Changes in the Behavioral Responses of Fishes Exposed to Petrogenic Contaminants

by

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Abstract

The extraction, transport and refining of crude oil generates a diverse group of contaminants that pose a risk to both fresh and saltwater fishes. Adult fish can detect and actively avoid exposure to these pollutants; however, the embryonic and larval fish cannot outswim a toxic plume and are more sensitive to narcotic contaminants like crude oil. Because of the diversity of oil-based contaminants as a group, the primary mechanisms of toxicity vary. In crude oil, polycyclic aromatic hydrocarbons (PAHs) are thought to be responsible for the lethality and cardiotoxicity observed. Each type of crude oil has a unique PAH fingerprint that contributes to differences in toxicity, and crude oils with uncharacteristic PAH composition like diluted bitumen (dilbit), are not well characterized. Unlike crude oil, the toxicity of by-products of oil extraction such as oil sands process-affected water (OSPW) is thought to be driven primarily by naphthenic acids (NAs). OSPW also contains high concentrations of salts and some heavy metals that could contribute to its toxicity to fishes. In my thesis, I studied the impact of a broad range of oil-related contaminants on the development and behavior of both fresh and saltwater fishes. My study included various unweathered, weathered and dispersed crude oils, dilbit, and raw and treated (ozonated) OSPW.

Dilbit, an unconventional crude oil, is made by diluting raw bitumen with natural gas condensates, which is a common practice in the oil sands region to facilitate pipeline transport. The toxicity and environmental fate of dilbit is poorly understood. In chapter two, the developmental toxicity of dilbit was compared to two conventional crude oils that are commonly shipped via pipeline transport. Water accommodated fractions (WAFs) of dilbit caused lower levels of mortality and pericardial edema than conventional crude oil; the prevalence of yolk sac edema was similar across all exposures. All WAF exposures decreased border dwelling/shelter

seeking behavior and eliminated a ‘steady state’ swim phenotype that was present in the control population. The monoaromatic hydrocarbon content in the WAF was the primary predictor of lethality and pericardial edema. In my study, dilbit toxicity was less than or equal to that of conventional crude oils.

The Deepwater Horizon oil spill was the largest spill in U.S. history and the effect a spill of this magnitude could have on pelagic and coastal fish species in the Gulf of Mexico is not well understood. In chapter three and four I compared the toxicity of weathered, unweathered and dispersed crude oil to both fresh and saltwater model fishes. Weathering decreased the PAH content in both fresh and saltwater WAFs; dispersant application increased the PAH concentration in saltwater WAFs exponentially cf. the freshwater equivalent. The WAF LC_{50} (lethal loading rate) values for the freshwater zebrafish and sheepshead minnow 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. Acute WAF exposure increased the heart rate in zebrafish and decreased the heart rate in sheepshead minnow, and altered the mRNA expression of biotransformation enzymes, vitellogenin and neurodevelopmental genes in both species. As adults, developmental exposure to unweathered oils increased the variability in swim activity and decreased the variability in anxiety-based behavioral responses in sheepshead minnow.

OSPW, a by-product of bitumen extraction in Northern Alberta, has acute and chronic effects on aquatic life. Ozonation, a potential treatment technology, has been shown to effectively remove or greatly reduce NAs from OSPW. The OSPW used in my study was practically non-toxic to zebrafish embryos, and changes in mRNA expression in cardiac development and biotransformation enzymes were not associated with tissue level effects in exposed fish. Developmental exposure had no impact on breeding or prey capture success, but

both raw and ozonated OSPW altered the response of fish to hypoxanthine-3-n-oxide (an alarm cue) and decreased the variability of responses to the induction of prey. Unexposed and exposed F1 embryos had different border dwelling, maximum swim speed and activity levels than their unexposed counterparts, and there were transgenerational effects of exposure on VTG and NKX2.5 expression. Ozonation was able to mitigate some, but not all the effects of exposure.

Developmental exposure to oil-based contamination permanently alters the behavioral responses of both fresh and saltwater fishes. The impact these behavioral changes could have on wild fish populations during a spill or release is yet to be determined.

Preface

This thesis is an original work by Danielle Philibert. Research ethics approval from the University of Alberta Animal Care and Use Committee (ACUC) was given for this research project under the animal use protocol AUP00052 – Chemicals, effluents, and fishes.

Chapter two of my thesis is a journal article published in *Environmental Science & Technology*. The published citation is:

Philibert, D.A., Philibert, C.P., Lewis, C., and Tierney, K.B. (2016). Comparison of diluted bitumen (dilbit) and conventional crude oil toxicity to developing zebrafish. *Environ. Sci. Technol.*50: 6091- 6098.

I was responsible for writing this article and all the data collection and analysis for this publication, Carlie Lewis and Clara Philibert shared the work of breeding fish, and embryo exposure and care.

Chapter three of my thesis is a journal article published in *Science of the Total Environment*. The published citation is:

Philibert, D.A., Lyons, D., Philibert C.P., and Tierney, K.B. (2019). Field-collected crude oil, weathered oil and dispersants differentially affect the early life stages of freshwater and saltwater fishes. *Sci. Total Environ.* 647: 1148- 1157.

I was lead author of the article, generating all the water- accommodated fractions of oil, analyzing all the chemistry data, scoring heart rate (assisted by Clara Philibert) and muscle deformities, toxic unit calculations for both the zebrafish and the sheepshead minnow larvae. Danielle Lyons was responsible for all the steps in collected gene expression data, and she also wrote a rough draft of all the information on gene expression in the article. Embryo exposures and care were shared between Danielle Lyons, Clara Philibert and myself.

Chapter four of my thesis is an article under review in the journal *Toxicological Sciences*. The manuscript number is TOXSCI-19-0169.

I collected and analyzed all the behavioral data for this paper and wrote the article. Clara Philibert helped run the prey capture and male aggression trials, and Danielle Lyons extracted the DNA and ran the DNA methylation kit.

Chapter five of my thesis is an article published in the journal of *Environmental Pollution.* The published 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.*241: 959-968.

Danielle Lyons 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. I was responsible for collecting and analyzing heart rate and jaw morphology. The work of breeding fish, exposing and caring for embryos, and counting embryo survival was shared between Danielle Lyons and I. Taylor Zablocki, under my supervision, 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 six of my thesis is a journal article under review by the journal *Environmental Science & Technology.* The manuscript number is es-2019-01213h.

I was responsible for writing this article in its entirety. Larval exposures, larval care and fish breeding was shared between Danielle Lyons and I. I conducted the transgenerational behavior experiments, prey capture experiments, and alarm compound experiments. Danielle Lyons extracted RNA, ran qPCR, and ran the DNA methylation kit. Rui Qin and Rongfu Huang measured the chemical composition of both types of OSPW and ozonated the OSPW for the exposures.

Dedication

Toxicology, it's a bastard science!

-Dr. Mike Belosevic

Acknowledgements

The first person I would like to thank would be supervisor and mentor Dr. Keith Tierney. Thank you for taking a chance on a student with a mediocre GPA and the vocabulary of a drunken sailor. You have given me the opportunity to challenge myself, you have taught me how to critically think, and I have had 5 years of happy memories and good laughs in your lab. I wouldn't trade this experience for the world, and I am sad to leaving a place that truly felt like home.

I would also like to thank my committee members Dr. Mohamed Gamal El-Din, Dr. Greg Goss, and Dr. Sarah Hughes for their advice and support throughout my degree.

Lastly, I would like to thank my friends, family and lab mates who supported me throughout this degree, in particular:

Dani Lyons, I feel privileged to have worked (and lived) with someone who is talented, ambitious and fun as you. You are the molecular to my behavior and I will likely never find another scientist that I sync with as well as you.

Christie Morrison, you are wise beyond your years. You made the lab a fun place to be and working and drinking with you was a real privilege.

My mom and dad, thank you for always being there throughout this whole experience. You have kept me well fed and grounded, and without your guidance and I would have never become the scientist let alone the person I am today.

My sister, fish breeder extraordinaire, I wouldn't have half the n values I do without you. Thank you for being my right-hand man and counting so many damn fish.

My cousin bestie, Hailie Carnegie. You are a person I could turn to in my darkest meltdowns and who would celebrate my victories as if they were your own. I am truly blessed to have you as my best friend.

Doug Kelly, though you are a new addition to my life compared to a lot of the people on this list, I love you beyond words, and you brought the peace and balance to my life that I desperately needed to finish writing this thesis.

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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).

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Figure 6.6. Changes in the activity of fish in the control (A, B) raw OSPW exposed (C, D) and ozonated OSPW exposed (E,F) population before and after the introduction of alarm compound hypozanthine-3-n-oxide. Heat maps represent populations of fishes and their swimming activity metrics. Control fish had two activity types present in the population: high distance travelled and high velocity fish (indicative of active exploring) and low velocity moderate distance travelled fish (indicative of a steady state swim). After exposure to alarm cue fish maintained the higher max velocity but travelled less distance (freezing and darting phenotype-indicative of anxiety). Fish exposed to raw and ozonated OSPW were less active than control fish, and in response to the alarm cue displayed a steady state swim phenotype.

Figure 6.7. Effect of developmental exposure of raw and ozonated OSPW exposure on the percent prey captured and distance travelled during the prey capture trial. No differences were found across treatment groups.

Abbreviations list

ACUC- Animal care and use committee
ANOVA – Analysis of variance
AOP – Advanced oxidation processes
BOD5 – 5 day biochemical oxygen demand
BTEX – Benzene, toluene, ethylbenzene, xylene
C.I. – Confidence intervals
COD – Chemical oxygen demand
Dilbit – Diluted bitumen
Dpf – Days post fertilization
EM – Embryo media
EPA – Environmental protection agency
GC/FID – Gas chromatography with flame ionization detection
GC/MS – Gas chromatography-mass spectrometry
H3NO – Hypoxanthine-3-n-oxide
K_{ow} – Octanol water partitioning coefficient
LC_{50} – Lethal concentration for 50% of the population
MOA – Mechanism of action
MSB – Mixed sweet blend
MSC – Medium sour composite
NA – Naphthenic acid
NTU – Turbidity
OSPW – Oil sands process-affected water
PAH – Polycyclic aromatic hydrocarbon
PBS – Phosphate buffered saline
PBT - Phosphate-buffered saline with Tween
PCA – Principal component analysis
qPCR – Quantitative polymerase chain reaction

RF – Response factor
SDL – Sample detection limits
SEM – Standard error of the mean
SHC – Saturated hydrocarbons
SIM – Selection ion mode
TLM – Target lipid model
TOC – Total organic carbon
TPAH – Total polycyclic aromatic hydrocarbon content
TPH – Total petroleum hydrocarbons
TRAP – Toxicity relationship analysis program
Tu – Toxic units
TUNEL - Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling
UPLC-TOF-MS - Ultra performance liquid chromatography time-of-flight mass spectrometry
VOC – Volatile organic component
VTG – Vitellogenin
WAF – Water accommodated fraction
ΣPAH – Sum of PAHs in solution

Chapter 1: General Introduction

Background information

Crude oil and its associated by-products are ubiquitous in many aquatic environments due to both natural (i.e. oil seeps and surface deposits) and anthropogenic sources (i.e. pipeline ruptures, grounded ships, storage tank leaks and tailings pond seepage). This diversity in sources gives rise to a large family of complex contaminant mixtures, including unweathered and unweathered oil, unconventional oil, such as diluted bitumen (dilbit), and crude oil extraction effluents, such as oil sands process water (OSPW). To reduce oil-associated contaminants and any adverse effects they may have on aquatic life, remediation efforts have focused on facilitating the breakdown of the contaminants and treating contaminated water. Historically, chemical dispersants have been applied to large marine oil spills to assist biological and physical weathering (Lehr et al., 2010). Such methods may emulsify oil, which may have uncertain ramifications to aquatic life (Couillard et al., 2005). Oil extraction-based contaminants, on the other hand, respond well to treatment, such as advanced oxidation processes (AOPs), and previous studies have found that AOPs can reduce the toxic effects of OSPW (He et al., 2012; Wang et al., 2016; Lyons et al., 2018). Overall, a question is, can water-soluble components of oil, oil-extraction and oil emulsions be related to specific toxic effects?

The Exxon Valdez oil spill of 1989 was the first large scale oil release to occur in U.S. waters, and the funding and research that occurred as a result of the spill laid the foundation for our current understanding of crude oil toxicity. 42 million liters of Alaskan North Slope crude oil was released into Prince William Sound and spread over 750km along the Alaskan coast (Peterson et al., 2003). The impact the Exxon Valdez oil spill had on marine ecosystems in the area persist to this day (Peterson et al., 2003). Shortly after the spill, comparative work began on other crude oil sources, and it became clear that oils from different geological sources can vary greatly in both physical behavior and chemical composition (Christensen et al., 2005). The ratios of aliphatic hydrocarbons, aromatic hydrocarbons, resins, and asphaltenes determine the chemical characteristics and the toxicological impact of a crude oil sample (Christensen et al., 2005). Heavy crude oils like Alaskan North Slope crude from the Exxon Valdez spill, and diluted bitumens produced in Northern Alberta, tend to have a higher proportions of asphaltenes,

long chain aromatic hydrocarbons, and sulfonated compounds than most lighter crude oils. Medium and light crude oils tend to have higher levels of monoaromatic and short chain aromatic compounds. These crude oil varieties come in both "sweet" and "sour" varieties, sour indicating a high sulphur content easily identified by a sour odor. How sulphur content and variations in chemical composition contribute to different toxicological effects is not well understood. Of all the compounds present in a crude oil sample, the toxicity of a given crude oil has been attributed primarily to polycyclic aromatic hydrocarbons (PAHs) (Carls et al., 2008). These compounds have high solubility, are environmentally persistent, and are readily taken up by oil-exposed organisms (Incardona et al., 2013). Cardiotoxicity has thought the be the most sensitive phenotype associated with crude oil exposure (Incardona et al., 2013).

Despite the compositional variability of crude oil and crude oil extraction by-products, their exposure has been frequently associated with changes in cardiac function and embryo survival. Cardiotoxicity has been the major research focus of crude oil toxicity testing in fishes (Incardona et al., 2004; Incardona et al., 2006; Carls et al., 2008; Incardona et al., 2009; Incardona et al., 2011; Incardona et al., 2015; Marentette et al., 2015; Mohseni et al., 2015; Brown et a. 2017). Oil exposure can cause a wide range of defects that include changes in heart shape, abnormal vessel branching, impaired circulation and severe edema (de Soysa et al., 2012; Incardona et al., 2013; Mager et al., 2014). Exposure to the water-soluble components in crude oil also decreased heart rate and survival of fish embryos (Linden et al., 1976; Middaugh et al., 1996; Incardona et al., 2009; Shen et al., 2010; Zhang et al., 2014; Tissier et al., 2015). Unconventional crude oils (e.g. bitumen) are not as well studied, though they are comparably lethal and cardiotoxic to the conventional crudes (Philibert et al., 2016). Commercial naphthenic acid (NA) mixtures that mimic OSPW, and organic fractions of OSPW, have also been shown to cause pericardial edema, decrease heart rate and decrease survival in the early life stages of fishes (Scarlett et al., 2013; Wang et al., 2015; Marentette et al., 2015; Marentette et al., 2015; Li et al., 2017). The effects of exposure on more environmentally relevant endpoints such as prey capture ability and breeding success have yet to be determined.

Relatively few studies have been conducted on the effects of crude oil and oil extraction-based contaminants on complex behaviors in fishes, despite the merits of including these sensitive and ecologically relevant endpoints in toxicological studies. Exposure to crude oil may

decrease spontaneous swimming activity, decrease feeding activity, increase prey capture latency, and decrease border dwelling (Schulman et al., 2000; Kochlann et al., 2015; Frantzen et al., 2015; Lari et al., 2016; Philibert et al., 2016). Oil exposure may also change the swimming activity of larval fish by altering variation in behavioral phenotypes (i.e. 'personality'), vs. outright impairment of function (Philibert et al., 2016). This particular study offered no mechanism for the behavioral changes. In a novel tank assay, behavioral phenotypes have been associated with differences in whole body cortisol (Egan et al., 2009). Fish exhibiting high levels of anxiety-based locomotor activity were found to have higher levels of whole body cortisol relative to the less anxious fish (Egan et al., 2009). Cortisol levels during embryological development have also been associated with behavioral phenotypes of adult fish (Wilson et al., 2016). Fish that were embryologically treated with a synthetic glucocorticoid had a higher frequency of bold behavioral phenotypes as adults (Wilson et al., 2016). These studies suggest cortisol can be associated with behavioral phenotypes, and that developmental cortisol levels may pre-determine the behavioral phenotypes found in a population of exposed fishes.

Research objectives

The main objective of my research is to explore the effects of crude oil and oil extraction-related contaminants on ecologically relevant behaviors in both fresh and saltwater fishes. My research objectives are distributed across three contaminant-based studies: i) a comparison of diluted bitumen (unconventional crude oil) and conventional crude oil toxicity to freshwater fish, ii) the impact of weathering and dispersant application on crude oil toxicity to fresh and saltwater fishes, and iii) the effects of raw and ozonated OSPW on freshwater fish. These objectives can be broken into two areas: 1) characterization of toxicity of various crude oils and oil extraction-based contaminants to early life stages; and 2) exploration of any lasting impacts of crude oil and oil extraction-based contaminant exposures on a diverse array of ecologically relevant behaviors.

First, I related oil constituent composition to embryo lethality and cardiotoxicity. Oil-based contaminants encompass a wide range of constituents with highly variable chemical characteristics. OSPW contains metals, salts, un-recovered bitumen and naphthenic acids (Allen et al., 2008; Headley et al., 2013). Their presence can all vary with the age of the storage pond (Rowland et al., 2012; Marentette et al., 2015), and the physicochemical extraction process used (Grewer et al., 2010; Rowland et al., 2012), and can also be altered by ozonation. Ozonation is a

remediation method that has been previously shown to decrease the toxicity of OSPW (He et al., 2012). Similarly, the impact of a crude oil release can vary depending on the oil source (Anderson et al., 1974; Philibert et al., 2016), degree of weathering (Chapman et al., 1995; Shelton et al., 1999), and the use of chemical dispersants, which increase crude oil toxicity (Couillard et al., 2005; Ramachandran et al., 2004; Anderson et al., 2009; Schein et al., 2009; Adeyemo et al., 2015; Mu et al., 2014; Finch et al., 2017). In my research, I planned to explore the compositional differences of the mixtures and relate them to differences in early-life stage toxicity. Because of the wide range of crude oils and oil extraction-based contaminants used in my study, I would expect that oil mixture related-constituents would be differentially toxic due to the compositional diversity of the mixtures, and the ozonation and dispersants used.

Second, I explored the effects of exposure on various ecologically relevant behaviors. Regulatory guidelines put in place for the protection of fishes and their habitat are largely based on acute lethality testing. These guidelines often fail to take into account 'ecological death', which is an organism's inability to successfully compete for resources owing to subtle sublethal effects (Scott et al., 2004). Complex behaviors (i.e. foraging, predator avoidance, prey capture and reproduction) are superior to simple 'behaviors' (e.g. coughs, ventilation, body tremors) because they link physiological changes to population level effects (Scott et al., 2004). Despite the value of complex behaviors, few crude oil and OSPW studies include these endpoints. Because of the diverse compounds found in crude oil and oil extraction-based contaminants and the sensitivity of neural development early in development, I would expect exposure to alter ecologically relevant behaviors in fishes. Mechanisms through which behaviors could be affected include altered development of neural networks, total body cortisol in early development, and sensory systems.

Chapter 2: Comparison of diluted bitumen (dilbit) and conventional crude oil toxicity to developing zebrafish

Abstract

To facilitate pipeline transport of bitumen, it is diluted with natural gas condensate, and the resulting mixture, 'dilbit', differs greatly in chemical composition to conventional crude oil. Despite the risk of accidental dilbit release, the effects of dilbit on aquatic animals are largely unknown. In this study, we compared the toxicity of water accommodated fractions (WAFs) of dilbit and two conventional crude oils, medium sour composite and mixed sweet blend, to developing zebrafish. Mortality and pericardial edema was lowest in dilbit WAF-exposed embryonic zebrafish but yolk sac edema was similar in all exposures. Shelter-seeking behavior was decreased by dilbit and conventional crude WAF exposures, and continuous swimming behavior was affected by all tested WAF exposures. Regardless of WAF type, monoaromatic hydrocarbon content (largely made up of benzene, toluene, ethylbenzene, and xylene (BTEX)), was a more accurate predictor of lethality and pericardial edema than polycyclic aromatic hydrocarbon (PAH) content. Our results suggest that the toxicity of dilbit to a model fish is less than or similar to that of conventional crudes.

Introduction

The oil sands in northern Alberta cover a total of 142 200 km², reserves totaling an estimated 168 billion barrels of oil, and is the third largest oil reserve in the world (Government of Alberta). The bitumen reserves, unlike conventional oil reserves, are not preserved through bactericidal subterranean temperature spikes. In the absence of heat preservation, oil-degrading microorganisms metabolize the lighter chain hydrocarbons, leaving behind only the heavy chain hydrocarbons typically found in crude oil (Crosby et al., 2013) The viscosity of bitumen produced from heavy chain hydrocarbons is not naturally conducive to pipeline transport, and so the addition of diluent (i.e. natural gas condensate) is common practice. Diluent is typically added at a 30% diluent and 70% bitumen ratio, creating what is referred to as dilbit (Crosby et al., 2013). With increased development oil sands and transport of dilbit, there is risk for accidental release and environmental exposure. Relatively recently, a dilbit spill in the Kalamazoo River, MI, of an estimated 843,000 gallons posed a unique environmental threat as droplets of dilbit coated sediment in the water column and sank to the river bed (EPA). Despite

146 the risk dilbit spills pose to aquatic life, data on its toxicity is lacking. What little is known
147 suggests that while it shares some toxicological characteristics with crude oil, it may pose a
148 unique threat to aquatic species if a spill were to occur due to its composition and environmental
149 fate (Dew et al., 2015; Madison et al., 2015)

150 Spurred by major catastrophes such as *Exxon Valdez* and *Deepwater Horizon* oil spills,
151 research in the field of conventional-crude oil toxicology has made rapid progress in assessing
152 the impacts of exposure on fish health and development. Embryological exposure to crude oil
153 can induce a wide range of cardiac defects. Sublethal cardiac defects occur across a spectrum of
154 severity that ranges from subtle defects in cardiac form and function to defects that impact the
155 swimming performance, vasculature development, and circulatory proficiency along the trunk of
156 the fish (de Soysa et al., 2012; Incardona et al., 2013; Mager et al., 2014). In the most severe
157 cases, early developmental exposure can lead to loss of circulation, severe pericardial edema and
158 death (Incardona et al., 2013). Fish that exhibit cardiac defects on the milder end of the spectrum
159 may survive to adulthood under controlled laboratory conditions, but will have permanent
160 cardiac impairment that is associated with reduced swimming performance (de Soysa et al.,
161 2012; Incardona et al., 2013; Mager et al., 2014; Kennedy et al., 2006; Hicken, 2011; Yu et al.,
162 2015). Changes in cardiac morphology and cardiac output are considered sensitive indicators of
163 crude oil exposure (Incardona et al., 2015; Incardona et al., 2004; Incardona et al., 2006; Carls et
164 al., 2008; Incardona et al., 2009; Incardona et al., 2011; Jung et al., 2013), however, they are not
165 the only effects that have been noted. Studies suggest that exposure to water accommodated
166 fractions (WAFs) of oil can affect the development of the peripheral neuron axon projections (de
167 Soysa et al., 2012), reduce acetylcholinesterase activity (Kochhann et al., 2015), cause brain
168 hemorrhaging (Kochhann et al., 2015), decrease response to alarm substance (Kochhann et al.,
169 2015), decrease spontaneous swimming activity (Kochhann et al., 2015), and cause abnormal
170 swimming and locomotor behavior (de Soysa et al., 2012).

171 The principal goal of this study was to compare the toxicities of dilbit and two other
172 conventional crude oils in a model fish species, the zebrafish (*Danio rerio*). In comparing
173 toxicities, we chose to use a variety of endpoints, including those of cardiac and behavioral
174 relevance. A secondary goal was to ascribe differences in the oil compositions to any observed
175 differences in effects.

Materials and Methods

Fish

Embryos were collected from adult AB strain zebrafish, that were kept on a 14h:10h light dark cycle and were fed a custom mixture of a 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).

Preparation of water accommodated fractions and exposures

Two conventional crude oils, mixed sweet blend (MSB) and medium sour composite (MSC), and a bitumen natural-gas condensate blend (dilbit) were used in this study. Both MSB and MSC were benchmark blended aggregates of light, medium and heavy hydrocarbons that originate from various oil wells drilled in the Western Canadian Sedimentary Basin. Oil samples were stored at 4°C in amber glass bottles capped with argon gas under a Teflon plug. The WAFs were prepared in a 1:10 oil:water ratio using the non-vortexing method as previously described (Singer et al., 2000). In brief, in 2L aspirator bottles, 180 mL of crude oil was added to 1.8L of embryo media using a gas tight glass syringe (leaving 20% headspace in the bottle). The bottle was then capped and set to stir at approximately 100 rpm for 20 h. Following stirring, the bottle was left to settle for 4 h and the oil-less portion of WAF was collected unfiltered. After collection, WAF was pH adjusted to 7.2 ± 0.05 using a 0.1M HCl to eliminate potential pH dependent effects. Fresh WAFs were made every 48 h.

Groups of 70 embryos were exposed within 30 min post-fertilization to 7 days post fertilization (dpf) to 0, 10, 20, 40, 60, 80 or 100% WAF in glass Petri dishes. Petri dishes contained 40 mL of exposure media of which ~95% were refreshed daily. Exposure groups were replicated 3-5 times with 1-2 plates per replicate, per oil type.

Analytical chemistry

To measure polycyclic aromatic hydrocarbons (PAHs), alkylated PAHs and petroleum biomarkers the WAF samples were processed using liquid/liquid extraction according to the 3510C EPA method (www.3epa.gov). In brief, the PAH/alkylated PAH and petroleum were measured using gas chromatography-mass spectrometry (GC/MS) in the selection ion mode (SIM). Saturated hydrocarbons (SHC) and total petroleum hydrocarbons (TPH) were measured using the same liquid/liquid extraction, 3510C EPA method. The analytes were then measured

using gas chromatography with flame ionization detection (GC/FID). Volatile hydrocarbons (VOC) were measured in the WAF samples using gas chromatograph-mass spectrometer (GC/MS) equipped with a purge-and-trap autosampler and concentrator unit. The method used is a modified version of the EPA Method 8260C. For more detail on the analytical chemistry, refer to the supplemental information.

Morphological assessment and heart rate recordings

Pericardial edema was recorded as present/absent based on fluid accumulation around the heart and lack of epidermal pulsation adjacent to the heart (Incardona et al., 2013); yolk sac edema was recorded as present/absent based on obvious morphological variance as previously described (Fraysse et al., 2006). Abnormal tail curvature was scored based the presence of a dorsal or lateral curvature in the tail after 3 dpf. All deformities, along with hatch rate, were counted daily and daily totals were summed to determine the incidence of each deformity over the course of the 7 d exposure period. To examine relationships between WAF toxicity and class of chemical compounds, survival and pericardial edema were plotted against ΣPAH, toxic units of PAH and total monoaromatic hydrocarbon content. Toxic units of PAHs were calculated using the target lipid model to normalize for the differing toxicities of individual PAHs in the WAFs (refer to supplemental information). For 2 dpf embryos, between 14:00-16:00, 30 s videos were taken of embryo hearts and heart rates were determined manually. The videos were randomized and scored blind.

Behavioral assessments

Behavior was measured in embryos at 7 dpf, as at this stage, the embryos had exhausted their yolk sac and would need to swim to forage. For these measurements, embryos were placed in 2 mL of their respective exposure solution, randomly distributed across the 12 central wells of a 24 well plate (Costar®, Corning, NY), and left undisturbed for 20 min. After acclimation, the embryos were video recorded overhead for 10 min using a CCTV camera (WV-CL930; Panasonic, TX). Maximum swimming speed, total distance traveled and time spent in the border (3 mm around the 12mm diameter wells) were determined using EthoVision XT 10 (Noldus, NE). The time spent in the border can serve as a measurement of anxiety-like behavior in adult and larval fish, as an anxious fish will search the periphery in order to find shelter (Richendrfer

2012). We chose to examine the 0, 60 and 100% WAF-exposed embryos because this was sufficient to capture the range of effects. Due to poor survival of the 100% MSC WAF-exposed embryos (>3%), this group was omitted. To visualize if behavioral phenotypes ('personalities') were affected by WAF exposures, the maximum speed, distance traveled and time spent in the border were plotted using 3D mesh plots.

Statistical analysis.

The LC_{50} values were determined using the Toxicity Relationship Analysis Program (TRAP) developed by Russell Erickson and the U.S. Environmental Protection Agency (Duluth, MN). Statistical differences between replicates were first tested using a one-way analysis of variance (ANOVA). As no differences were detected, replicates were pooled. Differences between the oil types were tested using a two-way (oil type x %WAF) ANOVA followed by a Holm-Sidak *post hoc* test. All percentage values (incidence of pericardial edema, yolk sac edema, abnormal tail curvature, and hatch rate) were arcsine transformed. As border seeking data were non-normal (which was tested using a Shapiro-Wilks test for normality) and had unequal variance, and as all transformations failed to normalize the data, differences between each treatment group were tested using a Kruskal-Wallis ranked one-way ANOVA with Dunn's methods for pairwise comparisons. We ran a second analysis on the nonparametric data using the permutation test approach (David C. Howell, University of Vermont, Burlington, VT) with R (R Foundation for Statistical Computing, Vienna, Austria). Significant difference was accepted at $p<0.05$ and all statistical tests (with the exception of the permutation tests) were performed using SigmaPlot 11 (Systat, San Jose, CA).

Analytical chemistry.

To measure polycyclic aromatic hydrocarbons (PAHs), alklylated PAHs and petroleum biomarkers the WAF samples were processed using liquid/liquid extraction according the 3510C EPA method (www.3epa.gov). In brief, the PAH/alkylated PAH and petroleum were measured using gas chromatography-mass spectrometry (GC/MS) in the selection ion mode (SIM). The initial calibration was performed using target analytes to demonstrate the linear range of the analysis. Every 24 hours calibration was verified to ensure the consistency of the analysis. Concentrations of each respective compound were calculated vs. internal standards. Analyte

levels were quantified using the average response factor (RF), which were generated using the values from the initial calibration. The alkylated PAHs were assigned RF based off of the parent PAH, triterpanes were assigned the RF parent value of moretane, and steranes were assigned the RF parent value of cholestane. Laboratory control spikes were prepared to ensure analytic accuracy.

Saturated hydrocarbons (SHC) and total petroleum hydrocarbons (TPH) were measured using the same liquid/liquid extraction, 3510C EPA method. The analytes were then measured using gas chromatography with flame ionization detection (GC/FID). Initial calibration was performed at the beginning of every 24 hour batch of samples to demonstrate the linear range of analysis. SHC and TPH concentrations were calculated using the internal standard method as described previously for PAH, alkylated PAH and petroleum biomarkers. Normal alkanes were quantified using the RF from the initial calibration, and the isoprenoid hydrocarbons were quantified using the RF of the n-alkanes immediately preceding and following each target isoprenoid hydrocarbon. Laboratory control spikes were used to calculate analytical accuracy and to ensure precise measurement of all analytes.

Volatile hydrocarbons (VOC) were measured in the WAF samples using gas chromatograph-mass spectrometer (GS/MS) equipped with a purge-and-trap autosampler and concentrator unit. The method used is a modified version of the EPA Method 8260C. In brief, the WAF sample was bubbled with inert gas at an elevated temperature to force the VOCs out of the aqueous solution into the gas phase. The VOCs were separated from other compounds in the gas by a sorbent trap, the sorbent trap was then heated, and the isolated VOCs were resolubilized in an inert gas that was collected in a non-polar fused silica capillary chromatography column. The components were separated using gas chromatography. The compounds were detected using the full scan mode of the mass spectrometer. Calibration was verified every 12 hours of sample analysis. The concentration of paraffins, isoparaffins, aromatics, naphthenes, and olefins (PIANO) were calculated using RF generated from the initial calibration. Samples that contained the analytes at concentrations above the highest range of the calibration value were re-analyzed at a smaller sample size as needed until all values rested within the calibration range.

Target lipid model.

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 (Di Toro 2000; Kipka 2009; McGrath 2005; Di Toro 2007) 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 LC_{50} and K_{ow} (Di Toro 2000) The formula can be broken down to:

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

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 LC_{50} 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 LC_{50} is the inverse Log of the numbers for each compound derived from formula (1) (McGrath 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) (Di Toro 2000), and the critical target lipid burden coefficient for zebrafish (1.920) (Kipka 2009), the chemical class correction (ΔC_i) for PAHs (-0.263)(Di Toro 2007), and the $\log(K_{ow})$ (Di Toro 2007), for each of the individual compounds from the literature.

Results and Discussion

Water accommodated fractions

The MSC WAF had the highest benzene, toluene, ethylbenzene and xylene (BTEX) content, followed by MSB and dilbit WAFs (Table 2.1). The MSB WAF had the highest concentration of total (Σ) petroleum hydrocarbons and ΣPAHs, followed by MSC, and then dilbit. Dilbit had a fourfold-lower ΣPAH content and the PAH profile differed from the conventional crudes; dilbit WAF had lower levels fluorenes, phenanthrenes/anthracenes and comparatively high levels of sulphur based PAHs. The sulphur based PAHs in MSC WAF (benzothiophenes and dibenzothiophenes) were comparable to dilbit. Compared to conventional crudes, dilbit WAF had unique elevations in select volatile organic compounds (VOCs), including pentane, isopentane, and dimethylbutane (Figure 2.1).

