentiation, was downregulated by both raw and ozonated OSPW exposure (Figure 2.8, $F_{2,20} = 9.11$, $p<0.001$ and $p<0.05$, respectively). *atp2a2a*, a gene involved in calcium transport and cardiac function, was downregulated by raw but not ozonated OSPW exposure (Figure 2.8, $F_{2,11} = 7.25$, $p<0.05$). Heart rate, cardiac area and arrhythmia in 2 dpf embryos was also measured in order to further elucidate the potential effects of OSPW on cardiac development (Figure 2.9a). Raw OSPW increased the heart rate of 2 dpf zebrafish embryos ($p<0.05$). Though there was a statistical difference between the treatment groups, heart rates for all the treatment groups were within an expected range for zebrafish embryos (Garrity et al., 2002) (Figure 2.9a). Exposure to both raw and ozonated OSPW had no effect on the pericardial area, the time blood spent in the atrium and time the blood spent in the ventricle of the heart (Figure 2.9b, c, d).

***gli2a* expression and jaw morphology**

Craniofacial development appears to be relatively unaffected by OSPW exposure. The expression of *gli2a*, a neurodevelopment gene shown to be highly involved in craniofacial development (Chang et al., 2016; Mo et al., 1997; Schwend et al., 2010), was not significantly affected by exposure to either type of

OSPW, though there was a downward, non-significant, trend with raw OSPW exposure (Figure 2.10a, p=0.07). Jaw morphology was also unaffected by exposure to either OSPW treatment group (Figure 2.10b). This indicates that developmental OSPW exposure likely did not affect craniofacial development in zebrafish embryos.

Apoptosis biomarker expression and TUNEL assay

Our results show no indication of increased apoptotic activity in OSPW exposed zebrafish embryos. The expression of *p53* and *casp9*, genes involved in apoptosis, were relatively unaffected by both raw and ozonated OSPW exposure (Figure 2.11a). The raw exposed treatment group had a very slight downregulation in *p53* expression (Figure 2.11a, $F_{2,12} = 4.45$, $p<0.05$). This downregulation (approximately 0.8 fold compared to control) is likely not a biologically relevant as it is within the range of normal variability. The occurrence of apoptotic cells did not differ between treatment groups when measured in whole embryos and in the tail region of embryos (Figure 2.11b).

Discussion

Ozonation

The reduction of the ratio of O_2 NAs and increase of the ratio of O_4 NAs among all of the NAs after ozonation indicated a shift of the distribution of NA organic species to more oxygen-rich species, which is consistent with a previous study (Wang et al., 2016). The increase in oxygenated NAs, O3 and O4, also normally leads to an increase in BOD5. The distribution of NA species also shifted to lower carbon number after ozonation. This indicates that the ozonation cleaved the large molecules with more carbon numbers and break them into smaller molecules (Pérez-Estrada et al., 2011; Wang et al., 2016). The enhanced reactivity of high carbon number compounds may be due to the increment of hydrogen atoms and/or alkyl groups, resulting in higher reactivity towards hydroxyl radicals (•OH). As for the –Z numbers, ozonation preferentially degraded the concentration of NAs with higher –Z number. The increasing numbers of tertiary carbon atoms could be an explanation for this result. Higher –Z numbers may indicate more ring structure with more tertiary carbon atoms in their molecules. H atom abstraction happening on tertiary carbon generates more stable carbon centered radical, which make this process more favored to occur. Thus, NAs with higher –Z number showed higher reactivity (Pérez-Estrada et al., 2011). In general, 30mg/L ozone used/required to increase OSPW biodegradability for subsequent treatment (Xue et al., 2016). However, the ozone concentration of 80 mg/L was chosen for this study in order to reduce classical NAs in the treated OSPW to below 1 mg/L.

Survival and cytochrome P450 expression

Previous studies have found contradicting results with respect to the survival of OSPW-exposed fishes. Some found that exposure decreased survival of embryos (He et al., 2012a; Marentette et al., 2015a), while others did not find a change in survival (Colavecchia et al., 2004; Wiseman et al., 2013b). Our study found that exposure to both raw and ozonated OSPW had no effect on zebrafish embryo survival throughout the 1-7 dpf exposure period. Though ozone treated OSPW had a 92% reduction in total NAs, there was no difference in lethality between the two exposures since raw OSPW exposure did not affect survival in the first place. Despite the fact that we didn't find a reduction in survival with raw OSPW exposure, some studies have found that NAs themselves do cause mortality in exposed zebrafish (Scarlett et al., 2013; Wang et al., 2015). Findings regarding survival likely differ between studies due the use of different OSPW sources and the variable heterogeneous nature of OSPW, leading to the variation of effects observed (Frank et al., 2016). Some of these studies may also differ in their findings due to the use of different fish species and life stages (Hughes et al., 2017).

Previous studies have found slightly differing findings in regards to CYP induction with exposure to OSPW. Wiseman et al. (2013b) found a slight induction of *cyp1a* in fathead minnows exposed to OSPW, while others have found little to no effect on CYP expression (Alharbi et al., 2016; He et al., 2012a). The CYP enzymes play a major role in phase 1 biotransformation and are also involved in the metabolism of endogenous substrates. CYP expression is commonly used as a biomarker of exposure to PAHs (Goksøyr, 1995; Payne, 1976; Payne and Penrose, 1975). In our study, we found that raw OSPW exposure induced both *cyp1a* and *cyp1b* expression, while ozonated OSPW only slightly induced the expression of *cyp1b* and did not affect *cyp1a*, potentially indicating that ozonation decreased the amount of aryl hydrocarbon receptor (AHR) inducing compounds in the OSPW. The expression pattern found in raw OSPW exposed embryos was different from what other studies have found with exposures to different AHR agonists (ex. TCDD, methylcholanthrene, PCB126) (Dorrington et al., 2012; Jönsson et al., 2007, 2010; Zanette et al., 2009). *cyp1b* expression is often induced to a lesser extent by environmental contaminants than *cyp1a*. However, our study found that OSPW exposure induced *cyp1b* to a larger extent than *cyp1a*. The induction pattern of CYP1 genes found in our study could be useful for monitoring exposure to complex mixtures. In the future, with more validation, the expression levels of these two genes may be useful as a biomarker of OSPW exposure, since this pattern of induction has only been found from exposure to OSPW. Many studies measure only one CYP gene, however, measuring multiple CYP genes will clearly be beneficial, especially when studying complex hydrocarbon mixtures as it could lead to a greater understanding of the compounds the organism is being exposed to.

nkx2.5 and *atp2a2a* expression and heart rate

For fish, decreased cardiac output could lead to decreased swim performance, which can have an effect on a fish's fitness (Hicken et al., 2011; Incardona et al., 2015). Cardiac deformities and impaired cardiac function are common effects caused by oil exposure in fishes (Hicken et al., 2011; Incardona et al., 2004, 2005). Previous studies have shown that developmental exposure to OSPW can lead to cardiovascular defects and inhibited cardiogenesis in fishes (He et al., 2012a; Peters et al., 2007) . Both *nkx2.5*, an important transcription factor in cardiomyocyte differentiation, and *atp2a2a*, which encodes a protein involved in calcium regulation in the heart, are required for proper development and function of the heart(Staudt and Stainier, 2012; Zhang et al., 2013). In our study we found *nkx2.5* and *atp2a2a* expression were decreased by raw OSPW exposure. We also found a slight increase in heart rate due to raw OSPW exposure, but found no signs of arrhythmia. However, the slight increase in heart rate was within a normal range for zebrafish, which is approximately between 135-165 beats per minute for 2 dpf zebrafish (Garrity et al., 2002; Lin et al., 2007; Rana et al., 2010). Our findings indicate that though raw OSPW exposure had slight effects on gene expression, that these changes were likely not adversely affecting the fish since they appeared to have normally functioning hearts. Our work supports that conclusions drawn from changes in gene expression or biomarkers should be used with caution, as they may not necessarily lead to effects at the tissue, or whole organism level (Forbes et al., 2006).

Embryos exposed to ozonated OSPW had downregulated expression of *nkx2.5* though it did not lead to a change in any cardiac function/morphology endpoints. Since exposure to raw OSPW led to a change in heart rate, though not likely a biologically relevant change, and ozonated OSPW did not have an impact on heart rate, ozone treatment of the OSPW may have decreased its potential for impacting cardiac function.

gli2a expression and jaw morphology

Some previous studies have found that exposure to crude oil, oil sands sediment and NAs extracted from OSPW lead to craniofacial abnormalities in fishes (Colavecchia et al., 2004; Incardona et al., 2004; Marentette et al., 2015b; Raine et al., 2017). Craniofacial deformities could have an impact on fish later in life, perhaps by reducing their ability to capture prey. Gli zinc finger transcription factors are involved in craniofacial development (Chang et al., 2016; Mo et al., 1997; Schwend et al., 2010) . We did not find any significant changes in *gli2a* expression or craniofacial abnormalities in 7 dpf zebrafish exposed to OSPW. Our findings on craniofacial development do not correspond with what some previous studies have found, though many studies on OSPW do not analyze craniofacial morphology.

Apoptosis biomarker expression and TUNEL assay

Previous studies have found that exposure to OSPW in fathead minnows increased expression of genes involved in apoptosis (He et al., 2012a; Wiseman et al., 2013a, 2013b). However, our study did not find an increase in apoptotic cells, nor did we find increased transcript levels of genes involved in apoptosis. Though there was a slight, non-significant upward trend in the occurrence of apoptotic cells in raw OSPW exposed embryos, there was no increase in *p53* or *casp9* gene expression. The lack of change in the occurrence of apoptotic cells in the tail region is congruent with the lack of tail malformations found in the exposed embryos. The different findings between our study and previous studies could be due to the use of different species and OSPW sources. Other studies have also found no change in expression of apoptosis-related genes with exposure to OSPW and diluted bitumen (Madison et al., 2017; Marentette et al., 2017). The lack of increased apoptotic activity is promising in respect to the toxicity of the OSPW sample used in this study, as it indicates that this particular sample has no negative effects on the embryos in regards to apoptosis. Considering the lack of responses found in our study, it is perhaps not surprising that we did not find an induction of apoptosis.

Conclusion

Developmental OSPW exposure in our study did not appear to be overtly toxic to zebrafish embryos. Ozonation decreased the amount of total NAs (O_2 +O_3 + O_4) present in the OSPW sample from 34.6mg/L to 2.9mg/L, a decrease of 92% and slightly reduce the impact of exposure for some endpoints (e.g. heart rate), though raw OSPW had very few effects on zebrafish embryos. Exposure to either type of OSPW did not affect survival, heart area, or jaw morphology and did not induce cardiac arrhythmia, or the occurrence of apoptotic cells. There was a slight increase in heart rate due to raw OSPW exposure, but it remained well within the normal heart rate range for zebrafish embryos. Heart rate remained unaffected by ozonated OSPW exposure. Though we did find changes in the expression of some target genes in our study, these alterations at the transcriptional level were not necessarily linked to changes at the cellular or organism level. The *cyp1a* and *cyp1*b expression pattern that we observed is, to our knowledge, a novel expression pattern that, with more research and validation, could potentially be used as a biomarker of OSPW exposure.

In general, we did not find that the OSPW sample used in this study caused many negative effects in developing fish. Zebrafish, however, are considered relatively tolerant compared to some fish species (ex. rainbow trout) native to the Athabasca watershed (Fogels and Sprague, 1977). Though they

tend to be more tolerant than some other species, zebrafish still make a good model species, as they are easy to study their use enables many different endpoints to be studied. Many different fish species, with differing tolerances to OSPW exposure, live in the Athabasca watershed, meaning that it is important to study many different fish species and their responses to OSPW exposure to allow for comparisons.

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Tables

Table 2.1. Characterization of raw and ozonated OSPW.

Parameter	Raw OSPW	Ozonated OSPW
pH	7.10	7.15
Turbidity (NTU)	128	129
Alkalinity (mg/L as $CaCO_3$)	288	275
Total organic carbon (TOC) (mg/L)	51.5 ± 2.8	44.7 ± 0.7
Chemical oxygen demand (COD) (mg/L)	114 ± 0.6	83.7 ± 5.2
Biochemical oxygen demand (BOD_5) (mg/L)	1.4±0.3	8.7 ± 0.1
O_2-NAs (classical NAs) (mg/L)	16.9	0.6
O_3-NAs (mg/L)	8.6	0.9
O_4-NAs (mg/L)	9.1	1.4
Acid extractable fraction (mg/L)	40.8±0.5	9.60±1.3
Selected analytes		
Li (mg/L)	0.131	0.133
Na (mg/L)	251	252
Mg (mg/L)	13.5	13.5
Fe (mg/L)	<DL	<DL
Ca (mg/L)	27.6	27.7
Mn (mg/L)	0.0676	0.0595
Cu (mg/L)	0.0225	0.0108
Ba (mg/L)	0.156	0.154

DL: Detection limit.

Table 2.2. qPCR primers and accession number or reference.

Abbreviation	Target Gene name	Forward Sequence (5'-3')	Reverse Sequence (5'-3')	Accession Number or Reference
βactin	Beta-Actin	CGA GCA GGA GAT GGG AAC C	CAA CGG AAA CGC TCA TTG C	AF057040(Rodríguez et al., 2008)
cyp1a	Cytochrome P4501a	AGG ACA ACA TCA GAG ACA TCA CCG	GAT AGA CAA CCG CCC AGG ACA GAG	NM_131879(Timme-Laragy et al., 2007)
cyp1b	Cytochrome P4501b	CCA CCC GAA CTC TGA AAC TC	AAA CAC ACC ATC AGC GAC AG	NM_001013267(Timme-Laragy et al., 2007)
gli2a	GLI family zinc finger 2a	AAA AAC AGG GCG GGA CTA CT	ATG CTG GGT TGG AGG TAC AG	Paule *et al*.(Kanungo et al., 2013)
nkx2.5	Homeobox protein *nkx2.5*	GTC CAG GCA ACT CGA ACT ACT C	AAC ATC CCA GCC AAA CCA TA	NM_131421(Zhang et al., 2012)
atp2a2a	Cardiac muscle ATPase, Ca++ transporting, slow twitch 2a	GCA GGT TAG AGC CGT TTC TG	CTG TGC CTT GTG CAA TGA CT	CR407563(Nesan and Vijayan, 2012)
p53	Tumor suppressor protein 53	ACC ACT GGG ACC AAA CGT AG	CAG AGT CGC TTC TTC CTT CG	NM131327(Luzio et al., 2013)
casp9	Caspase 9	CTG AGG CAA GCC ATA ATC G	AGA GGA CAT GGG AAT AGC GT	NM001007404 (Luzio et al., 2013)

Figures

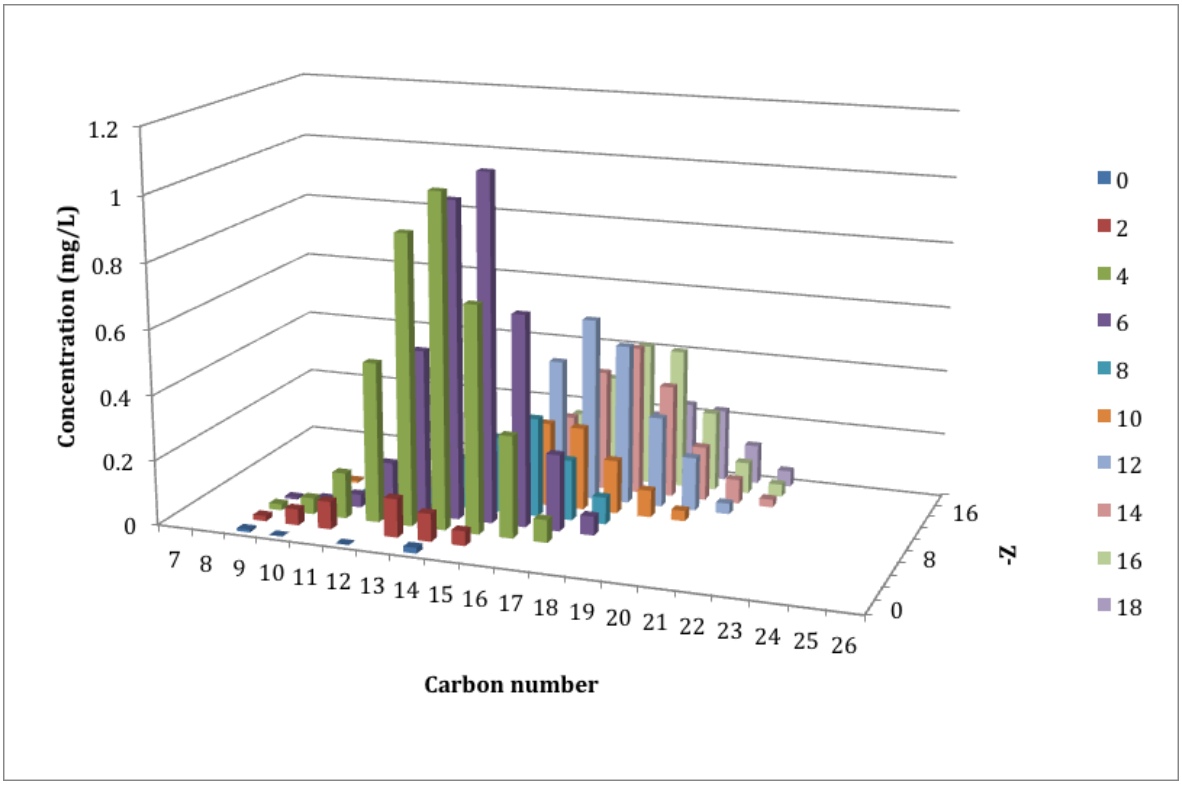

Figure 2.1. The distribution profile of O_2-NAs in raw OSPW, in terms of carbon and Z numbers. The concentration of O_2-NAs is 16.94 mg/L.

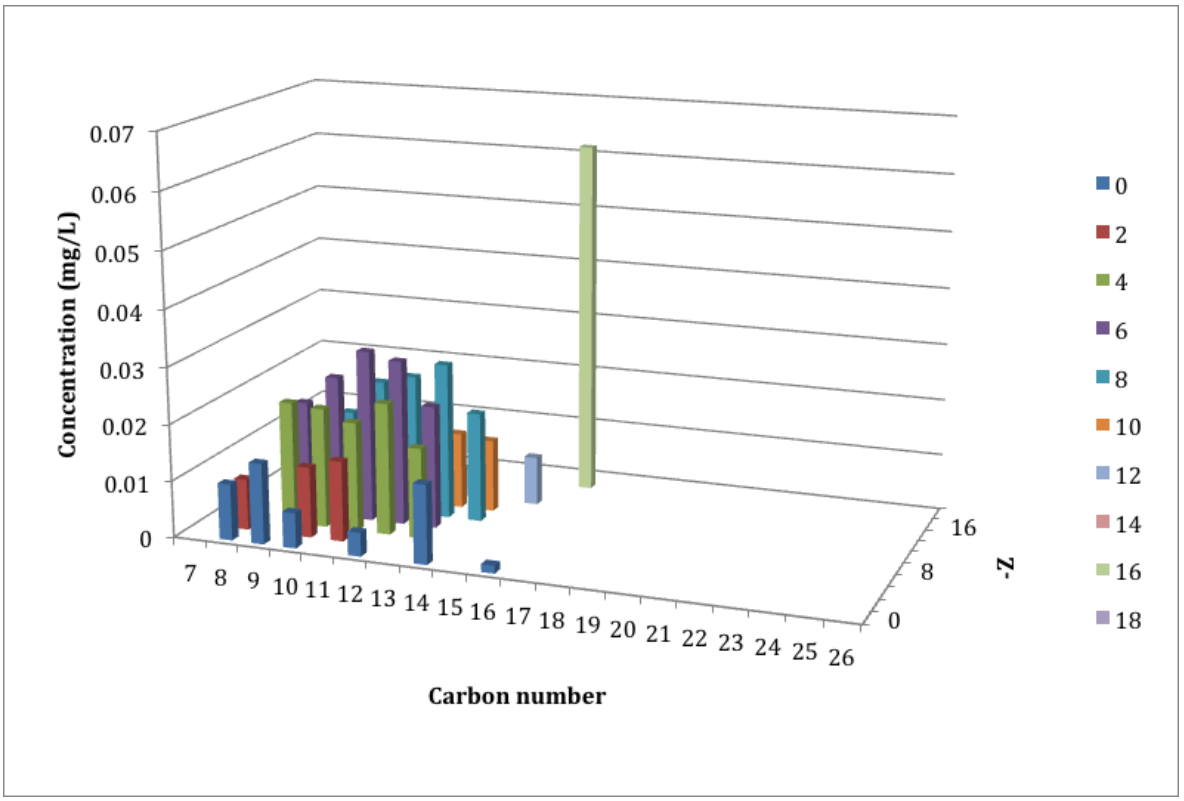

Figure 2.2. The distribution profile of O_2-NAs in OSPW after ozonation treatment, in terms of carbon and Z numbers. The concentration of O_2-NAs is 0.56 mg/L.

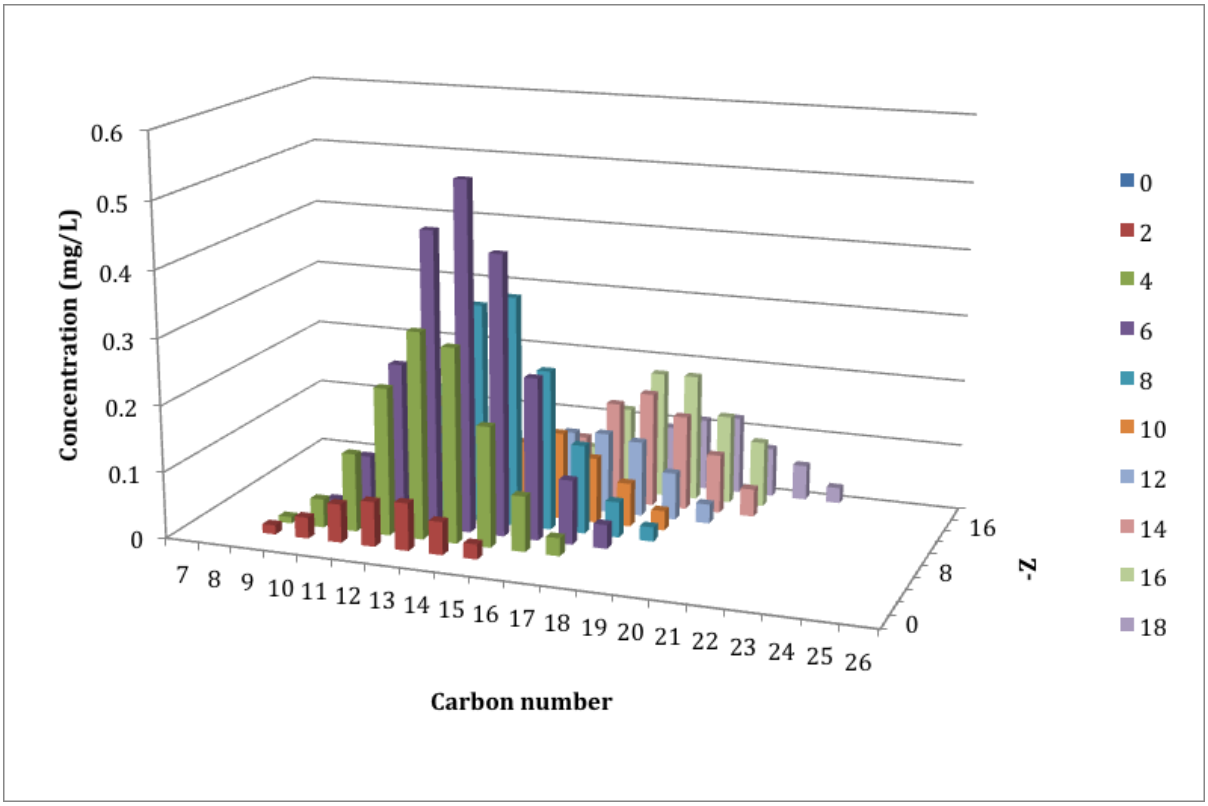

Figure 2.3. The distribution profile of O_3-NAs in raw OSPW, in terms of carbon and Z numbers. The concentration of O_3-NAs is 8.59 mg/L.

