Effects of model freshwater diluted bitumen spills on wild small-bodied fish at the IISD-Experimental Lakes Area, northwestern Ontario

by

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Abstract

Diluted bitumen (dilbit) is one of the primary exports from the Alberta Oil Sands Region and is transported across much of North America by pipelines and rail. The effects of this petroleum product on freshwater environments are still poorly understood. This thesis addresses the potential effects of a freshwater dilbit spill on small-bodied fish through a combination of lab and limnocorral experiments. The sensitivity of wild fathead minnows (FHM; *Pimephales promelas*) to residual concentrations of the water accommodated fraction (WAF) of dilbit in order to determine the effects of a spill after applying remedial. FHM were exposed to a treatment of 1:100,000 and 1:1,000,000 WAF to water or 100% pure reference lake water in a laboratory for 21 days followed by a 14-day depuration. A combination of confinement and handling stress resulted in over 50% mortality across all treatments and significantly lower body condition compared to an unconfined reference sample from the source lake. In order to create a less stressful environment for the toxicity testing of wild fish, the following year a large-scale limnocorral study was conducted at IISD-Experimental Lakes Area in northwestern Ontario. Seven 10 m diameter limnocorrals were treated with a regression of dilbit spill volumes resulting in mean daily total petroleum hydrocarbon (TPH) of 169-1646 μg/L. Two limnocorrals served as reference systems. Adult finescale dace (FSD; *Phoxinus neogaeus*) were released in the limnocorrals 21 days after dilbit addition and were exposed for two months. No FSD were recaptured from the three highest dilbit treatments. Despite biliary metabolites indicating increased interaction with the dilbit with increased exposure, there were no significant trends in condition factor, somatic indices, liver cell size or gonadal development with increased mean TPH. The basal epithelial layer on the secondary lamellae of the gills was thinner in fish from limnocorrals compared to the lake indicating the continued potential for confinement stress. The results of both studies emphasize the importance of assessing the effects of multiple stressors in the context of dilbit spills.

Preface

This thesis is an original work written in its entirety by Lauren Timlick. Ethics approval for this project was granted through the University of Manitoba Animal Care and Use Committee under animal use protocol AUP F17-010 and F18-008. This thesis is written using the "sandwich method" and due to the style of this format there may be redundancies in the introductory paragraphs of the two manuscripts as well as the introductory chapter.

A shortened version of Chapter 2 in this thesis is a manuscript under review by the Bulletin of Environmental Contamination and Toxicology (BECT-D-20-00306). I was responsible for writing this manuscript as well as all data collection, processing, and the majority of the analyses. Dr. Heather Dettman and NRCan supplied the WAF and funded the subsequent ALS Laboratories GC-MS analysis. Dr. Valérie Langlois supplied the lab space and consumables for the qPCR analyses. Sarah Wallace was indispensable in the completion of the targeted gene expression analysis as was Dr. Lisa Peters in the completion of the histology. Spectrofluorescence analysis was completed at Dr. Stephen Brown's lab at Queens University by Johanna Mason.

Chapter 3 of this thesis is a manuscript that will be submitted for review to Environmental Toxicology and Chemistry in September 2020. I was responsible for writing this manuscript as well as all data collection related to fish biology including the capture, deployment, collection, and processing of fish (although this happened with the assistance of an army of students, see Acknowledgements), as well as the majority of the histology and bile fluorescence. The analysis of data and creation of all figures was also my responsibility. The BOREAL project was designed and led by Drs. Jules Blais, Mark Hanson and Diane Orihel in collaboration with Drs. José-Luis Rodríguez-Gil, Bruce Hollebone and Vince Palace. Chemistry analysis was completed by Sawyer Stoyanovich at University of Ottawa. Dr. Lisa Peters completed histological measurements on the gills. Jamie Dearnley provided vital assistance in the fluorescence analysis of the bile in lab space supplied by Dr. Gregg Tomy.

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Thank you also to the staff at IISD-ELA who are the backbone of the incredible work that is done out there: *Chandra Rogers, Cyndy Desjardins, John Neall, Ken Sandilands, Lauren Hayhurst, Lee Hrenchuk, Mike Rennie, Michael Paterson, Paul Fafard, Pauline Gerrard, Roger Mollot, Sarah Warrack, Sonya Havens,* and *Scott Higgins.* Massive thank you in particular to John Neall for literally keeping the engines of science running.

Of all the wonderful people at ELA I am most grateful for my Tox Crew family: *Adam Scott, Aidan Guttormson, Alyssa Ball, Cody Jackson, Jamie Dearnley, Katarina Djordjevic, Kelsey Friesen, Madeline Stanley, Patrique Bulloch,* and *Sonya Michaleski*. Thanks for every late night followed by an early morning, for the enthusiastic pursuit of cowboy science, and for being my minnow nannies. Extra huge thank you to Sonya for being my work wife/field partner and taking on assisting me with this project like it was her own. Also, to Madeline for maintaining my sanity with morning coffee dates in the office and then over the internet when the apocalypse happened.

My fellow BOREAL project students have been an absolute joy to work with: *Daniel Denton, Danielle Desrochers, Jeff Cederwall, Johanna Mason, Jon Seguin, Pepe Rodríguez-Gil, Sam Patterson, Sawyer Stoyanovich,* and *Tyler Black.* I'd like to particularly thank Pepe for being my science "dad", tolerating my initial illiteracy in statistics, and always answering my silliest questions in the most meaningful answers. You're the best and I'm sorry I like mixing serif and sans-serif fonts.

Thank you to the principle investigators of the BOREAL project for including me on such a massive endeavour: Bruce Hollebone, Diane Orihel, Jules Blais, and Mark Hanson. I am also grateful to the Langlois Lab for welcoming me to Kingston and Quebec City, dealing with my mediocre French, and helping me understand mRNA. Particular thanks to Sarah Wallace for being

the best possible mentor, Easter dinner date, and for joining me on the confusing, somewhat disappointing adventure that was the last season of Game of Thrones.

Thank you as well to members of the COGRAD and Treberg labs at University of Manitoba for the use of their equipment and assistance. I'm also grateful to my committee for their thoughtful review and support of this thesis. Particular thanks to my advisor Vince for taking a chance on a simple geologist who thought just maybe she could be a toxicologist. So… can I?

I am very appreciative of the financial support that this project from an NSERC grant (STPGP 493786-16) awarded to J. Blais, M. Hanson and D. Orihel and also by the National Contaminants Advisory of Fisheries and Oceans Canada. (MECTS-3713639) to V. Langlois and V. Palace. Additionally, I am grateful for funding support awarded directly to me from the IISD-ELA Graduate Fellowship and the Manitoba Graduate Scholarship.

I also need to thank the people who have supported me outside of the scope of this project. Thank you to my family for loving me, feeding me, and keeping me sane. Thank you to my friends for always being there for a glass of wine and understanding that, for the last three years, hanging out with me could only happen as multitasking. Particular thanks to Emily and Hanna for living with me through this and dealing with the fact that I never know where my keys, phone, or wallet are. Finally, immeasurable gratitude to Pat. You know what you are to me and I love you for it, every day.

Dedication

The world in which I am finishing this MSc is incredibly different from the one in which I began it. In this time of the global COVID-19 pandemic, I would like to dedicate this work to the frontline and essential workers who are the engine that keeps society running. Without you there would be no capacity for the advancement of science during normal times or in times of crisis. Thank you.

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Abbreviations List

ANOVA: Analysis of Variance

AOSR: Alberta Oil Sands Region

BOREAL: Boreal-lake Oil Release Experiment by Addition to Limnocorrals

BTEX: Benzene, Toluene, Ethylbenzene, and Xylene

CAPP: Canadian Association of Petroleum Producers

cDNA: Complementary DNA

CEPA: Canadian Energy Pipeline Association

CLB: Cold Lake Blend

CPUE: Catch Per Unit Effort

cyp1a: Cytochrome P450 A

Dilbit: Diluted Bitumen

DO: Dissolved Oxygen

EC50: 50% Effect Concentration

ECCC: Environment and Climate Change Canada

ECRC: Eastern Canada Response Corporation

ef1α: elongation factor 1α

Ex/Em: Excitation/Emission

FAC: Fluorescent Aromatic Compounds

$FF_{EX/EM}$: Fixed Fluorescence and wavelengths

FHM: Fathead Minnow (*Pimephales promelas*)

FSD: Finescale Dace (*Phoxinus neogaeus*)

GC-MS: Gas Chromatograph Mass Spectrometry

gst: Glutathione-S-Transferase

H & E: Hematoxylin and eosin

HEWAF: High Energy WAF

HPLC-MS: High Performance Liquid Chromatography Mass Spectrometry

HVI: Hepatocyte Volume Index

IISD-ELA: International Institute for Sustainable Development - Experimental Lakes Area

K: Condition Factor

LC50: 50% Lethal Concentration

LMM: Linear Mixed Models

LMW: Low Molecular Weight

HSI: Hepatosomatic Index

mRNA: Mitochondrial RNA

MS-222: Tricaine methanesulfonate

NAs: Naphthenic Acids

NEB: National Energy Board

NRBD: Northern Red Belly Dace (*Phoxinus eos*)

NRCan: Natural Resources Canada

OECD: Organization for Economic Cooperation and Development

OLR: Ordinal Logistic Regression

OSPW: Oil sands processed waters

PACs: Polycyclic Aromatic Compounds

ppm: parts per million

ppb: parts per billion

qPCR: Qualitative Polymerase Chain Reaction

rpl8: 60S ribosomal protein L8

RNA: Ribnucleic Acid

RSC: Royal Society of Canada

SARA: Saturates, Aromatics, Resins, Asphaltenes

TPAC: Total PACs

TPH: Total Petroleum Hydrocarbons

US-EPA: US Environmental Protection Agency

WAF: Water Accommodated Fraction

Chapter 1: Introduction & Background

1.1 General Introduction

One of the main petroleum products transported by pipeline is diluted bitumen (dilbit), an unconventional crude oil and common export from the Canadian oil sands (CAPP, 2017; NEB, 2018). It is created by adding diluent such as natural gas condensate to highly viscous bitumen, the primary product of the Alberta Oil Sands Region (AOSR), in order to facilitate transport by pipelines (Alsaadi et al., 2018). In 2010, the oil sands surpassed conventional oil production in Canada (NRCan, 2016). In parallel with production, the use of pipelines to transport petroleum products across Canada and to the United States has also increased (NEB, 2018; NRCan, 2016). Pipelines transport upwards of 3.9 million barrels per day from the AOSR with relatively few incidents (NEB, 2018; NRCan, 2016); however, since it is the dominant method of oil transportation in Canada that means that there are still accidental releases annually.

In 2015, after requests for information from the National Energy Board (NEB), the Canadian Association of Petroleum Producers (CAPP) and the Canadian Energy Pipeline Association (CEPA) commissioned the Royal Society of Canada (RSC) to conduct an expert panel review on the behavior and environmental impacts of oil products in aquatic environments (Lee et al. 2015). They noted a high-priority research to "*better understand the environmental impact of spilled oil in high-risk and poorly understood areas (inland rivers, lakes, and wetlands)*" (Lee et al. 2015). Increased reliance on rail transport of petroleum products and Canada's investment in new pipeline project developments despite regulatory approval delays suggest that the boreal biome fits the criteria of a high risk and poorly understood area. Existing pipelines currently transport dilbit and other oil products across thousands of kilometers of Canada's boreal ecozone, an area that encompasses ~60% of the Canadian landmass and a significant portion of our

freshwater (Brandt, 2009; CAPP, 2017). The average inland crude oil pipeline spill in North America between 2008 and 2017 released 8.98 m^3 but the largest spill during that timeframe was the Enbridge Line 6B pipeline spill, which released 3,320 m^3 of dilbit into the Kalamazoo River in Michigan (J.L. Rodríguez-Gil, unpublished research). Larger spills, like Kalamazoo, attract significant attention from the public and regulatory agencies but there has still been relatively little research on the effects of freshwater oil spills compared to what has been done for marine environments (Lee et al. 2015). Dilbit is of concern because of the high percentage of toxic and soluble compounds in the diluent, the high compositional variability, and the different behaviours of the bitumen versus diluent portions of the blends (Lee et al. 2015). These characteristics make the behaviour of dilbit in water difficult to predict based on chemical composition (Fingas, 2015a).

In order to address this research need, an ecosystem level study to examine the effects of dilbit in freshwater. began at the International Institute for Sustainable Development's Experimental Lakes Area (IISD-ELA) in 2017, led by researchers from the University of Ottawa, Queens University, University of Manitoba, and Environment and Climate Change Canada. Included in the Boreal lake Oil Release Experiment by Additions to Limnocorrals (BOREAL) project are smaller scale, low level exposure, pilot project in the summer of 2017 and a large (10-m diameter) mesocosm study using *in situ* limnocorrals in the summer of 2018. IISD-ELA offers an excellent venue for this project as it has the ability, afforded by special legislation at the provincial (O. Reg 60/14) and federal (SOR/2014-95) level, to conduct large-scale whole ecosystem manipulations, including the addition of oil. Additionally, having been operational for more than fifty years, IISD-ELA has a comprehensive dataset of baseline environmental conditions in the 58 lakes under their jurisdiction, something that the RSC noted was often absent in studies of unanticipated spills in the environment.

My research within this overarching project addresses the potential toxicity effects of a simulated dilbit spill on the physiology and reproductive potential of small bodied freshwater fish. Fathead minnows (FHM, *Pimephales promelas*) were selected as the test species for the laboratory pilot project due to their historical use in toxicity testing by the United States Environmental Protection Agency (US-EPA), Environment and Climate Change Canada (ECCC) and the Organization for Economic Cooperation and Development (OECD); widespread distribution; well characterized reproductive physiology and behavior; ease of differentiating sexes; complete sequenced genome; available reference data; and common use as a surrogate species for larger bodied fish (Ankley et al. 2010). Finescale dace (FSD, *Phoxinus neogaeus*) were selected as the test species for the limnocorral exposures due to their widespread distribution in the boreal ecozone; very apparent sexual dimorphism; large population in the chosen experimental lake, and early spawning period that had been completed by the time of the full BOREAL field experiment. This final point was important in trying to mitigate the potential for disparate populations between the enclosures due to reproduction. FSD and FHM are both environmentally and economically important as commonly used bait fish and prey for recreational fishing species including lake trout (*Salvelinus namaycush*; Scott & Crossman, 1973).

1.2 Diluted Bitumen

1.2.1 Composition

Dilbit is an unconventional crude oil created by diluting bitumen with 20-30% natural gas condensate, naphtha, or other low molecular weight (LMW) oil products in order to decrease viscosity and facilitate transport through pipelines (Dew et al., 2015; Alsaadi et al., 2018). For transport by rail the bitumen is often diluted with less (~15%) of the same types of diluents because the product can be warmed at the time of loading or offloading, lowering its viscosity. It is

primarily a shipping product from the AOSR and spills are of toxicological concern due in part to the composition and variability of these diluents (Lee et al. 2015). Although the general composition of both bitumen and diluent is known, petroleum companies hold proprietary information on the composition of dilbit blends including the type and amount of diluent. This, coupled with the fact that the amount of diluent must change with respect to the ambient temperature to offset increased viscosity at lower temperatures, limits the available information on the exact composition of a specific dilbit, hindering response planning and execution following a spill (Lee et al., 2015; Madison et al., 2017).

Crude oil is composed of thousands of different compounds, but generally comprises four main groups, represented by the acronym SARA: saturates, aromatics, resins, and asphaltenes. The saturates and aromatics are the lighter, more volatile components and are generally associated with the diluent in dilbit. Saturates (primarily alkanes) contain the maximum number of hydrogen atoms bound to each carbon and are considered to be less toxic because they are the most biodegradable and least water soluble (Dupuis & Ucan-Marin, 2015; Lee et al., 2015).

Aromatics are the most toxic and include monoaromatics, associated with acute toxicity, and polycyclic aromatic compounds (PACs), which contribute most to sublethal, or chronic, toxicity (Dupuis and Ucan-Marin 2015). Monoaromatics are the most water-soluble hydrocarbons and include benzene, toluene, ethylbenzene and the xylene isomers (BTEX). BTEX compounds move through the water phase by diffusion and partition through biological membranes (Lee et al. 2015). PACs are more persistent than monoaromatics as they are formed from two or more fused aromatic rings and often include alkyl side groups when present in oil (Dupuis and Ucan-Marin 2015; Lee et al. 2015). These alkyl PACs are of concern as the addition of carbon chains to parent compounds decreases their solubility in water and increases lipophilicity, which in turn increases

the potential for bioaccumulation. Alkyl PACs with 3-5 rings are the primary component of oil associated with chronic toxicity and vastly outnumber their parent compounds (Martin et al., 2014). Oil sands products, including dilbit, typically contain an even higher proportion of these alkyl PAHs compared to conventional crude oils (Yang et al., 2011). The asphaltenes and resins are heavier components, generally thought to be less toxic because they are less water soluble and have low bioavailability. They are operationally defined on the basis of solubility in hexane and are generally associated with the bitumen portion of dilbit.

Bitumen is thought to have originally been conventional crude oil that was biodegraded and thermally altered *in situ* over geological time resulting in the depletion of the LMW fraction and concentration of higher molecular weight resins and asphaltenes as residue (Fustic et al., 2012). Resins are typically smaller (500-1,000 Da estimated molecular weight) than the asphaltenes (~1,200 Da molecular weight), but both are highly persistent in the environment and resistant to biodegradation (Lee et al. 2015). In addition to the main SARA components, dilbit may also contain smaller fractions of biologically or geologically derived substances including metals, minerals, sulphur and naphthenic acids, and heterocyclic compounds containing sulphur, nitrogen and oxygen (Lee et al. 2015). Metals include iron, copper, vanadium, chromium, and nickel and are often associated with asphaltenes and are thus more abundant in heavier oils including bitumen (>100 ppm; Yang et al., 2017).

1.2.2 Fate and Behaviour

Changes in petroleum properties begin immediately when exposed to the physical, chemical and biological factors of open aquatic environments through the process of weathering. The key physical features relating to weathering of petroleum products in water are viscosity and density of the oil, and temperature, salinity and oxygenation of the water body (Lee et al. 2015). Density

dictates the sinking behaviour of the oil wherein a density greater than 1.00 g/mL in water results in the sinking of a slick (Demirbas et al., 2015). Viscosity is the feature that controls how dilbit spreads across water – higher viscosity correlates with higher bitumen content (Lee et al., 2015). Between the two, these features control the majority of the weathering processes acting on a dilbit spill in water: dispersion, sedimentation, spreading and emulsification (Yang et al., 2017; Fig. 1.1). Dispersion is the suspension of oil droplets in water, sedimentation is the aggregation of particles and suspended particles in the water column followed by subsequent sinking and spreading is the lateral movement of the oil slick at the water surface (Lee et al. 2015). Emulsification, the formation of oil-in-water or water-in-oil mixtures, occurs by physical mixing from wave action at the surface (Lee et al. 2015). Dissolution and evaporation are controlled primarily by the type and amount of diluent in the blend and are the fastest acting processes, resulting in losses of large amounts of the volatile phase of dilbit within a short time of the spill (Lee et al. 2015). Photo-oxidation is the main aspect of photic interaction with the spill itself and is a product of the interactions of PACs, oxygen, and sunlight to produce more water soluble, resistant products (Lee et al. 2015).

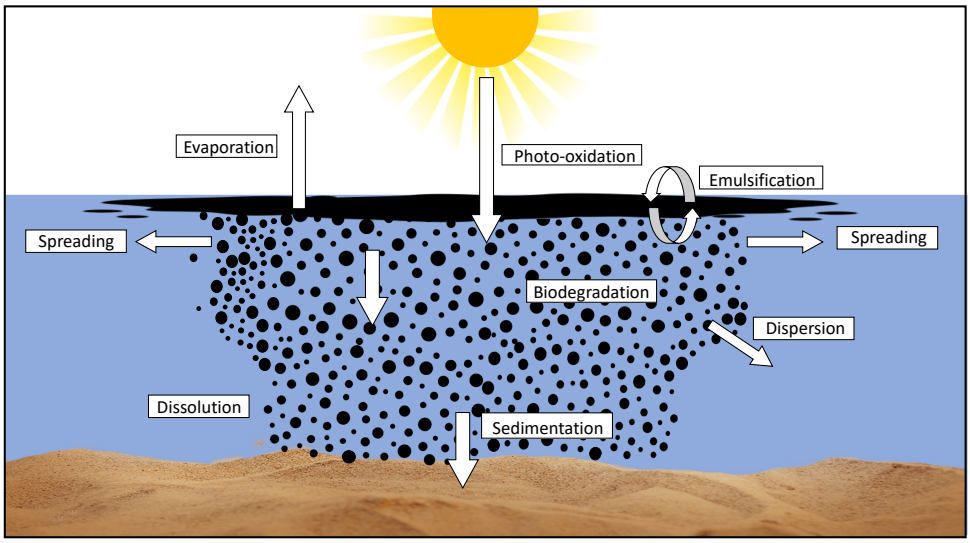

Figure 1.1: Summary of the weathering processes affecting the fate and behaviour of oil spilled in water (after Lee et al., 2015).

Temperature, salinity, and dissolved oxygen (DO) content of a water body can also affect the fate and behaviour of oil, impacting resident microbial communities involved in the biodegradation of oil. Hundreds of species of microorganisms with the capability to degrade hydrocarbons and other oil products have been found in marine systems, freshwater and soil (Lee et al. 2015). These microorganisms concentrate in areas where there is an historical source of hydrocarbons and thereby expediting the biodegradation of accidental spills in these areas (e.g. the Gulf of Mexico; Hazen et al., 2010). The optimal temperature for hydrocarbon degradation by microbes in freshwater is 20 to 30°C but, if the microbial community is accustomed to a perennially cooler environment, significant degradation can still occur at lower temperatures (Brakstad & Bonaunet, 2006; Colwell et al., 1978; Kristensen et al., 2015). Salinity has been shown to have an inverse relationship with the solubility of PACs, which may lead to more bioavailability and higher rates of biodegradation (Whitehouse 1984). Moreover, when there is a change in the natural salinity of a system accompanying an oil spill this may alter the composition of the microbial community and lower biodegradation rates (e.g. Ulrich et al., 2009; Ward & Brock, 1978). Aerobic conditions (high DO) increase rates of biodegradation and are widely considered vital to the long-term degradation of hydrocarbons, particularly when settled into sediment (NRC, 1985; Lee et al., 2015).

The behaviour of a dilbit spill will be different based on the type of water body that it has entered. In a small lake, oil would likely cover a significant portion of the surface over a relatively short period of time with any residual surface oil eventually being deposited on the windward side of the lake (Lee et al., 2015). If oil is spilled during seasonal turnover in the spring or fall, it may be mixed into the water column and carried into the colder, less oxygenated hypolimnion (Lee et al., 2015).

1.3 Toxicity Effects of Oil on Fish

Exposure to petroleum compounds can cause cell damage, necrosis, spinal and cardiovascular deformities, reduced fecundity and mortalities in fish (Fig. 1.2). These effects may result in less recruitment in years following a spill, less young of year, and a declining population trajectory in affected areas. The endpoints selected for use in this thesis specifically to assess these potential effects (Fig 1.3). Oil toxicity in fish is primarily associated with the less than 1% of oil mass associated with PACs in the water accommodated fraction (WAF) rather than direct contact with suspended droplets or the slick itself (Carls et al., 2008). The WAF of oil includes hydrocarbons and small oil droplets that have been incorporated into the water column by stirring and/or mixing (Lee et al., 2015). Acute oil toxicity in fish is primarily associated with the LMW compounds and is often brief and localized due to weathering and dilution of oil in a water body (Lee et al., 2015). This is of particular concern since proprietary diluents in dilbit blends are often an oil-gas condensate comprising a large, usually unknown, amount of these LMW saturates and mono- and di-aromatics (Alsaadi et al. 2018).

Oil spills can cause extensive fish mortalities, particularly when entering a small water body where the ability of fish to avoid the slick is limited (e.g. Pine River, BC; Goldberg, 2011).

