Evaluating the photo-enhanced toxicity of diluted bitumen and conventional heavy crude spills to freshwater organisms.

by

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Abstract

The majority of Canada's oil is extracted from the Alberta Oil Sands region, with the main products being diluted bitumen (dilbit) and conventional heavy crude (CHV). These products are transported across North America primarily by pipelines and rail. The effects of these petroleum products on freshwater environments after accidental spills are still poorly understood, even though the risks of oil spills into fresh water are increasing with more pipeline and rail transportation. Information regarding the efficacy of alternative remediation strategies for freshwater environments, particularly shorelines, are also lacking. This thesis addresses the effects of freshwater dilbit and CHV spills alone and in combination with ultraviolet (UV) radiation to evaluate the photo-enhanced toxicity (PET) of these oils. Model oil spills were conducted as a part of the IISD-Experimental Lakes Area (IISD-ELA) Freshwater Oil Spill Remediation Study (FOReSt) to evaluate minimally invasive remediation methods for shoreline environments after oil spills. Species with established sensitivities were chosen to evaluate the water accommodated fraction (WAF) of these oils before and after remediation, including *Hyalella azteca* and wild fathead minnows (FHM; *Pimephales promelas*). Juvenile *Hyalella azteca* were exposed to WAFs of weathered dilbit and CHV with various remediation treatments, including the surface washing agent (SWA) Corexit EC9580A and nutrient additions, referred to as enhanced Monitored Natural Recovery (EMNR). Dilbit and CHV both exhibited PET to *Hyalella azteca* by decreased growth and increasing mortality at lower lethal concentrations, measured as the concentration required to reach 50% mortality (low UV LC_{50s} of 14,162 ng L^{-1} and 4761 ng L^{-1} compared to high UV LC_{50s} of 9466 ng L^{-1} and 484 ng L^{-1} for dilbit and CHV respectively). Individuals exposed to Corexit EC9580A and its active surfactant dioctyl sodium sulfosuccinate (DOSS) incorporated bubbles internally and externally, disrupting buoyancy and increasing mortality. Early life-stages of wild FHMs were also exposed to WAFs of dilbit to evaluate its PET to a wild collected species at lower concentrations after remediation. Three exposure rounds were conducted at increasing post-oil spill intervals (12-, 20-, and 38-days post spill). FHM mortality was similar among all treatments, including the reference treatment, and lethal concentrations did not significantly differ between UV groups, however, the high UV group did have a lower LC_{25}. In the last round, individuals in the high UV group had increased mortality compared to their low UV group counterpart, suggesting increased PET. Individuals in dilbit and dilbit with high UV treatments did exhibit significantly upregulated *cyp1A* expression and increases in malformations, particularly yolk sac edemas, cardiac malformations, and deflated swim bladders, indicating potential PET at sub-lethal levels. However, individuals exposed to the more weathered dilbit in later rounds showed phototoxic trends of increased mortality, upregulated *cyp1A* and thyroid related genes, and increased malformations, indicating dilbit photodegradation and weathering may potentially cause more PET. The results of these studies emphasize the importance of conducting toxicity testing under environmentally-relevant conditions, as real-world factors, such as UV radiation, can impact the toxicity to organisms in natural environments where accidental spills occur.

Dedication

To the brave, resilient people of Ukraine. Thank you for your sacrifices protecting such a beautiful culture I am so proud to share.

Glory to Ukraine

Glory to the heroes

May your memory be eternal

Слава Україні

Героям слава

Вічная Пам'ять

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Table of Contents

List of Figures

List of Tables

Chapter 1: Introduction & Background

1.1 General Introduction

Canada has the fourth largest oil reserves and is the sixth greatest energy producer in the world (Lee et al. 2015; NRCan 2021). Canadian oil production has steadily increased, with current production at over 1.5 times the domestic demand, which is anticipated to climb by 1.27 million barrels per day over the 2.84 million barrels per day currently being produced (CAPP 2019; 2023). One of the main petroleum products of the Canadian oil industry is diluted bitumen (dilbit), which is an extremely viscous unconventional crude oil that is typically diluted by about 30% with a diluent, such as a natural gas condensate, to reduce its viscosity so that it can be transported in pipelines or other transportation modes (Alsaadi et al. 2018b; CAPP 2019). Dilbit is the main product of the Alberta oil sands region (located in northern Alberta and Saskatchewan), which covers approximately 142,200 km^2 and holds an estimated 165.4 billion barrels of bitumen reserves (Government of Alberta 2022; Philibert et al. 2016). Production of bitumen in the Alberta oil sands region surpassed that of conventional oil in 2010 (NRCan 2021). Although dilbit is now produced in greater quantities, conventional crude is still a prominent product and a significant contributor to Canadian oil production and exports (CER 2022).

The majority of dilbit and conventional heavy crude (CHV) are transported through pipelines. As of 2020, over 87% of crude oil exports were transported through pipeline, with rail and marine tanker transport making up the remaining approximately 13% (CER 2023). The transport capacity for Canadian oil pipelines is upwards of 4.3 million barrels per day, with few small spill incidents occurring (CER 2023). Spill occurrences from Canadian crude pipelines are down by approximately 85% since the 1970s, with spill volume from this time also down by 92% (Etkin 2023). However, oil spills do occur, and many pipelines and proposed pipelines are located near freshwater potentially threatening this important resource (Lee et al. 2015; Gray et al. 2002). Another concern is that current post-spill cleanup methods focus on aggressive removal of contaminated soil which increases erosion, damages vegetation and can affect the productivity of littoral regions (Lee et al. 2015; Gray et al. 2002). With more proposed shipping routes and pipelines that border fresh water, there is a need to evaluate new and more effective oil spill clean-up methods. Non-invasive shoreline cleanup methods are needed to eliminate disturbance to sensitive riparian areas that occur with typical cleanup operations. Optimizing methods to treat residual oil that remains after an oil spill cleanup and to assess the potential impacts of residual oil in freshwater systems are both high priorities. In 2015, the Royal Society of Canada (RSC) conducted an expert panel review on the behaviour and environmental impacts of oil in aquatic environments, and identified high priority research needs surrounding oil products (Lee et al. 2015). One of these priorities included understanding the impact of spilled oil in high-risk and poorly understood areas, and conducting controlled field research in these environments to better understand the interactions of oil to advance spill response (Lee et al. 2015). These poorly understood areas include freshwater rivers, lakes, and wetlands, all of which have little information regarding oil spill impacts and remediation measures. Other priorities included increasing the understanding of different oil and spill-control agents, as well as testing remediation methods for freshwater shorelines to ensure rapid recovery and optimum balance between oil cleanup and habitat protection (Lee et al. 2015).

In response to the RSC report (Lee et al. 2015), the International Institute for Sustainable Development Experimental Lakes Area (IISD-ELA) developed the Freshwater Oil Spill Remediation Study (FOReSt) research program to examine the effectiveness of minimally invasive shoreline cleanup methods. The FOReSt project represents a large-scale effort to conduct a chemical and ecological effects assessment on the part of non-government organizations, industry, and the regulatory community. The overall objective of the FOReSt study is to compare the efficacy of non-invasive methods to remediate freshwater shoreline systems and minimize effects to aquatic biota after a spill.

My research is one component of the FOReSt project and examines the effects of both CHV and dilbit and their photo-enhanced toxicity on early-life stages of fish and amphipods by examining growth and development through malformations, length, and gene expression indicative of oil exposure. Additionally, it evaluates the potential effects of shoreline remediation methods on these organisms using these same endpoints. The core purpose of this thesis is to advance scientific knowledge regarding the impacts of dilbit and CHV spills, cleanup and remediation methods, and photo-enhanced toxicity to at-risk early-life stages of fish and amphipods.

1.2 Oil Compositions

Dilbit is a mixture of natural oil sand deposits, called bitumen, and lighter diluents that are added to lower the viscosity to enable transportation. Bitumen has a sand like texture that is too viscous to be transported via pipelines. In Canada, it is extracted from the Alberta oil sands region through one of two processes: open pit mining or in-situ production via steam injections (NRCan 2016). The majority of the Canadian oil sand reserves, approximately 80%, are in deep deposits that require in-situ removal, while the other 20% are accessible via surface open-pit mining (NRCan 2016). To transport bitumen, it is typically diluted with a natural gas condensate at a 30% diluent to 70% bitumen ratio (Lee et al. 2015). These diluents contain a higher proportion of low molecular weight compounds that lowers the viscosity and density, and ultimately changes the chemical composition of the final dilbit product (Lee et al. 2015). The compositions of the bitumen and diluent are generally known, however the composition information and mixture ratios of these dilbits vary due to cost and availability. This results in different dilbits with varying compositions, meaning general response plans do not necessarily apply to all products (Lee et al. 2015; Madison et al. 2017). Cold Lake Blend (CLB) dilbit is the most common blend transported in Canada and is approximately 30% diluent by mass (King et al. 2015).

There are various types of crude oil produced within Canada, including multiple conventional and unconventional types (Lee et al. 2015). Crude oils can also be split into different categories, including light, medium and heavy crude oils, based on their physical and chemical properties (Conmy et al. 2017). Crude oils contain thousands of different compounds, but they can all be categorized into four chemical classes: saturates, aromatics, resins, and asphaltenes (SARA;Lee et al. 2015). Saturates are hydrocarbon molecules with the maximum number of hydrogens bound to the carbons (Lee et al. 2015). They comprise the majority of the compounds present in light and medium crude oils, but in heavy crude oils and bitumen, they make up a much smaller proportion (Conmy et al. 2017). Saturates are lighter, volatile, biodegradable, and are the least water soluble, meaning they are generally considered to be less toxic (Lee et al. 2015). Aromatics, including monoaromatics and polycyclic aromatic compounds (PACs), have low molecular weights and are associated with acute localized toxicity, that is usually brief and localized in the area of the oil spill and weathering (Dupuis & Ucan-Marin 2015, Lee et al. 2015). Monoaromatics are the most water-soluble hydrocarbons, and most notably include benzene, toluene, ethylbenzene and xylenes (BTEX), which can move via diffusion through biological membranes and impart acute toxicity after a spill (Lee et al. 2015). Polycyclic aromatic compounds (PACs) are a group of organic compounds with two or more fused benzene rings arranged in various configurations (Zhang et al. 2016). PACs can also include fused rings and include alkyl groups, which are called alkylated PACs, the structure of which makes them more persistent than monoaromatics (Dupuis & Ucan-Marin 2015; Lee et al. 2015). Alkyl PACs are particularly concerning because of the associated bioaccumulation risk due to reduced water solubility, which increases their fat or lipid solubility (Lee et al. 2015). In oil, alkyl PACs with 3-5 rings are present in amounts greater than their parent compounds, and they are the primary driver of chronic toxicity (Lee et al. 2015; Hodson et al. 2007; Martin et al. 2014; Adams et al. 2014). Conventional crude oils typically have a lower proportion of alkylated PACs compared to oil sands products like bitumen or dilbit (Yang et al. 2011). The final two components, asphaltenes and resins, have higher molecular weights, and therefore thought to be less toxic due to lower water solubility and bioavailability. These four SARA chemical classes contribute to the viscosity, specific gravity, and biodegradation rates of oil, and when you compare these categories to dilbit, it is most similar to CHVs based on BTEX composition, polycyclic aromatic hydrocarbons (PAHs), sulphur and metals (Lee et al. 2015; Zhou et al. 2015).

1.3 Fate & Behaviour of Oil in the Environment

CHV and CLB dilbit have varying chemical composition and physical properties. As such, how they interact in the environment also varies with changing compositions and how the product weathers. Weathering refers to how an oil is degraded by exposure to sunlight, waves, weather, and microorganisms in the environment that causes physical and chemical changes to the oil (Lee et al. 2015). The physical, chemical, and biological properties of the receiving environment water body can also affect how spilled oil changes over time (Lee et al. 2015). Density, salinity, temperature, and viscosity are particularly important when it comes to how oil behaves in the aquatic environment, including fresh waters (Lee et al. 2015). These parameters drive the majority of weathering processes, including evaporation, spreading, dispersion, photodegradation, emulsification, biodegradation, and sedimentation (Zhong et al. 2022). Evaporation is when light weight compounds, such as condensates, evaporate to the atmosphere causing up to 75% of mass losses (NRC 2003; Yang et al. 2017). Evaporation is rapid and usually the most immediate weathering process. Spreading is when oil moves unhindered on the surface of water, which can lead to dispersion, which is when oil droplets become suspended in water (Lee et al. 2015). Photodegradation is the reaction of PACs, oxygen, and ultraviolet (UV) radiation to produce oxygenated products that are typically more water-soluble and resistant to biodegradation (Yim et al. 2012). Smaller PACs are more biodegradable and susceptible to evaporation and less susceptible to photodegradation, whereas the opposite is true for larger PACs with more aromatic rings (Lee et al. 2015). Photodegradation occurs earlier in the weathering process, within fresh oil, more than in weathered oil (Yim et al. 2012). Emulsification is when physical mixing like waves causes oil-in-water or water-in-oil mixtures to form. Biodegradation is when living organisms break down organic material in the oil, and sedimentation is the process where oil becomes submerged through weathering processes and eventually sinks (Lee et al. 2015). These processes can occur in order, simultaneously, or not at all, depending on environmental conditions, geography, oil properties, and other factors (Yang et al. 2017).

The density of oil is ultimately determined by the composition of the oil. For mixtures like dilbit, this will depend on the ratio of diluent to bitumen, with greater proportions of asphaltenes and resins creating more dense compositions. When oil interacts with an aquatic environment, the lighter compounds (e.g. aromatics and alkanes) in the oil evaporate, leaving a greater proportion of heavier compounds, like resins and asphaltenes. This causes the oil to become more dense, and in heavy oils that have initial densities that are already close to water, the increase in density causes them to sink (Stoyanovich et al. 2021). Weathering can increase the density of oil by approximately 7-8% from fresh oil, and evaporation alone can cause oil to sink, depending on the proportion of lower weight compounds present (Zhou et al. 2015). To compare oil types, the industry standard is to use American Petroleum Institute (API) gravity, which is the inverse measure of density (Lee et al. 2015). Oils with an API gravity >10% will float on freshwater at 15°C, therefore when oil density falls below this, it will sink.

Temperature also alters the behaviour of oil in freshwater environments, because the density of water is driven by temperature. The density of water is 1 g mL^{-1} at 4℃, and decreases at both higher and lower temperatures, with ice being the least dense. Freshwater has an API gravity of 10% API at 15°C, which is approximately 0.9990 g/mL (Lee et al. 2015). Freshwater density decreases even more when higher summer temperatures are reached, notably 0.9982 g/mL at 20°C and 0.9970 g/mL at 25°C. This temperature-density relationship is also what drives stratification, lake turnover and mixing. If mixing occurs when oil is present in a waterbody, this can drastically change its fate in that system. Mixing, either in the water column or on the surface, can cause emulsification to occur, which is when oil-in-water or water-in-oil suspensions form (Lee et al. 2015). Temperature driven mixing into colder, less oxygenated regions can also limit the degradation of the oil (Lee et al. 2015). Temperature can also impact microbial communities that drive the biodegradation of oil. There are naturally occurring microbial communities and microorganisms that consume and degrade hydrocarbons in the environment (Lee et al. 2015). The optimal temperatures for these organisms in freshwater is thought to be 20 to 30℃, but can persist and continue to degrade hydrocarbons in cooler environments (Brakstad & Bonaunet 2006; Kristensen et al. 2015).

Salinity and dissolved oxygen also play a role in the weathering and fate of oil. Higher salinity increases the density of water, causing oil to sink slower than in freshwater. Sinking in saline waters tends to be in droplets instead of entire slicks as in freshwater (King et al. 2014; Stoyanovich et al. 2021). However, salinity also has an inverse relationship with the solubility of PACs.

Therefore, while changes in salinity to a system may alter microbial community composition and hinder biodegradation, PACs in a higher salinity environment may remain more bioavailable, which facilitates biodegradation rates of oil (Ulrich et al. 2009; Ward & Brock 1978; Whitehouse 1984). Dissolved oxygen works similarly in the sense that aerobic conditions stimulate biodegradation (Lee et al. 2015). Aerobic conditions are particularly important for long-term biodegradation after hydrocarbons have undergone sedimentation (Lee et al. 2015).

Viscosity dictates how oil spreads across the water surface and its resistance to dispersion. Greater proportions of low molecular weight aromatics in an oil lowers viscosity, whereas heavier asphaltenes and resins increase the viscosity (Lee et al. 2015). The viscosity of dilbit is higher than light and medium conventional crude oils because of its greater concentrations of asphaltenes and resins, but is similar to heavy conventional crude oil (Zhou et al. 2015). However, the diluent found in dilbit has a much higher volatility, meaning much of it evaporates quite rapidly and changes the viscosity of the oil (Stoyanovich et al. 2021; Zhou et al. 2015). This can have strong connotations for spill response because different responses are required for sunken oil.

Photodegradation is another method of weathering and breakdown of oil in the environment and includes photic interaction in the form of UV radiation and oxygen with the oil (Lee et al. 2015). Photodegradation causes the breakdown of aromatic portions of the oil, creating higher proportions of resins and asphaltenes (Maki et al. 2001; Lee et al. 2015). This process also increases the oxygen content of the oil, and this oxygenation makes the biodegraded products increase in polarity, and therefore become more bioavailable, and potentially more toxic (Lee et al. 2015). This interaction also produces more water soluble and resistant products (Lee et al. 2015). However, the bioavailability and toxicity of by-products is dependent on the oil composition and the impacted species sensitivity (Lee et al. 2015). Based on these reactions, the likely concentration of these oxygenated products in the environment would be low, but they do have the potential to cause harm when this reaction occurs within aquatic organisms (Lee et al. 2015; Conmy et al. 2017; McConkey et al. 2002).