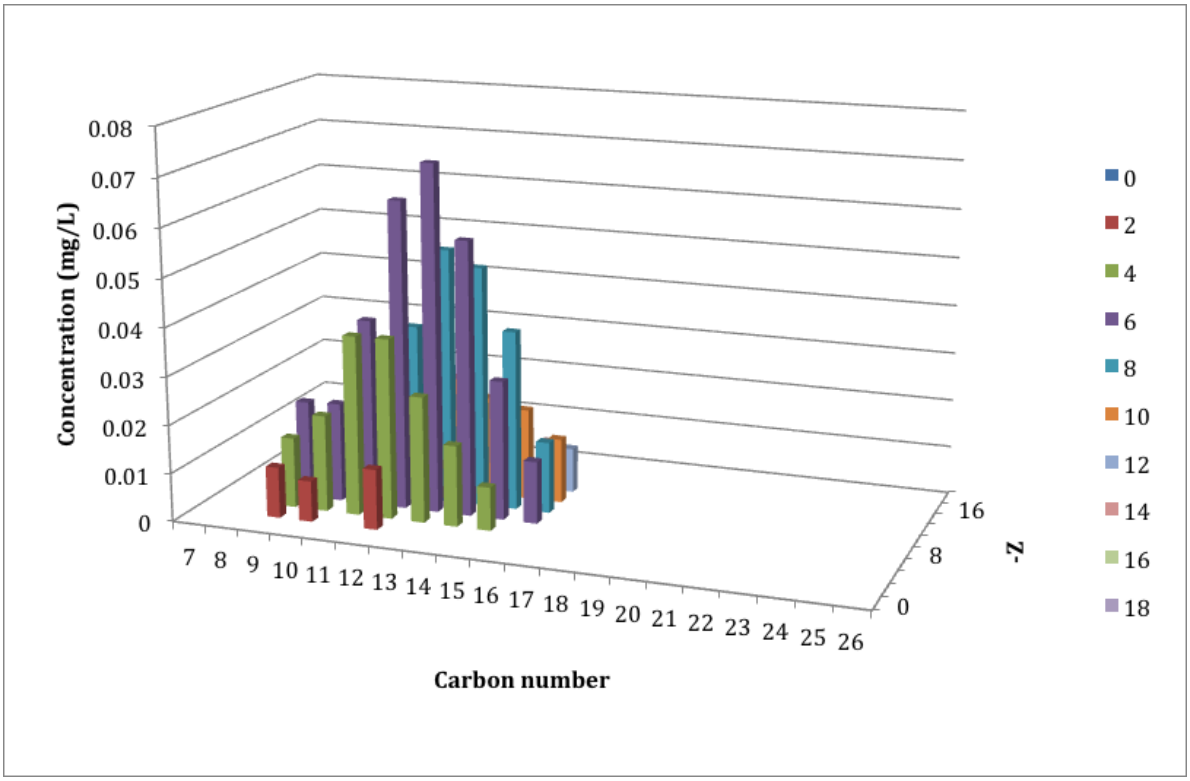

Figure 2.4. The distribution profile of O_3-NAs in OSPW after ozonation treatment, in terms of carbon and Z numbers. The concentration of O_3-NAs is 0.89 mg/L.

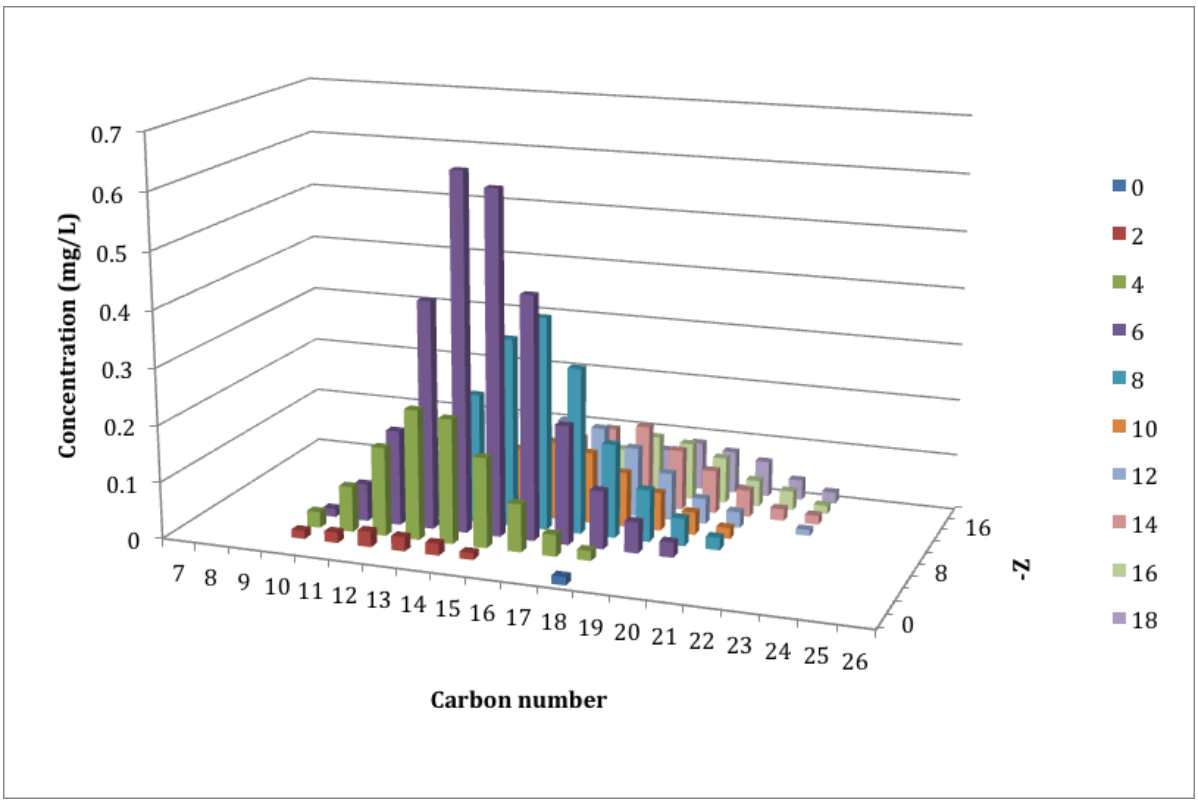

Figure 2.5. The distribution profile of O_4-NAs in raw OSPW, in terms of carbon and Z numbers. The concentration of O_4-NAs is 9.09 mg/L.

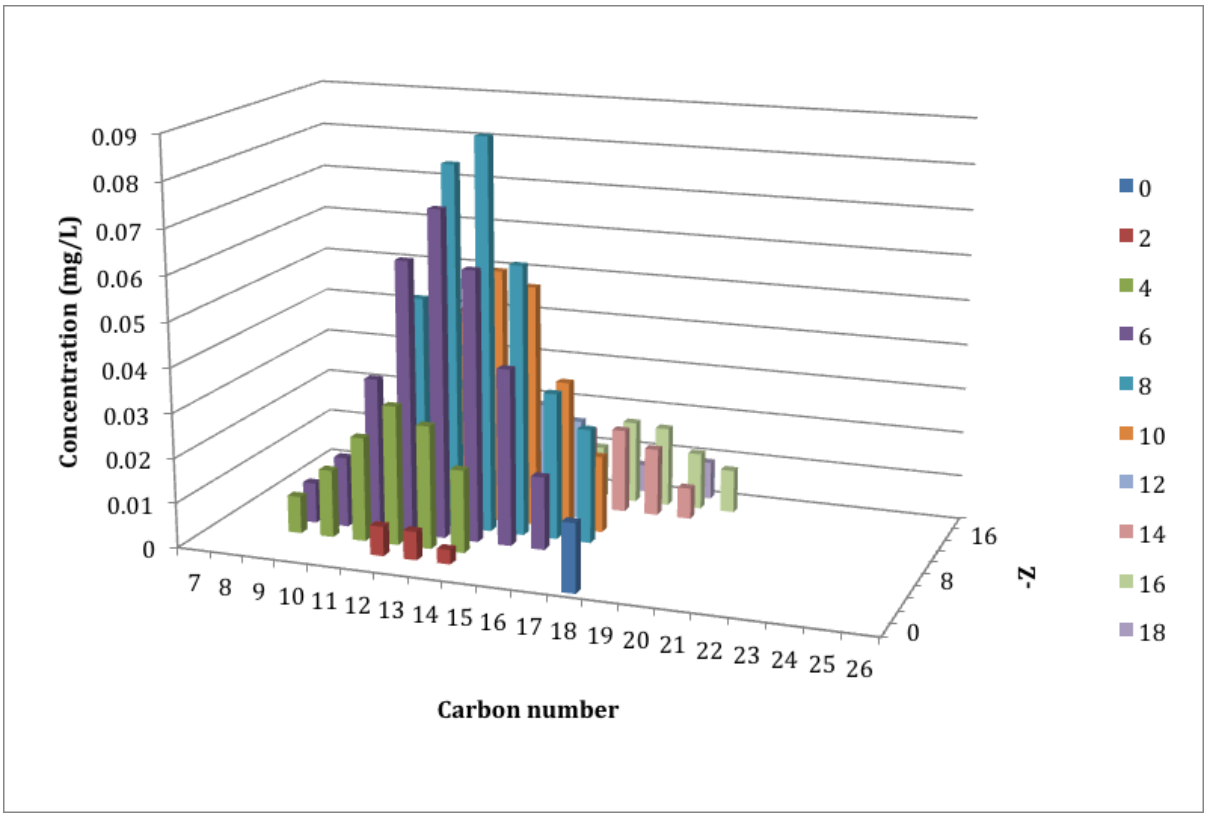

Figure 2.6. The distribution profile of O_4-NAs in OSPW after ozonation treatment, in terms of carbon and Z numbers. The concentration of O_4-NAs is 1.38 mg/L.

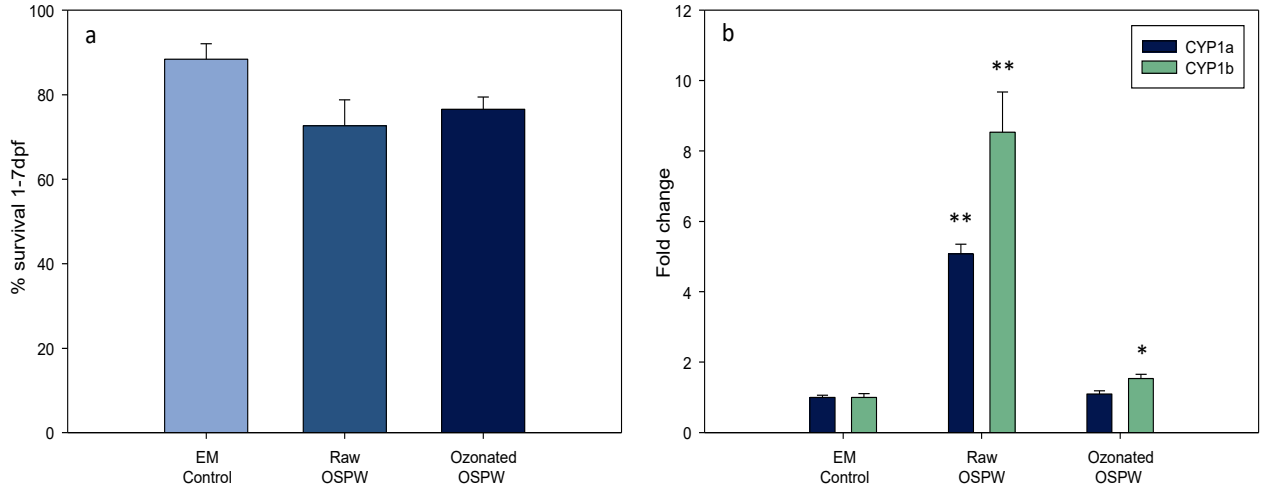

Figure 2.7. Percent survival of embryos exposed to raw and ozonated OSPWs and embryo media (EM) control from 1-7 dpf (a) and expression levels of biotransformation enzymes *cyp1a* and *cyp1b* at 7dpf (b). (a) Embryo survival was not affected by raw or ozonated OSPW exposure (one way ANOVA and Holm Sidak post hoc, n=3-6 replicates). (b) Both *cyp1a* and *cyp1b* were upregulated by exposure to raw OSPW. *cyp1b* was slightly upregulated by exposure to ozonated OSPW; however, *cyp1a* expression was not affected (one way ANOVA and Tukey's post hoc, * indicates $p<0.05$, ** indicates $p<0.001$, n=4-6).

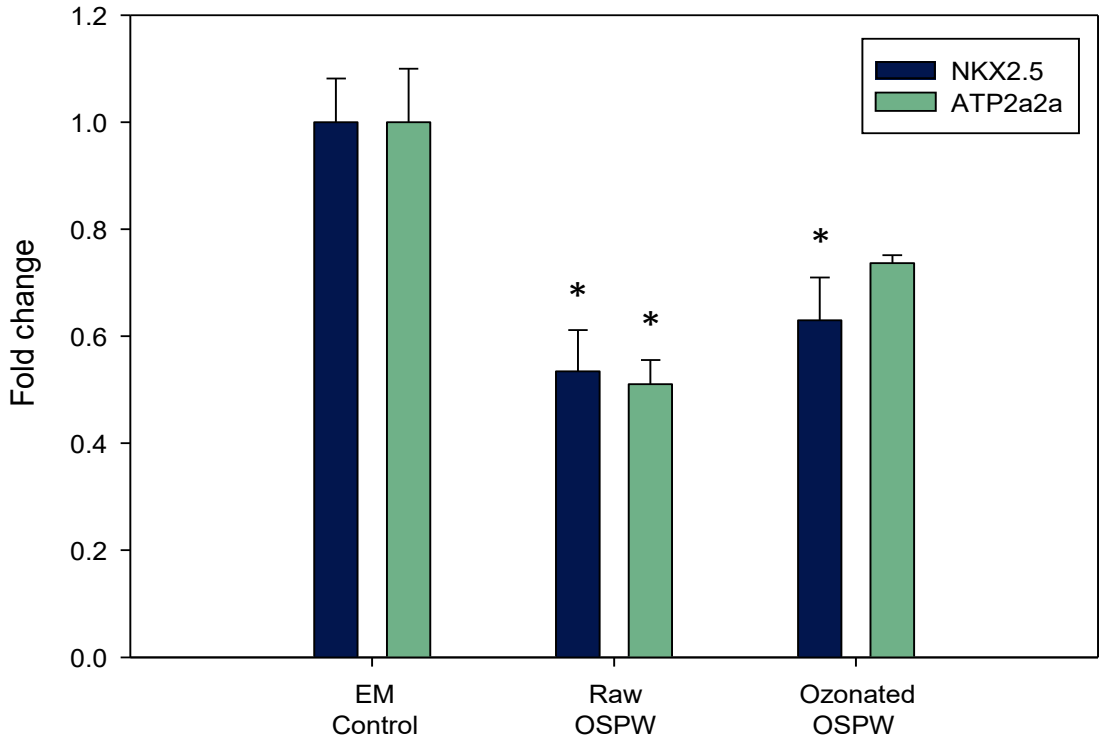

Figure 2.8. Expression levels of cardiac development genes *nkx2.5* (a) and *atp2a2a* (b) after exposure to raw and ozonated OSPW. (a) *nkx2.5* was downregulated by raw and ozonated OSPW (one way ANOVA and Tukey's post hoc). (b) *atp2a2a* was downregulated by raw OSPW exposure (one way ANOVA and Tukey's post hoc test, * indicates $p<0.05$, n=4-6).

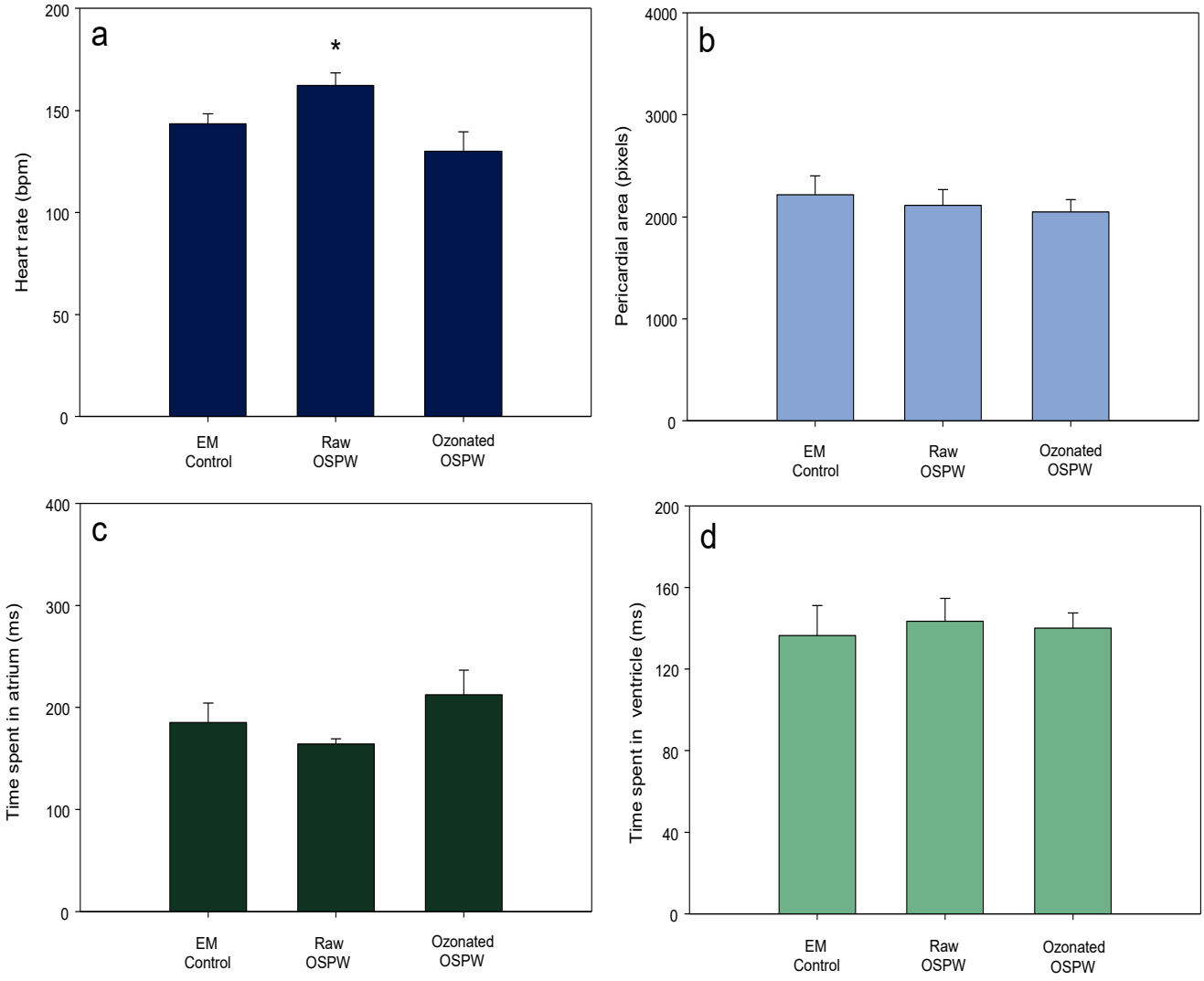

Figure 2.9. The effect of raw and ozonated OSPW exposure on the heart rate (n=20-35) (a), pericardial area (n=10-15) (b), time blood spent in the atrium of the heart (n=6-10) (c), and time blood spent in the ventricle of the heart (n=6-10) (d) of 2 dpf zebrafish embryos. Heart rate was higher in the raw OSPW exposed embryos (one-way ANOVA and Tukey's post hoc, $p<0.05$). Exposure had no effect on the pericardial area, time the blood spent in the atrium or ventricle of the heart.

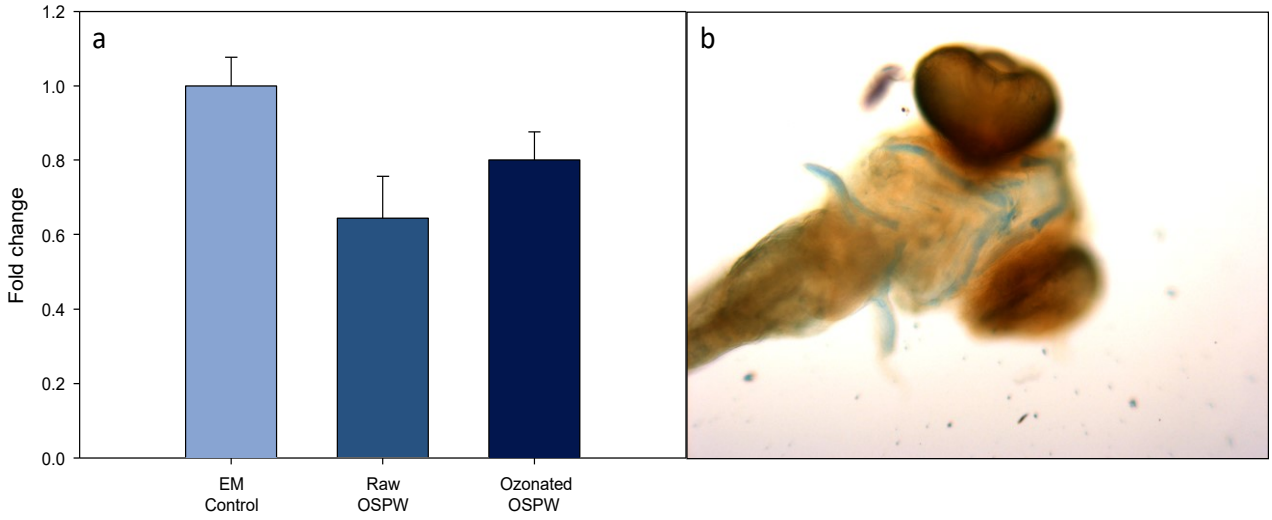

Figure 2.10. Expression levels of neurodevelopment gene *gli2a* (a) and an example of normal embryo jaw morphology (b) after exposure period at 7dpf. (a) *gli2a* expression was unaffected by OSPW exposure (one way ANOVA, n=4-6). (b) Image shows normal jaw structure in a control embryo. There were no observed changes to jaw morphology between treatment groups (n=25-30 per treatment).

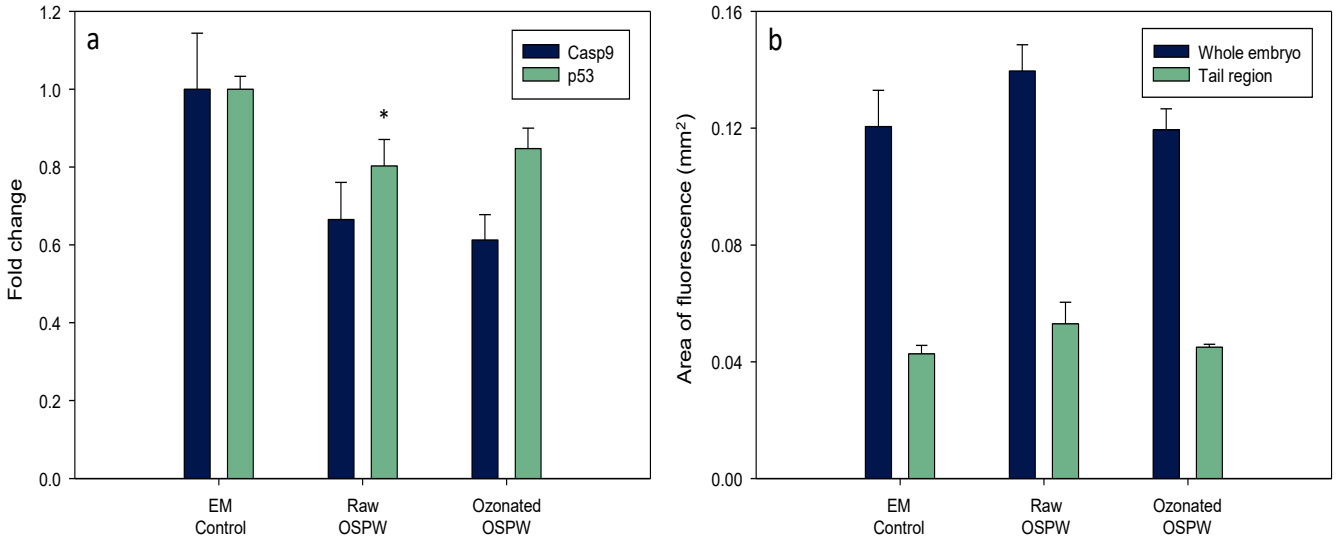

Figure 2.11. Expression levels of apoptosis markers *casp9* and *p53* in 7dpf embryos (a) and occurrence of apoptotic cells in whole embryos and the tail region of 3dpf embryos using the TUNEL assay (b). (a) *casp9* expression remained unaffected by exposure, while *p53* expression

was slightly downregulated by exposure (one way ANOVA and Tukey's post hoc, * indicates $p<0.05$, n=4-5). (b) Occurrence of apoptotic cells was unaffected by OSPW exposure (one way ANOVA, n=3 trials of 4-5 embryos per treatment).

Chapter 3: Growth and recovery of zebrafish embryos after developmental exposure to raw and ozonated oil sands process-affected water

Abstract

Due to the increasing volume of oil sands process-affect water (OSPW) and its toxicity to aquatic organisms, it is important to fully understand its effects and study remediation processes that will enable its release to the environment. Ozone treatment is currently being considered as a tool to expedite remediation, as it is known to degrade toxic organic compounds present in OSPW. In this study, we aimed to measure the effects of OSPW exposure on the growth, development and recovery of zebrafish (*Danio rerio*) embryos. We also used ozone-treated OSPW to determine whether ozonation negated any effects of raw OSPW exposure. As biomarkers of exposure, we assessed the expression of genes involved in neurodevelopment (*ngn1, neuroD*), estrogenicity (*vtg*), oxidative stress (*sod1*), and biotransformation (*cyp1a, cyp1b*). Our study found that exposure to both raw and ozonated OSPW did not impair growth of zebrafish embryos, however, otoliths of exposed embryos were smaller than those of control embryos. The expression levels of both *cyp1a* and *cyp1b* were induced by raw OSPW exposure. However, after the exposure period, expression levels of these genes returned to control levels within two days of residence in clean water. We found no changes in the expression levels of *ngn1*, *neuroD* and *vtg* genes with exposure to treated or untreated OSPW. Overall, our study found that raw OSPW exposure did not have many negative effects on zebrafish embryos and embryos appeared to recover relatively quickly after exposure ended. Furthermore, ozone treatment decreased the induction of *cyp1a* and *cyp1b*.

Introduction

A wastewater commonly referred to as oil sands process-affected water (OSPW) is produced during the extraction of bitumen from the Canadian oil sands and has been found to be toxic to aquatic organisms. The bitumen extraction process requires extensive amounts of water, resulting in large quantities of OSPW, which is stored in tailings containment structures and recycled for various processes. Approximately 1.67 barrels of fresh water is used per barrel of bitumen extracted from the oil sands (Shell Canada Ltd., 2016). Although 85-95% of the water

used in the extraction process is recycled, OSPW is continuously accumulating in tailing ponds (National Energy Board, 2006; Shell Canada Ltd., 2016).