Survival and morphometrics

At WAF concentrations below 60% there was no impact on the survival of the embryos (Figure 2.2A). At 80 and 100% WAFs, both of the conventional crudes had a larger impact on survival than dilbit. The MSC WAF had the lowest 7day LC_{50} value at 72.7% (C.I. 70.8-74.7%), followed by MSB at 77.8 % (C.I. 76.1-80.1%), and dilbit at 88.4% (C.I. 85.5-90.5%). The frequency of pericardial edema reflected the survival of the embryos over the course of the exposure period (Figure 2.3B). The MSC WAF exposure induced the highest rates of pericardial edema at both 80 and 100% WAF ($F_{2,86} = 15.3, p < 0.001$); the MSB WAF exposure was similar ($F_{2,86} = 15.3, p < 0.001$).It should be noted that the 100% Dilbit WAF exposure group had high levels of variability in both survival and frequency of pericardial edema.

Across all oil types, toxicity was better explained by the monoaromatic content and not ΣPAH content or total toxic units of PAHs (Figure 2.2C; Figure 2.3D). Normalizing ΣPAHs to the toxic units of PAHs from the target lipid model accounted for differences in toxicity of individual PAHs, however even after applying the model, monoaromatic content best explained toxicity. A variety of studies have shown that PAHs can contribute a significant portion to cardiotoxicity and lethality of WAFs (Incardona et al., 2004; Carls et al., 2008; Incardona et al., 2009; Incardona et al., 2011; Jung et al., 2013). However, the monaromatic hydrocarbon content of WAFs is generally underreported and its toxicity under studied (Madison et al., 2015;

Incardona et al., 2013; Mager et al., 2014; Incardona et al., 2015; Carls et al., 2008; Incardona et al., 2011; Brown-Peterson et al., 2015; Carls et al., 2010; Tissier et al., 2015; Shen et al., 2010).

The MSC WAF decreased hatch rate for the embryos at 2 dpf ($F_{3,36} = 5.96, p < 0.001$)(Figure 2.4B). This delay was only temporary, as by 3 and 4 dpf the difference in hatch rate was no longer present. Hatch rate was unaffected by the other WAFs. Exposure to PAHs can cause severe edema, which may slow hatching, and may also delay development (Tissier et al., 2015).

The relative frequencies of pericardial edema did not correspond with changes in heart rate in the different treatment groups (Figure 2.4A). The 80 and 100% MSB WAF exposures decreased heart rate, dilbit WAF increased heart rate at 60 and 80%, showing no real dose dependent effect of oil exposure on heart rate ($F_{2,331} = 7.27, p < 0.001$). Previous studies have found that oil exposure during sensitive windows of growth decreased heart rate (Incardona et al., 2009; Shen et al., 2010; Linden et al., 1976; Middaugh et al., 1996; Incardona et al., 2012; Zhang et al., 2014; Brette et al., 2014), and PAHs have been linked to decrease cardiac function (Hicken 2011; Yu et al., 2015; Incardona et al., 2004; Incardona et al., 2006), arrhythmia (Incardona et al., 2009; Incardona et al., 2011; Jung et al., 2013), and impaired excitation-contraction coupling of cardiomyocytes (Kalueff et al., 2013). These data argue that there are multiple factors in each of the different WAFs and within the WAFs that are contributing to various changes in heart rate.

To show what proportion of deaths were attributable to pericardial edema, the frequency of pericardial edema was plotted vs. mortality and the slopes of linear regression models were compared (Figure 2.3A). For the two conventional crudes, the occurrence of pericardial edema was a good predictor of lethality (slope = 0.966 [i.e. close to a 1:1 ratio], p <0.001, R^2 = 0.965, and 0.783, p <0.001, R^2 = 0.921, for MSC and MSB WAF; respectively); for dilbit it was not (slope = 0.526, p <0.001, R^2 = 0.803). None of the WAFs impacted the frequency of dorsal and lateral tail curvature (data not shown).

All three WAFs induced the same increases in the occurrence of yolk sac edema at 80 and 100% WAF exposure ($F_{6,86} = 25.87, p < 0.001$) (Figure 2.4C). Dilbit WAF was less lethal, and because yolk sac edema was the same across oil types, relative to mortality dilbit induced a

higher frequency of yolk sac edema. This suggests that chemicals within dilbit WAF were specific to this form of edema. Previous studies yolk sac edema was found to be a side effect of circulatory impairment and is commonly observed in crude oil-exposed zebrafish embryos (Incardona et al., 2013; Jung et al., 2013; Brown-Peterson et al., 2015).

Behavioral metrics

There was no difference in the average distance traveled and the maximum speed across exposure groups (p>0.05, Q<2.01) (S2.2). Both 100% MSB and dilbit WAF exposures altered shelter seeking behavior, as embryos exposed to these WAFs spent little to no time in the border vs. unexposed and 60% WAF exposed embryos (p<0.05, Q=4.45; Dunn's method; p<0.05, $F_{5,187}$=4.29; permutation test). Border seeking behavior is considered an anxiety-like response in zebrafish, as fish will naturally pursue the border in search of refuge from potential predators (Treit et al., 1988). Border dwelling is a very common behavioral endpoint that is highly conserved across a variety of model species including rodents (Belzung et al., 2007; Lopex-Patino et al., 2008), and fish (Colwill et al., 2011; Prut et al., 2003). Border dwelling is characterized as anxiety-like behavior because it can be repressed and enhanced with the use of anxiolytic and anxiogenic drug, respectively (Treit et al., 1988; Li 2015; Schnorr et al., 2012). The use of the thigmotaxis as a metric for anxiety-like behavior in zebrafish larvae has been validated as young as 5 dpf (Coleman et al., 1998). Regardless of mechanism, if an oil exposure were to cause a decrease in self-preservation behavior, there could be significant ramifications for survival in the case of accidental oil release.

In control embryos there were two different highly active behavioral phenotypes: embryos that had high maximum speeds of ~120-140 mm/s (the 'long distance-high speed' phenotype), and embryos that had lower maximum speeds of ~60 mm/s (the 'long distance-medium speed' phenotype) (Figure 2.5A). In the 100% dilbit and MSB WAF treatment groups, the 'long distance-high speed' phenotype was present, but the 'long distance-medium speed' phenotype was absent (Figure 2.5B, C). This phenotype is indicative of steady swimming, which would be more aerobically demanding. Previous studies have found aerobic capacity and swim performance may be hindered by oil exposure in juvenile and adult fish (de Soysa et al., 2012; Incardona et al., 2013; Mager et al., 2014; Kennedy et al., 2006; Hicken et al., 2011). The data from the present study indicate that swimming ability can be affected almost immediately post-hatch.

In our study the use of 3D mesh plots created a behavioral 'landscape' that facilitated identification of behavioral phenotypes in control and oil exposed embryos. Because of the inherent variation in behavioral data, categorizing behavioral phenotypes, or 'personalities', has been used to better analyze fish behavior, often in the form of binary comparisons of 'bold' and 'shy' individuals (Treit et al., 1988; Frost et al., 2007; Smith et al., 2009; Pasquet et al., 2015; Shamchuk et al., 2012). However, binary comparisons may be limited and not capture the spectrum of behaviors that exist. For example, a study exploring the stimulus-evoked responses of zebrafish embryos revealed 18 repeatable and discrete behavioral phenotypes (Shamchuk et al., 2012). In the current study, phenotypic differences across treatment groups served as a way to assess the swimming ability of an embryonic fish, one which could not be assessed in a more conventional manner using a swim tunnel respirometry.

Overall, comparing diluted bitumen to two conventional crude oils, monoaromatic hydrocarbon content was a better predictor of survival and pericardial edema than ΣPAH. The ΣPAH content likely related to bradycardia as heart rate was decreased by MSB WAF, which had the highest ΣPAH. Relative to the conventional crudes, dilbit produced a unique WAF chemical 'fingerprint' and had a lower impact on the survival of zebrafish embryos. Exposure to WAF, independent of oil type, impacted shelter-seeking behavior as well as the behavioral phenotypes of the embryos. Future studies should focus on increasing our understanding of the toxicity of monoaromatic compounds to fish early life stages and the role of complex mixtures containing monaromatics and PAHs.

Acknowledgements

The authors thank Vicki Lightbown and John Zhou. This study was funded by grants from Alberta Innovates Energy and Environment Solutions and NSERC to KBT.

Tables

Table 2.1. BTEX, ΣPAH, and total petroleum hydrocarbon content of mixed sweet blend (MSB), medium sour composite (MSC) and dilbit WAF. The ΣPAH includes the 16 EPA priority PAHs. (Sample Detection Limits (SDL) are given in parentheses)

	MSB (mg/L)	MSC (mg/L)	dilbit (mg/L)	(SDL) (ug/L)
Benzene	10.6	12.7	10.9	(0.0760)
Toluene	7.36	6.72	5.23	(0.0294)
Ethylbenzene	0.455	0.631	0.0908	(0.0118)
Xylene	2.26	1.86	1.05	(0.0409)
Total BTEX	20.7	21.9	17.3	n.a.
Total Petroleum Hydrocarbons	4.36	3.64	2.76	(0.0574-0.108)
ΣPAH	0.206	0.163	0.0461	(0.000594 - 0.00372)

Figures

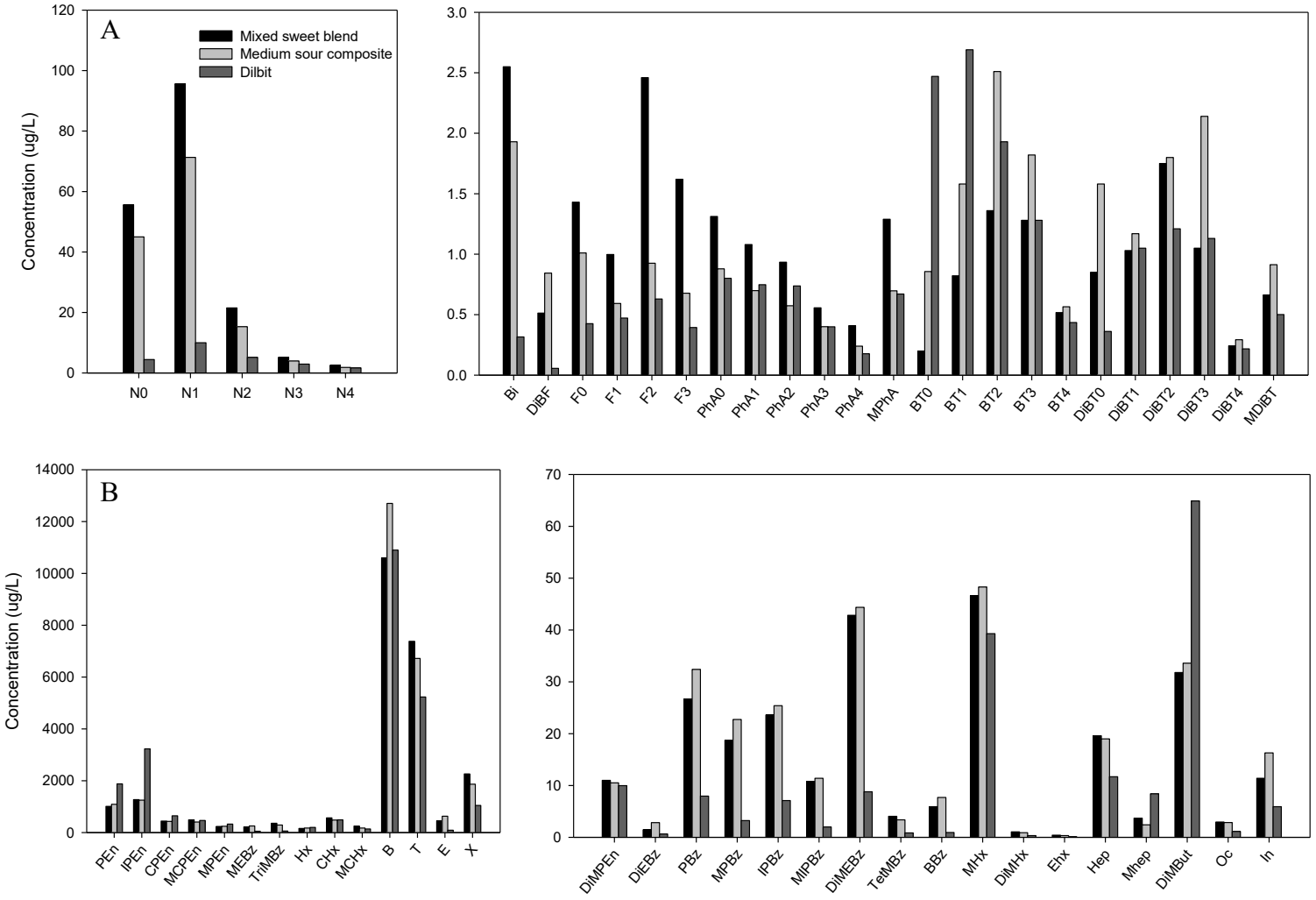

Figure 2.1. PAH (a), and VOC (b) content of MSB, MSC, and dilbit WAF. Compounds were grouped according to relative concentration and structural similarity. N, naphthalenes; Bi, biphenyl; DiBF, dibenzofuran; F, fluorene; PhA, phenanthrenes/anthracenes; MPhA, methylphenanthrene; BT, benzo[b]thiophene; DiBT, dibenzothiophene; MDiBT, methyldibenzothiophene; D, decalins; AcTy, acenaphthylene; Ac, acenaphthene; MA, methylanthracene; BbF, benzo[b]fluorine; FP, fluoranthene/pyrene; BNt, benzonaphthathiopene; BaC, benzoanthrocenes/chrysenes PEn, pentane; IPEn, isopentane; CPEn, cyclopentane; MCPEn, methylcyclopentane; MPEn, methylpentane; MEBz, methylethylbenzene; TriMBz, trimethylbenzene; Hx, hexane; CHx, cyclohexane; MCHx, methylcyclohexane; B, benzene; T, toluene; E, ethylbenzene; X, xylene; DiMPEn, dimethylpentane; DiEBZ, diethylbenzene; PBz, propylbenzene; MPBz, methylpropylbenzene; IPBz, isopropylbenzene; MIPBz, methylisopropylbenzene; DiMEBz, dimethylethylbenzene; TetMBz, tetramethylbenzene; BBz, butylbenzene; MHx, methylhexane; DiMHx, dimethylhexane; Ehx, ethylhexane; Hep, heptane; Mhep, methylheptane; DiMBut, dimethylbutane; Oc, octane; In, indane

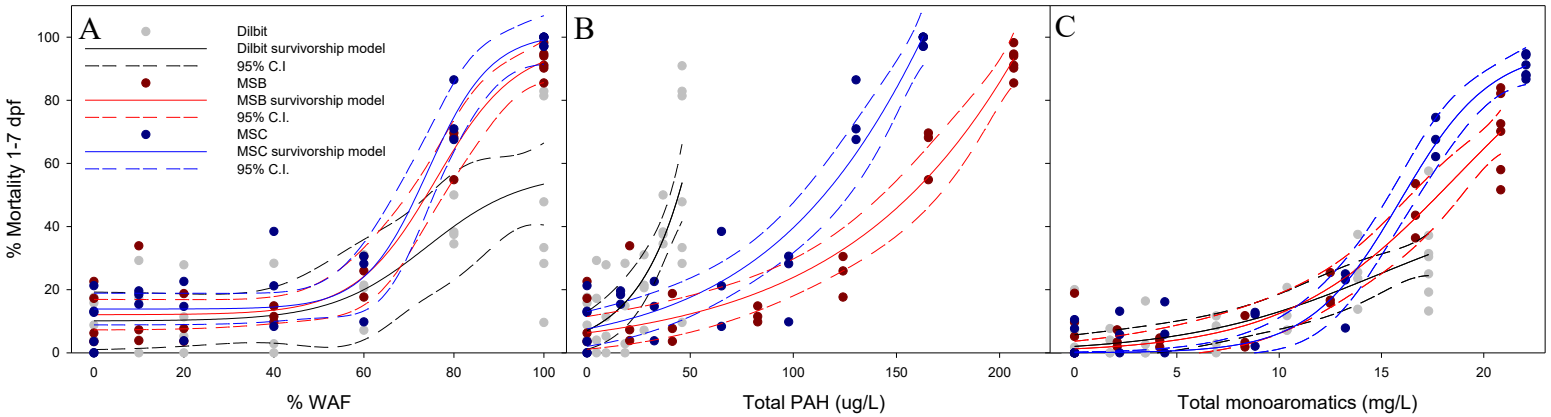

Figure 2.2. The percent mortality from 1-7 days post fertilization from 0-100% WAF (a) as well as the percent mortality from 1-7dpf in relation to the total PAH content (b), and total monoaromatic content (c). 95% confidence intervals (C.I) were included with each curve.

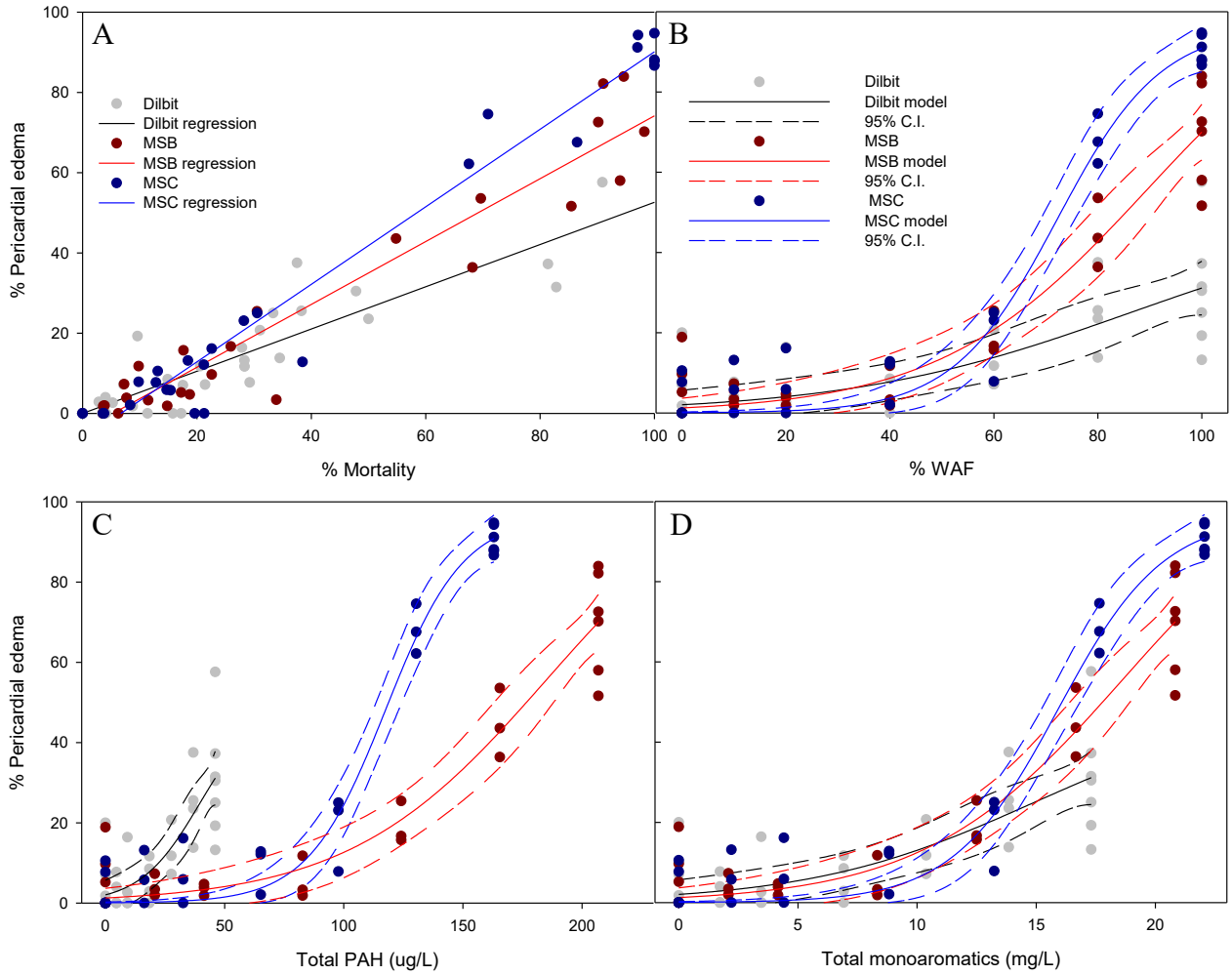

Figure 2.3. The effect of WAF exposure on the frequency of pericardial edema relative to the %WAF (b), PAH (c), and total monoaromatic content (d). The frequency of pericardial edema was compared to the relative mortality for each of the WAFs using linear regression, to determine how pericardial edema contributed to the cause of death of the WAF. The closer to 1 the slope of the line is, the stronger the relationship between pericardial edema and mortality. MSC WAF had a slope of 0.96; MSB WAF had a slope of 0.78; dilbit WAF had a slope of 0.53.

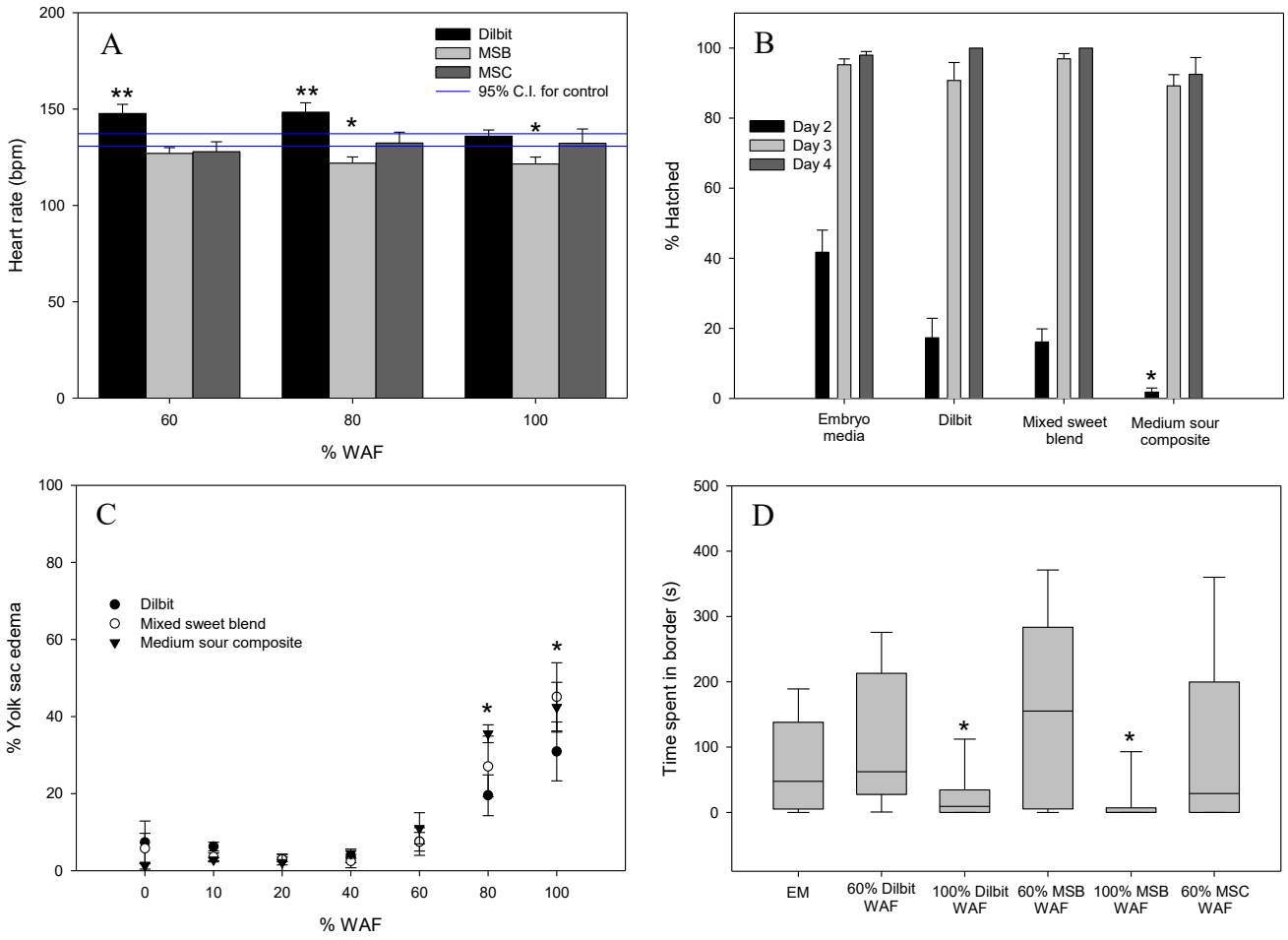

Figure 2.4. The impact of 60-100% WAF exposure on heart rate (a). Heart rate was recorded at 2 dpf, 10-40% WAF exposure groups had no impact on heart and were not included. Blue lines were used to denote the 95% confidence interval (C.I) of the 0% WAF (EM) mean. Hatch rate between the 100% dilbit, MSB, and MSC WAF from 2-4 dpf for the 100% WAF (b). Frequency of yolk sac edema form 0-100% dilbit, MSC, and MSB WAF (c). The impact of 60 and 100% WAF on border dwelling behavior in 7dpf embryos (d). 100% MSC WAF exposed embryos were not included because of poor (<3%) survival. "*" and "**" denote statistical differences between treatment groups.

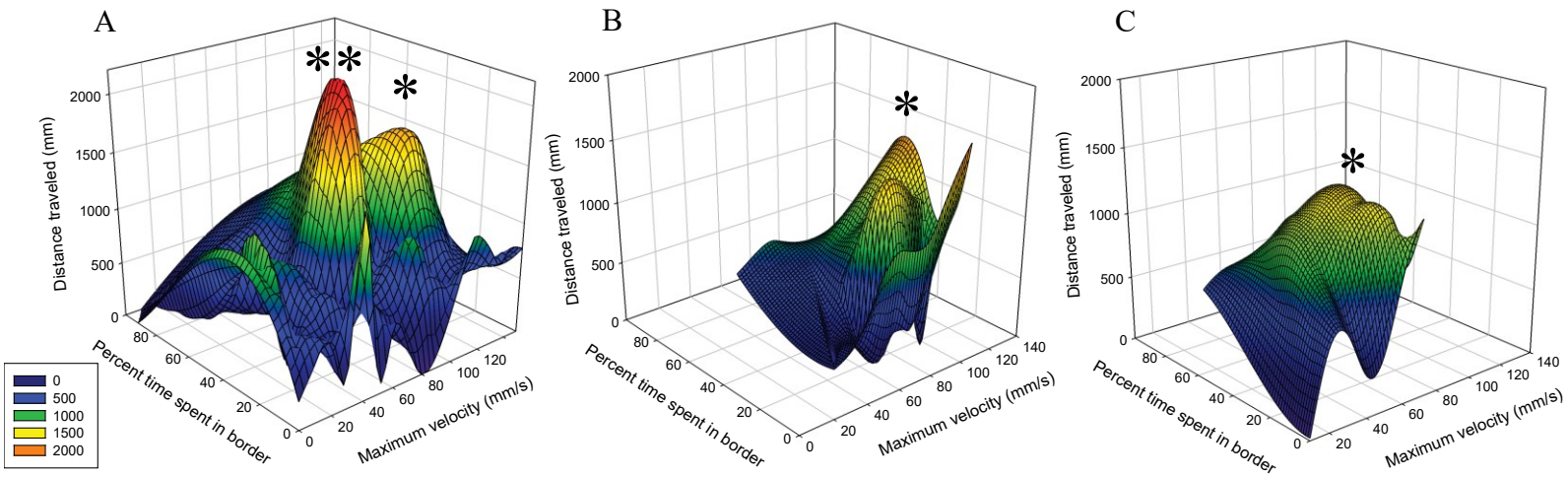

Figure 2. 5. Behavioral phenotyping of the activity of zebrafish embryos using 3D mesh plot analysis. Fish were classified as 'active' if they traveled over 1000mm over the course of the 10 min trial. The presence of the active fish creates peaks in the embryo media (a), 100 % dilbit WAF (b), and 100% MSB (c) treatments that can be used to phenotype the fish based on either a high maximum velocity (*) or fish with a medium maximum velocity (**). The 100% MSC WAF exposed embryos could not be concluded because of poor (<3%) survival.

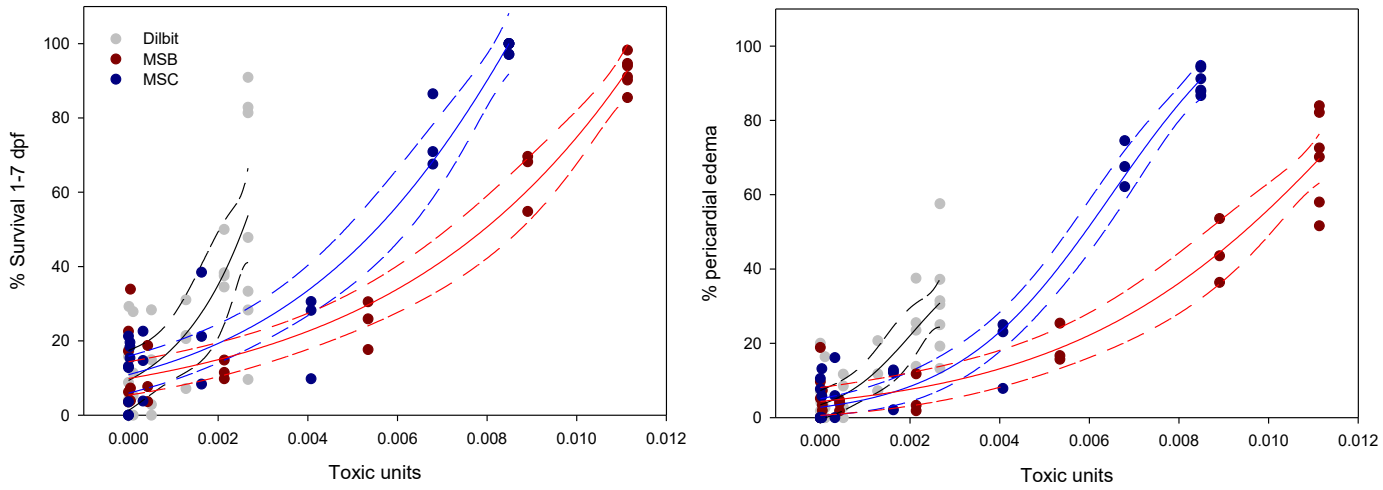

Figure 2.6. The percent mortality from 1-7 days post fertilization from 0-100% WAF (a) as well as the percent pericardial edema (b), in relation to the number of toxic units of PAHs per WAF. 95% confidence intervals (C.I) were included with each curve.

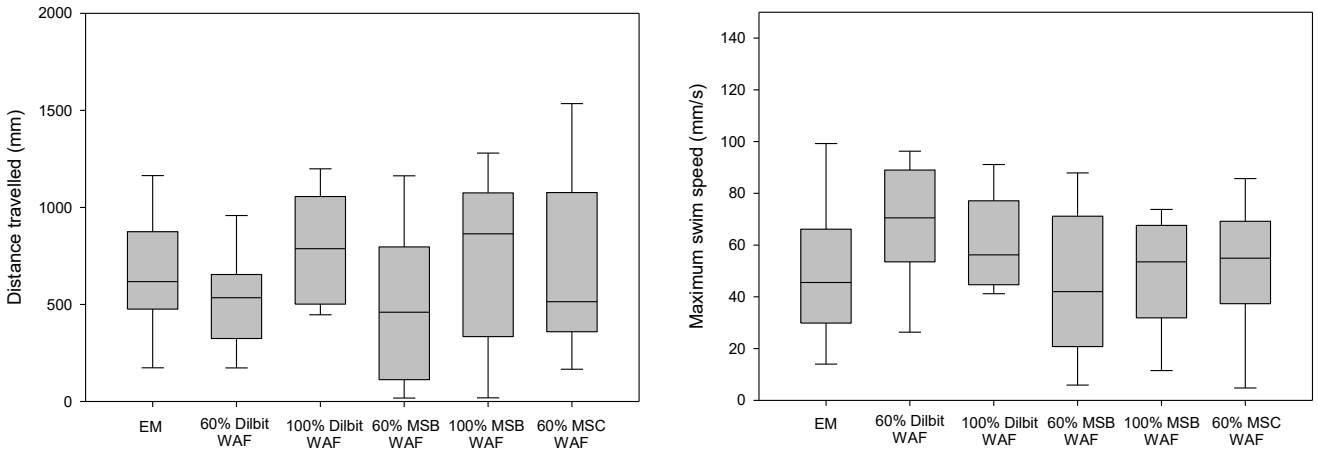

Figure 2.7. The impact of 60 and 100% WAF on the swimming activity of 7 dpf zebrafish embryos. WAF exposure had no impact on the average distance travelled (A) and maximum swim speed (B) of the various treatment groups.