1.4 Oil Toxicity

Water accommodated fractions (WAFs) refers to oil components, including hydrocarbons and small oil droplets, that have dissolved into the water column by stirring and/or mixing (Lee et al., 2015). PACs and BTEX compounds present in the WAFs constitute the majority of toxicity to fish, despite representing a small percentage of oil mass (Carls et al. 2008; Lee et al. 2015). For example, the average volume of BTEX found in CLB dilbit and CHV is $1.06 \pm 0.17\%$ and $0.93 \pm 0.14\%$ respectively (ECCC et al. 2013; CrudeMonitor 2023). Both PACs and BTEX are associated with acute and chronic toxicity, mortality, narcosis, reduced reproduction, and various pericardial, craniofacial, and spinal malformations in aquatic organisms, along with being known carcinogens (Lee et al. 2015; Madison et al. 2015; Madison et al. 2017; Barron 2017; Incardona et al. 2014). These petroleum compounds and their associated impacts can also cause lower fish survival, fecundity, and lower recruitment, contributing to declining populations (Alsaadi et al. 2018b). Heterocyclic aromatics, or aromatics that have carbons substituted with a different atom (commonly sulphur, nitrogen, or oxygen), have been shown to exhibit embryotoxicity (e.g., S-containing dibenzothiophene; Rhodes et al. 2005), but additional studies are required to determine how sulfur, oxygen, or nitrogen in aromatic rings change toxicity (Alsaadi et al. 2018a). Low molecular weight compounds, such as the two-ringed PAC naphthalene, are more likely to cause acute necrosis and lethality, but they evaporate quickly as oil products weather (Redman & Parkerton 2015). This means the acute toxicity effects from low molecular weight compounds can be brief and localized, depending on where oil enters the water body and the weathering process (Lee et al. 2015). Larger and heavier oil constituents, like chrysenes, are less bioavailable and more likely to persist in the system and are more associated with chronic toxicity (Lee et al. 2015; Lin et al. 2015).

PACs exert their effects via several pathways. Among these, aryl hydrocarbon receptor (AhR) binding and associated developmental toxicity and oxidative stress is a major driver of toxicity (Alsaadi et al. 2018b). Genes that are regulated in this pathway include phase I xenobiotic metabolism (cytochrome P450/*cyp1A*), phase II biotransformation enzymes (glutathione S-transferase/*gst*), and oxidative stress defence enzymes (superoxide dismutase/*sod*, catalase/*cat*; Alsaadi et al. 2018b; Holth et al. 2014; Kim et al. 2013; Olsvik et al. 2012). Specifically, the expression of cytochrome P4501A (*cyp1A*) is strongly correlated with exposure to dilbit and crude oils in fish, because *cyp1A* is associated with initial metabolism (Madison et al. 2015; Madison et al. 2017; Carls et al. 2005). This correlation makes it a useful and sensitive biomarker of embryotoxicity after oil exposure and PAC accumulation (Alsaadi et al. 2018a; Carls et al. 2005). The alkylated congeners of PACs, for example phenanthrenes, more readily bind to the AhR, making them more likely to upregulate *cyp1A* (Barron et al. 2004; Billiard et al. 2002). The expression of *cyp1A* is quite sensitive to PAC exposure, activated at total PAH concentrations as low as 0.4 μg/L (Madison et al. 2017) and CLB bitumen concentrations as low as 3.5 μg/L (Alderman et al. 2017). This increased *cyp1A* expression following oil exposure can result in blue sac disease (BSD), which can cause impaired cardiac function and yolk sac malformations (Billiard et al. 1999; Madison et al. 2017). BSD is characterized by a yolk sac edema that appears blue, due to edema fluid with high concentrations of proteins that fluoresce (Alsaadi et al. 2018b). Additionally, early life stages of fish have limited PAC metabolism capacity, leading to increased concentrations of dissolved PACs and *cyp1A* expression from those PACs that are metabolised (Incardona 2017). Alternatively, adult and larger juvenile fish more readily detoxify and eliminate PACs from their system (Jung et al. 2015). Therefore, embryotoxicity is an important and sensitive life stage to evaluate, for the sensitivity in this stage as well as developmental toxicity that can continue into adulthood.

PAC exposure can also have an adverse effect on the development and function of the thyroid gland in fish (Movahedinia et al. 2018). Thyroid hormones (TH) are essential for regulating development, growth, metabolism, reproduction, and behavior in fishes (Movahedinia et al. 2018; Johnson & Lema 2011; Porazzi et al. 2009). Therefore, a disruption in normal thyroid function can lead to issues in any of these areas, which is why it is important for thyroid function to be examined. Normally, the thyroid synthesizes and secretes TH when it is stimulated by the hypothalamus (Movahedinia et al. 2018). The hypothalamus, influenced by environmental factors, induces the pituitary gland to secrete thyroid-stimulating hormones, which activates synthesis of thyroxine (T4, four iodine residues) and triiodothyronine (T3, three iodine residues) in the thyroid gland

(Movahedinia et al. 2018). Produced by the thyroid gland, T4 is converted into T3 in target tissues, most importantly in the liver, but also in some other peripheral tissues (Hersikorn & Smits 2011). In most freshwater fish, T4 concentrations are typically greater than T3 concentrations in eggs, which is why evaluating both THs is important (Tagawa et al. 1990). It has been reported that PACs (e.g. benzo[a]pyrene; Movahedinia et al. 2018) can significantly decrease plasma T4 levels and the circulating and tissue levels of thyroid hormones through interference with thyroid gland function, change in the structure of the thyroid gland leading to hormone synthesis disruption, thyroid hormone metabolism, or by attaching to the thyroid hormone binding proteins in the bloodstream (Movahedinia et al. 2018; Teles et al. 2005).

Changes to TH availability, metabolism, and disposal may be caused by changes in hormone-converting iodothyronine deiodinase enzyme activity (Deal & Volkoff 2020). In teleost fish, there are 3 types of iodothyronine deiodinase enzymes that regulate TH levels; type I (*dio1*), type II (*dio2*), and type III (*dio3*) (Köhrle 1999, 2000; Orozco & Valverde-R 2005). These thyroid-related transcripts (as well as *thrα* and *thrβ*) are found in high levels in fathead minnow (FHM) eggs 2-3 days post-fertilization (dpf), suggesting that THs are maternally transferred (Vergauwen et al. 2018). The products *dio1*, *dio2* and *dio3* each have a different role in regulating TH activity via activation and inactivation. In fish, *dio2* works to convert T4 to T3 by removing iodine from the outer ring (activation), while *dio3* removes iodine from the inner ring to convert T4 and T3 to the inactive forms (inactivation), such as reverse triiodothyronine (rT3) and diiodothyronine (T2) (Johnson & Lema 2011). The role of *dio1* in fish is more difficult to define, but is thought to play a role in catalysis of both outer- and inner-ring deiodination (Johnson & Lema 2011; Orozco & Valverde-R 2005; Deal & Volkoff 2020). In fish, it is known that deiodinase activity is sensitive to stress, hormones (e.g. 17*β*-estradiol), nutrition and feeding, as well as various environmental contaminants, including metals, pesticides, polychlorinated biphenyls, and PACs (Picard-Aitken et al. 2007; Brown et al. 2004). Overall, PAC exposure can cause decreases in the level of circulating and tissue TH, which could be caused by direct thyroid toxicity or interfering with TH protein expression and *dio2* synthesis (Boas et al. 2009; Duntas 2015; Rurale et al. 2022).

The thyroid receptors (TRs) *thrα* and *thrβ* are also important to thyroid signalling and mediation of T3 and T4 (Power et al. 2001; Mortensen & Arukwe 2006) and are responsible for initiating thyroid hormone action (Vergauwen et al. 2018). In adults, the expression of *thrα* and *thrβ* is tissue specific, suggesting they might hold different functions (Liu et al. 2000; Power et al. 2001; Deal & Volkoff 2020). However, in early-life stage fish, the expression of *thrα* and *thrβ* has been detected prior to the maturation of the thyroid gland (Power et al. 2001; Liu et al. 2000). The TR *thrα* is expressed earlier and at greater levels, remaining high through hatch, whereas *thrβ* peaks at hatch and decreases after (Liu et al. 2000; Power et al. 2001). These different isoforms are present in different tissues at differing times due to their roles in development, *thrα* being predominant in heart and brain development, whereas *thrβ* is more predominant in muscle, kidney, and liver development (Deal & Volkoff 2020). The expression of these TRs also appears to be sex- and species-specific, with ovaries having higher levels compared to testis in developing FHMs (Filby & Tyler 2007). In FHMs, *thrα* is crucial for both ovary and testis development, but *thrβ* may only be required for ovary development, or be required in lower amounts for testis development (Filby & Tyler 2007). When exposed to various oil compounds and PACs, there is the potential for fish, specifically early-life stages of fish, to experience adverse effects to thyroid receptors, such as reduced *thrα* and *thrβ* transcription, which has the potential to disrupt the function of thyroid hormones (Sun et al. 2008). Both *dio* and *thr* genes have been used to demonstrated impact to the thyroid and development systems in fish exposed to varying pollutants (Picard-Aitken et al. 2007; Morgado et al. 2009; Mortensen & Arukwe 2006; Johnson & Lema 2011; Truter et al. 2017; Mousavi et al. 2022; He et al. 2012).

1.5 Photo-enhanced Toxicity

Photo-enhanced toxicity (PET) is the interaction between a contaminant and UV radiation that causes it to become more toxic (Barron 2007; Barron et al. 2018; Little et al. 2000). It occurs when an organism takes up photoreactive compounds and is then exposed to light sources with the right quantity and quality of UV radiation (Barron 2007). The extent of PET varies based on the type and concentrations of a contaminant and the conditions of the environment affecting the attenuation, quantity, and quality of UV radiation (Barron 2007). The attenuation of UV radiation is affected by factors such as turbidity, dissolved organic matter, and morphometry (Tartarotti et al. 2022; Williamson 1995). In clear lakes, over 10% of UVB radiation can penetrate to substantial depths (7-20 m), resulting in a zone of influence weighted toward shallower, near shore habitats (Tartarotti et al. 2022; Williamson 1995). Decreasing wavelengths result in decreased UV transmittance, with UVB (280-320 nm) having greater attenuation than UVA (320–400 nm) and visible light (400-700 nm) (Barron et al. 2000; 2008). However, the degree of PET is dependent on the spectrum of UV, with wavelengths in the UVA region (320–400 nm) being most associated with PET in aquatic organisms (Barron 2017). Because of this, the PET of PACs is a function of the product of tissue residues of PACs and UVA dose (Ankley et al. 1997; Barron 2017). The quality and quantity of UV radiation also varies spatially and temporally, with the risk for PET greatest at solar noon, the time of day where the sun is at its highest point in the sky, which varies spatially and seasonally (Barron et al. 2000). Solar noon also has the lowest loss of UV through surface reflection (Barron 2017). Even with sun angles that are ideal for PET and photodegradation, this weathering process is slow and typically accounts for $< 0.1\%$ of total losses per day (WSP Canada 2014). However, because photodegradation produces oxidized products that have the potential for PET, this means this relatively small contribution to weathering can have significant contributions to overall toxicity impacts (Lee et al. 2015).

UV exposure alone in the natural environment can impose stress to biota, even before the addition of a pollutant (Tartarotti et al. 2022). The interaction between the two can result in significantly higher mortality rates, reduced growth, and increased malformations (Barron et al. 2008; 2018; 2021). In petroleum-based products, UV radiation can interact with specific PACs in vivo, increasing the toxicity of those specific compounds (Barron 2007). Certain 3-5 ring PACs, including anthracenes, fluoranthenes, pyrenes, and benzopyrenes, are responsible for PET due to their ability to interact and absorb specific wavelengths of incoming UV to generate radicals even at part per billion concentrations (Barron 2017).

PET is not limited to petroleum-based products, as many chemicals can exhibit PET, including drugs (e.g., tetracyclines), pesticides (e.g., diquat), natural compounds (e.g., citrus oils, psoralens;

Barron 2007; 2017). These compounds share the ability to be activated by UV radiation through photodegradation/photomodification/photooxidation or photosensitization (Barron 2017). Photodegradation breaks down a contaminant into various oxidized compounds, which can exhibit greater toxicity than the parent compound (Barron 2017; McConkey et al. 2002). These oxidized compounds can by themselves cause malformations, oxidative stress, and mortality in aquatic organisms (Barron 2017; Knecht et al. 2013; Lampi et al. 2006). Photosensitization differs from photodegradation by accumulating a contaminant into an organism's biological membranes and then modifying it upon exposure to UV radiation (Barron 2017; Little et al. 2000). This occurs when incoming UV energy from specific wavelengths is absorbed by the PACs, causing an excitation of an electron in the outer orbital to a higher energy orbital (Barron 2017). This energy is passed to oxygen molecules, which causes the formation of reactive oxygen species or free radicals, which can cause oxidative damage, lipid peroxidation, cell death, and ultimately, with prolonged exposure, mortality (Barron 2017; Barron et al. 2018).

The attributes of aquatic organisms are also important determinants of the potential for PET. Those organisms with the highest potential sensitivity to PET include small organisms with transparent or semitransparent life stages, such as the early life stages of invertebrates and fish (e.g. *Hyalella azteca* and FHMs; Barron 2017; Barron & Ka'aihue 2001). Translucent organisms are at a high risk because of their lack of pigmentation and armouring, and an epidermis of only a few layers that allows for substantial UV penetration, causing vital internal organs and tissues to be targeted (Barron 2017; Barron et al. 2018). Behavioral avoidances, such as sediment burial or daily migration, may reduce UV exposure, and therefore reduce the risk of PET (Barron 2007). Overall, the risk of PET may be reduced if a species possesses characteristics that protect it from UV radiation, such as pigmentation, UV absorbing compounds, or avoidance behaviours (Hatch & Burton 1999a).

Toxicological studies, especially laboratory studies, often occur in isolation, controlling for environmental factors, meaning few of these studies include the impact of UV radiation and PET. It is important to test the toxicological effects of dilbit and CHV paired with UV exposure, considering natural environments where spills may occur would have unavoidable UV exposure. Without the inclusion of PET in toxicity testing, mortality estimates, projected lethal concentration values, spill response plans and impact assessment models can underestimate the additive or synergistic impact of PET to at-risk species (Barron 2017). Additionally, many toxicants have not had their PET evaluated in freshwater systems, including various oil constituents and mixtures. These in tandem pose significant concerns for at-risk organisms, for their individual populations as well as their significant roles in food-webs and within ecosystems. Therefore, it is important that environmentally-relevant PET studies be included in testing protocols to produce accurate and reliable toxicity information to include in spill response plans, impact assessment models and environmental policy (Barron 2017). Having environmentally-relevant interactive studies will help regulatory agencies recognize the complexity of nature and contribute to the development of appropriate environmental policy.

1.6 Study Setting

The Experimental Lakes Area (49°40′N, 93°44′W, now IISD-ELA), in the boreal forest region of Northwestern Ontario, Canada, was established in 1969 to serve as a natural laboratory for studies relevant to the Great Lakes. IISD-ELA has special provincial and federal legislation that designate 58 small lakes in the area for whole lake and ecosystem scale science, experimentation, and long-term ecological monitoring. For more than five decades, IISD-ELA has used large ecosystem level studies to research human impacts on freshwater, including evaluating the effect of algal blooms (Findlay & Kasian 1987), mercury (Sandilands et al. 2005), acidification through acid rain (Schindler et al. 1980), artificial estrogen (Kidd et al. 2007), and climate change on freshwater ecosystem health (Guzzo & Blanchfield 2017). Each of these studies, although conducted in single or few lakes, have been internationally recognized as more influential than laboratory tests, due to evaluating the entire ecosystem simultaneously (Schindler 2009). These studies have had a profound influence on North American policy related to aquatic resource management (Schindler 2009). For these reasons, the IISD-ELA is an optimal facility to conduct and answer large scale research questions related to oil spill remediation.

Lake 260 (49°42′N, 93°46′W) at the IISD-ELA was chosen as the site of multiple large scale oil spill projects, including the FOReSt project, previously described, the companion FLOating Wetlands To Enhance Remediation (FLOWTER), and the Boreal lake Oil Release Experiment by Additions to Limnocorrals (BOREAL) projects. The FLOWTER project was designed to optimize the microbiome associated with floating wetland rhizomes to enhance degradation of oil to develop non-invasive strategies for remediation of oil spills. BOREAL was conducted in 2018 prior to the FOReSt project and evaluated the fate and effects of dilbit spills in 10 m diameter pelagic mesocosms sealed to the lake bottom (Rodriguez-Gil et al. 2021).