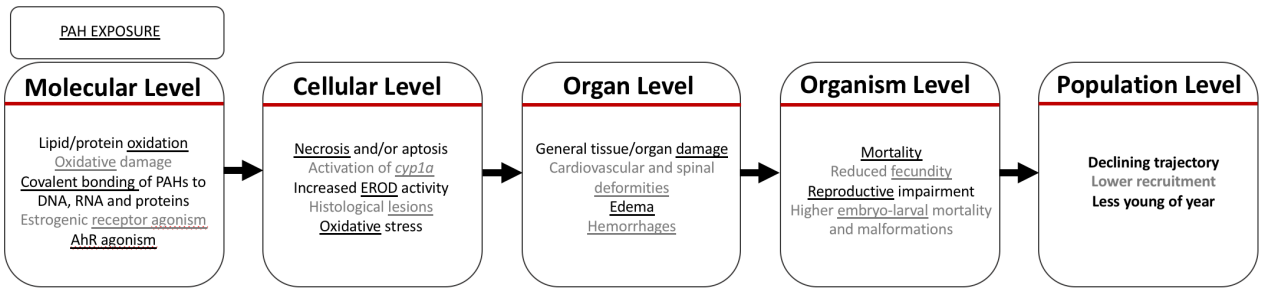

Figure 1.2: Proposed adverse outcome pathway (AOP) of dilbit toxicity for fish. This AOP is specifically focused on reproductive effects and direct sublethal to chronic toxicity associated with PAH exposure (compiled from Colavecchia et al., 2007; Ankley et al., 2009; Dupuis & Ucan-Marin., 2015 and Lee et al., 2015)

The lethality of dilbit and other oil products is difficult to quantify, however, because of the variability of the oil composition and the proprietary nature of the blends (Dupuis and Ucan-Marin 2015). For example, estimates for 96-hr LC_{50} based on total petroleum hydrocarbons (total mass of all hydrocarbons; TPH) range over two orders of magnitude from 0.045 to 40.20 mg/L (NRC, 2005). Narcosis caused by hydrophobic chemicals partitioning into cell membranes and disrupting central nervous system functions is the main mode of action leading to acute toxicity from oil exposure (Barron et al., 2004). Narcotically active chemicals are diverse within oil blends and are often additive in their toxicity but are generally limited to the first 48 hours following a spill (NRC, 2005).

The main components in dilbit and other oil products that are known to cause chronic toxicity are alkyl PACs, as they partition out of water more slowly than monoaromatics and, once

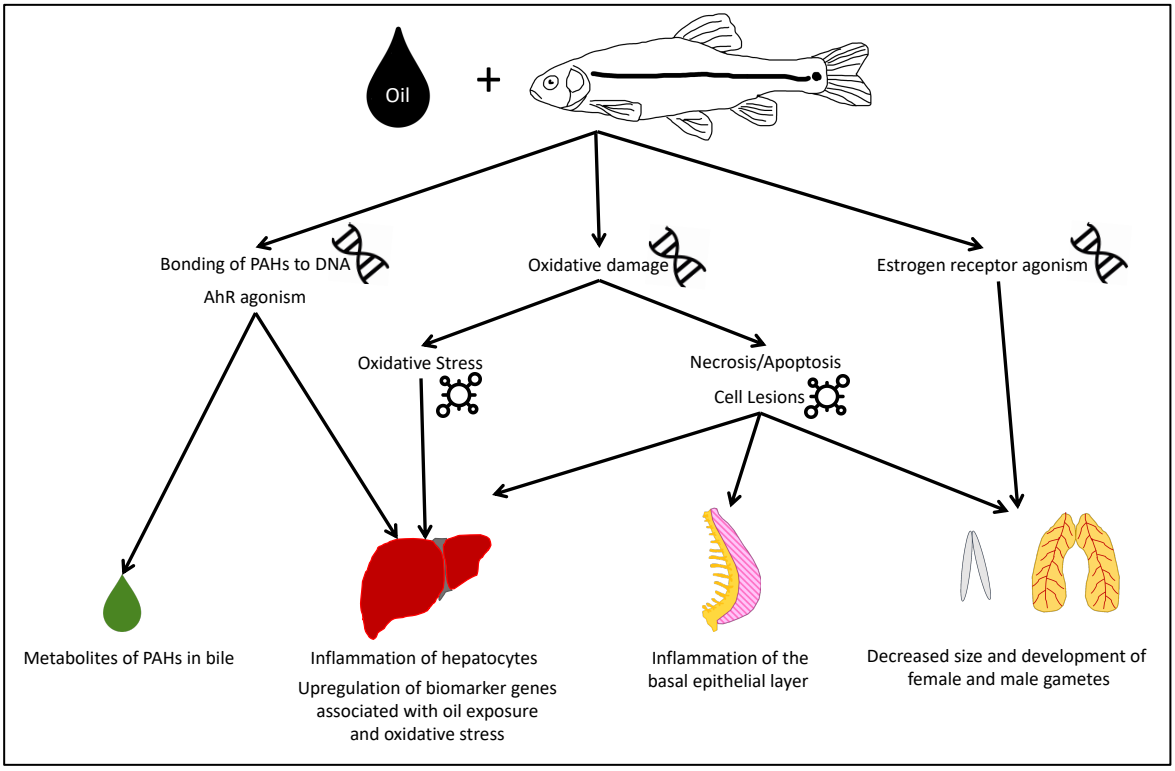

Figure 1.3: Conceptual model outlining the pathways that were used to determine which endpoints should be included in this thesis in order to assess the overall effects of dilbit exposure on the health and reproductive potential of wild minnows (compiled from Colavecchia et al., 2007; Ankley et al., 2009; Dupuis & Ucan-Marin., 2015 and Lee et al., 2015).

stranded in sediments, will continue to partition into the water (Lee et al., 2015). Chronic and population level effects include genetic and molecular responses of the cells, changes in fecundity, growth impairment, and mortality. Changes in fecundity can result from lower concentrations of sex steroids (e.g. plasma estradiol), slower gonad maturation, and delayed time to sexual maturity (Brown-Peterson et al., 2014). These effects are difficult to link directly to oil exposure due to the variability between the effects on different stages of maturation. An important delayed effect of exposure to PACs in dilbit is that once they are metabolized their toxicity may increase (e.g. retene metabolite toxicity on trout embryos *in* Hodson 2008; 1-methylphenanthrene derivatives' toxicity to Japanese medaka (*Oryzias latipes*) embryos *in* (Fallahtafti et al., 2012)).

An important aspect of dilbit toxicity that a field based study can address is the increased toxicity of PACs when exposed to UVB (280-320 nm) and UVA (320-400 nm) radiation (Calfee et al., 1999; Pelletier et al., 1997). This toxicity is thought to be a result of activation of chemical residues bioaccumulated by fish and invertebrates rather than direct photic interaction with the oil products in the water column (Little et al., 2000). The organisms at greatest risk for photo-enhanced toxicity are those living in clear, shallow water and those who have transparent or semitransparent life stages (Barron and Ka'Aihue 2001). Photo-enhanced toxicity *in vivo* is of higher concern because it initiates the production of reactive oxygen species in cells and their rapid destructive reaction with lipids, proteins, and nucleic acids (Barron et al. 2005; Lee et al. 2015). This can initiate or propagate lipid peroxidation, inactivation of enzymes, mutations, apoptosis, tissue necrosis, and subsequent mortality. Induction of the CYP1a protein is a reliable indicator that a fish has been exposed to PACs and is often associated with increased toxicity since it works to metabolize the PACs and can also give rise to oxidative radicals. Metabolites in the bile of the fish's gallbladder can also be used as an indicator of PAC exposure using either scanning or fixed

wavelength fluorometry (Pampanin et al., 2016). The produced metabolites can be even more toxic than the original parent substrate molecule (Madison et al., 2017) and cause sublethal effects on adults and juveniles including impaired swimming (Mager et al. 2018) and suppression of feeding behaviour (Lari et al., 2015).

1.4 Study Area and Species

1.4.1 Experimental Lakes Area

IISD-ELA is a research facility encompassing 58 small lakes in Northwestern Ontario and has been operational since 1968. IISD-ELA has special provisions in provincial and federal legislation allowing for whole lake and other ecosystem scale experimentation and over 50 years of long-term ecological monitoring data to provide important background for these experiments. Previous projects at the facility include work on the role of phosphorus in toxic algal blooms (Findlay and Kasian 1987), acid rain (Schindler et al., 1980) , mercury (Sandilands et al. 2008), artificial estrogen from wastewater effluents (Kidd et al. 2007), and the effects of climate change on freshwater fish (Guzzo and Blanchfield 2017) . Currently, in addition to the BOREAL project, IISD-ELA is supporting experimental research on the effects of cannabis, selenium, metformin, and microplastics on freshwater ecosystems.

Lake 260, where the 2018 BOREAL project was carried out, is a small oligotrophic lake. It is located 52 km east southeast of Kenora at approximately 370 m above mean sea level (Brunskill and Schindler 1971).It is relatively hydrologically isolated, as it is a bedrock lake, but does connect through wetlands to a large downstream lake (Winnange Lake). The surface area of Lake 260 is 34 ha with a maximum depth of 14.4 m and a volume of 17.64×10^5 m^3. The climate in the area is characterized by colder, drier winters and wetter warmer summers (Table 1.2). The local bedrock in the Experimental Lakes Area is primarily Dryberry Lake pink granodiorite, which

has a mineralogy resistant to dissolution and does not contribute much to the overall water chemistry (Brunskill and Schindler 1971). No formal investigations into the quality and composition of soils in the Experimental Lakes Area have been completed as of 2019. The substrate of Lake 260 is loosely categorized into three main types: Sand/organic, sand/cobble, and wetland/peat (Fig. 1.4).

The fish in Lake 260 consist of several well-studied species including FHM, white sucker (*Catostomus commersonii*) and lake trout. In the early 2000s Lake 260 housed the estrogen addition project to observe how ethinylestradiol, the active component of birth control pills, affected fish populations (Kidd et al., 2007). FHM within the lake were all but eradicated however, in the years since, their population and health has rebounded (Blanchfield et al., 2015). Although the 2016-2017 survey of the lake found more FHM than FSD using trap nets, they were difficult to capture using our methods of Gee style minnow traps and pole seines, particularly the males during breeding season. For this reason, and the fact that FHM are fractional spawners and may have continued to spawn throughout the experiment causing variability in the fish population between enclosures, FSD were chosen as the species for the BOREAL experiment.

1.4.2 Fathead Minnow (Pimephales promelas)

FHM are one of the most common fish in the boreal ecozone and are widespread throughout North America (Fig. 1.5; Scott and Crossman 1973). They are a schooling minnow that are important prey of northern pike, yellow perch, lake trout, largemouth bass (Chivers et al., 1996, Stewart & Watkinson, 2004). FHM are opportunistic omnivores, but commonly operate in the food web as benthic filter feeders (Ankley and Villeneuve 2006). They have deep, compressed bodies

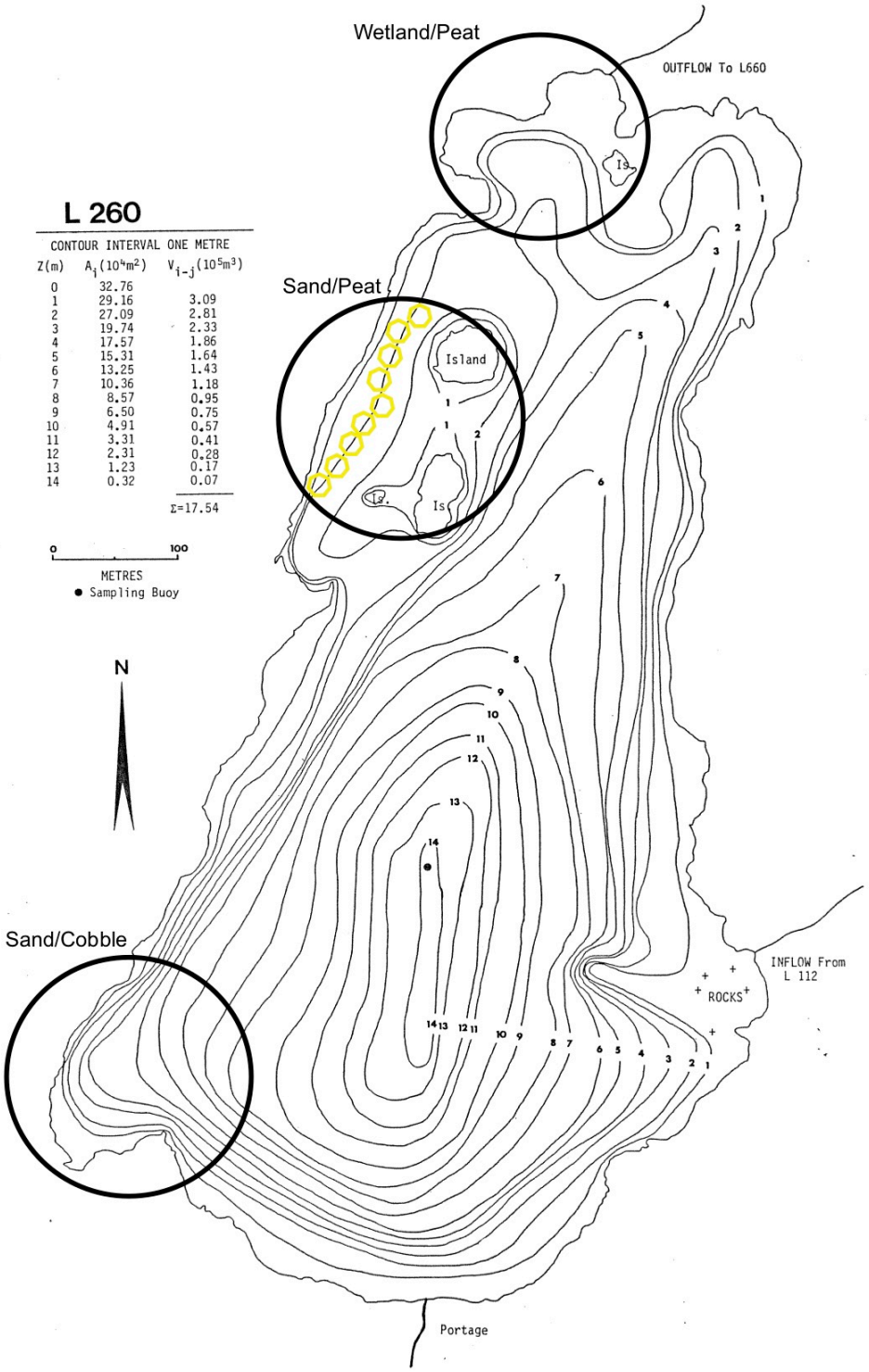

Figure 1.4: Bathymetric map indicating the locations where the three different substrate types in Lake 260 were sampled as well as the location of the 2018 limnocorral study (modified with the permission of IISD-Experimental Lakes Area).

and are typically between 5-8 cm in length (Ankley et al., 2001). Their most distinguishing feature is the short, blunt head from which they get their name, and they are generally a dark olive colour with a dusky dorsal side and yellow-white underbelly (Ankley et al., 2001). They have an incomplete lateral line and a dark lateral stripe. Sexual maturity is reached by the age of 4-5 months under optimal conditions, and they typically live 2-3 years although high post-spawning mortality occurs, and they typically live 2-3 years although high post-spawning mortality occurs, particularly in the males (Ankley et al., 2000; Wisenden 2009).

FHM males are generally larger than females and have distinct sexual dimorphism during their late-May to mid-August spawning period (Wisenden et al., 2009). Males have dark vertical bands on their sides, nuptial tubercles on the nose, and a fleshy dorsal pad extending back from their head (Ankley et al., 2001). Females develop an ovipositor, a fleshy lobe near the urogenital opening, approximately a month before spawning begins (Ankley et al., 2001). FHM spawning is temperature dependent, and is triggered when the water reaches approximately 18°C (Scott and Crossman 1973). Spawning usually takes place at night, when males court females to visit the nest space they've cleaned on the underside of rocks or branches near the sediment (Ankley et al., 2000; Wisenden, 2009). Local observations at IISD-ELA support the notion that FHM spawning occurs within 20 cm of the surface at less than 1 m deep (V. Palace, pers. comm.). Males roll the females over and adhesive eggs are laid onto the nest space, which the male will then protect aggressively until hatch 4-5 days later (Ankley et al., 2000). FHM are polygynandrous fractional spawners and

Figure 1.5: Male (left) and female (right) fathead minnows. Male is displaying the secondary sex characteristics associated with spawning, including nuptial tubercles, and a dorsal fat pad (©L. Hayhurst).

females can participate in over 20 spawning events during the season (Ankley et al., 2000; Wisenden 2009). Toxicity assays often use FHM as a test species due to their short lifespan and continuous, temperature-controlled breeding (Ankley & Villeneuve, 2006). In this project, I used FHM for the lab-based, low level exposure pilot experiment.

1.4.3 Finescale Dace (Phoxinus neogaeus)

FSD are a common minnow in the AOSR as well as in the Great Lakes Basin, Manitoba, northwestern Ontario, Quebec, the Maritimes, and across the northern United States from Maine to Michigan (Scott and Crossman 1973). Like the FHM they are also a common schooling baitfish and prey for larger recreational fishing species like northern pike or lake trout (Stewart & Watkinson, 2004). FSD have a very diverse diet composed of macroinvertebrates, zooplankton, diatoms, and green algae but their larger mouth and pharyngeal teeth lend them to being preferentially predatory (Cochran et al., 1988). FSD range from 6.5-9 cm in length (Stewart and Watkinson 2004), although the IISD-ELA populations are generally smaller than this average (L. Hrenchuk, pers. comm.). They have small, embedded scales that are difficult to see with the naked eye, a dusky dorsal area and lighter white-yellow ventral surface (Stewart and Watkinson 2004). They are distinguished from their close co-occurring relative northern red belly dace (NRBD; *Phoxinus eos*) exteriorly by their longer, horizontal mouth and internally by their shorter gut and silvery, black speckled peritoneum (Cochran et al., 1988; Stewart & Watkinson, 2004). FSD display clear sexual dimorphism outside of spawning season (Fig. 1.6). The males have thick opaque pectoral fins all year, whereas the females have delicate almost transparent pectoral fins clearly differentiated from above (Scott and Crossman 1973). During the spawning season males also have bright red and yellow ventral colouration and nuptial tubercles, while the females develop ovipositors (Scott and Crossman 1973).

FSD are commonly associated with shoreline structures, including reeds and bog mats, for both feeding and spawning (Cochran et al., 1988). Spawning begins in late April to early May and extends until the water temperatures reach approximately 19°C (Stasiak 1978). Ripe females leave the school for the cover of reeds or brush and are followed by several males who then use their oversized pectoral fins to hold a female to them (Stasiak and Cunningham 2006). They curl their tails together and the male then rubs his nuptial tubercles against the females urogenital opening. This occurs for about ten seconds while the milt and 20-30 eggs are released (Stasiak and Cunningham 2006). The eggs are then abandoned and the adult FSD, usually in their 2nd-3rd year, display no parental behaviour (Stasiak and Cunningham 2006). When FSD and NRBD cooccur, they will hybridize, producing fertile female offspring (Stewart and Watkinson 2004). These offspring can reproduce normally with either parental species or asexually in entirely female populations (Stewart and Watkinson 2004). In cases where this hybridization occurs it is impossible to physically distinguish between species or hybrids in the field.

1.5 BOREAL Project Overview

The BOREAL project consisted of two reference mesocosms containing no dilbit and seven experimental mesocosms with a logarithmic regression of dilbit volumes between 1.6 and 160 L added to 7 enclosures (Table 1.3). Dilbit volumes were chosen to be environmentally

Figure 1.6: Male (left) and female (right) finescale dace (FSD) specimens. Note the clear difference in the size and opacity of the pectoral fins, and the yellow and pink ventral markings on the male. These specimens are likely an unknown generation of northern redbelly dace hybrids but display more physical characteristics associated with FSD. (©L. Hayhurst).

relevant and representative of the 50th to 99th percentile of historical inland freshwater spills in Canada and the United States over the last ten years (Fig. 1.7). The 50th percentile is equal to a spill of ~10 m^3 while the 99th percentile of this regression is equal to ~3 000 m^3 of dilbit, or almost equivalent to the 2010 Kalamazoo River Spill stemming from the Enbridge Line 6B pipeline in Michigan. Oil:water ratios were calculated based on a hypothetical spill scenario where the historical spill volume would enter a typical boreal lake (~ 2 million m^3).

The location chosen for the limnocorrals was a relatively flat-bottomed bay that is sheltered by several small islands (Fig. 1.4). Limnocorrals were manufactured by Curry Industries in Winnipeg, MB. The limnocorrals were installed at a depth of 1.5-2 m on the sandy-organic substrate 1-5 meters apart. Each 10 m diameter limnocorral comprised an impermeable curtain with a skirt at the base and floatation collar at the surface (Fig. 1.8). A double layer of sandbags was used to seal the skirt to the sediment and a reinforcement ring of PVC pipe was located midway up the curtain. At the surface of each enclosure there was a 5.85 m^3 area boomed off from the surface slick in order to facilitate sampling. Surface booming was installed around the enclosures as well as at the lake outflow and around known lake trout spawning shoals as secondary and tertiary containment in case of a rupture. An underflow dam was also installed in the Lake 260 outflow stream.

The BOREAL project was divided into four subthemes in order to address current knowledge gaps with respect to freshwater dilbit spills. Each subtheme was managed by a principal investigator and had between one and three graduate students working on the objectives.

<u>Subtheme 1: Behaviour, weathering and compartment mass balance of dilbit in enclosures</u>

This subtheme investigated the changes in dilbit components from the spill onwards, with specific interest in how the compounds partitioned within the system and the overall mass balance of the dilbit itself. The first order degradation rates of the SARA constituents of the

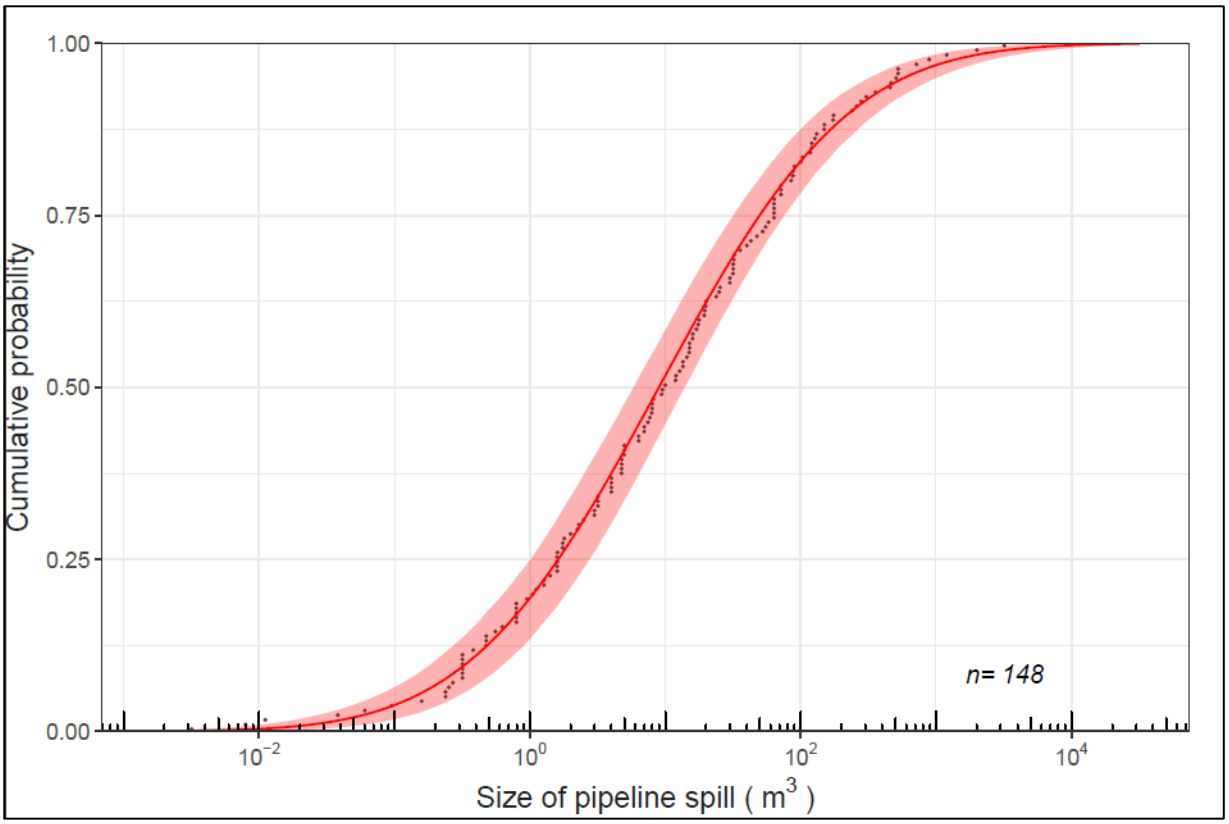

Figure 1.7: Cumulative probability curve for inland pipeline crude oil spill sizes in the United States and Canada between 2008 and 2017 (J.L. Rodríguez-Gil, unpublished data).