OSPW is composed of water, silt and clay, residual bitumen, and other organic and inorganic material and has been shown to be both acutely and chronically toxic to aquatic organisms (Allen, 2008; Anderson et al., 2012a; Clemente and Fedorak, 2005; He et al., 2012a; Wiseman et al., 2013b, 2013a). The water soluble organic fraction of OSPW, naphthenic acids (NAs) in particular, has been attributed to the toxicity of exposure to aquatic organisms (Anderson et al., 2012b; Garcia-Garcia et al., 2011; He et al., 2010; Hughes et al., 2017). NAs are complex carboxylic acids that are a natural component of bitumen (Mahaffey and Dubé, 2016). Other organic and inorganic constituents of OSPW such as metals and salts may also contribute to its observed toxicity, however, the toxicity of each component present in OSPW is not yet fully understood (Klamerth et al., 2015; Leclair et al., 2013; Li et al., 2014; Morandi et al., 2015). The complexity of OSPW makes it challenging to determine the full effects of exposure and its mechanisms of toxicity. Among other effects, OSPW exposure to fishes has been shown to cause endocrine disruption (He et al., 2012b; Lister et al., 2008; Wiseman et al., 2013b), impaired immune function (Leclair et al., 2013), impaired olfaction (Lari and Pyle, 2017; Reichert et al., 2017), and apoptosis (He et al., 2012a; Wiseman et al., 2013a).

Reclamation efforts are required to permit eventual integration of this water back into the environment. Recent studies have focused on the use of ozonation as a tool to assist in remediation efforts, as it has been found to reduce negative effects of OSPW exposure (Anderson et al., 2012b; He et al., 2010). The principle aim of this study was to evaluate the effects of OSPW exposure on the growth, development and recovery of embryonic zebrafish (*Danio rerio*). A secondary aim was to determine whether ozone treatment of OSPW decreased its toxicity. We selected growth as an endpoint as it is an indicator of general health, and it has been found to be affected after OSPW exposure (Marentette et al., 2015). We also measured yolk sac area and absorption as an indicator of delayed development and/or growth.

To assess growth in fishes, otoliths (ear stones) are often used to determine daily or yearly growth and size-at-age as calcium rings are continuously deposited on the otoliths and this permits age and size calculations as well as detection of growth defects due to environmental factors (Pisam et al., 2002). The back-calculation technique of using otoliths to determine size-

at-age has been widely used in many different fish species and ages, including larval fish (Vigliola and Meekan, 2009). However, this method for measuring growth has yet to be validated in zebrafish embryos/larvae. Zebrafish otoliths are formed by 24-30 hours post fertilization (Pisam et al., 2002) and day and night behavior/activity cycles are established by 3 days post fertilization (dpf), therefore, daily growth rings should begin forming by 3 dpf (Cahill, 2002). We analyzed daily growth rings in embryonic zebrafish in order to determine if they could be used to estimate size-at-age for each day after hatch *in lieu* of measuring fish length on a daily basis. We also analyzed zebrafish otoliths as an indicator of metabolic rate, as otolith size has been found to be correlated with metabolic rate in embryonic fish (Bang and Grønkjær, 2005; Bochdansky et al., 2005).

To probe any developmental effects from OSPW exposure, we elected to look at genes related to neurotoxicity, as many environmental contaminants are neurotoxic to fishes (Akaishi et al., 2004; Bailey et al., 2013; Brown et al., 2016). Neurotoxicants can act in many different ways including disruption of neural determination, differentiation, proliferation and synapse formation. These effects can lead to functional impacts due to changes in locomotion and behavior (Bailey et al., 2013). The genes targeted in our study, *ngn1* and *neuroD*, are both basic helix loop helix transcription factors (Korzh et al., 1998). *ngn1* is responsible for development of all cranial ganglia and is expressed before and more extensively than *neuroD* (Andermann et al., 2002; Mueller and Wullimann, 2002). While *ngn1* is involved in neural determination, *neuroD* is involved in neural differentiation (Korzh et al., 1998). We measured the expression levels of these genes as markers of whether neural determination or differentiation was potentially affected by OSPW exposure.

We also measured the expression of vitellogenin (*vtg*) to examine whether OSPW exposure was having an estrogenic effect on zebrafish embryos. The expression of *vtg* is commonly used as a biomarker of exposure to estrogenic compounds (Chow et al., 2013; Gagné et al., 2012; Heppell et al., 1995; Kidd et al., 2007). By measuring this, we could determine whether OSPW might be causing endocrine disruption or have estrogenic effects. Another biomarker we measured in this study, *sod1*, is commonly used as a biomarker of oxidative stress in aquatic organisms (Alharbi et al., 2016; Basha and Rani, 2003; He et al., 2012a; Kwang Wook An et al., 2008). An increase in *sod1* expression is linked with an increase in the presence of

reactive oxygen species (ROS) (Alharbi et al., 2016; Basha and Rani, 2003; He et al., 2012a; Kwang Wook An et al., 2008). The expression of *sod1* was measured in this study to probe whether OSPW was causing an increase in ROS production in zebrafish embryos.

Lastly, to explore recovery, we measured transcript levels of cytochrome P450 (CYP) at the end of the exposure period and during a four-day recovery period. CYP enzymes play a major role in phase 1 biotransformation and are commonly used as biomarkers of exposure to organic contaminants (Goksøyr, 1995; Payne, 1976; Payne and Penrose, 1975). Few studies of sub-lethal exposures examine recovery despite this likely being the reality in the environment. Our goal here was to determine how long zebrafish showed signs of exposure and determine whether or not there may be long-term effects from OSPW exposure.

Materials and Methods

Ozonation of OSPW

The OSPW used in this study was collected in 2015 from Shell Canada Ltd's Muskeg River Mine (located ~60 km north of Fort McMurray, Alberta, Canada now under operation by Canadian Natural as of June 2017) and was stored at 4°C in 200L polyvinyl chloride barrels. OSPW was ozonated as described in Lyons et al. (2018). The raw OSPW sample contained16.9 mg/L of classical NAs and the ozonated sample contained 0.6 mg/L. Further characterization of both the raw and ozonated OSPW can be found in Lyons et al. (2018).

Analysis of naphthenic acids

Raw and ozonated OSPW samples were analyzed using ultra performance liquid chromatography time-of-flight mass spectrometry (UPLC-TOF-MS) as described in Lyons et al. (2018).

Fish Care

Embryos used for this study were collected from a breeding colony of approximately 500 adult AB strain wild type zebrafish. Adult and embryonic zebrafish were housed at 28°C±0.5°C on a 14h:10h light dark cycle. Adult fish were fed a mixture of TetraMin® flakes (Tetra Holding, Blacksburg, VA), Cobalt™ Aquatics spirulina flakes (Cobalt, Rock Hill, SC), and Omega One™ freeze dried bloodworms (Omegasea, Sitka, SK).

Embryo exposures

Zebrafish embryos were exposed to dilutions of raw and ozonated OSPW within 30 minutes post-fertilization until 7dpf. Embryos were held in groups of 70 in glass Petri dishes containing 40mL of treatment water, with ~95% of the exposure water exchanged daily. Control groups of embryos were held in embryo medium (EM) (Westerfield, 2000). After exposure (at 7 dpf) embryos were either collected for analysis or transferred to EM for a two or four-day recovery period.

Daily growth and yolk sac measurements

Embryos were imaged daily between 14:00-15:00 from 2-7 dpf under an OMANO dissecting microscope (Roanoke; VA, USA). The lengths of embryos in each daily image were measured using ImageJ (National Institute of Health). Yolk sac area was used as a measure of yolk sac absorption and was measured in 2 and 3 dpf embryos using ImageJ (whole visible outline of yolk sac, including extension). Three trials of embryos were measured, each trial consisting of 7-10 embryos per treatment.

Otolith measurements

Embryos were collected immediately after exposure at 7 dpf and stored in methanol at -20°C until use. Otolith pairs (ear stones) were extracted from zebrafish on an OMANO stereomicroscope (Roanoke; VA, USA) under birefringence (polarized lenses above and below the embryo at 90° to each other), as this makes them appear bright and enables their identification. Once extracted from the embryo, otoliths were glued to a microscope slide, polished, and imaged under oil immersion at 100× magnification on a Leica DMRXA microscope (Meyer Instruments; Houston, TX, USA). In order to determine fish size-at-age, the biological intercept model (Campana, 1990) was used to back-calculate fish length from otoliths. In brief, the hatch check was determined by locating the first and thickest ring. Then daily increment rings were counted and the widths between daily increments were measured. These measurements, along with fish size at 7dpf and fish size at hatch were included in the biological intercept equation (Campana, 1990) to determine size-at-age for any given day. Otoliths were extracted from 10 embryos per treatment group for three replicates.

RNA extraction, cDNA synthesis, and qPCR

At 7, 9 and 11 dpf, embryos were preserved in RNAlater® (Thermo Fisher; Waltham, MA, USA) and stored at -20°C until RNA extraction. Each sample of total RNA was extracted from 20-35 pooled embryos using TRIzol® (Ambion; Carlsbad, CA, USA) according to the manufacturer's instructions. Purification of extracted RNA was done using an RNeasy® Mini Kit (Qiagen; Hilden, Germany) according to manufacturer's protocol for RNA cleanup. Potential genomic DNA contamination was removed by an on-column DNase incubation using an RNase Free DNase Set (Qiagen).

Purified RNA was stored at -80°C until analysis. Quality and concentration of RNA samples were measured using a Nanovue (General Electric Healthcare; Chicago, IL, USA). Each sample of cDNA was synthesized from 2 µg of total RNA using SuperScript® First-Strand Synthesis System for RT-PCR (Invitrogen; Carlsbad, CA, USA) as described by the manufacturer using a Mastercycler Pro S (Eppendorf; Hamburg, Germany).

Primer efficiencies were calculated prior to real-time PCR (qPCR) reactions. Acceptable primer efficiencies were between 90-110%. qPCR was run in 96 well PCR plates on an Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems; Foster city, CA, USA). Each 10 µL qPCR reaction contained 5 µL custom SYBR Green master mix, 2.5 µL of forward and reverse gene specific primers, and 2.5 µL cDNA. Each individual target cDNA amplification for qPCR was run in triplicate. Expression levels of target genes were quantified relative to the endogenous control gene, Beta-actin. The relative fold changes of target genes were calculated using the $2^{-\Delta\Delta Ct}$ method. The reactions were denatured at 95°C for 2 min then cycled through 95°C for 15 s and 60°C for 1 min for a total of 40×. After amplification cycles were complete, the qPCR products underwent dissociation steps and curves were generated to ensure the amplification of a single product. The dissociation steps were 95°C for 15 seconds, 60°C for 1 min, 95°C for 15 s, and finally 60°C for 15 s. See Table 3.1 for a full list of target genes and primer sequences.

Statistical analyses

Statistical differences between treatments were detected using one-way analysis of variance (ANOVA) followed by either Tukey's or Holm Sidak post-hoc tests. Tukey's post-hoc test was used for all gene expression data and Holm Sidak was used for all other data. T-tests were used

to compare otolith back-calculations to actual growth. When needed to meet the assumptions of parametric tests, gene expression data was transformed using a $\log_{10}$ transformation. All data are expressed as the mean ± standard error (SE) and a p-value<0.05 was accepted as significant. All statistical analyses and graphing were carried out using SigmaPlot 11 (Systat; San Jose, CA).

Results

Water chemistry

OSPW parameters are published in Lyons et al. (2018). In brief, total NAs were reduced by 92% after the 80mg/L ozone treatment (from 34.6mg/L to 2.9mg/L). NAs with more oxygen were degraded less by ozonation as indicated by the degradation efficiencies for O_2, O_3, and O_4 NAs (97, 90, and 85%, respectively). This also reflects the formation of O_4 species from O_2 species. Ozone treatment resulted in a shift in NA distribution towards those that are more oxygen-rich and with a lower carbon number (Lyons et al., 2018).

Daily growth and yolk sac measurements

Overall growth of zebrafish embryos from 2-7 dpf was unaffected by OSPW exposure (Figure 3.1). Raw OSPW exposed embryos were shorter in length when compared to the control group at 3 and 4 dpf ($F_{2,86} = 4.15$, $p<0.05$ and $F_{2,89} = 4.01$, $p<0.05$, respectively). However, the lengths of the raw OSPW exposed embryos were the same as those of the control by 5 dpf and remained the same until 7 dpf when the exposure ended. The length of ozonated OSPW exposed embryos did not differ from control throughout the exposure period.

Yolk sac area at 2 and 3 dpf was not altered by exposure (Figure 3.2a). There appeared to be a trend towards decreased absorption of the yolk sac between 2 and 3 dpf with raw OSPW exposure, though it was not significant (Figure 3.2b). Developmental OSPW exposure did not affect yolk sac area and absorption or overall fish length; therefore, embryo development did not appear to be delayed by exposure.

Otolith measurements

Otolith back-calculations resulted in a similar size-at-age when compared to actual length measurements from 2-5 dpf in all three treatments, though there were some differences. Comparing otolith back-calculations to actual length measurements (Figure 3.3), there was a

significance difference in size-at-age for EM control at 3 dpf ($p<0.001$) and 4 dpf ($p=0.026$), for raw OSPW exposed embryos at 3 dpf ($p=0.002$), and for ozonated OSPW exposed embryos at 5 dpf ($p=0.038$). Otolith area at hatch (2 dpf) was the same between all treatments, however, at 7 dpf, otolith area was smaller in exposed embryos compared to control (Figure 3.4; $F_{2,112} = 18.8$, $p<0.001$).

Neurodevelopment gene expression

The expression of neurodevelopment genes *ngn1* and *neuroD* were not significantly affected by OSPW exposure (Figure 3.5). There was a downward trend in expression levels of *neuroD* with raw OSPW exposure, but it was not statistically significant. These findings show no indication that neurodevelopment was affected by raw or ozonated OSPW developmental exposure, though it does not rule out that some other effects may be occurring to impact neurodevelopment.

***vtg* and *sod1* gene expression**

The expression levels of *vtg*, a biomarker of exposure to estrogenic compounds, and *sod1*, a marker of oxidative stress, were not affected by OSPW exposure (Figure 3.6a and b). The *vtg* findings suggest that developmental OSPW exposure did not have an estrogenic effect on the embryos. Similarly, OSPW exposure did not induce *sod1* expression, indicating that exposure was likely not increasing production of ROS or causing an increase in oxidative stress.

Cytochrome P4501A and 1B expression

The biotransformation enzyme genes *cyp1a* and *cyp1b* were upregulated by raw OSPW exposure, but were only slightly affected by ozonated OSPW exposure (Figure 3.7, data for 100% raw and ozonated OSPW exposures from Lyons et al. (2018)). The 25, 50 and 100% raw OSPW exposures led to an upregulation of both *cyp1a* and *cyp1b* ($F_{6,34} = 81.3$, $p<0.001$ and $F_{6,34} = 67.9$, $p<0.001$, respectively). Exposure to 100% ozonated OSPW slightly induced the expression of *cyp1b* to a fold change of less than 2 ($p<0.05$) and did not affect the expression of *cyp1a*. Both the 1% raw and 50% ozonated OSPW exposures did not induce CYP transcription. The threshold exposure level for significant CYP induction for raw OSPW exposure was between 1 and 25%, while for ozonated OSPW it was closer to 100% exposure.

Recovery of *cyp1a*/1B expression

The expression levels of biotransformation enzymes *cyp1a* and *cyp1b* in larval zebrafish decreased to normal levels relatively quickly after the termination of OSPW exposure. After 2 d of recovery in clean water, at 9 dpf, the expression of *cyp1a* and *cyp1b*, which were upregulated by 50 and 100% raw OSPW exposure at 7 dpf, had a significantly lower expression ($p<0.001$) that was similar to the control (Figure 3.8b). After 4 d of recovery in clean water, expression of these genes was again very similar to those of control, but not different from the levels measured at 9 dpf (Figure 3.8c).

Discussion

Daily growth and yolk sac measurements

In our study, we found that by the end of the exposure period, neither type of OSPW exposure affected the overall length of embryos. Growth and size are both critical for survival and are parameters routinely used to gauge fish health (Pepin, 1991; Quinn and Peterson, 1996). However based on this study, it appears that OSPW exposure during the embryonic stage of a fish's lifecycle does not necessarily affect growth. This indicates that development may not be delayed by OSPW exposure. Another study also found that growth over 7 d in fathead minnows was unaffected by OSPW exposure (Siwik et al., 2000).

We found no difference in yolk sack area between treatment groups at 2 or 3 dpf and no difference in yolk absorption between the two days. However, previous studies have found that exposure to metals and hydrocarbons affect yolk absorption (Johnson et al., 2007; Yamauchi et al., 2006). These studies found that yolk sac area was larger in exposed groups, suggesting a delay in yolk sac absorption and development, though fish in these studies were exposed to higher concentrations of OSPW constituents than in our study. Yolk sac absorption and length are often linked since reduced nutrient uptake from the yolk sac to other tissues often leads to a decrease in length/size of an embryo (Yamauchi et al., 2006). The lack of an effect on both overall length and yolk sac absorption in exposed fish in our study suggests that exposure did not delay zebrafish development during the embryonic stage.

Otolith measurements

Otolith area at hatch and embryo length at 7 dpf in our study were not different between treatment groups, though, embryo length at hatch and otolith size at hatch are indirectly correlated and, therefore, do not necessarily coincide, as the area inside the otolith hatch check is more of a measure of metabolic rate than fish size (i.e. faster metabolism leads to larger area at hatch) (Bang and Grønkjær, 2005; Bochdansky et al., 2005). Others have found that there is often large variation in otolith size at hatch, even between the left and right otoliths in a pair within an individual fish (Bang and Grønkjær, 2005), making it difficult to establish differences in sizes between treatment groups.

Though there was no difference in otolith area at hatch in our study, otolith area at 7 dpf was slightly smaller in exposed groups (both raw and ozonated OSPW) vs. control. This could be due to different calcium deposition rates between exposure groups. Previous studies have found that stress causes a reduction in otolith ring widths (Molony and Choat, 1990). The stress of OSPW exposure may be the reason we found a reduced otolith size in 7dpf exposed fish. Ozone treatment did not appear to negate the effect that raw OSPW exposure had on otolith area. This indicates that total NAs in OSPW were not responsible for the effect. This slight reduction may be an indication of lowered metabolic rate, but its impact on fish health would likely be small or absent.

With respect to the use of otolith growth as a growth metric in larval zebrafish, our results indicate the potential to use otoliths to back-calculate size-at-age in zebrafish embryos/larvae. However, more data needs to be collected to determine its applicability to generate accurate estimates. Because the otoliths used in this study are from such young fish, it would be beneficial to compare them with otoliths from older fish to be able to better determine the hatch check as well as whether fish growth and otolith growth are linear or not. The back-calculation equation that was used in this study is based on a linear relationship between fish growth and otolith growth. It is possible for the relationship in these otoliths/embryos to be allometric/non-linear, as previous studies have indicated that for the first few days of life, otoliths may not grow in a linear fashion (Vigliola et al., 2000; Wilson et al., 2009). Because the back calculation did not completely agree with the actual length of embryos throughout the exposure period, more research is needed to determine if using an allometric back-calculation equation would provide more accurate estimates. We are confident in the technique used in this

study to determine size-at-age since it's a commonly used model and is also commonly used in larval fish (Vigliola and Meekan, 2009), yet we found that some back-calculated sizes did not match actual fish size. For this reason, more data needs to be collected to determine the correct formula to use to calculate size-at-age based on otoliths for these fish during their embryonic stage.

While daily measurements from fish size were taken from 2-7 dpf, we could only be confident in otolith measurements from hatch (2 dpf) to 5 dpf as no daily structures were observed for 6 or 7dpf. There is possibly a lag time between otolith deposition and fish growth in zebrafish embryos, as has been found in other fish species (Molony and Choat, 1990; Yokouchi et al., 2011). More research would be needed to determine the daily deposition rates in zebrafish otoliths to understand the implications of time lag. Another limiting factor in embryonic daily back-calculations is that back calculating over a short time period using average values may not be accounting for individual variability in size-at-hatch, which affects the confidence in back-calculated estimates. Also, since the time frame for these back calculations was so short, any error in measuring distance between rings would have lead to a large error in calculations, where as when back calculations are made over a longer period of time, these slight errors in measuring are not as significant since the measurements coincide with longer amounts of time and larger lengths.

Neurodevelopment gene expression

Though many environmental contaminants, including crude oil, are neurotoxic to fishes (Akaishi et al., 2004; Bailey et al., 2013; Brown et al., 2016; de Soysa et al., 2012), we did not find any significant changes in expression levels of the neurodevelopment genes *ngn1* or *neuroD*. This indicates that the OSPW sample used in this study did not have a neurotoxic effect on embryonic zebrafish, though these findings do not completely rule out the possibility of neurological impacts of OSPW exposure. Despite there being no significant changes, there appeared to be a dose-dependent downward response in the expression levels of these genes with increasing raw OSPW exposure. Future study should investigate the expression of these genes earlier in development (i.e. 0-3 dpf) when these genes would be more highly expressed (Korzh et al., 1998; Mueller and Wullimann, 2002). Furthermore, functional tests should also be carried out to determine if there is any change in behavior due to OSPW exposure. Behavior is often an

overlooked endpoint, though it has important implications on a fish's survival in the environment and is an indicator of neurological effects (Tierney, 2011).

vtg and *sod1* gene expression

Our study did not find a change in *vtg* expression with exposure to either raw or ozonated OSPW. The expression of *vtg* is commonly used as a biomarker of exposure to estrogenic compounds and has been linked to fish population decline (Chow et al., 2013; Gagné et al., 2012; Heppell et al., 1995; Kidd et al., 2007). Previous studies have found endocrine disruption in fishes exposed to OSPW (Gagné et al., 2012; He et al., 2012b; Wang et al., 2015; Wiseman et al., 2013b). Wang et al., (2015) found upregulation of *vtg* from NAs extracted from the oil sands in 7 dpf zebrafish. However, Reinardy et al., (2013) found that total NAs (up to 2 mg/L) extracted from OSPW did not induce *vtg* expression, though subfractions of OSPW NAs did very slightly induce the expression of *vtg*. The differences in compounds found in OSPW samples and differing effects between species may be a reason for differing results between studies (Frank et al., 2016; Hughes et al., 2017).

We also didn't find a change in *sod1* gene expression levels between treatment groups. An increase in *sod1* expression would indicate an increase in reactive oxygen species (ROS) production resulting in oxidative stress. Previous studies have found an increase in the expression of *sod1* in fishes exposed to OSPW (Alharbi et al., 2016; Gagné et al., 2012; He et al., 2012a). However, some studies, like ours, have found no change in *sod1* expression due to OSPW exposure (Marentette et al., 2017). This would likely indicate that exposure to the OSPW used in our study was not causing an increase in oxidative stress at the end of the exposure period, and again, the use of different OSPW samples between studies and the variable heterogeneous nature of OSPW can lead to differing results (Frank et al., 2016; Hughes et al., 2017).

Cytochrome P4501A and 1B expression and recovery

Our study found that *cyp1a* and *cyp1b* were upregulated by raw OSPW but less so by ozonated OSPW, indicating that ozonation significantly reduced the fraction of the OSPW that causes induction of CYP enzymes. Some studies have found that *cyp1a* is upregulated by exposure to OSPW and its constituents (Gagné et al., 2012; Marentette et al., 2017; Wiseman et al., 2013a), however, others have found that *cyp1a* remains unaffected by OSPW exposure (Alharbi et al.,

2016; He et al., 2012a). The upregulation of *cyp1a* and *cyp1b* found in our study indicates that there were agonists (inducers) present in the OSPW of the aryl hydrocarbon receptor (AhR), though the expression levels of these genes are often used simply as biomarkers to determine whether exposure has occurred, rather than markers of effect.

We used multiple dilutions in our exposures to mimic the OSPW concentrations that could occur in a spill scenario as well as to determine at what dilution biological responses arose. The 1% raw OSPW exposure did not induce *cyp1a* or *cyp1b* expression levels, indicating that the threshold for biological effects is at lesser dilutions (between 1% and 25% raw OSPW).

The expression levels of cytochrome P450s have not previously been monitored during a recovery period after OSPW exposure. The quick return to control expression levels that we found after exposure ended indicates that embryos may be able to rapidly metabolize OSPW constituents during acute OSPW exposure, at least with exposure to our OSPW sample. Some environmental studies found that *cyp1a* remained elevated in fish near marine oil spills compared to reference sites for up to 10 years after the spill occurred (Dubansky et al., 2013; Jewett et al., 2002; Jung et al., 2011). However, direct measures of CYP recovery in clean water after toxicant exposure has never been monitored before. The recovery and long term effects of developmental exposure in fish are important when characterizing the effects of a toxicant, though they are often overlooked due to time and space challenges. This recovery of CYP expression in zebrafish embryos indicates that compounds in OSPW itself are potentially not having long-lasting effects on embryos, though this does not rule out that irreversible effects may have occurred during the exposure period.

Conclusion

Developmental OSPW exposure was not overtly toxic to zebrafish embryos. Growth, yolk absorption and otolith size at hatch were unaffected by exposure and the expression of many target genes remained unaffected. At 7 dpf, otoliths were smaller in exposed embryos, perhaps due to a lag time in deposition, reduced metabolic rate, or thinner layers being deposited on the otolith. Though otolith size was slightly reduced, which indicated a small, likely harmless, physiological effect, the actual size of embryos was the same at 7 dpf. The embryos appeared to recover relatively quickly after the exposure ended (using biotransformation enzyme expression as a measure). This is a promising result, indicating that larval fishes may be able to rapidly

metabolize and excrete compounds present in OSPW. The caveat is that other sources of OSPW may contain higher concentrations of biologically active compounds and there would be a threshold where biotransformation and excretion would be overwhelmed.