Lake 260 is an oligotrophic boreal lake that has an area of 330,000 m^2, a volume of 2,000,000 m^3, an average depth of 7 m, a maximum depth of 16 m, and is approximately 370 m above mean sea level (Brunskill & Schindler 1971). It has bedrock geology that is quite resistant and does not contribute much to overall water chemistry (Brunskill & Schindler 1971). The bedrock also makes it relatively hydrologically isolated, with an outflow through wetlands to the larger downstream Winnange Lake. Lake 260 at the IISD-ELA was selected as the study site for several reasons. First, this lake provides a range of physical shoreline habitats including organic wetland, sand-cobble, and rock-cobble substrates (Figure 1.1). Examining the efficacy of the selected remediation techniques in each of these different shoreline environments was intended to facilitate extrapolation of results to other systems. Lake 260 has also had extensive baseline chemical and biological information collected prior to oil addition. Lake 260 was previously used in the early 2000s to evaluate the effect of ethinylestradiol, the active component of birth control, to fish populations (Kidd et al. 2007). The population of the FHM was diminished during this experiment, however the health and size of the population has recovered (Blanchfield et al. 2015). This experiment and long-term monitoring provide decades of necessary background information. This, paired with ongoing monitoring of reference conditions within (i.e., untreated enclosures and lake reference sites outside the enclosures) and outside the study lake (i.e. nearby reference lakes), allowed us to discern oil-related effects from confounding influences using a Before-After-Control-Impact (BACI) approach.

1.7 FOReSt Project Chronology

The FOReSt project was created to study the effects of oil spills and compare the efficiency of minimally invasive methods to remediate freshwater shorelines after oil spills. The shoreline environment was chosen due to its high productivity and because oil can be deposited on the shoreline (i.e. the “bathtub ring” effect) and continuously resuspend into the aquatic environment (Palace et al. 2021a). The project was conducted in Lake 260 at the IISD-ELA, with the enclosures installed in two areas with different physical characteristics, a wetland and a rock-cobble environment (Figure 1.1). The organic wetland environment included soft organic sediment and detritus. The rock-cobble environment consisted of various sizes of rocks, from pebbles to boulders, on top of a sandy-cobble substrate. For the purpose of this thesis, only the organic wetland environment was evaluated. This was due to the overall FOReSt project logistical constraints of enclosures in the rock-cobble environment, which caused the rock-cobble environment to not be tested in 2021 with CHV. The FOReSt project evaluated both CLB dilbit and CHV in three phases; a pilot project with both dilbit and CHV in 2018, followed by full enclosure studies of dilbit and CHV in 2019 and 2021 respectively. Briefly, the pilot project was performed in 2018 and evaluated the effectiveness of Monitored Natural Recovery (MNR) as a secondary remediation method for CLB dilbit and CHV after primary removal using absorptive media. Results from this project are outlined in (Palace et al. 2021a) and were used to guide the development of the larger FOReSt project field study in 2019.

Enclosures used in all three years of the study were manufactured by Curry Industries in Winnipeg, Manitoba. They were made of an impermeable curtain that followed the approximate contour of the lake bottom from the shore to the distal end, 10m from shore. The curtain extended from the lake bottom up to a floatation collar at the surface of the water (Figure 1.2). The 5 m wide enclosures were installed starting at the shoreline and extended to a length of 10 m with an approximate maximum depth of 1.5 to 2 m at the distal end, enclosing an approximate 20,000-25,000 L aquatic littoral environment. A double layer of sandbags was used to seal the curtain to the sediment and shoreline, with additional sandbags in areas that needed extra reinforcement. As a secondary containment measure, surface booming was installed around the enclosures, the lake outflow, and known lake trout spawning shoals in case of an enclosure was breached resulting in oil releases from any of the enclosures. An underflow dam was also installed in the Lake 260 outflow as a tertiary containment measure. Full methods of the experimental oil spill, primary and secondary remediation are outlined in Palace et al. (2021b).

The FOReSt project evaluated model spills of weathered oil, instead of fresh oil. The approach simulated oil entering shoreline environments after weathering and being re-deposited onto the shoreline (Palace et al. 2021a). CLB dilbit (2019) and CHV (2021) were weathered according to the methods described in (Palace et al. 2021a). In their respective years, 7 kg of each oil was weathered for 36 h on 25 cm of Lake 260 water in large 1.2 m diameter stainless steel evaporation pans. After weathering, the oil was skimmed from the surface using stainless steel utensils and collected into light protected glass jars.

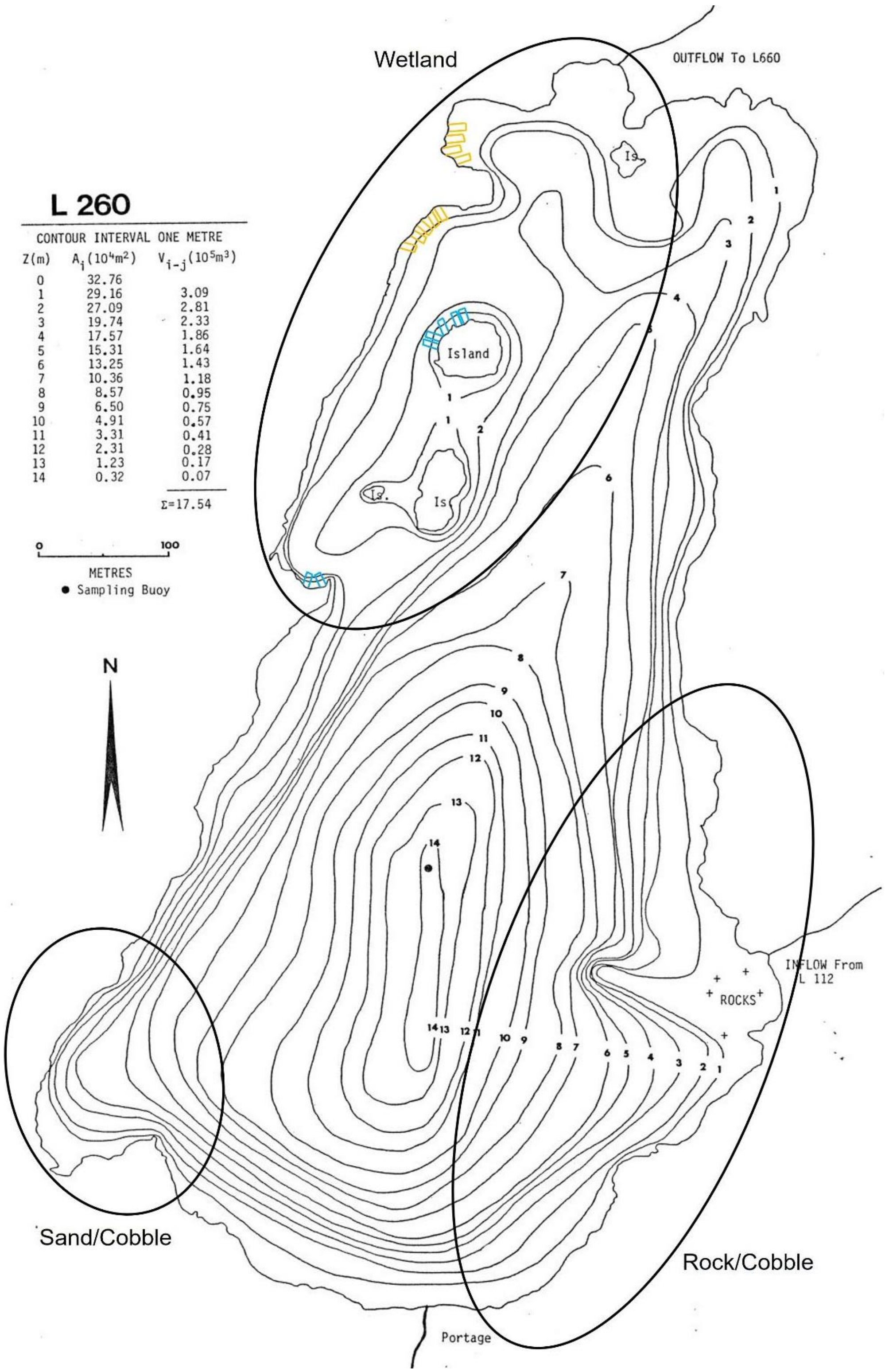

Figure 1.1. Bathymetric map of the experimental lake, Lake 260 at the IISD-Experimental Lakes Area (IISD-ELA), with different shoreline environments circled. Enclosures used in the Freshwater Oil Spill Remediation Study (FOReSt) are circled; diluted bitumen enclosures from 2019 in yellow, and conventional heavy crude enclosures from 2021 in blue.

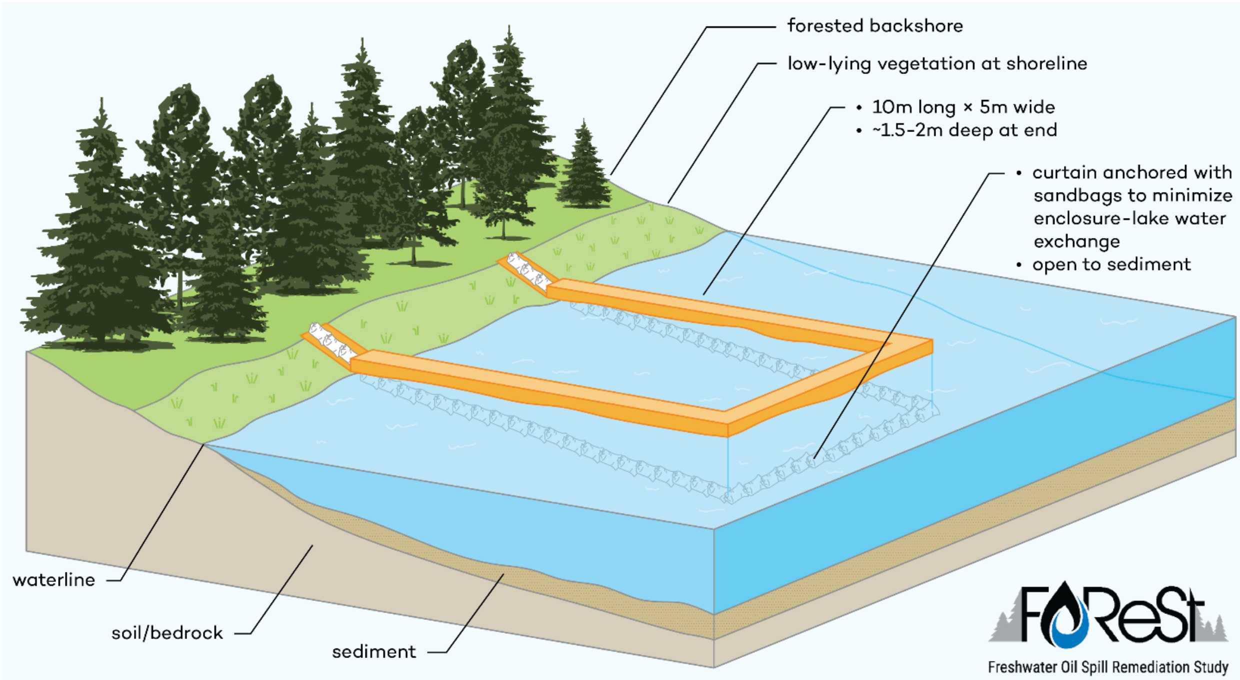

Figure 1.2. Schematic of the Freshwater Oil Spill Remediation Study (FOReSt) enclosures used in 2019 with diluted bitumen and 2021 with conventional heavy crude.

In 2019, model spills of CLB dilbit were conducted in 18 enclosures split among two physically different shoreline environments: nine in an organic wetland environment and the other nine in a rock-cobble substrate area. Model spills included ~1.25 kg (approximately 1.25 L) of dilbit or CHV that was poured directly on the water surface ~50 cm from the shoreline. The oil remained in place for 96 h prior to primary remediation to simulate potential response times for spill response in remote locations. The spill volume was chosen to represent moderate to heavy shoreline oiling, i.e. 1-3 mm thickness based on heavy wave action (20cm height) in boreal lakes at the IISD-ELA (Palace et al. 2021a). Two of these enclosures in each shoreline region were not treated with oil to serve as reference systems (i.e. (7 oil treated enclosures + 2 reference enclosures) × 2 shoreline environments = 18 total enclosures). All enclosures had a primary oil recovery that involved polypropylene sorbent pads (Spill Ninja, MEP Brothers, Winnipeg, Canada), 12 minutes of shoreline washing using a low-pressure washer (approximately a 1600 L flushing), followed by another round of free product capture using sorbent pads. After primary clean up, we applied three secondary remediation methods to treat and remove residual oil. First, Enhanced Monitored Natural Recovery (EMNR), which includes nutrient additions to stimulate microbial activity and bacterial degradation of soluble oil constituents and consume excess carbon contained in the oil. For the EMNR secondary remediation, approximately 50-60 g of fertilizer (Scotts-Osmocote, Everris, NA INC., Fusion 19-6-9, Nitrogen 19%, Phosphate 6%, Potassium 9%) was added into the enclosure. The second remediation method used was the petroleum-based surface washing agent (SWA), Corexit EC9580A (a surfactant-based SWA), which was added to the oil on the shoreline and its vegetation. This allowed the oil to be rinsed into the aquatic environment to be captured using absorptive media. The Corexit EC9580A, and other shoreline cleaners, help loosen the oil from the shoreline, therefore making low-pressure washing of the shoreline more efficient.

Corexit EC9580A contains a surfactant, dioctyl sodium sulfosuccinate (DOSS), that consists of a polar, water-soluble group and a non-polar oil-soluble group, that removes the oil from the surface and prevents redeposition (Canevari et al. 1995). Corexit EC9580A was applied according to the manufacturer's recommended rate of 265 g to an approximate area of 2.5 m^2 following primary clean up. The Corexit EC9580A was sprayed using a garden chemical sprayer to the shoreline of the enclosures. The Corexit EC9580A was left to interact with the residual oil for 30 minutes, followed by rinsing for another 12 minutes using a low-pressure washer. Finally, one oil treated enclosure in each shoreline type was augmented with an Engineered Floating Wetland (EFW) platform. The EFWs contained cattails (*Typha spp.)* and other wetland plants (*Carex spp.*) that have vast root systems. These root systems, collectively referred to as the rhizosphere, provide a large surface area that hosts a rich microbial community that can consume carbon from the oil.

In 2021, model spills of CHV were conducted in seven shoreline enclosures identical to the 2019 studies, but limited to the organic wetland environment. Oil additions, primary recovery and EMNR were also applied as in 2019. Three of the enclosures served as reference systems (i.e. 4 oil treated enclosures + 3 reference enclosures = 7 total enclosures). Of the 4 oiled enclosures, 3 received the EMNR secondary remediation, and 1 received the EFW treatment. The Corexit EC9580A SWA was not repeated in 2021 due to physical impacts to organisms seen in the 2019 exposure, outlined below (Chapter 2) and in Black et al. (2020), and based on limited effectiveness in the organic wetland environment (Palace et al. 2021b).

The enclosures were monitored for water, oil and nutrient chemistry, wave action, temperature, light, dissolved oxygen (DO), soil and sediment chemistry, phytoplankton, periphyton, zooplankton, benthic invertebrates, emerging insects, and effects on fish throughout the summer field season (approximately 90 days). Each enclosure was set up to allow for sampling that would cause the least disruption to the system as possible. This included remotely accessed sampling ports (set up at the surface and 1 m depths), used to collect water and zooplankton samples. Periphyton strips were deployed inside and outside the enclosure and were collected at various time points. Minimally invasive soil and sediment samples were collected monthly during the exposure, as well as more intensive sampling prior to decommission of the enclosures. Zooplankton and emerging insects were collected weekly, along with temperature, DO, and light measurements. Prior to oil exposure, fish were removed from the enclosures. Then 5 male and 5 female (10 individuals) FHMs were added to each enclosure prior to oil addition. In 2019, the FHMs were left to swim freely in the enclosure. Although negligible mortality was detected, recapturing free swimming fish was difficult. Therefore, in 2021, fish were housed in mesh cages in their respective enclosures and at reference locations.

1.8 Study Species

1.8.1 Hyalella azteca

Hyalella azteca are epibenthic, sediment burrowing amphipods that feed on detritus and live in brackish and freshwater environments across North America (ECCC 2017). They are so widely dispersed, that *Hyalella azteca* are considered to be the most widely dispersed invertebrates in North America (Witt & Hebert 2000). Amphipods are a major, but vulnerable, component of many freshwater, estuarine, and marine food webs, including those of the Great Lakes (Borgmann et al. 1989; ECCC 2017). Amphipods make up a considerable amount of biomass of the benthic community, and in favourable habitats, *Hyalella azteca* can have densities greater than 10,000 individuals/m^2 (ECCC 2017). *Hyalella azteca*, and amphipods as a whole, are typically a more sensitive species when it comes to acute toxicity tests (Borgmann et al. 1989). They are particularly relevant when it comes to evaluating toxicity, considering they are often a dominant part of the benthos in well-oxygenated near-shore freshwater environments, sensitive to a variety of contaminants, and culture extremely well in the lab (Borgmann et al. 1989; ECCC 2017; Major et al. 2013). As a result, *Hyalella azteca* have been widely used in acute and chronic toxicity tests to determine regulatory toxicity standards, including for wild populations of *Hyalella azteca* (Major et al. 2013).

Because of the inherent isolation of laboratory cultures, a shift of phenotypic and genetic differences has been noted in the *Hyalella azteca* species (Major et al. 2013). There is a variety of species-level phenotypic and genetic variation within wild *Hyalella azteca* populations, and many lab cultures cannot be traced back to the same wild populations (Major et al. 2013). Analyses comparing wild to cultured populations, found that the cultured populations can be similar to wild populations, while others are quite divergent (Babin-Fenske et al. 2012). *Hyalella azteca* in lab cultures are distinguished through clade groupings. In North America, the clades typically used for toxicity testing include clade 1 and 8 (Leung et al. 2016). Clade 1 is specifically cultured out of Environment and Climate Change Canada (ECCC, Canada Centre for Inland Waters, Burlington, Ontario), and has been referred to as the Burlington clade (Major et al. 2013). Clade 8 is a common clade found across Canada and the United States and has been referred to as the US Laboratory Clade (ECCC 2017; Major et al. 2013). In the literature, clade 8 is considered to be more tolerant than clade 1, with slight differences in LC_{50s} of test contaminates (Leung et al. 2016). In this thesis, clade 1 *Hyalella azteca* provided by ECCC in Burlington were studied.