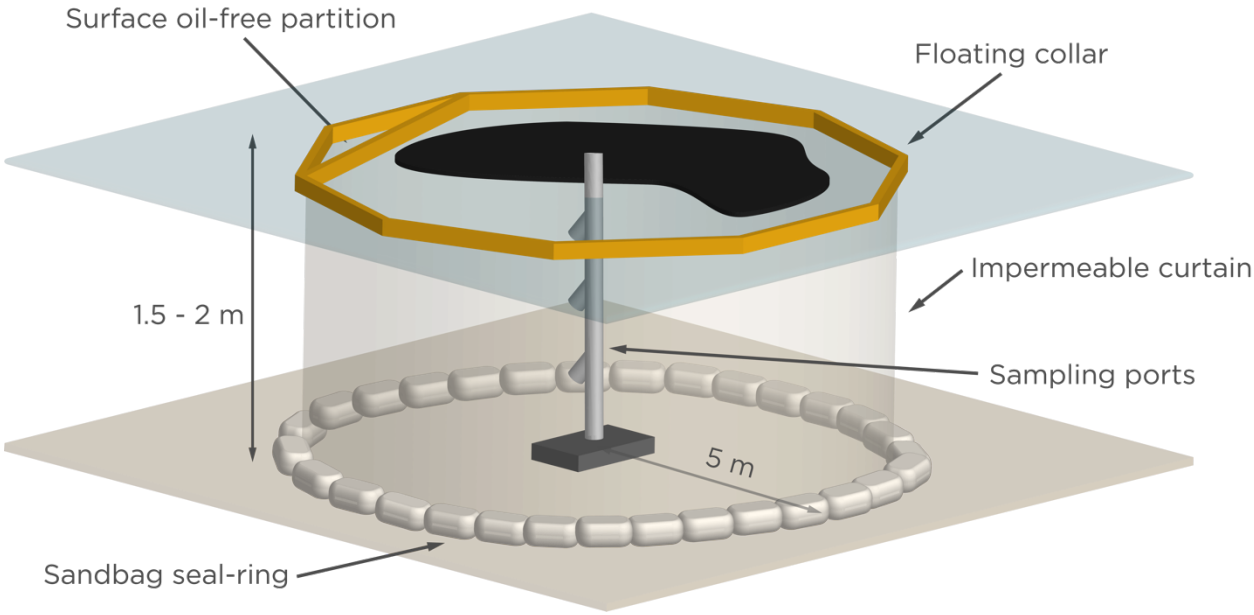

Figure 1.8: Design and set up for the BOREAL limnocorrals (© J.L. Rodríguez-Gil)

dilbit were examined as well as comparing how this occurs in a freshwater versus oceanic environment. A post doc on the project evaluated the mass balance partitioning of oil between air, slick, water column, and sediments. Mass balance was estimated using the physical and chemical results from the full 12 weeks of the BOREAL study.

Subtheme 2: Impacts of dilbit additions to aquatic ecosystem function

One of the high priority research needs brought forward in the Royal Society of Canada report in 2015 was the need to better understand the effects of dilbit on trophic interactions and ecosystem function. This subtheme examined the community changes in zooplankton, emergent insect and benthic invertebrates exposed to dilbit, as well as the responses of planktonic algal populations, productivity, and function. An additional component of this subtheme was the use of stable isotope analysis to assess changes in trophic interactions caused by the dilbit addition. Radiocarbon was also assessed as a possible method of studying oil-driven carbon incorporation into organisms, including compound specific radiocarbon ratios in phospholipid fatty acids from the limnocorral sediments and changes in the bulk carbon reservoirs.

Subtheme 3: Bioaccumulation of dilbit in mussels and frogs

Bioaccumulation, how organisms uptake and concentrate compounds by all possible means, and depuration, how compounds are excreted, was examined in both woodfrog (*Lithobates sylvaticus*) tadpoles and giant floater mussels (*Pyganodon grandis*). This an is important step in understanding the route of exposure in organisms interacting with dilbit spills.

Subtheme 4: Classic toxicological endpoints in fish and frogs

This is the subtheme of the project that this thesis covers. Chronic toxicity of dilbit to small bodied freshwater fish was evaluated using a combination of histological endpoints, health metrics,

bile fluorometry, and targeted gene expression analyses. Additionally, a second MSc student covered similar toxicological endpoints in developing woodfrog tadpoles.

1.6 Thesis Questions and Objectives

Research Question 1: Is there a change in the health and reproductive potential of wild adult FHM exposed to residual amounts of dilbit over three weeks? (Pilot Project)

Objectives

1) Expose wild adult FHM to extremely low concentrations of dilbit low energy WAF, similar to what remains in the environment after a spill has been cleaned up (1:100,000 and 1:1,000,000 parts WAF to water).
2) Assess the fecundity of exposed FHM using gonadosomatic indices (GSI), a reproductive assay, and histological analysis of the ovaries and testes.
3) Evaluate the overall health of the FHM using metrics including condition factor (K), hepatosomatic index (HSI), histology of gill, hepatocyte volume index (HVI), and targeted gene expression of biomarkers including cyp1a and glutathione-s-transferase (GST).

Main Hypothesis: There will be no significant difference in the health or reproductive potential of FHM exposed to residual amounts of dilbit over three weeks.

Research Question 2: Is there a relationship between oil:water exposure and adverse health and reproductive measures in wild adult FSD? (Limnocorral Study)

Objectives

1. Expose wild adult FSD to a regression of environmentally relevant concentrations of dilbit at both near surface and lake bottom conditions (dilutions between 1:100,000 and 1: 1,000 parts dilbit to water).

2. Assess the fecundity of exposed FSD using GSI and histological analysis of the ovaries and testes.
3. Measure toxicological endpoints including metabolic enzymes (cyp1a and GST), exposure indictors (fluorescence of PAC metabolites in bile) and health metrics (CF, HSI, HVI, gill histology) in tissues of FSD exposed to dilbit.

Main Hypothesis: Higher oil:water ratios will not be correlated with adverse health or reproductive measures in exposed wild adult FSD

1.7 Study Significance

The effects of oil spills in freshwater ecosystems remains a high priority research question due to the relatively low amount of existing information and the importance of freshwater to the health of humans and the environment (Lee et al. 2015). It is important to not simply quantify behaviour and fate of oil in freshwater but also examine the direct and indirect toxicity effects on multiple levels within the food web. Deleterious effects in even one trophic level can initiate serious repercussions for the entire biome. As output from the AOSR continues to increase, the boreal biome crossed by pipelines transporting oil across Canada and the United States becomes more at risk for potential spills (CAPP, 2017). The proposal for TransCanada's Energy East pipeline – which would have crossed a large region of the boreal biome between Hardisty, AB and St. John, NB – was withdrawn in October 2017 but the Canadian federal government's recent takeover of the Transmountain Pipeline Expansion is indicative of what a priority exporting AOSR products including dilbit will continue to be in years to come.

Any transport of oil from Alberta will traverse a portion of the boreal ecozone and consequently a great deal of Canadian freshwater (Brandt, 2009). Any pipeline spill entering freshwater is of concern to environmental and local human health. Freshwater fishing in the boreal

ecozone is a popular recreational pastime but is also relied upon by lodge owners for their livelihood and is of particular importance to local First Nations peoples. Many First Nations communities in the Canadian north rely on country foods, including freshwater fish, both as a source of nutrients since market food is much more expensive as well as for the socio-cultural importance of hunting, fishing and the traditional diet (Paci et al., 2004). Larger predatory fish such as lake trout are a common recreational and indo-cultural fishing species and pelagic minnows, including our study species FHM and FSD, are an important part of the diet of these important top predator fishes (Guzzo et al., 2017). Reproductive effects of oil spills on these fish populations are of particular concern due to the potential for population level impacts depending on life-cycle timing of the spill (Lee et al. 2015). This thesis will provide controlled, field-based data on the effects of diluted bitumen on the reproductive health of fathead minnows from a well-studied lake ecosystem. Information from this thesis will be complementary to previous lab-based (e.g. Alderman et al., 2017; Barron et al., 2018; Madison et al., 2017) and "spill of opportunity" (de Santiago-Martín et al., 2016; Kennon & Bouldin, 2015) studies and help to address the high priority research need identified by the Royal Society of Canada as "*better understanding the environmental impact of spilled oil in high-risk and poorly understood areas (inland rivers, lakes, and wetlands)*" (Lee et al. 2015).

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Chapter 2: Potential effects of environmentally relevant residual levels of diluted bitumen on wild fathead minnows (*Pimephales promelas*)

Abstract

Continued reliance on pipelines to transport crude oil across Canada's boreal ecozone creates the potential for petroleum products to enter freshwater ecosystems. The sensitivity of wild fathead minnows (FHM) to residual concentrations of the water accommodated fraction (WAF) of diluted bitumen (dilbit) was assessed by exposing them in a laboratory setting for 21 d followed by a 14 d depuration. Target concentrations were well below detection limits for GC-MS, but were estimated by dilution factor (1:100,000 and 1:1,000,000 WAF:water) to contain less than 0.0003 μg/L of polycyclic aromatic compounds. Even at such low concentrations, FHM of both sexes exhibited statistically lower body condition, females had smaller cortical alveolar oocytes, and males had larger testicular lobe lumen sizes (ANOVA, $p<0.05$). There were no significant differences in *cyp1a* or *gst* expression in the livers of these same fish at the same time after the 14-day depuration period. The results of this experiment delineate the compounded effects of low-level dilbit contamination and additional stressors on a sensitive wild strain of a common model species in ecotoxicology.

2.1 Introduction

Canada has the world's third largest crude oil reserves, the majority of which are held in the Alberta oil sands and the use of pipelines to transport petroleum products across Canada and to the United States continues to increase (NRCan, 2017). Despite advances in both prevention and response to pipeline breaches, oil spills continue to occur across Canada and the United States. Efficiency and speed of pipeline spill clean-up efforts in Canada are regulated by the National Energy Board (NEB) but remain the liability of the responsible company. For this reason, pipeline operators must maintain specified amounts of available resources to respond quickly to incidents. For example, it is federally regulated in Canada that major transmission pipeline operators, meaning a company or aggregate of companies with the capacity to transport 250,000 barrels per day, must maintain a reserve of $1B USD for incident response (2015). The industry standard is to have all necessary response equipment on site and containment and clean up measures commenced within 72 h of discovering the spill (CEPA 2015). Such rapid response times are established to ensure that the majority of the oil will be removed from the water in a relatively short period of time. Inevitably there will be some amount of oil components, in the low ppm (Noskov, 2018) to ppb range (Agostinis, 2017), left in the water even after a cleanup is completed to industry standards.

It is important to understand how these residual concentrations of oil affect aquatic biota after an initial cleanup. Studying these ppb levels of oil toxicity may also prove useful for delineating trajectories of recovery in small bodied resident fish. Wild fathead minnows (FHM; *Pimephales promelas*) are often used in toxicity testing by the US-Environmental Protection Agency (US-EPA), Environment and Climate Change Canada (ECCC), and the Organization for Economic Cooperation and Development (OECD) due to their widespread distribution, well

characterized reproductive physiology and behavior, sexual dimorphism, complete sequenced genome, and common use as a surrogate species for larger bodied fish (Ankley et al. 2010)

This study focuses on the potential toxicity of residual dilbit constituents left after the cleanup of a spill, thus the focus is on very low exposure concentrations. Reproductive and overall health was examined in FHM exposed to two low concentrations of dilbit water accommodated fraction (WAF) using basic meristic measures, histological differences, and targeted gene expression methods. The two main genes of interest were cytochrome p450 1A (*cyp1a*), associated with xenobiotic metabolism of PACs, and glutathione-s-transferase (*gst*), associated with oxidative stress and phase II metabolism (Alsaadi et al., 2018). Results from this study will provide information for risk assessors regarding exposure and effects markers in wild FHM following the rehabilitation of a real-world spill site.

2.2 Materials and Methods

2.2.1 Dilbit WAFs

Dilbit is created by adding one part diluent (e.g., natural gas condensate) to three parts highly viscous bitumen in order to reduce overall viscosity of the product and facilitate transport in pipelines (Crosby et al. 2013; Alsaadi et al. 2018). Low energy dilbit WAF was sourced from Natural Resources Canada's lab in Devon, Alberta. The WAF was made from Cold Lake Blend dilbit using a wave pool approximating natural wave action and was removed after 2.5 h of mixing. The total polycyclic aromatic compound (TPACs) content of the WAF was determined using Gas Chromatograph Mass Spectrometry (GC-MS) at ALS Laboratories in Calgary, Alberta. Analysis followed procedures adapted from the US-EPA (Test Methods for Evaluating Solid Waste, SW-846, Methods 3510C & 8270) with the exception that samples exceeded the recommended ALS holding time by 3 days. FHM were exposed to dilutions of 1: 1 000 000 ("very low") or 1:100 000

("low") of this WAF in water obtained from a reference Lake 114 (49° 40' 21.108 "N, 93° 45' 20.88" W; 12.1 hectare surface area; 5 m max depth) at the International Institute for Sustainable Development-Experimental Lakes Area (IISD-ELA) in Northwestern Ontario, Canada. This research laboratory conducts large scale and whole ecosystem experiments to better understand how emerging risks to freshwater may affect the boreal biome and other ecosystems. The WAF was diluted at the low or very low ratio daily and was reapplied to the tanks with consistent, gentle mixing at the same time as the daily water change. WAF was drawn up into a glass pipette and the added to a 1-L PYREX™ measuring jug filled with clean lake water, which is then used to mix the WAF into the tanks.

Water samples from the low treatment tank were analyzed using a secondary method: scanning spectrofluorometry (Quanta-Master Fluorescence Spectrometer, PTI Ltd., London, ON, Canada) using the protocol of Adams et al., 2014. This method is quick and relatively inexpensive and offers qualitative estimates of the concentration of PACs in a water sample (Adams et al. 2014). Three replicate samples were taken from the 1:100,000 treatment tank on exposure day two and then transported back to Queen's University for analysis. A Cold Lake Blend (CLB) Winter blend dilbit as well as a sample of the WAF used in this project were used to create standard dilution curves. An excitation wavelength of 300 nm was used across emissions of 310-460 nm and spectra are all background corrected (50/50 lake water and 99% ethanol).

2.2.2 Fathead minnow collection and exposures

Adult fathead minnows were collected in late May 2017 from the reference Lake 114 using 10 baited, 'Gee' style minnow traps. Specimens were selected based on the presence of secondary sex characteristics – tubercles and banding in males and ovipositors in females – to ensure specimens were sexually mature and to attain a balanced sex ratio. FHM were transported in

aerated lake water from the lake to the IISD-ELA field station (15-20 min) in coolers and transferred into 40 L glass aquaria covered with opaque plastic sheets to reduce environmental stress. The aquaria were kept in coolers in order to stabilize the temperature and minimize stress by exposure to external stimuli. A static renewal exposure was maintained where 50% of the water in the aquaria was refreshed manually with Lake 114 water once a day throughout the 60-day acclimation period. Ammonia, dissolved oxygen, and temperature were recorded daily for the first 30 days, and then every other day when it was evident that the 50% static renewal maintained ammonia levels stably < 2 ppm. Four days after capture the minnows were fed *ad libitum* using Tetrafin™ fish flakes. Uneaten food and feces were removed from each aquarium with a low flow siphon 10 minutes after food was introduced.

The exposure phase (21 days) was conducted in three aerated 40-L aquaria inside of coolers, one reference, one very low (1:1 000 000) and one low (1:100 000) treatment. The aquaria were cooled with circulating lake water flowing through each cooler on the outside of each aquaria in order to maintain constant temperature (~23 °C) in the range of FHM spawning temperature (Ankley and Villeneuve 2006). Monitoring and water changes remained on the same schedule as during acclimation. After the 21-day exposure, a 2-week recovery phase began when breeding triplicates of 2 females and 1 male FHM were randomly selected from each of the treatment aquaria and were moved to 9-L breeding chambers. The breeding chambers contained gravel substrate and a 4” PVC half-pipe breeding tile. Each of the chambers was housed in a commercial zebrafish bioassay unit that provided consistent circulating water quality (21-26 °C, >8.0 mg/L DO, 6.5-7.5 pH). All of the breeding pairs were isolated from external stimuli by suspending an opaque curtain around the perimeter of the bioassay unit.

Fish were individually euthanized at the end of the recovery period by anesthetizing them in 0.4 g/L tricaine methanesulfonate (MS-222) pH buffered (at 7.0) and then severing the spine. Each fish was weighed, measured and dissected to remove gonads and livers. Condition factor (K), hepatosomatic index (HSI), and hepatocyte volume index (HVI) were determined as measures of the overall health of the exposed FHM. Livers and gonads were weighed, and the gonads and half of each liver were fixed in 10% formalin pH buffered (at 7.0). The remaining half of the liver was frozen between slabs of dry ice and stored at -80 °C prior to gene expression analysis.

2.2.3 Histology

Histological slides were prepared at the University of Manitoba veterinary pathology laboratory. Briefly, the tissues were trimmed and embedded in paraffin and sectioned (7 μm). They were dehydrated and stained using hematoxylin and eosin (H&E) and mounted on microscope slides for analysis. Digital images were obtained and analyzed using Zeiss Zen Blue software (Carl Zeiss, Brussels). To assess the reproductive potential of the female FHM, gonads were analyzed for number, size, and developmental stages of the oocytes. Perinucleolar oocytes were identified by the presence of nucleoli at the periphery of the nucleus, cortical alveolar were identified by the appearance of yolk vesicles within the ooplasm, vitellogenic were identified by obvious spherical yolk granules, and atretic cells were designated based on compromised cell membrane structure (Fig. 2.1A-C). I also compared the sizes of each oocyte at each developmental stage. In order to randomize the selection of oocytes chosen for this analysis, I superimposed a grid on the same microscopic fields of view that were used for the oocyte counts. Proceeding from top left to bottom

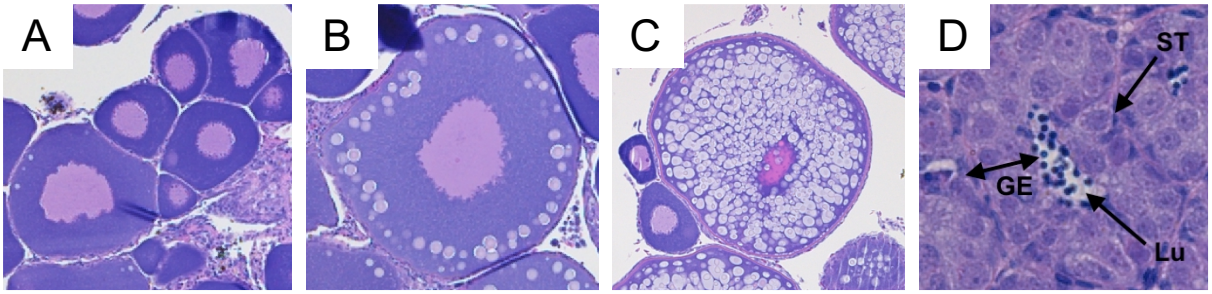

Figure 2.1: Histological slides, stained with hematoxylin and eosin, of different stages of oocytes and the structure of the seminiferous tubule in testes. Images are not to scale and all are from the reference treatment. **A.** Perinucleolar oocytes, **B.** Cortical alveolar oocyte**, C.** Vitellogenic oocyte, **D.** Male testes with the perimeter of the seminiferous tubule (ST) and lumen (Lu) as well as the diameter of the germinal epithelium (GE) indicated in black.

right, I measured oocyte diameter in two directions, north-south and east-west, for the first 5 oocytes per slide of each type that were intersected by a crosshair of our grid. If there were not five oocytes of that type intersected by the grid, I analyzed all oocytes of that stage in the field of view. Only cells with visible nuclei were counted and measured to ensure similar measurements along the cross sections of the oocytes.

Male reproductive health was also assessed using similar methods. The diameter of each seminiferous tubule and tubular lumen were measured and results are presented as a ratio (Fig. 2.1D). Developmental stages of the testes were also assed using the morphologic criteria set out by the US-EPA histology guidelines for FHM. Liver health was assessed using hepatocyte volume indices (Leatherland and Sonstegard 1984). Three randomly selected areas of 100 μm^2 were selected from three sections of the livers of each fish. The number of hepatocyte nuclei within those areas were counted and the average number of nuclei per 100 μm^2 was calculated.

2.2.4 qPCR targeted gene expression

Targeted gene expression responses in pathways related to phase I and phase II metabolism were examined as indicators of FHM exposure to the WAF following methodology described in Alsaadi et al. 2018b. RNA was isolated from each liver tissue using a E.Z.N.A Total RNA mini

kit (Omega Bio-Tek, Norcross, GA, USA) and the Turbo DNA-free kit (Thermofisher, Ottawa, ON) at the Royal Military College of Canada (Kingston, ON). RNA purity and nucleic acid concentrations were quantified using a Nanodrop-2000 spectrophotometer (260/230 ratio accepted as pure RNA, Thermofisher, Ottawa, ON). GoScript Reverse Transcription System (Promega, WI) was used to synthesize cDNA from 1 µg RNA for qualitative Polymerase Chain Reaction (qPCR) analysis with GoTaq Mastermix (Promega, WI) and the primers for *cyp1a* (Alsaadi et al., 2018b), *gst* alpha (Mager et al. 2008), and the housekeeping genes 60S ribosomal protein L8 (*rpl8*) and elongation factor 1α (*ef1α;* Martyniuk and Houlahan 2013). mRNA levels were assessed using a CFX96 Real Time System qPCR (BioRad, Mississauga, ON, CA) and all analyses were performed following Alsaadi et al. (2018b). Briefly, a standard curve in duplicate with a serial dilution (1:4) of pooled cDNA, no template controls, and no reverse transcriptase controls were included for each plate and considered acceptable with an efficiency of 100 ± 10% and $R^2 > 0.985$. mRNA levels were normalized to the average expression of the housekeeping genes and data were reported as fold changes relative to the reference tank.

2.2.5 Statistical analyses

Heteroscedasticity and normality of the data was confirmed using a Levene's test (normality accepted if $p>0.05$) and examining a Q-Q plot before using a one-way analysis of variance test (ANOVA). ANOVAs were used to test for differences across all treatments, while a Tukey's post hoc analysis determined differences among the 3 treatment groups. If data were non-parametric, as in the case of oocyte counts, a Kruskal-Wallis test was used to test for differences across all treatments. Where that difference was significant a Mann-Whitney U-Test was used to determine if the treatments were significantly different from the reference. Differences in all tests

were considered significant if $p < 0.05$. All statistical analyses were performed and accompanying figures created using R Studio (RStudio Team, 2016).

2.3 Results and Discussion

2.3.1 Chemistry results

GC-MS analysis indicated that the original WAF contained approximately 26 µg/L TPACs at the beginning of the experiment before it was diluted either 1:1 000 000 (Very Low) or 1:100 000 (Low). These ultra-low exposure levels were chosen to represent residual PAC levels at a spill site after cleanup is completed when most of the lower molecular weight compounds have volatilized. The secondary method of scanning spectrofluorometry assessed a serial dilution of a WAF sample at the end of the experiment as well as samples from the low treatment tank. Only one of the triplicate samples from the first day of the experiment contained PACs above the detection limit. This sample indicated that the exposure medium contained approximately 4% WAF. This outlier may be the result of poor mixing at the time of application or sampling and suggests that the WAF is delivering PACs into the water, but not homogenously. Here, fluorescence results are only referred to as a percentage of the fluorescence measurement of 100% WAF as the estimates for TPAC estimated from the spectrofluorescence analysis were far higher than those of the GC-MS.

Many of the WAF exposures in the literature are applied to embryo-larval stages of fish due to increased sensitivity during development (McKim 1977). In exposures using these more sensitive life stages, LC50s were found to be in the mg/L range (Barron et al., 2018; Colavecchia et al., 2004). Toxicity effects concentrations of TPACs on fish embryos are also higher than the dilutions used in the present work. Range of TPACs causing toxicity effects in fish embryos have been reported at <1 – 18 µg/L TPAC (Carls et al., 2008; Madison et al., 2017). EC50 for

developmental malformations in FHM embryos exposed to CLB has been reported as 500 µg/L TPH-F (total petroleum hydrocarbons measured with spectrofluorescence; Alsaadi et al., 2018).