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Tables

Table 3.1: qPCR primers and accession number or reference.

Abbreviation	Target Gene name	Forward Sequence (5'-3')	Reverse Sequence (5'-3')	Accession Number or Reference
βactin	Beta-Actin	CGA GCA GGA GAT GGG AAC C	CAA CGG AAA CGC TCA TTG C	AF057040
cyp1a	Cytochrome P4501a	AGG ACA ACA TCA GAG ACA TCA CCG	GAT AGA CAA CCG CCC AGG ACA GAG	NM_131879 (Timme-Laragy et al., 2007)
cyp1b	Cytochrome P4501b	CCA CCC GAA CTC TGA AAC TC	AAA CAC ACC ATC AGC GAC AG	NM_001013267 (Timme-Laragy et al., 2007)
sod1	Cu/Zn-Superoxide dismutase 1	CGT CTA TTT CAA TCA AGA GGG TG	GAT GCA GCC GTT TGT GTT GTC	Behrendt et al., (2010)
vtg	Vitellogenin	CTG CGT GAA GTT GTC ATG CT	GAC CAG CAT TGC CCA TAA CT	AF406784.1 (Kanungo et al., 2012)
ngn1	Neurogenin1	TGC ACA ACC TTA ACG ACG CAT TGG	TGC CCA GAT GTA GTT GTG AGC GAA	NM_131041 (Fan et al., 2010)
neuroD 1	Neurogenic differentiatio	CAG CAA GTG CTT CCT TTT CC	TAA GGG GTC CGT CAA ATG AG	Kanungo et al., (2013)

	n1

Figures

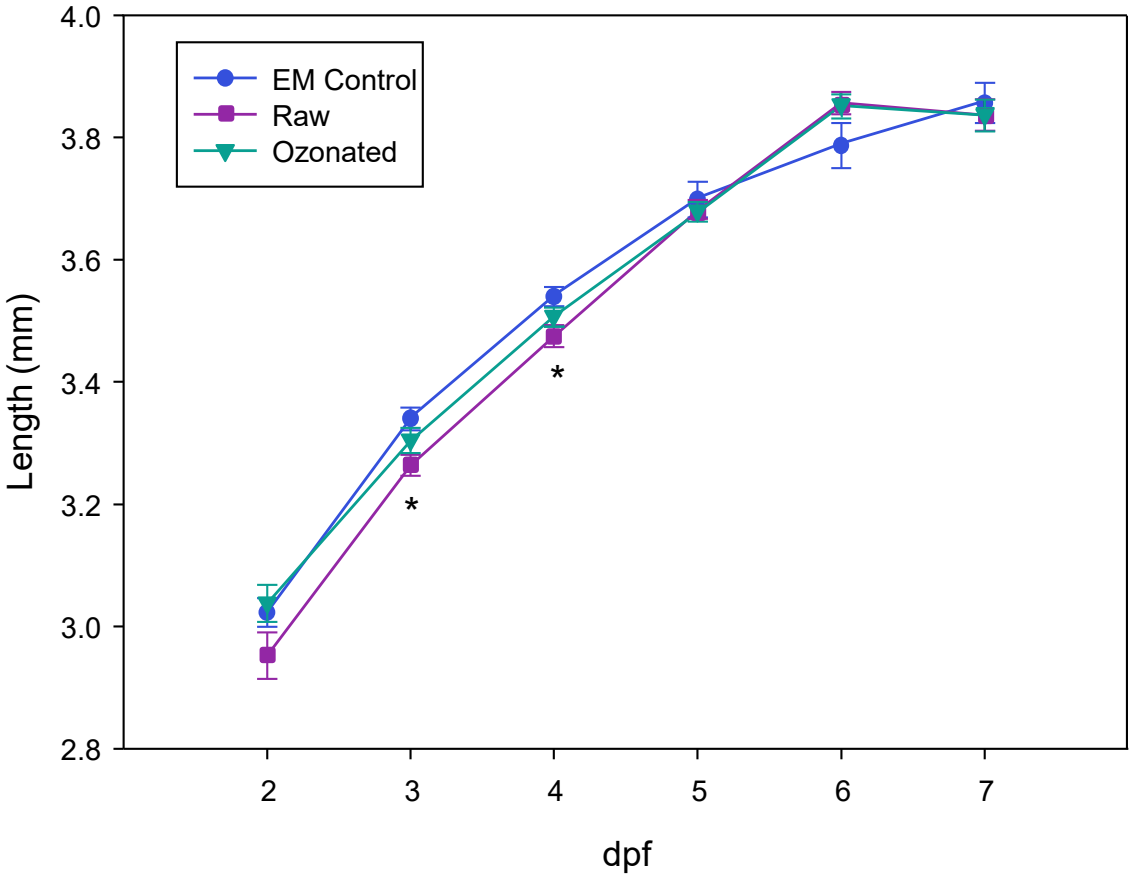

Figure 3.1: Daily growth of embryos exposed to raw and ozonated OSPW from 2-7 days post fertilization (dpf). Raw OSPW exposed embryos were slightly shorter in length than EM control embryos at 3 and 4dpf, while ozonated OSPW exposed embryos were no different in length compared to control (one way ANOVA and Tukey's post hoc, * indicates $p<0.05$ for raw OSPW compared to control).

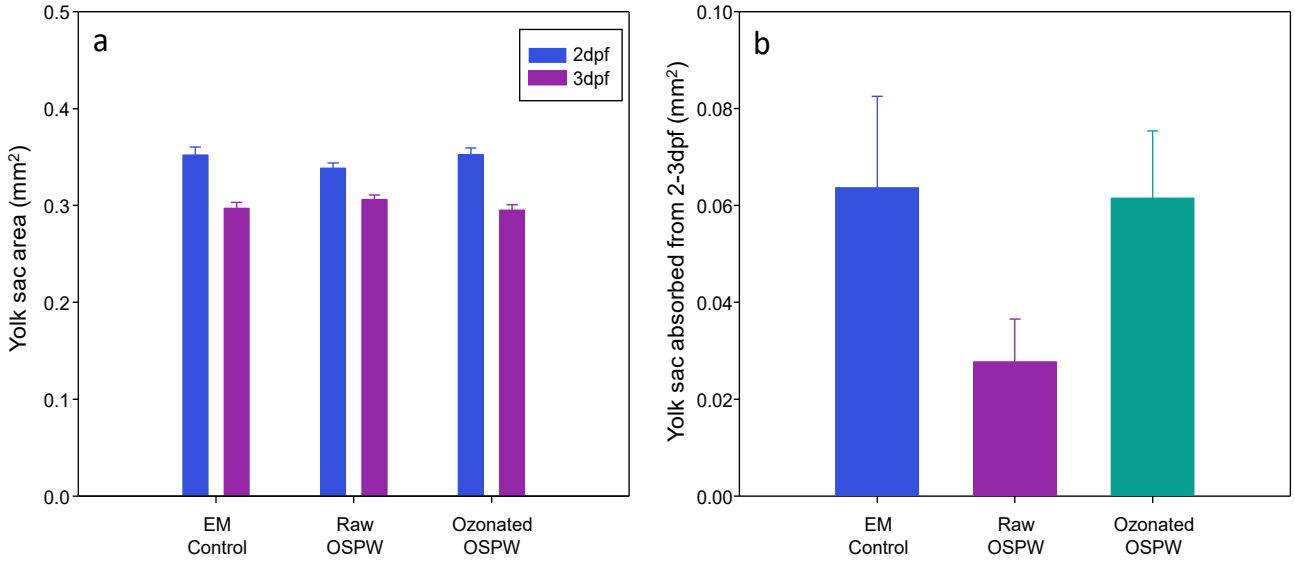

Figure 3.2: Yolk sac area of 2 and 3dpf embryos exposed to raw and ozonated OSPW. (a) Yolk sac area at 2 and 3 dpf; (b) area of yolk sac absorbed from 2 to 3 dpf (one way ANOVA).

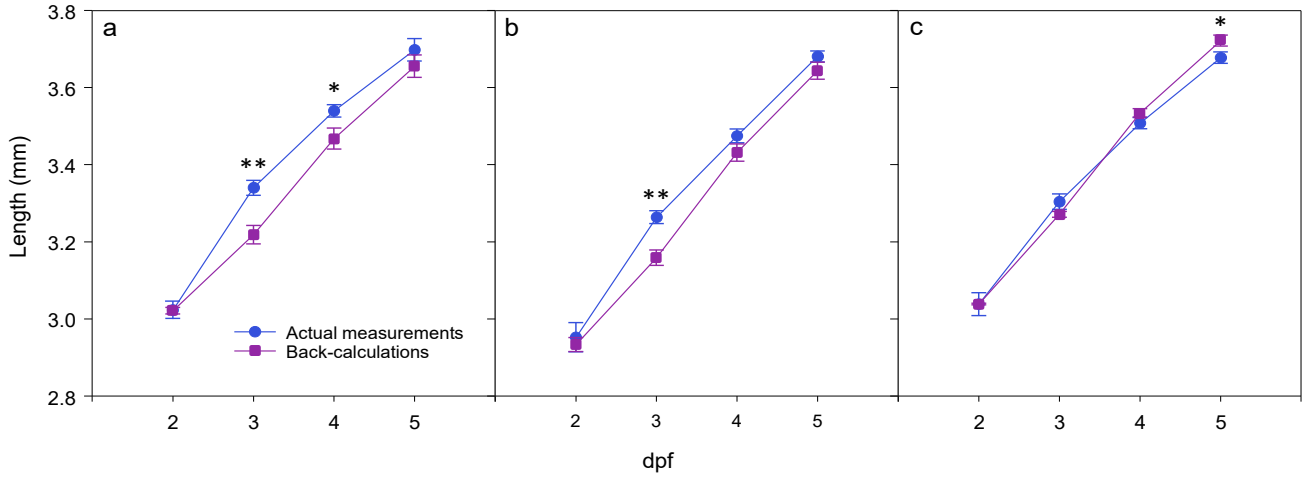

Figure 3.3: Embryo otolith back-calculations used to measure size-at-age and actual embryo length measurement for a) EM control; b) raw OSPW exposed embryos; and c) ozonated OSPW exposed embryos (t-test, $p<0.05$, * indicates $p<0.05$ and ** indicates $p<0.001$ for back-calculations compared to actual measurements).

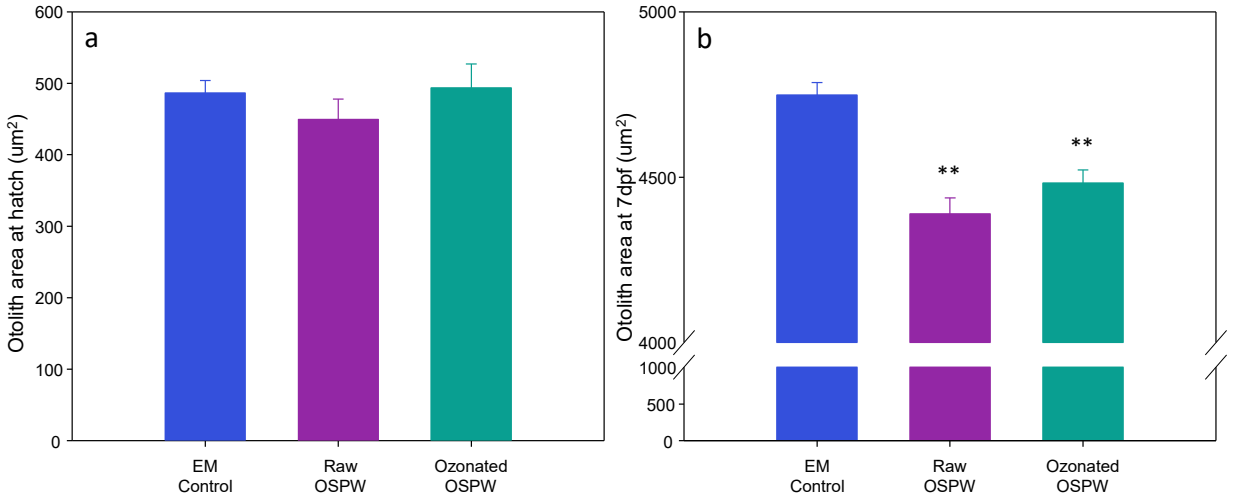

Figure 3.4: Area of zebrafish embryo otoliths exposed to raw and ozonated OSPW. a) Otolith area at hatch; b) otolith area at the end of the exposure period (7dpf) (one way ANOVA, Holm-Sidak post-hoc, ** indicates difference from control $p<0.001$).

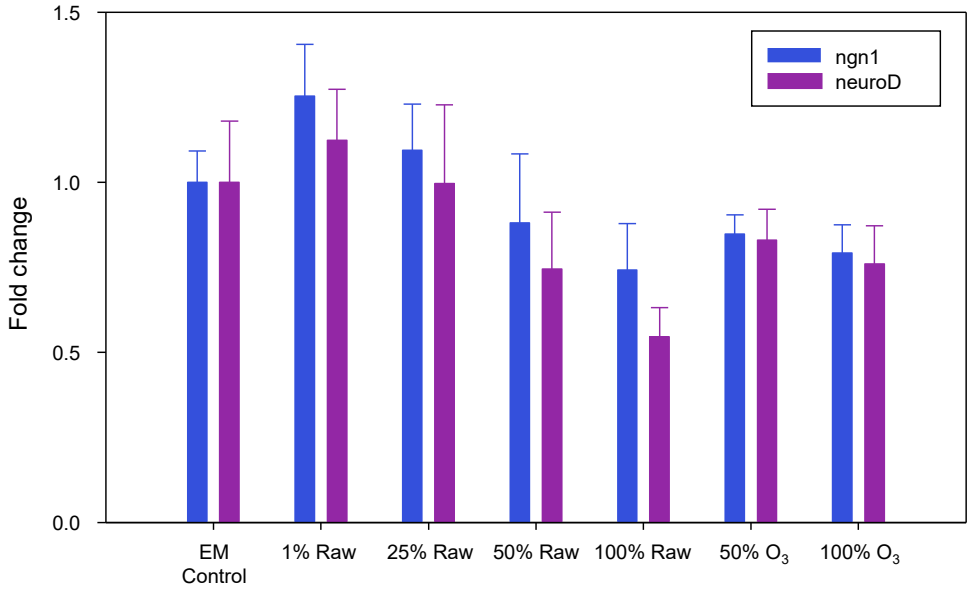

Figure 3.5: Expression levels of neurodevelopment genes *ngn1* and *neuroD* in 7dpf embryos exposed to 1, 25, 50, and 100% raw OSPW and 50 and 100% ozonated OSPW (one way ANOVA).

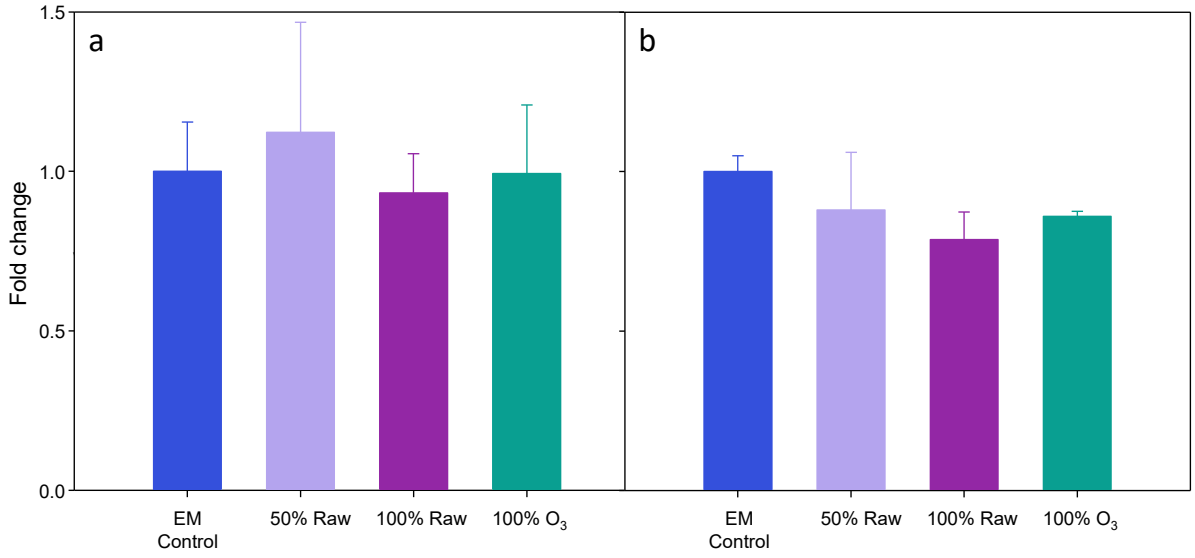

Figure 3.6: Expression of *vtg* (a), a marker of exposure to estrogenic compounds, and *sod1* (b), a marker of oxidative stress, in 7dpf embryos exposed to 50 and 100% raw and 100% ozonated OSPW (one way ANOVA).

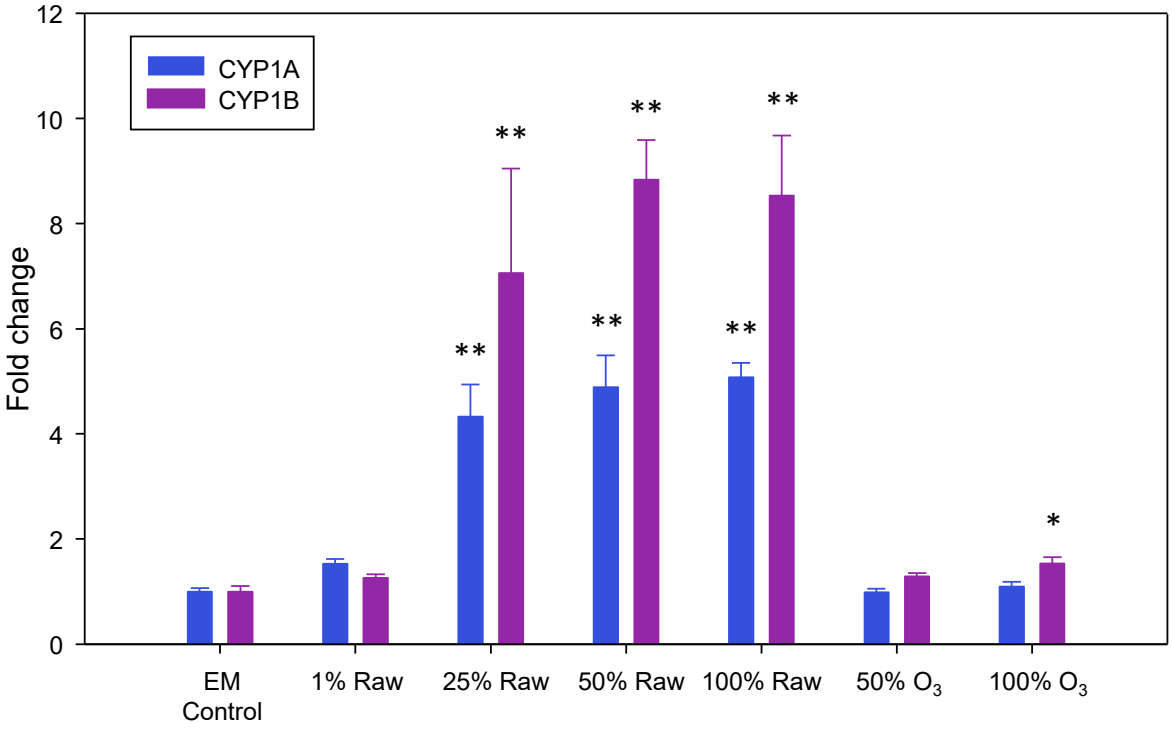

Figure 3.7: Expression levels of biotransformation enzymes *cyp1a* and *cyp1b* in 7 dpf embryos exposed to 1, 25, 50 and 100% raw OSPW and 50 and 100% ozonated OSPW (data for EM Control, 100% raw, and 100% ozonated also shown in Figure 1 in Lyons et al. (2018), shown here for comparison to other exposures; one way ANOVA and Tukey's post hoc, * indicates $p<0.05$, ** indicates $p<0.001$).

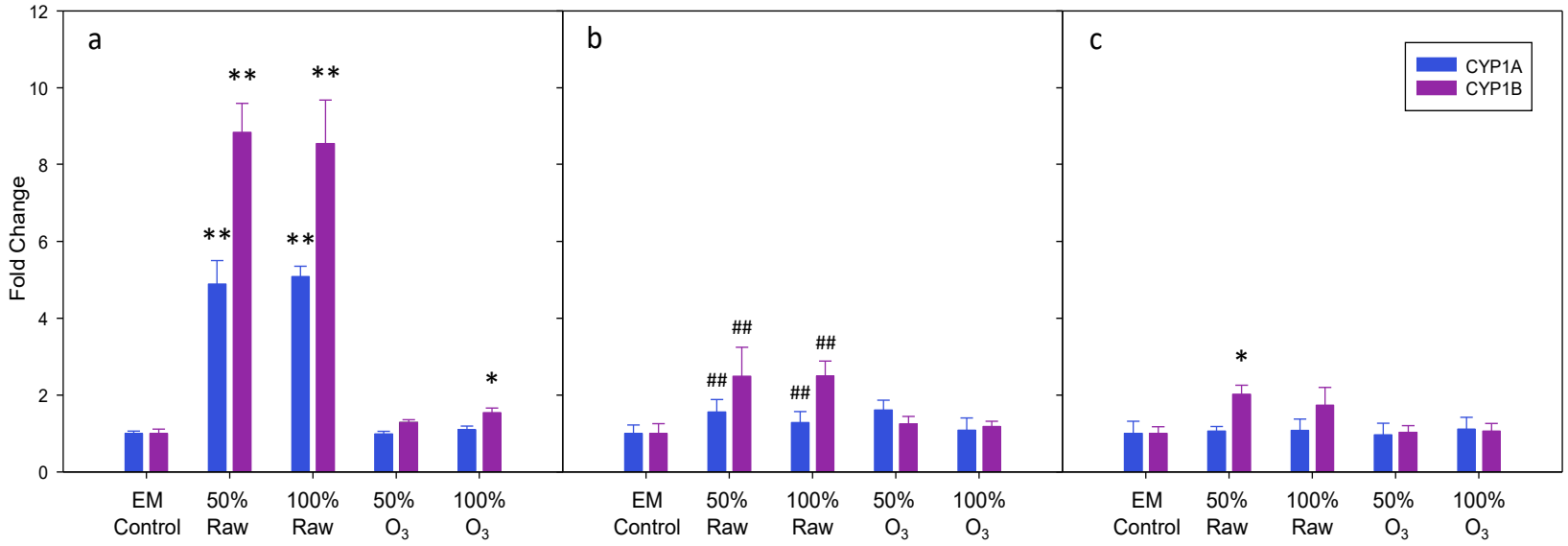

Figure 3.8: Expression levels of biotransformation enzymes *cyp1a* and *cyp1b* at 7 (a), 9 (b), and 11 days post fertilization (dpf) (c) after exposure to 50 and 100% raw and 100% ozonated OSPW from 0-7dpf (data in 6a from Figure 4, shown here for comparison; one way ANOVA and Tukey's post hoc, * indicates $p<0.05$ and ** indicates $p<0.001$ when compared to control within time point; two way ANOVA, ## indicates $p<0.001$ when compared to previous day.

Chapter 4: Effects of field-collected crude oil, weathered oil and dispersants on the early life stage of model fresh and saltwater fishes

Abstract

The Deepwater Horizon (DWH) oil spill was the biggest in US history and released large volumes of light crude oil into the Gulf of Mexico. The effect a spill of this magnitude would have on aquatic life was largely unknown. In this study, we compared the toxicity of water accommodated fractions (WAFs) of naturally weathered crude oils, source oil, and source oil with dispersant mixtures to developing sheepshead minnow and zebrafish. Although a freshwater fish, zebrafish have been used as a model for marine oil spills owing to the molecular and genetic tools available, their ability to have developmental consistency, and their amenability to lab care. Our study not only aimed to determine the effect of crude oil on early life stages of these two fish species, but also whether dissolved crude oil constituents were similar in fresh and saltwater, and if freshwater fish might be a suitable model to study marine spills. The chemistry of the WAFs differed across oil types but were consistent across water matrices except for the saltwater, source oil plus dispersant WAF. The addition of dispersant increased PAH concentration in the saltwater WAF to a larger extent than in freshwater. WAF exposure had varied effects on survival, heart rate and the expression of biotransformation biomarkers, vitellogenin and neurodevelopment genes in both species. Muscle deformations were only found in oil-exposed zebrafish. This is one of the most comprehensive studies to date on crude oil toxicity; it includes unweathered, moderately and heavily weathered crude oil and dispersants and directly compares the PAH and VOC profiles of these WAFs and their effects on the early life stage of two model fishes. This study highlights the species-specific differences in cardiotoxicity, estrogenic effects, biotransformation enzyme induction and potential neurotoxicity of crude oil exposure.