The impacts of various petroleum products and compounds (i.e. individual PAHs) have been studied using *Hyalella azteca* as a test species. Typical parameters measured include growth or length, reproductive output, behaviour, and mortality (Everitt et al. 2020; Gauthier et al. 2016; Hatch & Burton 1999a). When exposed to various petroleum products and PAHs, *Hyalella azteca* are more likely to experience reduced growth (Barron et al. 2021; Bartlett et al. 2012; Tani et al. 2021; Indiketi et al. 2022; Ingersoll et al. 1996), oxygen consumption and ventilation (Everitt et al. 2020; Gauthier et al. 2016), activity (Everitt et al. 2020; Gauthier et al. 2016), feeding (Hatch & Burton 1999b), pleopod (swimming limbs) beating frequency (Everitt et al. 2020), reproduction and associated behaviour (Satbhai et al. 2017), acetylcholinesterase (AChE, neurotransmission enzyme) activity (Gauthier et al. 2016), and greater respiration (Everitt et al. 2020; Gauthier et al. 2016), hyperstimulation (i.e. uncoordinated muscle movements; Gauthier et al. 2016), and mortality (i.e. lower LC_{50} values) compared to controls (Hatch & Burton 1999a; Barron et al. 2021; Verrhiest et al. 2001; Bartlett et al. 2012; Tani et al. 2021; Indiketi et al. 2022; Ingersoll et al. 1996). Most of the results are for individual PAHs, but there are few examples of *Hyalella azteca* being exposed to whole crude oil and dilbit blends (Indiketi et al. 2022; Ankley et al. 1994 and Wernersson 2004 used samples collected from contaminated environments). *Hyalella azteca* have also been used to evaluate PET, but again, studies are limited to specific PAHs instead of whole products, which are more likely to be encountered in real-world PET scenarios (Ankley et al. 1994; Clément et al. 2005; Duan et al. 2000; Hatch & Burton 1999a; 1999b; 1999c; Verrhiest et al. 2001; Wernersson 2004; Wilcoxen et al. 2003). Because of the known impacts of PAHs to *Hyalella azteca*, their position as a standardized toxicity test species, and the gap of information using whole oil products and around PET, *Hyalella azteca* are an ideal test species to evaluate the impacts of dilbit, crude oil and PET to growth and development.

1.8.2 Fathead Minnows (Pimephales promelas)

Fathead minnows (FHM, *Pimephales promelas*) are one of the most widely utilized fish species for toxicity testing, due to their extensive presence across North America, sexual dimorphism, amenability to laboratory culturing, completely sequenced genome, and well-defined reproductive output (Ankley & Villeneuve 2006). The FHM has also been adopted as a model test species by the European Union Organisation for Economic Co-operation and Development (OECD) and the United States Environmental Protection Agency (USEPA; Alsaadi et al. 2018a, 2018b). FHMs are typically between 5-8 cm in length and dark olive in colour with yellow to white underbellies, and have distinguishable short, blunt heads, an incomplete lateral line, and a dark lateral stripe (Ankley et al. 2001). They are benthic filter feeders and play a bridging role in many ecosystems as important prey for larger fish species, including lake trout, bass, yellow perch, northern pike, and more (Stewart & Watkinson 2004). FHMs reach their sexual maturity at 4-5 months, and have a lifespan of approximately 2-3 years, although post-spawning mortality is common (Jensen et al. 2001). They are fractional spawners, meaning they release mature eggs in intervals, and in good conditions, female minnows can produce clutches of 50-150 eggs every 3-5 days (Ankley et al. 2001; Jensen et al. 2001). Spawning is temperature driven, starting at approximately 18°C, with males attracting females to nests they build, rolling over the female so their adhesive eggs stick to the nest surface, fertilizing the eggs via the release of milt, and aggressively protecting the eggs until they hatch 4-5 days later (Ankley et al. 2001). FHM embryos have well-defined development stages, hatch within 4-5 days, and do well in culture (Ankley & Villeneuve 2006). Seven-day FHM early-life stage toxicity tests, which include egg, embryo, and early larval stages, have been utilized since 1985, and have since been standardized (Lewis et al. 1994; Ankley & Villeneuve 2006). These early-life stage tests are some of the most sensitive bioassays for examining toxicity in fish (OECD 1992; Hutchinson et al. 1998; Parrott 2005). During the early-life development phases of a typical FHM (< 7-days), individuals will undergo eye development, pigmentation, swim bladder inflation, heart and spinal development, hatching, and rapid growth. Since they are being exposed during the development period, the results of these tests can apply to survival, hatching success, malformations, growth, and various behavioural endpoints, such as swimming and feeding, all of which project future success and fecundity (Valenti et al. 2009; Parrott 2005). Additionally, FHMs lack pigment and are transparent during their embryo and early larval stages, which make them particularly susceptible to PET. Because of this, FHMs are a suitable species for studying both adult reproduction and early-life development, as well as PET.

Early-life stage FHMs have been extensively used to test the impacts of various contaminants across numerous disciplines. FHMs have also had their entire genome sequenced, which has provided an abundance of molecular tools for studying this species. When it comes to evaluating the impacts of petroleum products, various PACs and other oil constituents have been examined using early-life stage FHMs, particularly using genetic analyses (Alsaadi et al. 2018b; Colavecchia et al. 2007; Lara-Jacobo et al. 2019, 2021; Loughery et al. 2019). When exposed to dilbit, early-life stage FHM are more likely to experience reduced growth (weight, length, tail length; Vignet et al. 2019), premature or delayed hatching (Colavecchia et al. 2004), craniofacial malformations (Colavecchia et al. 2004, 2007; Lara-Jacobo et al. 2021), yolk sac edemas (Colavecchia et al. 2004, 2007; Lara-Jacobo et al. 2021; Vignet et al. 2019), cardiotoxicity (pericardial edemas, tube heart, hemorrhaging, altered heart rates; Alsaadi et al. 2018b; Colavecchia et al. 2004, 2007; Lara-Jacobo et al. 2021; Vignet et al. 2019), spinal curvatures (Colavecchia et al. 2004, 2007; Lara-Jacobo et al. 2021), induction of *cyp1A* (Alsaadi et al. 2018b; Bérubé et al. 2021; Colavecchia et al. 2004, 2007; Lara-Jacobo et al. 2021), and greater mortality compared to controls (Alsaadi et al. 2018b; Bérubé et al. 2021; Colavecchia et al. 2004; Vignet et al. 2019). Non-inflation of the swim bladder has been identified in other species exposed to petroleum, which can cause future impacts on development, swim performance and overall fitness and survival (Price & Mager 2020). Some examples include Japanese Medaka (*Oryzias latipes*) exposed to dilbit (Madison et al. 2015, 2017) and several PAHs and alkyl PAHs (Lin et al. 2015), 7 PAHs in zebrafish (*Danio rerio*; Incardona et al. 2004), and the topic of PAHs impacting swim bladder inflation reviewed as a whole (Price & Mager 2020). Because of the known impacts of dilbit to embryotoxicity, as well as the establishment of FHMs as a standardized toxicity test species, FHMs are an ideal test species to evaluate the impacts of dilbit and PET to development on the thyroid axis and compare these findings to the wealth of literature available.

At the IISD-ELA, FHMs are also abundant in numerous lakes and are easily collected in both adult and egg stages. Typically, toxicity tests use cultured FHM eggs. Using wild collected FHM eggs allows for an opportunity to see how potentially more sensitive native populations may be impacted by oil spills.

1.9 Thesis Objectives & Hypotheses

In this thesis, I contributed to the FOReSt project by evaluating the impact of two oil types, CLB dilbit and CHV, as well as the role of ultraviolet (UV) radiation to the toxicity of two species; *Hyalella azteca* and early-life stages of wild FHMs. These species were selected due to their use as a common species in toxicity testing by the USEPA, ECCC, and the OECD, as well as their widespread distributions, well characterized physiology and behavior, sensitivity to contaminants, and the wealth of information available for reference and comparison (Ankley et al. 2010; Major et al. 2013). Because the toxicity of certain constituents of oil can be greatly increased when organisms are exposed to oil and UV radiation, I also evaluated the effects of oil exposure in combination with UV radiation. Specifically, I used targeted gene expression along the thyroid axis, malformation analyses, and growth metrics to determine the toxicity of dilbit, CHV, and UV radiation alone and in combination.

For my thesis, I addressed two major research questions:

1. Do constituents of diluted bitumen and/or conventional heavy crude exhibit photo-enhanced toxicity to *Hyalella azteca* and early-life stage wild fathead minnows in freshwater environments under environmentally-relevant conditions?
2. Is there a change in the health of juvenile *Hyalella azteca* and early-life stage wild fathead minnows (*Pimephales promelas*) exposed to heavy crude oils (Cold Lake Blend diluted bitumen and conventional heavy crude)?

I tested these research questions through the following hypotheses:

1. Ultraviolet (UV) radiation will alter the toxicity of diluted bitumen and conventional heavy crude to *Hyalella azteca* and early-life stages of fathead minnows (*Pimephales promelas*), as indicated by:
    a. Mortality.
    b. Growth.
    c. Physical effects.
2. Early-life stage fathead minnows exposed to diluted bitumen will have significantly altered condition, as indicated by:
    a. Malformations.
    b. *cyp1A* expression.
    c. Thyroid axis gene expression (*dio2, dio3, thrα,* and *thrβ*).

The overall approaches to address concerns to *Hyalella azteca* were to:

1. Expose juvenile *Hyalella azteca* to WAFs from remediated simulated spills of diluted bitumen and conventional heavy crude of environmentally-relevant concentrations, high-concentration positive control WAFs, and two UV radiation treatments, for 5-day exposures.
2. Expose *Hyalella azteca* to WAFs of the Corexit EC9580A surface washing agent and its active surfactant dioctyl sodium sulfosuccinate (DOSS) for 5-day exposures, representing their early juvenile stages.
3. Assess the physical effects associated with diluted bitumen, conventional heavy crude, Corexit EC9580A, dioctyl sodium sulfosuccinate (DOSS), and photo-enhanced toxicity.
4. Evaluate the growth of *Hyalella azteca* using length measurements.

The overall approaches related to fathead minnows were to:

1. Expose early-life stage wild fathead minnows to WAFs from remediated simulated spills of diluted bitumen of environmentally-relevant concentrations, and two UV radiation treatments, for 7-day exposures.
2. Assess the physical malformations, including yolk sac, cardiac, cranio-facial, spinal, and swim bladder categories, of fathead minnows exposed to simulated spills of crude oil.
3. Evaluate the molecular responses of fathead minnows using gene expression biomarkers, particularly those related to oil exposure and the thyroid, which is important for growth and development (including *cyp1A, dio2, dio3, thrα,* and *thrβ*).

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<u>Chapter 2: Photo-enhanced toxicity and physical effects of diluted bitumen, conventional heavy crude oil, and Corexit EC9580A to *Hyalella azteca*.</u>[1]

Abstract

Diluted bitumen and conventional heavy crude are the two major products of the Canadian oil and natural gas industry. Spills into aquatic environments posing risk to biota, particularly in freshwater environments where there are still knowledge gaps on the effects of oil and remediation strategies. Oil spill remediation methods applicable to low energy freshwater environments are limited but we investigated the efficacy of the surface washing agent Corexit EC9580A, which contains the surfactant dioctyl sodium sulfosuccinate (DOSS). Previous studies have evaluated the direct toxicity of diluted bitumen, heavy crude, or SWAs on biota, but many overlook potential interactive effects of photo-enhanced toxicity. To examine the photo-enhanced toxicity of diluted bitumen and conventional heavy crude, we exposed *Hyalella azteca* to varying water accommodated fractions of diluted bitumen and conventional heavy crude with various remediation treatments under low (~15%) and high (~90%) UV exposure. When exposed to diluted bitumen or conventional heavy crude and UV radiation in combination, *Hyalella azteca* had significantly reduced growth (on average ~0.35 mm shorter with both oils) and increased mortality. When Corexit EC9580A or its active surfactant DOSS were included, *Hyalella azteca* experienced physical effects resulting in the disruption of their buoyancy which were related to increased mortality. We highlight the need to evaluate photo-enhanced toxicity and physical effects when examining the effects of oil spills and spill mitigation strategies.

2.1 Introduction

Canada holds some of the largest proven reserves of oil and is one of the top five oil producers globally, producing over 1.5 times its domestic demand (CAPP 2019; Daisy et al. 2022; Lee et al. 2015). The oil and natural gas industry has been steadily growing in Canada and across the world, with global demand estimated to increase by over 106 million barrels per day by 2040 and Canadian production anticipated to climb by 1.27 million barrels per day over the 2.84 million barrels per day currently being produced (CAPP 2019; 2023). In the Canadian oil industry, bitumen, and conventional heavy crude oil (CHV) represent the majority of the oil reserves and production (CAPP 2019; Lee et al. 2015). Bitumen and heavy crude oils have a high viscosity and density and must be mixed with natural gas condensates (in the case of diluted bitumen (dilbit), approximately 20-30% condensate and 70-80% bitumen) for transport in pipelines (Lee et al. 2015). Due to their ability to transport large quantities of oil with statistically the lowest accident rate, the majority of oil is transported through pipelines in North America, with excess transport mostly relying on rail (CAPP 2019; Crosby et al. 2013). Spill occurrences from Canadian crude pipelines are down by approximately 85% since the 1970s, with spill volume from this time also

[1] This chapter is in preparation for publication but has not yet been published. Sonya Michaleski conducted the experiment, analyses, and wrote the original manuscript. Lauren Timlick helped conduct all experiments. Adrienne Bartlett supplied organisms for all studies and assisted with experimental design. Lisa Peters conducted chemical analysis for all experiments. Mace Barron helped conceive the study, with experimental design, and with ultraviolet measurements. Ken Jeffries assisted with analysis. Vince Palace helped conceive the study, with experimental design, with conducting the experiment, and funded the experiment. All authors participated in editing the manuscript.

down by 92% (Etkin 2023). However, spills into aquatic environments are a concern especially in freshwater where less is known about the effects compared to marine systems (Lee et al. 2015; Crosby et al. 2013). This is a concern because freshwater systems provide essential resources, including water supplies and fish, which have high value to humans (Devlin et al. 2016), and pipelines and rail networks are often located near fresh water, posing spill risks to these systems (Alsaadi et al. 2018; Dew et al. 2015; Levy 2009).

Studies considering the potential impacts of oil spills and remediation methods environments are usually focused only on direct oil toxicity in freshwater, but do not consider photo enhanced toxicity (PET). Photo-enhanced toxicity refers to an interaction between a contaminant and ultraviolet (UV) radiation that increases the toxicity of the contaminant (Barron 2007; Barron et al. 2018; Little et al. 2000). Photo-enhanced toxicity can increase toxicity between 2- and 1000-fold in aquatic organisms, depending on the potency and concentration of the contaminant and the quantity and quality of UV radiation (Barron 2007). Freshwater environments often have sufficient UV radiation from solar exposure, making the potential for photo-enhanced toxicity relevant (Barron 2017). Toxicological studies, especially laboratory studies, often occur in isolation, controlling for environmental factors, meaning few of these studies include the impact of UV radiation and PET. It is important to test the toxicological effects of dilbit and CHV paired with UV exposure, considering natural environments where spills may occur would have unavoidable UV exposure. UV attenuation is dependent on turbidity, dissolved organic matter, morphometry, and other parameters, but in clear lakes, over 10% of UV radiation, specifically UVB radiation, can penetrate to substantial depths (7-20 m) resulting in a zone of influence weighted toward shallower, near shore habitats (Tartarotti et al. 2022; Williamson 1995). Without the inclusion of PET in toxicity testing, mortality estimates, projected lethal concentration values, spill response plans, and impact assessment models can underestimate the additive or synergistic impact of PET to at-risk species (Barron 2017). Additionally, many toxicants have not had their PET evaluated in freshwater systems, including various oil constituents and mixtures. These in tandem pose significant concerns for at-risk organisms, for their individual populations as well as their significant roles in food-webs and within ecosystems. Therefore, it is important that environmentally-relevant PET studies be included in testing protocols to produce accurate and reliable toxicity information to include in spill response plans, impact assessment models and environmental policy (Barron 2017). Having environmentally-relevant interactive studies will help regulatory agencies recognize the complexity of nature and contribute to the development of appropriate environmental policy.