2.3.2 Mortality and morphometrics

There was significant mortality across all three treatments, the majority of which occurred during the initial acclimation (Fig. 2.2). No spawning events occurred when the fish were split into breeding triplicates during the recovery period despite tank temperature being in the range for FHM spawning. Since these effects were pervasive across both the oiled and reference treatments, they cannot be attributed to the oil exposure and are more likely symptomatic of confinement stress. Confinement stress has been primarily studied in salmonids (Wendelaar Bonga 1997) due to their extensive use in aquaculture (i.e., Pottinger & Carrick, 1999; Schreck et al., 2001). It has been shown that confinement stress and its inhibitory effects (e.g. smaller vitellogenic follicles,

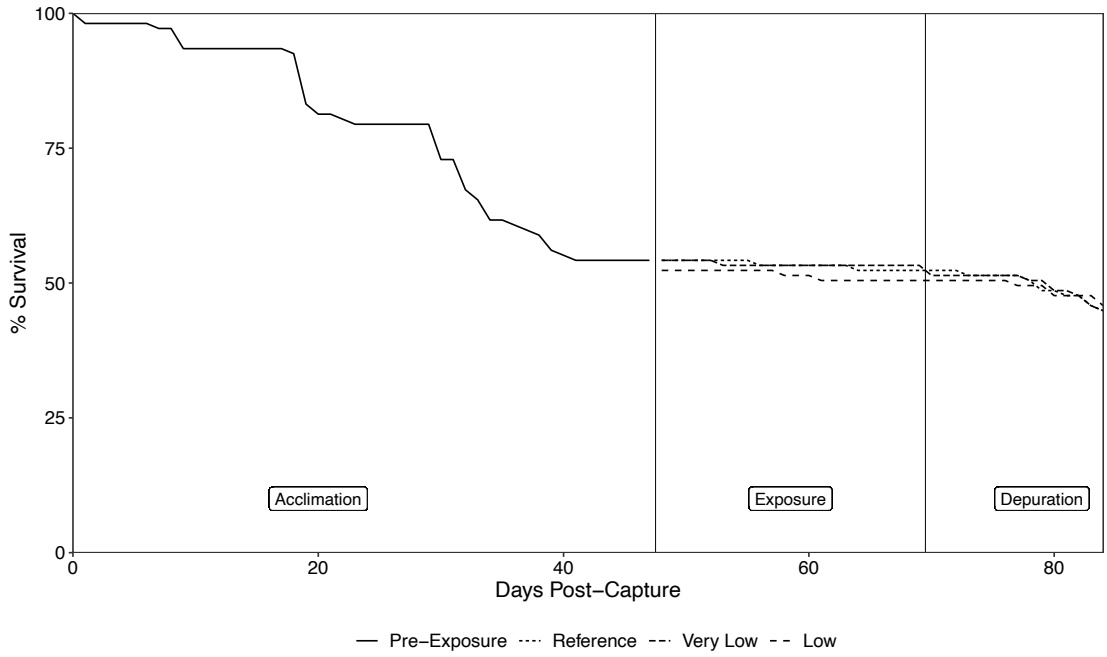

Figure 2.2: Percent survival of wild adult fathead minnows during the acclimation, exposure, and depuration phases of the experiment. The majority of the mortalities occurred during acclimation, after which percent survival was consistent between treatments.

high levels of atresia) on reproduction are enhanced in species that are non-salmonids, have asynchronous ovarian development, and have been removed from their native wild habitat (Pankhurst 2016). FHM are cyprinids with notably asynchronous ovarian development (Jensen et al., 2001; Wallace & Selman, 1981) and the ones used in this experiment were sourced from a pristine natural environment, creating an experimental population vulnerable to confinement stress. Fish were collected during early spawning season in order to be able to externally determine sex, but it is possible that this timing further increased stress. FHM males in particular experience high post-spawning mortality due to the energetic exertion of defending their nests, but females are also more vulnerable at this time as a result of their physical reproductive investment (Unger and Sargent 1988; Divino and Tonn 2008).

Fulton's condition factor (K, Eq. 2.1) is a commonly used calculation to assess comparative health and nutritional status of fish.

$$K = \frac{Weight\ (g)}{Length^3\ (cm)} \times 100 \qquad \text{Eq. 2.1}$$

A subsample of healthy adult FHM taken from the source lake in June 2018 had an average K of 1.18 (± 0.16, N=221) for females and 1.24 (± 0.10, N=223) for males. When the current experiments were completed average K was significantly lower than these values across all three treatments (Fig. 2.3-A). K has long been known to vary seasonally and with relation to spawning events (Nash et al., 2006) and this may be partially responsible for the decreased K in FHM during August compared to June. Increased handling and stimuli, and a new environment can cause stress in wild fish and, although great care was taken to minimize this interaction, this may have further contributed to lower K in the FHM. Observationally, the fish were consuming very little of their daily feedings. The stress of confinement, in addition to changes in their circadian rhythms and natural feeding schedules (Kulczykowska and Sánchez Vázquez 2010), is likely the main cause of

their diminished condition. There is no consensus on the effects of oil exposure on fish condition in the literature. Some studies have observed significant increases in condition of FHM exposed to TPAC concentrations much higher than those in this experiment compared to reference sites (Kavanagh et al., 2012; Van den Heuvel et al., 2012), while others report no effect on condition at all (Parrott et al. 2019). One study in particular observed an increase in the condition of pearl dace downstream of oil sands deposits, but no change in the condition of brook stickleback from the same location (Raine et al. 2017).

In this study, there was a nonsignificant trend towards lower condition in exposed FHM compared to reference fish, which may be indicative that low-level exposure to dilbit has the potential to exacerbate the effect of confinement stress and dietary changes. Wild fish can be more sensitive than lab cultures of the same species (Wendelaar Bonga 1997) and experience various natural, non-toxic stressors (i.e. spawning, predation, overwintering) throughout their lives, which could result in similar effects in the wild. Domesticated or cultured strains of fish have been shown to have a less sensitive stress response and lower cortisol levels than wild fish undergoing the same capture or confinement (Wendelaar Bonga 1997).

Similar to K, hepatosomatic index (HSI, Eq. 2) is an important measure of fish health and nutritional status. An elevated HSI can be diagnostic of exposure to contaminants and induction of metabolism enzyme systems (Everaarts et al., 1993; Huuskonen & Lindström-Seppä, 1995).

$$HSI = \frac{Liver\ Weight\ (g)}{Body\ Weight\ (g) - Liver\ Weight\ (g)} \times 100 \qquad \text{Eq. 2.2}$$

The livers of the fish from the low treatment were significantly larger than those of the reference fish (p = 0.027). There were no significant differences between sexes, or between the low and very low or reference doses (Fig. 2.3-B), suggesting that HSI in these fish was not consistently altered by the WAF.

Hepatocyte volume index (HVI) is a count of hepatocyte nuclei in a known area of the liver as a proxy for liver cell size. HVI is indicative of liver condition and enlargement or shrinking of hepatocytes in relation to nutrition or contaminant exposure (Leatherland and Sonstegard 1984; Palace et al. 2002). There was no significant difference between hepatocyte volume across treatments in females (Fig. 2.3-C). This supports the HSI results indicating that any changes to liver health in these fish is unrelated to low level WAF exposure. In the males however, the cells from the treatment fish are significantly smaller than those from the reference tank (ANOVA< $p<0.05$).

2.3.3 Gonad histology

Assessing reproductive condition is important from an adverse outcome pathway (AOP) perspective, because diminished reproductive potential is a major factor in lowering recruitment and expediting population decline. Histology of the gonads is a useful tool for assessing the reproductive health of fish both on its own and in conjunction with hormone, morphology and fecundity measurements (Blazer 2002). Contaminant or stressor induced changes at the cellular level could be associated with reproductive impairment and act as a warning sign for potential long-term population effects (Thomas 1990; Blazer 2002). Histological assessment of reproductive potential is particularly useful in cases where time or space constraints prevent a full reproductive assay (i.e., US-EPA, 2002). Larger oocytes with more vitellogenin are indicative of sexual development in female fish. Histology of the testes provides a means of estimating the sexual development of each fish based on the width of the germinal epithelium (i.e., thinner germinal epithelium means less spermatocysts in various stages of development and more spermatozoa in the lumen).

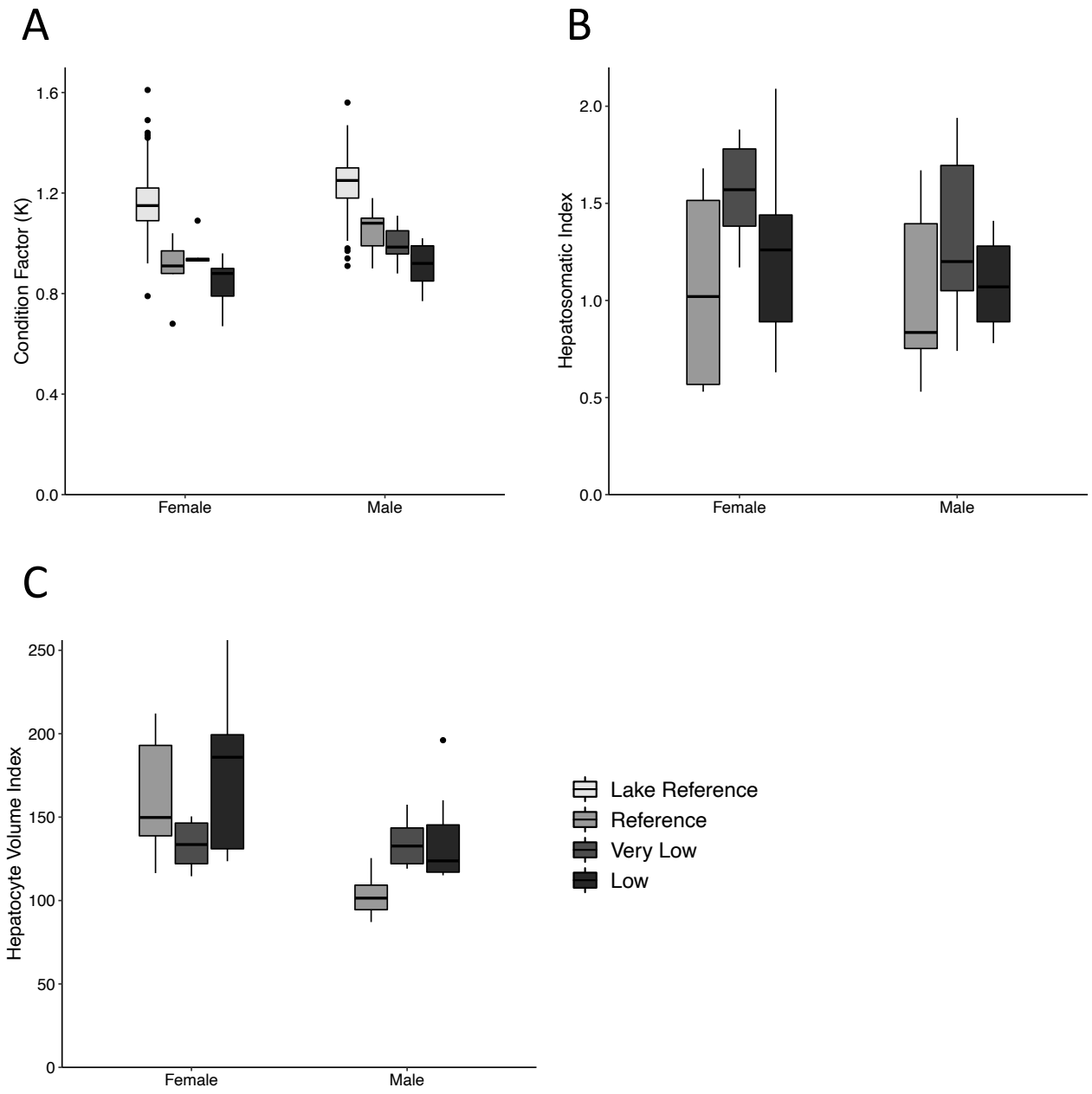

Figure 2.3: Several common health metrics in adult fathead minnows (FHM). Only condition factor (K) has a lake reference as the fish were weighed and measured, but not sacrificed. **A.** K values of experimental fish and lake references. All fish from the experiment had significantly lower condition than those from the lake (ANOVA, $p=0.00$), and there is a non-significant trend towards the fish from the high treatment having the lowest body condition. **B.** FHM from the experiment have no significant differences in hepatosomatic indices between sexes or treatments (ANOVA, $p>0.05$). **C.** Hepatocyte volume indices results for experimental FHM where there is no significant difference between treatments in the females but the males from the treatment tanks have significantly smaller cells than those from the reference (ANOVA, $p<0.05$).

The fish from the low treatment had more vitellogenic cells than the very low and reference treatments, but there were too few to run accurate statistics on the measurements (Fig. 2.4-A). The cortical alveolar cells from the reference treatment are significantly larger than the low treatment oocytes ($p < 0.001$). All female fish had significantly more perinucleolar oocytes than any other oocyte stage (Fig.2.4-B**)**. The size of the lumen relative to the seminiferous tubule is indicative of development in the testes, with a larger lumen size indicating a more developed fish. The testes of male fish from the low treatments were significantly more developed than those of the very low ($p < 0.05$) and reference ($p < 0.001$) treatment fish (Fig. 2.5). All male FHM displayed a Grade 4 (affecting >80% of the tissue; US-EPA 2006) increase in the proportion of spermatogonia compared to other stages in the testes.

Other studies have found similarly incongruous effects of PAC exposure on gonadal development between the sexes, but the literature remains divided on what these effects entail. A 1999 review of the effects of PAC exposure on female fish gonads indicated that most studies in the past decade found a reduction in egg development and subsequently lower reproduction, but this inhibition was both found to be reversible and inconsistently reported (Nicolas 1999). More recent studies have found that exposure to PACs and other petroleum components including naphthenic acids may also have effects on male gonad development. Oil sands processed waters (OSPW) have been shown to have a strong effect on male gonad development in yellow perch (*Perca flavescens*), but reduction or expansion in relative gonad size varied based on the pond (Van den Heuvel et al., 2012). Another study on oil sands related compounds in an AOSR

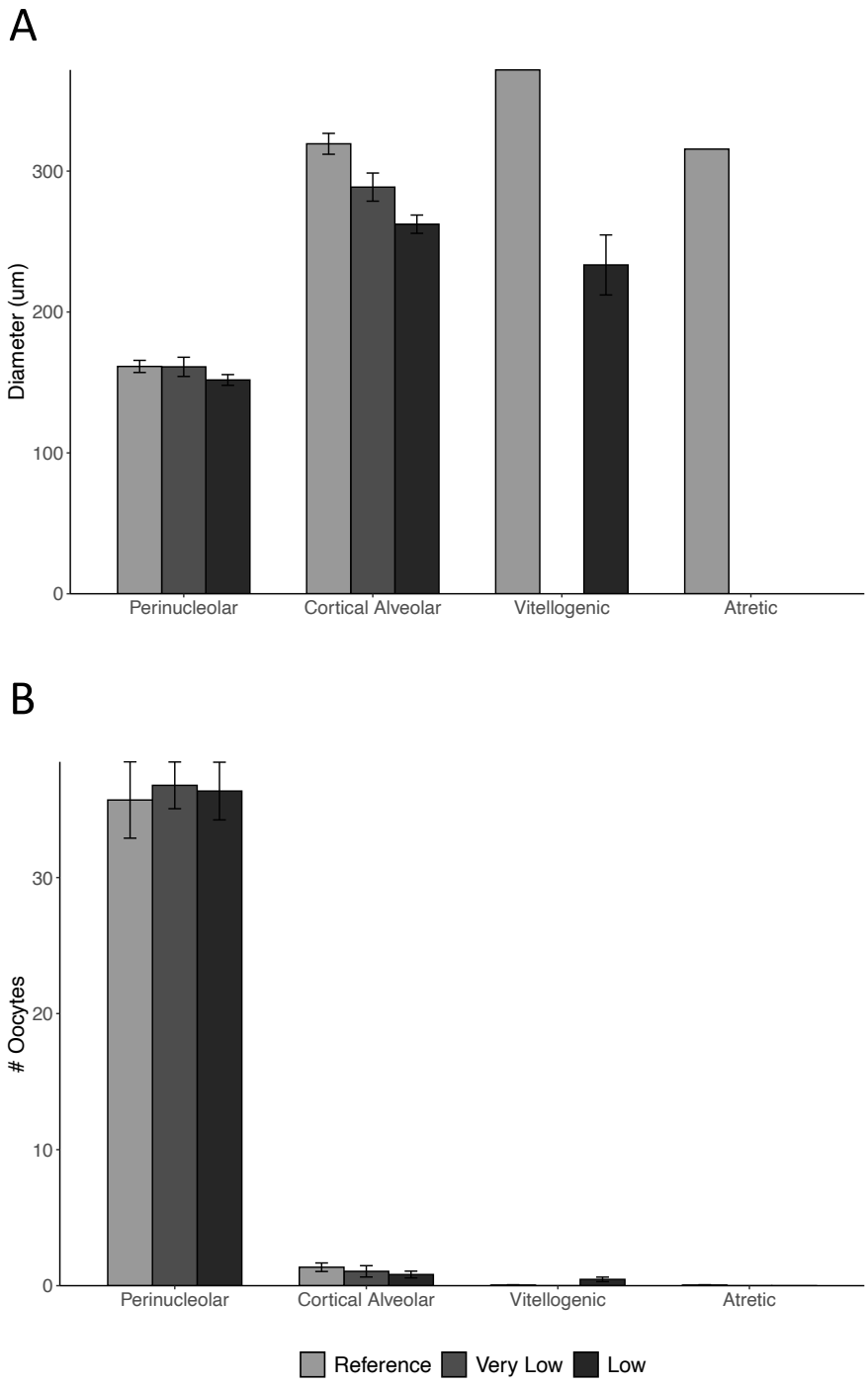

Figure 2.4: The size and relative number of different developmental stages of oocytes in the gonads of female fathead minnows from this experiment. **A.** Average diameter of oocytes at different stages. Cortical alveolar cells from the high treatment are significantly smaller than those from the low and reference treatments (ANOVA, $p<0.001$) and this may also be the case for vitellogenic cells but there were not enough oocytes to run statistical tests on the significance. **B.** Total number of oocytes counted within a slide. All treatments had significantly more perinucleolar cells than any other stage (Kruskal Wallis, $chi^2=13.92$, $p<0.05$).

watershed indicated that male slimy sculpin (*Cottus cognatus*) have increased gonadal development;whereas, pearl dace (*Margariscus margarita*) showed reduced gonadal development in both sexes (Tetreault et al., 2003). When exposed to OSPW female FHM had significantly smaller ovaries than the fish from the reference site (Kavanagh et al. 2011). Although the FHM in this experiment were exposed to residual levels of dilbit our results mirror these inconsistencies, with indicators of slightly more developed male gonads and smaller vitellogenic oocytes in the high exposure.

It is also important to note that the stress of confinement on wild fish species has been shown to have a similar deleterious effect on female gonad development (Pankhurst 2016). Exposure to confinement and handling stress during early vitellogenesis, similar to our collection time at the beginning of the FHM spawning season, has led to the production of smaller eggs in rainbow trout (*Oncorhynchus mykiss*; Contreras-Sánchez et al., 1998). Similarly, a loss of larger vitellogenic

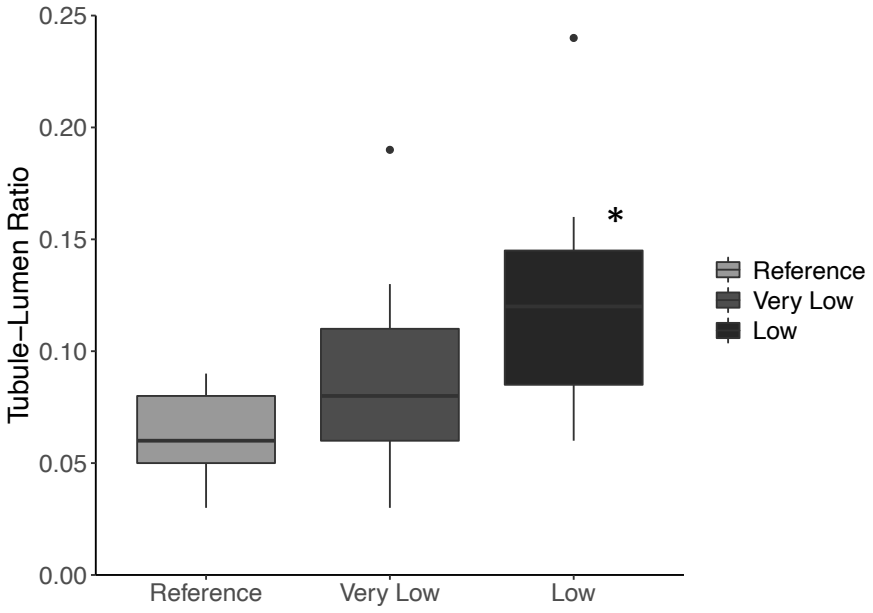

Figure 2.5: This figure illustrates the differences in the ratio between the area of the seminiferous tubules and lumens in male fathead minnow gonads. These results indicate that the fish from the high treatment have significantly larger lumens and are more developed than in the low and reference treatments (ANOVA, $p<0.001$).

follicles occurred in female wild striped trumpeter (*Latris lineata*) that had been captured, confined, and repeatedly disturbed over the course of 9 days (Morehead 1998). When fish are undergoing stress energy expenditure is reallocated to accommodate the stress response and this can compromise the functionality of other systems, including reproduction in general and female vitellogenesis in particular (Contreras-Sánchez et al., 1998). In male rainbow trout, disturbance stress has led to repressed spermatogenesis in males (Campbell et al., 1992), which is similar to our underdeveloped FHM testes. Additionally, the FHM in this experiment were observationally noted to be underfeeding which may lower their resilience to dealing with both the compounded effects of confinement stress and low level dilbit exposure (Schreck 2010). The compounded effects of residual oil exposure and external stress stimulated by confinement did not produce a consistent effect on the reproductive health of these already fragile wild fish.

2.3.4 Hepatic gene expression

The expression of *cyp1a* and *gst* were measured in FHM livers to determine if up-regulation occurred at these dilute dilbit WAF concentrations and was maintained after a 14 day depuration period. These genes have been shown to be sensitive biomarkers of PAH exposure in FHM embryos with *cyp1a* up-regulation reaching 56-fold and *gst* response at 2.5 fold at 2895 µg/L TPH-F (Alsaadi et al. 2018b). Exposure of adult FHM to these extremely low concentrations caused no significant difference in the up-regulation of either *gst* or *cyp1a* compared to the reference fish (Fig. 2.6). In Japanese medaka embryos, up-regulation of *cyp1a* has a linear relationship with increased exposure to TPACs, but *cyp1a* mRNA levels do not increase significantly below 0.4 µg/L TPACs (Madison et al. 2017). Similarly, in FHM embryos *cyp1a* and *gst* expression have been shown to have a positive sigmoidal relationship with increased exposure to CLB WAF (Alsaadi et al., 2018b). Up-regulation of *cyp1a* has also been shown to prelude

EC50s for embryotoxicity, offering a more sensitive measure of TPAC exposure (Carls et al. 2008; Alderman et al. 2017; Madison et al. 2017; Alsaadi et al. 2018; McDonnell et al. 2019).