Introduction

The subsea release of oil from the MC252 well in the Gulf of Mexico in 2010 was the first incident where dispersants were injected directly into the rising flow of oil and gas (Place et al., 2010). The amount of oil released during the 87 day blow out was determined to be 3.19 million barrels based on a ruling by the U.S District Court for the Eastern District of Louisiana (United States District Court for the Eastern District of Louisiana, 2015). After two test periods, after May 15th dispersant was injected continuously to reduce oil droplet size and prevent the formation of large surface slicks until the well was capped 61 days later July 15th (Kujawinski et al., 2011). Macondo oil, a light non-viscous crude, travelled 1500 m up the water column and underwent a series of compositional changes due to both biological and physical weathering, characterized by dissolution, emulsification, evaporation, biodegradation, photo-oxidation and dispersion (Wang et al., 2013). During the spill, 1.1 million gallons of the chemical dispersant Corexit 9500 was aerially applied to surface slicks and 0.77 million gallons were applied directly at the wellhead to assist biological and physical weathering (Lehr et al., 2010). Dispersant has previously been used on large scale releases, like Exxon Valdez, but was only applied to the surface and was not injected at depth. Dispersant application, as used in the DWH spill, increases the exposure of aquatic organisms to dispersed oil droplets in the water column (National Research Council (U.S.), 2005). Laboratory studies have found that dispersants increase the toxicity of crude oil to fishes via increasing the aquatic concentration of polycyclic aromatic hydrocarbons (PAHs) (Adeyemo et al., 2015; Anderson et al., 2009; Couillard et al., 2005; Mu et al., 2014; Ramachandran et al., 2004; Schein et al., 2009). Most evidence suggests that PAHs are the primary source of aquatic toxicity in crude oil (Hodson, 2017).

Dispersants are not the only factor that could influence the toxicity of a given crude oil, the type and degree of weathering can also influence the exposure of PAH profiles and other oil constituents in the water column. The majority of weathered oil studies use artificially weathered oil, in which oil is weathered in the laboratory using heat (Brette et al., 2014; Carls et al., 2008; Frantzen et al., 2015; Heintz et al., 1999; Incardona et al., 2006, 2009, 2014) or the oil sample is sparged until approximately 20% of the sample volume is lost (Couillard et al., 2005; Ramachandran et al., 2004). The natural weathering of crude oil includes not only heating and ‘sparging’, but a wide range of processes that include emulsification, microbial degradation,

evaporation and photo-oxidation (Wang et al., 2013). Oil type, dispersant application and weathering can all impact the PAH profile in the water and it is very unlikely that all PAHs exhibit toxicity through the same mechanism of action (Hodson, 2017).

Of the studies on oil toxicity, many have focused on the sublethal effects of oil exposure on developing fishes. Of these, studies of effects related to cardiac morphology and cardiac output are prominent (Carls et al., 2008; Incardona et al., 2004, 2006, 2009, 2011, 2015). Cardiac impairment caused by oil exposure has also been linked to alterations in swimming behavior and decreased swimming performance (Hicken et al., 2011; Incardona et al., 2013; Kennedy and Farrell, 2006; Mager et al., 2014; de Soysa et al., 2012; Yu et al., 2015). Whole organism cardiac effects have been linked to gene expression changes in cardiac biomarkers (ex. *nkx2.5*) (Incardona et al., 2015). Other biomarkers commonly used in crude oil studies, such as cytochrome P450 (e.g. Cyp1a), not only play a role in cardiotoxicity, but also participate in PAH biotransformation (Barron et al., 2004).

In our study, we directly compared the toxicity of field-collected naturally weathered oil, source oil (without weathering), and source oil and dispersant mixtures between zebrafish (*Danio rerio*) and a common saltwater model, sheepshead minnow (*Cyprinodon variegatus variegatus*). Both fresh and saltwater fishes have been used to study crude oil toxicity despite marine spills being both larger in magnitude and vastly more common. Using zebrafish as a model fish species has many advantages to typical saltwater models; they have a short generation time, are highly fecund, are developmentally well-characterized and more genetic tools are available (Driever et al., 1994). There have been many studies that use zebrafish to study the effects and mechanisms of crude oil toxicity (Incardona et al., 2013; Jung et al., 2013; Pauka et al., 2011; Perrichon et al., 2016; Raimondo et al., 2014; de Soysa et al., 2012), however, the applicability of these studies to large scale marine spills is largely undetermined. Our study aimed to determine if water accommodated fractions (WAFs), gene expression changes and the physiological effects remained consistent across both fresh and saltwater model species, and if there was merit to using a freshwater model to investigate toxicity in marine oil spills.

Results

Water accommodated fractions

There were differences in the WAF composition across water types (fresh- and saltwater) and treatment groups (oil type and dispersant). In the freshwater WAF, source oil and the source oil + dispersant had comparable concentrations of naphthalenes, with abundance decreasing as the number of side chains increased (Figure 4.1A). A similar trend was present in saltwater WAFs, except that the addition of dispersant resulted in higher concentrations of branched naphthalenes (N2-N4) (Figure 4.1B). This indicates that there was an interaction between saltwater and dispersant that solubilized larger naphthalenes. All weathered oil WAFs had low naphthalene concentrations.

The concentration of polycyclic aromatic hydrocarbons (PAHs) differed across both water and oil types. Moderately weathered oil (weathered oil A) had a higher PAH content than the heavily weathered oil (weathered oil B) in both the fresh and saltwater WAFs (Figure 4.2A, B). Parent compounds and lightly branched PAHs (0-1) were more prevalent than highly branched PAHs (2-4) for fresh and saltwater WAFs. Biphenyl, fluorene, and phenanthrene/anthracene concentrations were highest in the source oil and source oil + dispersant freshwater WAFs (Figure 4.2A). The saltwater source oil + dispersant WAF had more than a 2-fold increase in PAH content compared to the freshwater counterpart. Weathered oil A (WO A) + dispersant (saltwater treatment only) had higher PAH levels than WO A alone and was comparable to the source oil treatment. This trend also existed for benzothiophenes, dibenzothiophenes, and many other PAHs that were found in the WAFs (Figure 4.3, 4.4). This again indicates that the dispersant was better at solubilizing compounds in salt- vs. freshwater.

Volatile organic compounds (VOCs; dimethylbutane, methylpentane, hexane, methylhexane, heptane, dimethylhexane, methylheptane, ethylhexane, octene, and octane), were only detected in the source oil and source oil + dispersant WAFs for both freshwater and saltwater exposures (Figure 4.5A, B, Figure 4.6). The benzene, toluene, ethylbenzene and xylene (BTEX) content was relatively constant across the oil mixtures and water matrices (Figure 4.5A, B). Pentane, hexane and benzene-based compound concentrations were relatively consistent between source oil and source oil + dispersant treatments for both fresh and saltwater WAFs.

Survival

Source oil + dispersant was 100% lethal to both zebrafish and sheepshead minnow embryos; the other treatment groups had no effect on survival over the course of the 0-7 and 1-10 dpf exposures; respectively (data not shown). The LC50 for the zebrafish and sheepshead minnow exposures were 44.9 % WAF (95% confidence interval (C.I.) 42.1-47.9) and 16.8 % WAF (95% C.I. 13.7-20.5); respectively. Across all three target lipid models (Kow I, Kow I,II, and Kow pp-TLM), there was no overlap between the zebrafish and sheepshead minnow survival curves (Figure 4.7). According to all models, sheepshead minnow had a lower toxic unit LC50 than zebrafish, which means that on a per toxic unit basis, sheepshead are more sensitive to exposure. The critical body burden value used to calculate the toxic units in the WAFs was derived from literature, and should have adjusted for the differences in sensitivity between these two species. The mortality curves for the species did not overlap, which suggests that the critical body burdens derived from the literature is not optimized for early life stages in these species.

Gene expression

There were differences in the gene expression levels across species and treatments. The biotransformation enzymes Cyp1a and CYP1b were upregulated by all WAF and B[a]P (positive control) exposures; source oil exposure induced the highest Cyp1a and CYP1b fold change in the zebrafish embryos ($F_{7,33} = 55.27, p < 0.05$; $F_{7,33} = 53.04, P < 0.05$; respectively) (Figure 4.8A). The Cyp1a expression in sheepshead minnow was only affected by exposure to B[a]P, weathered oil B, source oil, and source oil + dispersant exposure ($F_{8,29} = 7.25, p < 0.05$) (Figure 4.8B). Source oil + dispersant exposure induced the highest level of Cyp1a expression in the sheepshead minnow. Weathered oil B, B[a]P, source oil + dispersant WAF exposure induced Cyp1a expression to comparable levels between the two species; source oil induced a larger fold change in Cyp1a in zebrafish than in the sheepshead minnow.

In zebrafish, WAF exposure had no effect on the expression of *vtg* (Figure 4.8C). In contrast, for sheepshead, source oil WAF increased *vtg* expression ($F_{8,29} = 7.19, p < 0.05$) (Figure 4.8D), indicating that sheepshead may have been more sensitive to estrogenic compounds, or that saltwater increased estrogenic constituents or their uptake.

WAF exposure had no effect on the expression of the neural development gene *neuroD* in either species (Figure 4.8E, F). However, in zebrafish but not sheepshead, *ngn1* was downregulated in source oil + dispersant exposures (; $F_{7,33} = 11.67, p < 0.05$). *gli2a* expression was also measured in zebrafish and found that it was upregulated by weathered oil B exposure ($F_{7,33}$ = 3.8 $p<0.05$).

Heart and muscle development

Cardiac responses differed between the zebrafish and sheepshead minnow embryos. WAF exposure increased the heart rate of zebrafish embryos ($F_{5,172} = 15.39, p < 0.001$) (Figure 4.9A), whereas in sheepshead, source oil exposure decreased the heart rate (Q=2.79, $p<0.05$) (Figure 4.9B). *nkx2.5* expression was not altered by exposures. (Figure 4.9C).

Muscle deformities were observed in freshly hatched 2 dpf fish exposed to weathered oil, source oil and source oil with dispersant WAFs (Figure 4.10). The deformities ranged from abnormal myoseptal divides (Figure 4.10E), hemorrhaging along the trunk of the tail (Figure 4.10B, C) and gaps in the muscle striations (Figure 4.10D). The deformities were observed at low frequencies (≤4%) and were only present in the oil-exposed treatment groups, and were most prevalent the moderately weathered oil (weathered oil A) and the source oil + dispersant WAF treatment groups (Figure 4.10F)

Discussion

Our study compared the toxicity of fresh crude oil, moderately and heavily field-collected weathered oil, and oil and dispersant mixtures on the early life stage model freshwater and saltwater fishes. This study compared the WAF chemistry composition, lethality, toxic units, gene expression changes, and heart rate between sheepshead minnow and zebrafish embryos/larvae to determine if a freshwater model could be an effective tool to study marine spills. We found that water salinity contributed to only slight variance in WAF composition except for when a dispersant was used. Dispersant use increased the measured concentrations of PAHs in the saltwater WAF considerably, which likely due to increased oil droplet emulsion (Sandoval et al., 2017). Source oil + dispersant WAF was also the only treatment that impacted embryo/larval survival in both species. Exposure to fresh crude oil was estrogenic to sheepshead minnow, but not zebrafish. On the other hand, exposure altered neurodevelopment gene

expression in zebrafish but not the sheepshead minnow. Exposure also had opposing effects on heart rate; heart rate was increased in zebrafish and decreased in the sheepshead minnow. The absence of change in cardiac gene expression did not correlate with changes in heart rate in the zebrafish, though changes in heart rate have previously been seen in tandem with changes in *nkx2.5* expression (Sun and Liu, 2017). Because of the different WAFs produced in fresh and saltwater and the varying effects seen across these species, this study highlights the importance of using ecologically relevant models to study oil spills.

WAFs and Mortality

Following a spill, the concentrations of dissolved hydrocarbons will depend on many factors, including environmental degradation, the use of a dispersant, and the salinity of the water. Dispersants are designed for use in marine spills and there is very little literature on the effectiveness of dispersant in freshwater as they are not currently approved for use in inland spills (Wrenn et al., 2009). Our study found that the addition of dispersant to source oil increased the naphthalene and PAH content in saltwater more so than in freshwater (Figure 4.1, 4.2). Research has shown that dispersants can be modified to be suited for freshwater conditions, and in the case of a largescale freshwater spill, could potentially be used to minimize the effects of oil slicks on birds and shoreline habitat (Wrenn et al., 2009). The use of dispersants remains controversial, is under scientific and regulatory review, approvals are evaluated in a case by case basis by a variety of stakeholders and interest groups, and come with both environmental and economic tradeoffs. Previous studies have found that dispersants increase the bioavailability of compounds within crude oil, resulting in increased toxicity to fishes (Couillard et al., 2005; Finch et al., 2017; Ramachandran et al., 2004; Schein et al., 2009), though more recent studies have concluded that the increase in measured PAHs is likely due to oil drop emulsions in the WAF (Stefansson et al., 2016).

The analytical chemistry techniques used in most crude oil studies cannot differentiate between dissolved hydrocarbons and oil droplets (Sandoval et al., 2017), which could artificially increase the perceived bioavailability of PAHS in source oil + dispersant WAFs. Regardless, in our study, source oil + dispersant generated the most lethal WAF to both zebrafish and sheepshead minnow. Previous work using a very similar WAF preparation method on the same oil samples for a 48h photo-enhanced toxicity testing of 3 dpf sheepshead minnow found that the %WAF

LC50 was approximately 40% (Finch et al., 2017). Our LC50 %WAF concentration was much lower, likely due to a longer exposure period that began 24hpf and continued onto the free swimming larval stage. The study also ranked various organisms according to their sensitivity as follows; mysid shrimp> inland silverside > sheepshead minnow> gulf killifish (Finch et al., 2017). The study suggests that sheepshead minnow embryos are moderately hardy in response to acute exposures, our studies suggest that they are much more sensitive to longer exposure periods.

In both the fresh and saltwater, source oil and source oil + dispersant exposures introduced more naphthalenes and PAHs to the WAFs than the weathered oils, and weathered oil + dispersant mixtures (Figure 4.1, 4.2). The weathering of crude oil varies between oil spills due to different physical and biological conditions in the spill zone. For most crude oils, 50-70% of the amount of oil released is depleted in the first 10-12 h through spreading of the slick and evaporation of the volatile components (Mackay and McAuliffe, 1989). Weathering of oil has also been shown to decrease the PAH content entering the water column relative to the volume of oil added (Heintz et al., 1999), and can decrease toxicity (Chapman et al., 1995; Shelton et al., 1999). Our study, as with past work, found that weathering decreased the toxicity of the WAF due to the decreased PAH and VOC content in the WAF. On a g/L basis, weathered oil is less toxic than fresh crude.

Because of the availability of molecular tools to study the mechanism of toxicity are unrivaled in the zebrafish, there are many benefits to using zebrafish to study environmental releases. Our study highlights a major pitfall in using zebrafish to study marine spills through a direct side by side comparison with a saltwater model fish with a wide range of oil exposure types. Exposure had a more significant effect on neurodevelopment in zebrafish, was less estrogenic and increased (as opposed to decreased) heart rate. The species also had differing levels biotransformation enzyme upregulation across the exposure groups suggesting the two species are differentially sensitive to PAH exposure.

Oil exposure studies in the literature are very inconsistent, which makes cross study comparisons challenging, and until there is a universal exposure method there is a need for more side by side

comparative studies between species and oil types. A previous study conducted on Inland silverside exposure to the same oil samples included toxic units in their comparison of source oil and weathered oil toxicity (Echols et al., 2016). In the case of complex mixtures like crude oil, detailed analytical chemistry and toxic unit modelling like the target lipid model (TLM) are needed to compare across species, across oil types and across studies to better under the impact of large scale marine spills lie Exxon Valdez and Deepwater Horizon.

In our study we attempted to use toxic units (TUs) in the TLM to account for the different PAH content found in our source oil + dispersant WAFs to directly compare the sensitivity of our two species (Figure 4.7). Critical body burden may vary with age, and because our study used embryos/larvae as opposed to adult fish the critical body burden in literature for these species may not have been appropriate. In our study, we also found that zebrafish and sheepshead minnow embryos/larvae exposed to the same concentration of BaP had very different levels of *cyp1a* expression (Figure 4.7A, B). Because fish species have differing sensitivities to individual compounds, the target lipid model may be optimized for comparing multiple mixtures across the same species as opposed to comparing a mixture across multiple species.

Gene expression

As expected, *cyp1a* and *cyp1b* were upregulated by WAF exposure. Previous studies suggest that CYP upregulation is a concentration dependent response that may serve as a marker of potential immunosuppression, vitamin and hormonal imbalance, and reproductive failure (Safe, 1994; Sanni et al., 2017). Interestingly, sheepshead minnows and zebrafish had significantly different *cyp1a* fold change inductions in response to source oil exposures, though both species had significant upregulation. This may suggest that AhR has a slightly different affinity to binding specific PAHs or that *cyp1a* is generally more inducible in zebrafish than in sheepshead minnow. A previous study found that biomarker responses may be similar between species, however the response magnitude and the concentration required for induction may be species dependent (Sanni et al., 2017). Weathered oil B exposure upregulated *cyp1a* to a higher extent than weathered oil A in sheepshead minnow. This indicates that though weathered oil A and B contain approximately the same PAH content, weathered oil B must contain compounds that more specifically bind to sheepshead AhR than those found in weathered oil A. PAHs can be taken up by many different routes including the gills, skin, and mouth. Sheepshead minnow have

a very thick sticky polysaccharide coating excreted with the eggs' chorion that is used for adhesion to surfaces in nature, however this coat could affect the permeability of the egg to PAHs pre-hatch. Fish accumulate hydrocarbons quickly, and these compounds can concentrate in tissues at concentrations 10-100 times the concentrations found in the surrounding water (Ramachandran et al., 2006). There may be a difference in the permeability of the skin of these embryos/larvae that could also contribute to the differing *cyp1a* induction levels in these two species.

In terms of estrogenicity, WAF exposures did not affect the expression of *vtg* in zebrafish. In contrast, source oil WAF upregulated *vtg* expression in sheepshead. PAHs are only mildly estrogenic compared to contaminants such as organochlorines and dioxins. However, PAHs have the potential to have an agonistic or antagonistic effect on the estrogen receptor (ER), and could artificially induce transcription of the vitellogenin gene (Nicolas, 1999; Stancel et al., 1995; Thomas, 1990; Thomas and Smith, 1993). Life stage and species-specific variation have also been shown to impact the response of *vtg* to a contaminant (Nicolas, 1999), and could explain the differences in the zebrafish and sheepshead minnow responses. Early life *vtg* induction has also been associated with changes in sex ratio in adults (Liao et al., 2009). Whether WAF exposure may be considered and endocrine disrupting compound in embryonic and larval fish remains for study.

In zebrafish only, source oil + dispersant WAF affected *ngn1* expression, while weathered oil B affected *neuroD* expression.. The lack of changes in sheepshead may be due to the large variation in expression between replicates (Figure 4.8F). *ngn1* and *neuroD* are expressed very early on in development with *neuroD* being downstream of *ngn1* in lateral line ganglion (Sarrazin et al., 2006). *neuroD* is also expressed in lateral line neuromasts (Sarrazin et al., 2006), and we found no change in *neuroD* expression in any of our exposures. Supporting these findings, we also found that the exposures had no effect on the number of neuromast hair cells or number of neuromasts (data not shown). The downregulation of *ngn1* expression in the source oil + dispersant WAF exposure could indicate that sensory neuron development was affected, though the downregulation was small and perhaps not biologically significant.

Cardiac effects

nkx2.5 is an essential transcription factor involved in cardiac development (Staudt and Stainier, 2012). *nkx2.5* along with other cardiac development genes have previously been found to be downregulated in fish larvae exposed to PAHs (Incardona et al., 2015; Zhang and Yan, 2014). Our results show no change in *nkx2.5* expression with exposure to WAFs. In terms of heart rate, WAF exposure had opposing effects between the two model fishes: source oil WAF increased heart rate in zebrafish and decreased it in sheepshead. The sheepshead results align with the literature, as previous studies have found that PAH exposure decreased cardiac function (Incardona et al., 2009, 2012; Linden, 1976; Middaugh et al., 1996; Shen et al., 2010; Tissier et al., 2015; Zhang and Yan, 2014). The increase in heart rate observed in the source oil-exposed zebrafish was unexpected, however it was also reported in another study that compared dilbit toxicity to conventional crude oil (Philibert et al., 2016). Species-based differences in cardiac sensitivity to these complex mixtures could play a role in determining the effect of WAF exposure.

Muscle deformities

A study from de Soysa *et al.* found that oil exposure caused muscle deformities in zebrafish larvae, but they did not report the frequency of deformities (de Soysa et al., 2012). In our study, we found various deformities in muscle structure and hemorrhaging along the trunk of the tail of 2 dpf zebrafish embryos/larvae exposed to WAFs, but these deformities occurred at low frequency ($\leq$4%). This finding highlights the importance of reporting frequency when examining effects of exposure. Frequency is rarely reported in histological endpoints in crude oils studies, which may misrepresent findings and toxicological effects as more severe than they are. Regardless, these data indicate that exposure to both weathered and unweathered crude oil can increase the frequency of muscle malformations in early life stages of fishes.

In conclusion, there are many challenges faced when studying the biological effects of complex mixtures that crude oil spills can generate. The rapid weathering of crude oil, potential strategies to mitigate spill effects and the various ecosystems affected can provide considerable uncertainty on predicting effects. Overall, we found that the PAH content in WAFs from weathered oil was very low and associated with no obvious adverse effects. The addition of dispersant to source oil amplified observed toxic effects through the increased dissolution of PAHs and oil droplet

emulsions, which was more pronounced in saltwater than freshwater. However, laboratory WAF preparations do not allow for the dispersion of droplets that occurs in an actual marine spill. The TLM model did not account for the differences in toxicity seen across fish species, likely because it has not been optimized for embryological/larval fish models. Through the various endpoints included in the study we found that zebrafish respond similarly to exposure as sheepshead in some ways, but there was very little consistency, and so researchers should consider prioritizing environmentally relevant models in their study design.

Materials and Methods

Oil sources

Exposures were prepared using three different oil types: an un-weathered Macondo oil collected directly from the subsea containment system directly above the well-head (source oil), an ~65% weathered oil collected from a large skimming vessel (weathered oil (WO) A), and an ~83% weathered oil sample collected from an alternate slick (WO B). Weathering was determined using the PAH depletion relative to the compound hopane as previously discussed (Prince et al., 1994).

Polycyclic aromatic hydrocarbons (PAHs), alkylated PAHs and petroleum biomarkers were measured using liquid/liquid extraction according to the 3510C EPA method (www.3epa.gov) as previously described (Philibert et al., 2016).

WAF preparation

The oil samples were stored in glass amber bottles at 4°C, and were capped with argon and a Teflon seal. The water accommodated fractions (WAFs) of crude oil were prepared in a 1:1000 oil:water ratio and dispersant was added at a 50 µL/g of oil loading rate. The preparation was done using a non-vortexing method as previously described (Singer et al., 2000). In brief, 1.8 mL of crude oil was added to 1.8L of laboratory made saltwater or embryo media (freshwater) in 2L aspirator bottles (leaving approx. 20% headspace in the bottle). The bottle was then capped with a Teflon plug and set to stir at approximately 100rpm for 20h, left to settle for 4h and then the oil-less fraction of the WAF was collected without any filtration. For the freshwater exposures, the WAF was pH adjusted to 7.2 ± 0.05 using a 0.1M HCl, the saltwater WAF required no pH adjustment. As a positive control, embryos/larvae were exposed to benzo[a]pyrene (BaP), of

which a stock solution was made using 0.07% (v/v) dimethyl sulfoxide (DMSO). Both sheepshead minnow and zebrafish embryos/larvae were exposed to the same concentration: 50μg/L of B[a]P.

Zebrafish and exposures

Embryos were collected from AB strain zebrafish adults kept on a 14h:10 h light dark cycle. The adults were fed a custom mixture of commercial juvenile trout chow, TetraMin® flakes (Tetra Holding, Blacksburg, VA), Cobalt™ Aquatics spirulina flakes (Cobalt, Rock Hill, SC), and Omega One™ freeze dried blood worms (Omegasea, Sitka, SK). 3-5 replicate groups of 70 embryos were exposed to various WAF treatments: embryo media control (no WAF), source oil, moderately weathered oil, heavily weathered oil, source oil with dispersant, and dispersant only. Exposures began within 30 min post-fertilization (dpf) and lasted until 7 dpf which spans both the embryological (0-2dpf) and larval (2-7dpf) life stages, mortalities were counted daily.

Sheepshead minnow exposures

Freshly fertilized embryos were purchased from Aquatic Biosystems (Fort Collins, CO). Eggs were shipped from the facility to the University within 1 dpf and were exposed immediately upon arrival. Exposures groups of 100 embryos were exposed to various WAF treatments: saltwater control (no WAF), source oil, moderately weathered oil, heavily weathered oil, source oil with dispersant, moderately weathered oil and dispersant, and dispersant only. Exposures began at 1 dpf and lasted until 10 dpf which spans both the embryological (0-5dpf) and larval (5-10dpf) life stages, mortalities were counted daily.

Heart rate and muscle deformities

Heart rate recordings were made on 2 dpf zebrafish and 5 dpf sheepshead minnow, between 14:00-16:00, for which 30 s videos were taken of embryo hearts and heart rates were determined manually. The videos were randomized and scored blind. Birefringence is a non-lethal imaging technique that depends on the natural structure of muscle fibers and their ability to reflect polarized light, and was used to examine muscle deformities in 2 dpf hatched zebrafish larvae using a technique previously described (Smith et al., 2013). In brief, larvae were live imaged on a glass slide with 2-3 drops of water using a Leica DM RXA microscope (Concord, ON) with a polarized filter and a polarized lens placed on the light source 90° to the polarized filter on the

microscope. 20-58 individuals from 6 replicate trials were imaged, and scored blindly for deformities to determine the frequency of abnormalities.