Along with the potential toxicity issues surrounding spills of crude oil in freshwater systems, there are also concerns surrounding remediation measures. Current freshwater remediation measures, mainly dredging and mechanical removal, can also impact the system and impair recovery. For example, when >3320 m^3 of dilbit was spilled into the Kalamazoo River in July 2010, impacts of the spill were difficult to differentiate from effects of the remediation measures (Lee et al. 2015; Fitzpatrick et al. 2015). Compared to marine systems, freshwater environments are lower energy and have less volume for oil dispersion and breakdown, limiting the availability of effective remediation methods (Fitzpatrick et al. 2015). Surface washing agents (SWAs) containing surfactants have been proposed to treat oiled shorelines as they facilitate removal of oil so it can be rinsed from the riparian area into the open water to be physically removed or degraded (Chen et al. 2020). There are a variety of SWAs approved for marine use in Canada including Corexit EC9580A, CytoSol, and PES-51, with another 54 SWAs approved for use in the United States (Chen et al. 2020; Black et al. 2020). In terms of spill-treating agents, only Corexit EC9500A and EC9580A make the list of those approved in Canada (Government of Canada 2016). However, neither dispersants or SWAs are currently approved for use in fresh water systems due to the limited literature regarding their impacts (Barron et al. 2018; Stroski et al. 2019). Additional information is needed to consider the efficacy of using SWA in near shore freshwater environments.

We conducted toxicity tests incorporating the effects of UV radiation and photo-enhanced toxicity on sensitive amphipod species (*Hyalella azteca*) exposed to environmentally-relevant model dilbit and CHV spills. We also evaluated the impact of the SWA Corexit EC9580A and its active surfactant dioctyl sodium sulfosuccinate (DOSS) on *Hyalella azteca* lethal and sublethal endpoints. To our knowledge, this is the first laboratory study to assess the photo-enhanced toxicity of dilbit, CHV, and a SWA to an invertebrate under a realistic model spill exposure scenario. Results from this study will support risk assessments focused on invertebrates and other at-risk aquatic species in near shore freshwater environments.

2.2 Methods

2.2.1 Freshwater Oil Spill Remediation Study (FOReSt) Project

This experiment was conducted at the IISD-Experimental Lakes Area (IISD-ELA), located in Northwestern Ontario in the boreal forest region of Canada (49°40′N, 93°44′W). This study was part of the Freshwater Oil Spill Remediation Study (FOReSt) that was conducted on Lake 260 (49°42′N, 93°46′W; Palace et al. 2021a). Lake 260 is an oligotrophic boreal lake that has an area of 330,000 m^2, a volume of 2,000,000 m^3, an average depth of 7 m, and a maximum depth of 16 m (Brunskill & Schindler 1971). The FOReSt project was created to study the effects of oil spills and compare the efficiency of minimally invasive methods to remediate freshwater shorelines after oil spills. Full methods of the experimental oil spill, primary and secondary remediation are outlined in Palace et al. (2021). Briefly, model oil spills were conducted in 5 m wide × 10 m long shoreline enclosures that contained approximately 20,000-25,000 L of littoral aquatic habitats that were then exposed to ~1.25 kg of oil weathered for 36 h prior to being applied. In 2019, model oil spills were with Cold Lake Blend (CLB) diluted bitumen, and in 2021, conventional heavy crude (CHV) was used. The weathered oil was left to interact with the shoreline in the FOReSt enclosures for 96 h following the spills to simulate conservative response times for spills in remote locations. After this interaction, period primary cleanup began. Free floating oil was removed from the surface of the water inside the FOReSt enclosures using polypropylene sorbent pads (Spill Ninja, MEP Brothers, Winnipeg, Canada), followed by 1200 L of freshwater flushing using a low-pressure manifold, followed by another round of removal of floating product using sorbent pads. No manual removal of oil from the shoreline substrates or vegetation was attempted. After primary clean up, secondary remediation methods were applied to address residual oil remaining in the FOReSt enclosures. The secondary remediation methods included Enhanced Monitored Natural Recovery (EMNR) and the SWA, Corexit EC9580A. The EMNR refers to the addition of nutrients, specifically nitrogen and phosphorus, which are added to approximate the Redfield ratio (106:16:1 C:N:P) with the carbon coming from oil. Nutrient addition is intended to stimulate microbial activity to assimilate carbon by degrading oil. In the FOReSt enclosures, approximately 50-60 g of fertilizer (Scotts-Osmocote, Everris, NA INC., Fusion 19-6-9, Nitrogen 19%, Phosphate 6%, Potassium 9%) was introduced into the near shore area ~50 cm from the shoreline. Corexit EC9580A is a petroleum-based product that contains a surfactant and coagulating agent to remove oil from the shoreline so it can be rinsed into the aquatic environment and collected from the surface. Corexit EC9580A was applied to the oiled area of the shoreline in each enclosure using a garden chemical sprayer according to manufacturer recommendations with an application rate of 265 g to an area of ~2.5 m^2. After a contact time of 30 minutes, shorelines were rinsed as noted above and any liberated oil was collected. Reference enclosures, to which had no oil applied, also underwent freshwater flushing.

2.2.2 Oil Weathering & Collection

Dilbit (2019) and CHV (2021) were weathered according to the methods described in (Palace et al. 2021b). Briefly, in 2019, 7 kg of Cold Lake Blend dilbit was weathered in 1.2 m diameter stainless steel evaporation pans containing 25 cm of Lake 260 water for 36 h. After weathering, the oil was collected from the surface of the water using stainless steel spoons. The weathered oil was applied at a rate of 1.25 kg (approximately 1.25 L) to each of the FOReSt enclosures. This oil weathering and exposure was repeated in 2021 using CHV instead of dilbit.

Water samples were collected from the FOReSt enclosures at 32 days post oil addition (28 days post recovery; July 25th, 2019) in 2019 (dilbit) and 45 days post exposure (41 days post recovery; August 11th, 2021) in 2021 (CHV). This timeline represents the effects that individuals are experiencing after remediation. The WAF treatments are representative of more immediate exposures without secondary treatment measures. We collected WAF water from the FOReSt enclosures and oil weathering experiments occurring at the IISD-ELA. Surface water (approximately 10 cm depth) was collected from each FOReSt enclosure using a peristaltic pump in 4 L amber glass bottles, avoiding any active surface slicks. Water from the weathering experiments was collected by draining off the water underneath the surface oil slick into 4 L amber glass bottles. Each of the triplicate exposure vessels was 250 mL and contained 150 mL of water from the appropriate treatment.

2.2.3 Low Energy WAF

Water accommodated fractions (WAFs) refer to the water-soluble constituents of oil and includes low molecular weight hydrocarbons released when in contact with water. In 2019, a sample of the water accommodated fraction (WAF) was collected from under the weathered oil in the stainless-steel evaporation pan. This was used as the high-concentration WAF treatment in 2019, which served as a positive control. In 2021, the WAF treatments were not collected from the primary oil weathering pan but were created through smaller oil weathering experiments. Briefly, 23×33 cm stainless steel pans were filled with 4 L of Lake 260 water and were then dosed with varying weights of CHV (Figure 2.1).

2.2.4 Surface Washing Agent & Surfactant

In the 2019 dilbit experiment, the FOReSt enclosure SWA treatment using Corexit EC9580A yielded physical effects in the form of external and internal bubbles (see sections 2.2.8 & 2.3.5). This result led to the inclusion of more treatments involving Corexit EC9580A and its active surfactant DOSS. DOSS is a chemical emulsifying surfactant that is used in the pharmaceutical, food and cosmetic industries and is classified as "Generally Recognized as Safe" by the US FDA (González-Penagos et al. 2022; Temkin et al. 2016; US FDA 1998). There is little known about the effects of DOSS in freshwater environments and to freshwater organisms, due to it mainly being evaluated in marine systems (González-Penagos et al. 2022; Goodrich et al. 1991; Corrie et al. 2021).

In the 2021 CHV experiment, some of the CHV low energy WAFs were secondarily treated with Corexit EC9580A (labeled SWA) or DOSS (Figure 2.1). The amounts of Corexit EC9580A (36.7 mg) and DOSS (5.5 mg) applied represent the recommended application rate and the rate that was applied to the FOReSt enclosures in 2019. In the case of DOSS, the volume refers to the representative fraction that would be present within Corexit EC9580A when using the recommended application rate (approximately 15%).

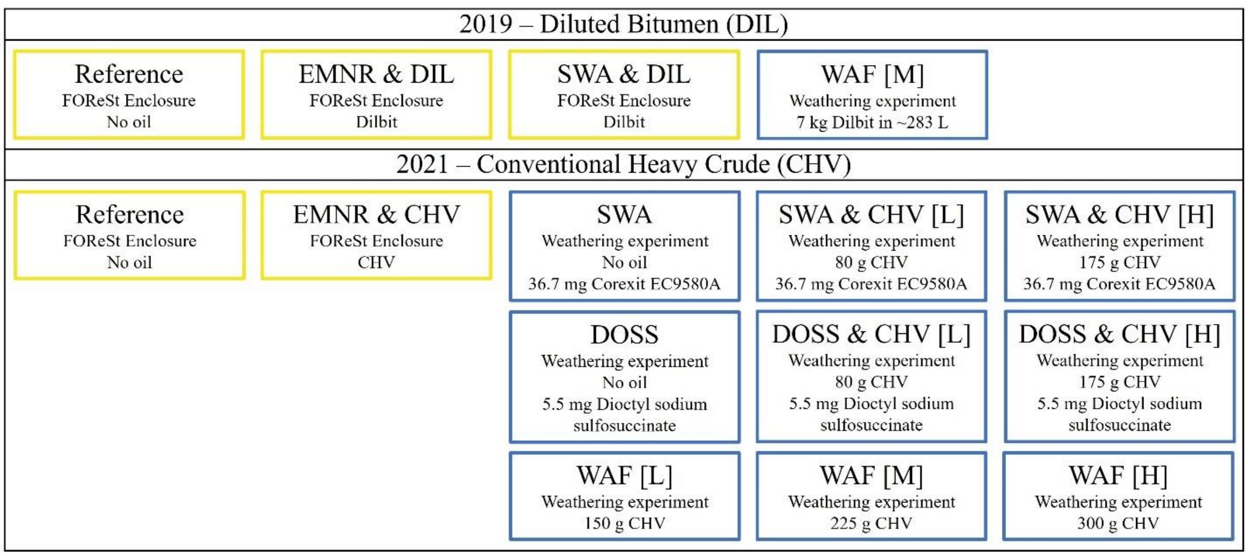

Figure 2.1. Treatments for the exposure of *Hyalella azteca* to treatments of diluted bitumen, conventional heavy crude, and remediation measures from the Freshwater Oil Spill Remediation Study (FOReSt) and weathering experiments. Treatments include unoiled reference enclosures from the FOReSt project (Reference), nutrient additions through Enhanced Monitored Natural Recovery (EMNR), the surface washing agent (SWA) Corexit EC9580A, the active surfactant in Corexit EC9580A dioctyl sodium sulfosuccinate (DOSS), and various concentrations (L – low, M – medium, H – high) of water accommodated fraction (WAF) of the two test oils; diluted bitumen (DIL) and conventional heavy crude (CHV).

2.2.5 Hyalella azteca Culture

Hyalella azteca used in this experiment were from the laboratory culture maintained at Environment and Climate Change Canada (Canada Centre for Inland Waters, Burlington, Ontario).

Culturing methods are described in detail by Borgmann et al. (1989). In brief, cultures were maintained with dechlorinated municipal tap water (Burlington, Ontario, Canada, originating from Lake Ontario; hardness 120–140 mg L^{-1}, alkalinity 90–110 mg L^{-1}, pH 8.2–8.6) at 25°C on a 16-hour light:8-hour dark schedule. Each culture container had cotton gauze at the bottom to provide a substrate. *Hyalella azteca* were fed powdered TetraMin fish food flakes (Tetra GMBH, Melle, Germany) three times per week. Once weekly, juvenile *Hyalella azteca* (0-7 days old) were collected and separated from the culture containers for use in experiments.

The *Hyalella azteca* used in this experiment belong to clade 1, termed the Burlington clade (Major et al. 2013). The two clades cultured in North America and typically used for toxicity testing include clade 1 and 8 (Leung et al. 2016). In the literature, clade 8 is considered to be more tolerant to metals and contaminants than clade 1, with slight differences in LC50s of test contaminants (Leung et al. 2016).

Juvenile (0-7 days old) *Hyalella azteca* were shipped to the IISD-ELA field station overnight and were examined for general condition, fed powdered TetraMin fish food flakes, aerated and left to recover for 24 h prior to exposures. The juvenile life stage was selected to determine the potential for oil and photo-enhanced toxicity to affect growth, a more sensitive endpoint than survival (Barron et al. 2021). Juveniles were 3–10 days old at the start of the exposure.

2.2.6 Experimental Setup

Hyalella azteca individuals were randomly selected and placed in jars until n=12 individuals per jar was reached. A single piece of approximately 5 cm^2 presoaked cotton gauze was placed in each jar as a substrate for the *Hyalella azteca* to perch on. During the exposures, *Hyalella azteca* were fed approximately 5 mg of powdered TetraMin fish food per container every 2 days. Individual vessels were incubated at 22°C (± 2°C) for 24 h before the first UV exposure. An 80% static water renewal was completed every second day. Individuals were inspected for mortality (lack of movement and opaque colour) once daily for a 5-day exposure. Mortalities were documented and removed daily. In the 2021 CHV experiment, individuals suspended at the surface were also documented during daily inspections.

The exposure vessels were kept in a mobile cart that held a circulating water bath to maintain temperatures (Figure 2.2). They were also stored in a climate-controlled room when not being exposed to UV radiation and had constant low level of aeration in the vessels to ensure sufficient oxygen (DO > 95%). HOBO® temperature and light loggers were placed on both sides of the exposure system to monitor and ensure the exposure cooling system maintained water temperatures at 22°C (± 2°C). The mobile system allowed all exposed vessels to be transported outside every day during solar noon (approximately 13:00-14:00 during July 2019, and 13:25-14:25 during August 2021).

Differing UV radiation exposures were accomplished by placing acrylic sheets over the corresponding treatment vessels that either reduced penetrating UV to ~15% or allowed ~90% UV radiation (confirmed through measurements outlined in Table 2.3 and shown in Figure 2.2). Space was left between the exposure vessels and the acrylic sheets to allow for air exchange. High UV exposures (~90% UV) represent full UV radiation and sunlight exposure that may occur in shallow, littoral zones. Low UV exposures (~15% UV) represent attenuated UV exposure in deeper water or in waters with lower UV penetration. Test vessels were distributed randomly to eliminate potential shading differences and were labeled with codes to ensure endpoint assessments were conducted blindly. To reduce the potential for different jars to be exposed to different shadowing or shading, the setup was lined with non-specular aluminum (Figure 2.2).

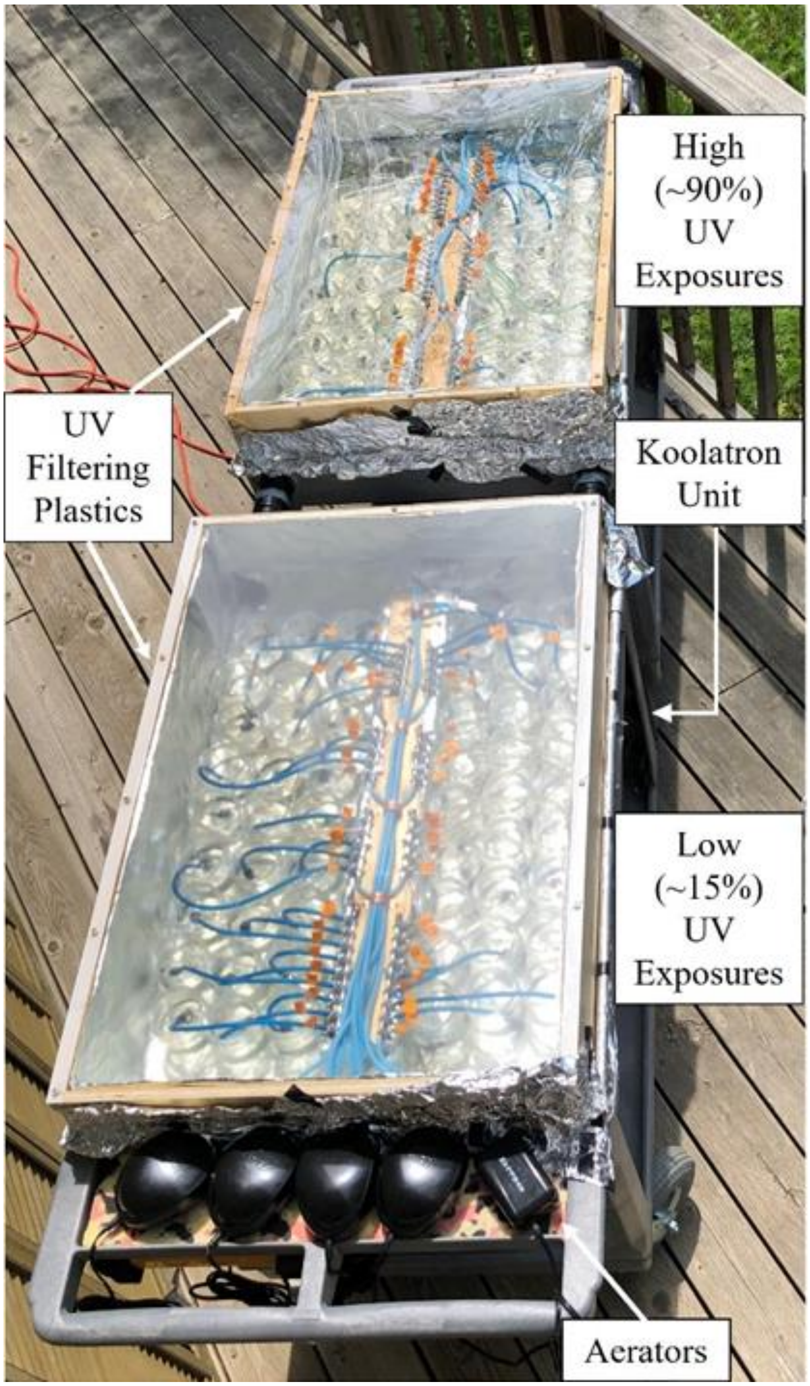

Figure 2.2. Experimental setup for the exposure of *Hyalella azteca* to diluted bitumen, conventional heavy crude, and water from Freshwater Oil Spill Remediation Study (FOReSt) enclosures and oil weathering experiments treated with remediation measures in combination with low or high UV exposure.