2.4 Summary and Conclusions

This study provides important information regarding the potential toxic effects of realistic low level dilbit exposures to fish after an initial spill cleanup has occurred. The TPAC values in this study were in the low ppb range and effects were measured in wild adult fathead minnows from a pristine Canadian boreal lake system. The WAF used for these exposures was derived using a low energy wave pool replicating wave action and mixing more typical of natural exposure scenarios that laboratory-based exposures with higher energy or chemically enhanced WAF preparations. Despite the residual level concentrations applied here, several statistically significant results were observed including lower body condition in exposed fish, smaller cortical alveolar oocytes in exposed females, and larger lumen size in males from the low exposure. Across all

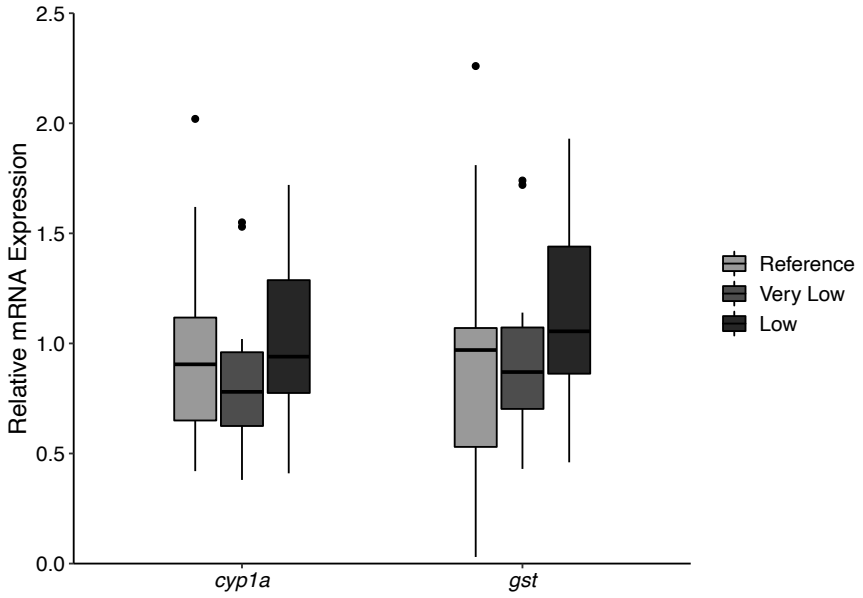

Figure 2.6: There is no significant difference in the relative mRNA expression of *cyp1a* and *gst* in the livers of exposed FHM compared to the reference fish (ANOVA, p>0.2). Expression was normalized using the average of *ef1a* and *rpl8* housekeeping genes.

treatments body condition of individuals was low, mortality was high, and no spawning events occurred in either the reference or the treatment tanks during the recovery period. These are all indications that the dilbit-exposed adult fish were likely less capable to cope with the stress of the new environment. Endpoints related to the stress of handling and housing wild fish are very similar to those from exposure to contaminants, including the effects on reproductive health noted in this experiment.

Using wild fish in contaminant studies can be difficult, but is important, as they can be more sensitive than more robust laboratory cultures and potentially provide greater insight into the effects of multiple stressors and low exposures on a wild population (Wendelaar Bonga 1997). In order to separate the stress of confinement from the stress of exposure, it may be prudent to house wild small-bodied fish in an environment more closely resembling their natural environment (i.e., mesocosms or limnocorrals) for future, similar experiments. Low-level exposures, like the ones simulated in this study, are more enduring and common in the environment than the high volumes of dilbit found at active spill sites, and important to study as a complement to lab exposures focused on higher concentrations of dilbit.

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Chapter 3: Limnocorral study to examine potential impacts of diluted bitumen on wild freshwater fish at IISD Experimental Lakes Area, northwestern Ontario

Abstract

The Boreal lake Oil Release Experiment by Additions to Limnocorrals (BOREAL) project began in 2018 at the IISD-Experimental Lakes Area (Ontario, Canada) to study the fate, behaviour, and potential toxicological impacts of diluted bitumen (dilbit) in fresh water. Model spills were contained within seven 10 m diameter, littoral limnocorrals (~2 m deep) and dilutions ranged from 1:100,000 to 1:1,000 dilbit:water. These values were chosen to represent a regression of real-world spills equivalent to the 50th to 99th centiles in North America over the last two decades and resulted in TPH exposures ranging between 169 μg/L/d and 1646 μg/L/d. Two additional limnocorrals untreated with dilbit, and fish caught from the open lake serve as references. Adult male and female finescale dace (*Phoxinus neogaeus*) were released in the limnocorrals 21 days after oil addition, while incidental juvenile fathead minnows (*Pimephales promelas*) were present in the enclosure from oil addition onwards. Here the effects of chronic exposure (>21 days) on reproductive health of adult fish as well as physiological responses are reported. Assessed metrics of reproductive health and metabolism include calculation of male and female gonadosomatic indices, egg diameter, histological development of gonads in both sexes, and condition factor. Gills were evaluated using the basal epithelia width of the secondary lamellae and hepatocyte volume indices were examined. Results indicate that in the lower exposures (< 309 μg/L/d) there are no cohesive effects on the health of adult small-bodied fish, but in the higher exposures (> 676 μg/L/d) there is a significant decrease in live fish recapture.

3.1 Introduction

Diluted bitumen (dilbit) is one of the main exports from Canada's Alberta Oil Sands Region (AOSR) and is primarily transported by pipeline across North America. (AER, 2018). Dilbit composition varies by season but is generally between 70-80% bitumen and 20-30% diluent such as gas condensate (Alsaadi et al, 2018a; Crosby et al., 2013). Diluent is added to reduce viscosity and to facilitate transport by pipeline and this fraction is typically enriched in lower molecular weight petroleum components e.g. saturates, BTEX). Pipelines disperse in all directions from the AOSR and all traverse a portion of the boreal ecozone, which is home to the majority of accessible Canadian freshwater (Brandt, 2009; CAPP, 2017). Although pipelines remain the statistically safest method of transporting petroleum products, accidental releases do occur and a better understanding of the effects of dilbit on freshwater ecosystems is paramount in the development of effective and efficient remediation methods (Lee et al., 2015; NEB, 2018; NRCan, 2017).

In 2015, the Royal Society of Canada conducted an expert panel review on the behavior and environmental impacts of crude oil products in aquatic environments (Lee et al. 2015). One of their high-priority research needs identified was to "*better understand the environmental impact of spilled oil in high-risk and poorly understood areas (inland rivers, lakes, and wetlands)*" (Lee et al. 2015). Dilbit is a concern because of the high percentage of toxic and soluble compounds in the diluent, the high compositional variability, and the different behaviours of the bitumen versus diluent portions of the blends (Crosby et al. 2013; Lee et al. 2015; The National Academies of Sciences Engineering and Medicine 2016).

Several studies have been conducted in the last decade to investigate the fate and behaviour of dilbit in model freshwater systems (SL Ross Engineering 2012; Stoyanovich et al. 2019) and in

the same time period lab studies have gathered information on the effects of dilbit exposure on freshwater fish species (Alderman et al., 2017a; Alderman et al., 2017b; Alsaadi et al., 2018b; Barron et al., 2018; Madison et al., 2015, 2017; Philibert et al., 2016). Wild fish can be more sensitive to contaminants and stressors than their lab culture counterparts and are more vulnerable to the effects of compounded stress (Wendelaar Bonga 1997). Depending on the timing of oil spills, fish populations may be under existing stress in their life cycle including spawning, overwintering, or food shortages creating a situation of compounded stressors (Schreck et al., 2001).

In 2017, the Boreal-lake Oil Release Experiment by Additions to Limnocorrals (BOREAL) project was undertaken to fill some of the primary knowledge gaps in the understanding of petroleum product spills in a freshwater environment (identified in Lee et al., 2015). This study represents a component of the larger BOREAL study and is the first report of the effects of *in situ* model dilbit spills on wild adult freshwater fish. This study focuses on the toxicity of spilled dilbit on the reproductive potential and overall health of a common small-bodied freshwater fish. Survivorship, basic meristic health measures, alterations to liver and germ cell size and structure, and presence of polycyclic aromatic compound (PAC) metabolites in the bile were all examined. Results from this study will increase understanding of the chronic effects of dilbit spill volume on freshwater fish within a range of environmentally relevant spill scenarios.

3.2 Materials and methods

3.2.1 Study Setting

The study was conducted at the IISD-Experimental Lakes Area, located in the pristine boreal forest of northwestern Ontario and 4 hours from Winnipeg MB. IISD-ELA has attained special provincial (O. Reg 60/14) and federal (SOR/2014-95) legislation that allows the

organization to conduct whole ecosystem manipulations in the 58 lakes under their administration. The IISD-ELA data set has over 50 years of background information on these lakes, providing a comprehensive understanding of changes that occur in manipulated ecosystems, including Lake 260, the site for the 2018 BOREAL project. Lake 260 is a small oligotrophic lake with a surface area of ~34 ha and a maximum depth of 14.4 m. Water chemistry of Lake 260 is typical of boreal lakes in the ELA region (Cleugh and Hauser 1971). Nutrient and chemistry results from Lake 260 before, during, and in the two years following the BOREAL project are presented elsewhere (Rodríguez-Gil, in prep).

3.2.2 Model Spill Enclosures

In May of 2018 the BOREAL project installed nine (*9*) limnocorrals (10 m diameter) in the littoral section of Lake 260 (Fig. 3.1). Two of the limnocorrals contained only lake water and were used as reference mesocosms. In June, seven (*7*) mesocosms were amended with a logarithmic regression of dilbit volumes ranging between 1.5 and 180 L (Table 3.1), representative of the range of spills (oil to water ratios) from the 50^{th} to 99^{th} percentile that have occurred in Canada and the United States over the last ten years (Rodríguez-Gil et al., in prep). This regression design was chosen over replication in order to be able to apply the results to multiple future spill scenarios and provide risk assessors with a tool to help predict the effects of different spill sizes on freshwater lakes. Logistically replication could not also be added to the regression due to spatial and fiscal restrictions.

Dilbit was chosen because it is one of the most common petroleum products transported across North America and little is known about the differences in fate and behaviour changes caused by the combination of bitumen and condensates (Crosby et al., 2013; Lee et al., 2015; The National Academies of Sciences Engineering and Medicine, 2016). The Cold Lake Blend (Winter,

2017) used in the BOREAL project was supplied by Environment and Climate Change Canada (ECCC) and applied to the enclosures by a trained team from ECCC and the Eastern Canada Response Corporation (ECRC). Predator deterrents were attached around each oiled limnocorral. Throughout the duration of the BOREAL project water chemistry and nutrients were monitored on a weekly basis (Rodríguez-Gil et al., in prep) and the water volume and any subsequent changes were monitored using the addition of a known volume of a benign tracer; tritiated water (H_3; Orihel, 2005).

3.2.3 Fish collection, housing and dissections

Finescale dace (FSD; *Phoxinus neogaeus*) were selected as a sentinel species for this study because they are a common small-bodied fish across North America, were prevalent in the study lake, have an early spawning season which finished prior to dilbit additions, and adults are perennially sexually dimorphic (Scott and Crossman 1973). Prior to dilbit application but after

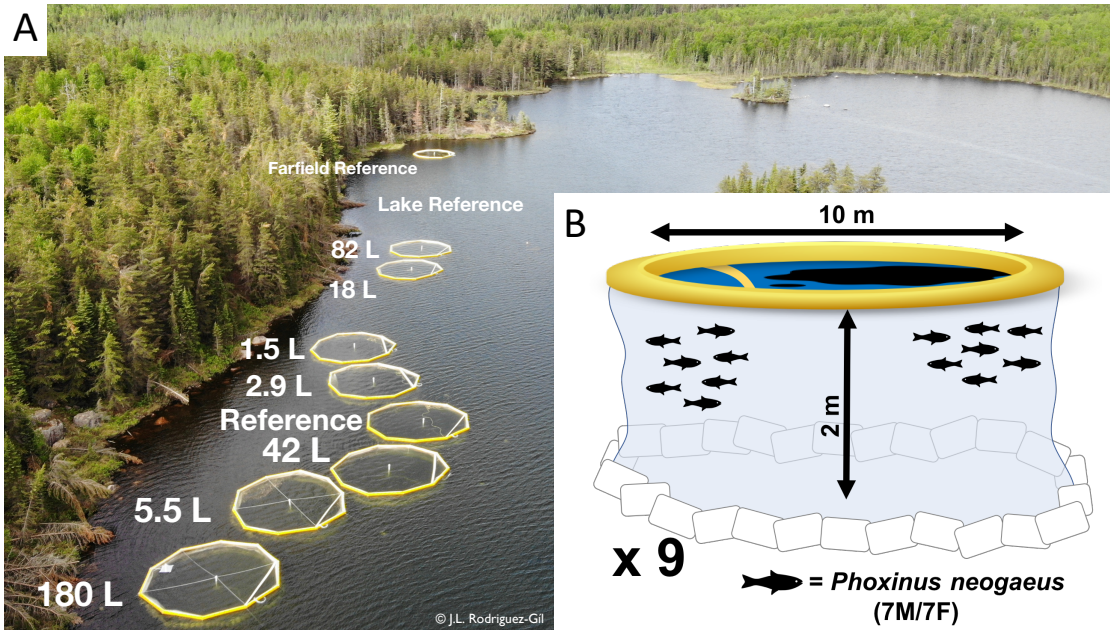

Figure 3.1: A. Location of the limnocorrals in Lake 260 at the Experimental Lakes Area. All limnocorrals are labeled with the applied volumes of dilbit or their reference designations. B. The dimension (10 m diameter by 2 m depth) and setup of each individual limnocorral.

Table 3.1: Applied dilbit volumes, corresponding to limnocorral references in text, and the associated target spill volumes from the original regression calculations as well as the mean daily total petroleum hydrocarbon (TPH) and total polycyclic aromatic compounds (TPAC) from each limnocorral during finescale dace exposures.

Applied Dilbit Volume	Lake	Farfield	Reference	1.5 L	2.9 L	5.5 L	18 L	42 L	82 L	180 L
Target Dilbit Volume (L)	0	0	0	1.60	3.45	7.43	16.0	34.5	74.3	160
Mean TPH (µg/L/day)	10.1	30.4	37.4	169	207	309	203	676	738	1646
Mean TPAC (ng/L/day)	NA	33.6	NA	NA	187	300	357	1058	1121	2002

limnocorral installation, native fish were removed from each limnocorral in order to minimize non-target population disparity between treatments. Application of dilbit occurred in late June 2018 and FSD were added three weeks later to ensure they were not exposed to the more acutely toxic volatile compounds. Gee Style minnow traps were set in the near-shore area of Lake 260 in order to capture FSD, which were then anesthetized (0.1g/L pH buffered (=7.0) tricaine methanesulfonate (MS-222)), weighed and measured, then released to swim freely in the limnocorral. Each limnocorral was stocked with seven males and seven females. Gee traps were also used to recapture FSD and any additional bycatch after a two-month exposure period. The same number of traps were deployed in each limnocorral to maintain a consistent unit of effort and comparable catch per unit effort (CPUE) results across treatments. Traps were checked every 24 - 48 hours and captured fish were transported back to the IISD-ELA field station in bags of fresh lake water that had the head space filled with oxygen and were packed securely in a cooler. Fish were individually euthanized within 2 hours of confinement in the bags by first anesthetizing them with MS-222 (0.4 g/L buffered to pH = 7.0) and then severing the spine. Each fish was weighed, measured and dissected to remove gonads, livers, gill, and gallbladder. Fulton's condition factor (Eq. 3.1), hepatosomatic index (Eq 3.2), gonadosomatic index (Eq. 3.3) and hepatocyte volume index were determined as measures of the overall health of the exposed FSD.

$$K = \frac{Weight\ (g)}{Length^3\ (cm)} \times 100 \qquad \text{Eq. 3.1}$$

$$HSI = \frac{Liver\ Weight\ (g)}{Body\ Weight\ (g) - Liver\ Weight\ (g)} \times 100 \qquad \text{Eq. 3.2}$$

$$GSI = \frac{Gonad\ Weight\ (g)}{Body\ Weight\ (g) - Liver\ Weight\ (g)} \times 100 \qquad \text{Eq. 3.3}$$

Livers and gonads were weighed, and the gonads, gill, and half of each liver were fixed in 10% pH buffered (=7.0) formalin for histological analyses. The remaining half of the liver and the gallbladder were frozen between slabs of dry ice then stored at -80°C.

3.2.4 Spectrofluorometry

Following exposure to petroleum products, fish quickly metabolize the PACs to which they are exposed and excrete the metabolites into bile in the gallbladder where the metabolite concentrations can be quantified (Lin et al., 1996). Our analysis focused specifically on 2-3 ring PACs. The fused aromatic ring structures of PACs and their metabolites are known to fluoresce, with the wavelength of maximal excitation increasing as the number of rings in the compound grows (Lin et al. 1996). For 2-3 ring PAC metabolites, the optimal fixed wavelength fluorescence signal occurs at an excitation wavelength of 290 nm, and emission wavelength of 335 nm ($FF_{290/335}$; Beyer et al., 1998; Pampanin et al., 2016). In our analysis, 2-naphthol was used as the standard against which biliary metabolites were quantified, with PAC concentrations reported as 2-naphthol equivalents. 2-naphthol (>99.0%) was acquired from TCI America and all samples were evaluated on a Cary Eclipse Fluorescence Spectrophotometer (Agilent, CA) and accompanying software. A standard curve was created using dilutions of a stock solution of 2-naphthol in 50:50 H_2O:CH_3OH (HPLC Grade, Fisher; v/v) and examined at $FF_{290/335}$. The majority of studies quantifying PAC biliary metabolites involve larger fish, whose larger gallbladders allow for an exact amount of bile to be harvested from each fish and diluted to a pre-determined concentration, however this was not feasible for this study due to the exceptionally small size of the experimental fish. Thus, the entirety of bile contained in each of the experimental gallbladders

was extracted and diluted to a final volume of 2.1 mL, to maintain a dilution of 1:850. A lower dilution than the typical 1:1000 was chosen because of the potential for error associated with transferring the whole gallbladder and to ensure detectable fluorescence. In order to accurately quantify the volume of bile extracted from each fish and obtain the exact dilution, I determined the mass of bile in each gallbladder and calculated the volume using the estimated density (see SI-I). Briefly, the full gallbladder was weighed in its centrifuge tube, then ~400 μL of 50:50 methanol/water solution was added. The tube was then reweighed, vortexed, and centrifuged (60s, 20 000 rpm) to extract the bile. The bile-solution supernatant was then transferred to a second pre-weighed microcentrifuge tube and further diluted for analysis. The original microcentrifuge tube with the now empty gallbladder was dried (to eliminate any residual supernatant) and reweighed, and the volume of bile estimated using a density of 1.00 g/mL. Each diluted sample was run at $FF_{290/335}$ with slit widths at 2.5 nm and then fit to the 2-naphthol standard curve to provide a quantitative measure of 2-naphthol equivalents in each of the bile samples. All samples were normalized to a 50:50 methanol/water solvent standard.

3.2.5 Histology

Slides were prepared at the University of Manitoba veterinary pathology laboratory where the tissues were trimmed and embedded in paraffin and sectioned (7 μm). They were then dehydrated and stained using hematoxylin and eosin and mounted on microscope slides for analysis. Digital images were obtained and analyzed using Zeiss Zen Blue software (Carl Zeiss, Brussels). All cell types were preserved in histocassettes but some samples were small enough to slip through the slats in the cassette. For this reason, there may be uneven numbers of tissue samples between analyses for a given treatment.

Liver health was assessed using hepatocyte volume indices (HVI; Leatherland & Sonstegard, 1984). Three randomly selected areas of 100 μm^2 were examined from three sections of livers from each individual fish. The number of hepatocyte nuclei within those areas were counted and averaged, providing the HVI for that fish. The thickness of the basal epithelial layer on the secondary lamellae of the gills was measured as an indication of gas exchange potential. Each individual had three images were examined from gill sections for each fish and from these, three random areas of the basal epithelia on the secondary lamellae were measured on either side for thickness, to obtain mean values.

I analyzed the gonads for number, size and developmental stages of the oocytes in order to assess the reproductive potential of the female FSD. Perinucleolar oocytes were identified by the presence of nucleoli at the periphery of the nucleus, cortical alveolar were identified by the appearance of yolk vesicles, vitellogenic were identified by the prevalence of spherical yolk granules, and atretic cells were designated based on compromised cell membrane structure. I also compared the sizes of each oocyte at each developmental stage. Selection of oocytes for measurement was randomized by superimposing a grid on the microscopic fields of view that were used for the oocyte counts. Proceeding from top left to bottom right, I measured oocyte circumference for the first 5 oocytes per slide of each type that were intersected by a crosshair of our grid. If there were not 5 oocytes of that type intersected by the grid, I analyzed all oocytes of that stage in the field of view. Only cells with visible nuclei were counted and measured to ensure similar placement of the cross sections. In the male FSD, the diameter of the seminiferous tubule and tubular lumen were measured and then presented as a ratio to assess the development of male gonads. The random selection of three tubules per slide was completed in the same way as the

oocytes. Developmental stages of the testes were also assed following the US Environmental Protection Agency (US-EPA) histology guidelines for fathead minnows.

3.2.6 Statistical analyses

For the health indices and bile fluorescence an ANOVA followed by Tukey's post-hoc test was used to determine differences between treatments. Where necessary to adhere to assumptions of normality and heteroscedasticity, data were log transformed. Linear regression was then applied to the results and the TPH concentrations from the limnocorrals to assess any trends in health indices and bile fluorescence related to increasing TPH exposure. The lake reference was excluded from the regression analysis as it represented a significantly different environment to the limnocorrals and was not included in the original regression design of limnocorral treatments.

For histology measurements of the gills, livers, oocytes, and testes linear mixed models (LMMs) were used to assess if there were any differences related to increasing TPH exposure. LMMs were chosen in order to account for variability within individual fish by adding this into the model as a random variable. For the counts of oocyte stages ordinal logistic regression (OLR) was used to assess the odds of a cell being a given developmental stage in each exposure. Normality of the residuals for all models were confirmed visually using a Q-Q plot and heteroscedasticity was tested visually and using a Levene's test (accepted where $p>0.05$). All statistical analyses were performed and accompanying figures created using R Studio (RStudio Team, 2016). Additional information regarding statistical analyses are provided in the attached supplementary information.

3.3 Results and discussion

3.3.1 Chemistry

The volumes of dilbit applied to the limnocorrals represent a regression of the dilution factors from the 50th-99th percentile of historical inland pipeline spills in North America. Both TPAC and TPH were measured after one-week, and later at two-week intervals from 20 cm below the surface and 20 cm above the sediment in all enclosures (Fig. 3.2, 3.3). The FSD were added three weeks post dilbit application to allow the acutely toxic components of the dilbit to dissipate and so that the focus of this assessment could remain on the potential for chronic toxicity among the exposed fish (Stoyanovich et al., 2019). TPAC results have not been processed for the reference and 1.5 L enclosures at this time so TPH results will be used to delineate exposure within the fish (Stoyanovich et al., in prep). The results follow similar patterns and relationships between limnocorrals, with one noticeable difference. The 18 L limnocorral had more TPACs than the 2.9 and 5.5 L limnocorrals but less TPHs. This may have to do with differences in weathering regimes and solubility of PACs compared to other dilbit components as the 18 L limnocorral had a slow leak, identified using tritium, while the other two limnocorrals did not (Rodríguez-Gil et al, unpublished data). TPACs are the primary petroleum compounds of concern in chronic toxicity studies and have been shown to have adverse effects on fish (e.g. Barron et al., 1999). Therefore, while concentrations of TPH were below those observed in the limnocorrals amended with lower dilbit volumes, it is important to note that fish in the 18 L limnocorral were still exposed to these chronically toxic compounds. Fish retrieved from the limnocorrals were exposed to <400 μg/L TPH and <500 ng/L TPACs while the limnocorrals that produced no fish contained between 250-2000 μg/L TPH and 500-2200 ng/L TPACs during the fish exposures (discussed in Section 3.3.2).

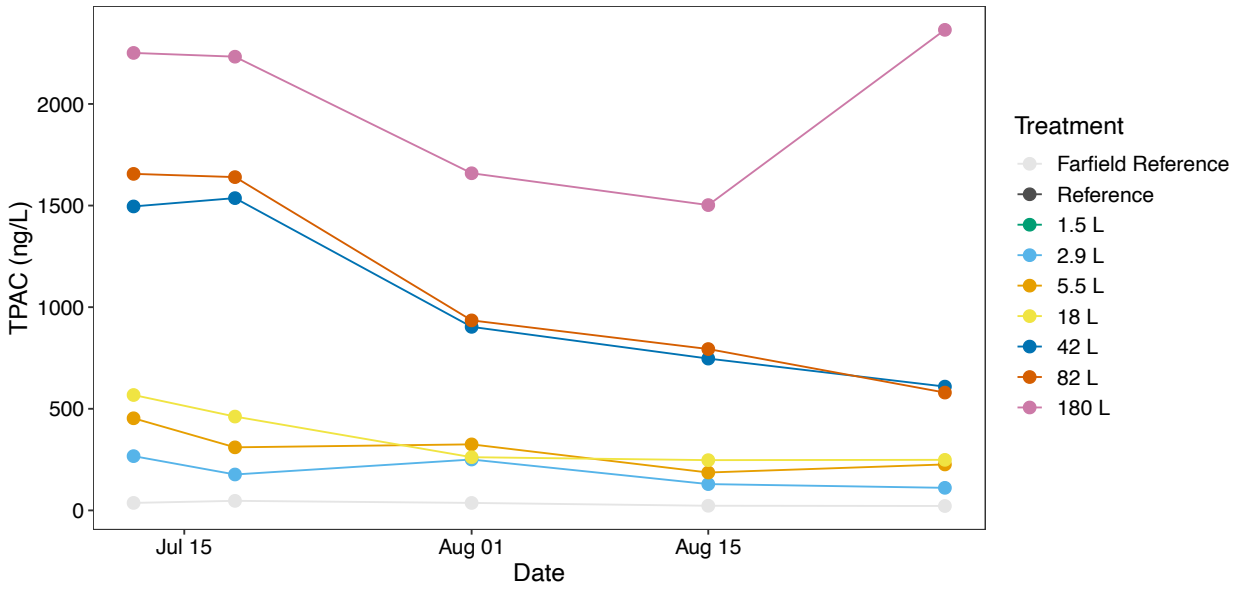

Figure 3.2: Total polycyclic aromatic compounds (TPAC) in the enclosures during the fish exposures. Results from the reference and 1.5 L enclosures have not yet been finalized. Fish were added July 11 and removed in the last week of August and first week of September. Results are averaged between the top and bottom sampling ports to represent the access that the FSD had to the entire enclosure. The 18 L enclosure has TPAC values that remain elevated, despite evidence of water exchange in that enclosure (Rodríguez-Gil, unpublished data).