Chapter 3: Field-collected crude oil and dispersants differentially affect the early life stages of freshwater and saltwater fishes

Abstract

The Deepwater Horizon (DWH) oil spill was the biggest in US history and released 3.19 million barrels of light crude oil into the Gulf of Mexico. 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 and their effects on developing sheepshead minnow and zebrafish. Although a freshwater fish, zebrafish has been used as a model for marine oil spills owing to the molecular and genetic tools available 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 aimed to determine whether dissolved crude oil constituents were similar in fresh and saltwater, and if freshwater fish might be a suitable model to study marine spills. Weathering and dispersant had similar effects on WAF composition in both fresh and saltwater, except that the saltwater source oil + dispersant WAF had markedly higher PAH levels than the freshwater equivalent. WAF exposure differentially affected survival, as the LC_{50} values in %WAF 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. Exposure increased heart rate of zebrafish embryos, whereas in sheepshead, source oil exposure had the opposite effect. WAF exposure altered mRNA expression of biotransformation makers, 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, and 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 . After May 15th, following two test periods, dispersant (Corexit 9500) was injected continuously to reduce oil droplet size and prevent the formation of large surface slicks until the well was capped 61 days later, on July 15th (Kujawinski et al., 2011). Macondo oil, a light non-viscous crude, travelled 1500 m up the water column

554 and underwent a series of compositional changes due to both biological and physical weathering,
555 characterized by dissolution, emulsification, evaporation, biodegradation, photo-oxidation and
556 dispersion (Wang et al., 2013).

557 Most evidence suggests that polycyclic aromatic hydrocarbons (PAHs) are the primary source of
558 aquatic toxicity in crude oil (Hodson, 2017). Laboratory studies have found that dispersants increase the
559 toxicity of crude oil to fishes via increasing the aquatic concentration of PAHs. Effects include increased
560 lethality, decreased heart rate, increased incidence of cardiac malformations, increased incidence of blue
561 sacs disease, various effects on sex differentiation gene expression, increased expression of cytochrome
562 P450 (eg. Cyp1a and Cyp1b), and increased EROD activity in various fish species including inland
563 silversides (*Menidia beryllina*), topsmelt silverside (*Atherinops affinis*), mummichog (*Fundulus*
564 *heteroclitus*), marine medaka (*Oryzias melastigma*) and rainbow trout (*Oncorhynchus mykiss*)
565 (Adeyemo et al., 2015; Anderson et al., 2009; Couillard et al., 2005; Mu et al., 2014; Ramachandran et
566 al., 2004; Schein et al., 2009).Weathering, on the other hand, has been found to decrease the PAH
567 content entering the water column (Heintz et al., 1999), and decreased toxicity on a volume per volume
568 basis (Chapman et al., 1995; Faksness et al., 2015; Shelton et al., 1999). Oil type, dispersant application
569 and weathering can all impact the PAH profile in the water and it is very unlikely that all PAHs exhibit
570 toxicity through the same mechanism of action (Hodson, 2017).

571 Of the studies on oil toxicity, many have focused on the sublethal effects of oil exposure on developing
572 fishes. Of these, studies of effects related to cardiac morphology and cardiac output are prominent (Carls
573 et al., 2008; Cox et al., 2017; Incardona et al., 2009; Incardona et al., 2015; Incardona et al., 2004;
574 Incardona et al., 2006; Incardona et al., 2011). Cardiac impairment caused by oil exposure has also been
575 linked to alterations in swimming behavior and decreased swimming performance (Brown et al., 2017;
576 de Soysa et al., 2012; Hicken et al., 2011; Incardona et al., 2013; Kennedy and Farrell, 2006; Mager et
577 al., 2014; Nelson et al., 2017; Yu et al., 2015). Whole organism cardiac effects have been linked to gene
578 expression changes in cardiac biomarkers (ex. NKX2.5) (Incardona et al., 2015; Xu et al., 2017). Other
579 biomarkers commonly used in crude oil studies, such as CYP1a, not only play a role in cardiotoxicity,
580 but also participate in PAH biotransformation (Barron et al., 2004).

581 In our study, we directly compared the toxicity of field-collected naturally weathered oil, source
582 oil (without weathering), and source oil and dispersant mixtures between zebrafish (*Danio rerio*) and a
583 common saltwater model, sheepshead minnow (*Cyprinodon variegatus variegatus*). Both species are
584 well established model species in the field of toxicology, and they are both considered relatively tolerant

to exposure (Hill et al., 2005; Woltering, 1984). Both saltwater and freshwater model fishes have been used to characterize potential effects of large scale marine spills under the assumption that responses will be conserved across species. 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 (de Soysa et al., 2012; Incardona et al., 2013; Jung et al., 2013; Pauka et al., 2011; Perrichon et al., 2016; Raimondo et al., 2014), however, the applicability of these studies to large scale marine spills is largely undetermined. Our study aimed to determine the impact of weathering and dispersant application on oil toxicity in fresh and saltwater systems, and the effects of the resulting water accommodated fractions (WAFs) have on gene expression changes and physiology in fishes. The study called in to question the merit of using a freshwater model fish (zebrafish) to investigate marine oil spills.

Materials and Methods

Oil sources

Exposures were prepared using three different oil types: an un-weathered Macondo oil named "MASS" collected by the "Massachusetts" barge from the subsea containment system above the well-head (source oil), an ~65% weathered oil named "CTC" collected from a barge that received oil from various skimming vessels (weathered oil (WO) A), and an ~83% weathered oil sample named "Juniper" collected from an alternate surface slick from the United States Coast Guard (USCG) skimming vessel (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). Samples were collected from both 6-36 hour old 100% WAF, 1-4 replicates per sample. The total polycyclic aromatic hydrocarbon (TPAH) content of each WAF is available in Table 3.1.

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 as described by the Florida International University test protocol. The preparation was carried out 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 (equivalent to full-strength seawater) 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). A solvent control of DMSO only was included. Both sheepshead minnow and zebrafish embryos/larvae were exposed to the same concentration: 50µg/L of BaP.

Zebrafish 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). For exposures, 3-5 groups of 70 embryos held at 27.5°C were exposed to 40mLs of various WAF treatments in glass petri dishes: 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 (mpf) and lasted until 7 days post fertilization (dpf), which spans both the embryological (0-2dpf) and larval (2-7dpf) life stages, mortalities were counted daily. 100% WAF was used for WO A, WO B, and source oil only exposures, and 25% WAF was used for the source oil + dispersant exposures. Different concentrations of WAF were used for the saltwater and freshwater exposures to account for the differences in lethality across the two species.

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. 3-5 replicate groups of 100 embryos held at 25-26°C were exposed to 200mLs of various WAF treatments in 500mL glass beakers: 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. 100% WAF was used for WO A, WO B, WO A + dispersant and source oil only exposures, and 15% WAF was used for the source oil + dispersant exposures.

Heart rate and muscle deformities.

Heart rate recordings were made on 2 dpf zebrafish and 5 dpf sheepshead minnow, between 14:00-16:00 to make sure the recordings were made at the same developmental stage. 30 s videos were taken of embryo hearts and heart rates were determined manually. The videos were randomized and scored blind. To examine muscle development, birefringence was used as it is a non-lethal imaging technique that depends on the natural structure of muscle fibers and their ability to reflect polarized light (Smith et al., 2013). For this, muscle deformities were examined in 2 dpf hatched zebrafish larvae. 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.

Fish survival 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 (Di Toro et al., 2000; Kipka and Di Toro, 2009). 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 LC_{50} and K_{ow} (Di Toro et al., 2000).

RNA extraction and cDNA synthesis.

In brief, zebrafish (7 dpf) and sheepshead (10 dpf) minnow were euthanized on ice, preserved in RNAlater (Thermo Fisher; Waltham, MA), 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. Refer to Table 3.2 and Table 3.3 for qPCR primer tables.

Statistics and LC_{50} 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). LC_{50} values were calculated using free online software based on the Finney method of Probit analysis (Finney, 1952).

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 3.1A). A similar trend was present in saltwater WAFs, except that the addition of dispersant resulted in higher concentrations of branched naphthalenes (N2-N4) (Figure 3.1B). 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 3.2A, B). Parent compounds and lightly branched PAHs (0-1 branches) were more prevalent than highly branched PAHs (2-4 branches) for fresh and saltwater WAFs. Biphenyl, fluorene, and phenanthrene/anthracene concentrations were highest in the source oil and source oil + dispersant freshwater WAFs (Figure 3.2A). 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 3.8, 3.9). 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 3.3A, B, 3.10). The benzene, toluene, ethylbenzene and xylene (BTEX) content was relatively constant across the oil mixtures and water matrices (Figure 3.3A, 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. The 7 day LC_{50} in %WAF for the zebrafish and 10 day LC50 for the 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. The 7 and 10 day LC50 in total polycyclic aromatic hydrocarbons (TPAH) for the zebrafish and sheepshead minnow were 265.1 µg/L (95% C.I. 248-282) and 207 µg/L (95% C.I. 169-253), 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 3.4). However, the TPAH LC50 was similar for these two species (Figure 3.11). According to all models, sheepshead minnow had a lower toxic unit LC_{50} than zebrafish, which means that on a per toxic unit basis, sheepshead were more sensitive to exposure. The critical body burden value used to calculate the toxic units in the WAFs was derived from literature (Kipka and Di Toro, 2009), and should have accounted 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. In zebrafish, 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 larvae ($F_{7,33} = 55.27, p < 0.05$; $F_{7,33} = 53.04, P < 0.05$; respectively) (Figure 3.5A). 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 3.5B). 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 3.5C). In contrast, for sheepshead, source oil WAF increased VTG expression ($F_{8,29} = 7.19, p < 0.05$) (Figure 3.5D), indicating that sheepshead may have been more sensitive to estrogenic compounds, or that saltwater increased estrogenic constituents or their uptake. Interestingly, WAF + dispersant did not increase the

741 expression of VTG mRNA, this could be due to the dilution of WAF used (15%) to limit lethality for
742 our sublethal endpoints.

743 WAF exposure had no effect on the expression of the neural development gene NeuroD in either
744 species (Figure 3.5E, F). However, in zebrafish but not sheepshead, Ngn1 was downregulated in source
745 oil + dispersant exposures ($F_{7,33} = 11.67, p < 0.05$). Gli2a expression was also measured in zebrafish,
746 and transcript expression was upregulated by WO B exposure ($F_{7,33} = 3.8, p < 0.05$).

747 *Heart and muscle development.*
748 Cardiac responses differed between the zebrafish and sheepshead minnow embryos. WAF
749 exposure increased the heart rate of zebrafish embryos ($F_{5,172} = 15.39, p < 0.001$) (Figure 3.6A),
750 whereas in sheepshead, source oil exposure decreased the heart rate (Q=2.79, p<0.05) (Figure 3.6B).
751 Nkx2.5 expression was only induced by exposure to weathered oil B (Figure 3.6C).

752 Muscle deformities were observed in freshly hatched 2 dpf fish exposed to weathered oil, source
753 oil and source oil with dispersant WAFs (Figure 3.7). The deformities ranged from abnormal myoseptal
754 divides (Figure 3.7E), hemorrhaging along the trunk of the tail (Figure 3.7B, C) and gaps in the muscle
755 striations (Figure 3.7D). The deformities were observed at low frequencies (≤4%) and were only present
756 in the oil-exposed treatment groups, and were most prevalent in the moderately weathered oil
757 (weathered oil A) and the source oil + dispersant WAF treatment groups, though they were not
758 statistically significant (Figure 3.7F)

759 ## Discussion
760 Our study compared the toxicity of fresh crude oil, moderately and heavily field-collected
761 weathered oil, and oil and dispersant mixtures on the early life stage model freshwater and saltwater
762 fishes. This study is the first to directly compare salt and freshwater crude oil exposures and includes
763 one of the largest ranges of oil exposures ever included in a side-by-side comparison. This study
764 compared the WAF chemistry composition, lethality, toxic units, gene expression changes, and heart
765 rate between sheepshead minnow and zebrafish embryos/larvae to determine if a freshwater model could
766 be an effective tool to study marine spills.

767 *WAFs and Mortality*
768 Following a spill, the concentrations of dissolved hydrocarbons will depend on many factors,
769 including environmental degradation, the use of a dispersant, and the salinity of the water. In both the
770 fresh and saltwater, source oil and source oil + dispersant exposures introduced more naphthalenes and

771 PAHs to the WAFs than the weathered oils, and weathered oil + dispersant mixtures (Figure 3.1, 3.2).
772 The weathering of crude oil varies between oil spills due to different physical and biological conditions
773 in the spill zone. For most crude oils, 50-70% of the amount of oil released is depleted in the first 10-12
774 h through spreading of the slick and evaporation of the volatile components (Mackay, 1989).
775 Weathering of oil has also been shown to decrease the PAH content entering the water column relative
776 to the volume of oil added (Heintz et al., 1999), and can decrease toxicity on a volume per volume basis
777 (Chapman et al., 1995; Faksness et al., 2015; Shelton et al., 1999). The more water soluble, lower
778 molecular weight components of crude oil are lost with weathering and are a potential source of acute
779 toxicity (Faksness et al., 2015). Weathered oil contains less water-soluble PAH's than source oil,
780 however, weathering increases the proportion of tricyclic PAHs. Tricyclic PAHS are thought to be the
781 major source of toxicity to fishes in crude oil spills (Barron et al., 2004; Esbaugh et al., 2016; Incardona
782 et al., 2009; Incardona et al., 2004). The increased proportion of these compounds makes weathered oil
783 appear more toxic then unweathered oil on a μg/L PAH basis (O'Shaughnessy et al., 2018), but not
784 necessarily on a gram per gram of oil comparison. In accordance with past work, our study found that
785 weathering decreased the toxicity of the crude oil on a gram of oil per liter of water basis (decreased
786 lethal loading concentration), due to decreased PAH and VOC content in the WAF.

787 Dispersants are designed for use in marine spills and there is very little literature on the
788 effectiveness of dispersant in freshwater as they are not currently approved for use in inland spills
789 (Wrenn et al., 2009). Our study found that the addition of dispersant to source oil increased the
790 naphthalene and PAH content in saltwater more so than in freshwater (Fig. 3.1, 3.2). Previous studies
791 have found that dispersants increase the bioavailability of compounds within crude oil, resulting in
792 increased toxicity to fishes (Couillard et al., 2005; Finch et al., 2017; Ramachandran et al., 2004; Schein
793 et al., 2009), though more recent studies have suggested that the increase in measured PAHs is likely
794 due to oil drop emulsions in the WAF (Stefansson et al., 2016) as opposed to just PAH solubility. The
795 analytical chemistry techniques used in most crude oil studies cannot differentiate between dissolved
796 hydrocarbons and oil droplets (Sandoval et al., 2017), which could artificially increase the perceived
797 bioavailability of PAHS in source oil + dispersant WAFs. Dispersant use remains controversial, and is
798 under scientific and regulatory review, approvals are evaluated in a case by case basis by a variety of
799 stakeholders and interest groups and come with both environmental and economic tradeoffs. Regardless,
800 in our study, source oil + dispersant generated the most lethal WAF to both zebrafish and sheepshead
801 minnow. Previous work using a very similar WAF preparation method on the same oil samples for a 48h
802 photo-enhanced toxicity testing of 3 dpf sheepshead minnow found that the %WAF LC50 was

803 approximately 40% (Finch et al., 2017). The Finch *et al.* study also ranked various organisms according
804 to their sensitivity as follows; mysid shrimp> inland silverside > sheepshead minnow> gulf killifish
805 (Finch et al., 2017). The study suggests that sheepshead minnow embryos are relatively hardy in
806 response to acute exposures, our studies suggest that they are much more sensitive to longer exposure
807 periods. Our LC_{50} %WAF concentration was much lower, likely due to the exposure period in our study
808 starting earlier (1 dpf) and the longer total exposure period. Our study also found that the TPAH LC_{50}
809 was very similar between the 7 day zebrafish and the 10 day sheepshead minnow exposure (Table 3.1).
810 Both zebrafish and sheepshead minnow are considered toxicologically tolerant species(Hill et al., 2005;
811 Woltering, 1984), and though the sheepshead minnow were exposed longer than the zebrafish, both
812 species were exposed for the same number of free swimming days (zebrafish hatched at 2dpf and the
813 sheepshead minnow hatched at 5 dpf) which could account for the similar sensitivity to WAF exposure.
814 Eggs tend to have a lower rate of uptake of xenobiotics than free swimming larvae due to the lower rate
815 of transport across the chorion, decreased circulation of fluids inside the chorion, and eggs generally
816 having a smaller surface area compared to larvae (Petersen and Kristensen, 1998).

817 Because of the availability of molecular tools to study the mechanism of toxicity are currently
818 unrivaled in the zebrafish, there are many benefits to using zebrafish to study environmental releases.
819 Our study highlights a major pitfall in using zebrafish to study marine spills through a direct side by side
820 comparison with a saltwater model fish with a wide range of oil exposure types. Exposure had a more
821 significant effect on neurodevelopment in zebrafish, was less estrogenic and increased (as opposed to
822 decreased) heart rate. The exposures started at different developmental stages which could play a role in
823 the different responses the fishes had to WAF exposure. Zebrafish ethanol toxicity studies have found
824 that exposure windows within an hour of each other could alter the severity of deformations, and that
825 some developmental windows are inherently more sensitive to exposure (Ali et al., 2011). The species
826 also had differing levels of biotransformation enzyme upregulation across the exposure groups
827 suggesting the two species are differentially sensitive to PAH exposure.

828 Oil exposure studies in the literature are very inconsistent, which makes cross study comparisons
829 challenging, and until there is a universal exposure method there is a need for more side by side
830 comparative studies between species and oil types. A previous study conducted on Inland silverside
831 exposure to the same oil samples included toxic units in their comparison of source oil and weathered oil
832 toxicity (Echols et al., 2016), but only compared samples across one model species and found the model
833 to be effective. 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 understand the impact of large scale marine spills like 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 3.4). The TLM is a model used to calculate the toxic potential of a mixture and was developed for complex PAH mixtures like crude oil WAFs. 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.

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 minnow 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 aryl hydrocarbon receptor (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 act both AhR dependantly and independently, and they can be metabolized and excreted, metabolized into a reactive intermediate, or accumulate in tissues and chronically activate the AhR pathway (Incardona et al., 2005). PAHs can also be taken up by many different routes including the gills, skin and mouth. Fish accumulate hydrocarbons quickly, and these compounds can concentrate in tissues at levels 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. The zebrafish and sheepshead minnow exposures began at different developmental stages. A study on the effect of an AhR agonist β-naphthoflavone on 8, 32, 56, 80, 104 and 128hpf zebrafish found that responses to exposure can vary temporally, and CYP1 expression and activity can begin as early as during the gastrula stage (Duan et

al., 2018). The role of the chorion in toxicokinetics and the differences in the developmental staging at the beginning of exposure could account for the different responses to WAF exposure in the sheepshead minnow and zebrafish larvae.

In terms of estrogenicity, WAF exposures did not affect the expression of VTG mRNA in zebrafish. In contrast, source oil WAF upregulated VTG mRNA 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 future 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 3.5F). 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 the biological significance is not known.

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; Incardona et

al., 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 we performed 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, and the developmental stage at which heart rate is recorded could also play a role.

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 (≤4%). This finding highlights the importance of reporting frequency when examining exspoure effects. 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.

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, and was more pronounced in saltwater than freshwater. 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 across species.

Acknowledgements

We would like to acknowledge the BP Exploration and Production Inc. and the BP Gulf Coast Restoration Organization for funding and support. The support by NSERC for Dr. Tierney is also acknowledged.

928 **Tables**

929

930 Table 3.1. The total polycyclic aromatic hydrocarbon content (TPAH) of the fresh and saltwater WAFs.
931 Only 1 WAF sample was analyzed for the source oil saltwater treatment group, the rest were samples 2-
932 6 times.

	Freshwater	Saltwater
WO A	26.6 ± 0.9	17.4 ± 3.1
WO B	8.6 ± 0.7	4.9 ± 2.8
WO A + disp.	-	31.8 ± 8.0
Source oil	460.7 ± 59.1	355.8
Source oil +disp.	590.4 ± 49.2	1235.4 ± 324.9

933

934 Table 3.2. Zebrafish qPCR primers.

Target Gene	Gene name	Forward Sequence (5'-3')	Reverse Sequence (5'-3')	Accession Number/ Reference
Bactin	Beta-Actin	CGA GCA GGA GAT GGG AAC C	CAA CGG AAA CGC TCA TTG C	AF057040[9]
Cyp1a	Cytochrome P4501a	AGG ACA ACA TCA GAG ACA TCA CCG	GAT AGA CAA CCG CCC AGG ACA GAG	NM_131879[8]
Cyp1b	Cytochrome P4501b	CCA CCC GAA CTC TGA AAC TC	AAA CAC ACC ATC AGC GAC AG	NM_001013267[8]
VTG1	Vitellogenin	CTG CGT GAA GTT GTC ATG CT	GAC CAG CAT TGC CCA TAA CT	AF406784.1[10]
NKX2.5	Homeobox protein nkx2.5	GTC CAG GCA ACT CGA ACT ACT C	AAC ATC CCA GCC AAA CCA TA	NM_131421[4]
Ngn1	Neurogenin1	TGC ACA ACC TTA ACG ACG CAT TGG	TGC CCA GAT GTA GTT GTG AGC GAA	NM_131041[7]
NeuroD 1	Neurogenic differentiation1	CAG CAA GTG CTT CCT TTT CC	TAA GGG GTC CGT CAA ATG AG	Paule *et al*.[5]
Gli2a	GLI family zinc finger 2a	AAA AAC AGG GCG GGA CTA CT	ATG CTG GGT TGG AGG TAC AG	Paule *et al*.[5]

935

936 Table 3.3. 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[6]
VTG1	Vitellogenin	ATG TCA CTG TGA AGG TCA ACG AA	ACC TGT TGG GTG GCG GTA A	AF239720[3]
Nng1	Neurogenin1	ACC GCG CAT GTG GTA	CTG TGG GAT GCT CAG TCA CC	XM_015371488.

		AAG AA		1
NeuroD 1	Neurogenic differentiation1	GTC TCA GCC GAC CAC TAA CC	GGC ATC TGA CAC CAG GAC TC	XM_015400246.1
NKA1a 1	Na/K-ATPase 1a1	GCC ACA CAG CCT TCT TCA C	ACA ATA GAG TTC CTC CTG GTC TTG	GE337281.1[6]

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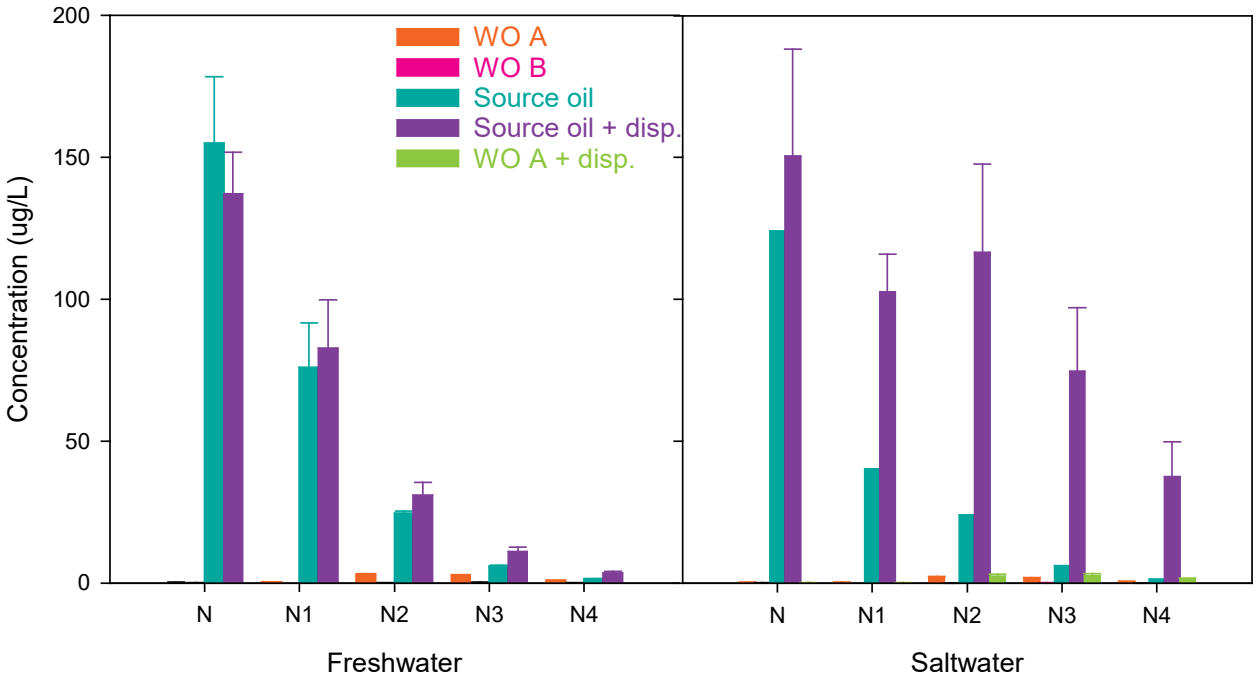
938

Figures



A B

Figure 3.1. 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, n=1-4 replicates per treatment).

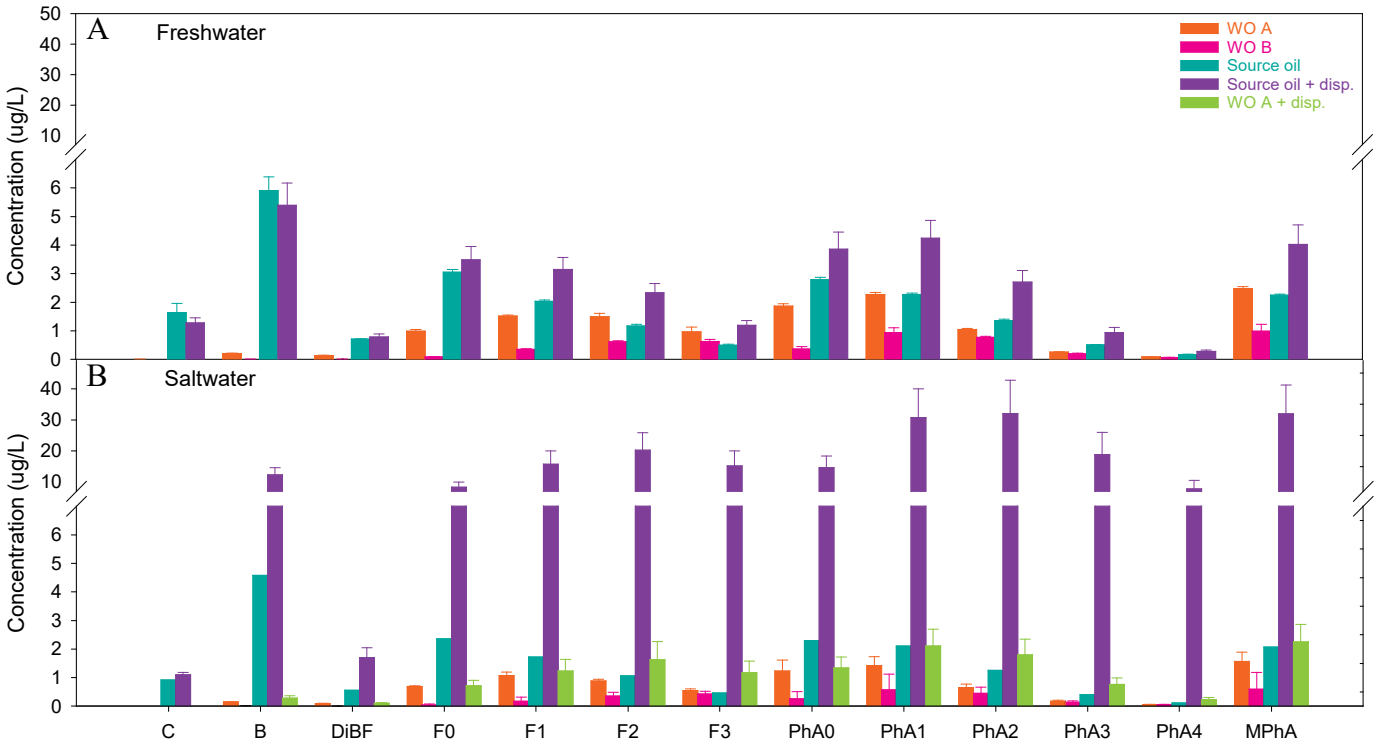


Figure 3.2. PAH content of the fresh (A) and saltwater (B) WAFs made with weathered oil A (WO A), weathered oil B (WO B), source oil, source oil with dispersant (Source oil + disp.), and weathered oil A + dispersant (WO A + disp.; saltwater only). C, carbazole; B, biphenyl; DiBF, dibenzofuran; F, fluorene; PhA, phenanthrenes/anthracenes; MPhA, methylphenanthrene. Error bars represent standard error (SEM, n=1-4 replicates per sample)

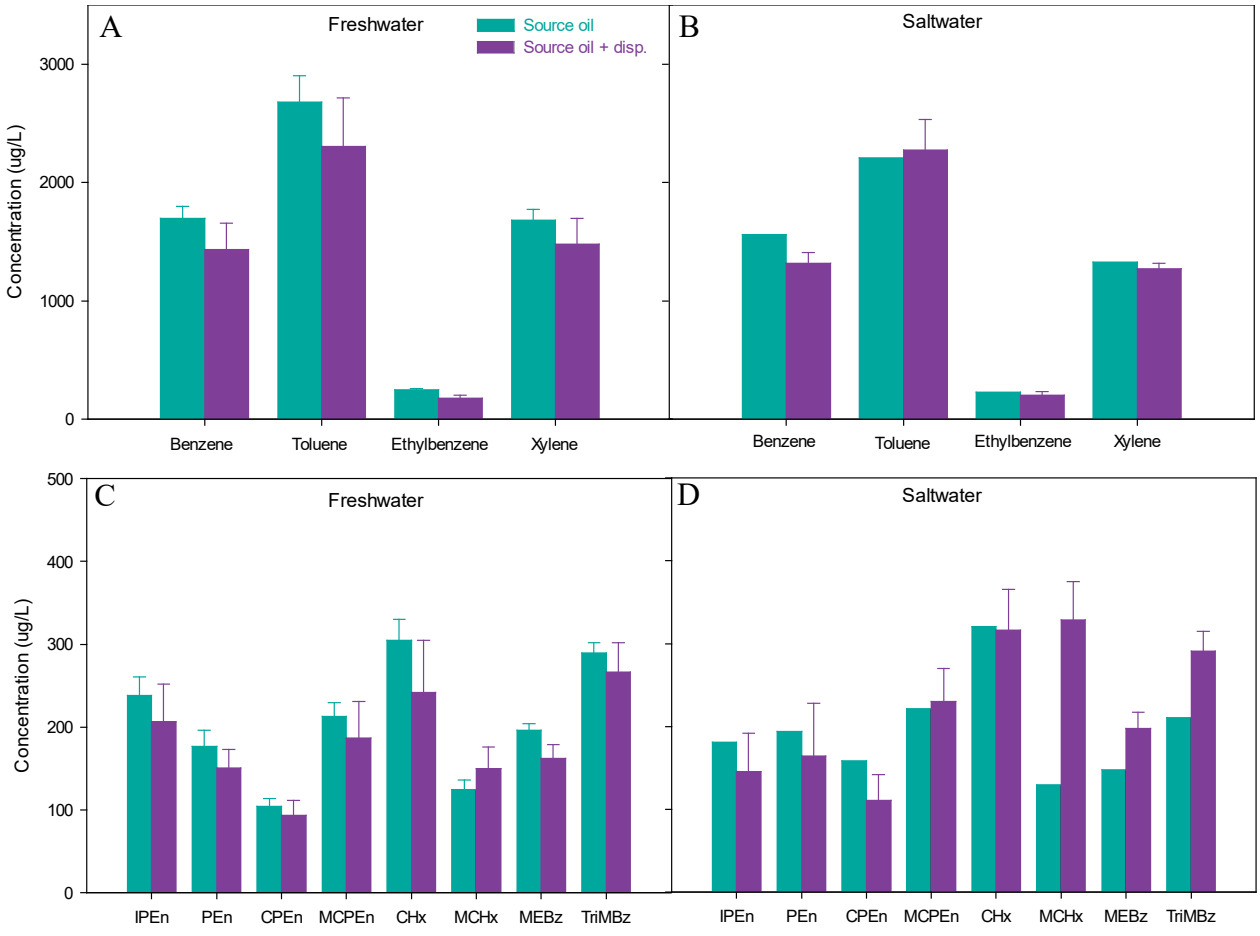
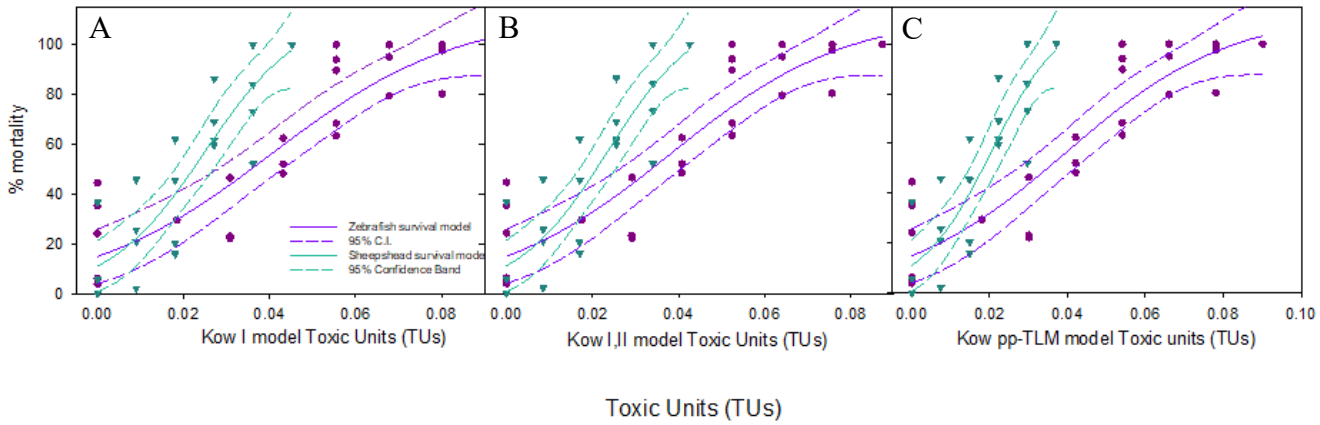


Figure 3.3. BTEX and Volatile organic compound (VOC) content of the fresh (A,C) and saltwater (B,D) WAFs made with source oil, and source oil with dispersant (Source oil + disp.). None of the weathered oil WAFs had measurable amounts of volatile compounds, so they were not included. IPEn, isopentane; PEn, pentane; CPEn, cyclopentane; MCPEn, methylcyclopentane; CHx, cyclohexane; MCHx, methylcyclohexane; MEBz, methylethylbenzene; TriMBz, trimethylbenzene. Error bars represent standard error (SEM, n=1-4 replicates per sample)



968

969 Figure 3.4. The percent mortality from 1-7 dpf (zebrafish) and 1-10 days post dpf (sheepshead minnow)
970 in relation to the number of toxic units of PAHs per source oil + dispersant WAF. Toxic units were
971 evaluated using three different models: K_{ow} I (A); K_{ow} I, II (B); K_{ow} pp-TLM (C). 95% confidence
972 intervals (C.I.) were included with each curve. n=4-6 replicate per treatment.