Toxic unit calculations

The survival and incidence of pericardial edema was scaled based on the target lipid model (TLM) approach to account for the difference in toxicity of individual PAHs within the total PAHs present in the solution, as previously described (Kipka et al., 2009; Toro et al., 2000). In brief, the TLM is used to predict the toxicity of compounds that act primarily through narcosis. The model is based on the inverse relationship between LC50 and K_{ow} (Toro et al., 2000). The formula is described as:

$$\log(LC50) = -0.945\log(K_{ow}) + \Delta C_i + \log(C_L) \quad (1)$$

where -0.945 is the universal slope of the linear relationship between octanol and the target lipid of an organism, ΔC_i is the chemical class correction factor, and C_L is the critical target lipid body burden that is associated with a particular species (different species have different tolerance levels for narcotic compounds). To predict the toxicity of a complex mixture WAFs can be evaluated using the concept of toxic units (TUs). A $TU_i$is the ratio of the exposure concentration and the LC50 for an individual compound:

$$TU_i = \frac{C_w}{LC50} \quad (2)$$

where Cw is the concentration of the compound found in the WAF and the LC50 is the inverse Log of the numbers for each compound derived from formula (McGrath et al., 2005). The sum of all the TU_i from each compound found within the WAF gives the TU value for the solution, which can be used to scale PAHs and account for the differing toxicities of individual compounds within the WAF.

$$TU = \Sigma TU_i \quad (3)$$

For our calculations we sourced the universal slope (-0.945) (Toro et al., 2000), and the critical target lipid burden coefficient for zebrafish and sheepshead minnow (1.920 and 1.795; respectively) (Kipka et al., 2009), the chemical class correction (ΔC_i) for PAHs (-0.263) and

the $\log(K_{ow})$ for each of the individual compounds from the literature (Di Toro et al., 2007). A similar methodology with mathematically adjusted values were used for all 3 models.

RNA extraction and cDNA synthesis

In brief, zebrafish (7 dpf) and sheepshead (10 dpf) minnows were euthanized on ice, preserved in RNAlater (Thermo Fisher; Waltham, MA, USA), and stored at -20°C until RNA extraction. Total RNA was extracted from 20-25 pooled whole larvae. Total cDNA was synthesized from 2µg of total RNA for each sample using SuperScript ® First-Strand Synthesis System for RT-PCR (Invitrogen; Carlsbad, CA, USA) as described by the manufacturer. qPCR was performed in 96 well PCR plates on an Applied Biosystems 7500 Fast Real-Time PCR System. Individual target cDNA amplifications were run in triplicate. Transcript levels of target genes were quantified by normalization to an endogenous gene (relative quantification). The endogenous genes used in this study were Beta-actin for zebrafish and *18s* rRNA for sheepshead minnow.

Target genes were chosen based on previous findings in studies on WAF exposure in fish (Edmunds et al., 2015; Incardona et al., 2009). Our focus is mainly on neurodevelopment and biotransformation enzymes. For symmetry, we attempted to target similar genes in both species of fish, however, genes for sheepshead minnows were limited by the availability of sequenced genes. For all genes, primers were designed based on sequences available in the NCBI Genbank database or from previously published (Fan et al., 2010; Kanungo et al., 2012, 2013; Knoebl et al., 2004; de Polo et al., 2014; Rodríguez et al., 2008; Timme-Laragy et al., 2007; Zhang et al., 2012). We chose to target biotransformation enzymes and PAH exposure biomarkers *cyp1a* and *cyp1b*; a marker of estrogenic exposure (vitellogenin; *vtg*); the neurodevelopment genes *neuroD*, *ngn1*, and *gli2a*; a cardiac development gene (*nkx2.5*); and *nka1a1*, a gene encoding sodium-potassium pumps.Refer to Table 4.1 and Table 4.2 for qPCR primer tables.

Statistics and LC50 calculations

Statistical differences between treatment groups were tested using a one-way ANOVA followed by a Tukey's post hoc test (gene expression data only) or a Holm-Sidak post hoc test. For non-normal data a Kruskal-Wallis one-way ranked ANOVA was used. Significant difference was accepted at $p<0.05$ and all tests were performed using SigmaPlot 11 (Systat, San Jose, CA). LC50 values were calculated using free online software based on the Finney method of Probit analysis (Finney, 1952).

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Tables

Table 4.1. Zebrafish qPCR primers.

Target Gene	Gene name	Forward Sequence (5'-3')	Reverse Sequence (5'-3')	Accession Number/ Reference
βactin	Beta-Actin	CGA GCA GGA GAT GGG AAC C	CAA CGG AAA CGC TCA TTG C	AF057040(Rodríguez et al., 2008)
cyp1a	Cytochrome P4501a	AGG ACA ACA TCA GAG ACA TCA CCG	GAT AGA CAA CCG CCC AGG ACA GAG	NM_131879(Timme-Laragy et al., 2007)
cyp1b	Cytochrome P4501b	CCA CCC GAA CTC TGA AAC TC	AAA CAC ACC ATC AGC GAC AG	NM_001013267(Timme-Laragy et al., 2007)
vtg1	Vitellogenin	CTG CGT GAA GTT GTC ATG CT	GAC CAG CAT TGC CCA TAA CT	AF406784.1(Kanungo et al., 2012)
nkx2.5	Homeobox protein *nkx2.5*	GTC CAG GCA ACT CGA ACT ACT C	AAC ATC CCA GCC AAA CCA TA	NM_131421(Zhang et al., 2012)
ngn1	Neurogenin1	TGC ACA ACC TTA ACG ACG CAT TGG	TGC CCA GAT GTA GTT GTG AGC GAA	NM_131041(Fan et al., 2010)
neuroD1	Neurogenic differentiation1	CAG CAA GTG CTT CCT TTT CC	TAA GGG GTC CGT CAA ATG AG	(Kanungo et al., 2013)
gli2a	GLI family zinc	AAA AAC AGG GCG GGA	ATG CTG GGT TGG AGG	(Kanungo et

	finger 2a	CTA CT	TAC AG	al., 2013)

Table 4.2. Sheepshead minnow qPCR primers.

Target Gene	Gene Name	Forward Sequence (5'-3')	Reverse Sequence (5'-3')	Accession Number/ Reference
18s	18s ribosomal RNA	GCT GAA CGC CAC TTG TCC	ATT CCG ATA ACG AAC GAG ACT C	EF535030(de Polo et al., 2014)
cyp1a	Cytochrome P4501a	GCA GAT TAA CCA CGA CCC AGA G	GCA TCG CCT CCT TCC TAA GC	(Hendon et al., 2008)
vtg1	Vitellogenin	ATG TCA CTG TGA AGG TCA ACG AA	ACC TGT TGG GTG GCG GTA A	AF239720(Knoebl et al., 2004)
ngn1	Neurogenin1	ACC GCG CAT GTG GTA AAG AA	CTG TGG GAT GCT CAG TCA CC	XM_015371488.1
neuroD1	Neurogenic differentiation1	GTC TCA GCC GAC CAC TAA CC	GGC ATC TGA CAC CAG GAC TC	XM_015400246.1
nka1a1	Na/K-ATPase 1a1	GCC ACA CAG CCT TCT TCA C	ACA ATA GAG TTC CTC CTG GTC TTG	GE337281.1(de Polo et al., 2014)

Figures

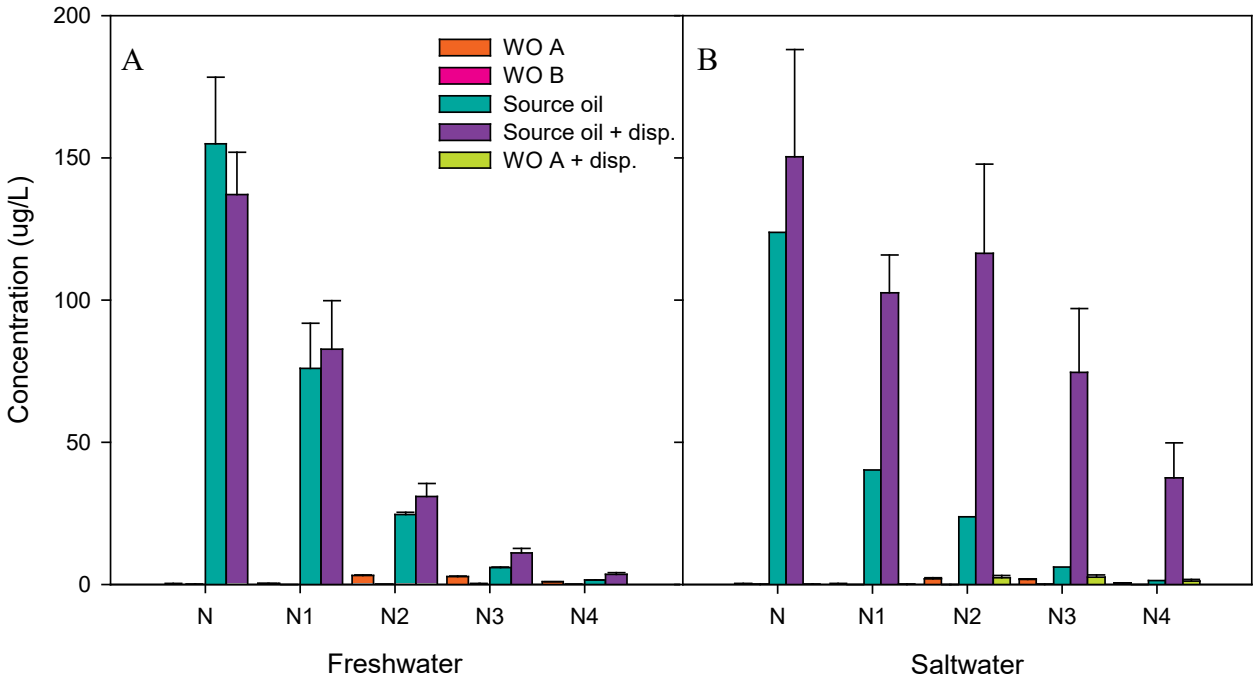

Figure 4.1. Naphthalene (N) and alkylated naphthalene (N1-N4) content of the fresh (A) and saltwater (B) WAFs made with weathered oil A (WO A), weathered oil B (WO B), source oil, source oil with dispersant (Source oil + disp.), and weathered oil A + dispersant (WO A + disp.; saltwater only). Error bars represent standard error (SEM).

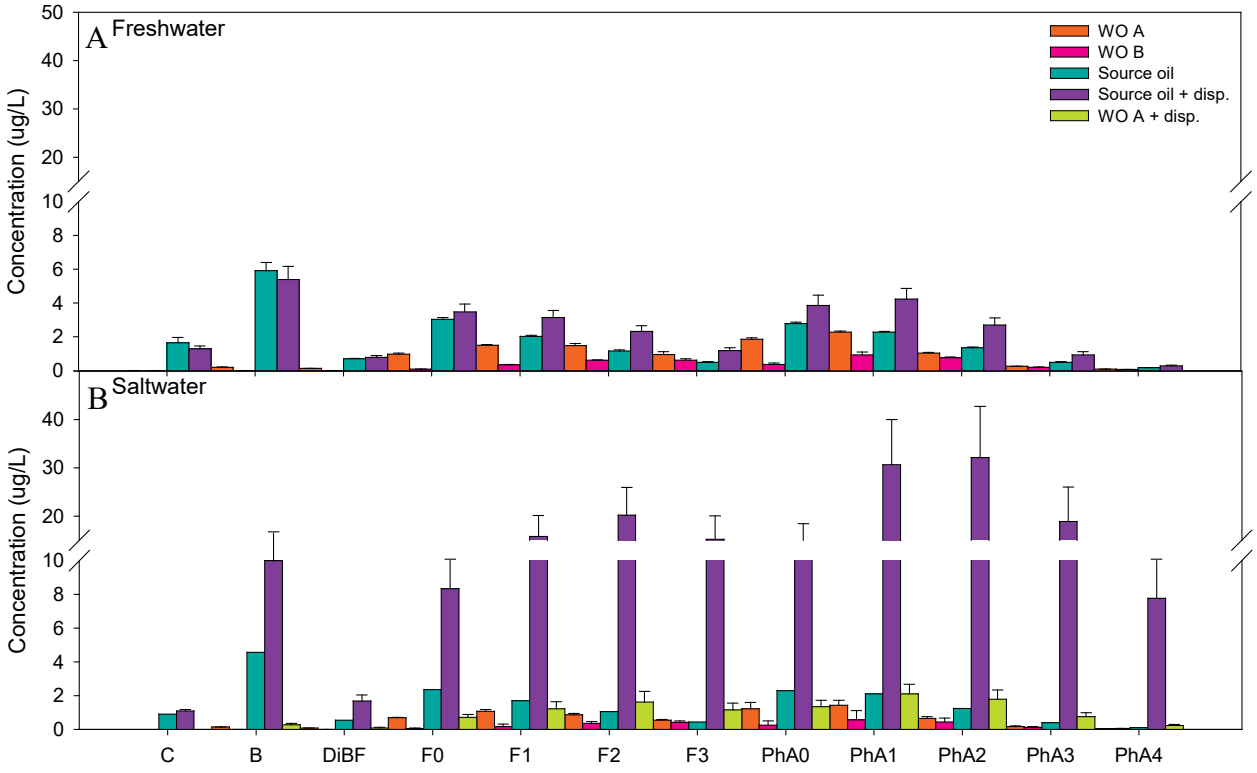

Figure 4.2. PAH content of the fresh (A) and saltwater (B) WAFs made with weathered oil A (WO A), weathered oil B (WO B), source oil, source oil with dispersant (Source oil + disp.), and weathered oil A + dispersant (WO A + disp.; saltwater only). C, carbazole; B, biphenyl; DiBF, dibenzofuran; F, fluorene; PhA, phenanthrenes/anthracenes; MPhA, methylphenanthrene. Error bars represent standard error (SEM).

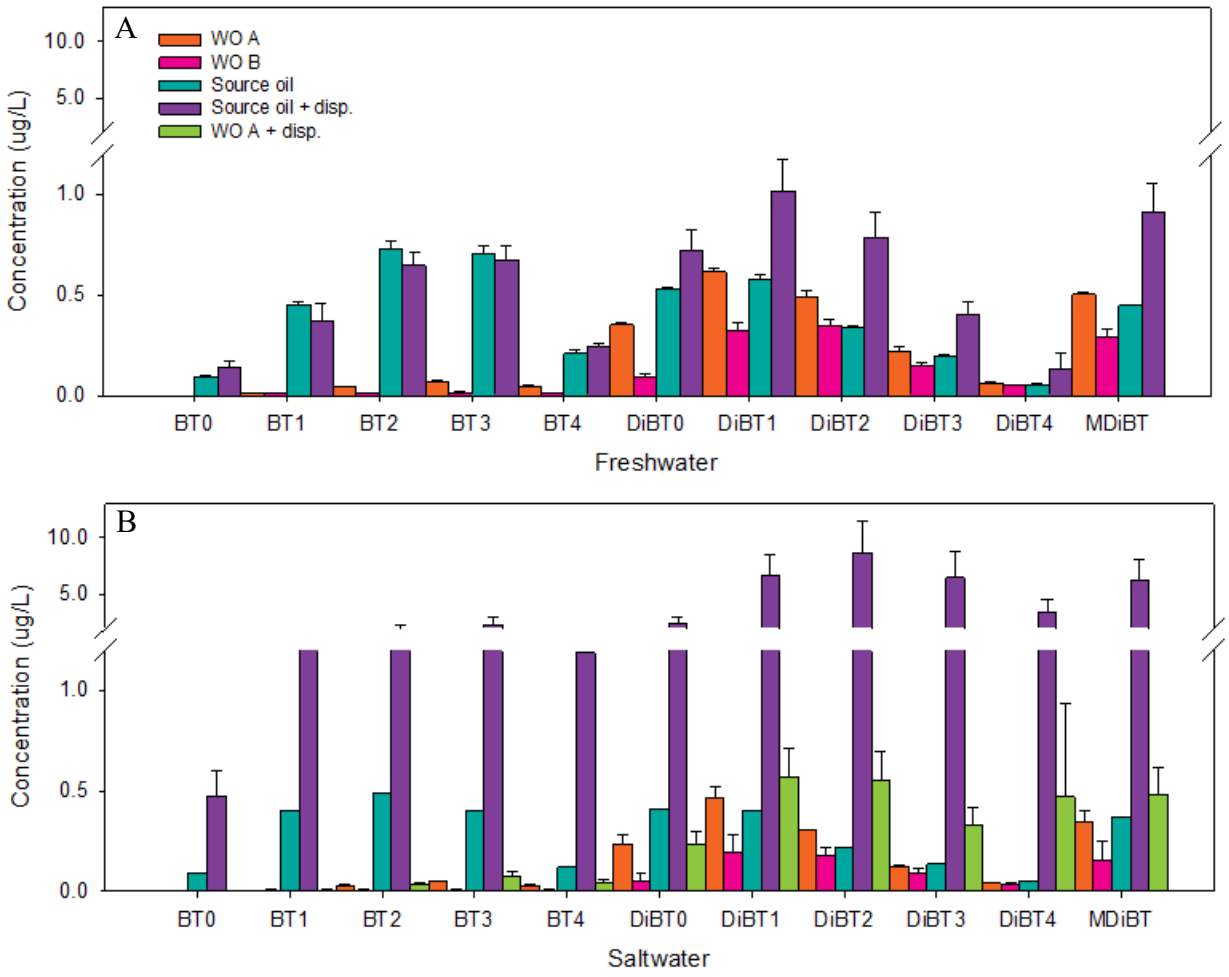

Figure 4.3. Benzothiophene based PAH content of the fresh (A) and saltwater (B) WAFs made with weathered oil A (WO A), weathered oil B (WO B), source oil, source oil with dispersant (Source oil + disp.), and weathered oil A + dispersant (WO A + disp.; saltwater only). BT, Benzothiophenes; DiBT, Dibenzothiophenes, MDiBT, Methyldibenzothiophenes. Error bars represent standard error (SEM).

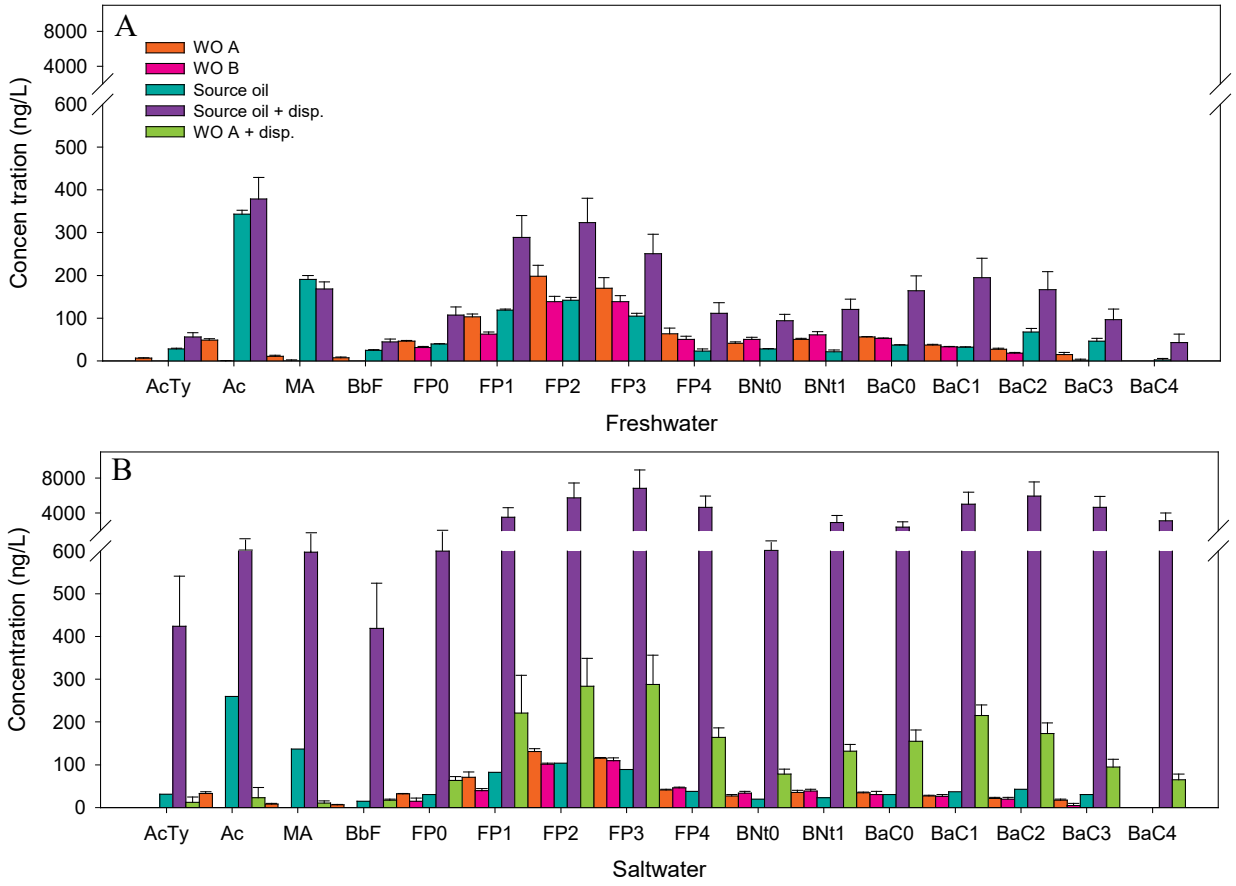

Figure 4.4. Low concentration PAHs in fresh (A) and saltwater (B) WAFs made with weathered oil A (WO A), weathered oil B (WO B), source oil, source oil with dispersant (Source oil + disp.), and weathered oil A + dispersant (WO A + disp.; saltwater only). AcTy, acenaphthylene; Ac, acenaphthene; MA, methylanthracene; BbF, benzo[b]fluorene; FP, fluoranthenes/pyrenes; BNt, benzo[a]naphthothiophenes; BaC, benzo[a]anthracenes/chrysenes. Error bars represent standard error (SEM).

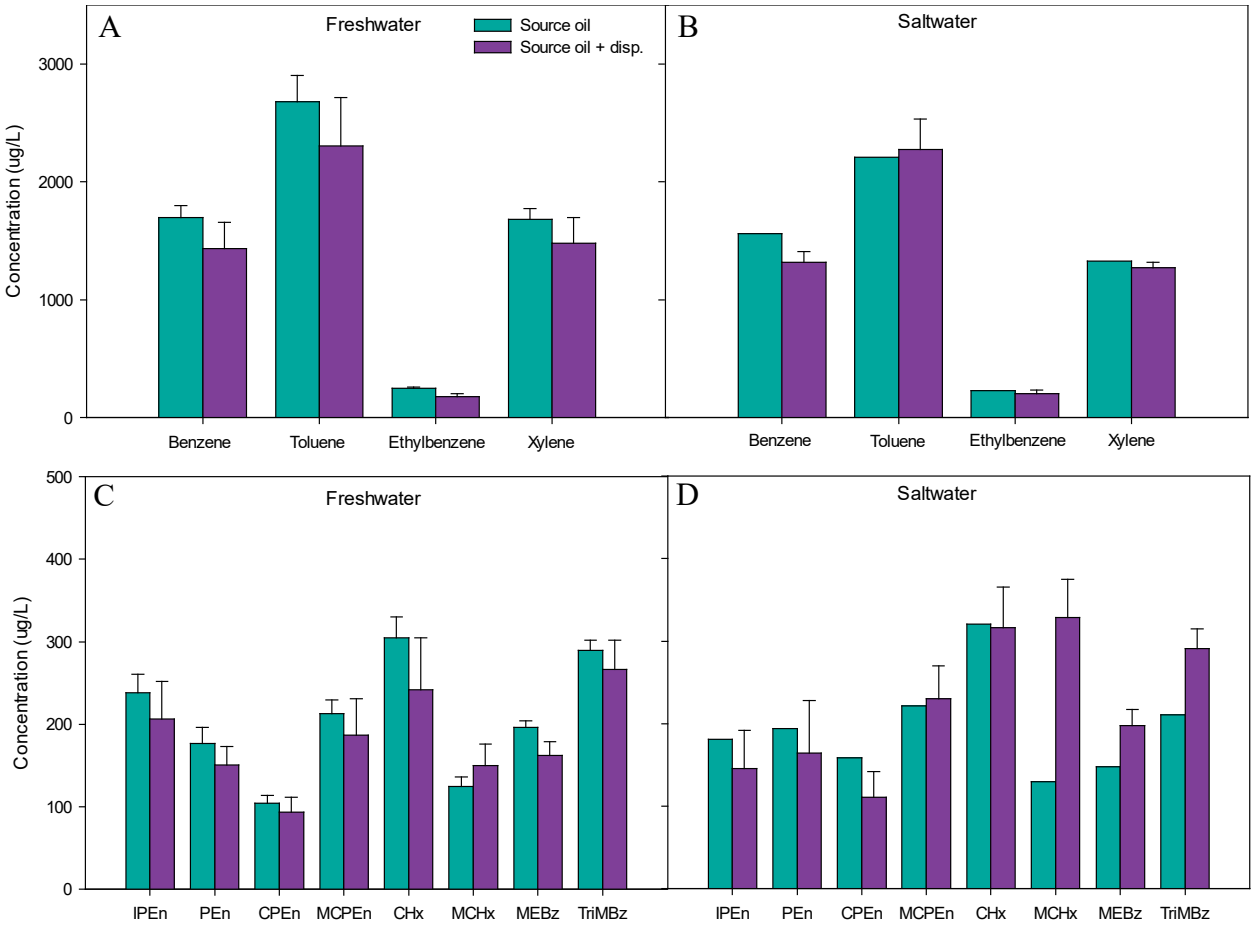

Figure 4.5. BTEX and Volatile organic compound (VOC) content of the fresh (A,C) and saltwater (B,D) WAFs made with source oil, and source oil with dispersant (Source oil + disp.). None of the weathered oil WAFs had measurable amounts of volatile compounds, so they were not included. IPEn, isopentane; PEn, pentane; CPEn, cyclopentane; MCPEn, methylcyclopentane; CHx, cyclohexane; MCHx, methylcyclohexane; MEBz, methylethylbenzene; TriMBz, trimethylbenzene. Error bars represent standard error (SEM).