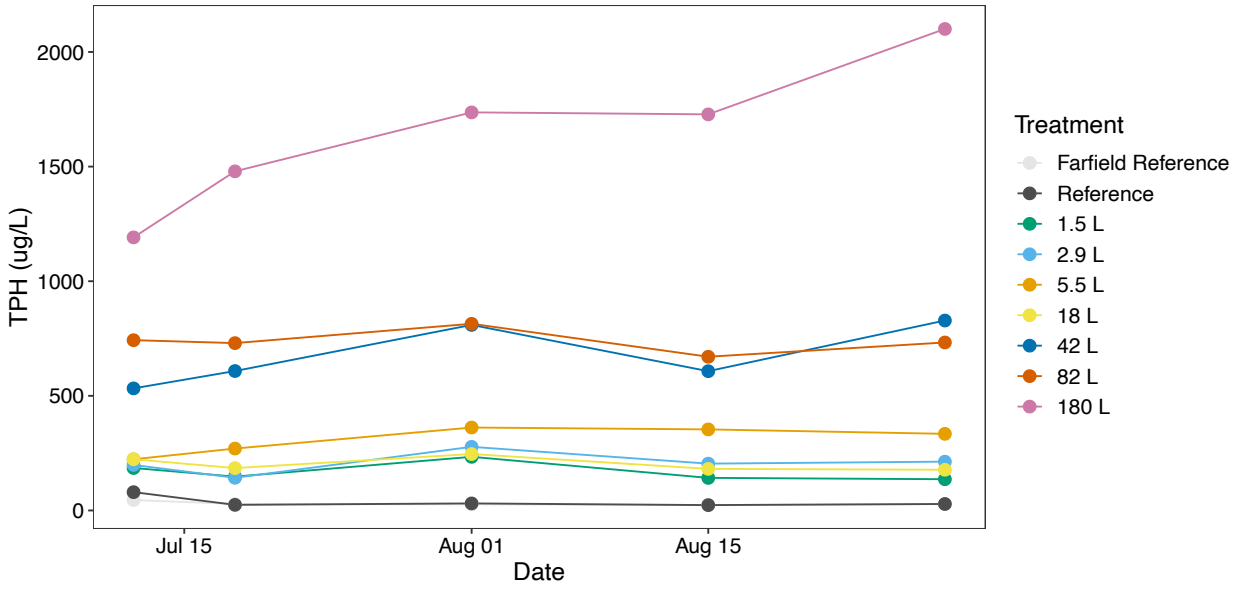

Figure 3.3: Total petroleum hydrocarbons (TPH) in the enclosures during the fish exposures. Fish were added July 11 and removed in the last week of August and first week of September. Results are averaged between the top and bottom sampling ports to represent the access that the FSD had to the entire enclosure. The 18 L enclosure had lower TPH than the 5.5 L and 2.9 L for the majority of the experiment and there is evidence of water exchange in that enclosure (Rodríguez-Gil, unpublished data).

Fish retrieved from the limnocorrals were exposed to an average of 37.4 μg/L TPH (*Reference*), 169 μg/L TPH (*1.5 L*), 207 μg/L TPH (*2.9 L*), or 309 μg/L (*5.5 L*) per day for the duration of the two-month exposure. Weekly lake sampling results indicate that wild fish from the lake were exposed to an average of 10.1 μg/L TPH per day. The EC_{50} for malformations in fathead minnow larvae exposed to CLB is 500 μg/L TPH determined by fluorescence (TPH-F) and 0.9 μg/L TPAC (Alsaadi et al., 2018b). These are low compared to what have been found for developing perch (3800 and 11.2 μg/L TPH-F and TPAC respectively; McDonnell et al., 2019) and Japanese medaka (4200 and 2.8 μg/L TPH-F and TPAC respectively; Madison et al., 2015, 2017).

3.3.2 Mortality and recapture

The catch per unit effort (CPUE) varied significantly between limnocorrals (Fig. 3.5), primarily as a function of the large numbers of juvenile fathead minnow (FHM) captured in the 1.5 and 5.5 L limnocorrals. I posit that these fish are young of year who were laid as eggs on the enclosure curtains as they soaked in the lake prior to being anchored to the sediment. Eggs from FHM are adhesive and often laid on the underside of rocks or branches (Stewart and Watkinson 2004). The sheltered folds of the curtain would have presented an ideal spawning substrate and if they were laid on the interior face of the curtain, once it was installed and anchored to the sediment the larval fish would have hatched inside the enclosure. At this size, they would not have been large enough to be trapped in the Gee style minnow traps during initial fish removal. Despite this, the main focus of this study will remain on the results pertaining to adult FSD.

No adult FSD were caught in the three highest treatments despite the same unit of effort being applied in all limnocorrals (Fig. 3.6). The only fish caught in the third highest treatment were retrieved already dead inside the traps. Observed mortalities explain this discrepancy in the 180 L limnocorral as over half of the FSD added were found dead during the experiment (Fig. 3.7).

Observed mortalities do not explain the CPUE of zero in the 82 L limnocorral as none were observed there. It is important to note that although there are no observed mortalities, this does not mean that no mortalities occurred. There is always the possibility that mortalities occurred that did not make it to the surface of the enclosure or that were simply not observed. Similar to the 82 L limnocorral, the farfield reference produced neither live fish nor mortalities by the end of the experiment. In this case unobserved mortality may be behind this discrepancy, but a second more likely explanation is that there were fewer predator deterrents on the reference enclosures compared to the oiled ones. There was a piscivorous bird regularly observed in the area of the farfield control and it is possible that predation caused this lack of production. There were no visual observations of fish in the farfield reference enclosure past week one of the experiment.

Overall, there was no statistically significant trend related to the volume of oil added to the limnocorrals and the number of mortalities. The nearfield reference enclosure had the second largest number of mortalities, equal to the number observed in the 5.5 L enclosure. This is unexpected but can be explained by the fact the three unintentional adult white sucker (*Catostomus commersonii)* were unable to be removed from that enclosure. These large fish are primarily benthic feeders but may have competed for resources and space in a relatively small area would be highly stressful to the smaller FSD. These observed mortalities may explain part of the low recapture rate in this limnocorral as well. Additionally, across all limnocorrals there was a shift in the zooplankton population resulting in a substantially depleted biomass the week prior to FSD addition (T. Black, *in prep*). This change would have caused some level of change in the source and quality of nutrients consumed by adult FSD as zooplankton make up a large portion of their diet (Scott and Crossman 1973; Stewart and Watkinson 2004). A shift in primarily predatory fish towards obtaining the majority of nutrients from phytoplankton and periphyton can both act as its

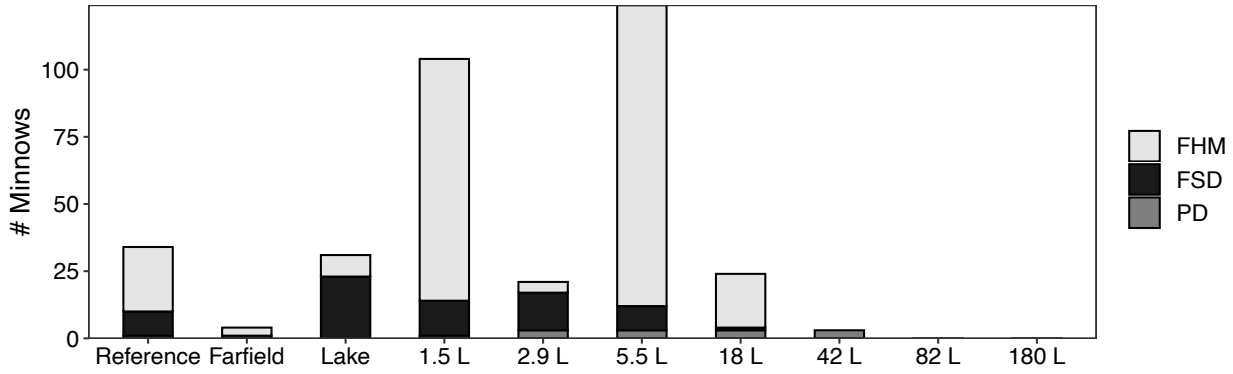

Figure 3.4: Total number of minnows recaptured from the limnocorrals after between 57 and 72 days of exposure (captured over a period of 2 weeks). The fish retrieved from the 42 L enclosure were dead in the traps.

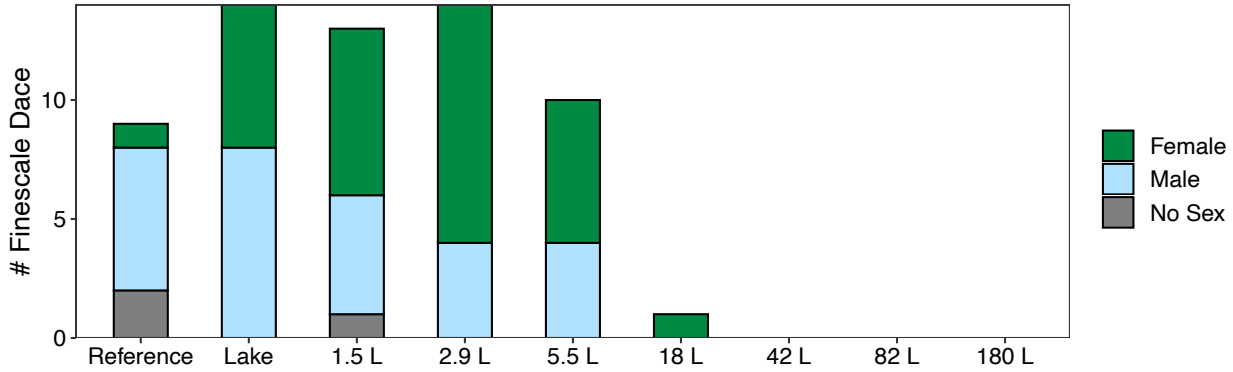

Figure 3.5: The number of adult finescale dace recaptured from the limnocorrals. The No Sex here represents those fish either too young and undeveloped to sex or those already dead and unable to be dissected.

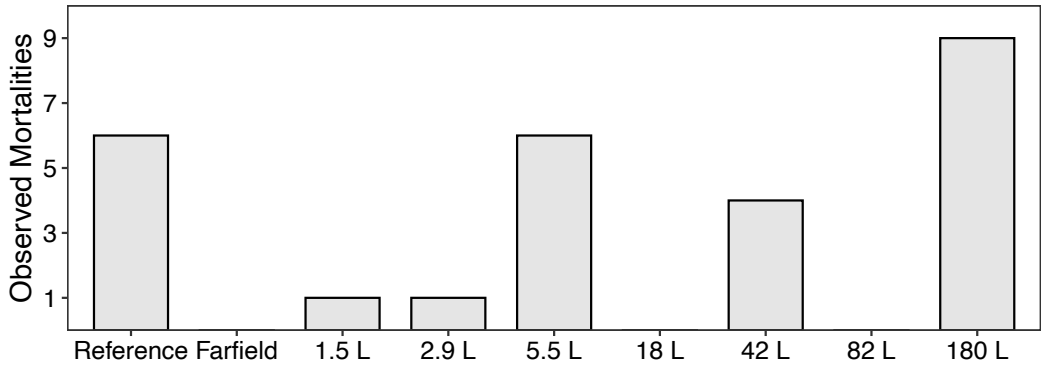

Figure 3.6: Total number of mortalities observed throughout the exposure period and during recapture of fish at the end of the experiment.

own source of stress and compound the effects of existing stressors (Wendelaar Bonga 1997). There are a lot of outlying factors that affect our ability to make conclusions based on these retrieval rates, however it is important to note that there were no live fish recaptured from the three highest treatments. In this case it is possible that the combined stress of exposure to dilbit, confinement, and dietary adaptation led to complete mortality within these enclosures but because trapping methods are not 100% effective there may have been live fish left within the limnocorral after terminating the exposures.

3.3.3 Gallbladder bile fluorescence

Fluorescence of bile from fish retrieved from the lake, reference enclosure, and lowest three treatment limnocorrals display a clear and significant ($p<0.05$) positive trend ($R^2 = 0.58$) at the peak associated with 2-3 ring PACs and increasing volumes of applied dilbit (Fig. 3.7). The naphthalene metabolite 2-naphthol was chosen for these analyses as naphthalene was still present above detection limits in all treatment enclosures at the time of retrievals and has previously been used in freshwater studies to better understand PAC exposures to petroleum sources (Cormier et al. 2000). This method is not necessarily specific to the chosen standard metabolite as metabolites of fluorene and phenanthrene are also known to contribute to the $FF_{290/335}$ peak (Lin et al., 1996). Therefore, the results are expressed as 2- naphthol equivalents. Fluorescence in FSD bile from the 1.5 L and 2.9 L enclosures, which had an average of 169 and 207 µg/L/day TPH respectively, was 2.5-3.5 times that of fish from the reference limnocorral and lake reference. The fish from the 5.5 L enclosure, exposed to a daily average of 309 µg/L TPH, had bile fluorescence that was another 2.3-2.4 times that of the FSD from the 1.5 L and 2.9 L limnocorrals.

Bile fluorescence is a good method of detecting PAC exposure in fish within 3-5 days of exposure and has been verified using HPLC-MS (Pampanin et al., 2016). Bile fluorescence

assessments allow for more rapid assessment of recent PAC exposure and are relatively inexpensive (Lin et al. 1996). It has been used over the last 25 years in this capacity, though for the majority of the experiments it was conducted on larger bodied fish than FSD. The method provides results that correlate well with upregulation of *cyp1a*, a known biomarker for exposure to PACs and indicator of phase I metabolism of xenobiotics (Aas et al., 1998, 2000) but has been primarily used in cod, flounder and other marine fish species (Aas et al. 2000; Pampanin et al. 2014). Other studies have examined freshwater fish including rainbow trout (*Oncorhynchus mykiss*), white sucker and common carp (*Cyprinus carpio*; Barra et al., 2001; Cormier et al., 2000). Our results are similar to other studies examining freshwater fish exposed to real world contaminated site measurements when expressed as relative increases in bile fluorescence compared to reference groups (Barra et al., 2001; Cormier et al., 2000). Small-bodied fish present a challenge for applying this method because of the size of their gallbladders. In larger species, an exact volume of bile can be extracted from the gallbladder and then solution added until the volume and dilution needed for the test are achieved. Small-bodied fish such as FSD have such low-volume

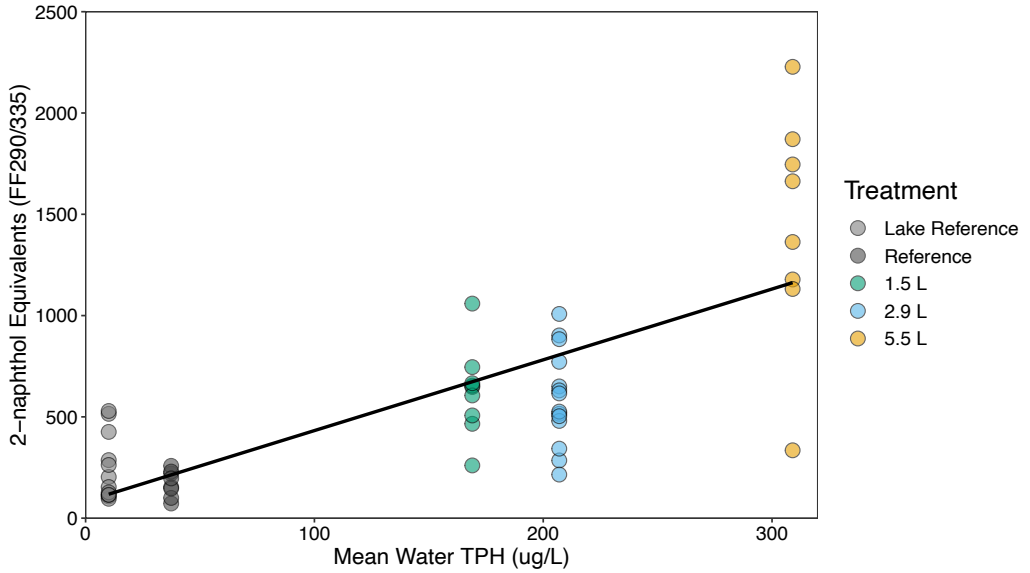

Figure 3.7: Bile fluorescence of finescale dace from the lake reference and limnocorrals. There is a significant trend (linear regression, $R^2 = 0.58$) of increasing fluorescence with increasing mean water total petroleum hydrocarbons.

gallbladders that it is necessary to use the entirety of the bile in the gallbladder to be above detection limits at the volume needed to run in the spectrometer. Future studies should consider pre-weighing all tubes prior to collecting gallbladders to minimize calculation errors during dilution.

3.3.4 Morphometrics

The morphometrics measured in the fish recovered from the lowest volume limnocorrals do not indicate any changes in health associated with increasing TPH exposure. FSD body condition was significantly lower in females compared to males across all treatments and there is no significant linear trend between increasing TPH exposure and a change in condition. The 1.5 L and 5.5 L treatments have fish with significantly lower body condition than the lake reference (ANOVA and Tukey Post-Hoc $p<0.05$; Fig. 3.8A). Other studies have shown decreasing condition in marine tilefish with increasing presence of biliary metabolites (Snyder et al., 2019). These fish burrow and interact with the sediment leading to increased PAH exposure compared to other marine species in the years following the Deepwater Horizon spill. A decreasing trend in condition ocurred primarily between 500 and 1500 μg fluorescent aromatic compounds (FACs) per gram bile (Snyder et al. 2019). It has been suggested that this may be due in part to the increased energetic cost associated with metabolizing PACs (Snyder et al. 2019). The relatively unchanged condition of FSD exposed to dilbit in the current study compared to the fish in the reference limnocorrals suggests that additional demand for metabolising PACs was not a significant change in energy output for these animals and that they were able to maintain nutrient intake.

The HSI for males was significantly lower than females in all treatments, but there were no significant differences between treatments ($p<0.05$; Fig. 3.8B). There was also no significant trend in HSI with increasing TPH exposure in the limnocorrals ($R^2=0.17$). Sheepshead minnows,

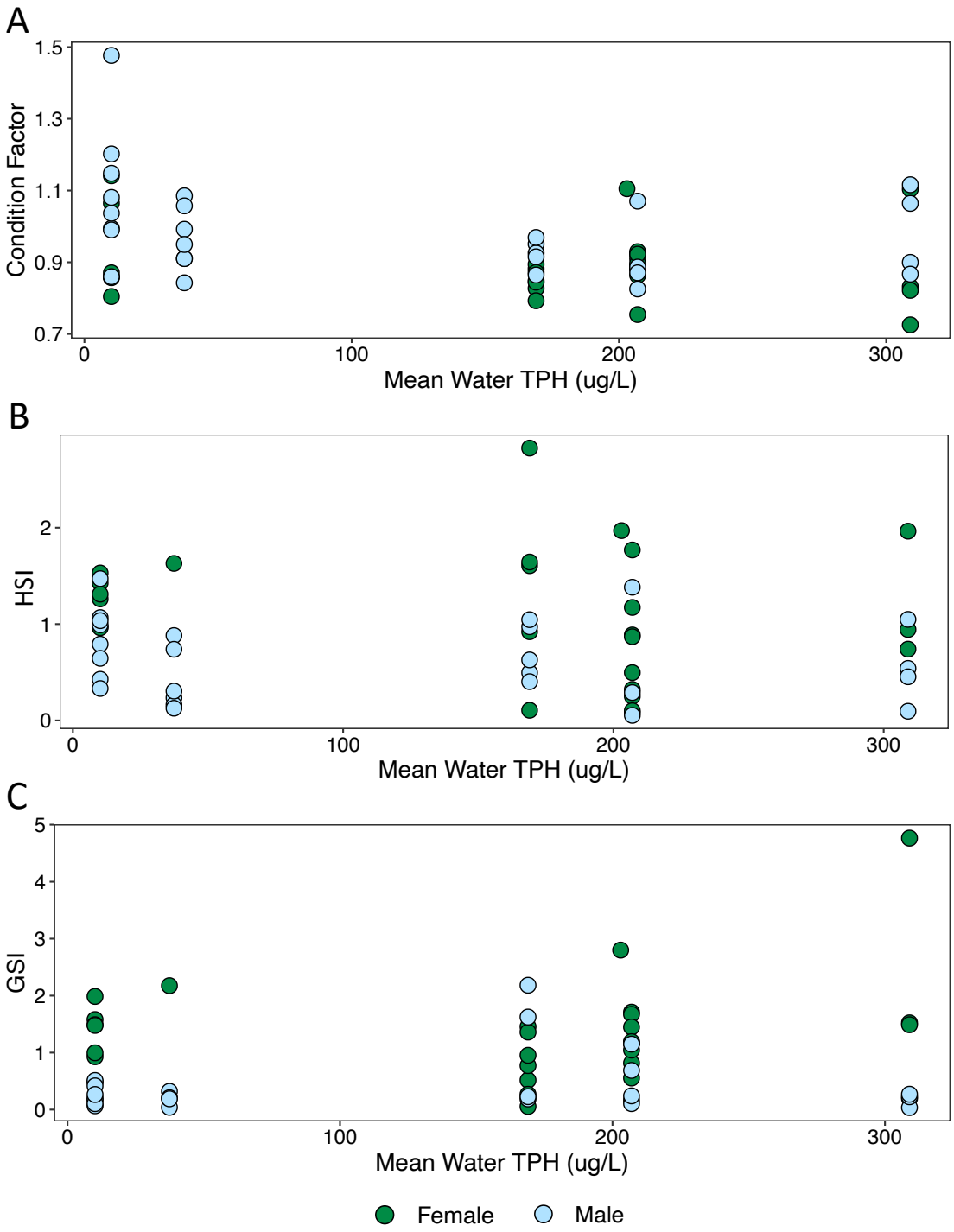

Figure 3.8: **A**. Condition factor in adult finescale dace (FSD). There was no significant change based on increasing exposure to total petroleum hydrocarbons (TPH) within the enclosures (linear mixed model, p=0.950). **B**. The female FSD had significantly higher hepatosomatic indices (HSIs) than the males (ANOVA, p=0.002) but neither sex has a significant change in HSI with increasing TPH (linear regression, p= 0.320, R^2=0.18). **C**. There was a non-significant increasing trend in the gonadosomatic index (GSI) of FSD with increased exposure to TPH (linear mixed models, p=0.320).

an estuarine species in the Gulf of Mexico, exposed to a dilution of high energy WAF with 1.7 μg/L TPAHs also did not exhibit significant changes in HSI, but a second exposure with 17 μg/L TPAHs caused a significant increase in HSI (Jasperse et al. 2019). However, this level of exposure is over 50x the highest treatment from which I recovered live fish in the BOREAL limnocorrals and approximately equal to the highest applied treatment. Field studies of fish in the Alberta Oil Sands Region have found that, when exposed to oil sands processed waters, the relative liver size of fish increases; however, there have also been cases that report no change, similar to what I have observed here, at much higher exposure levels (2.8 μg/L TPACs; Tetreault et al., 2003; van den Heuvel et al., 1999).

As expected, male FSD had significantly lower GSI than females (ANOVA, $p<0.05$; Fig. 3.8C). There was a non-significant increasing linear trend in GSI based on increasing TPH exposure ($R^2 = 0.25$; $p=0.73$). Sheepshead minnows exposed to a high energy WAF (HEWAF) had changes in fecundity of adults, primarily lower egg production and less fertilization, but this was not accompanied by any significant change in GSI (Jasperse et al. 2019).