973

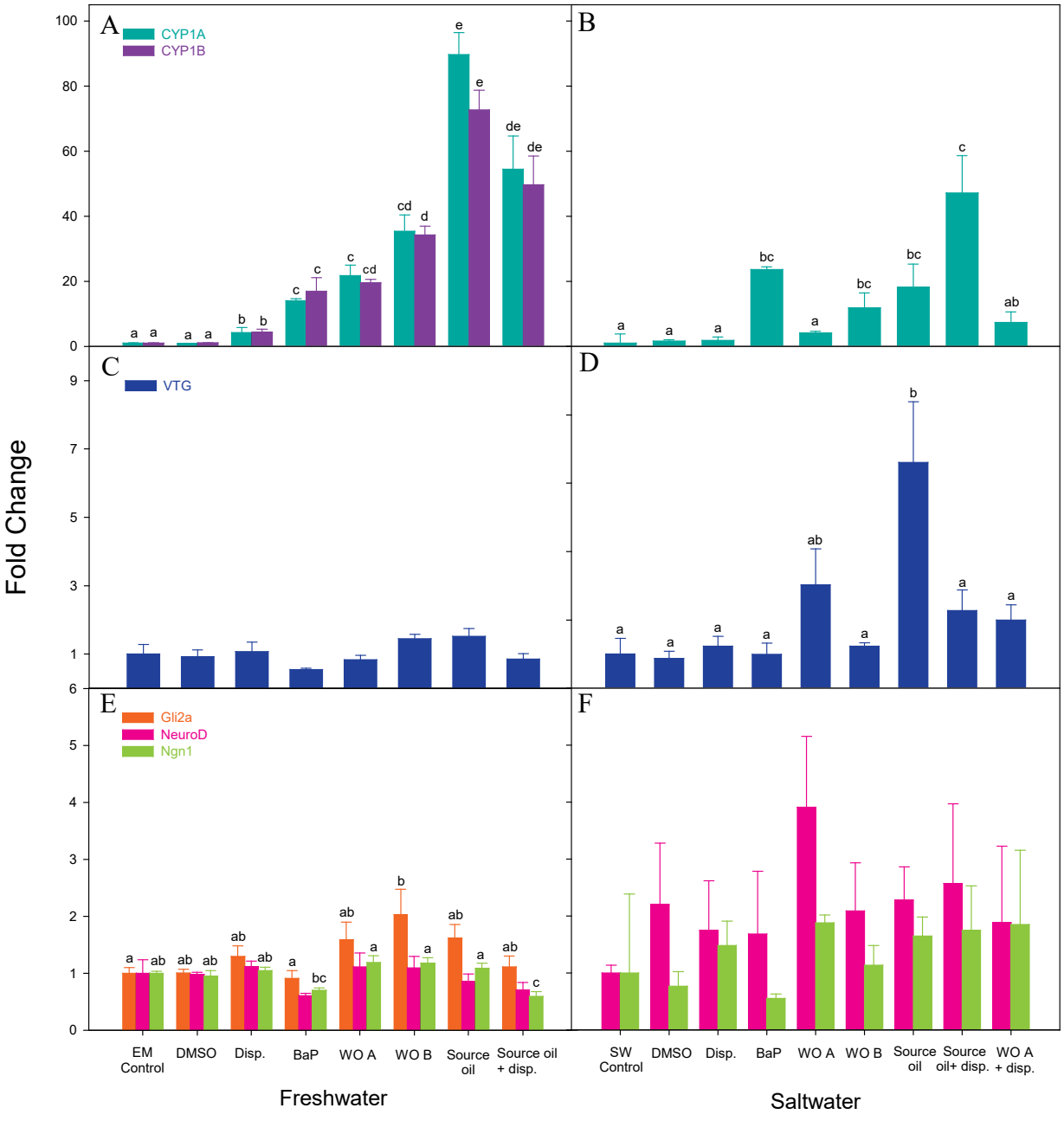


Figure 3.5. Effects of WAF exposures on the expression level (fold change from control) of the biotransformation enzymes CYP1A (A,B) and CYP1B (A), estrogenicity indicator VTG(C,D), and neurodevelopment markers Gli2a (E), NeuroD (E,F), and Ngn1 (E,F). Bars represent mean ± SE. The means of exposures 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. Error bars represent standard error (SEM, n=3-4 replicates of 20-25 pooled fish per treatment group)

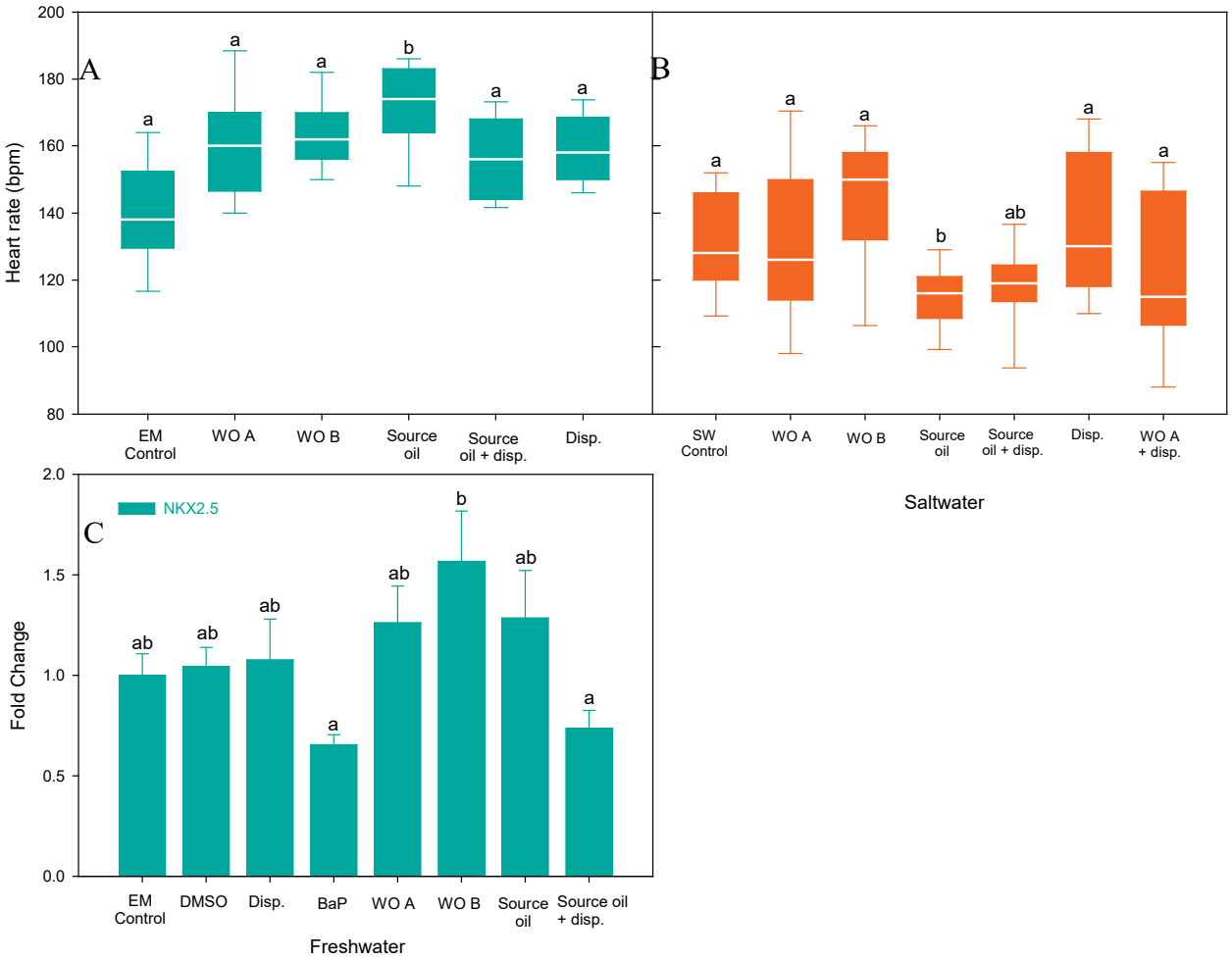


Figure 3.6. The impact of weathered oil A (WO A), weathered oil B (WO B), source oil, source oil with dispersant (Source oil + disp.), dispersant, and weathered oil A + dispersant (WO A + disp.; saltwater only) on the heart rate of 2dpf zebrafish (A) and 5dpf sheepshead minnow embryos (B), and the effect 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, heart rate had a n=25-50 individuals from 3-4 replicate trials, for gene expression data n=3-4 replicates of 20-25 pooled fish per treatment group)

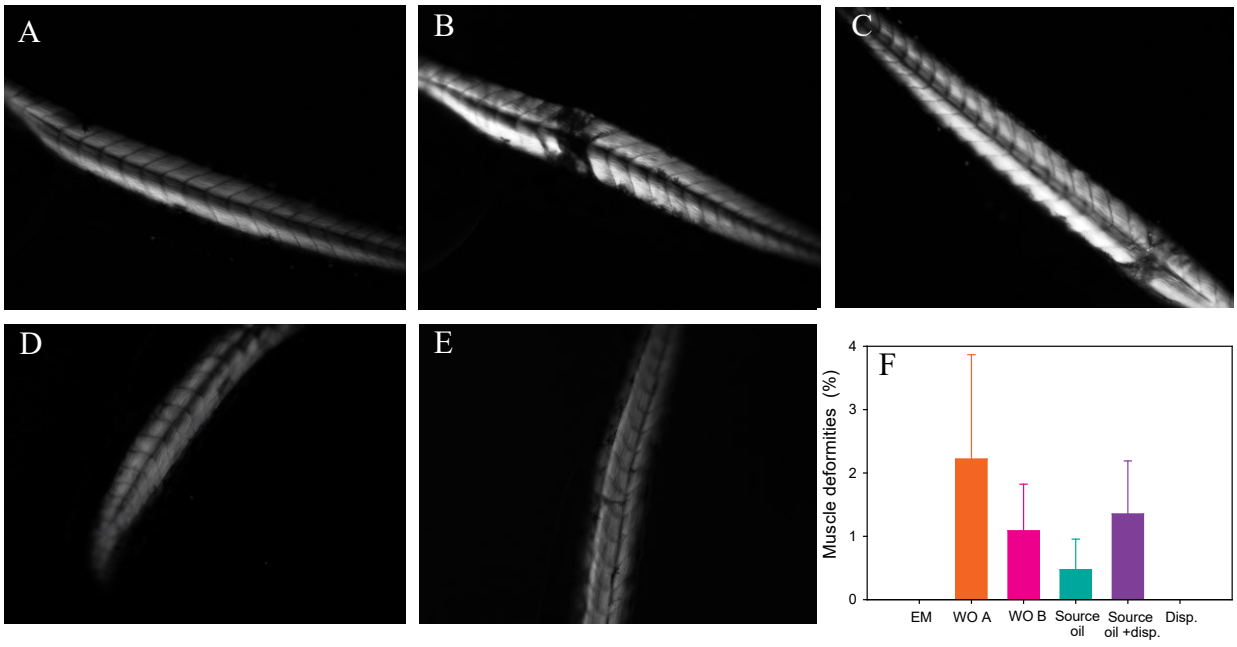


Figure 3.7. Birefringence images and frequency of muscle structure deformities in weathered oil A (WO A), weathered oil B (WO B), source oil, source oil with dispersant (Source oil + disp), and dispersant (Disp.) exposed 2dpf zebrafish larvae. Birefringence was used to examine normal myosepta, muscle 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.

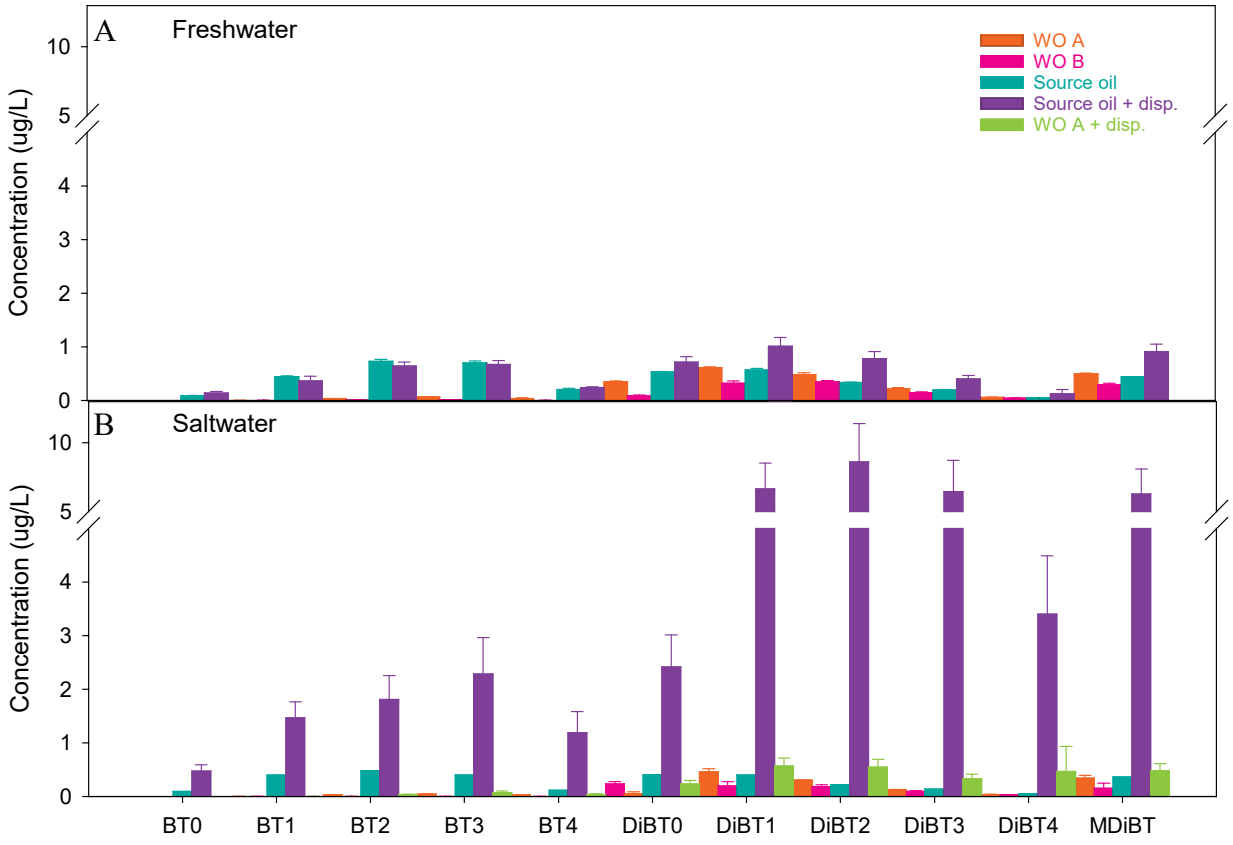


Figure 3.8. Benzothiophene based PAH content of the fresh (A) and saltwater (B) WAFs made with weathered oil A (WO A), weathered oil B (WO B), source oil, source oil with dispersant (Source oil + disp.), and weathered oil A + dispersant (WO A + disp.; saltwater only). BT, Benzothiophenes; DiBT, Dibenzothiophenes, MDiBT, Methyldibenzothiophenes. Error bars represent standard error (SEM, n=1-4 replicate samples per treatment group).

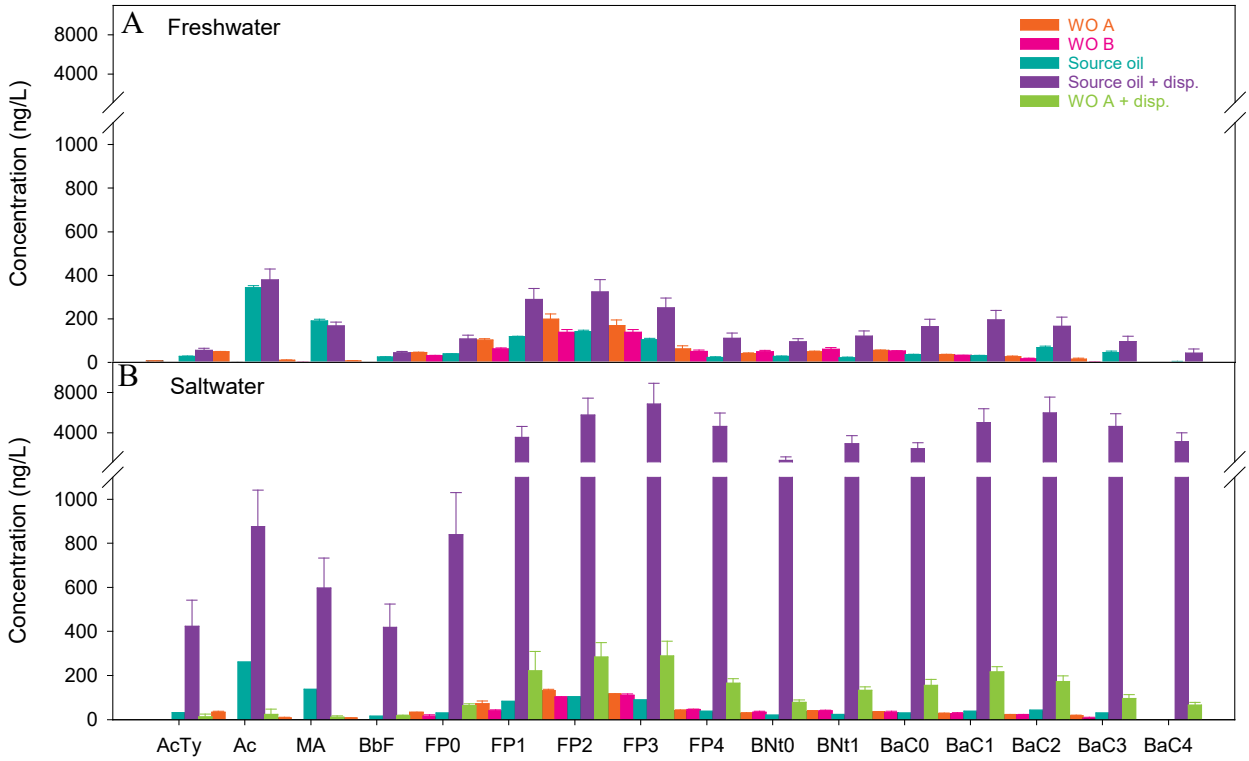


Figure 3.9. Low concentration PAHs in fresh (A) and saltwater (B) WAFs made with weathered oil A (WO A), weathered oil B (WO B), source oil, source oil with dispersant (Source oil + disp.), and weathered oil A + dispersant (WO A + disp.; saltwater only). AcTy, acenaphthylene; Ac, acenaphthene; MA, methylanthracene; BbF, benzo[b]fluorene; FP, fluoranthenes/pyrenes; BNt, benzo[a]naphthothiophenes; BaC, benzo[a]anthracenes/chrysenes. Error bars represent standard error (SEM, n=1-4 replicate samples per treatment group).

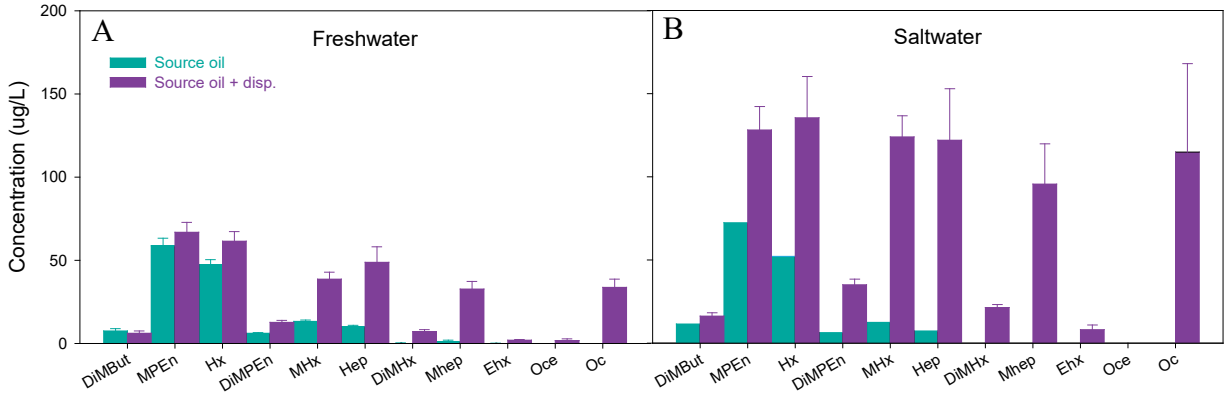


Figure 3.10. Other volatile organic compounds (VOC) found in fresh (A) and saltwater (B) WAFs made with source oil, and source oil with dispersant (Source oil + disp.). None of the weathered oil WAFs had measurable amounts of volatile compounds, so they were not included. DiMBut, dimethylbutane; MPEn, 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, n=1-4 replicate samples per treatment group).

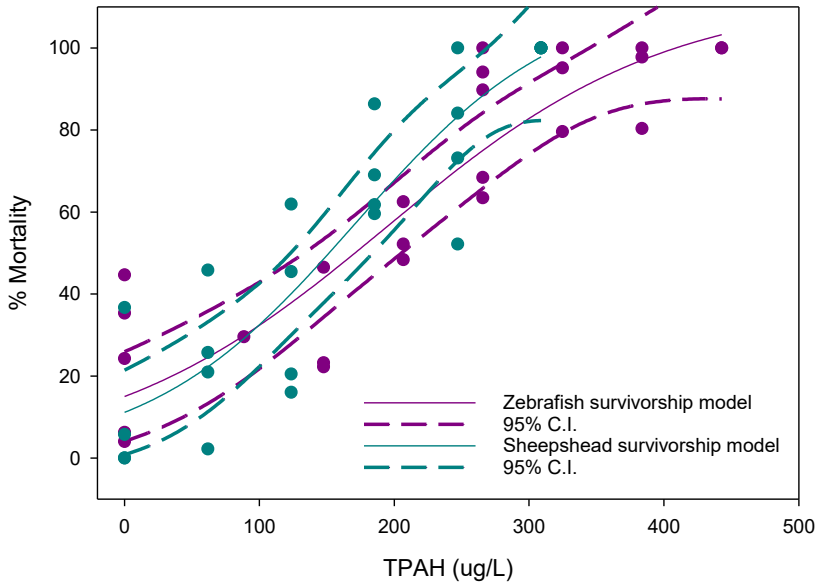


Figure 3.11. The percent mortality from 1-7 dpf (zebrafish) and 1-10 days post dpf (sheepshead minnow) in relation to the total PAH (TPAH) content of the source oil + dispersant WAF. 95% confidence intervals (C.I.) were included with each curve. The calculated LC50 for the zebrafish and sheepshead minnow were 265.1 ug/L (95% C.I. 248.6-282.8) and 207.5 ug/L (95% C.I. 169.3-253.3).

Chapter 4: Exposure to weathered, unweathered, and dispersed oil has persisting effects on ecologically relevant behaviors in sheepshead minnow

Abstract

The Deepwater Horizon oil spill released 3.19 million barrels of crude oil into the Gulf of Mexico, making it the largest oil spill in US history. Weathering and the application of dispersants can impact the acute toxicity of the crude oil. The cardiotoxicity of crude oil is well established, but few studies have been conducted assessing the effect exposure can have on complex behavioral responses in fishes. The primary aim of our study was to determine the lasting impact of developmental exposure to weathered, unweathered and dispersed crude oil could have on prey capture, male aggression and how fish interact with a novel object. Exposure to crude oil did not impair prey capture, but instead reduced the behavioral variability in responses to the addition of prey. Exposure to dispersed weathered oil also decreased border dwelling, a measure of anxiety, in response to a novel object. When all behavioral endpoints were compared using principal component analysis, we found that exposure to unweathered crude oil increased variability in the response to a novel object and exploratory behaviors, but decreased the variability in the anxiety-like behaviors. Further work is needed to understand the effects of oil exposure on fish behavior and the potential ecological impact of subtle behavioral changes in fishes.

Introduction

The Deepwater Horizon oil spill was the largest oil spill in U.S. history, lasting a total of 87 days. During the release, 3.19 million barrels of oil escaped from the damaged well-head into the Gulf of Mexico. To mitigate coastal effects and expedite natural degradation dispersant was injected at the well-head and applied on surface slicks (Kujawinski et al., 2011; Lehr et al., 2010).

Dispersant application and weathering can alter the toxicity of crude oil by impacting the polycyclic aromatic hydrocarbon (PAH) content released into the water column (Esbaugh et al., 2016). Dispersant application increases PAH levels in the water, thus increasing the toxicity of oil exposure to fishes (Anderson et al., 2009; Couillard et al., 2005; Mu et al., 2014; Ramachandran et al., 2004; Schein et al., 2009). Weathering of crude oil, which encompasses dissolution, emulsification, biodegradation, evaporation, photo-oxidation and dispersion, decreases the toxicity of crude oil to fishes (Chapman et al., 1995; Faksness et al., 2015; Shelton et al., 1999). The PAHs that remain after weathering are still toxic and are thought to be the primary driver of the cardiotoxic effects observed after crude oil exposure (Barron et al., 2004; Esbaugh et al., 2016; Incardona et al., 2009; Incardona et al., 2004). The

effect of crude oil exposure on cardiac morphology and cardiac output has been well studied (Carls et al., 2008; Incardona et al., 2009; Incardona et al., 2015; Incardona et al., 2004; Incardona et al., 2006; Incardona et al., 2011). In contrast, the impact of oil exposure on the ethology (i.e. the science of animal behavior) of fishes is not as well characterized.

Behavioral responses, despite their utility in linking physiological function with ecological effect, are rarely included in ecotoxicology studies (Scott and Sloman, 2004). Early life exposure to contaminants, such as crude oil, have the potential to alter behavioral responses to both environmental and physiological stimuli, which in turn could have an impact on population survival (Scott and Sloman, 2004). Predator avoidance, prey capture, reproductive behaviors and the exploration of a novel environment are complex behaviors that integrate many different tissues, physiological responses, and sensory systems (Sloman and McNeil, 2012). Ethological effects can be characterized throughout a fishes' life and are not limited to just sexually mature adults.

Development of the nervous system coincides with the development of complex behavioral responses in fishes (Weis, 2009). Abnormal behavioral patterns that are caused by developmental exposure to contaminants are referred to as 'behavioral teratology' (Weis, 2009). Studies on the developmental effects of methyl mercury exposure in fishes have found that early life exposure to the contaminant can permanently alter prey capture, basal activity, and predator avoidance ability well into adulthood (Samson et al., 2001; Weis et al., 2003; Weis and Weis, 1995a, b; Zhou and Weis, 1999). The few studies published on the behavioral teratology of crude oil have found exposure decreased distance travelled, decreased angular velocity, and decreased turn angles in juvenile zebrafish (*Danio rerio*) swim patterns (Wang et al., 2018). Dietary exposure to petrogenic PAHs has been associated with increased mobility, increased anxiety-like behaviors and lower levels of exploratory activity in zebrafish (Vignet et al., 2014). Exposure to water-accommodated fractions (WAFs) of crude oil also impaired the ability red drum (*Sciaenops ocellatus*) to compete with in a social hierarchy (Khursigara et al., 2018). The effect of oil exposure on other critical behaviors such as prey capture, predator avoidance and breeding is not well studied.

In our study, we aimed to determine the effect of larval exposure to unweathered, weathered and dispersed oil on complex behavioral responses in juvenile and adult sheepshead minnow (*Cyprinodon variegatus variegatus*). Sheepshead minnow are a resident species to the Gulf of Mexico and are a hardy species that is often used as a standard laboratory test organism for studying marine pollution and effluent toxicity (Choi et al., 2018). Embryos were exposed from 1-10 days post fertilization (dpf) and

then were raised to adulthood in artificial seawater to determine the lasting effect exposure had on prey capture, male aggression and exploration of a novel object.

Materials and Methods

Oil types and Water accommodated fractions.

Exposures were conducted with three different oil samples that were collected from the Deepwater Horizon spill. The unweathered oil sample (source oil) was a light crude oil collected from the subsea containment system situated about the well-head; the oil is referred to as 'MASS' because it was collected by the 'Massachusetts' barge. The moderately (~65%) weathered oil sample (WO A), referred to as 'CTC', was collected from a barge that received its oil from various skimming vessels. The heavily (~83%) weathered oil (WO B), referred to as 'Juniper', was collected from an alternate slick by a USGS skimming vessel. Weathering was determined using PAH depletion relative to the compounds hopane, as previously described (Prince et al., 1994) . All samples were stored at 4°C in argon capped amber class vials with Teflon caps. Water accommodated fractions were made with a 1/1000 oil to water ratio and100% WAF was used for all exposures except for the source oil plus dispersant treatment (because of lethality, 15% WAF was used in these exposures). WAF preparation and WAF chemistry is described in Philibert *et al.*(2019).

Exposures

Embryos were purchased from Aquatic Biosystems (Fort Collins, CO) and arrived at our facility (University of Alberta, Edmonton, AB, Canada) at 1 dpf and were exposed immediately. 3-5 replicates with 100 embryos per replicate were exposed to 200mL of 100% WAF (WO A, WO B, WO A + disp., source oil) or 15% WAF (source oil + disp.) in 500ml glass beakers held at 25-26°C. Exposures ran from 1-10 dpf, at 11 dpf embryos were transferred to a clean beaker with laboratory made saltwater until 12 dpf. At 12 dpf the embryos were transferred to a saltwater fish rack to be raised through to adulthood in 9L flow-through tanks. From 5-30 dpf fish were fed artemia twice daily, from 30dpf to adulthood fish were fed a fed a custom mixture of TetraMin® flakes (Tetra Holding, Blacksburg, VA), Cobalt™ Aquatics spirulina flakes (Cobalt, Rock Hill, SC), and Omega One™ freeze dried blood worms (Omegasea, Sitka, SK).

Prey Capture

The prey capture experiment was conducted on 30dpf sheepshead minnow juveniles. Fish were fasted for 48 h, then placed in a 60 mm petri dish to acclimate for 20 min. After the acclimation period 10 artemia were added with a pipette to the dish, video was recorded for 15 min, and was manually scored for latency to capture. The number of artemia captured was counted at the end of the trial.

Male aggression

1-1.5 year old male sheepshead minnow were placed in a sheltered isolation tent 20 L tank to acclimate for 20 min. After the acclimation period, a black barrier was removed to reveal a mirror on one side of the tank. Video was recorded for 15 min and was manually scored for latency to first strike and time spent attacking the reflection.

Novel Object

Without acclimation, 1.5-2 year old male fish were added directly to a blacked out 20 L tank containing a red cup in the center of the tank. Video was recorded for 15 min after the fish was introduced. EthoVision XT 10 (Noldus, NE) was used to track the fish's movement during the first 10 min of the trial to determine maximum velocity, distance travelled, time spent near the novel object, latency and frequency of approach and time spent near the border of the tank (anxiety-like behavior).

Global DNA methylation

DNA was extracted from the flash frozen muscle tissue of 1.5-2 year old male sheepshead minnow. A DNeasy Blood and Tissue Kit was used according the manufacturer's protocol for tissue samples. An Epigentek MethylflashTM Methylated DNA Quantification Kit (Colorimetric; Farmingdale, NY) was used according the manufacturer's protocol to measure global DNA methylation.

Statistics

Statistical differences between treatments were evaluated using one-way analysis of variance (ANOVA) followed by either Holm Sidak or Tukey's post-hoc tests. All data were expressed as the mean ±standard error of the mean (SEM) and p-values <0.05 were accepted as significant. SigmaPlot 11/14 (Systat, San Jose, CA) was used for the ANOVA. Principal component analysis (PCA) was performed using R (R Development Core Team, Vienna, Austria). Because the behavioral variables were measured on different scales, data for each variable were normalized by subtracting the mean and dividing by the standard deviation (Schrandt et al., 2012). This procedure was used to transform the behavioral endpoints into dimensionless variables and to eliminate the effect of units and scaling (Schrandt et al., 2012). The principal components included in the analysis all had an eigenvalue greater than 1. PCA was carried out for both activity and anxiety-based behavioral metrics (PCA1) and the anxiety-based behavioral metrics alone (PCA2). PCA1 principal component 2 (PC2) and principal component 3 (PC3) had very similar factor loading values, however, PC3 was chosen because the visual representation of the data was clearer. Eigenvalues and PCA outputs can be found in Tables 4.2-4.5.