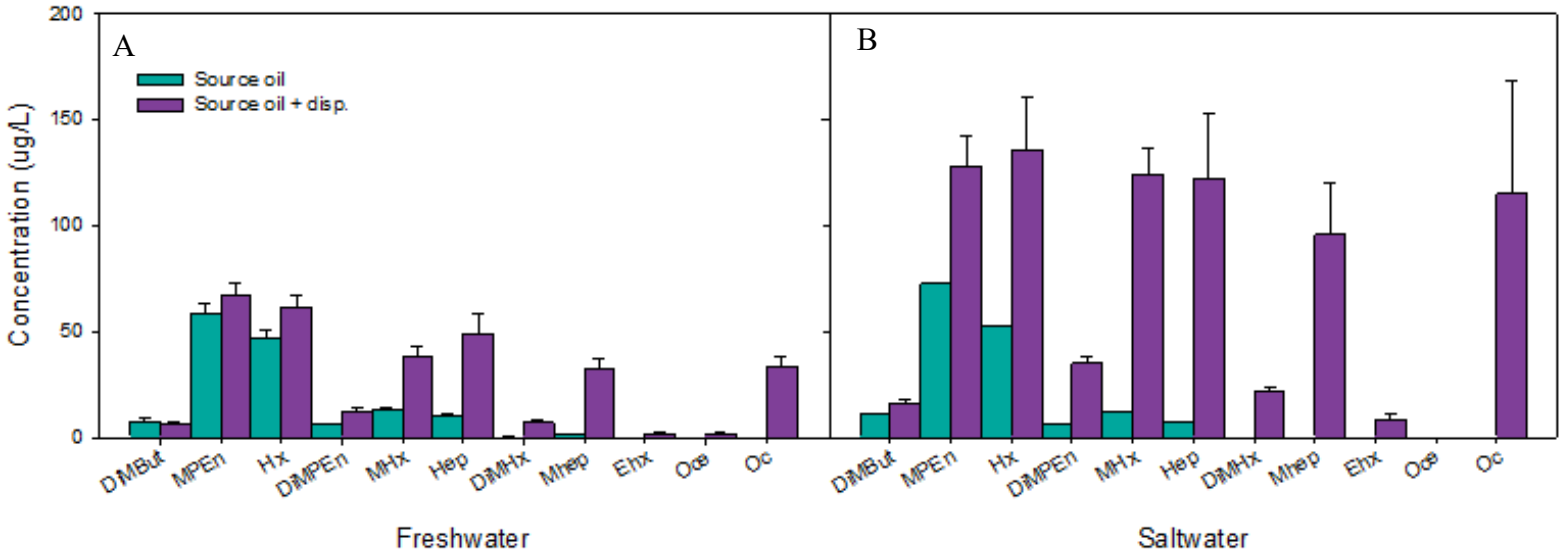

Figure 4.6. Other volatile organic compounds (VOC) found in fresh (A,C) and saltwater (B,D) WAFs made with source oil, and source oil with dispersant (Source oil + disp.). None of the weathered oil WAFs had measurable amounts of volatile compounds, so they were not included. DiMBut, dimethylbutane; MPEn, methylpentane; Hx, Hexane; DiMPEn, Dimethylpentane; MHx, methylhexane; Hep, Heptane; DiMHx, dimethylhexane; Mhep, methylheptane; Ehx, ethylhexane; Oce, octene; Oc, octane. Error bars represent standard error (SEM).

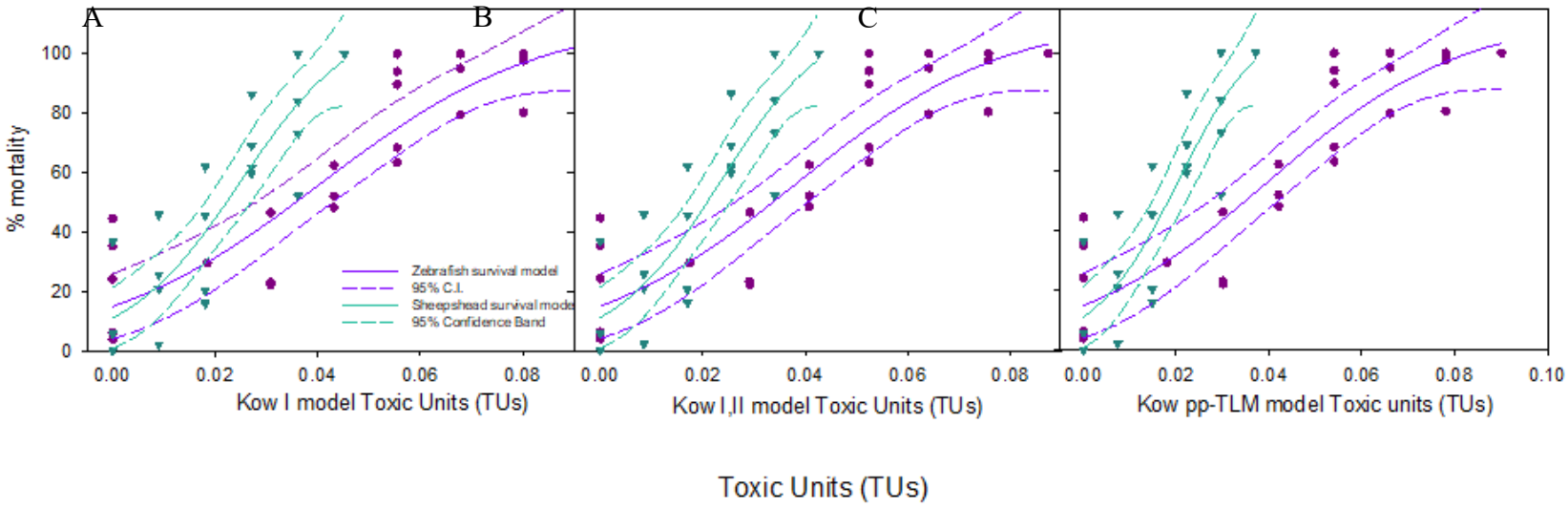

Figure 4.7. The percent mortality from 1-7 dpf (zebrafish) and 1-10 days post dpf (sheepshead minnow) in relation to the number of toxic units of PAHs per WAF. Toxic units were evaluated using three different models: K_{ow} I (A); K_{ow} I, II (B); K_{ow} pp-TLM (C). 95% confidence intervals (C.I.) were included with each curve. Error bars represent standard error (SEM).

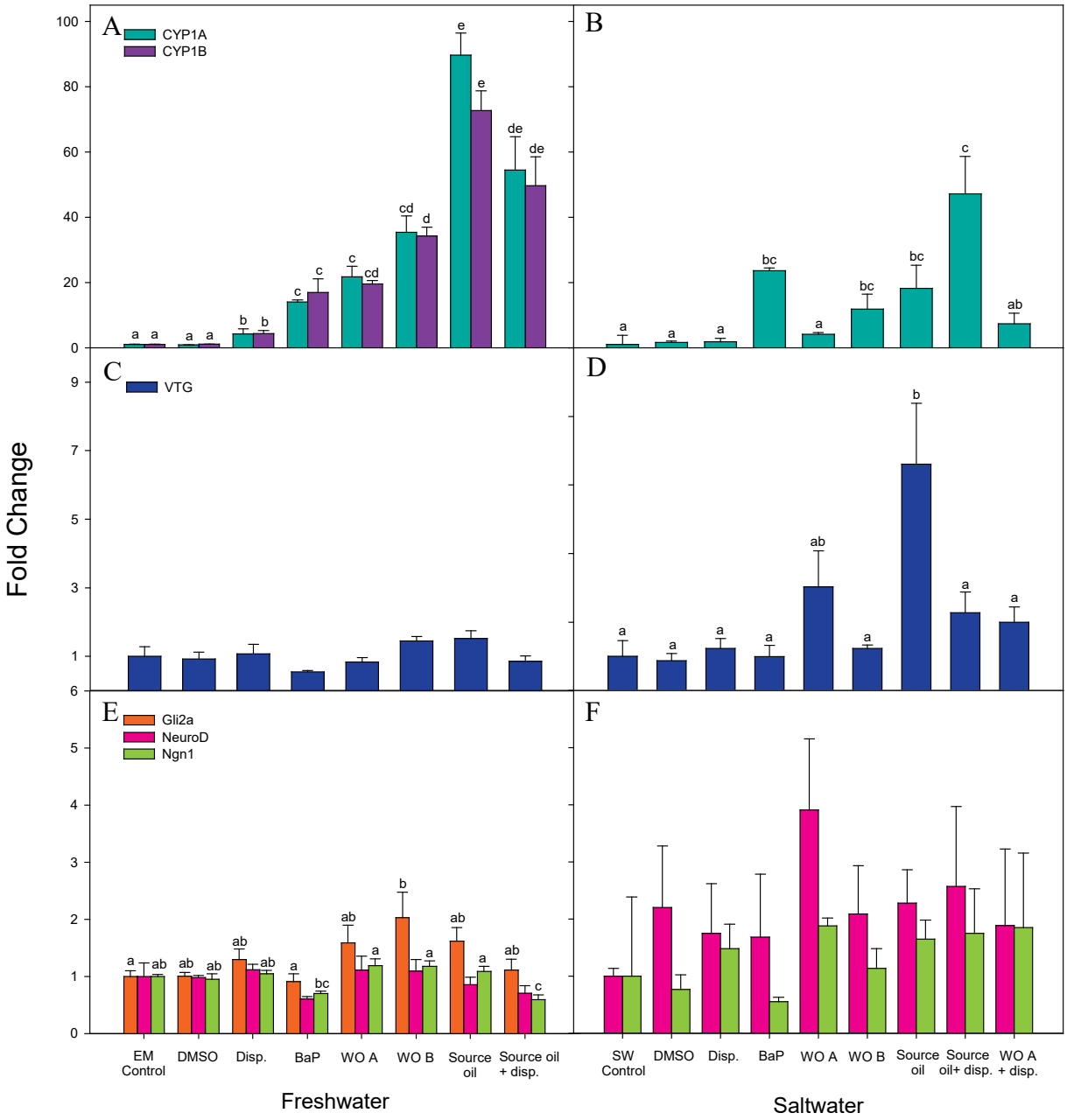

Figure 4.8. Effects of WAF exposures on the expression level (fold change from control) of the biotransformation enzymes *cyp1a* (A,B) and *cyp1b* (A), estrogenicity indicator *vtg*(C,D), and neurodevelopment markers *gli2a* (E), *neuroD* (E,F), and *ngn1* (E,F). Bars represent mean ± SE. The means of exposures that do not share a common letter are significantly different ($P<0.05$) as assessed by one-way ANOVA and Tukey's HSD test. Genes that have no symbols are

statistically the same across all treatment groups within each species. Error bars represent standard error (SEM).

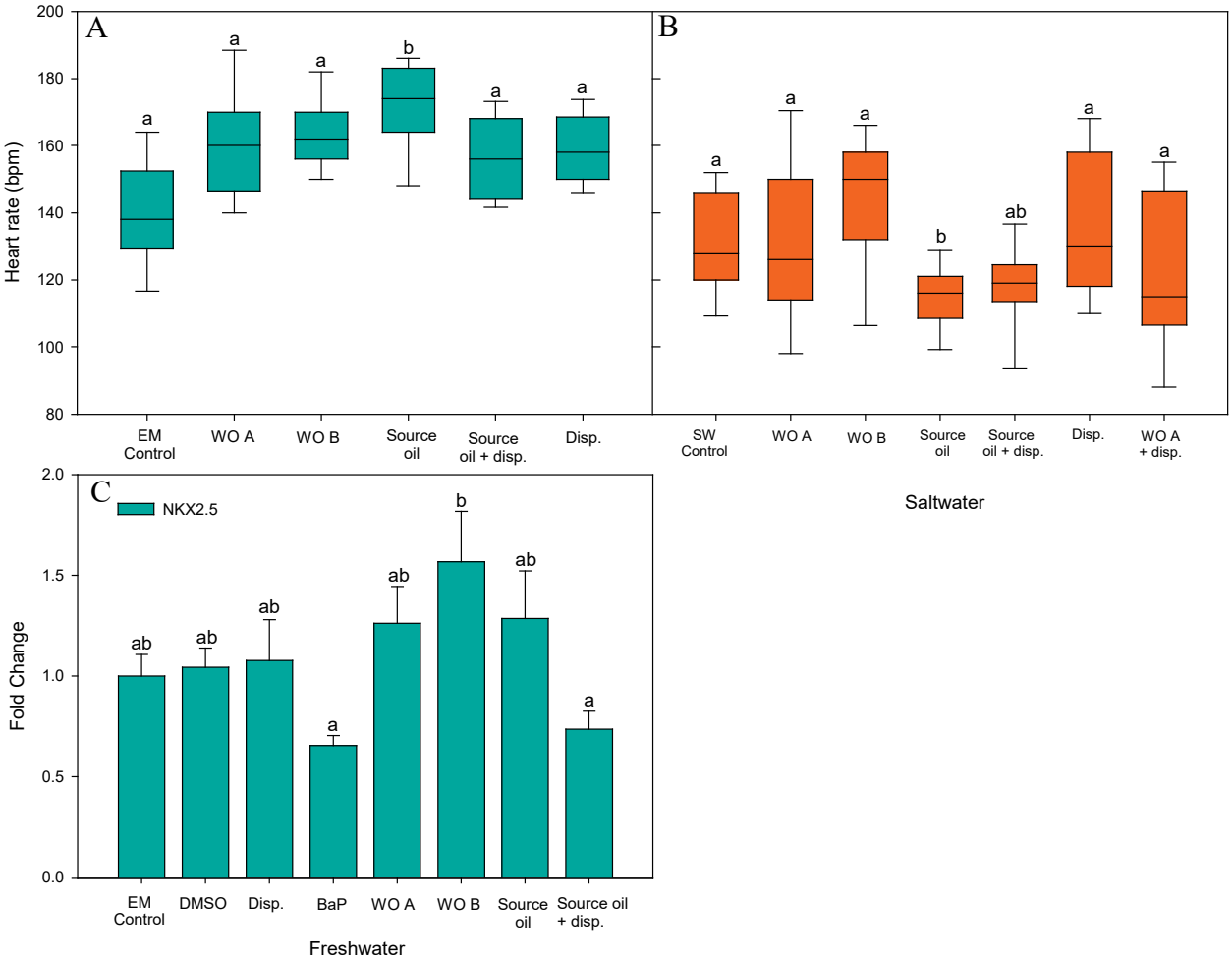

Figure 4.9. The impact of weathered oil A (WO A), weathered oil B (WO B), source oil, source oil with dispersant (Source oil + disp.), dispersant, and weathered oil A + dispersant (WO A + disp.; saltwater only) on the heart rate of 2dpf zebrafish (A) and 5dpf sheepshead minnow embryos (B), and the effect of WAF exposure on the expression levels of the heart development gene *nkx2.5* in 7dpf zebrafish (C). The treatment groups that do not share a common letter are significantly different ($p<0.05$). Error bars represent standard error (SEM).

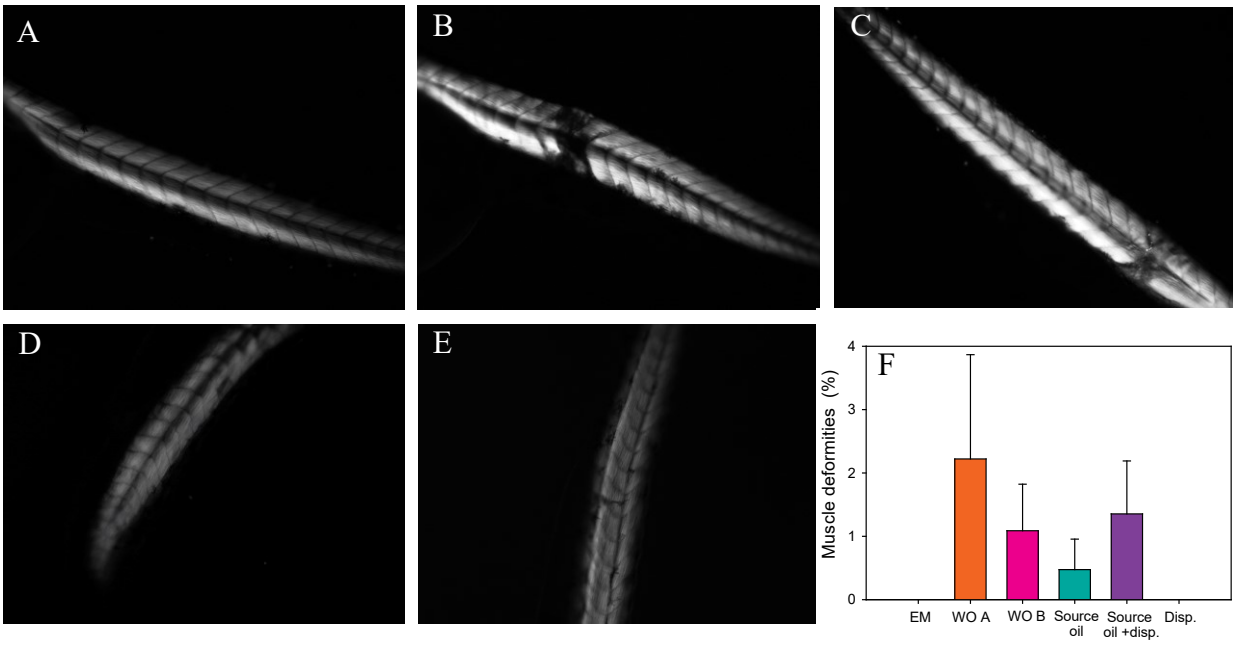

Figure 4.10. Birefringence images and frequency of muscle structure deformities in weathered oil A (WO A), weathered oil B (WO B), source oil, source oil with dispersant (Source oil + disp), and dispersant (Disp.) exposed 2dpf zebrafish larvae. Birefringence was used to examine normal myosepta, muscle striations and muscle structure along the tail of (A) control and to identify deformities which were found in (B) source oil WAF, (C) source oil + dispersant WAF, (D) WO A WAF and (E) WO B WAF. Deformity frequency (F) was very low (under 4%); deformities were only found in the oil exposed treatment groups. Error bars represent standard error (SEM). Images were taken from 20-58 individuals from 6 replicate trials for each treatment group.

Chapter 5: Summary and General Conclusions

Summary

Aquatic toxicology is the study of the effects of natural and anthropogenic compounds on aquatic species ranging from subcellular to ecosystem level effects. Adverse effects are commonly studied in fish, as they are often used as indicators of water quality and contamination. Aquatic contaminants include, but are not limited to, metals, effluent, pesticides, herbicides, and petroleum hydrocarbons. Increased oil production and transportation leads to spillage and the release of petroleum hydrocarbons into the environment and contamination of both terrestrial and aquatic ecosystems. Complex hydrocarbon mixtures produced by the oil industry such as oil sands process affected water (OSPW) and crude oil have been, are currently, and will be released both intentionally and unintentionally into the environment. OSPW and crude oil are the two complex hydrocarbon mixtures that I focused on throughout my thesis.

OSPW is a complex mixture with organic and inorganic components. Naphthenic acids (NAs) are thought to be the primary toxic component, though other compounds in OSPW likely contribute to its toxicity as well. Crude oil is mostly composed of polycyclic aromatic hydrocarbons (PAHs) as well as aliphatic compounds and BTEX. Both complex mixtures have been found to cause many adverse effects in fishes including reduced survival, endocrine disruption, and gross morphological defects (Arukwe et al., 2008; Heintz et al., 2000; Hughes et al., 2017; Incardona et al., 2004; Lister et al., 2008; Martin-Skilton et al., 2006; Peters et al., 2007).

Due to observed negative effects of exposure, the development and study of remediation and cleanup techniques is required to reduce those effects. Ozonation is currently being studied as a remediation process for OSPW. Ozone treatment degrades carbon-carbon bonds and, therefore, degrades the organic fraction of OSPW (including NAs) rendering ozonated OSPW potentially less toxic. Many clean up techniques exist for crude oil spills including the use of dispersants. Dispersants emulsify oil slicks to increase the rate of biodegradation and protect shoreline ecosystems.

Making comparisons between studies on these complex hydrocarbon mixtures is challenging due to the complexity and variability of samples. Comparisons are also challenging between these

studies due to differences in species and life-stages that are studied. The goal of my thesis was to use subcellular and whole organism endpoints to more fully characterize developmental effects of exposure to complex hydrocarbon mixtures and fill in some of the gaps in knowledge surrounding these types of exposures.

Chapter two of my thesis assessed the effects of raw and ozonated OSPW exposures in developing zebrafish and attempted to link gene expression with whole organism endpoints. Ozone treatment of the OSPW sample used in this study decreased the amount of classical NAs from 16.9mg/L in the raw OSPW to 0.6mg/L in the ozonated OSPW. Raw OSPW induced the expression of biotransformation genes *cyp1a* and *cyp1b*, while ozonated OSPW did not affect *cyp1a* expression and only slightly induced the expression of *cyp1b*. The expression pattern of these two CYP genes induced by raw OSPW exposure is, to my knowledge as far as published material, a novel expression pattern, with *cyp1b* being more upregulated than *cyp1a*. Exposure to crude oil causes the opposite pattern to occur with *cyp1a* induced more than *cyp1b*. OSPW exposure also led to a decrease in the expression of some genes related to heart development and function, though heart rate, size, and incidences of arrhythmia were not affected by exposure. This study found no effect on survival, indication of craniofacial abnormalities, or increase in apoptosis. Overall, exposure to both raw and ozone treated OSPW was not overtly toxic to embryonic zebrafish.

Chapter three evaluated the effects of OSPW exposure on the growth and recovery of developing zebrafish. Overall fish length at the end of the exposure period was not affected by exposure to either raw or ozonated OSPW. Otoliths were slightly smaller in exposed embryos, potentially indicating a slight physiological change. Exposure caused no alterations in the expression of neurodevelopment genes or vitellogenin, a marker of estrogenicity. The biotransformation genes *cyp1a* and *cyp1b* were upregulated by raw OSPW exposure, with *cyp1b* being upregulated to a larger degree. Within two days after exposure, the expression levels of these genes returned to a level similar to that of the control, indicating that the embryonic zebrafish were likely capable of excreting the exogenous compounds relatively quickly after the exposure ended. The expression of these genes was used as a proxy for recovery and an indication of when the fish were no longer exposed to parent compounds from the OSPW. This was not, however, an indication that the fish were not affected by the exposure after that period of time.

In chapter four, the toxicity of water accommodated fractions (WAFs) of naturally weathered crude oils, source oil, and source oil plus dispersant mixtures were compared in saltwater and freshwater species. The aim of this study was to determine the differences between the effects of oil exposures on developing sheepshead minnow, a saltwater species, and zebrafish, a freshwater species. A comparison within the same study between effects of crude oil exposure along with dispersants in both freshwater and saltwater species has never been done before. Zebrafish are often used as a model species due to their molecular tools and accessibility, but their use as a model for marine oil spills has not previously been validated. WAFs for each oil type varied little between saltwater and freshwater except the source oil WAF with dispersants, which increased the amount of PAHs in the WAFs to a large extent in saltwater, with only a moderate effect in freshwater. Exposure had varying effects depending on species and oil type. There were species-species differences in cardiotoxic effects as well as estrogenicity. The source oil plus dispersant exposure was the only one to have an impact on survival in both species. Source oil and weathered oils increased the expression of a biomarker of exposure to estrogenic compounds in sheepshead minnow but not zebrafish. Heart rate increased due to exposure in zebrafish but decreased in sheepshead minnow. The alterations in the expression of the heart development gene *nkx2.5* was not congruent with the changes in heart rate and the expression of neurodevelopment genes was altered in zebrafish but not sheepshead minnow. Overall, saltwater and freshwater WAFs varied greatly when dispersant was present and the effects due to exposures differed between species, leading to the conclusion that freshwater model species and systems should not be used to study marine oil spills.

My thesis shows that OSPW exposure was not overtly toxic to zebrafish embryos and that ozone treatment of the OSPW did not lead to a decrease in effects due to exposure. The lack of difference between the effects found from raw and ozonated OSPW exposures was likely due to the fact that raw OSPW exposure did not lead to many adverse effects. The expression of CYP enzymes was affected by raw OSPW exposure as well as crude oil exposures. The expression pattern of *cyp1a* and *cyp1b* was different in the OSPW exposed zebrafish compared to crude oil exposed zebrafish. The expression of other genes related to neurodevelopment and heart development/function was also affected by exposure these complex hydrocarbon mixtures but these changes were not always linked to effects found at the tissue or whole organism level.

Studying complex hydrocarbon mixtures is challenging due to the variability between samples. My thesis highlights the fact that it is important to study ecologically relevant species, as the effects caused by exposures can differ greatly between species based upon their inherent sensitivity as well as water type that they live in (i.e. saltwater vs. freshwater).

General Conclusions/discussion

Both OSPW and crude oil are complex mixtures that contain varying types of compounds. Ozone treatment changed the chemistry profile of the OSPW used in these studies. The ozonation did not degrade the organic fraction of the OSPW, but the original organic compounds were likely oxidized to different organic compounds. Ozonation changed the ratios of NAs present in the sample to more oxygen-rich species and also, in general, shifted the NAs to a lower carbon number.