2.2.7 Photo Analysis

At the end of the experiment, *Hyalella azteca* were sedated within their respective vessel with a 4 µg/L concentration of tricaine methanesulfonate (MS222). All individuals were aligned in a consistent orientation and photographed for future growth and deformity analysis. The *Hyalella azteca* were photographed individually using a Leica WILD M10 microscope fitted with a Leica MC170 HD camera. After all individuals from the vessel were photographed, they were placed in a 25 µg/L lethal dose of MS222 to be euthanized. Photos of individual *Hyalella azteca* were measured for length using ImageJ software (Rasband 2018). During early analysis we noted bubbles under the carapace and added an analysis of the affected area (i.e. area of bubbles) to our suite of endpoints. Length was recorded as the entire curved distance along the dorsal surface from the tip of the third uropod to the base of the first antennae (Figure 2.3A, as outlined in US EPA 2000). Lateral body area was recorded for individuals that had bubbles as the entire body area, including antenna and legs, and the cumulative area of bubbles was measured (Figure 2.3B & 3C). The severity of the bubbles was sorted into normal (0%), low (< 5%), medium (5-30%) and high (> 30%) categories based on the percentage of body area that was affected by the internal and external bubbles

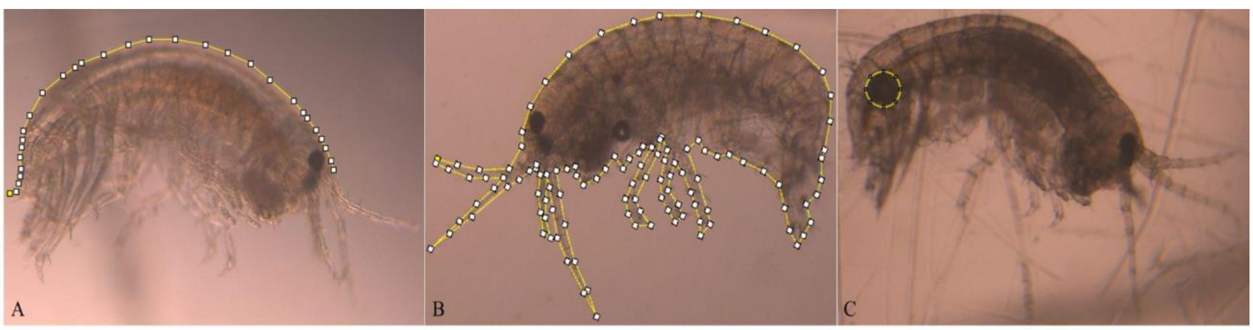

Figure 2.3. *Hyalella azteca* were measured to determine (A) length as the entire curved distance along the dorsal surface from the tip of the third uropod to the base of the first antennae, (B) lateral body area including antenna and legs and (C) cumulative bubble area at the end of a 5-day exposure to diluted bitumen, conventional heavy crude, and water from Freshwater Oil Spill Remediation Study (FOReSt) enclosures and weathering experiments treated with remediation measures in combination with low or high UV exposure.

2.2.8 Bubble Mode-of-Action Experiment

We conducted a 3-day study with *Hyalella azteca* exposed to the recommended application rate of DOSS with the air-water interface sealed off. This was to determine the contribution of in-water versus the surface to the development of internal and external bubbles in individuals. *Hyalella azteca* were received from the ECCC Burlington culture and left for 24 h to acclimate after shipping. Individuals were randomly selected and placed in jars until n=15 individuals per jar was reached. Four jars of the same DOSS concentration were used. A single piece of approximately 5 cm^2 presoaked cotton gauze was placed in each jar as a substrate for individuals to perch on. During the exposures, *Hyalella azteca* were fed approximately 5 mg of powdered TetraMin fish food per container every 2 days. Aerators were secured to the side of each jar to supply a small continuous amount of oxygen (> 92% dissolved oxygen). Each jar received 30 mL of a neutral (vegetable) oil on the surface to seal off the air-water interface that was gently added to not mix with the DOSS treated water. The number of suspended individuals and the location of each mortality was documented (i.e. in the water column versus suspended under the neutral oil barrier). Mortalities were removed daily, and photos were taken of all mortalities to document the count and location (internal versus external) of bubbles.

Live individuals were also photographed at the end of the experiment to document the count and location (internal versus external) of bubbles. Live individuals with external bubbles were also filmed for 30 seconds to see if attempts would be made to remove external bubbles once they were removed from the suspended surface and placed in clean water. An attempt to remove a bubble was defined as a body movement or convulsion in a direction that would push or remove the bubble.

2.2.9 Chemical Analysis of the Water Accommodated Fraction of Oil

Water samples were taken directly from the FOReSt enclosures that were treated with oil and secondary remediation measures, and from the low energy WAFs generated during oil weathering. The samples were analyzed for polycyclic aromatic compounds (PACs) and alkylated-PACs as described by Dearnley (2022). To obtain water from FOReSt enclosures, surface water was pumped from a depth of approximately 10 cm and approximately 3 m from the spill shoreline. Samples were collected into and stored in glass amber bottles with minimal headspace at 4°C prior to transport. In 2019 with dilbit samples, extractions occurred offsite within seven days of the sample collection, and in 2021 with CHV, extractions occurred onsite within 24 h. Samples were filtered by Whatman GF/C filters, added to a separatory funnel, spiked with 20 µL of a 5 ng/µL a recovery internal standard (d8-naphthalene, d8-acenaphthylene, d10-acenaphthene, d10-fluorene, d10-phenanthrene, d10-pyrene, d12-Benz(a)anthracene, d12-chrysene, d12-benzo(b)fluoranthene, d12-benzo(k)fluoranthene, d12-benzo(a)pyrene, d12-indeno(1,2,3-c,d)pyrene, d14-dibenzo(a,h)anthracene, and d14-benzo(g,h,i)perylene), and had 10 g sodium chloride added to aid in extraction. The dilbit samples (2019) were then double extracted using 50 mL (100 mL total) of dichloromethane (DCM) and added to a round bottom flask. The CHV samples (2021) were then extracted using 150 mL (100 mL) followed by 16 h at 6.8 rpm on a Bellco Digital Bench-top Roller Apparatus (Bellco Glass Inc., Vineland NJ). The volume of DCM was then reduced using rotary evaporation. The excess water was removed with sodium sulfate and the extract volume was further reduced to approximately 1 mL. Reduced extracts were fortified with an internal standard of d10-anthracene (20 µL of 5 ng/µL) and stored at 4°C in amber GC vials until instrumental analyses.

Native and d-labelled PAH and alkyl PAH quantification for the extracts was completed on an Agilent 7890 gas chromatograph coupled to a 7000C triple quadrupole mass spectrophotometer fitted with an electron ionization (EI) source. Helium was used as the carrier gas at a flow rate of 1.2 mL/min with an Agilent J&W HP-5ms ultra inert column (30 m × 0.25 mm, 0.25 µm film thickness). Sample volumes of 1 µl were injected by a PAL RSI 85 auto sampler at a temperature of 60°C. Final concentrations were corrected for internal standard recoveries. Gas chromatograph conditions and details of the product ion transitions for the 16 priority PAHs (identified by the US EPA as priority pollutants harmful to human health (Hussar et al. 2012)) and alkylated PAHs that were analyzed in the samples can be found in Idowu et al. (2018). PAC concentrations for each of the treatments are in the Appendix (Table 2.2, Figure 2.11).

2.2.10 Statistical Analysis

R Statistical Software (V4.1.2) and RStudio (version 2023.03.0) were used for all data and statistical analysis and visualization (R Core Team 2021; Posit Team 2022). The data and residuals were examined for homogeneity of variance using Q-Q plots and a Levene test, and for normal distributions using histograms and Shapiro tests (normality $p > 0.05$). No transformations were applied to the data prior to analysis. A 2-way analysis of variance test (ANOVA) with an interaction and a Tukey Kramer HSD test was used to examine the significance between groups. The percent final mortality (the total mortality of each treatment at the end of the 5-day exposure), length, and suspended individuals were evaluated in a linear regression model to assess how the interaction between UV and treatment variables impacted these variables. Across all tests, differences were considered significant if $p < 0.05$.

The lethal concentrations of dilbit and CHV were modeled using a four-parameter log-logistic dose-response function to define the sigmoidal concentration response, using the following formula:

$$f(x) = c + \frac{d - c}{1 + exp(b(log(x) - log(e)))} \qquad \textit{(Equation 1)}$$

The upper and lower asymptotes were fixed to the observed mortality values of 0% and 100% (parameter *c* and *d*). This results in the abbreviated formula:

$$f(x) = \frac{100}{1 + exp(b(log(x) - log(e)))} \qquad \textit{(Equation 2)}$$

This four-parameter log-logistic dose-response function is called LL.4 in the drc package in R (Ritz et al. 2015). The four-parameter log-logistic dose-response function was chosen so the limits (0 & 100%) could be fixed as the limits in the model.

2.3 Results

2.3.1 Ultraviolet Radiation

UV radiation exposures were achieved by moving the experimental setup outdoors to be exposed to natural sunlight during solar noon, between 13:00-14:00 during July 2019, and 13:25-14:25 during August 2021. Measurements of UVA, UVB, visible light, and photosynthetic active radiation (PAR) were recorded above and below the two plastics using a radiometer and HOBO loggers. The low UV (~15% UV radiation) exposure let 15.3% of UVA and 15.4% of UVB (15.3% total UV) penetrate, while the high UV (~90% UV radiation) exposure let 89.5% of UVA and 87.2% of UVB (89.4% total UV) through. The low UV group maintained 95.3% of visible light and 97.2% of PAR compared to the high UV exposures of 96.2% visible light and 99.6% PAR (Table 2.3, Appendix). There were no significant differences between visible light and PAR in the low and high UV plastics compared to without plastic.

In these experiments, natural sunlight was used as the UV radiation exposure, and therefore did not remain consistent day to day or between the different experiments (Figure 2.4). The average

UV radiation (including both UVA and UVB) in the 2019 dilbit study was 10.10 watts/m^2, and in the 2021 CHV study was 15.14 watts/m^2 (Table 2.4, Appendix).

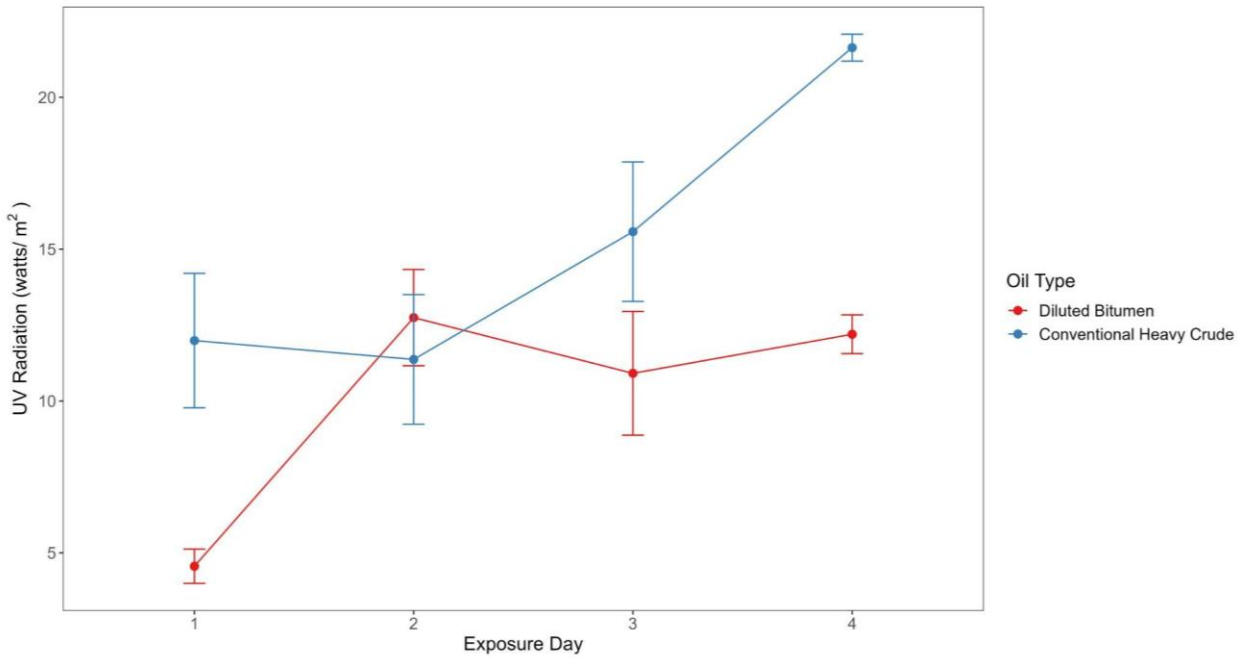

Figure 2.4. Daily solar noon ultraviolet (UV) radiation (watts/m^2) from exposures of diluted bitumen (2019), conventional heavy crude (2021), and water from Freshwater Oil Spill Remediation Study (FOReSt) enclosures and weathering experiments treated with remediation measures to *Hyalella azteca*. Points represent the average (with standard error) UV radiation over 1 h centered over solar noon (n=10-13).

2.3.2 Mortality

Dilbit and CHV alone and in combination with UV exposure significantly increased mortality compared to the reference treatments (Figure 2.5). Photo-toxic responses for the final mortality of *Hyalella azteca* exposed to dilbit and CHV were identified by modelling the interaction between the treatments and UV radiation in the statistical analysis (Tukey Kramer HSD, mortality ~ UV*Treatment). Full cumulative mortality for dilbit and CHV is displayed in Figure 2.12 in the Appendix. Individuals exposed to high UV radiation and the highest oil treatments from the dilbit and CHV WAFs all exhibited 100% mortality at the end of the experiment, while those exposed to low UV ranged from 85% to 100% final mortality. In the dilbit experiment, mortality was significantly higher in the positive WAF group than the reference group in the low UV group (Tukey Kramer HSD, p-value < 0.001, Figure 2.5). In the high UV group, all three dilbit treatments were significantly different from the reference group (Tukey Kramer HSD, p-value < 0.05, Figure 2.5). There were also significant differences among the dilbit treatments when comparing mortalities in low and high UV groups (Tukey Kramer HSD, p-value < 0.05, Figure 2.5). The interaction between dilbit treatments and UV radiation was significant (Tukey Kramer HSD, p-value < 0.05, Figure 2.5).

In the CHV experiment, mortality was significantly higher among the WAF treatments compared to the reference (Tukey Kramer HSD, p-value < 0.005, Figure 2.5). The SWA and DOSS treatments in both UV groups, and with or without oil, also significantly differed from the reference group (Tukey Kramer HSD, p-value < 0.05, Figure 2.5). There were significant increases from the reference to the EMNR remediation treatment in the high UV group (Tukey Kramer HSD, p-value < 0.05), but no significant differences in the low UV group (Figure 2.5). There were significant increases in mortalities across all SWA and surfactant treatments except the DOSS only treatment when comparing low to high UV groups (Tukey Kramer HSD, p-value < 0.05, Figure 2.5). The DOSS only treatment does not show a significant difference between low and high UV groups (Figure 2.5). The WAF treatments also do not have a significant difference between the low and high UV groups, as all were close to or at 100% mortality (Figure 2.5). In both experiments, the reference group had negligible mortality, and there was no significant difference between the low and high UV groups (Figure 2.5). The interaction between CHV treatments and UV radiation was significant (Tukey Kramer HSD, p-value < 0.05, Figure 2.5).