Results

Prey capture, male aggression, global DNA methylation

The exposure of sheepshead minnow from 1-10dpf to weathered, unweathered and dispersed oil had varying effects on the behavioral responses examined in this study. Prey capture ability was not impaired by exposure, however, the variability in the latency to capture observed in the control group was not present in any of the exposure groups (Figure 4.1A**)**, i.e. there was a reduction in behavioural phenotypes with exposure. Male aggression responses to their own reflection were also unimpaired in the exposed fish (Figure 4.1B,C).

Exposure to crude oil had no effect on the activity levels in the sheepshead minnow during the novel object experiment. Both the mean distance travelled and mean maximum velocity remained similar across all treatment groups (Figure 4.2A,B). There was an increase in inter-individual variability in activity levels in the source oil exposed group (Figure 4.2A,B). Exposure to dispersed weathered oil decreased the time spent in the border during the trial (Figure 4.2C) relative to the control population, suggesting these fish were less anxious. Exposure had no effect on the latency to approach the novel object, the percent time spent near the novel object or the frequency the fish approached the novel object (Figure 4.2D-F). Exposure to oil also had no effect on global DNA methylation (Figure 4.3).

Principal component analysis

Two principal component analyses were carried out, the first (PCA1; Figure 4.4) was run including both behavior and activity metrics (distance travelled and maximum velocity), and the second (PCA2; Figure 4.5) was run without including activity metrics. PCA1 reduced the eight measured variables down to two independent principal component (PC) axes that accounted for 46% of the observed variation (Figure 4.4). Principal component 1 (eigenvalue = 2.30) explained 28.7% of the variation in the behavioral responses, PC3 explained the additional 16.8% (eigenvalue = 1.51). Distance travelled (DT), maximum velocity (MV), frequency of novel object approach (FNO), and latency of novel object approach (LNO) clustered around the PC1 axis, and all have positive loadings on PC1 except LNO (Figure 4.4, Table 4.1). Time spent in the border of the tank (BD), time spent near novel object (TNO), latency to capture prey (LC) and latency to strike reflection (LS) clustered around the PC3 axis, and all had negative loadings on PC3 except LC (Table 4.1). PCA2 did not include the activity metrics (MV, DT), and reduced the six measured variables down to two PCs that accounted for 50% of the observed variability (Figure 4.5). PC1 (eigenvalue = 1.66) explained 27.6% of the variation, PC2 explained the 22.7% (eigenvalue = 1.36). FNO, LNO, TNO, and LC cluster along the PC1 axis; FNO and TNO varied together positively, LNO and LC varied together negatively (Table 4.1). BD and LS

clustered along the PC2 axis, and both varied together negatively. In the first analysis (Figure 4.4) activity levels were significant contributors to variability, once removed (Figure 4.5), novel object behaviors played a more prominent role in intra-treatment variation.

Discussion

The impact of Deepwater Horizon (DWH) oil spill on ecosystems in and around the Gulf of Mexico are unclear. Genotyping of populations of the marsh fish *Fundulus grandis* in coastal Mississippi has suggested that there is very little evidence that the spill had any effect on populations, likely due to the patchiness of oil exposure that occurred in the gulf (Schaefer et al., 2018). Alternatively, biogeochemical ecosystem models have suggested that as a result of the spill the biomass of reef habitat has decreased by 25-50% in hard hit areas, and the biomass of large demersal fish has decreased by 40-70% in the Gulf (Ainsworth et al., 2018). It has also been suggested that slower growing populations may take 30+ years to fully recover from the effects of the DWH spill (Ainsworth et al., 2018). Even with continual monitoring and research, there continues to be a disconnect between the results observed in laboratory studies and their applicability in predicting population/ecosystem level effects after a spill.

Going beyond traditional lethality-based tests in laboratory studies has the potential to bridge the gap from physiological to ecological to ecosystem level effects. Though toxicants may cause no discernable impairments in a laboratory setting, subtle behavioral adjustments may lower an organism's ability to find and compete for food, hold territory or escape predation in the environment (Rowe et al., 2001). In laboratory conditions there may be no abiotic or biotic stressors, which allows individuals with minor behavioral and physiological impairments to survive, even if the same effect in the wild could be lethal (Baird et al., 2007). Another consideration is the exposure window. Many studies focus on acute effects in both larval and adult fish. Adult fish can detect and avoid crude oil plumes in the water column (Lari et al., 2015), limiting the likeliness of exposure of fish in a spacious marine environment. Embryos and larvae, however, do not have the physical capability to outswim a toxicant plume. In this study, we exposed embryos to a range of weathered, unweathered and dispersed oil, and included behavioral endpoints critical to the ecological fitness of a wild fish to better understand the toxicity of crude oil from an ecologically-relevant perspective. We also looked at behavioral effects that were evident months/years after exposure occurred, filling another knowledge gap in the ecotoxicology of crude, weathered and dispersed oil.

Prey capture ability, which is obviously critical to growth and survival, was examined 30dpf in fasted juvenile fish. Prey capture is dependent on a complex network of neural pathways that include visual perception, recognition of the prey, decision-making, and a motor control response (Muto and Kawakami, 2013). Though we saw no direct impairment in the oil exposed fish, we did see a shift in the behavioral variability present in the population; there was an increased prevalence of a 'bold' response to the addition of prey in the exposed fish. This 'behavioral bottleneck effect' has been previously reported in studies on diluted bitumen, and oil sands process affected-water (Philibert et al., 2019; Philibert et al., 2016), and the multigenerational effects of fluoxetine exposure (Vera-Chang et al., 2018). Fluoxetine exposure has been shown to suppress cortisol levels and anxiety-like behavioral responses (Vera-Chang et al., 2018), implicating cortisol as a modulator of anxiety-like behavior in fishes. Changes in anxiety-like behaviors have not yet been linked to changes in ecological fitness.

Sheepshead minnow have a 'promiscuous' breeding system in which males compete for breeding territory that is then used to court multiple female fish (Kodricbrown and Mazzolini, 1992). Females evaluate the size and quality of the spawning territory and indicate their willingness to mate by approaching the male defended spawning territory (Kodricbrown and Mazzolini, 1992). Though there is no direct parental egg guarding behaviors observed in sheepshead minnow, protection of the eggs/embryos is a by-product of males effectively guarding their spawning territories (Craig and Bosman, 2013). In our study, oil exposure did not impact the aggression level in the adult sheepshead minnow males, but there was quite a bit of inherent variability within the treatment groups. Variability in aggression levels is important at the population level to allow for the development of functional social hierarchies, though subordinate male fish can have higher basal cortisol levels, which in turn, impairs neurogenesis (Tea et al., 2019).

Though fish intelligence is often underappreciated, fish have impressive and complex visual systems, and they are equipped with sophisticated visual repertoires (Newport et al., 2018). Archerfish (*Toxotes chatereus*) can be trained to recognize and discriminate between images of human faces, even when the images were rotated and presented at varying depth (Newport et al., 2018). The finely tuned visual system of fishes allows for rapid identification and interpretation of novel objects and potential threats in their environment. The response to a novel object can vary between fish who are quick to approach and acclimate to a novel stimulus (neophilic) and fish who are extremely cautious/anxious when confronted with a novel stimulus (neophobic). Even the same fish confronted multiple times with different novel stimuli can have a different response every time further complicating the interpretation of

exploration and novel object-type behavior in a laboratory trial (Fior et al., 2018). In our novel object experiment, we found very few differences between treatment groups apart from border dwelling responses. Border dwelling, a well characterized measure of anxiety behavior (Kalueff et al., 2013), was decreased in dispersed weathered oil (WO A + disp.) exposed fish, suggesting the fish were less anxious than the other exposure groups in the study. The WO A + disp. WAF had very comparable PAH levels to its undispersed counterpart (WO A), which suggests individual WAF PAH profile as opposed to total PAH (TPAH) content may be more predictive of exposure based ethological changes. It should also be noted that though there were no statistically significant differences in the other novel object metrics, there was higher variability in the activity metrics (distance travelled, maximum velocity) in the source oil exposed group. Activity during basal swimming can be indicative of the physical fitness and could serve as a marker of poor cardiovascular and neuromuscular condition in some source oil exposed individuals. Exposure to 3-ring PAHs, which are found in abundance in the source oil WAF, have been associated with cardiac impairment (Brette et al., 2014; Frantzen et al., 2015; Hodson, 2017; Incardona et al., 2009; Incardona et al., 2006; Incardona et al., 2014), as well as alterations in swimming behavior and decreased swimming performance (de Soysa et al., 2012; Hicken et al., 2011; Incardona et al., 2013; Kennedy and Farrell, 2006; Mager et al., 2014; Yu et al., 2015). Adverse outcome pathways for crude oil cardiotoxicity have been established, and changes in gene expression have been associated with whole organism endpoints (Xu et al., 2017). The molecular mechanism that modulates the behavioral effects of crude oil exposure are not as well understood.

DNA methylation, histone binding and other epigenetic mechanisms have been implicated as modulators of change in the central nervous system (CNS) (Lakstygal et al., 2018), and the epigenome has become an indispensable tool for understanding changes in behavior observed throughout the life of an animal (Lakstygal et al., 2018). For example, Schizophrenia-like behavior in fishes has been associated with decreased DNA methylation of the *gabrb2* promoter in zebrafish (Wang et al., 2016). Global DNA methylation assays, a more general approach to epigenetics, can provide clues on how an exposure effects the entire genome of a fish, though lacks specificity in the genes affected. In our study we found no statistical differences in global DNA methylation in our exposure groups, though it should be noted that the dispersed source oil exposed group trended towards a lower global methylation level than the other treatment groups. Links between DNA methylation and behavioral responses on the bold-shy spectrum have not yet been established.

All behavioral endpoints measured in this study can be divided into endpoints that describe activity levels, and endpoints that could serve as a measure of bold-shy behavioral phenotypes in exposed and unexposed fishes. Distance travelled (DT) and maximum velocity (MV) can serve as an indicator of a behavioral phenotype but the measurement is not direct. Border dwelling (BD), the frequency a fish approaches a novel object (FNO), the latency before a fish approaches a novel object (LNO), the time the fish spends in close proximity with a novel object (TNO), the latency to capture after the addition of prey (LC), and the latency to first strike when a fish sees their reflection in a mirror (LS) are all direct markers of bold/shy and anxiety-like behavior in fishes. Principal component analysis (PCA) can serve as a very useful tool to understand large and complex data sets, like the ones generated in ethological studies (Mazzamuto et al., 2019). When activity-based metrics were included in our PCA analysis (PCA1), we found activity metrics contributed to a large percent of our study-wide variability, and we found fish who travelled further during the 10 min novel object trial were also swimming faster. These more active fish were also approaching the novel object frequently which indicative of active exploration. The active and exploratory fish had a low latency to novel object approach, which suggests fish who are actively exploring a novel object will approach the object quickly to determine if it is food, shelter or a potential threat (Nomakuchi et al., 2009). Exploratory behavior has been previously used as an indicator of individual boldness (Verbeek et al., 1996). Fish who spent very little time pursuing shelter in the edges of the tank (i.e. fish with low anxiety levels) were also fish who were quick to act aggressively to their reflection and were slow to capture their first prey. You would expect fish that are aggressive and have low anxiety to fit the profile of a bold behavioral phenotype, and bold fish are typically aggressive feeders, but this was not evident in the PCA. Because prey capture was the only experiment done on juvenile fish, sexual maturation could have altered the bold-shy phenotype. In Brown trout (*Salmo trutta*), males perform 'bold' behaviors less consistently before sexual maturity, and many bold behaviors are thought to depend on sexually selected gene promoters during gonadal development(Johnsson et al., 2001). Activity levels in the source oil exposed fish were more variable than the weathered oil exposed and control fish, as evident by the high variability in PC1. This may be largely due to the cardiotoxicity of PAHs, which are found in abundance in unweathered crude oil (Brown et al., 2017; Incardona et al., 2004; Philibert et al., 2019).

When activity-based metrics were not factored into the PCA analysis, there was a more pronounced relationship between the various novel object related behavioral metrics. Fish who approached the novel object quickly after being introduced to the tank, visited the object more frequently also spent more time near the novel object, and were quick to capture prey during the prey

capture experiment, which is indicative of a bold exploratory behavioral response. There was high variability in the exploratory responses of source oil exposed fish. There is a relationship between fish with low anxiety levels (low levels of border dwelling) and aggression in response to a perceived male threat, and this behavioral response was much more variable in the control and weathered oil exposed fish. Without the confounding effects of physical fitness, unexposed and weathered oil exposed fish had more variable aggression and anxiety levels, where the source oil exposed fish had more variable exploratory behavioral responses. A study on wild caught atlantic cod (*Gadus* morhua) found that individuals that exhibited more exploratory/aggressive behaviors in a laboratory setting had a more expansive home-range and were less reactionary to changes in temperature in their natural environment than their less exploratory counterparts(Villegas-Rios et al., 2018).

A personality trait, like boldness, can shape how an animal responds to novelty and perceived risks in their environment. Bold individuals are (typically, but not always) more active and more exploratory than their shy counterparts (Conrad et al., 2011; Frost et al., 2013; Sneddon, 2003). The behavioral responses of fishes to novel stimuli are both complex and dynamic, but also represent an important part of ecological fitness. In our study we found that exposure to crude oil early in development impacted the behavioral responses of fish to novel stimuli. Populations of exposed fish were not impaired from capturing prey, defending territory, or responding to a novel environment but instead there was a decrease in the variability of responses. Fish exposed to unweathered oil had higher variability in exploratory behaviors but had decreased variability in anxiety and aggression responses. Weathered oil exposed fish behaved much more similarly to the control population, had was less ethological effects than unweathered crude oil. Further studies are needed to understand the ecological consequences of subtle behavioral shifts caused by early life exposure to crude oil contaminants. Overall, as variability and diversity within a species is at the core of species survival, factors that limit it should be treated with great caution.

Tables

Table 4.1. Factor loadings for the principal component analysis performed on the activity and anxiety-based behavioral metrics (PCA1) and the anxiety-based behavioral metrics alone (PCA2).

	PCA1		PCA2	
Variable	PC1	PC3	PC1	PC2
Distance travelled (DT)	**0.56**	0.15	-	-
Maximum velocity (MV)	**0.51**	0.07	-	-
Border dwelling (BD)	0.07	**-0.67**	0.09	**-0.69**
Frequency of novel object approach (FNO)	**0.44**	0.12	**0.52**	0.14
Latency of novel object approach (LNO)	**-0.39**	-0.004	**-0.62**	-0.12
Time spent near novel object (TNO)	0.12	-**0.32**	**0.51**	-0.11
Latency to capture (LC)	0.11	**0.21**	**-0.27**	0.04
Latency to strike (LS)	0.19	**-0.60**	0.07	**-0.69**

Table 4.2. The eigenvalues for the principal component analysis on the activity and anxiety-based behavioral metrics.

Principal Component	*Eigenvalue*
1	2.30
2	1.51
3	1.35
4	0.87
5	0.69
6	0.57
7	0.45
8	0.26

Table 4.3. Principal component analysis result summary on the activity and anxiety-based behavioral metrics.

Component:	*PC1*	*PC2*	*PC3*	*PC4*	*PC5*	*PC6*	*PC7*	*PC8*
Standard deviation	1.51	1.23	1.16	0.93	0.83	0.76	0.67	0.51
Proportion of variance	0.29	0.19	0.17	0.11	0.09	0.07	0.06	0.03
Cumulative proportion	0.29	0.48	0.64	0.75	0.84	0.91	0.97	1.00

Table 4.4. The eigenvalues for the principal component analysis on the anxiety-based behavioral metrics.

Principal Component	*Eigenvalue*
1	1.66
2	1.36
3	0.96
4	0.80
5	0.67
6	0.55

Table 4.5. Principal component analysis result summary on the anxiety-based behavioral metrics.

Component:	*PC1*	*PC2*	*PC3*	*PC4*	*PC5*	*PC6*
Standard deviation	1.28	1.17	0.98	0.89	0.82	0.74
Proportion of Variance	0.28	0.23	0.16	0.13	0.11	0.09
Cumulative proportion	0.28	0.50	0.66	0.78	0.91	1.00

Figures

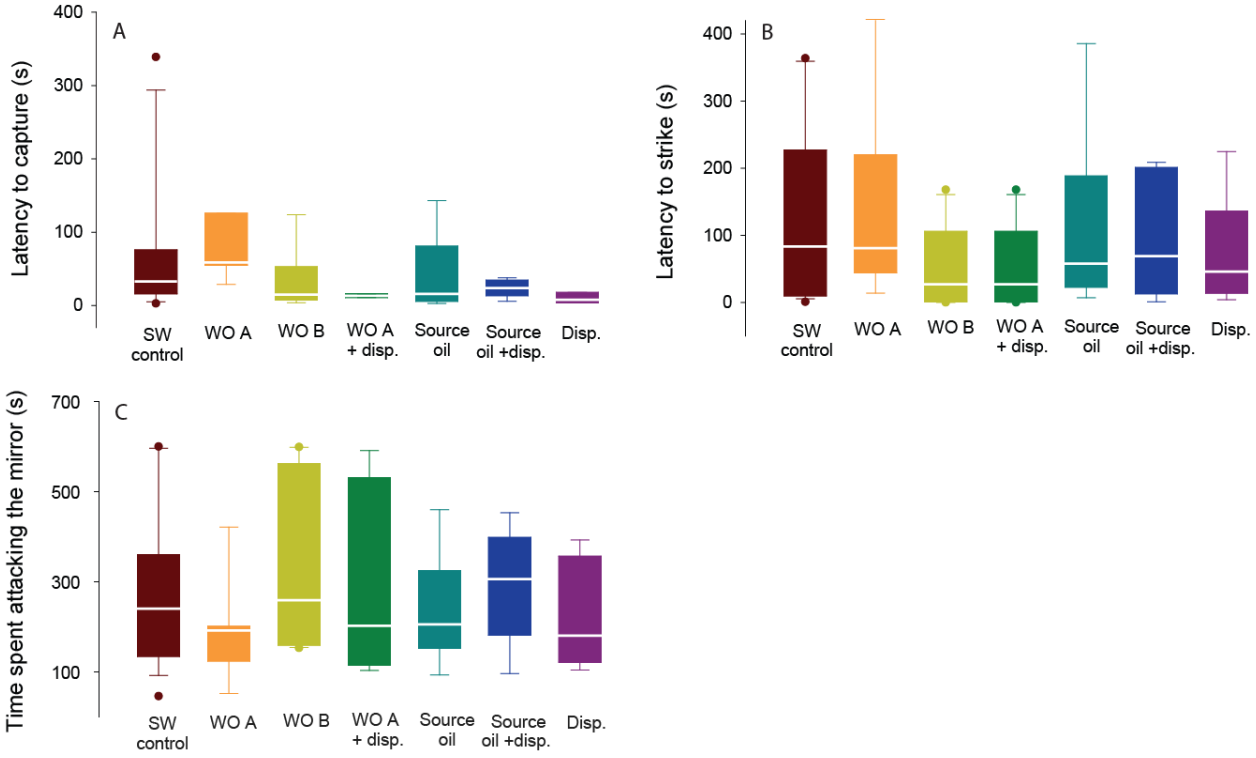


Figure 4.1. Effects of developmental exposure to weathered (WO A, WO B), unweathered (Source oil), dispersed oil (WO A + disp., Source oil + disp.), and dispersant alone on the prey capture and male aggression behavior in juvenile and adult sheepshead minnow; respectively. Prey capture success was measured in time it took to make the first capture (latency to capture), and male aggression was measured in the time it took to strike their reflection after the mirror was revealed (latency to strike) and total time after the first strike the males spent attacking their own reflection (time spent attacking the mirror). n=10-16 per treatment group. Boxes represent the 1st quartile, median and 3rd quartile, bars represent the standard error of the mean (SEM)

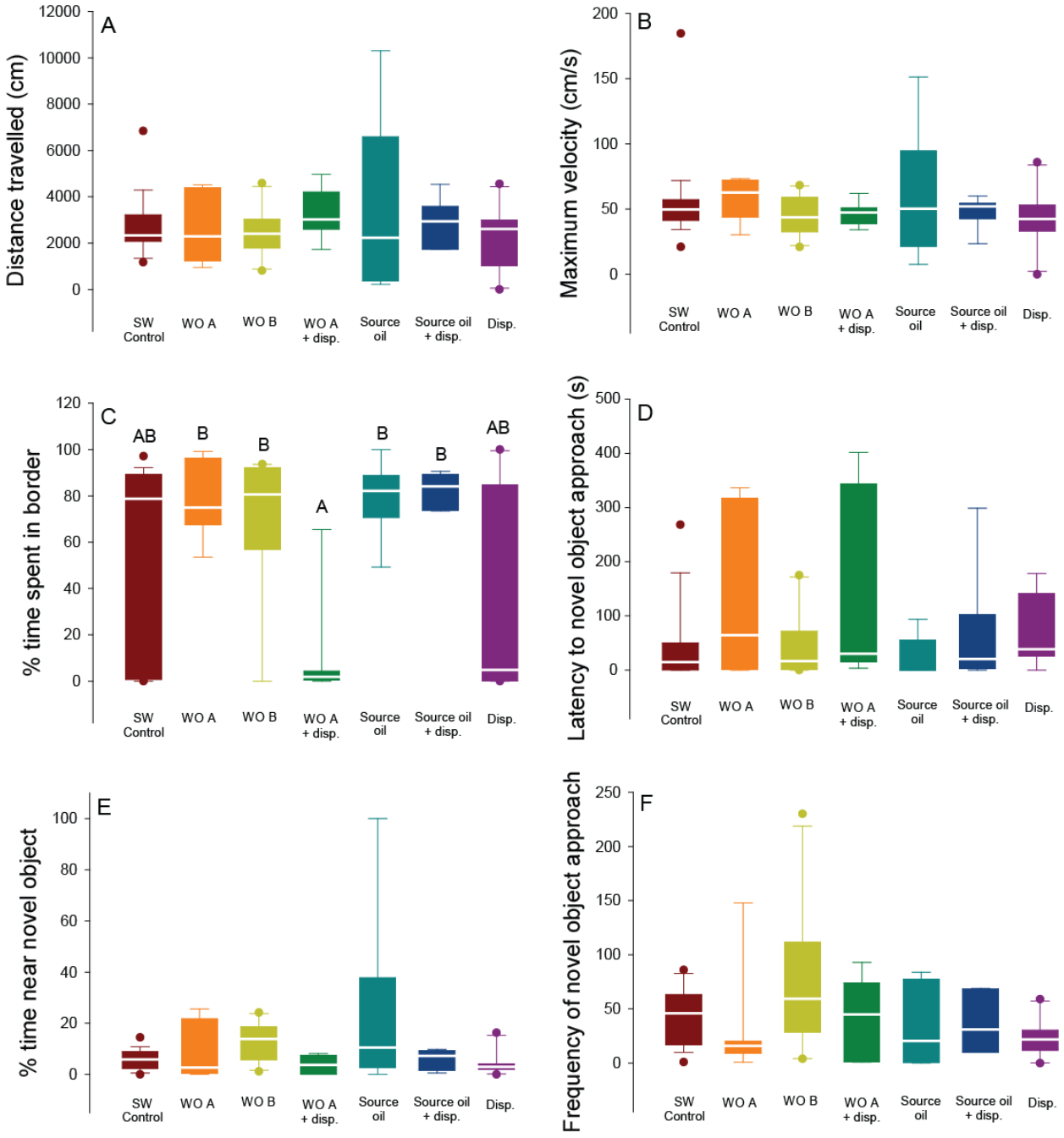


Figure 4.2. The effect of developmental exposure to crude oil on interactions with a novel object as an adult. Novel object behavior was measured in the first 10 minutes after entering the tank by measuring the distance travelled during the trial, maximum velocity, the percent time spent in the border of the tank (a measure of anxiety behavior), latency to novel object approach, the percent time spent near the novel object during the 10 minute trial, and the frequency of novel object approach. n=10-15 per treatment group. Boxes represent the 1st quartile, median and 3rd quartile, bars represent the standard error of the mean (SEM).

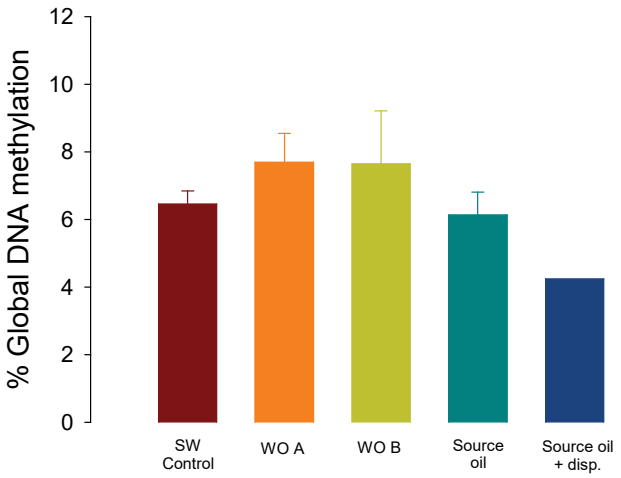


Figure 4.3. Change in the percent global DNA methylation in the muscle tissue of adult sheepshead minnow exposed from 1-10dpf to weathered, unweathered and dispersed oil. DNA methylation was measured in 2-2.5 year old adult fish. n= 1-3 per treatment group. Bars represent the SEM.

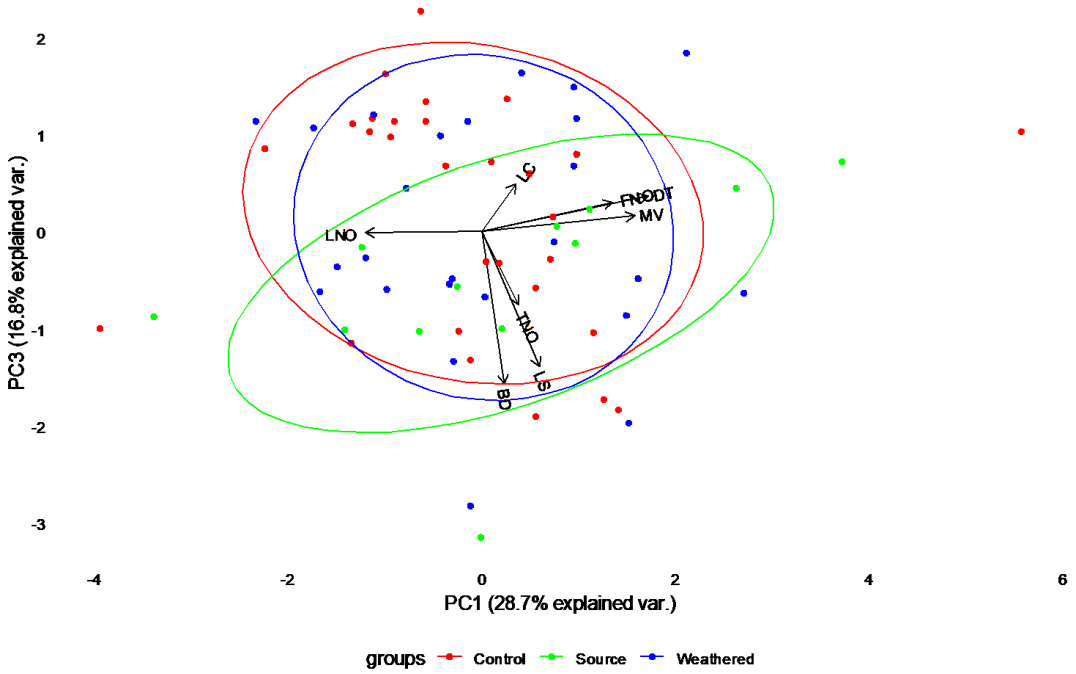


Figure 4.4. Principle Component Analysis (PCA) of all the behavioral endpoints from the prey capture, male aggression, and novel object assay. Metrics included were latency to prey capture (LC), latency to mirror strike (LS), distance travelled during novel object assay (DT), maximum swim speed during novel object assay (MV), time spent in the border of the tank during the novel object assay (BD), frequency of novel object approach (FNO), latency of novel object approach (LNO), and time spent near the novel object (TNO).

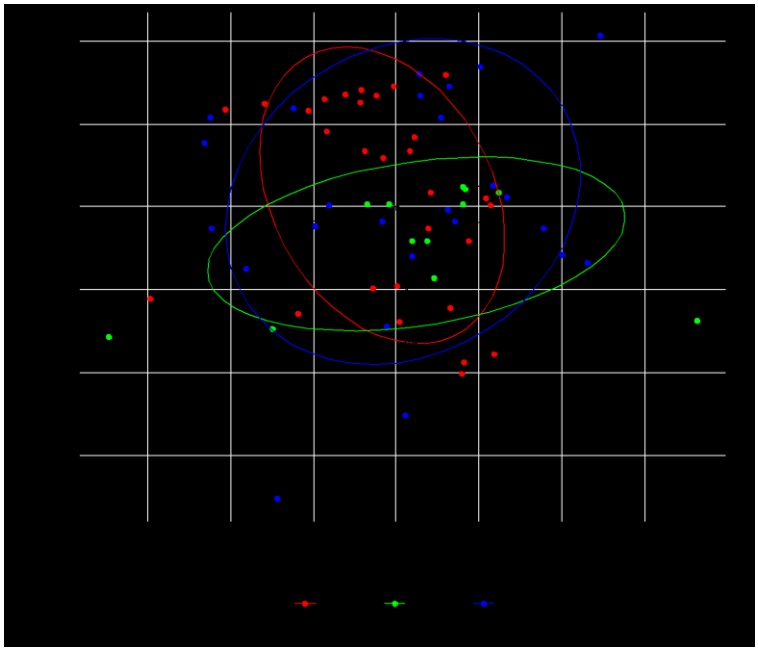


Figure 4.5. Principle Component Analysis (PCA) of the anxiety-like behavioral endpoints from the prey capture, male aggression, and novel object assay. Metrics included were latency to prey capture (LC), latency to mirror strike (LS), time spent in the border of the tank during the novel object assay (BD), frequency of novel object approach (FNO), latency of novel object approach (LNO), and time spent near the novel object (TNO).

Chapter 5: 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 associated 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 practically non-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 (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 5.1 as well as Figures 5.1-5.6. 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 manufacturer'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 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 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 5.2.

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 5.1) 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 5.1-5.6).

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 5.7A, 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 5.7B, *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 5.7b, $p<0.05$).

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 5.8, $F_{2,20} = 9.11$, $p<0.001$ 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 5.8, $F_{2,11} = 7.25$, $p<0.05$). 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 5.9A). 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 5.9A). 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 5.9B, 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 5.10A, p=0.07). Jaw morphology was also unaffected by exposure to either OSPW treatment group (Figure 5.10B). 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 5.11A). The raw exposed treatment group had a very slight downregulation in *p53* expression (Figure 5.11A, $F_{2,12} = 4.45$, $p<0.05$). 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 5.11B).

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 5 day biochemical oxygen demand (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 breaks 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 was practically non-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 and 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.

Acknowledgements

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Tables

Table 5.1. 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.

Figures

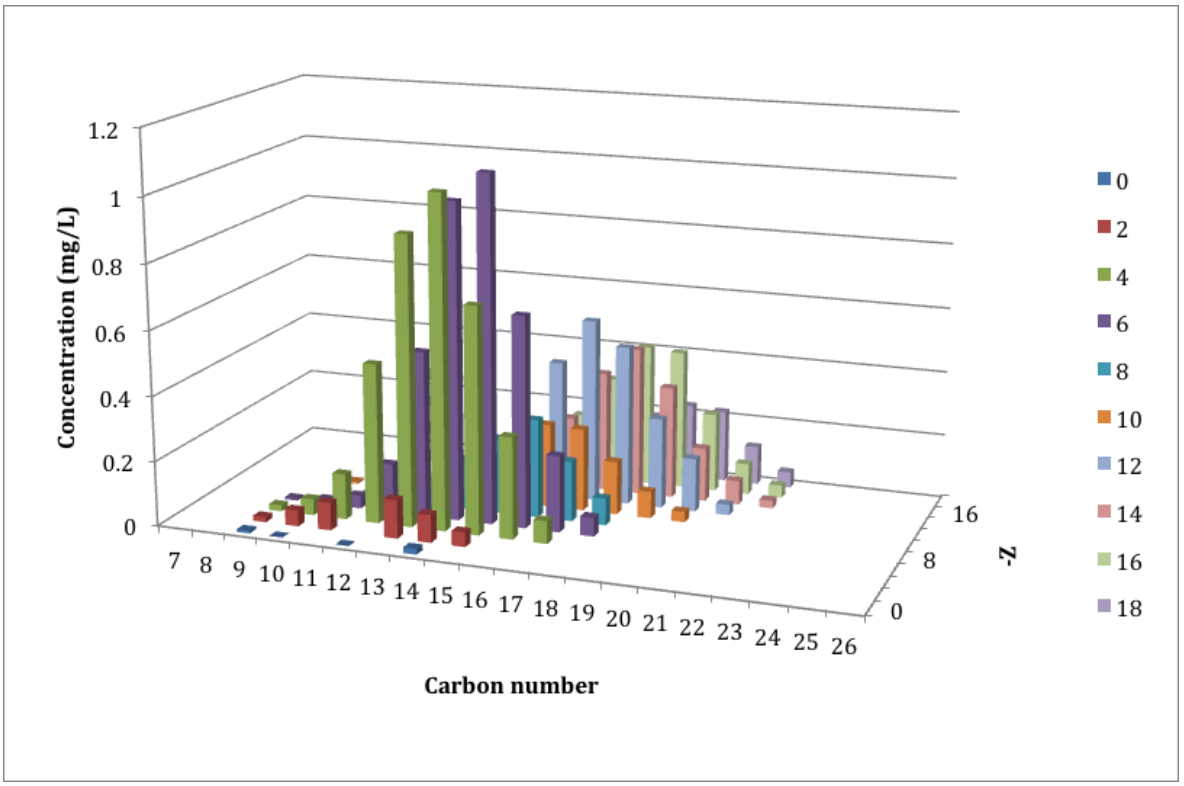

Figure 5.1. The distribution profile of O_2-NAs in raw OSPW, in terms of carbon and Z numbers. The concentration of O_2-NAs is 16.94 mg/L.