The weathered crude oils and source oil varied in their chemical composition, with source oil containing more of the smaller organic compounds such as VOCs and naphthalenes. The chemistry of the different oil types used in this study was comparable between fresh and saltwater except for source oil plus dispersant. The saltwater pulled more PAHs into solution compared to freshwater when dispersant was present with source oil. Though the compounds present in OSPW and crude oil vary greatly (NAs and metals vs. PAHs), exposure to these two environmental contaminants caused some similar effects in fishes in my studies. This may not be surprising, however, since both are petroleum-based contaminants.

In the studies included in my thesis, exposure to both types of OSPW (raw and ozonated) and all three types of crude oil (source, moderately weathered, and heavily weathered) had no effect no the survival of embryonic fishes. However, the source oil plus dispersant exposure did affect the survival of both zebrafish and sheepshead minnow. Though survival was not affected by most exposures in these studies, sublethal effects were observed.

Heart rate was affected by both raw OSPW and source oil exposures. In zebrafish, these exposures led to an increase in heart rate, though the change was not outside the normal range for heart rate in 2dpf zebrafish. On the other hand, source oil exposure in sheepshead minnow lead to a decrease in heart rate, rather than an increase. This divergence in effects between species highlights that exposure to toxicants can have differing effects on different species. The

expression of heart development gene *nkx2.5* was downregulated in OSPW exposure as well as the source oil plus dispersant exposure, however, heart rate was not affected in the source oil plus dispersant exposure group. This shows that changes at the gene expression level do not always lead to effects at the whole organism level. This serves as a reminder that changes gene expression should not always be used as biomarkers of adverse effects since alterations in gene expression may not result in or be indicators of actual adverse effects for the organism.

The expression of the neurodevelopment gene *ngn1* was downregulated by both OSPW and source oil plus dispersant exposures in zebrafish but was not affected in sheepshead minnow by exposure to any oil type. Conversely, the expression of *vtg*, a biomarker of exposure to estrogenic compounds, was upregulated in source oil exposed sheepshead minnow but was not affected by any exposure (either OSPW or crude oil) in zebrafish. These findings on the differences of gene expression between species exposed to the same contaminants highlights that species may respond differently to exposures.

Both OSPW and crude oil exposures induced the expression of CYP, however, the expression of these genes in zebrafish differed between exposure types. Crude oil exposure induced the expression of both *cyp1a* and *cyp1b* to a much larger extent than OSPW. Not only was the magnitude of induction different between exposures, but the expression pattern also differed. Crude oil exposures resulted in *cyp1a* being more highly expressed than *cyp1b*, while OSPW exposure resulted in the opposite pattern with *cyp1b* expressed to a greater extent than *cyp1a*. *cyp1a* is commonly used as a biomarker of exposure to organic compounds and is most often expressed to a greater extent than *cyp1b* (Dorrington et al., 2012; Jönsson et al., 2007, 2010; Zanette et al., 2009). To my knowledge, the expression pattern of *cyp1b* being expressed to a larger extent than *cyp1a* on a whole organism level has not previously been observed due to any exposure. My studies may show that the expression pattern of these two CYPs induced by OSPW exposure in zebrafish is specific to OSPW. This is supported by the crude oil exposures resulting in *cyp1a* being more greatly expressed than *cyp1b*. The crude oil study supports the idea that the CYP expression pattern found in the OSPW studies may be novel. With future validation and much more research, this expression pattern of CYPs may potentially be used in the future as a biomarker of exposure. However, since ozonation reduces the expression of these

CYP genes to nearly control levels, these genes would not be ideal for biomarkers of exposure to treated OSPW if ozonation were in fact used as a remediation/treatment process.

The induction *cyp1a* expression also differed between species with the crude oil exposures. The source oil plus dispersant exposure increased the relative fold change of *cyp1a* in zebrafish much more than it did in sheepshead minnow, despite the saltwater exposure having a larger amount of total PAHs. B[a]P, used as a positive control group between species, was also induced to a different amount between species, indicating that different compounds have different affinities for AHR in different species, and that different compounds will induce CYPs to different extents in depending on the species (Doering et al., 2013; Hahn, 2002). Different species may also have a different maximum amount of CYP induction relative to basal levels of expression.

These findings suggest that though there may be some similar responses to exposures, differing species will suffer differing adverse effects due to exposure to the same contaminants. This demonstrates that it is important to use ecologically relevant species when studying contaminants, since the species used may determine the effects found, and the effects found in one species may not be comparable to the effects found in another. Although it is still important to study the effects of exposure in multiple different species to get a more robust understanding of the contaminant, it is ultimately important to study species that are naturally found in the environment where these contaminants may be found. It is especially important to use ecologically relevant species when studying complex mixtures like OSPW and crude oil since they cause such diverse effects. However, it is also important to use model species, since they enable the study of many more endpoints. There is a give and take between using ecologically relevant species and model species. The molecular endpoints enabled by the use of zebrafish makes them a good choice for toxicological studies even though they are not found in the oil sands region or where crude oil is commonly spilled. Therefore, the study of both model species and ecologically relevant species is of importance to gather as much information as possible about these complex contaminants.

These complex environmental contaminants were found to cause many differing adverse effects, though most exposures did not cause outright lethality. The OSPW exposures were found to be relatively non-toxic, as many endpoints found no changes due to exposure and ozone treatment showed very slight reductions in the toxicity of exposures. Crude oil exposures caused some

adverse effects and the addition of dispersant greatly increase the toxicity of source oil exposures in fishes. The main findings of these studies include: that OSPW exposure may not cause as many adverse effects as previously thought; that dispersant greatly increases the lethality of source oil exposure; that changes in gene expression may not necessarily lead to adverse effects at higher levels of organization; and that it is important to use ecologically relevant species when studying environmental contaminants. Though survival was generally not affected by exposures, it cannot be ignored that, as humans, we are having a pronounced impact on our environment and the species within it.

Future Directions

The studies conducted in my thesis characterize the developmental effects of exposure to OSPW and crude oil. However, the legacy effects of developmental exposure have not been assessed. Developmental exposures are capable, in certain cases, of causing long-term sublethal effects, though they may not initially be evident. For example, Hicken et al. (2011) found that developmental exposure to crude oil in zebrafish resulted in changes in heart shape and swimming ability a full year after the exposure occurred.

These legacy or long-term sublethal effects of developmental exposure may affect the overall fitness of a fish. If breeding success is affected, then populations can change and perhaps decline. Breeding success can be measured in many different ways including number of pairs that breed, how often they breed, how many offspring they produce, and the survival of their offspring to adulthood. Zebrafish that were developmentally exposed to OSPW were raised in clean water until adulthood and measured their breeding success (Fig. A1). As measured by the percent of pairs that bred, the fertilization success, and the survival of embryos from 1-7dpf (see methods in appendix), breeding success was not affected by developmental exposure to either raw or ozonated OSPW.

Though breeding success is an important endpoint for determining whether exposure has long-term effects, the second-generation embryos should also be assessed. B[a]P, a PAH, has been found to cause trans-generational effects in the form of morphological deformities, decreased reproductive success, and decreased survival in offspring. It may be that the compounds in OSPW could cause effects in the next generation of embryos. How the second generation

embryos react to exposure to the same OSPW type as the first generation may shed light on any epigenetic trans-generational effects of the developmental exposure in the first generation.

I have measured the expression levels of a few genes (*cyp1a*, *cyp1b*, *nkx2.5*, and *vtg*) in the second-generation embryos. I found that the expression of *cyp1a* and *cyp1b* was not altered in the unexposed second-generation embryos and that the exposed second-generation embryos (exposed to the same OSPW type as their parents – see appendix for methods) had a very similar expression pattern as the first generation (Fig. A2). This would indicate that the second-generation embryos did not have any adaptations to the exposure and responded similarly to the first generation exposed embryos. *nkx2.5* and *vtg* had differing responses in the second-generation compared to the first (Fig. A3 and A4). In the second-generation embryos, the expression of both *nkx2.5* and *vtg* was significantly higher than control in one of the treatment groups. This would indicate that the second-generation embryos had a heritable change in basal gene expression levels, since these embryos were not exposed to anything but yet had differences in gene expression.

These changes in basal gene expression in the second-generation unexposed embryos indicates that there is a heritable change occurring due to the developmental exposure in the previous generation. This suggests that there may be changes in DNA methylation caused by the exposure. I measured global DNA methylation in these embryos (see appendix for methods) and found that there were no significant differences in percent DNA methylation, though there was a large decrease in global DNA methylation in the unexposed raw OSPW embryos (Fig. A5). Though there was no change in global DNA methylation, it is important to take into consideration that no change in DNA methylation does not mean that there was no change in the pattern of methylation. Using bisulfite sequencing to measure the specific methylation pattern of the promoter region of certain genes would be the next step in looking at whether developmental OSPW exposure has an epigenetic effect on these fish. It would also aid in determining what is causing the difference in basal gene expression levels in the second generation. Though a change in methylation may not lead to a discrete change in a measureable endpoint, it means that the population of exposed fish is changed in some way for the next generation, which could lead to multiple different negative consequences.

These results on breeding success and second generation embryos are the beginning of determining the long term, multi-generational effects of developmental exposure to OSPW, but in no way do they create a complete picture. Many more endpoints should be measured in the second- and third-generation embryos to determine the full effect of exposures in the first generation. These endpoints that I have looked at in adult and second-generation OSPW exposed fish should also be measured in crude oil exposed fish. This ides of epigenetic changes caused by exposure to complex organic mixtures should be researched further in the future. Multigenerational effects are often overlooked because they are very time consuming and use a lot of resources. However, in order to fully determine the effects that exposures can have, sublethal multigenerational endpoints must be measured.

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Appendix

Methods

Breeding success

Embryos exposed from 0-7 dpf were rinsed overnight and then housed in 2.8 L tanks in groups of 30 in clean rack water. At 90 dpf, when the fish reached adulthood, they were transferred to 6.0 L tanks. To breed, fish were sexed and paired, and left isolated by a barrier overnight. The following morning the pairs were released and eggs were collected after 4 h of spawning time. The number of spawning pairs, the fertilization success, number of eggs spawned, and survival of the progeny (raised in EM control water) were used to score breeding success.

Second-generation exposures and survival

Embryos from the same fish used for measuring breeding success were collected and exposed within 0.5 hours post fertilization until 7 dpf. These embryos were exposed to the same OSPW type as their parents were developmentally exposed to. Embryos were held in groups of 70 in glass Petri dishes containing 40 mL of exposure water. Approximately 95% of the exposure water was exchanged daily. Survival was counted daily from 1-7 dpf. Table 4 presents an explanation of differences between first-generation, second-generation unexposed and second-generation exposed embryos.

DNA extraction and Global Methylation

DNA was extracted from 7 dpf exposed first-generation embryos, and 7dpf unexposed and exposed second-generation embryos using a DNeasy Blood and Tissue Kit according to the manufacturer's protocol for tissue samples. Each DNA sample was extracted from 5-10 embryos each. An Epigentek Methylflash^{TM} Methylated DNA Quantification Kit (Colorimetric) was used to measure global DNA methylation in exposed first-generation embryos and unexposed and exposed second-generation embryos. This kit was used according the manufacturer's protocol.

Figures

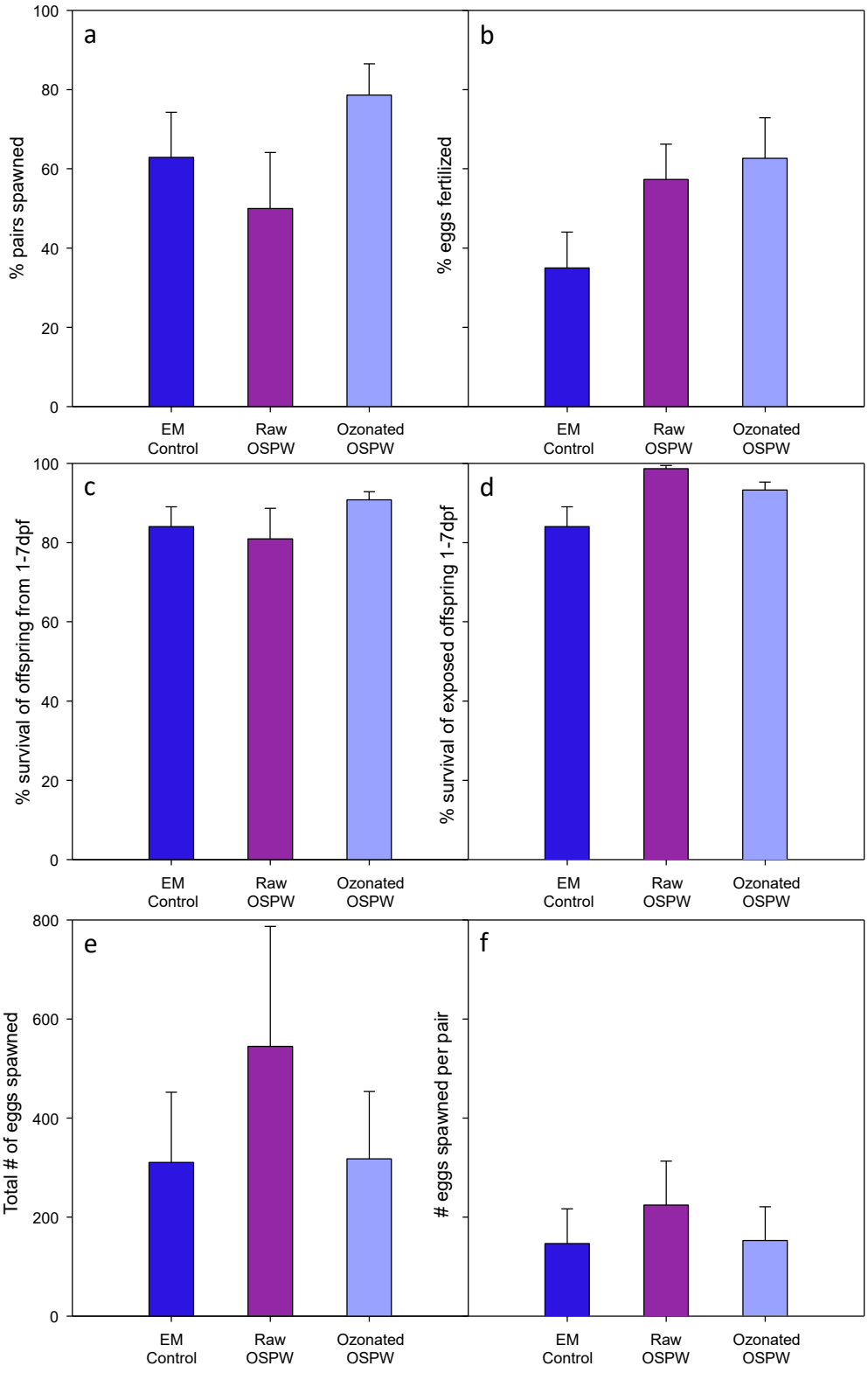
a
% pairs spawned
100
80
60
40
20
0
EM Control
Raw OSPW
Ozonated OSPW
b
% eggs fertilized
EM Control
Raw OSPW
Ozonated OSPW
c
% survival of offspring from 1-7dpf
d
% survival of exposed offspring 1-7dpf
e
Total # of eggs spawned
800
600
400
200
0
f
eggs spawned per pair

Figure A.1. Breeding success of embryos developmentally exposed to raw and ozonated OSPW as determined by percent pairs spawned (a), percent eggs fertilized (b), percent survival of offspring from 1-7dpf (c), percent survival of exposed offspring (d), total number of eggs spawned (e) and number of eggs spawned per pair that bred (f). (a-d) Percent pairs spawned, eggs fertilized, survival of unexposed and exposed offspring from 1-7 dpf did not differ between treatment groups (one way ANOVA, n=6-9). (e,f) The number of eggs spawned (both total and per pair) did not differ between treatment groups (one way ANOVA, n=6-8).

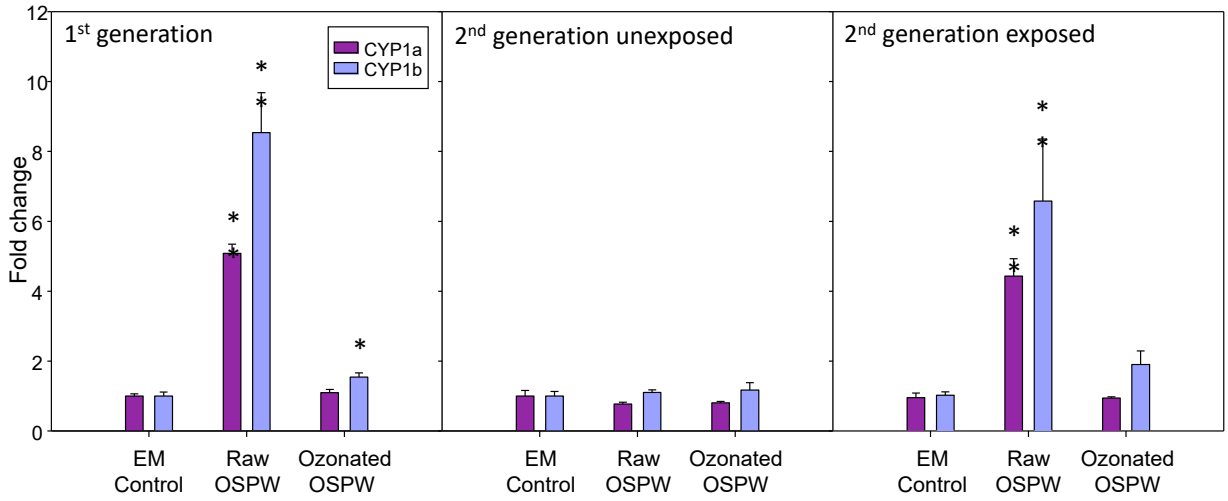

Figure A.2. Expression levels of *cyp1a* and *cyp1b* in unexposed and exposed second-generation embryos. Second generation unexposed embryos showed no alterations in expression levels of these genes. Raw OSPW exposed second-generation embryos had significantly increased expression in both Cyp1a and CYP1b (one way ANOVA, Tukey's post hoc, $p<0.001$). Ozonated OSPW exposed second-generation embryos had a slight increase in CYP1b expression but no change in the expression of Cyp1a (one way ANOVA, Tukey's post hoc, $p<0.05$, n=3-5).

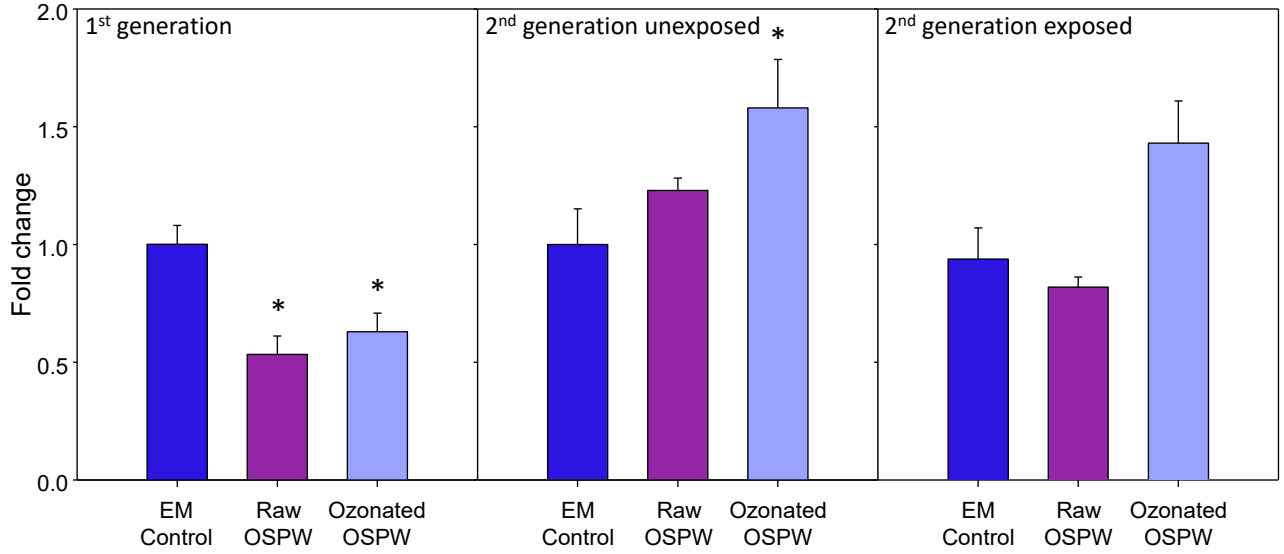

Figure A.3. Expression of *nkx2.5* in first generation and second-generation unexposed and exposed embryos. Second-generation unexposed embryos from parents developmentally exposed to ozonated OSPW had a significantly increased expression of *vtg* (one way ANOVA, Tukey's post hoc, $p<0.05$). Exposed second-generation embryos had no changes in *nkx2.5* expression (n=3-5).

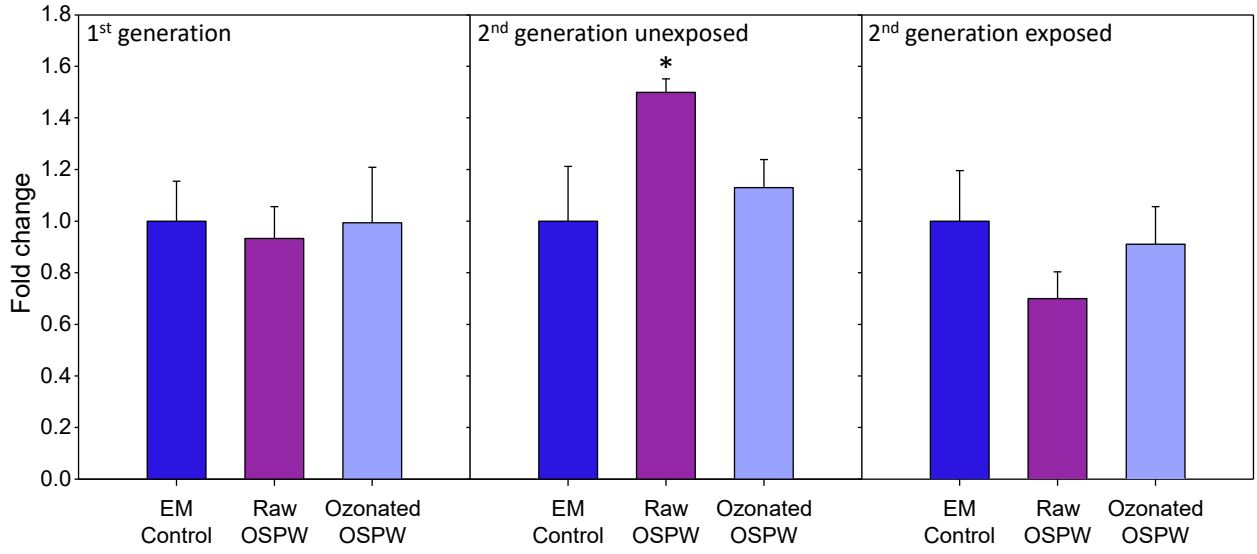

Figure A.4. Expression of *vtg* in unexposed and exposed second-generation embryos. Second-generation unexposed embryos from parents developmentally exposed to raw OSPW had a

significantly increased expression of *vtg* (one way ANOVA, Tukey's post hoc, $p<0.05$). Exposed second-generation embryos had no changes in *vtg* expression (n=3-5).

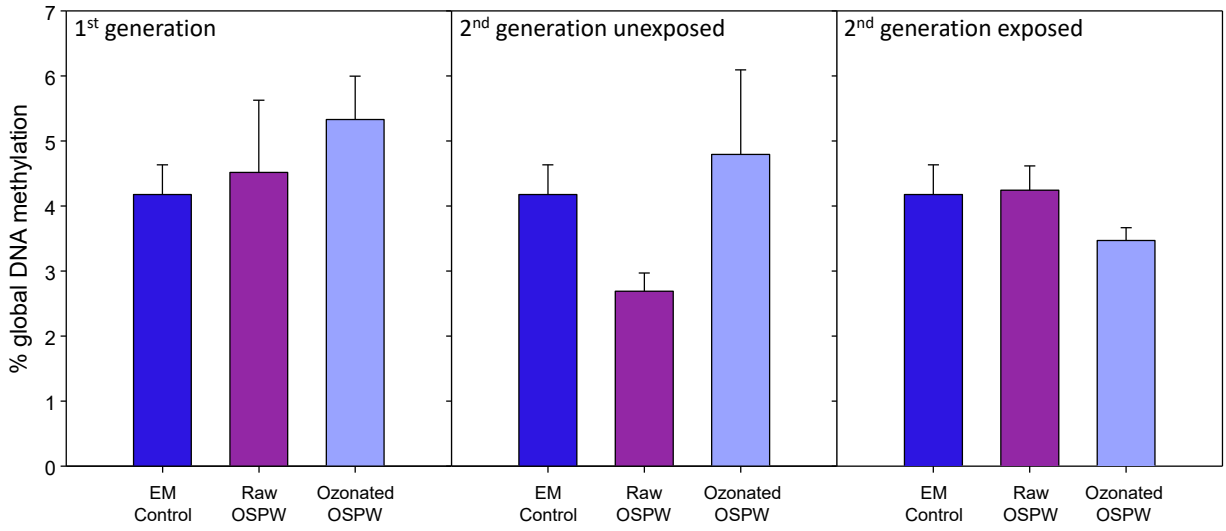

Figure A.5. Percent global DNA methylation of first-generation and second-generation embryos (unexposed and exposed). Global methylation was not altered by exposure in first-generation embryos or second-generation unexposed and exposed embryos (one way ANOVA, n=3-4 DNA samples per treatment extracted from 5-10 embryos each).