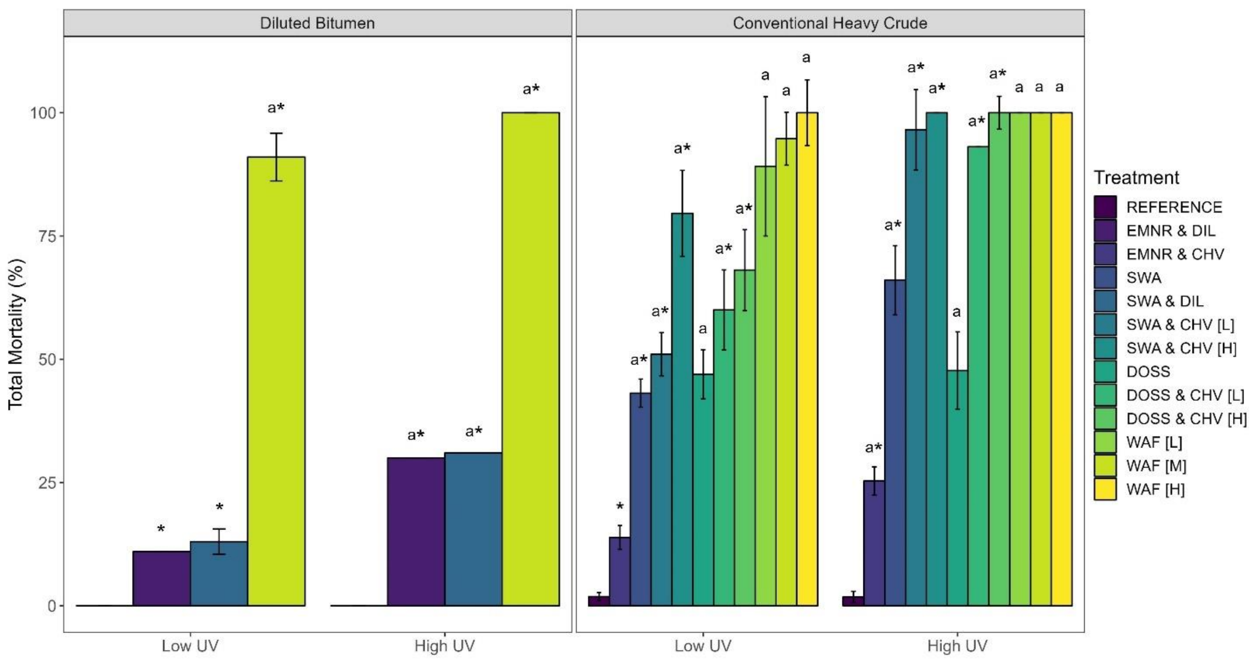

Figure 2.5. Mortality of *Hyalella azteca* at the end of a 5-day exposure of diluted bitumen and conventional heavy crude from the Freshwater Oil Spill Remediation Study (FOReSt) and weathering experiments treated with remediation measures in combination with low or high UV exposure. Treatments include unoiled reference enclosures from the FOReSt project (Reference), nutrient additions through Enhanced Monitored Natural Recovery (EMNR), the surface washing agent (SWA) Corexit EC9580A, the active surfactant in Corexit EC9580A dioctyl sodium sulfosuccinate (DOSS), and various concentrations (L – low, M – medium, H – high) of water accommodated fraction (WAF) of the two test oils; diluted bitumen (DIL) and conventional heavy crude (CHV). **a** indicates a significant difference from the reference, and ***** indicates a significant difference between the low and high UV groups of the same treatment.

2.3.3 Lethal Concentrations

The lethal concentration (LC) values were modeled using a four-parameter log-logistic dose-response function. The dilbit exposures at low UV concentrations resulted in the following model (Figure 2.6):

$$y = \frac{100}{1 + exp(-2.855(log(x) - log(e)))} \quad \textit{(Equation 3)}$$

For the high UV model, the model was described by (Figure 2.6):

$$y = \frac{100}{1 + exp(-2.752(log(x) - log(e)))} \quad \textit{(Equation 4)}$$

The LC_{25} and LC_{50} values of dilbit were significantly different between low and high UV groups, with no overlap of the 95% confidence intervals (Figure 2.6, Table 2.6 Appendix). The high UV group had a lower lethal concentration (LC_{50}) of 9466 ng L^{-1} [7891, 11,041 ng L^{-1}] compared to the 14,162 ng L^{-1} [11,609, 16,714 ng L^{-1}] LC_{50} of the low UV group, which is about 1.5 times greater (Figure 2.6, Table 2.7 Appendix).

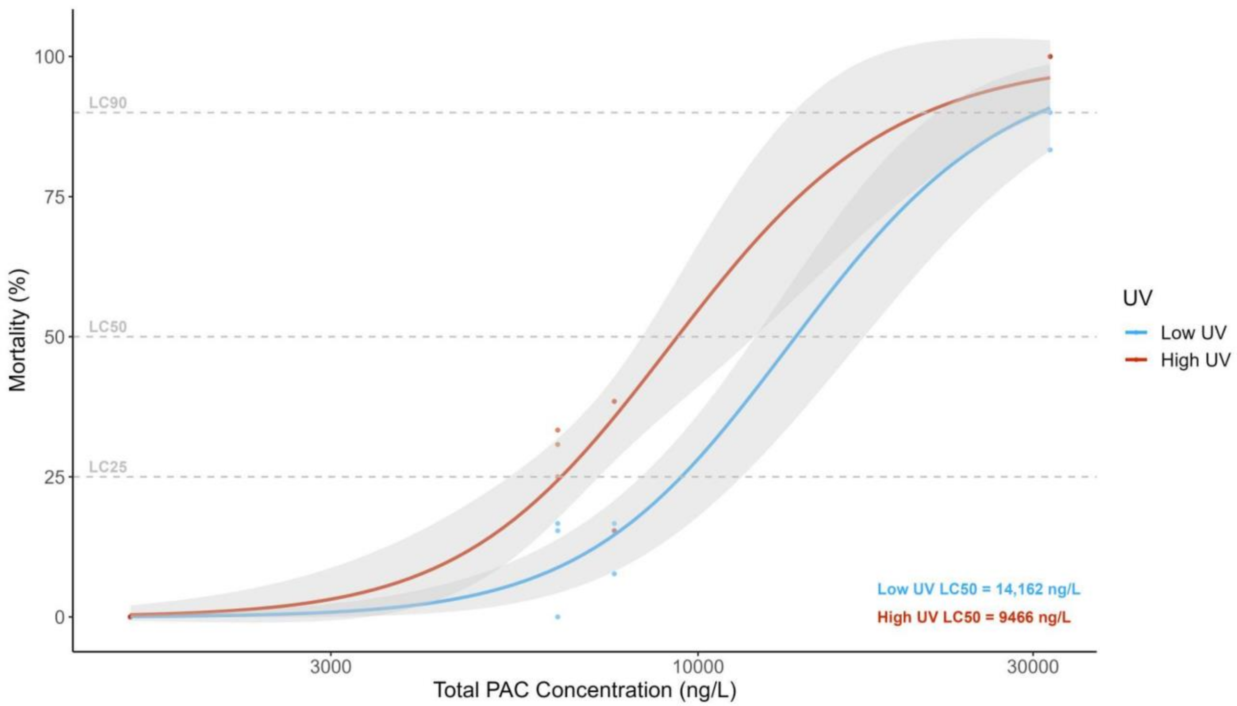

Figure 2.6. Regression of the 5-day cumulative mortality of *Hyalella azteca* against the total PAC concentration of diluted bitumen from the Freshwater Oil Spill Remediation Study (FOReSt) and weathering experiments treated with remediation measures, seperated by low and high UV exposure. The 50% lethal concentration (LC_{50}) values for the low (blue) and high (red) UV exposures are provided at the bottom right. Total PAC concentration (ng L^{-1}) on the x-axis is presented on a logarithmic scale.

The lethal concentration CHV model at low UV concentrations resulted in the following model (Figure 2.7):

$$y = \frac{100}{1 + exp(-0.585(log(x) - log(e)))} \qquad \textit{(Equation 5)}$$

For the high UV model, the model was described by (Figure 2.7):

$$y = \frac{100}{1 + exp(-0.899(log(x) - log(e)))} \qquad \textit{(Equation 6)}$$

The LC_{50} and LC_{90} values of CHV were significantly different between low and high UV groups, with no overlap of the 95% confidence intervals (Figure 2.7, Table 2.6 Appendix). The high UV group had a lower lethal concentration (LC_{50}) of 484 ng L^{-1} [246, 721 ng L^{-1}], compared to the 4761 ng L^{-1} [2305, 7216 ng L^{-1}] LC_{50} of the low UV group, which is nearly 10 times greater (Figure 2.7, Table 2.7 Appendix).

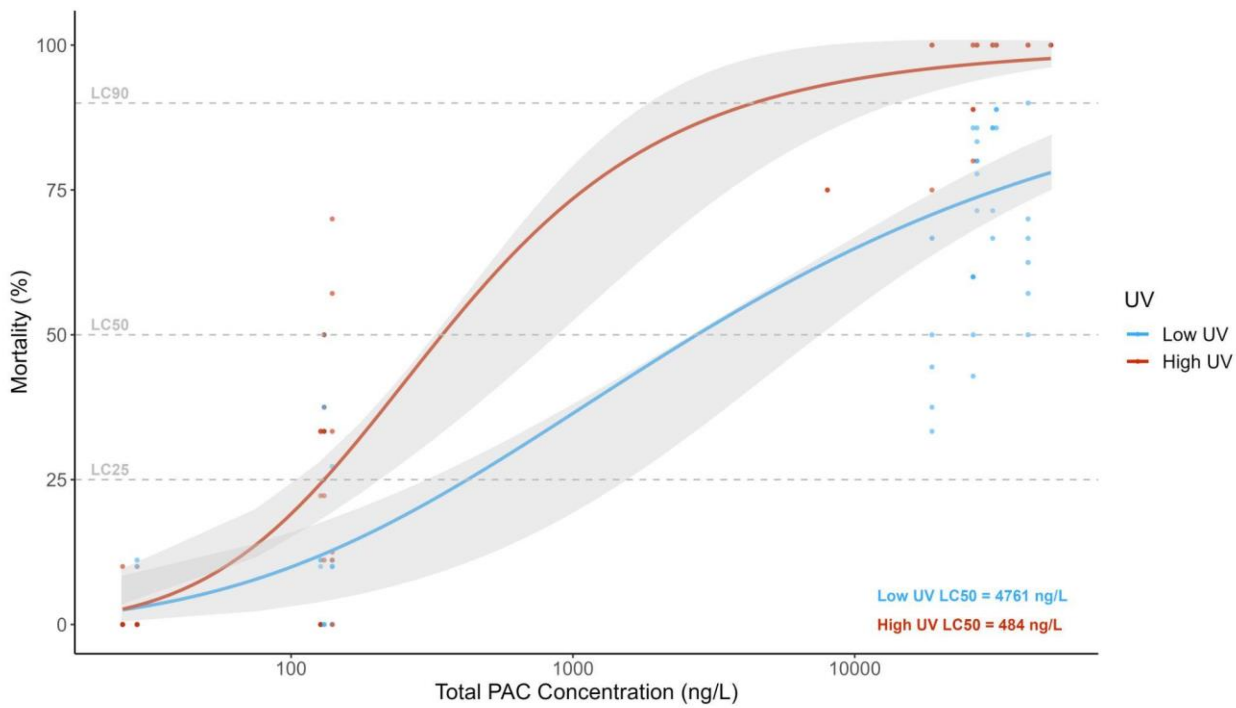

Figure 2.7. Regression of the 5-day cumulative mortality of *Hyalella azteca* against the total PAC concentration of conventional heavy crude from the Freshwater Oil Spill Remediation Study (FOReSt) and weathering experiments treated with remediation measures, seperated by low and high UV exposure. The 50% lethal concentration (LC_{50}) values for the low (blue) and high (red) UV exposures are provided at the bottom right. Total PAC concentration (ng L^{-1}) on the x-axis is presented on a logarithmic scale.

2.3.4 Growth

Hyalella azteca exposed to the treatments containing oil (either dilbit or CHV) were significantly shorter in both UV groups compared to the reference treatments (Tukey Kramer HSD, p-value < 0.005, Figure 2.8). Individuals exposed to dilbit and low UV were on average 0.17 mm shorter than their reference counterparts, and 0.35 mm shorter when exposed to high UV (Table 2.8, Appendix). When exposed to CHV, individuals were on average 0.26 mm shorter than their reference counterparts with low UV, and 0.35 mm shorter with high UV (Table 2.8, Appendix). Photo-toxic response (i.e. reduced length) of *Hyalella azteca* exposed to dilbit and CHV were identified by modelling the interaction between the treatments and UV radiation in the statistical analysis (Tukey Kramer HSD, length ~ UV*Treatment). These groups also had greater lengths in the low UV group compared to their counterparts in the high UV group (Tukey Kramer HSD, p-value < 0.05, Figure 2.8). When individually modelled, the interaction between dilbit treatments and UV radiation (Tukey Kramer HSD, p-value < 0.05) and CHV treatments and UV radiation (Tukey Kramer HSD, p-value < 0.05) were significant (Figure 2.8).

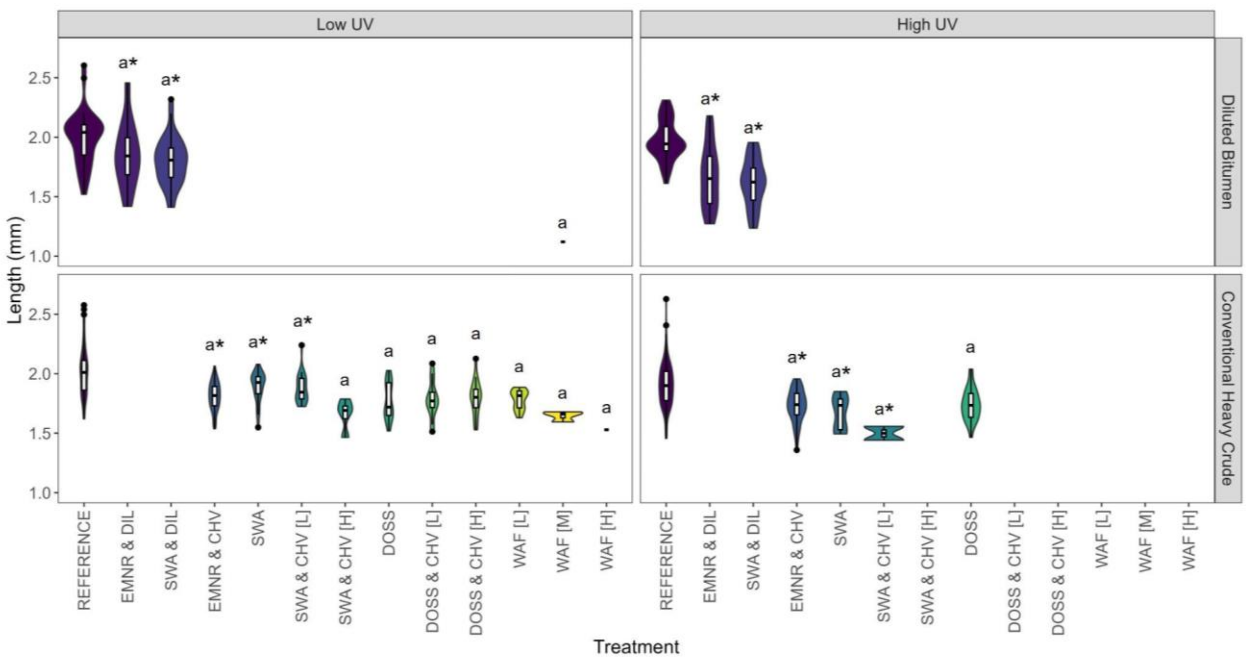

Figure 2.8. Length of *Hyalella azteca* at the end of a 5-day exposure to diluted bitumen and conventional heavy crude from the Freshwater Oil Spill Remediation Study (FOReSt) and weathering experiments treated with remediation measures in combination with low or high UV exposure. Treatments include unoiled reference enclosures from the FOReSt project (Reference), nutrient additions through Enhanced Monitored Natural Recovery (EMNR), the surface washing agent (SWA) Corexit EC9580A, the active surfactant in Corexit EC9580A dioctyl sodium sulfosuccinate (DOSS), and various concentrations (L – low, M – medium, H – high) of water accommodated fraction (WAF) of the two test oils; diluted bitumen (DIL) and conventional heavy crude (CHV). **a** indicates a significant difference from the reference, and ***** indicates a significant difference between the low and high UV groups of the same treatment. Treatments without observations lacked enough remaining individuals to present or were not evaluted.

2.3.5 Bubbling

2.3.5.1 Suspended Individuals

During photographing of *Hyalella azteca* from the dilbit experiment, we observed external and internal bubbles in some of the remaining live individuals from the SWA & DIL treatment group. Based on these observations, we hypothesized that individuals were becoming suspended at the surface. In the subsequent CHV experiment, the number of individuals suspended at the surface was recorded during daily inspections. We found significantly more suspended individuals in the SWA and DOSS treatments, with few in the WAF treatments and no suspended individuals in the references (Tukey Kramer HSD, p-value < 0.005, Figure 2.9). There were no significant differences between the low and high UV groups (Tukey Kramer HSD, p-value 0.45, Figure 2.9).