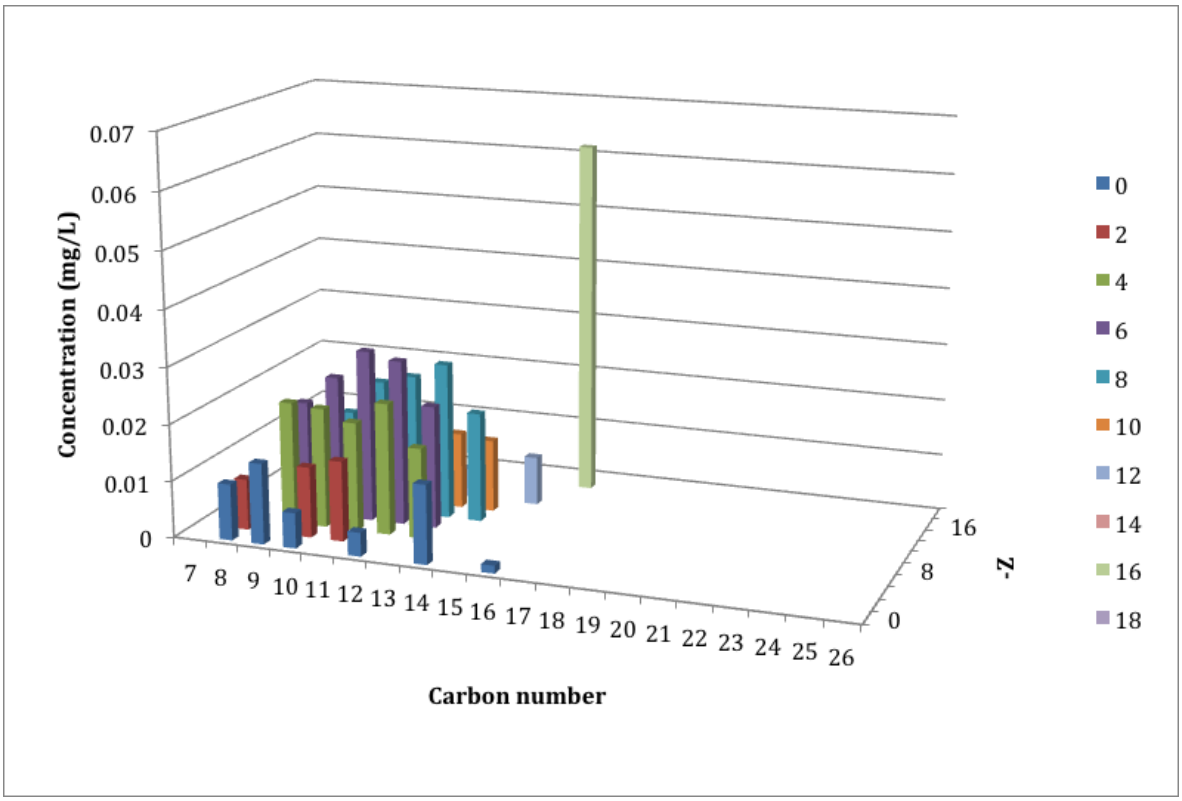

Figure 5.2. The distribution profile of O_2-NAs in OSPW after ozonation treatment, in terms of carbon and Z numbers. The concentration of O_2-NAs is 0.56 mg/L.

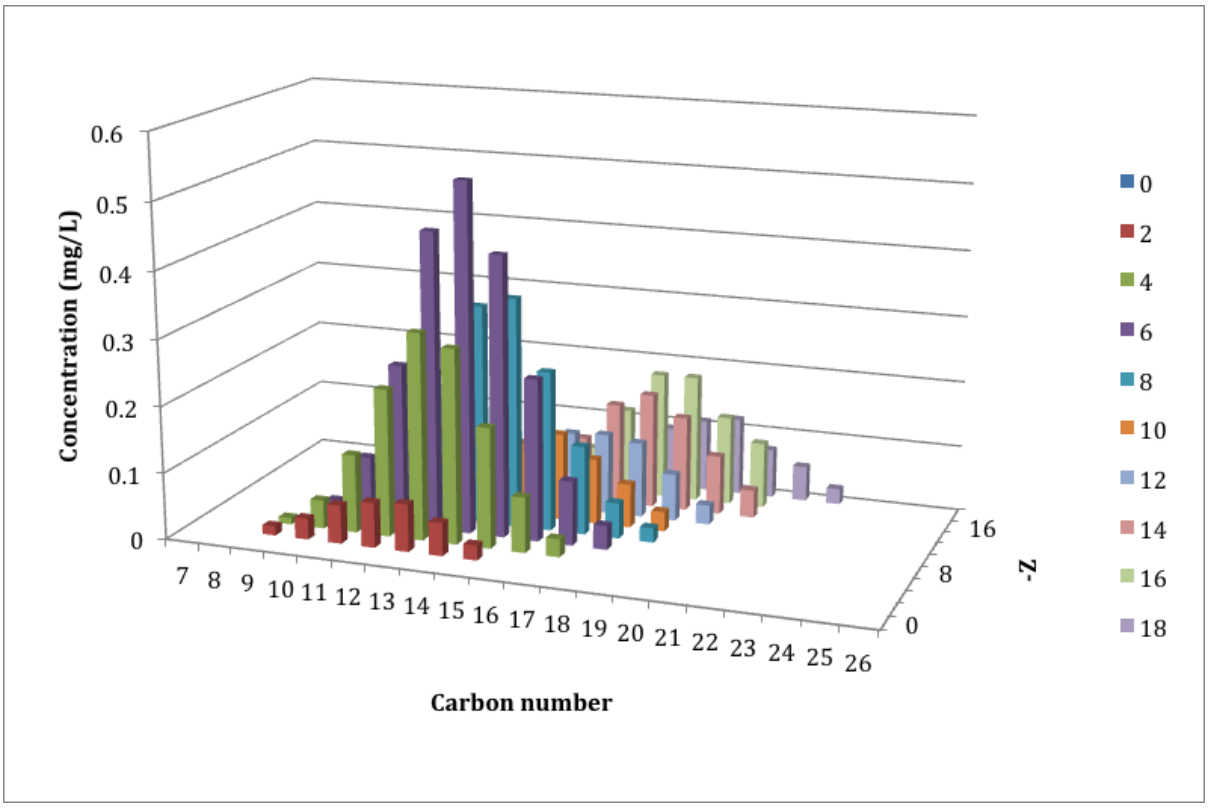

Figure 5.3. The distribution profile of O_3-NAs in raw OSPW, in terms of carbon and Z numbers. The concentration of O_3-NAs is 8.59 mg/L.

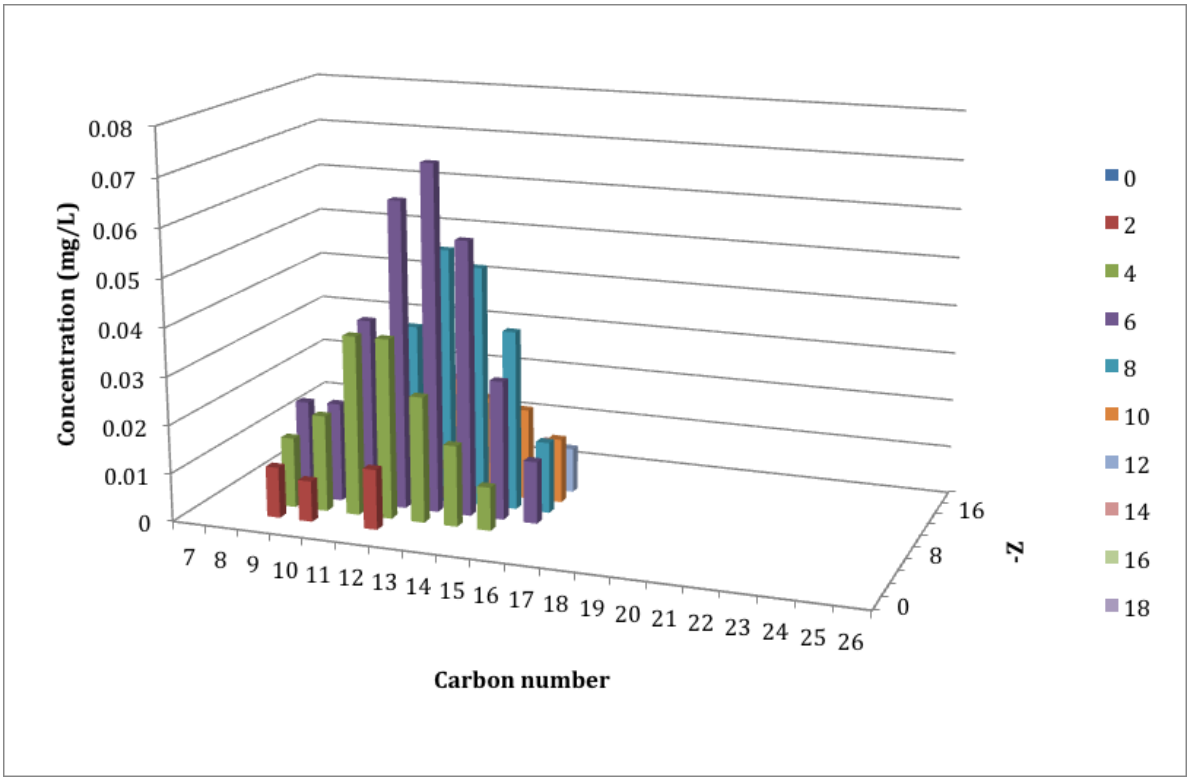

Figure 5.4. The distribution profile of O_3-NAs in OSPW after ozonation treatment, in terms of carbon and Z numbers. The concentration of O_3-NAs is 0.89 mg/L.

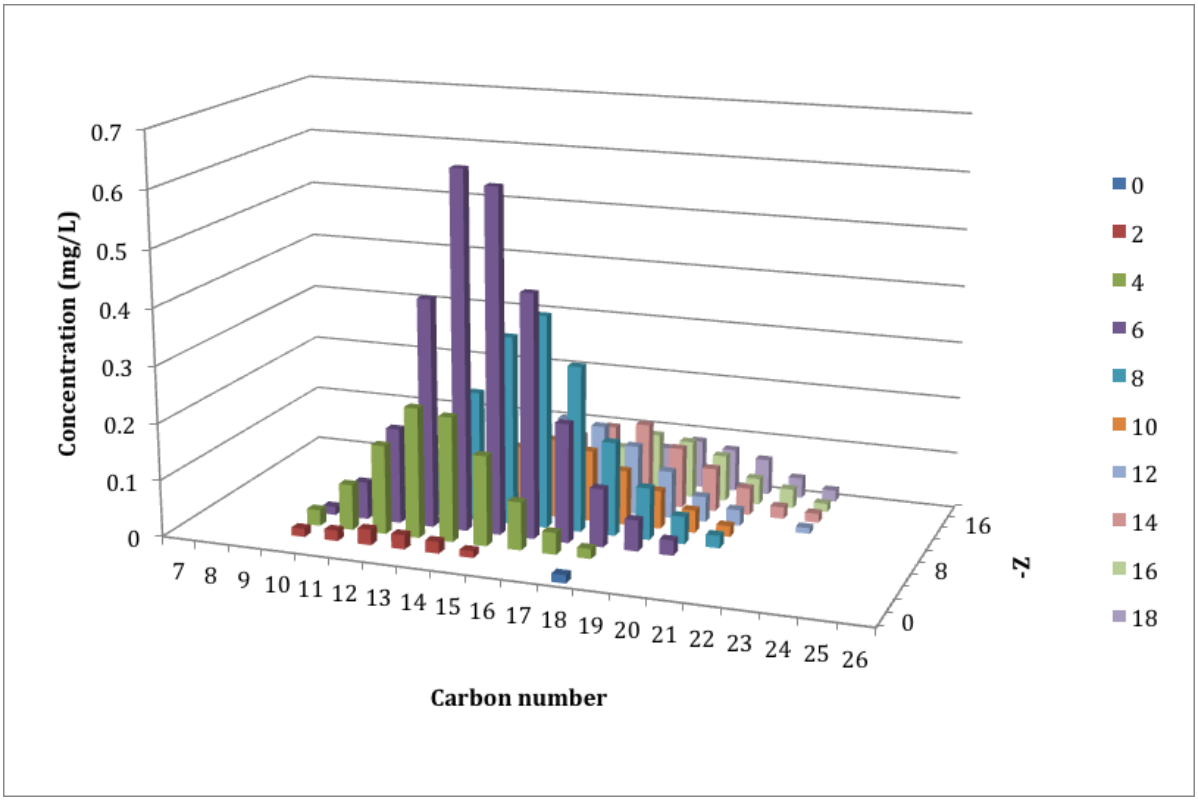

Figure 5.5. The distribution profile of O_4-NAs in raw OSPW, in terms of carbon and Z numbers. The concentration of O_4-NAs is 9.09 mg/L.

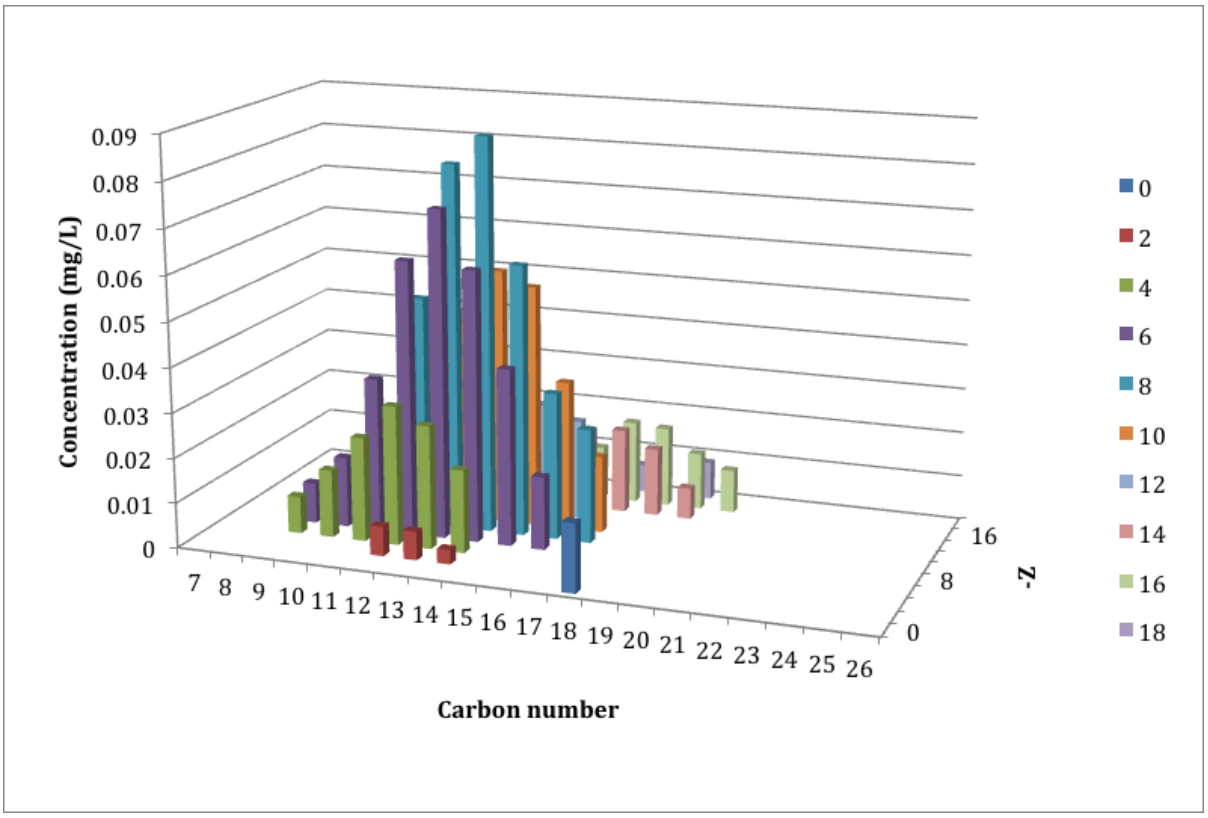

Figure 5.6. The distribution profile of O_4-NAs in OSPW after ozonation treatment, in terms of carbon and Z numbers. The concentration of O_4-NAs is 1.38 mg/L.

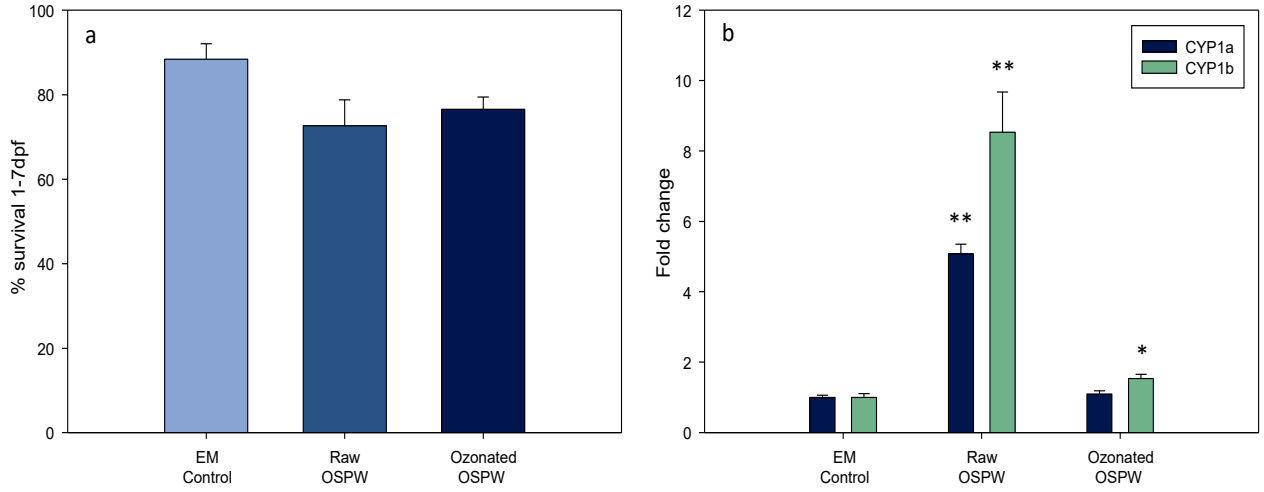

Figure 5.7. Percent survival of embryos exposed to raw and ozonated OSPWs and embryo media (EM) control from 1-7 dpf (A) and expression levels of biotransformation enzymes *cyp1a* and *cyp1b* at 7dpf (B). (A) Embryo survival was not affected by raw or ozonated OSPW exposure (one way ANOVA and Holm Sidak 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).

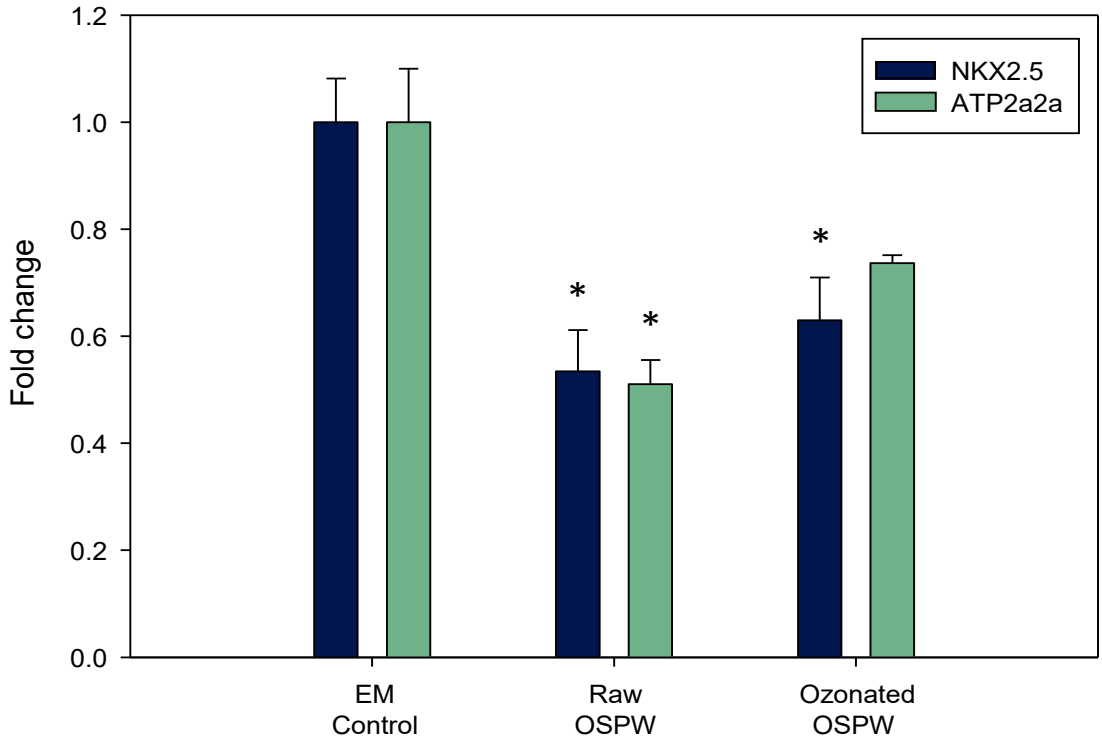

Figure 5.8. Expression levels of cardiac development genes *nkx2.5* and *atp2a2a* after exposure to raw and ozonated OSPW. *nkx2.5* was downregulated by raw and ozonated OSPW (one way ANOVA and Tukey's post hoc). *atp2a2a* was downregulated by raw OSPW exposure (one way ANOVA and Tukey's post hoc test, * indicates $p<0.05$, n=4-6).

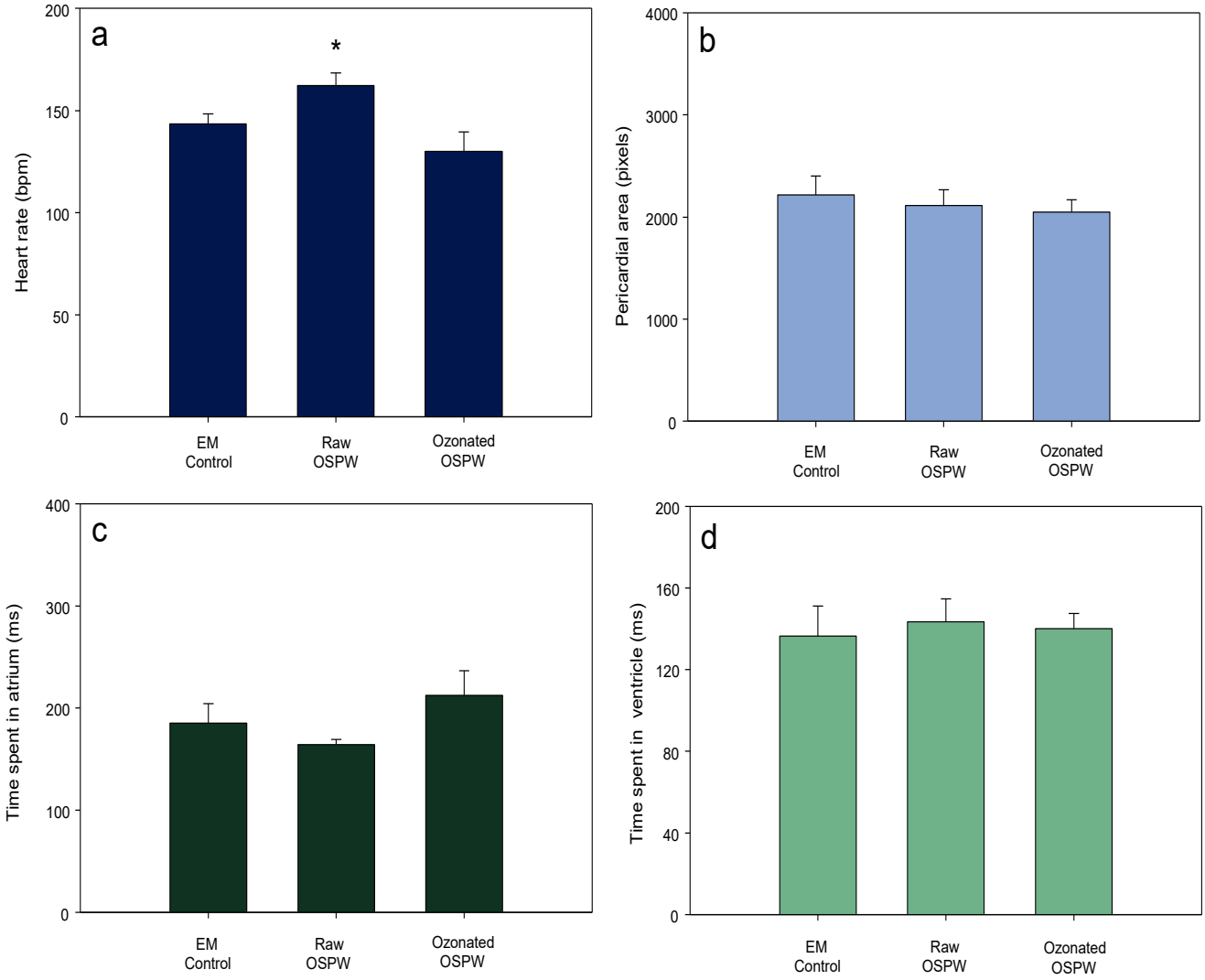

Figure 5.9. The effect of raw and ozonated OSPW exposure on the heart rate (n=20-35) (A), pericardial area (n=10-15) (B), time blood spent in the atrium of the heart (n=6-10) (C), and time blood spent in the ventricle of the heart (n=6-10) (D) of 2 dpf zebrafish embryos. Heart rate was higher in the 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.

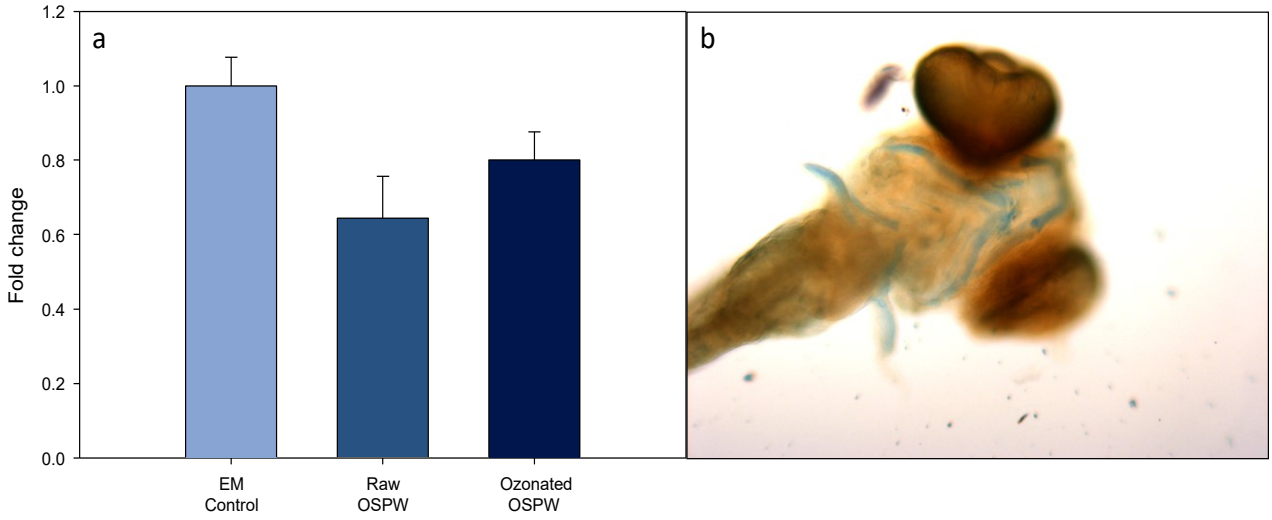

Figure 5.10. Expression levels of neurodevelopment gene *gli2a* (A) and an example of normal embryo jaw morphology (B) after exposure period at 7dpf. (a) *gli2a* expression was unaffected by OSPW exposure (one way ANOVA, n=4-6). (B) Image shows normal jaw structure in a control embryo. There were no observed changes to jaw morphology between treatment groups (n=25-30 per treatment).

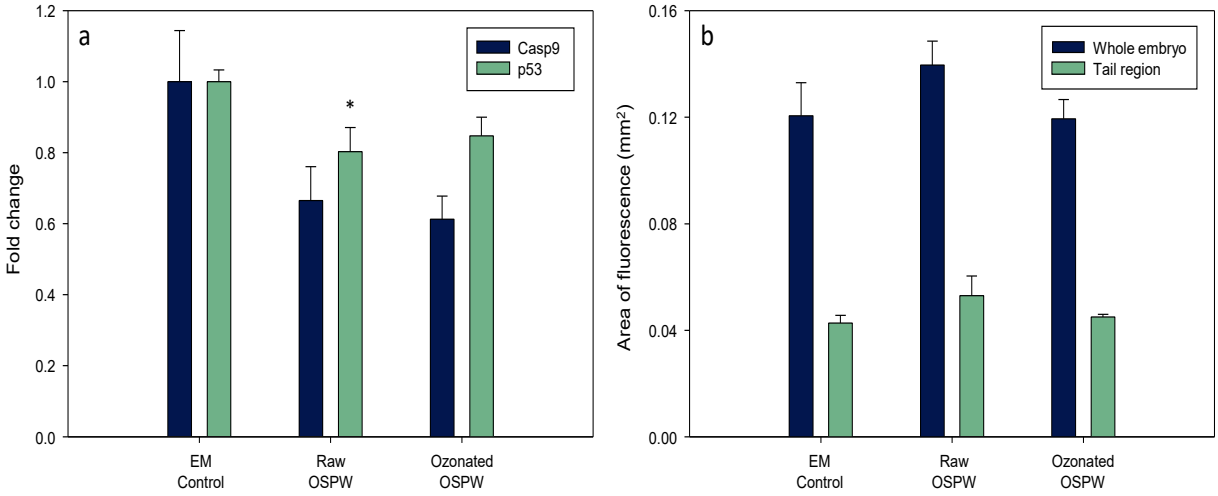

Figure 5.11. Expression levels of apoptosis markers *casp9* and *p53* in 7dpf embryos (A) and occurrence of apoptotic cells in whole embryos and the tail region of 3dpf embryos using the TUNEL assay (B). (a) *casp9* expression remained unaffected by exposure, while *p53* expression was slightly downregulated 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 6: Persistent and transgenerational effects of raw and ozonated oil sands process-affected water exposure on a model vertebrate: the zebrafish

Abstract

Exposure to oil sands process-affected water (OSPW), a by-product of Canadian oil sands mining operations, can cause both acute and chronic adverse effects in aquatic life. Ozonation effectively degrades naphthenic acids in OSPW, mitigating some of the toxicological effects of exposure. In this study we examined the effect of developmental exposure to raw and ozonated OSPW had on the breeding success, prey capture, and alarm cue response in fish months/years after exposure and the transgenerational effect exposure had on gene expression, global DNA methylation, and larval basal activity. Developmental exposure altered the expression of VTG and NKX2.5 in the unexposed F1 generation. Exposure to both raw and ozonated OSPW had a transgenerational impact on larval activity levels, anxiety behaviors, and maximum swim speed compared to the control population. Prey capture success was unaffected, however, the variability in the behavioral responses to the introduction of prey was decreased. Fish developmentally exposed to either treatment were less active before exposure and did not have an anxiety response to the alarm cue hypoxanthine-3-n-oxide. Though ozonation was able to mitigate some of the effects of OSPW exposure, further studies are needed to understand the transgenerational effects and the implications of exposure on complex fish behaviors.