3.3.5 Gill histology

Basal epithelia of the secondary lamellae in the gills from fish in all enclosures were significantly thinner than those from the lake reference site (ANOVA and Tukey Post Hoc, $p<0.001$) but there was no effect related to TPH concentration or sex on basal epithelial width (LMM; $p=0.867$, $p=0.262$, SI). A thinner basal epithelium in all fish confined to limnocorrals compared to the lake reference (Fig. 3.9) was unexpected because thicker basal epithelia are often associated with poor water quality or the presence of gill irritants (Leino & McCormick, 1993), low pH (Leino et al., 1987), or abrupt cooling (Farrell et al., 2004). Petroleum compounds

including PACs as well as heavy metals are known gill irritants (Kavanagh et al., 2013; Laurent & Perry, 1991). Previous studies have shown that irritation from PAC exposure primarily results in thickening of the basal epithelium in other minnow species (FHM; Farrell et al., 2004; Kavanagh et al., 2013).

This unexpected result is likely unrelated to the addition of dilbit as there is no significant difference between the basal epithelial widths in reference fish and all fish from the treated limnocorrals (ANOVA and Tukey Post Hoc, $p>0.3$). Hypoxia has been shown to cause thinning in the basal epithelium of the secondary lamellae of teleosts (Harper and Wolf 2009) however, the amount of dissolved oxygen in the enclosures was not significantly different from the lake reference (ANOVA, $p=0.086$). Changes to temperature and pH are also known to cause irritation of the secondary lamellae but result in swelling, not thinning, and there was no significant difference in either between the lake reference and the enclosures (ANOVA, $p>0.99$). Average conductivity, however, was significantly lower in the lake reference compared to all enclosures (ANOVA, $p<0.05$). Conductivity provides an estimate of the concentration of ions in the water

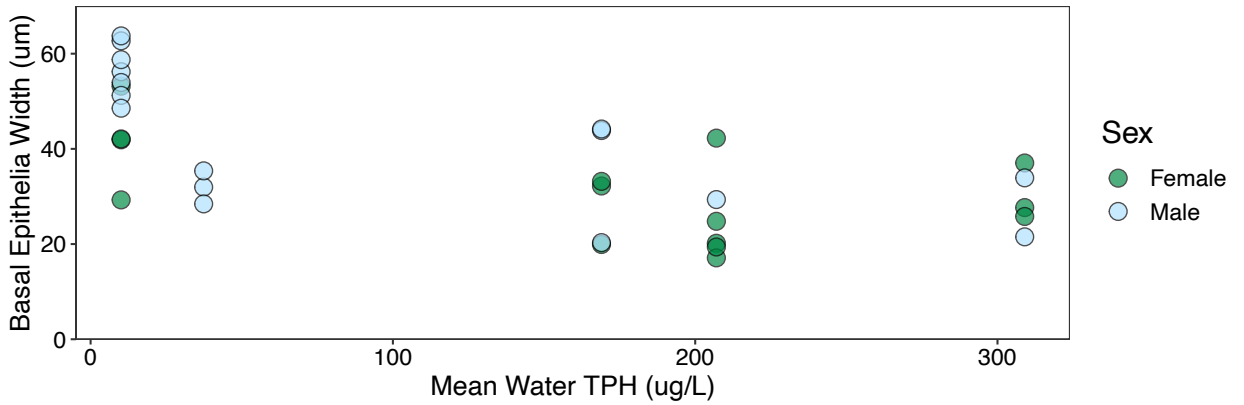

Figure 3.9: Mean basal epithelia width on the secondary lamellae of gills for finescale dace from the lake reference (10.7 µg/L total petroleum hydrocarbons (TPH)) and the limnocorrals. All fish from limnocorrals have significantly thinner basal epithelia than those from the lake (ANOVA, $p<0.05$) but there is no significant trend with increasing TPH within the limnocorrals (linear mixed model, $p=0.867$).

including dissolved salts. The primary function of the basal epithelium in the secondary lamellae is ion and gas exchange, however if a gill response associated with increased ions or salinity did occur, it would be expected that the layer would thicken in order to better regulate the flow through the gills (Blair et al., 2016; Harper & Wolf, 2009). Another possible cause for universal thinning amongst the limnocorrals would be associated with hyperventilation as a reaction to confinement and handling stress. Stress has been shown to have a significant effect on the gills, particularly the basal epithelia on the lamellae as noradrenaline acts as a branchial vasodilator during secondary stress response in the gill filaments (Mallat 1985). Additionally, reversable gill remodelling – in the form of epithelial thinning in cyprinids exposed to physical stressors, specifically hypoxia – has occurred in several species including goldfish (*Carassius auratus*) and crucian carp (*Carassius carassius*; Nilsson, 2007). Thus, it is possible that continuous stress from confinement in the limnocorrals after handling, caused the gills to react and adapt as though they were in a hypoxic environment.

3.3.6 Liver histology

Females from all treatments, including the lake reference, had a higher HVI than males by a significant margin (LMM, p=0.029). There was no significant difference between the lake reference and the unoiled reference limnocorral (ANOVA, p=0.999) and there was no significant linear relationship between increasing TPH exposure and HVI either as a total of all fish or delineated by sex (LMM, p=0.383; Fig. 3.10). HVI is inversely related to hepatocyte size and level of vacuolization in the liver tissue, which is directly related to glycogen levels (Nero et al. 2006). Glycogen levels and vacuolization both tend to decrease in the liver when fish are malnourished but has been shown to increase when the liver is metabolizing certain toxicants (Wolf and Wolfe 2005). Hepatocyte hypotrophy is indicative of a secondary inflammatory response in the liver,

often caused by microsomal enzyme induction after exposure to certain xenobiotics (Nero et al., 2006; van den Heuvel et al., 1999).

The liver does not come into direct contact with petroleum compounds like the gills but remains vulnerable to the effects of contaminants due, in part, to the efficiency of enterohepatic cycling (Harper and Wolf 2009). Goldfish exposed to oil sands process-affected water, which contains many of the same PACs and petroleum hydrocarbons as dilbit, had a higher percentage of hypertrophic hepatocytes than those exposed to water from a reference location (Nero et al. 2006). Other studies on oil sands processed waters (OSPW) have found that in some cases the hypertrophy was enough to affect the liver size resulting in higher HSIs in exposed fish (Van Den Heuvel et al. 1999). Results from the BOREAL project study indicate that there was no significant change in HVI between treatments and thus no evidence of an altered hypotrophy or vacuolization response related to dilbit exposure.

3.3.7 Gonad histology

Perinucleolar oocytes were by far the predominant cell type in ovaries of fish from this study. There was no significant difference in the proportions of different oocyte stages with increasing

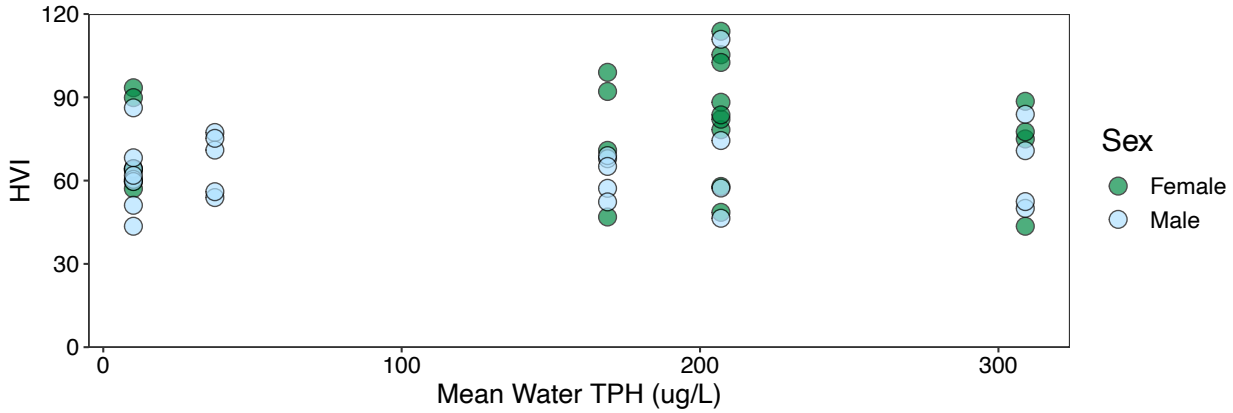

Figure 3.10: Hepatocyte volume index (HVI) of finescale dace from the lake reference (lowest total petroleum hydrocarbons (TPH)) and all limnocorrals. HVI is inversely related to hepatocyte size and number of vacuoles. There was no significant linear trend in hepatocyte size based on TPH exposure (linear mixed model, p=0.490).

daily TPH exposure (OLR, p=0.175; Fig. 3.11A; SI-II). There was a non-significant trend of increasing perinucleolar oocytes and decreasing cortical alveolar or vitellogenic oocytes with increasing TPH exposure (Fig. 3.11B). There was also no significant difference in the size any oocyte stage with increasing TPH exposure. However, there was a nonsignificant trend towards smaller oocytes in fish exposed to greater TPH (LMM, p=0.0569, SI). Although the increasing TPH concentration had no significant overall effect on the size of oocytes, how each stage was affected by increasing TPHs was significantly different (LMM, $p<0.001$, Fig. 3.12, SI-V). The nonsignificant decreasing trend in size is most prominent in the more developed stages.

The relatively undeveloped nature of the oocytes in FSD from this study is not surprising because this species spawns in early- to mid-spring when the water is ~15°C. Fish from this study were collected in late August and early September, well after their spawning season when the ovaries would have been in an early stage of recrudescence (Stewart and Watkinson 2004). The non-significant trend towards more perinucleolar stage oocytes with increasing TPH could be indicative of a trend towards delayed development with increasing dilbit exposure. PACs can inhibit vitellogenesis in wild and laboratory fish and may have an antiestrogenic effect by interfering with the aryl-hydrocarbon receptor (reviewed in Nicolas, 1999). This may be partially responsible for the trend toward decreasing oocyte size in more developed cells. In general, PAC exposure decreases maturity and fecundity of female freshwater fish (reviewed in Nicolas, 1999). These effects are most pronounced in fish interacting with the sediment, as some small-bodied freshwater fish (e.g. FHM) do during foraging (Stewart and Watkinson 2004). Some studies on specific PACs including phenanthrene have shown a similar trend of increasing the proportion of perinucleolar to vitellogenic cells with increased exposure levels in adult FHM (Loughery et al., 2018).

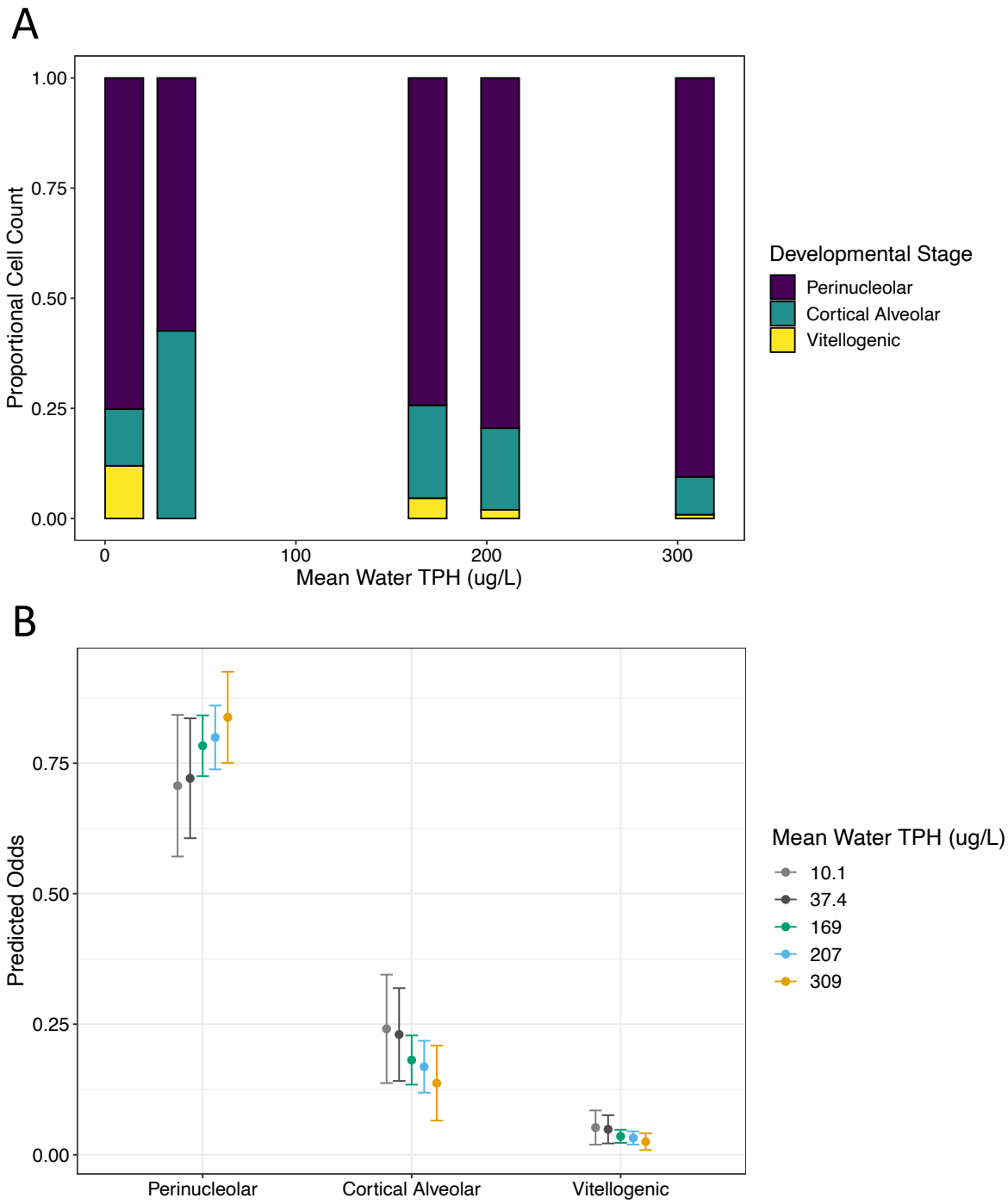

Figure 3.11: **A**. The proportional distribution of oocyte stages within finescale dace from the lake reference and dilbit treated limnocorrals. Each exposure level has more perinucleolar cells than any other stage, which is indicative of undeveloped ovaries. **B**. The predicted odds for occurrence of each stage across increasing levels of TPH exposure (ordinal logistic regression, p=0.175). There is no significant difference in the odds of any one stage occurring between treatments, but in general there are low odds of more developed cells (cortical alveolar and vitellogenic) being present in each treatment. Please note that results from the reference enclosure (37.4 µg/L) are representative of a single fish.

Although less developed cells in ovaries from fish exposed to higher levels of TPH is supported by previous studies, it is important to note that the extremely high proportion of perinucleolar cells exists in all fish, including the reference enclosure and lake reference. It is possible that this effect would have been more significant during or immediately following the spawning season when a larger proportion of vitellogenic and cortical alveolar cells would be expected. It is also possible that this is not an effect related to TPH exposure at all. There is less than complete consensus regarding the effects of petroleum compounds on fish reproduction. Despite the previously mentioned studies reporting deleterious effects on oocytes of fish exposed to PACs, other studies on OSPW, which contain PACs including naphthenic acids (13 mg/L), have shown no deleterious effects on female reproduction after chronic exposure (Van den Heuvel et al., 2012).

The potential impacts of confinement stress may have masked any effects of TPH exposure with respect to adult female reproductive success indicators. Confinement and handling stress impact female fish reproductive health primarily early during spawning or when the gonads are at peak maturity (Schreck et al. 2001; Jeffries et al. 2012) and the collection of the FSD for the project occurred at least one month post spawning. The deployment collection occurred in June and with

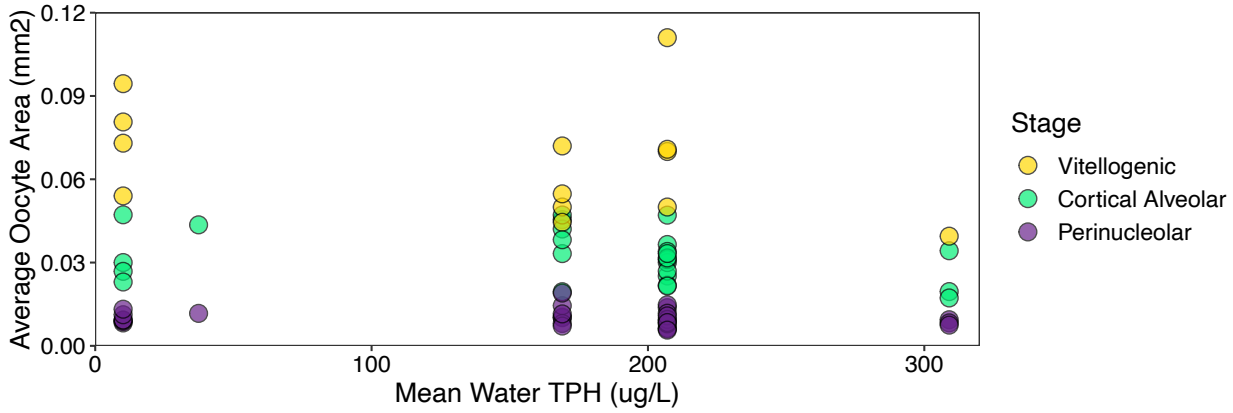

Figure 3.12: Average size of oocytes from finescale dace in the lake reference and dilbit treated limnocorrals. The reference enclosure (37.4 µg/L) has results from only one fish. There is a non-significant trend toward smaller cells with higher exposure to total petroleum hydrocarbons (linear mixed model, p=0.057).

exposures lasting until late August to early September. It is, however, possible that the nonsignificant trend in decreasing oocyte size that is most prevalent in the vitellogenic and cortical alveolar cells could be related to or extrapolated by stress as it would be expected that this would be most clear in more mature oocytes (Schreck et al. 2001).

Similar to the female gonad histology, there is no significant trend in the development of the testes in relation to increasing TPH exposure, based on the ratio of lumen area to the seminiferous tubule (p=0.987, Fig. 3.13, SI-VI). The male gonads were also relatively undeveloped, with the majority of the cells being spermatocytes. A high proportion of spermatocytes and small lumens are indicative of a regressed germinal epithelium, which again is not unexpected because the spawning season of FSD had been over for months at the time of dissection. The stage of testes development is an important endpoint to study in PAC and TPH

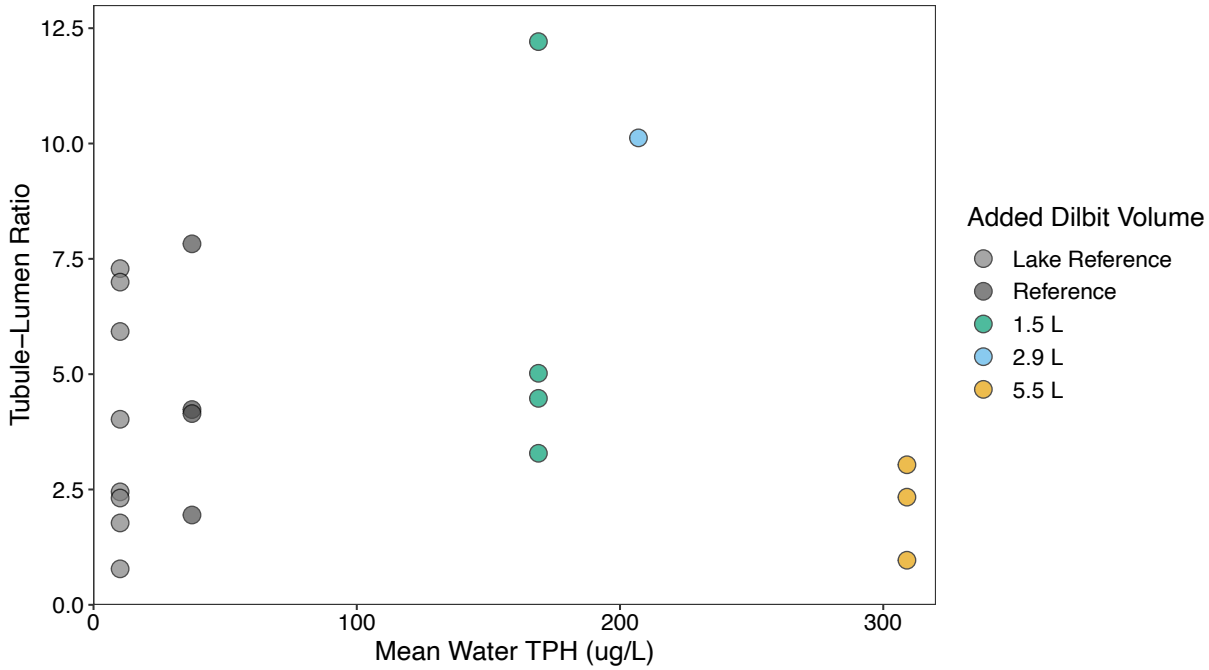

Figure 3.13: Tubule-lumen area ratios for the testes of male finescale dace in the lake reference and treatment limnocorrals. There is only one male fish from the 2.9 L limnocorral. Each point represents the average of all measured ratios for one fish. There is no significant trend in these ratios with increasing mean daily TPH exposure (linear mixed model, p=0.987).

exposures. Phenanthrene exposure in particular can lead to a disproportionate increase in the presence of spermatogonia relative to other stages (Loughery et al. 2018). Naphthenic acids (NAs) and other compounds associated with OSPW had mixed effects on male yellow perch, in some cases leading to a reduction in testes size but fish from the same study in a different pond having insignificantly more developed testes (13 mg/L NAs; (Van den Heuvel et al., 2012). It is possible that these effects may have been more evident in our exposed FSD if they were collected at peak fertility, however it is equally possible that the effects from the higher doses in these historical studies do not exist at these lower volume exposures.

3.4 Summary and Conclusions

There have been few studies that have examined the effects of model oil spills on wild fish species. The BOREAL project was designed to assess the potential impacts of dilbit exposure on the whole ecosystem over a range of simulated spill volumes. In general, survival was greatest among FSD, the sentinel small bodied freshwater fish selected for this study, in limnocorrals treated with lower volumes of dilbit. Among those limnocorrals where fish were successfully recaptured, there were no significant trends in deleterious effects on overall health and reproductive fitness related to increasing volumes of TPH exposure. However, there were trends towards smaller and less developed oocytes and smaller liver cells in FSD exposed to higher TPH concentrations. Any trends associated with reproduction may have been clearer in mature rather than regressed gonads, which is why I recommend that future studies focus on small-bodied fish species that are fractional spawners (e.g. FHM) while assessing the effects of oil exposure on gonad maturation. Due to the low CPUE in limnocorrals with higher TPH and the few subtle effects found in FSD exposed to lower simulated spill volumes I would recommend future studies on the effects of dilbit spills on wild freshwater fish focus on a range between 250-600 μg/L TPH

and 500 to 1000 ng/L TPAC. This will allow for better identification of the threshold of mortality as well as assessing whether the nonsignificant deleterious trends found in this study continue in higher exposures.

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Chapter 4: Summary and Lessons Learned

4.1 Summary and Lessons Learned

Historically, the majority of studies on the effects of oil spills on fish have been conducted in marine environments, but in the last decade there has been increased concern regarding the potential for releases in freshwater and the impact on the associated ecosystems (Lee et al. 2015). This continues to be a topic of concern due in part to regular industrial and government investment in pipelines in Canada and the United States (e.g. Keystone XL and Transmountain TMX). Increasing the number of pipelines crossing North America will in turn increase the risk of a spill occurring and entering freshwater. This thesis provides information to address knowledge gaps in our overall understanding of the effects of potential oil spills on freshwater fish populations. Firstly, in Chapter 2 I addressed the potential effects of low-level oil residues, equivalent to residual oil remaining in the aquatic environments after a spill, on the health of a sentinel small bodied fish species. In Chapter 3 I monitored effects of spilled diluted bitumen on fish using a regression of spill sizes representative of the 50^{th} to 99^{th} centile of spills occurring in North America over the past decade.