Introduction

A by-product of bitumen extraction in the Canadian oil sands, oil sands process-affected water (OSPW), can harm aquatic life following brief and longer-term exposures(Allen, 2008; Anderson et al., 2012a; Clemente and Fedorak, 2005; He et al., 2012a; Lari et al., 2016). For fishes, OSPW and compounds in OSPW have been shown to impair olfaction(Lari and Pyle, 2017; Reichert et al., 2017), alter behavioral responses(Reichert et al., 2017), impair immune function(Hogan et al., 2018; Leclair et al., 2013), and disrupt the endocrine system(Arens et al., 2015; He et al., 2012b; Wang et al., 2015a; Wang et al., 2015b). OSPW is a complex mixture composed of water, residual bitumen, silt, clay, and inorganic and organic materials(Allen, 2008; Huang et al., 2018; Li et al., 2017). The toxicity of OSPW is commonly attributed to the organic fraction, in particular, classical naphthenic acids (NAs), which are carboxylic acids naturally

found in bitumen deposits (Anderson et al., 2012b; He et al., 2012a; Hughes et al., 2017; Redman et al., 2018). The inorganic components of OSPW, consisting of metals and salts, may also be a potential source of toxicity to aquatic life, however, any mechanism of toxicity is poorly understood (Fu et al., 2017; Klamerth et al., 2015; Leclair et al., 2013). The Alberta Environmental Protection and Enhancement Act (1993) currently holds all oil sands operators accountable to a 'zero discharge policy', which has resulted in over 973 million m^3 of stored tailings (as of 2013)(Alberta Environment and Sustainable Resource Development. 2014. Reclamation Information System). To reclaim the area, stored water will need to be released back into the environment, as it is a requirement in all oil sands operations regulatory approvals. Though fractions of the organic contaminants in OSPW degrade naturally, this process is unlikely to be sufficient. Reclamation pond studies have found that the total NA content decreases over time but sublethal effects persist(Anderson et al., 2012a; Toor et al., 2013). Ozonation has shown promise as a possible remediation strategy for OSPW as it can oxidize any toxic organics in OSPW and hence decrease the toxicity of OSPW exposure(Anderson et al., 2012b; Meshref et al., 2017; Toor et al., 2013). The majority of OSPW toxicity research has focused on acute exposures and effects on physiological and molecular endpoints. However, the potential for effects to persist following an exposure during early development into adulthood, including effects passed on to progeny, remains unknown. Effects across generations may owe to epigenetic modifications, as has been seen in other contaminant-animal exposures(Lee et al., 2018; Novo et al., 2018), and could play a role in the transgenerational transmission of effects after OSPW exposure.

The epigenome's role in regulating the genes that drive cellular responses to toxicological exposure is undisputed (Brander et al., 2017). The three main forms of epigenetic modification are shared across most taxa: DNA methylation, non-coding RNAs and histone modifications (Brander et al., 2017; Vandegehuchte and Janssen, 2011). DNA methylation is the most well studied form of epigenetic modification; it can be transgenerational and non-transgenerational and can facilitate the occurrence of chronic effects after acute exposures (Brander et al., 2017). Most commonly, DNA methylation involves the addition of a methyl group by a methyltransferase to a cytosine upstream of a gene promotor, which blocks the access to the promotor, thereby decreasing the expression of the downstream gene. DNA methylation can be transient and dynamic, especially in response to a complex chemical mixture. Modifications in

the patterning of DNA methylation post-exposure could result in both molecular, whole organism and population-level changes(Munzbergova et al., 2019). Changes in DNA methylation observed in transgenerational and multigenerational studies in the field of ecotoxicology have been limited, and to-date, there have been no such studies performed with OSPW.

Similarly, there are very few investigations into changes in complex behaviors in OSPW studies. The development of behaviors coincides with the development of the nervous system, and behavioral abnormalities that occur as the result of a developmental exposure are referred to as "behavioral teratology" (Weis, 2009). Studies on methylmercury have found that developmental exposures can have lasting effects on fish behaviors such as prey capture ability, basal swimming activity and predator avoidance (Samson et al., 2001; Weis et al., 2003; Weis and Weis, 1995a, b; Zhou and Weis, 1999). The mechanisms behind these lasting behavioral changes are not well understood, furthermore, how they relate to environmentally-relevant exposures to complex contaminant mixtures like OSPW, is poorly studied.

The principle aim of this study was to determine if exposure to OSPW during early development could result in lasting impairment in fish prey capture, predator avoidance, as well as the ability to reproduce and produce viable offspring, and to see if any of these impairments were associated with epigenetic modifications. Our secondary aim was to determine if ozonation, a potential remediation strategy, could ameliorate any of the observed effects.

Materials and Methods

OSPW samples and ozonation.

The OSPW samples used in this study were collected in 2015 from the Muskeg River Mine operated by Shell Canada Ltd. located ~60 km north of Fort McMurray, AB, Canada. As of June 2017, this mine is now operated by Canadian Natural Resources. The samples were stored at 4°C in 200 L polyvinyl chloride barrels and the OSPW samples were ozonated as previously described in Lyons *et al.* 2018 (Lyons et al., 2018b). The naphthenic acid (NA) profile of the raw and ozonated OSPW samples were analyzed using ultra performance liquid chromatography time-of-flight mass spectrometry (UPLC-TOF-MS) (Huang et al., 2018). The raw and ozonated OSPW sample contained 16.9 and 0.6 mg/L of total classical NAs, respectively. Detailed

analytical methods and characterization results of the OSPW samples can be found in Lyons *et al.* 2018.

F0 Animals and exposures

Zebrafish embryos were collected from adult AB strain. All adults and embryos were housed at 28 ± 0.5°C on a 14h:10h light/dark cycle. Adult breeding stock were fed a mixture of TetraMin® flakes (Tetra, Blacksburg, VA), Cobalt™ Aquatics spirulina flakes (Cobalt, Rock Hill, SC), and Omega One™ freeze dried bloodworms (Omegasea, Sitka, SK). Embryos were exposed to raw OSPW, ozonated OSPW or embryo medium (EM; control) within 30 min post-fertilization until 7 days post-fertilization (dpf). Embryos were held in groups of 70 in glass Petri dishes containing 40 mL of exposure water; 95% of the exposure water was changed daily.

F0 breeding success and F1 exposure

Embryos exposed from 0-7 dpf, were placed in fresh water overnight and then 30-50 embryos from three replicate spawning events were grown up in 2.8 L polycarbonate tanks in groups of 20-30 in clean water. At 90 dpf, at which point the fish reached adulthood, they were transferred to 6 L tanks. For breeding trials, fish were sexed and paired, and then left isolated by a barrier overnight. The following morning the barrier was removed, and eggs were collected during spawning to raise in either embryo media or to be exposed to the same exposure media as their parents. Exposure of the F1 generation was done in the same manner as F0. The number of spawning pairs, fertilization success, number of eggs spawned and survival of the progeny from 1-7 dpf (raised in EM control water) were used to score breeding success.

F0 and F1 gene expression

Gene expression was measured in samples of 25-35 pooled embryos using the RNA extraction protocol and qPCR methods described in Lyons *et al.* 2018. A full list of target genes and primer sequences can be found in Table 6.1.

F0 and F1 basal swimming activity

Basal swimming activity was measured in first- and second- generation exposed embryos at 7 dpf using a previously published method(Philibert et al., 2016). In brief, embryos were placed in 1 mL of their respective exposure solution, randomly distributed across the 12 central wells of a 24 well plate (Costar, Corning, NY) left undisturbed for 20 min. After acclimation, the embryos were video recorded overhead for 10 min using a CCTV camera (WV-

CL930; Panasonic, TX). Maximum swimming speed, total distance travelled, and time spent in the border (3 mm periphery around the 12 mm diameter wells) were determined using the computer software EthoVision XT 10 (Noldus, NE). The time spent in the border can serve as a measurement of anxiety-like behavior in adult and larval fish, as an anxious fish will search the periphery to find shelter(Kalueff et al., 2013).

F0 and F1 DNA methylation assay

DNA was extracted from 7dpf exposed F0 embryos, and 7dpf unexposed and exposed F1 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 pooled embryos. An Epigentek Methylflash^{TM} Methylated DNA Quantification Kit (Colorimetric; Farmingdale, NY) was used according the manufacturer's protocol to measure global DNA methylation in the F0 and exposed and unexposed F1 embryos.

F0 prey capture

Fish were fasted 48 h prior to the trial and were placed in 60 mm plastic petri dishes and left to acclimate for 20 min. Video was then recorded for 3 min to capture the basal activity of the fish, 10 artemia nauplii (brine shrimp feed) were all added to the center of the petri dish, and then the prey capture activity was recorded for 10 min. At the completion of the trial the remaining artemia were counted to determine the number of artemia captured. Videos were manually scored to determine the latency to first capture, sample identification was blind (n=10-16 per treatment group).

F0 adult alarm cue response

The response of adult zebrafish to an alarm-inducing compound, hypoxanthine 3-n-oxide (H3NO) (Gallus et al., 2016; Speedie and Gerlai, 2008), was tested in 1.5-2-year-old developmentally-exposed fish. Alarm cue is a kin-specific mixture found in specialized epidermal cells that is released when skin is damaged(Speedie and Gerlai, 2008), and H3NO has been identified as a common compound in the alarm substance of many fishes (Parra et al., 2009). Individual fish were placed in an 8L tank to acclimate for 20 min, basal swimming activity was recorded for 10 min, then the alarm cue (H3NO) was added to the tank through a microtube (1.85 mm diameter) attached to the edge of the tank. The activity after the addition of

alarm cue was recorded for 10 min. The video was analyzed using the using EthoVision XT 10 (n=10-16 per treatment group).

Statistics

Statistical differences between treatments were evaluated using one-way analysis of variance (ANOVA) followed by either Holm Sidak, Tukey's post-hoc tests, or Dunn's test on ranks. Tukey's post-hoc test was used for all qPCR data; Dunn's test was used on data that failed the normality assumption (Q values were included), and Holm Sidak was used 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 were expressed as the mean +/- standard error of the mean (SEM) and p-values <0.05 were accepted as significant. SigmaPlot 11/14 (Systat, San Jose, CA) was used for all statistical analyses.

Results

F0 breeding Success.

Developmental exposure to raw and ozonated OSPW had no effect on the breeding success of 6 month to 1.5-year-old zebrafish (Figure 6.1). Breeding success was measured by recording the total number of eggs spawned per breeding event (Figure 6.1A), number of eggs spawned per breeding pair (Figure 6.1B), the percent of pairs that spawned per breeding event (Figure 6.1C), and the percent eggs that were fertilized out of all eggs that were produced (Figure 6.1D). Breeding was conducted over the course of a year to account for any potential seasonal variability.

F0 and F1 transgenerational mRNA expression

Exposure had varying effects on the mRNA expression of biotransformation enzymes, endocrine disruption markers and neurodevelopment genes in the F0, F1 exposed, and F1 unexposed fish (Figure 6.2). Raw OSPW exposure increased the expression of the biotransformation markers CYP1a and CYP1b in F0 and F1 exposed fish (Figure 6.2A, B) ($F_{2,10}$ = 57.1, p<0.001; $F_{2,10}$ = 26.0, p<0.05 respectively), exposure to ozonated OSPW had no effect and no effects were seen in the unexposed F1 fish (Figure 6.2C). A marker of endocrine disruption, vitelogennin (VTG), increased in the raw OSPW F1 unexposed treatment group (Figure 6.2F) ($F_{2,12}$ = 5.01, p<0.05). No effects were seen in any of the other exposure groups

(Figure 6.2D, E). The cardiac development gene *nkx2.5* was down regulated in the F0 raw and ozonated OSPW exposure groups (Figure 6.2G) ($F_{2,20} = 9.11$, $p<0.05$). No effects were seen in the exposed F1 population (Figure 6.2H), but there was an increase in expression in the unexposed F1 raw OSPW fish (Figure 6.2I) ($F_{2,12} = 4.11$, $p<0.05$).

F0 and F1 transgenerational basal activity

Raw and ozonated OSPW exposure affected the basal swimming activity of the F0, F1 exposed, and F1 unexposed fish (Figure 6.3). Specifically, in the F0 generation, raw OSPW exposure decreased maximum swim speed ($Q = 2.95$, $p<0.05$), while ozonated OSPW exposure had the opposing effect, which increased the maximum swim speed (Figure 6.3D) ($Q = 3.41$, $p<0.05$). Maximum speed was also increased in the ozonated OSPW F1 exposed fish ($Q = 4.15$, $p<0.05$), but no effect was observed in the raw OSPW group (Figure 6.3E). F1 raw OSPW exposed fish had an increased distance travelled and decreased border dwelling, compared to the control fish (Figure 6.3B, H) ($Q = 2.50$, $Q = 3.85$, $p<0.05$y). In the F1 unexposed fish, exposure led to increased distance travelled for raw and ozonated OSPW fish ($Q = 4.99$, $p<0.05$), and in increase in maximum swim speed for raw OSPW fish (Figure 6.3C, F) ($Q = 4.73$, $p<0.05$).

F0 and F1 DNA methylation

Though there were no statistically significant changes in global DNA methylation, there was a ~3% decrease in DNA methylation in the unexposed second generation of raw OSPW-exposed fish (Figure 6.4).

F0 prey capture

Developmental exposure to raw and ozonated OSPW did not alter distance travelled, the percent prey captured, the latency to capture, or time spent in the border (a measure of anxiety behavior) (Figure 6.5, S6.2). Juveniles developmentally exposed to ozonated OSPW had a higher maximum velocity during prey capture ($Q = 2.49$, $p<0.05$) (Figure 6.5C), mirroring results in the basal swimming activity (Figure 6.3). Exposure to both raw and ozonated OSPW decreased the variation in the latency to capture and border-dwelling behaviors of the fish during the prey capture trial (Figure 6.5A, B). There was a reduced frequency of the shy phenotype in both the raw and ozonated OSPW exposed populations, resulting in decreased behavioral variation within the exposed populations.

F0 alarm cue response

The control fish had two behavioural phenotypes: fish that had high maximum velocity and high distance travelled (quick moving active fish – indicative of active exploration), and fish that had a more moderate distance travelled and a low maximum velocity (steady state swim phenotype) (Figure 6.6A). After exposure to the alarm cue, unexposed fish maintained a high maximum velocity but travelled much less leading to a lower distance travelled (freezing then darting phenotype- indicative of an anxiety response) (Figure 6.6B). In contrast, fish developmentally exposed to raw OSPW were much less active than control fish, and instead of displaying a 'freezing then darting' phenotype after the addition of an alarm cue, the fish displayed an atypical 'steady state swim' phenotype (Figure 6.6C, D). Similar behavioral responses to the alarm cue was also seen in the ozonated OSPW exposed population (Figure 6.6E, F). This result suggests that while the ozonation of OSPW ameliorates some of the toxicological effects of exposure, the inorganic fraction of OSPW may play a role in the toxicity of this complex mixture.

Discussion

Previous studies have shown that free swimming adult fish can smell OSPW and will actively avoid exposure at concentrations as low as 0.1% (Lari and Pyle, 2017; Reichert et al., 2017; Sun et al., 2014). This innate avoidance response could limit the exposure of adult fish to an OSPW plume, however, embryos and larvae are less mobile, and thus at greater risk of exposure and the development of any adverse effects. For this reason, our study focused on the embryological and early developmental impact of OSPW exposure, and the lasting effects exposure could have on behavior, breeding success and offspring survival.

F0 breeding success and VTG expression

Previous work has suggested OSPW and/or naphthenic acids may act as endocrine disrupting compounds (EDCs). Research indicated that early life exposures can impact the abundance of steroid hormone transcripts (Wang et al., 2015a; Wiseman et al., 2013a), steroidogenesis (Wang et al., 2015b), and sex receptor binding affinity (Leclair et al., 2015). There is also work that suggested that ozone treatment mitigated these effects (He et al., 2010). However, the effects on exposure on breeding success has not been studied. We found that developmental exposure to raw and ozonated OSPW had no effect on the breeding success of the adult zebrafish. Many of the studies suggesting OSPW is a potential EDC were conducted on cell

lines or larvae that were not sexual mature. For zebrafish, sex determination occurs around ~25dpf and sexual maturity is reached at ~3 months of age (Siegfried and Nusslein-Volhard, 2008), so a marker of altered sex hormone concentrations, such as VTG expression at a larval stage, may have no meaningful relationship to reproductive success. Our study found that raw OSPW increased VTG expression in somatically exposed (F1 unexposed) embryos. Early life stages of zebrafish have a higher induction threshold than adult male fish, and zebrafish in general, are less sensitive to VTG induction than other test species (Brion et al., 2004), which must be taken into consideration when comparing this study to the literature. As such, a better marker is adult breeding success. Spawning also integrates many cues; it is affected by mate choice, chemical mating cues, visual stimuli and various social interactions, making breeding success a viable metric of population success (Nasiadka and Clark, 2012). The ecological relevance of breeding success makes it a very favorable endpoint to include in toxicological studies, however, the inherent variability can limit the usefulness of breeding success in laboratory based toxicological studies. Alternate molecular endpoints that are more sensitive and repeatable can be advantageous when studying the effects of sublethal exposures.

F0 and F1 transgenerational mRNA expression

Our study used markers of biotransformation, EDC exposure and cardiotoxicity in order to determine if raw and ozonated OSPW exposure had transgenerational effects on gene expression. These biomarkers, particularly biotransformation enzymes developed from laboratory toxicity studies, serve as tools to monitor acute and transgenerational effects of exposure. Previously, there have been mixed findings on the effect of OSPW exposure on biotransformation enzyme expression. Some studies have found that OSPW and/or NA exposure did not induce CYP expression (Alharbi et al., 2016; He et al., 2012a), while other studies have found that there is a slight induction of CYP1A (Wiseman et al., 2013b), yet other studies from our lab have found that both CYP1A and CYP1B were transiently upregulated in raw OSPW exposed fish (Lyons et al., 2018a; Lyons et al., 2018b). Our study suggests that raw OSPW exposure for both the F0 and exposed F1 embryos elevates the transcription of CYP1A and CYP1B. Because CYP expression is a transient response to exposure and can dissipate days after exposure (Lyons et al., 2018a), we did not expect to see any changes in the unexposed population. Ozonated OSPW has a very low classical NA content (<1 mg/L) and did not evoke a

response in any exposure group. We did, however, find changes in the expression of our other biomarkers in the unexposed F1 fish.

Previous research has found that exposure to OSPW can cause cardiovascular defects and inhibit cardiogenesis in fishes (He et al., 2012a; Peters et al., 2007). Transcription factors involved in cardiomyocyte differentiation, including NKX2.5, play a critical role in cardiac function and development (Staudt and Stainier, 2012). These transcription factors could serve as potential biomarkers of cardiotoxicity. Our study found that both raw and ozonated OSPW decreased the expression of NKX2.5 in 7dpf embryos, suggesting that not only the organic fraction could impact cardiac development, but components found in the ozonated OSPW sample (i.e. salts and metals) could impact NKX2.5 expression. Historically, cardiotoxicity is most commonly attributed to organic classes of compounds like PAHs (Incardona et al., 2009; Incardona et al., 2004; Incardona et al., 2011), dioxins (Antkiewicz et al., 2006; Plavicki et al., 2013), and pesticides (Simoneschi et al., 2014; Tryfonos et al., 2009). Previous findings from our lab suggest that changes in the expression of NKX2.5 may not translate into changes in functional cardiac endpoints (Lyons et al., 2018b), suggesting expression changes in this biomarker may not be relevant at the whole organism level. There was increased NKX2.5 expression in unexposed F1 fish, though as previously discussed, changes in NKX2.5 expression of this magnitude are unlikely to have negative effects on cardiac function. It is interesting to note that there appears to be a compensatory effect (having a higher baseline expression of a gene to compensate for OSPW exposure decreasing gene expression) in our exposed vs. unexposed second-generation embryos. Compensatory effects in the context of epigenetic RNA expression modifications has been poorly studied, which makes drawing conclusions in terms of mechanisms and potential effects limited.

F0 and F1 transgenerational basal activity

Early development is marked with increases in neural plasticity, and exposure to early life stressors such as raw and ozonated OSPW, may have long lasting and potentially detrimental effects on individuals and their offspring (Maccari et al., 2014). Our study found that exposure to raw and ozonated OSPW had transgenerational effects on fish behavior. Because of the complexity of behavior and the variable responses to exposure across the exposed and unexposed second-generation embryos, it is difficult to establish a cohesive narrative on the effects

observed. However, as with the changes in NKX2.5 expression, there was evidence of compensatory-like effects of repeat exposure to raw OSPW. Specifically, ozonated OSPW exposure caused an increase in max velocity and hyperactive-like effects in zebrafish embryos, which were not seen in the F1 unexposed fish. Hyperactivity, characterized by increases in swim velocity, are indicators of an innate type of escape response where fish attempt to 'out-run' further exposure to a chemical (Magalhaes et al., 2012). The mechanism behind these subtle behavioral changes is not well understood, however, early life stress and changes in glucocorticoids could play a role in behavioral programming. For example, in a rodent model (*Rattus norvegicus*), prenatal stress is associated with heightened anxiety in the adult offspring (Vallee et al., 1997), increased basal hypothalamic-pituitary-adrenal (HPA) axis activity (Koehl et al., 1999), impaired neural development (Lemaire et al., 2000) and cognitive deficits (Welberg et al., 2001). Glucocorticoids have been implicated as a 'programming factor', responsible for lasting changes in behavior, as pregnant rodents treated with dexamethasone (a synthetic glucocorticoid) produced offspring with the same behavior phenotype as experimentally stressed mothers(Kapoor and Matthews, 2005; Welberg et al., 2001). Studies on maternal stress in vertebrates are rarely presented in an ecotoxicological context and studies linking toxicological exposure to HPA programmed changes in behavior are limited.

F0 and F1 DNA methylation

Transgenerational changes in gene expression and larval behavior can be moderated by epigenetic changes (Lenkov et al., 2015). Though global DNA methylation kits lack specificity, global changes in DNA methylation can be used as indicators of epigenetic changes across the entire genome (Brander et al., 2017), and could serve as a mechanism for the transgenerational effects observed in our study. In our study we found a ~3% decrease in the unexposed 2nd generation embryos which was not found in the exposed first or second generation, though it was not statistically different from the control. The level at which changes in global DNA methylation are biologically relevant to the organism is currently unknown.

F0 prey capture

The successful capture of prey is a complex behavior that relies on several neural processes, including visual perception, recognition, decision-making and motor control (Muto and Kawakami, 2013). Prey capture is also an endpoint that is ecologically relevant, as it is essential for survival. In our study, we looked at both prey capture success as well as activity

metrics that were occurring during the prey capture trial. There was no difference in the distance travelled and the number of prey captured per treatment, suggesting that swimming ability and the exposed fish's ability to capture prey was not impaired. However, we did see a difference in the maximum velocity of the ozonated OSPW-exposed fish. Basal swimming activity at 7 dpf in OSPW-exposed fish had a higher maximum velocity, and it was surprising to see this effect persisting in the same population of fishes 53 days post-exposure. The mechanism by which ozonated OSPW exposure increased maximum swimming speed in zebrafish is unclear. There were no statistical differences in the mean latency to capture time of our control and exposed fish, however, there was a notable difference in the distribution of behavioral phenotypes within the population. In the control population there were two distinct groups, fish that were quick to make a capture after the introduction of prey, and there were fish that took over 2 min after prey introduction to capture their first prey. The fish that were hesitant after the addition of prey displayed traits of a 'shy' behavioral phenotype, whereas the fish that were quick to capture displayed a 'bold' behavioral phenotype. In the raw OSPW exposed fish there were no shy individuals, and in our ozonated OSPW-exposed fish, there were very few shy individuals. The same trend was observed in the time fish spent in the border versus center of the well, and so border dwelling may be a good indicator of anxiety and a shy phenotypic trait (Kalueff et al., 2013). This reduction in the variability of behavioral phenotypes within an exposed population, i.e. the 'behavioral bottleneck' effect (Philibert et al., 2016), has been under studied in the field of ecotoxicology because so few studies include complex behavioral endpoints. Bold and shy behaviors represent the willingness for an individual to take risks, and interindividual variation in this temperament could play a role in the ability of a population to adapt to different stressors and ecological challenges (Sneddon, 2003).

F0 alarm cue response

The legacy effects of developmental exposure are rarely studied, especially the lasting effect(s) exposure may have on fish behavior. In our study, we included not only prey capture but also a predator avoidance response to an established alarm cue (Gallus et al., 2016; Parra et al., 2009). We compared the basal swimming activity of fish before and after exposure to alarm cue to determine if developmental exposure to raw and ozonated OSPW could alter the predator avoidance behavior of adult zebrafish. In the control fish, fish were active and moving rapidly before the addition of the odorant (high max velocity and high distance travelled). After the

addition of alarm cue, fish maintained a high maximum velocity but covered less distance. This suggests their movement went from a continuous swim phenotype to darting and freezing type swim behavior, which is a well-characterized fear response in zebrafish(Kalueff et al., 2013). The bold fish in the raw and ozonated OSPW exposed population had a low maximum swimming speed and low distance travelled, suggesting they were not as active as control fish. In response to the alarm cue, the distance travelled increased but the swim speed of the fish was lower, suggesting a steady state swim type, a very atypical alarm response. Further study is needed to understand the ecological implications of behavioral changes in alarm responses in contaminant-exposed fish populations.

Many ecotoxicological studies focus on the acute effects of exposure using molecular and physiological endpoints, in part owing to the reproducibility of the results and the ease by which a toxic threshold can be determined. However, often these endpoints lack environmental relevance, which limits their predictive power in realistic in situ scenarios. Behavioral endpoints and trans- or multi-generational studies improve ecological relevance for predicting the survival of the fish in environmental exposures. Our study found that exposure to both raw and ozonated OSPW can have lasting effects on how fish behave and react to predators and prey, potentially across their unexposed progeny, and that though treatment may mitigate some of the effects of exposure it was not sufficient. More studies need to be done on the inorganic fraction of OSPW and its potential toxicity to fish. Additionally, studies that include behavioral endpoints often neglect the functional importance of interindividual variability and disregard outliers, even though population changes at the evolutionary scale can be driven by both behavioral and physical traits(Gosling, 2001). Further studies are needed to better understand the effect exposure to complex contaminants can have on fish behavior and their offspring, and the potential of inheritance of these changes.

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Tables

Table 6.1. qPCR primers and accessions number or reference

Abbreviation	Target Gene name	Forward Sequence (5'-3')	Reverse Sequence (5'-3')	Accession Number or Reference
Bactin	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
CYP1b	Cytochrome P4501b	CCA CCC GAA CTC TGA AAC TC	AAA CAC ACC ATC AGC GAC AG	NM_001013267
VTG	Vitellogenin	CTG CGT GAA GTT GTC ATG CT	GAC CAG CAT TGC CCA TAA CT	AF406784.1
NKX2.5	Homeobox protein nkx2.5	GTC CAG GCA ACT CGA ACT ACT C	AAC ATC CCA GCC AAA CCA TA	NM_131421

Figures

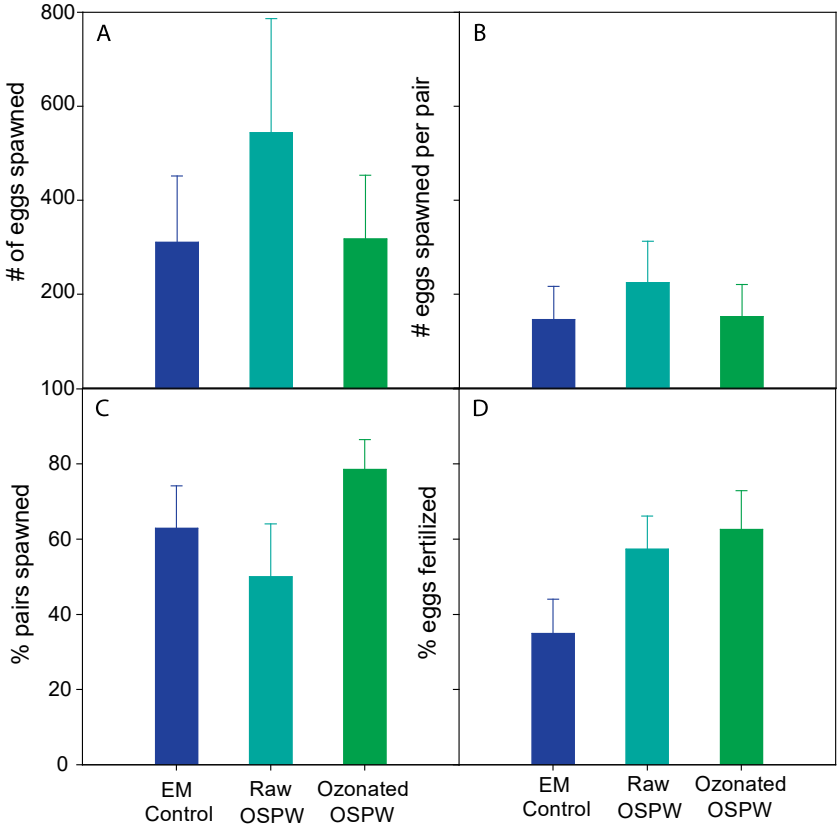


Figure 6.1. Breeding success of F0 fish exposed to raw and ozonated OSPW as determined by total number of eggs spawned (A), number of eggs spawned per pair that bred (B), percent pairs spawned (C), and percent eggs fertilized (D). The number of eggs spawned (both total and per pair) did not differ between treatment groups (one-way ANOVA, n=6-8) (A,B), percent pairs spawned and eggs fertilized also did not differ between treatment groups (one-way ANOVA, n=6-9) (C ,D).

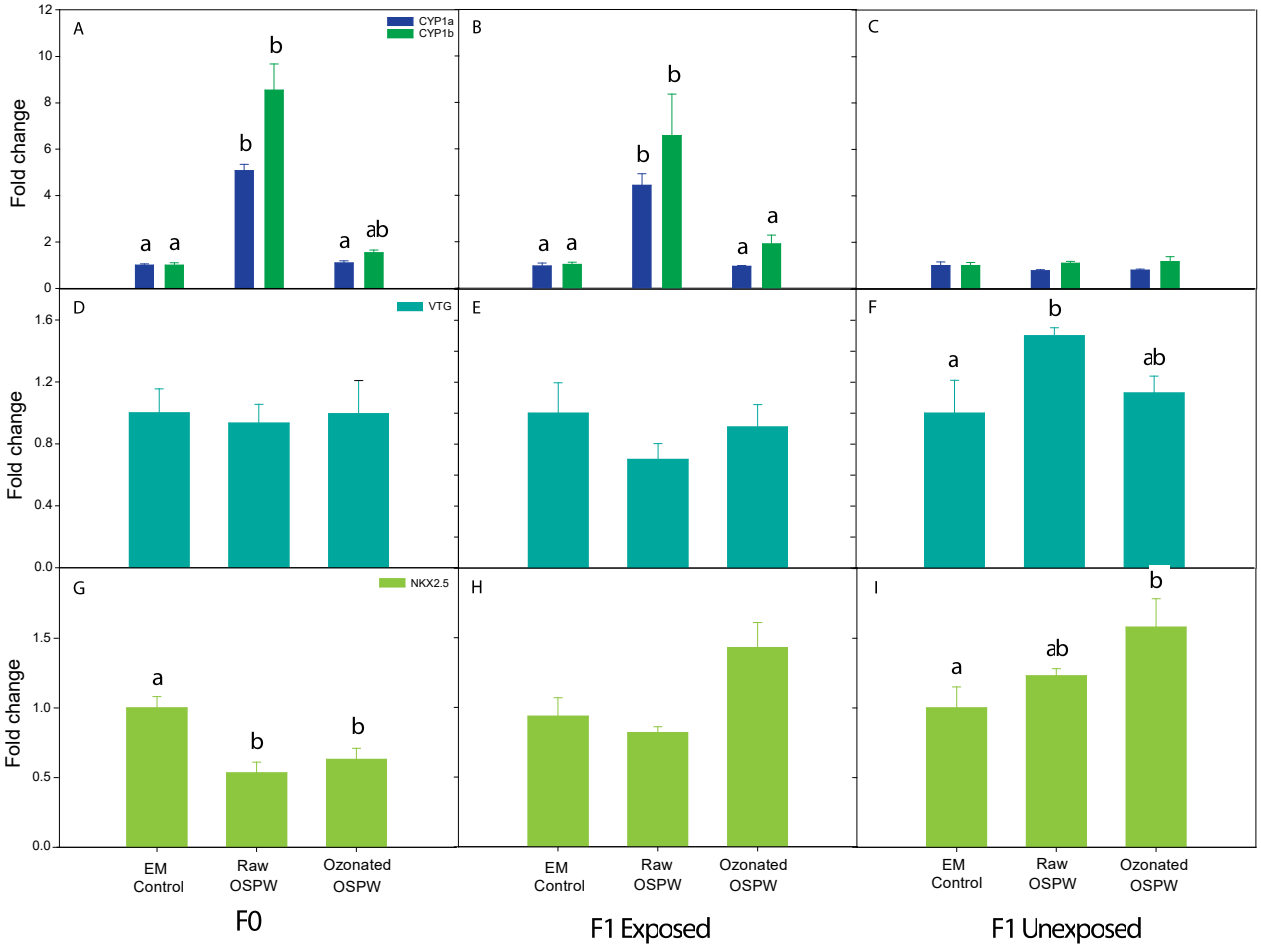
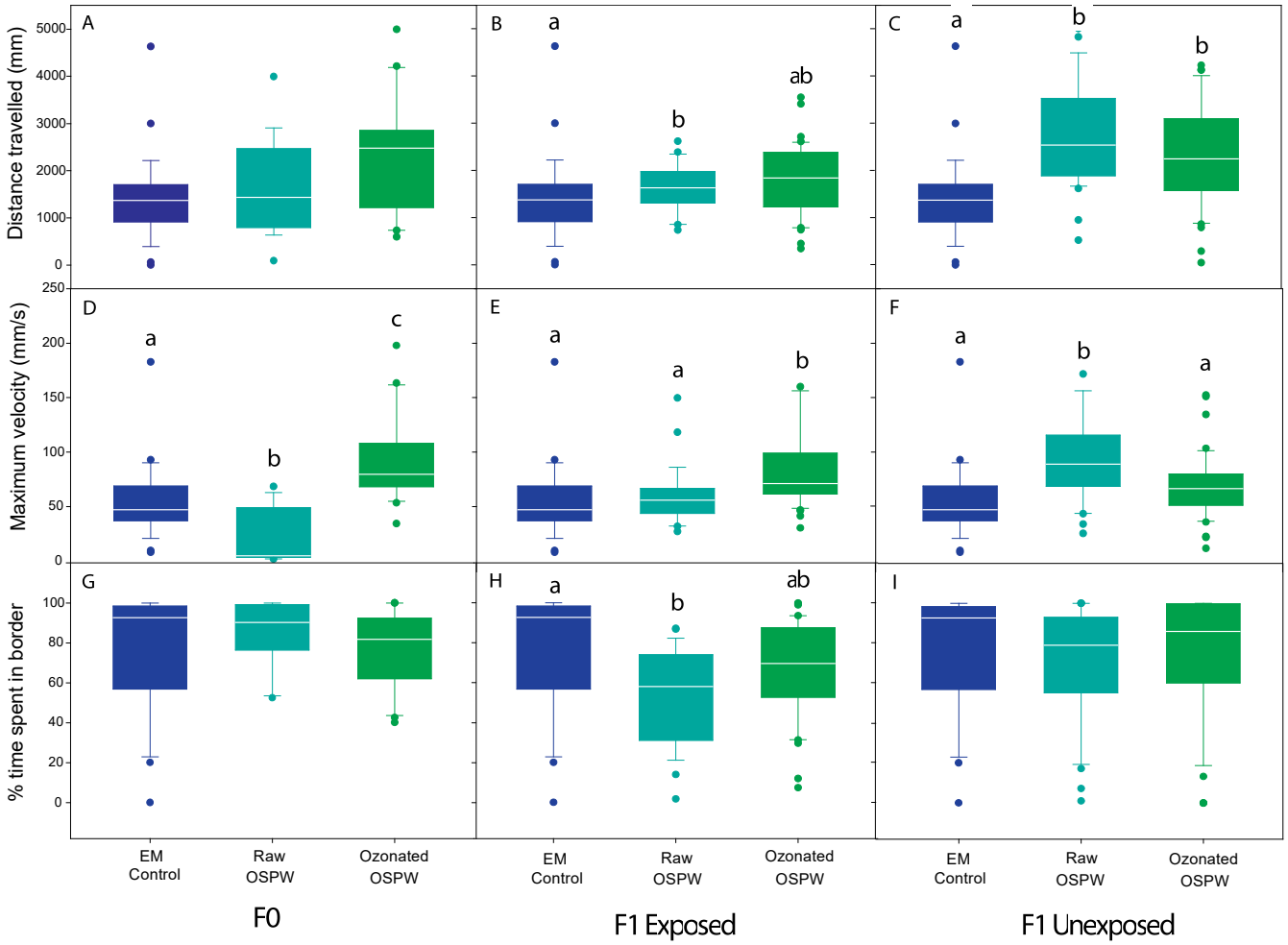


Figure 6.2. Differences in mRNA expression of CYP1a and CYP1b (A-C), VTG (D-F), and NKX2.5 (G-I) in fish exposed from 0-7 dpf (F0), and their exposed (F1 exposed) and unexposed (F1 unexposed) progeny. Second generation unexposed embryos showed no alterations in expression levels of these genes. Raw OSPW 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). 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). Second-generation unexposed embryos from parents developmentally exposed to ozonated OSPW had a significantly increased expression of NKX2.5 (one way ANOVA, Tukey's post hoc, $p<0.05$). Exposed second-generation embryos had no changes in NKX2.5 expression (n=3-5).



2314
2315

2316 Figure 6.3. Effect of exposure on the 7dpf basal activity of the first generation (F0), and second
2317 generation exposed (F1 exposed) and unexposed (F1 unexposed) progeny. Exposure to ozonated
2318 OSPW increased distance travelled in F1 exposed fish and increased max velocity in F0 and F1
2319 exposed fish (one way ANOVA, Dunn's post hoc, $p<0.05$). Exposure to raw OSPW increased
2320 distance travelled in the F1 exposed and unexposed fish, and decreased the maximum velocity in
2321 F0 and increased the maximum velocity in F1 unexposed (one way ANOVA, Dunn's post hoc,
2322 $p<0.05$). Exposure to raw OSPW also decreased border dwelling behavior in F1 exposed fish
2323 (one way ANOVA, Dunn's post hoc, $p<0.05$).

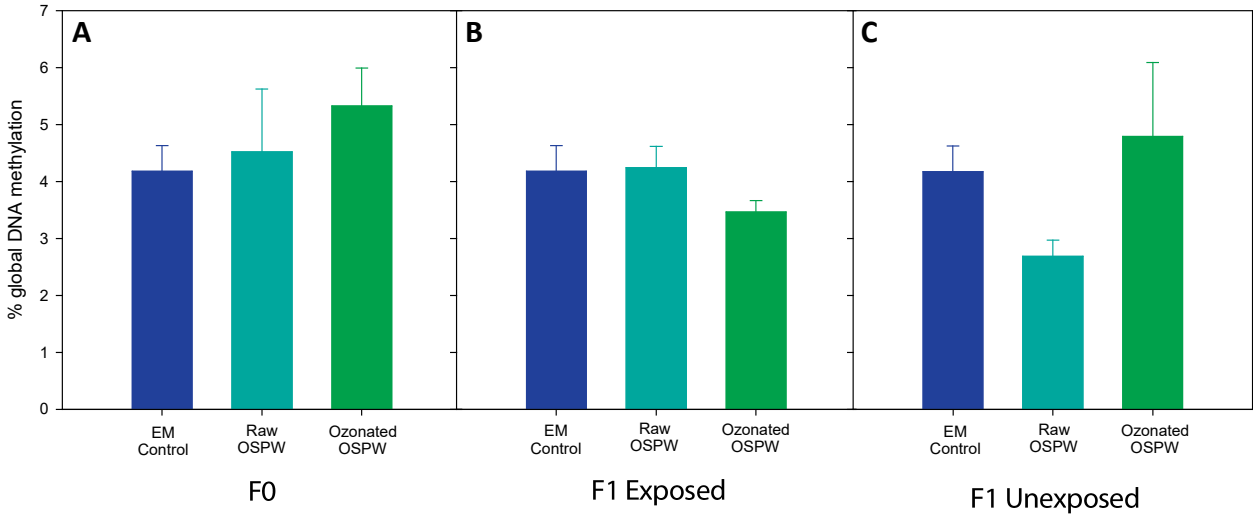


Figure 6.4. Percent global DNA methylation of first-generation and second-generation embryos (unexposed and exposed). Global methylation was not altered by exposure in first-generation embryos or second-generation unexposed and exposed embryos (one-way ANOVA, n=3-4 DNA samples per treatment extracted from 5-10 embryos each).

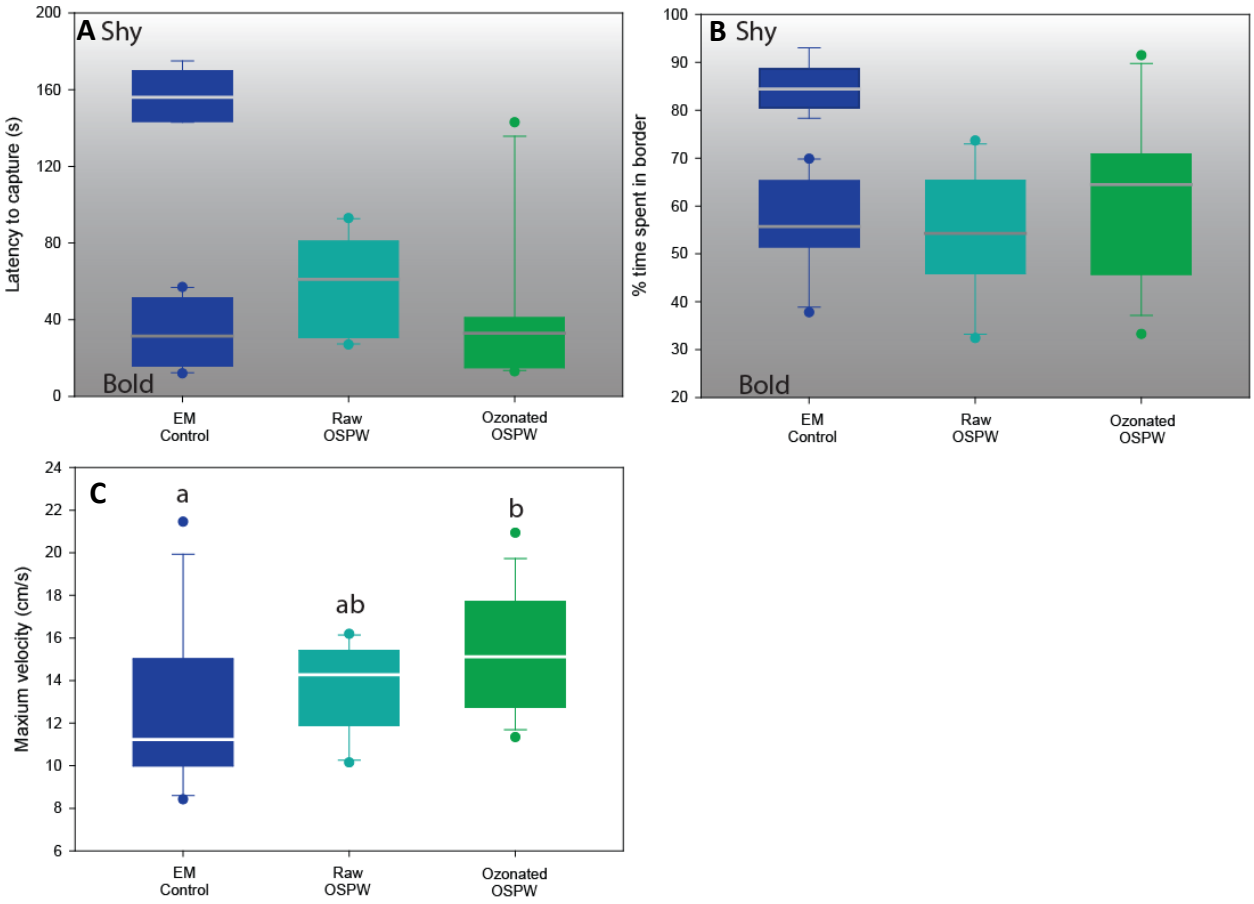


Figure 6.5. The effect of 0-7dpf exposure of raw OSPW and ozonated OSPW on the prey capture of 60dpf F0 zebrafish juveniles. Prey capture behavior was measured using the latency to capture (A), border dwelling behavior (anxiety-like behavior) (B), and maximum velocity (C). Exposure decreased the variability of behavioral phenotypes present in the population, ozonated OSPW exposure increased the maximum velocity of population (one-way ANOVA, n=10-16).

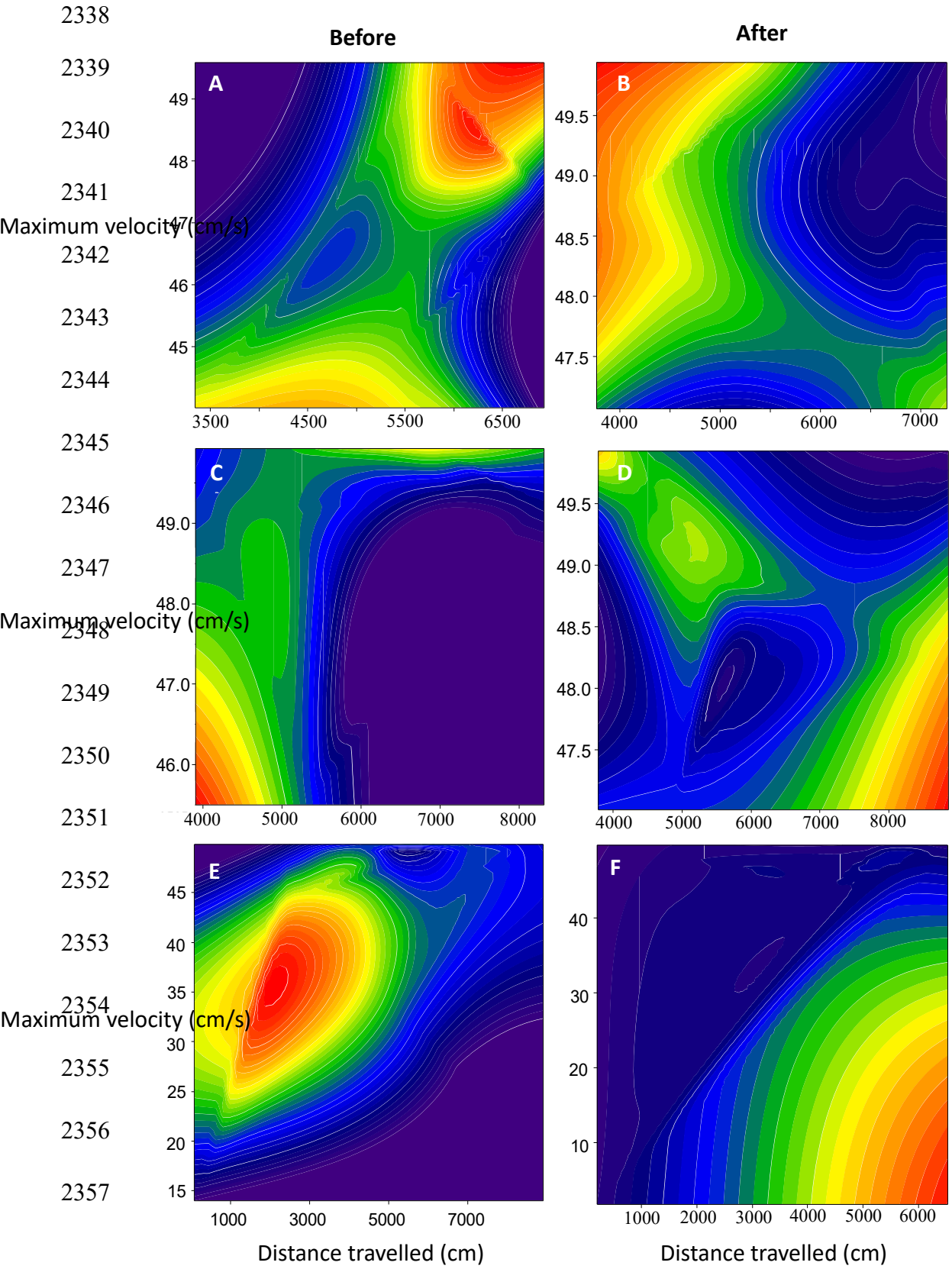

Before
After
A
B
C
D
E
F
Maximum velocity (cm/s)
Maximum velocity (cm/s)
Maximum velocity (cm/s)
Distance travelled (cm)
Distance travelled (cm)

Figure 6.6. Changes in the activity of fish in the F0 control (A, B) raw OSPW exposed (C, D) and ozonated OSPW exposed (E,F) population before and after the introduction of alarm compound hypozanthine-3-n-oxide. Heat maps represent populations of fishes and their swimming activity metrics. Control fish had two activity types present in the population: high distance travelled and high velocity fish (indicative of active exploring) and low velocity moderate distance travelled fish (indicative of a steady state swim). After exposure to alarm cue fish maintained the higher max velocity but travelled less distance (freezing and darting phenotype-indicative of anxiety). Fish exposed to raw and ozonated OSPW were less active than control fish, and in response to the alarm cue displayed a steady state swim phenotype.

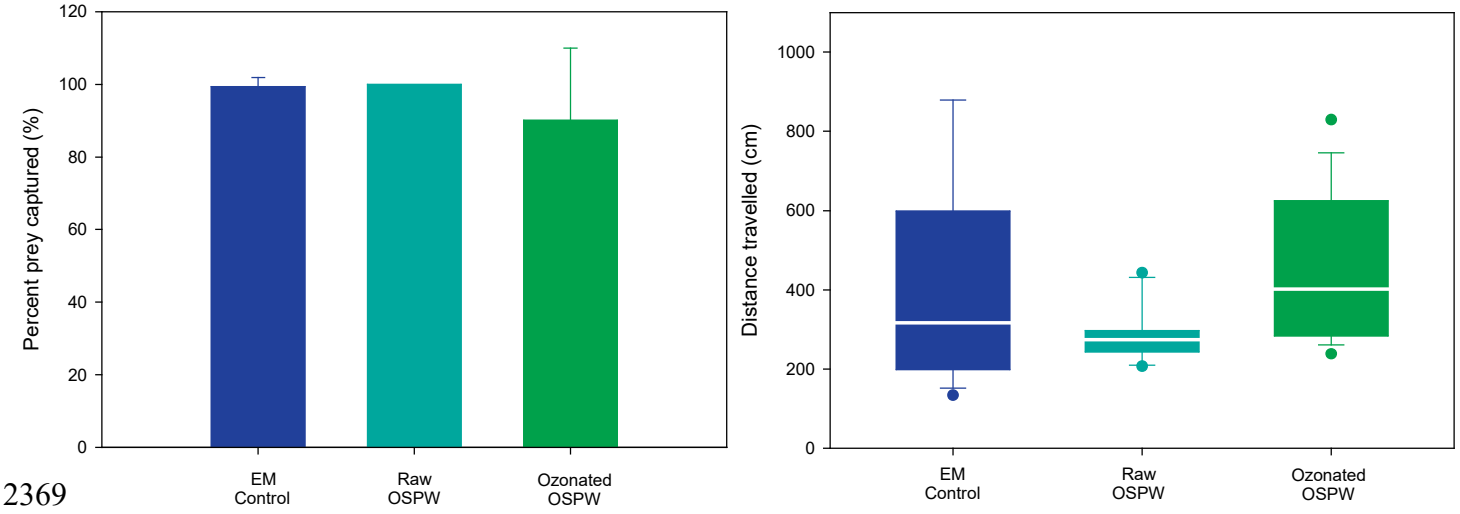


Figure 6.7. Effect of developmental exposure of raw and ozonated OSPW exposure on the percent prey captured and distance travelled during the prey capture trial. No differences were found across treatment groups

Chapter 7: Summary and General Conclusions

Summary

With an ever-growing global demand for petrogenic products, the extraction, transport and refining of crude oil will continue to pose a risk to aquatic life. Though oil economy predictions vary, it is estimated that oil demand will increase from 96 million barrels per day (mb/d) to 111 mb/d by 2040, largely driven by the transportation industry in developing countries (Sioshansi and Webb, 2019). Each stage of production carries different levels of risk and severity in the case of a spill (Eckle et al., 2012), and mitigation of risk and development of treatment technologies within the extraction, refining and transport of oil is key to limiting the industries impact on aquatic life. In my thesis, I examined oil-related contaminants and their toxicity, and the ability of select treatment technologies to mitigate effects of exposure for the early life stages of fishes. I included a very diverse group of contaminants including unweathered, weathered, and dispersed crude oil, the oil extraction by-product oil sands process-affected water (OSPW), and the diluted bitumen blend. Many oil-related contaminants have a unique chemical composition that drives their impacts on aquatic biota.

Diluted bitumens (dilbits) are blends of bitumen and natural gas condensates that are not as well characterized as many light and heavy crudes and have limited toxicity data available for hazard and risk assessment (Barron et al., 2018). Dilbits have higher concentrations of asphaltenes, sulphurous polycyclic aromatic hydrocarbons (PAHs), and are more viscous than conventional crude oils (Dew et al., 2015). Dilbits can also vary seasonally in composition(Dew et al., 2015). Though the toxicology of dilbits are not well studied, conventional crude oils have been, and the toxicity of conventional crude oil has been largely attributed to PAHs (Esbaugh et al., 2016; Incardona et al., 2005; Incardona et al., 2011). In my second chapter, I compared the toxicity of dilbit and conventional crudes to early life stages of a model fish, the zebrafish (*Danio rerio*). At high concentrations (1:10 oil to water ratio) dilbit was found to be less toxic than conventional crude oil, had lower total polycyclic aromatic hydrocarbon content (TPAH), lower concentrations of monoaromatics, and caused relatively lower rates of pericardial edema, a hallmark of oil toxicity. Yolk sac edema was a significant contributor to lethality in dilbit exposed fish, which may be due to the high concentrations of sulfonated PAHs in the WAF.

Monoaromatics contributed the most to the observed lethality in all the acute oil exposures. Exposure to high concentrations of crude oil decreased shelter-seeking behavior and eliminated a 'steady state swim' phenotype that was found in unexposed population. Steady-state swimming is more aerobically demanding, and loss of this phenotype may be an indicator of decreased aerobic performance commonly associated with crude oil exposure (Johansen and Esbaugh, 2017; Kennedy and Farrell, 2006; Nelson et al., 2017).

Chapter three and four also looked at the impacts of early life-stage exposures to crude oil, but instead focused on the impact weathering and dispersants have on crude oil toxicity in both freshwater (zebrafish) and saltwater (sheepshead minnow; *Cyprinodon variegatus variegatus*) fishes. The oil used was collected from the Deepwater Horizon oil spill that occurred in the Gulf of Mexico in April 2010. I found the water accommodated fractions (WAFs) of crude oil were comparable between the salt and freshwater treatments for the weathered and unweathered oil exposures, with the exception of dispersed source oil. Saltwater dispersed unweathered oil had markedly higher PAH content than its freshwater counterpart, and on a toxic unit and per PAH basis, sheepshead minnow were more sensitive to oil exposure than zebrafish. Sheepshead minnow and zebrafish had opposing changes in heart rate after exposure; heart rate decreased in the sheepshead minnow and increased in the zebrafish. Exposure to crude oil also cause deformations in the muscle structure of larval zebrafish. Chapter three highlights the variability in responses of two model fishes and the importance of using saltwater fish to study saltwater spills.

Chapter four focused on the lasting impact that developmental exposure can have on the behavioral responses of juvenile and adult fishes. The exposed sheepshead minnow from chapter three were raised to adulthood. Prey capture, male aggression and novel object responses were recorded in juvenile and adult fish. Though WAF exposure did not impair any of the ecologically relevant behaviors included in my study, there were decreases in the variability in the behavioral responses in the population of source oil exposed fish. When principal component analysis (PCA) was used, it revealed that exposure to unweathered crude oil increased the variability in the novel object responses, increased the variability of activity responses in the population, but decreased the variability in the anxiety responses observed during the trials. Variability at the population level is key for adaptation (Volff, 2005), and an increase in the

prevalence of bold behaviors in fishes and a decrease in the behavioral variability could limit the adaptability of a population of fishes to further stress. Changes in the behavioral variability of prey capture responses observed in sheepshead minnow in chapter four was also seen in oil sands process-affected water (OSPW) exposed zebrafish in chapter six.

Unlike crude oil exposures, OSPW contains very low concentrations of oil and petrogenic PAHs. A by-product of the extraction of oil from the oil sands region in Northern Alberta, OSPW is made up of naphthenic acids, salts, and metals and can vary in composition from mining site to mining site (Allen, 2008). Ozonation, historically used to treat municipal waste water, has shown promise as a treatment technology for OSPW (Wang et al., 2016). Chapter five and six of thesis focus on the developmental, behavioral and transgenic effects of OSPW exposure in zebrafish. In chapter five, changes in gene expression in the raw and ozonated OSPW exposed embryos were not associated with tissue level effects. Decreases in cardiac development genes were not associated with changes in heart rate, cardiac arrythmia or heart size. Biotransformation enzymes were induced, but there were no indications of craniofacial abnormalities or physical impairment. Raw and ozonated OSPW was found to be not overtly toxic to the early life stages of zebrafish.

Chapter six followed the exposed fish from chapter five into adulthood, and I found developmental exposure to OSPW had no lasting effect on the fertility and breeding success of adult zebrafish, but exposure to OSPW altered the gene expression and larval basal activity of both exposed and unexposed progeny. OSPW exposure decreased the interindividual variability of the prey capture responses in juvenile fish, similar to what was seen in sheepshead minnow in chapter four, and altered the responses of adults to an alarm odorant. Ozonation mitigated some of the molecular effects but did not reduce the behavioral effects observed in our study.

My thesis examined the lasting effects early life exposure oil-related contaminants can have on fishes and demonstrates the sensitivity of ecologically relevant behaviors in toxicity testing. Traditional toxicity tests focus on lethality, changes in gene expression, and simple physiological metrics that are not always environmentally relevant and do not always translate to effects at the whole organism level. Though behavioral assays can add complexity due to interindividual variation, they help bridge the gap between laboratory testing and ecological responses to spill/release. Studies that include endpoints months and years after an acute

exposure are rare and there is little understanding of the long-term effect of complex contaminant mixtures. The research in my thesis bridges these gaps and furthers our understanding of petroleum-related contaminants beyond cardiotoxicity and acute lethality tests.

General Conclusions and Discussion

Petroleum-based mixtures are a very complex and diverse group of contaminants. In my thesis there are 2 main groups: 1) weathered, unweathered and dispersed crude oils, and 2) OSPW. Dispersed crude oil was the most lethal exposure whereas weathered oil and OSPW caused no overt lethality during my developmental exposures. PAHs are considered to be the primary driver of crude oil toxicity (Carls et al., 2008), and the WAFs with the highest TPAH content tended to be the most lethal, however, comparing the target lipid model (TLM) of the 3 crude oils in chapter two revealed that monoaromatic content was a better predictor of acute lethality than the TPAH. A few studies have suggested that the volatile organic compounds in crude oil are responsible for the majority of the stress-based effects observed after acute exposure (Kennedy and Farrell, 2008; Thomas et al., 1997), however the majority of the research on crude oil only reports the PAH content of their WAFs. Acute exposures to crude oil WAF preparations represent chemical 'snap shots' of the weathering and dissolution process that occurs during a marine or freshwater spill, and the monoaromatics and naphthalene present are the first compounds to leave the spill site (Faksness et al., 2015). An environmental exposure to a crude oil would therefore be less likely to have the monoaromatic and naphthalene content that is present in laboratory prepared WAFs of unweathered crude oil, and in nature it is most likely persisting PAHs that drive toxicity. At high oil doses (1:10 oil to water ratio) of dilbit, embryos had higher incidences of yolk sac edema than any of the conventional crudes used in chapter two and three. Crude oils that have similar TPAH content may have very different PAH fingerprints(Jung et al., 2013), not all PAHs are equally toxic(Barron et al., 2004). The uniquely high levels of sulfonated PAHs could trigger different forms of lethality than the typical narcosis and cardiotoxicity observed in most crude oil studies(Brette et al., 2014; Brown et al., 2017; Cox et al., 2017; Incardona et al., 2009). The toxicity of sulfonated PAHs has never been studied in isolation.

The TLM was used to compare dilbit to conventional crudes in chapter two, and was used to compare interspecies sensitivity to dispersed unweathered crude oil in chapter three. TLM and

toxic unit calculations (TUs) were very effective at comparing different crude oil exposures to one model organism, but the model was less effective at comparing crude oil toxicity between species. For the target lipid model calculations, the critical body burden (aka the species sensitivity) was derived from the literature (Di Toro et al., 2007; McGrath et al., 2005), and the stage of development used to derive these values was never specified.

Cardiotoxicity, though not the focus of my thesis, was included in my larval studies as a point of comparison with the literature. Exposure to crude oil increased heart rate in zebrafish, decreased heart rate in sheepshead minnow and OSPW had no effect in either species. Most studies have found oil exposure to decrease heart rate in larval fish (Edmunds et al., 2015; Incardona et al., 2009; Incardona et al., 2004; Incardona et al., 2013), but oil exposed fish have been found to compensate for decreases in cardiac output with a higher heart rate during a swim test (Nelson et al., 2017), and this compensation could explain the increased heart rate observed in Chapters 2 and 3. Because of the different developmental stages used for each study and the varying methodology used to collect heart rate measurements, cross study comparisons were a challenge. This could be remedied by trying to use the methodology used in other studies, but our lab was not equipped to do so.

Differences in bold, risk-taking, aggression and stress coping mechanisms have been documented in species across the animal kingdom (Coleman and Wilson, 1998; Conrad et al., 2011; Cote and Clobert, 2007; Dall et al., 2004; Johnson and Sih, 2005; Stamps, 2007). There are two models used to describe interindividual behavioral variability, the first model is based around a genetic pre-disposition for a behavioral phenotype (Stamps, 2007). In the 'genetic' model, after a specific developmental stage, behavioral phenotypes are fixed and an individual's responses to any external stressor will conform to that behavioral phenotype. The 'genetic' model implies that there is an intraindividual behavioral stability that facilitates the interindividual variation observed in behavioral tests (Stamps, 2007). An alternate model to describe behavioral variability that is more difficult to understand is the 'behavioral plasticity' model (Stamps, 2007). Behavioral plasticity implies an individual will behave 'optimally' according to the stressors at hand, and if a wide range of behavioral responses yield the same expected ecological fitness, this model could account for the wide-range of interindividual variability and low individual consistency in behavioral responses (Stamps, 2007). Even

individuals from line bred laboratory strains have subtle variations in their physiological, behavioral, and morphological characteristics and these characteristics could determine the 'optimal' behavioral response in any given situation. Because behavioral traits can be heritable (Conrad et al., 2011; Patrick et al., 2013), and behavioral responses from the same individual can vary over time (Frost et al., 2013; Frost et al., 2007), likely both genetics and behavioral plasticity contribute to an individuals response to a stimuli. Social hierarchies could also play a role in increasing the variability of responses to chemical exposure (Ivanova et al., 2017). There is a direct relationship between social dominance and physiological responses in fish, and subordinate fish tend to be impacted more severely by contaminant exposure (Ivanova et al., 2017). In my thesis exposures to both high and low doses of oil-related contaminants did not impair but instead altered the variability of behavioral responses in larval, juvenile and adult fishes. Exposed zebrafish had decreased variability in larval swim responses, juvenile prey capture response times, and adult alarm cue responses, and the changes in larval behavior were heritable to their exposed and unexposed progeny. Unweathered crude oil exposed sheepshead minnow had altered variability in their exploratory and anxiety-like behaviors that persisted years after exposure. Exposure to a contaminant during neural development has the potential to permanently and transiently alter genetic potential, physiology, and neural networking, which all plays in to determining an individual's response in a behavioral assay, and the heritability of this response, as observed throughout my thesis (Frost et al., 2007).

Elevated cortisol during developmental could be mediating behavioral changes in my exposed fish. In humans, early life stress in the form of childhood abuse and neglect has been shown to cause social, emotional, and cognitive impairment that persist into adulthood (Targum and Nemeroff, 2019). Studies on mice have shown prenatal stress is associated with decreased serotonin metabolism and decreased oxytocin receptor expression in pups, which results in decreased social behavior in adults (Gur et al., 2019). Some of the behavioral changes observed in my studies parallel prenatal stress studies in mice. Mice exposed to elevated cortisol levels during the last week of gestation were found to have increased exploratory behaviors and decreased anxiety like behaviors (Pallares et al., 2007), which mirrors the results found in my sheepshead minnow PCA analysis in Chapter 4. Studies on the effect of embryonic stress on fish have found exposure to a conspecific alarm cue decreased the prevalence of anxiety behaviors in novel environment (Poisson et al., 2017), similar to effects I observed in fish exposed to

contaminants during the same developmental window. Though it is well established that exposure to toxicants can induce stress responses in fishes (Canli et al., 2018; Sajjad et al., 2018; Sandoval-Gio et al., 2019; Sarasamma et al., 2018; Ullah et al., 2019), there has yet to be a study directly linking the stress of exposure to alterations in behavioral responses.

Future Directions

Though lethality and biomarker-based tests remain useful for risk assessment and safe water concentration guidelines, transgenerational and multigenerational effects, which are largely understudied, are the next frontier in our understanding of toxicity. As an endpoint, decreased behavioral variability is more sensitive than lethality, as no lethality was observed in most studies that found behavioral changes. There remains controversy as to whether connections can be made between a pollutant release and population decline in fishes, and multigenerational studies are needed to understand population level changes after a spill. In my thesis I examined some of transgenerational effects of OSPW exposure, and though that fills a valuable knowledge gap, the multigenerational effects of exposure to oil-related contaminants and the epigenetic mechanisms that make these changes heritable are poorly understood. The changes observed in both behavior and gene expression in F1 unexposed fish suggests epigenetics could be modulating basal gene expression and behavior, but because these fish were somatically exposed, this connection is not clear.

Epigenetic modulation is a dynamic and complex, and the global DNA methylation assays used in my study are limited in their ability to describe what is occurring at the epigenetic level. Bisulfite sequencing, a tool that determines if a specific gene is methylated, would be a superior tool to use to investigate the heritability of epigenetics and potential mechanisms behind the transgenerational changes in fish behavior. Methylation of both neural and endocrine targets are likely to play a role in the modulation of behavioral responses to stressors in both an exposed individual and their progeny, however, the cortisol response system seems a very favorable target.

The hypothalamus-pituitary-interrenal (HPI) axis in fish controls the excretion of glucocorticoids from interrenal cells, and glucocorticoids, like cortisol are responsible for modulating the stress response in a tissue specific manner (Bonga, 1997). In teleosts, the magnitude of a stress response is driven by both the severity of the stressor, as well as the genetic

predisposition to be more or less sensitive to stress (Tucker et al., 2019; Vallejo et al., 2009). Glucocorticoids, including cortisol, also play an important role in brain development and behavioral programming early in life (Moisiadis and Matthews, 2014). Higher levels of maternally transferred cortisol during early development can increase the frequency of bold behavioral phenotypes in larval zebrafish (Best et al., 2017), and zebrafish larvae at 3 dpf exposed to waterborne cortisol displayed atypically high activity in both light and dark cycle exposure (Best and Vijayan, 2018). The stress of contaminant exposure may have a similar effect, and changes in early life cortisol during exposure needs to be investigated as a mechanism behind changes in behavioral variability.

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