The pilot study described in Chapter 2 was designed to examine potential health and reproductive effects of wild adult fathead minnows (FHM) exposed to environmentally relevant levels of dilbit in a lab setting. Despite very low-level exposures, statistically significant changes in the fish were observed, including lower body condition, smaller cortical alveolar cells in females, and larger lumens in males. The effects were different between sexes, which agrees with the literature where effects on gonad development are inconsistently reported between sexes of fish across multiple studies (Nicolas 1999; Tetreault et al. 2003; Van den Heuvel et al. 2012). However, all experimental groups including the control, had lower body condition compared to

free ranging fish in the lake. Mortality was also high, and no spawning events occurred among fish split into breeding trials. Ultimately, this led us to the conclusion that wild fish exhibiting signs of stress may have been more susceptible to the toxicity arising from even these very low concentrations of dilbit. Despite the modest dilbit concentrations, these wild fish were more susceptible to toxicity due to the compounded stress of adapting to captivity. The potential for oil spills to affect fish populations are often judged based on the results of toxicity tests using laboratory cultures of fish but the effects of multiple stressors may be overlooked when not testing on these more sensitive wild fish populations.

The studies in Chapter 3 examined the impacts of oil exposure and multiple stressors by releasing adult finescale dace into 10 m diameter limnocorrals installed in the littoral zone of a boreal ecozone lake. Free ranging fish are likely subject to less confinement stress than the fish held in aquaria in Chapter 2, but an average of ~20% mortality still occurred in the limnocorrals, suggesting that some confinement stress was still evident. Recapture rates were higher among lower level exposures (<250 TPH) compared to treatments with more added dilbit (>600 TPH) but mortalities were not significantly correlated with increasing dilbit exposure. The histology metrics and health indices of fish from the lower level exposures did not show any significant trends associated with increasing TPH exposures, although the female gonads did display trends towards underdevelopment. There was evidence in the gills that, although these fish were being kept in an environment much more similar to their natural habitat, the limnocorrals themselves may have had an effect on the respiratory health of the finescale dace. The enclosure effects in this case may have been more a factor of forced dietary adaptation than actual confinement stress, or an interactive effect. Zooplankton populations, the main food source for finescale dace, were almost entirely depleted in all treatments one week prior to FSD addition,

which would have forced a significant shift in nutrient sourcing for the fish. This may have compounded the effects of the TPAC exposure as there is an increased energetic cost associated with metabolizing them (Snyder et al. 2019). Despite the presence of these enclosure effects, this study provides important information on the effects of oil spills on freshwater fish. Laboratory studies have found that an EC_{50} for fathead minnow embryo malformation is 500 μg/L TPH (Alsaadi et al. 2018) but that the 96 hr LC_{50} is in the mg/L TPH range (Barron et al. 2018). However, no fish were recaptured at concentrations above 500 μg/L/d TPH exposure in adult specimens, suggesting deleterious effects in adult wild specimens at much lower concentrations than predicted by laboratory studies. Future studies should focus on examining effects in wild fish at concentrations between 300 and 600 μg TPH /L/day, which is the lower end of the range that resulted in no recaptured fish during this study. This is especially true for wild fish experiencing multiple stressors. Future work should also include histological assessments of reproductive health. Such assessments would be particularly useful if they examined effects on fractional spawners like the fathead minnow during the spawning period when effects on development of maturing oocytes could be determined.

A particularly challenging aspect of using wild fish is that endpoints related to handling and confinement stress can be very similar to those associated with exposure to PAC contamination (Wendelaar Bonga 1997; Nicolas 1999; Lee et al. 2015). To minimize the effects of stress, prolonging exposure periods should be avoided to minimize confinement and/or a change in food availability, which can confound the apparent effects of target contaminants. Despite this, it remains important to study the effects of contaminants on these wild populations due to their higher sensitivity relative to laboratory strains (Wendelaar Bonga 1997). Although caged wild fish have been historically used successfully in contaminants studies (e.g. Doebel et al. 2004;

Klaverkamp et al. 2006) the negative effects of confinement are well known (e.g. Campbell et al. 1992, 1994; Wendelaar Bonga 1997; Schreck et al. 2001; Jeffries et al. 2012) and can have a negative synergistic effect with PACs on the survival of freshwater fish (Farrell et al. 2004). There are cycles of stress throughout the lives of freshwater fish arising from food limitation, overwintering, the physical strain of a spawning season, or pressure from climate change and other anthropogenic generated stressors. It is important to account for the compounded effects of multiple stressors in oil spill studies, as these effects may exist in the context of a real spill site.

Working with wild fish presents challenges due to their sensitivity, but it is precisely this attribute that justified our studies and that makes the results important for application to real spill scenarios. Research into the effects of oil spills on freshwater communities is essential in order to increase understanding of the potential impacts and subsequently inform more efficient response methods in the event of real world accidental releases. This need has been assessed and reviewed by numerous groups including the National Academy of Sciences, Fisheries and Oceans Canada, and the Royal Society of Canada as a high-priority area in toxicological research during this coming decade (Lee et al. 2015; The National Academies of Sciences Engineering and Medicine 2016; Canadian Science Advisory Secretariat 2017). By addressing this knowledge gap, this thesis underlines the importance of continued monitoring of spill sites after the completion of remedial strategies due to the potential for cellular level reproductive effects, especially where extraneous stressors may have increased vulnerability in the fish populations. It also serves to remind risk assessors of the importance of accounting for these environmental and perceived stressors in spill scenarios, and offers insight into the ranges of spill volumes with the potential to cause total mortality versus those that will have low-level effects that may be more difficult to quantify. The

results from this thesis will help to direct future research by identifying the potential for even low-level dilbit exposure to affect sensitive wild populations when they are under extraneous stressors.

4.2 References

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Supplementary Information

I. Results from statistical analyses. All intercepts are the reference tank, except in the case of condition factor where the comparisons are between the lake reference and all tanks. All analyses are ANOVAs except in the case of oocyte counts where the analysis was a Kruskal-Wallis test followed by a Wilcoxon rank sum test.

Sex	Exposure	Estimate	Standard Error	t-value	p-value
Condition Factor					
Female	Intercept	1.16	0.01	162.86	0.000*
	Reference	-0.26	0.05	-5.46	0.000*
	Very Low	-0.20	0.04	-4.50	0.000*
	Low	-0.32	0.04	-8.80	0.000*
Male	Intercept	1.24	0.01	180.26	0.000*
	Reference	-0.20	0.04	-5.65	0.000*
	Very Low	-0.25	0.03	-8.03	0.000*
	Low	-0.33	0.04	-9.40	0.000*
HSI					
Female	Intercept	1.06	0.22	4.93	0.000*
	Very Low	0.50	0.28	1.79	0.093
	Low	0.20	0.26	0.77	0.452
Male	Intercept	1.02	0.13	7.91	0.000*
	Very Low	0.33	0.17	1.93	0.065
	Low	0.07	0.18	0.38	0.710
HVI					
Female	Intercept	162.02	16.55	9.79	0.000*
	Very Low	-28.37	21.67	-1.31	0.209
	Low	13.17	21.67	0.61	0.552
Male	Intercept	103.54	9.83	10.53	0.000*
	Very Low	30.79	12.87	2.39	0.029*
	Low	33.55	12.87	2.61	0.019*

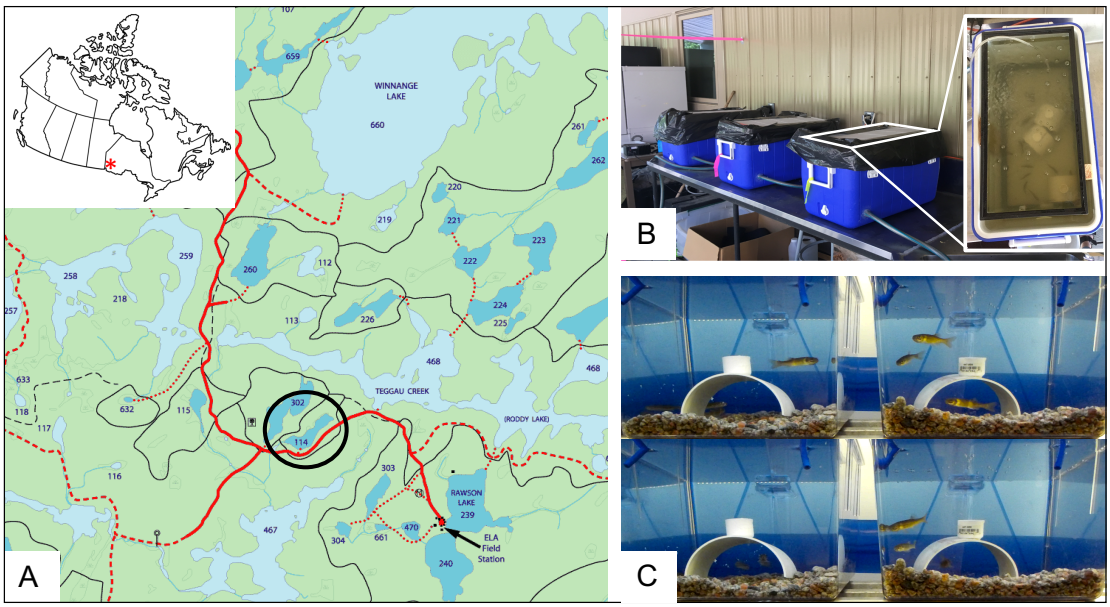
Oocyte Measurements					
Stage	**Exposure**	**Estimate**	**Standard Error**	**t-value**	**p-value**
Perinucleolar	Intercept	161.37	4.03	40.00	0.000*
	Very Low	-0.29	7.97	-0.04	0.971
	Low	-9.60	5.76	-1.67	0.096
Cortical Alveolar	Intercept	319.39	6.50	49.11	0.000*
	Very Low	-30.74	15.59	-1.97	0.053
	Low	-57.06	10.09	-5.66	0.000*

Oocyte Counts		
chi-squared	**df**	**p-value**
13.92	2	0.001*
Reference-Low		0.021*
Reference-Very Low		0.001*
Low-Very Low		0.099

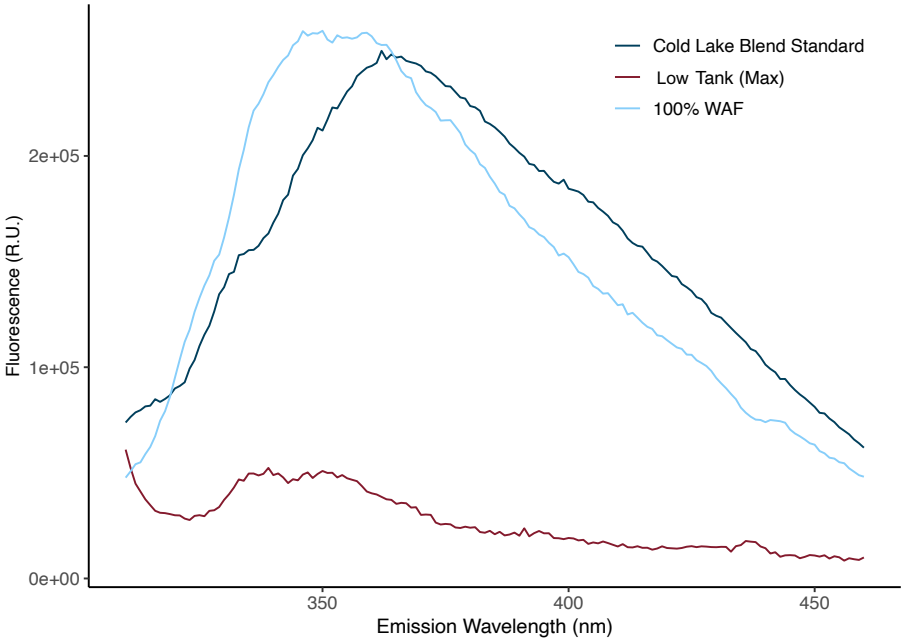
Testes				
Exposure	**Estimate**	**Standard Error**	**t value**	**p-value**
Intercept	0.06	0.01	6.13	0.000*
Very Low	0.03	0.01	1.93	0.060
Low	0.06	0.01	4.49	0.000*

Relative mRNA Expression					
Gene	**Exposure**	**Estimate**	**Standard Error**	**t value**	**p-value**
cyp1a	Intercept	1.04	0.13	8.37	0.000*
	Very Low	-0.19	0.18	-1.10	0.281
	Low	-0.04	020	-0.22	0.831
gst	Intercept	1	0.17	5.88	0.000*
	Very Low	-0.05	0.23	-0.21	0.834
	Low	0.17	0.23	0.74	0.463

P Value Significance: <0.05

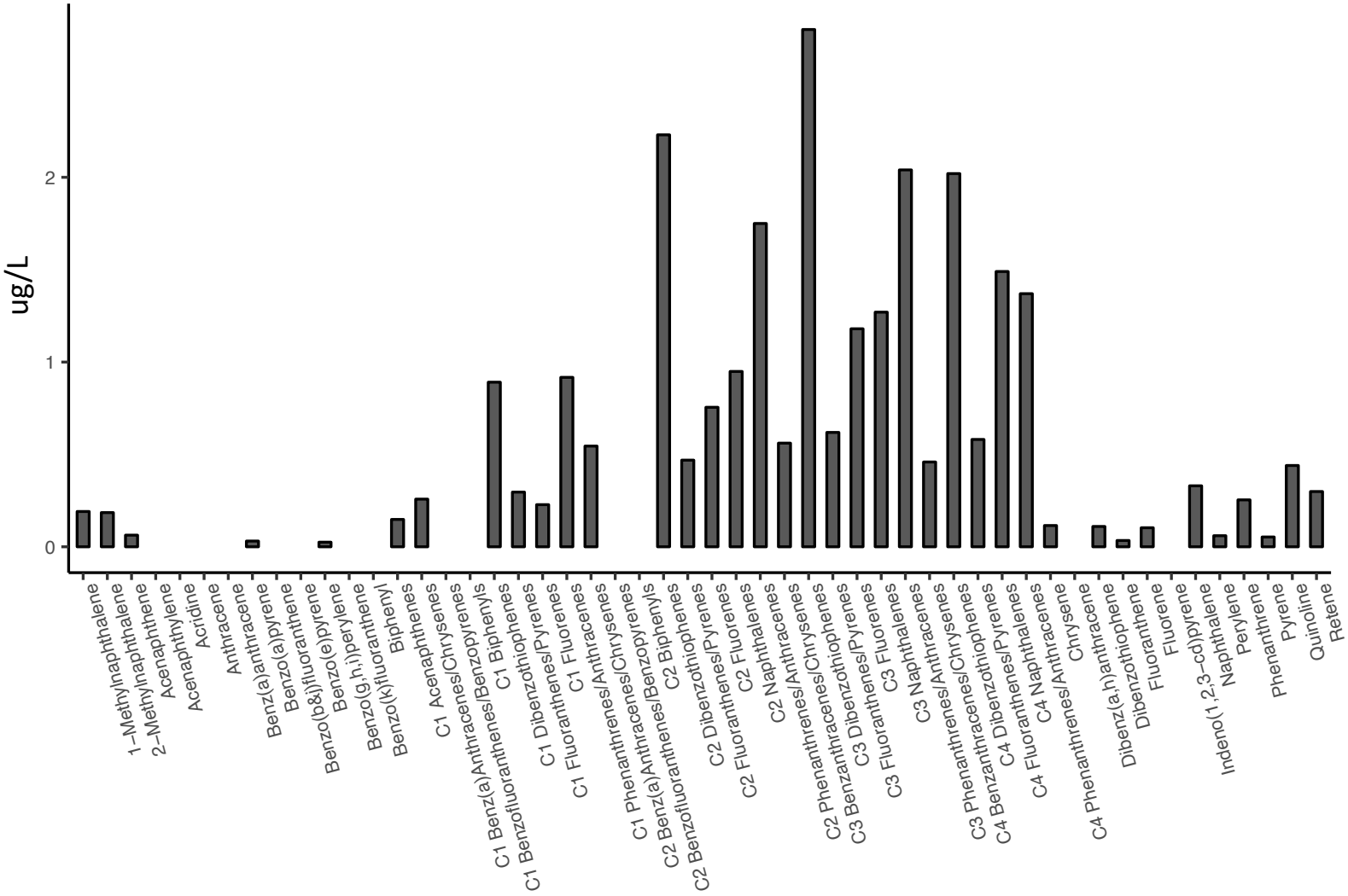


II. Images of the project location and set up. A. Map of the southern section of the IISD-Experimental Lakes Area – location in Canada shown in the inset – with the source lake for fathead minnows circled in black and the ELA field station where the experiment was conducted indicated with a black arrow. B. The acclimation and exposure cooler set up. Tanks were kept inside of coolers to keep the temperature more consistent and minimize external stimuli. Cold water was pumped in between the tanks and the coolers to provide additional temperature control. C. Recovery tanks were kept in a temperature controlled constant flow bioassay unit where the fish were split into breeding triplicates and filmed for 15 minutes a day for spawning behaviour. No spawning events occurred.



III. Spectrofluorescence results for an older Cold Lake Blend Winter (CLB-W) dilbit standard, 100% water accommodated fraction (WAF) collected at the end of the experiment, and the one occasion where there was a detectable signal from the 1:100 000 (Low) tank. The WAF sample has enhanced lower wavelength (lower

molecular weight) components compared with the CLB-W standard. This is consistent with more soluble polycycle aromatic components preferentially transferring to water during the mixing needed to create a WAF.



IV. Gas chromatograph mass spectrometer results from the total polycyclic aromatic compounds analysis. Analytes are listed left to right in alphabetical order

V. Equations for bile calculations from Chapter 3

Step	Associated Equation	Significant Figures
Weigh original tube and full gallbladder (CT_{fullGB})	NA	5
Add 400 uL 50:50 H_2O:CH_3OH	$CT_{GB+50:50}$ (g) = A + 400 uL H_2O:CH_3OH	5
Reweigh the tube with 50:50 solution ($CT_{GB+50:50}$)	NA	5
Vortex then centrifuge (60 s @ 20,000 rpm)	NA	NA
Transfer bile/solution supernatant to a new tared tube and weigh (Ext_{Bile})	NA	5
Air dry the original tube and empty gallbladder and reweigh ($CT_{emptyGB}$)	NA	5
Calculate extracted bile weight	$B_{wt} = CT_{fullGB} - CT_{emptyGB}$	5
Use known density of 50:50 H_2O:CH_3OH* to determine the dilution factor (DF) of Ext_{Bile}	$DF = Ext_{Bile} + ((CT_{GB+50:50} - CT_{fullGB}) / 0.9272\ g/mL) / Ext_{Bile}$	3
Calculate the amount of Ext_{Bile} needed for a dilution of 1:850 in the 2100 uL cuvette	$Vol_{Bile} = (2100 \times DF)/850$	3
Calculate the amount of 50:50 H_2O:CH_3OH needed to complete the dilution	$Vol_{50:50} = 2100 - Vol_{Bile}$	3

* Density of 56:44 Methanol:Water from Perry's Chemical Engineers Handbook

VI. Statistical results from Chapter 3

Condition Factor
ANOVA

	DF	Sum Squares	Mean Squares	F Value	p-value
TPH Concentration	5	0.210	0.042	3.413	0.010
Sex	1	0.109	0.109	8.887	0.004
Residuals	49	0.603	0.012	-	-

Linear Regression

R^2 = 0.19, Adj = 0.13	Estimate	Standard Error	t-value	p-value
Intercept	0.88	0.06	13.83	0.000
TPH Concentration	-0.00	0.00	-0.07	0.950
Sex	0.08	0.07	1.03	0.310
Sex by TPH Concentration	0.00	0.00	0.01	0.990

Hepatosomatic Index
ANOVA

	DF	Sum Squares	Mean Squares	F Value	p-value
TPH Concentration	5	3.117	0.624	2.265	0.066
Sex	1	2.870	2.870	10.423	0.002
Sex by TPH Concentration	4	1.032	0.258	0.937	0.452
Residuals	40	11.012	0.275	-	-

Linear Regression

R^2 = 0.18, Adj = 0.13	Estimate	Standard Error	t-value	p-value
Intercept	1.29	0.21	6.07	0.000
TPH Concentration	-0.00	0.00	-1.01	0.320
Sex	-0.60	0.27	-2.25	0.030
Sex by TPH Concentration	0.00	0.00	0.46	0.650

Gonad Somatic Index
ANOVA

	DF	Sum Squares	Mean Squares	F Value	p-value
TPH Concentration	5	5.073	1.015	2.089	0.086
Sex	1	7.831	7.831	16.12	0.000
Sex by TPH Concentration	4	6.450	1.612	3.319	0.019128
Residuals	41	19.917	0.486	-	-

Linear Regression

R^2 = 0.25, Adj = 0.21	Estimate	Standard Error	t-value	p-value
Intercept	1.21	0.29	4.17	0.00
TPH Concentration	0.00	0.00	0.35	0.73
Sex	-0.88	0.38	-2.35	0.02
Sex by TPH Concentration	0.00	0.00	0.15	0.88

Bile Fluorescence

Linear Regression (Standard Curve)

R^2 = 1.00, Adj = 1.00	Estimate	Standard Error	t-value	p-value
Intercept	1.21	0.56	2.15	0.05
Standard	0.07	0.00	95.55	0.00

ANOVA

Log Transform	DF	Sum Squares	Mean Squares	F-value	p-value
TPH Concentration	1	24.43	24.43	89.36	0.00
Residuals	49	13.40	0.273	-	-

Linear Regression

R^2 = 0.58, Adj = 0.57	Estimate	Standard Error	t-value	p-value
Intercept	96.96	80.69	1.20	0.24
Standard	0.07	0.00	95.55	0.00

Hepatocyte Volume Indices

Linear Mixed Model

	Estimate	Standard Error	t-value	Chi Squared	DF	p-value
Intercept	74.25	6.94	10.7	-	-	-
TPH Concentrations	0.02	0.04	0.55	0.477	1	0.490
Sex	-10.67	8.62	-1.24	6.12	1	0.013*

Gill Basal Epithelial Widths

ANOVA

	DF	Sum Squares	Mean Squares	F Value	p-value
TPH Concentration	4	22060	5515	37.62	0.000*
Sex	1	2490	2490	16.98	0.000*
Residuals	185	27122	147	-	-

Tukey Post-Hoc Test

TPH Concentration (μg/L)	Difference	Lower Limit	Upper Limit	p-value
37.4 - 10.1	-19.24	-28.04	-10.44	0.000*
169 - 10.1	-18.90	-25.72	-12.07	0.000*
207 - 10.1	-25.66	-32.49	-18.84	0.000*
309 - 10.1	-21.98	-29.25	-14.72	0.000*
169 - 37.4	0.34	-9.29	9.97	1.000
207 - 37.4	-6.42	-16.05	3.21	0.356
309 - 37.4	-2.74	-12.69	7.20	0.942
207 - 169	-6.76	-14.62	1.10	0.128
309 - 169	-3.08	-11.33	5.16	0.841
309 - 207	3.68	-4.57	11.92	0.735

Linear Mixed Model (Limnocorrals Only)

	Estimate	Standard Error	t-value	Chi Squared	DF	p-value
Intercept	28.134	5.985	4.700	-	-	-
TPH Concentrations	-0.004	0.024	-0.168	0.0281	1	0.8668
Sex	4.615	4.117	1.121	1.2565	1	0.2623

Oocyte Stage Counts
Ordinal Logistic Regression

Coefficients	**Estimate**	**Standard Error**	**z-value**	**p-value**
TPH Concentration	-0.003	0.002	-1.355	0.175
Perinucleolar | Cortical Alveolar	0.855	0.350	2.441	-
Cortical Alveolar | Vitellogenic	2.877	0.356	8.094	-

Oocyte Stage Measurements
Linear Mixed Model

	Estimate	Standard Error	t-value	Chi Squared	DF	p-value
TPH Concentration	-	-	-	3.63	1	0.057
Stage	-	-	-	1808	2	0.000
TPH By Stage	-	-	-	25.76	2	0.000
Intercept	38066	2334	16.31	-	-	-
TPH Concentration	-33.99	12.44	-2.73	-	-	-
Perinucleolar	-27236	1745	-15.61	-	-	-
Vitellogenic	36798	2332	15.78	-	-	-
TPH : Perinucleolar	27.41	9.15	3.00	-	-	-
TPH : Vitellogenic	-46.02	16.19	-2.84	-	-	-

Testes Lumen : Seminiferous Tubule Ratios
Linear Mixed Model

	Estimate	**Standard Error**	**t-value**	**Chi Squared**	**DF**	**p-value**
TPH Concentration	-	-	-	0.00	1	0.987
Intercept	4.57	0.95	4.79	-	-	-
TPH Concentration	-0.00	0.01	-0.02	-	-	-