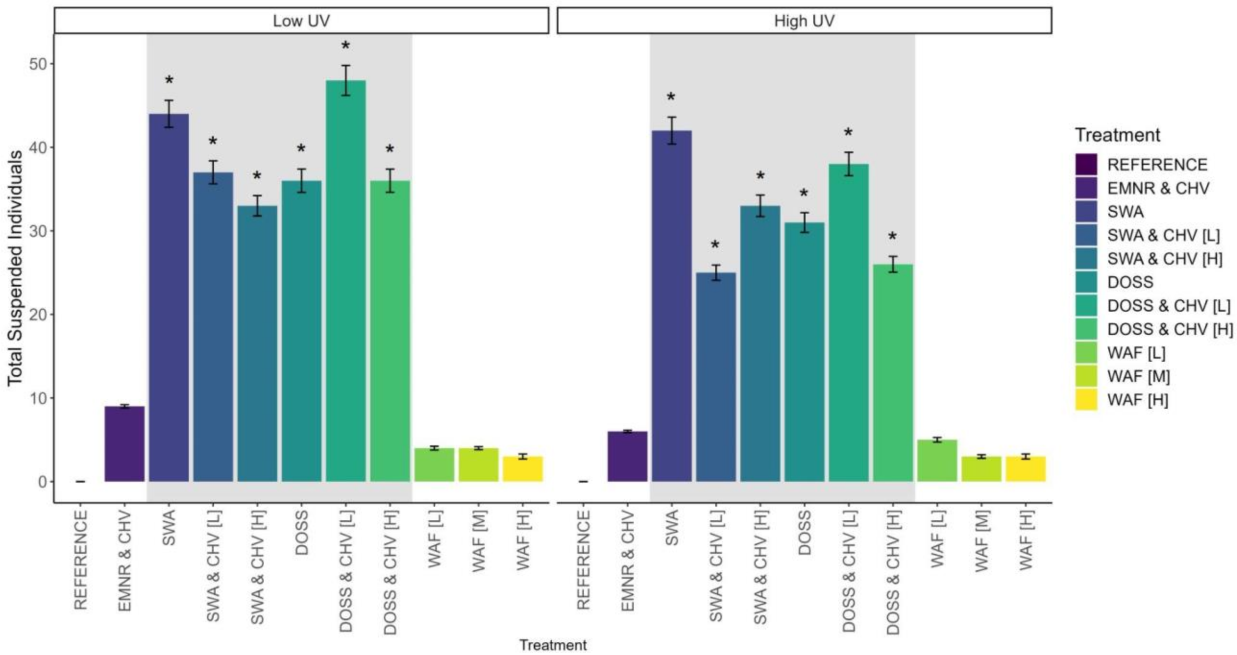

Figure 2.9. Observations of total suspended individual *Hyalella azteca* during a 5-day exposure of conventional heavy crude from the Freshwater Oil Spill Remediation Study (FOReSt) and weathering experiments treated with remediation measures in combination with low or high UV exposure. Treatments include unoiled reference enclosures from the FOReSt project (Reference), nutrient additions through Enhanced Monitored Natural Recovery (EMNR), the surface washing agent (SWA) Corexit EC9580A, the active surfactant in Corexit EC9580A dioctyl sodium sulfosuccinate (DOSS), and various concentrations (L – low, M – medium, H – high) of water accommodated fraction (WAF) of conventional heavy crude (CHV). Surface washing agent and surfactant treatments treatments are highlighted in grey. ***** indicates a significant difference from all treatments without surface washing agents or surfactants.

2.3.5.2 Bubble Severity

We observed the bubbles in both dilbit and CHV experiments but only in the treatments that also contained Corexit EC9580A (SWA) or DOSS. The severity of the bubbles was sorted into normal (0%), low (< 5%), medium (5-30%) and high (> 30%) categories based on the percentage of body area that was affected by the internal and external bubbles. In the CHV experiment, SWA was evaluated in the presence of oil (similarly to the dilbit experiment) and alone. The active surfactant within Corexit EC9580A, DOSS, was also evaluated with and without CHV. We observed that 11% to 65% of individuals among the SWA and DOSS treatments did not experience any bubbles (Figure 2.10). Meanwhile, among the SWA and DOSS treatments, 12 to 78% of the individuals in each treatment group experienced bubble deformities classified as low (< 5% body area impacted), 4 to 50% as medium (5-30% body area impacted), and 4 to 19% as high (> 30% body area impacted; Figure 2.10, Table 2.9).

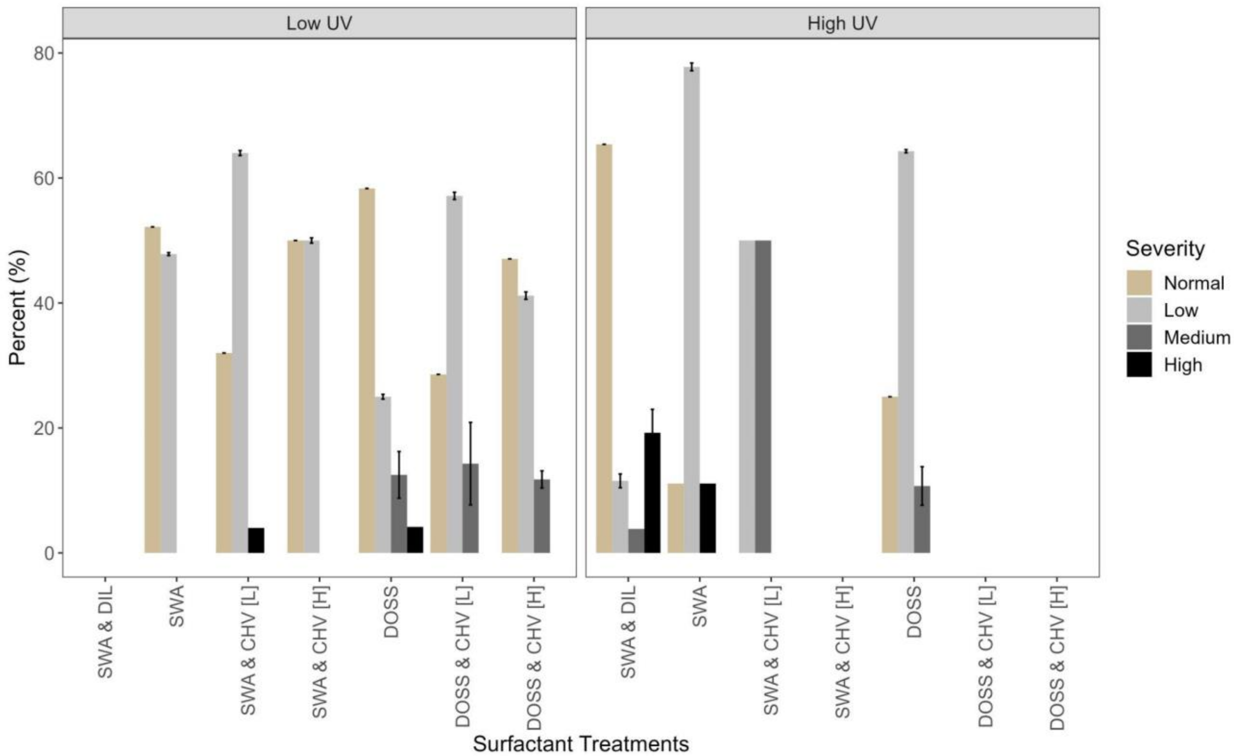

Figure 2.10. Severity of bubbles* in *Hyalella azteca* at the end of a 5-day exposure of diluted bitumen and conventional heavy crude from the Freshwater Oil Spill Remediation Study (FOReSt) and weathering experiments treated with surface washing agents or surfactants remediation measures in combination with low or high UV exposure. Treatments include the surface washing agent (SWA) Corexit EC9580A, the active surfactant in Corexit EC9580A dioctyl sodium sulfosuccinate (DOSS), and various concentrations (L – low, H – high) of the two test oils; diluted bitumen (DIL) and conventional heavy crude (CHV). Treatments without observations had no remaining individuals to present or had no individuals with bubbles. *Normal: 0% body area affected by bubbles, Low: < 5%, Medium: 5-30%, High: > 30%.

2.3.5.3 Bubble Mode-of-Action Experiment

Individuals in this experiment did not develop internal bubbling, only external bubbling that resulted in the suspending of individuals to the underside of the surface barrier. Those that had their buoyancy disrupted and were suspended in most cases were unable to shake off the external bubbles (Table 2.10, Appendix). A significant (two-way t-test, $p < 0.05$) proportion of mortalities in this experiment were those that were suspended under the air-water interface barrier, with the average being 77.6% (Table 2.1).

Table 2.1. Observations of total suspended individual *Hyalella azteca*, as well as total and suspended mortalities at the end of a 3-day exposure to the active surfactant in Corexit EC9580A, dioctyl sodium sulfosuccinate (DOSS), with the air-water interfaced blocked off to determine the mode-of-action of bubble physical effects after exposure to Corexit EC9580A and DOSS.

Jar	Total Individuals	Total Mortalities	Total Mortalities (%)	Total Suspended Mortalities	Total Non-Suspended Mortalities	Proportional Suspended Mortalities (%)	Total Suspended Individuals
1	15	9	60.0	8	1	88.9	9
2	11	4	33.4	3	1	75.0	5
3	14	6	42.9	4	2	66.7	6
4	16	5	31.3	4	1	80.0	5

2.4 Discussion

The trends observed in mortality, growth and LC_{50} all suggest that the effects of CLB dilbit and CHV are greater with higher UV radiation, indicating photo-enhanced toxicity. The LC_{50s} indicate that individuals in high UV environments, such as shallow littoral areas, would be affected at 1.5 to 10 times lower concentrations of dilbit or CHV than those in a low UV environment, such as deeper pelagic areas (Table 2.7, Appendix). We also saw a sequential elevation of toxicity with UV exposure so that LC_{25} values under low UV conditions were similar to the LC_{50} values under high UV conditions, and low UV LC_{50s} were similar to high UV LC_{90s} (Figures 2.6 & 2.7). Therefore, the lethal concentrations exponentially decrease when higher or environmentally-relevant concentrations of UV are considered.

Spill response times typically depend on the type and quantity of oil, the geographic location, the preparedness of the responsible organization, and the emergency response plans of the country involved (Daisy et al. 2022). These factors greatly influence how long oil remains in the system, spanning from quick responses on the day of the incident for smaller spills, to oil remaining in the system for a year in larger spills (US EPA 1999). We allowed the oil to remain on the water surface for 48 h. Based on data from the exposures in the current studies, organisms could have already been impacted by oil toxicity, and may also be impacted by photo-enhanced toxicity in high UV environments. We observed immediate mortality in the high WAF treatments, particularly in the high UV exposure group. Therefore, spills left without a cleanup response for more than a day could cause irreversible harm to *Hyalella azteca* or similar populations.

We used natural sunlight as our UV radiation exposure in this experiment, meaning the UV exposure was not consistent each day. This resulted in an average UV radiation exposure slightly higher in the CHV than the dilbit exposure (Figure 2.4). Natural sunlight was chosen as the UV exposure due to environmental relevance. The UV freshwater environments are experiencing is from the sun, which has daily variability. While this environmental variability is important to note, the outcome of this experiment was to compare a low UV exposure to a high UV exposure. The low and high UV exposures that are being compared occurred simultaneously under the same environmental UV radiation prior to the UV filtering.

Corexit EC9580A and DOSS were also evaluated on their own to determine if the bubble deformities we saw would also happen in the absence of oil (labeled SWA and DOSS treatments). What we saw was that both the only SWA and DOSS treatments saw bubble deformities. This confirms the notion that it is these products, and more specifically the presence of a surfactant, that is driving the presence of bubbles in the *Hyalella azteca*. However, what was unexpected was that Corexit EC9580A alone also exhibited a slightly significant photo-toxic response in both mortality and growth (Figure 2.5 & 2.8). While further research would be needed to evaluate a dose-response and confirm these findings, it appears that Corexit EC9580A by itself might be photo-toxic or increase the photo-enhanced toxicity of the oil products. In contrast, DOSS on its own did not exhibit a photo-enhanced toxicity response.

We used length relative to the reference treatments to evaluate growth of the juvenile *Hyalella azteca.* Length and growth are considered to be more sensitive, sub-lethal endpoints compared to mortality (Barron et al. 2021; US EPA 2000; Ingersoll et al. 1998), which is why we selected juvenile *Hyalella azteca*. We found that all oil, SWA and DOSS treatments had shorter lengths compared to the reference treatments. As well, the high UV group had shorter lengths compared to their low UV group counterparts across all treatments, including the reference group (Figure 2.8). This implies that dilbit, CHV, Corexit EC9580A, DOSS and UV are all individually, as well as in combination, impacting growth in *Hyalella azteca*. This could have impacts on *Hyalella azteca* fitness and survival in a natural environment. *Hyalella azteca* have a minimum size needed for reproduction, with larger amphipods tending to have higher reproductive output, and therefore growth in early juvenile stages is important to overall fitness (Ingersoll et al. 1998; US EPA 2000).

The oil exposures used in this experiment were designed to examine long term effects of residual oil after cleanup (SWA and EMNR treatments) and short-term exposures to high doses (WAF treatment) that are likely to occur immediately after an oil spill but before cleanup. SWAs as a remediation measure are designed to facilitate the removal of oil from the shoreline so it can be rinsed into open water to be physically removed or degraded (Chen et al. 2020). There has been some speculation that SWAs could be used as a remediation tool in freshwater systems, but little is known about their efficiency and impacts in these systems (Page et al. 2000; Canevari et al. 1995). In the FOReSt project, the use of Corexit EC9580A only marginally increased the recovery of oil from wetland systems in the FOReSt study (Palace et al. 2021a). While the recovery in the Corexit EC9580A treatment was marginally greater and there was reduced mortality compared to the WAF positive control group, the physical effects manifested via bubble formation in the Corexit EC9580A and DOSS groups may indicate reduced long-term survival if SWAs were used as an oil remediation method.

In 2021, Corexit EC9580A and the surfactant DOSS caused two separate bubble deformities, internal and external bubbles. Based on photos taken during the mode-of-action experiment when the air-water interface was blocked off, individuals did not accumulate internal bubbles, only external bubbles, which implies there are gaseous bubbles in the water column of DOSS treatments that individuals can get stuck to. When this happened, an individual's buoyancy became disrupted, causing them to become suspended under the neutral oil surface, or at the surface in the case of the main experiment. This resulted in over two-thirds of mortalities from this experiment occurring when individuals were suspended under the neutral oil, which supports the hypothesis that individuals in the SWA and DOSS treatments had increased mortality due to becoming suspended to the surface (Table 2.1). Without the ability to regulate their own buoyancy, we hypothesize that individuals remained suspended, where internal bubbles could then accumulate due to increased ingestion of air. Internal bubbles were seen in the overall experiment where the SWA and DOSS only treatments had high mortality and suspended individuals with internal bubbles, despite not having any oil exposure (Figures 5 & 9). However, we saw individuals in the main experiment with only internal bubbles, implying individuals may become suspended by methods other than external bubbles or can shake off the external bubbles and regain buoyancy. It is possible that when an individual vertically migrates in the water column, for example to feed, they may also get caught in the slick, as Corexit EC9580A is a petroleum-based product that produces its own sort of slick (Black et al. 2020). We observed that individuals were infrequently able to remove external bubbles, despite most exhibiting efforts to do so (Table 2.10, Appendix). In the overall experiment, the only chance for individuals to be released from the slick or to remove external bubbles was during water changes. In a real-world scenario, there is a possibility of individuals being released through wind, wave action, turnover, etc., which could allow for the expulsion of the soluble gasses incorporated in their cavities. However, individuals that get caught up in a surface slick would be more exposed to surface oil slicks, as well as being unable to avoid predation and UV radiation, which would likely cause mortality before recovery is possible.

The individuals affected by bubbles in the SWA and DOSS treatments, especially those in the high occurrence category, have a large percentage of their body area affected that may affect survival. The high occurrence category represented between 4 and 19% of individuals in the SWA treatment across both years respectively, and 4.2% in the 2021 DOSS treatment, and the majority of the treatments had more than 50% belonging to one of the three affected categories (Table 2.9). Therefore, the long-term success and survival of *Hyalella azteca* exposed to scenarios where dilbit or CHV was remediated with a SWA, regardless of UV exposure, could be less than what the LC_{50} values suggest. Incorporating these physical effects, which most toxicity tests do not, has the potential to alter the lethal concentrations and thresholds of the population.

Oil spills can present unique effects to the benthic and epibenthic communities in freshwater systems, especially in remote areas where oil spill response efforts may be delayed. The density of oil increases the longer it remains in the system, due to the volatilization of lighter weight hydrocarbons, resulting in a thicker oil that can become negatively buoyant (Rodriguez-Gil et al. 2021; Stoyanovich et al. 2019, 2021). This can make the oil more difficult to remove from the system, and result in a layer of thick, heavy oil on the sediment (Rodriguez-Gil et al. 2021; Stoyanovich et al. 2019, 2021). *Hyalella azteca* are epibenthic, so their desired habitat could be smothered under such a scenario. While *Hyalella azteca* could vertically move in the water column to avoid sunken oil, they would be entering higher UV environments during a time of higher PAC exposure, potentially affecting their fitness and survival.

Photo-enhanced toxicity is an increasingly important mechanism of toxicity, especially in the context of continuing climate change. With climate change, UV radiation to aquatic systems is increasing, potentially exacerbating the relative effects of photosensitive pollutants to aquatic biota (Hooper et al. 2013; Kim et al. 2010). This may result in lower toxicological tolerance, increased sensitivity, and higher mortalities when pollutant exposure occurs (Hooper et al. 2013). This may be particularly relevant for invertebrate species that undergo daily vertical migration patterns such as *Hyalella azteca* that move within the water column to avoid predation. Behavioural patterns could be affected due to this exponentially increasing stress when exposed to UV radiation. This has been seen as a decrease in upwards migrations and increased sediment burial, which could result in lower food uptake and higher predation risk (Barron 2007; Hatch & Burton 1999a, 1999b).

2.5 Conclusion

Dilbit and CHV exhibit photo-enhanced toxicity to *Hyalella azteca* in freshwater systems at varying concentrations. For these two oils, photo-enhanced toxicity lowered the LC_{50} values by 1.5- to 10-fold, which significantly differs from standard toxicity testing conducted under low UV conditions. These results have important ramifications for oil spill remediation in freshwater environments emphasizing the need to consider photic environment, time to response, and chemical characteristics of the spilled oil. We also highlighted the importance of considering physical toxicity endpoints for affected invertebrate species. While the cause of bubble formation in *Hyalella azteca* exposed to Corexit EC9580A or the surfactant DOSS are not fully understood, the physical impact could be an important factor disrupting buoyancy and potentially causing invertebrate population impacts. Additional studies to examine the potential for other oils, SWAs, surfactants, detergents, and surface-active contaminants to induce similar physical effects in *Hyalella azteca* and other invertebrates are warranted.

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2.8 Appendix

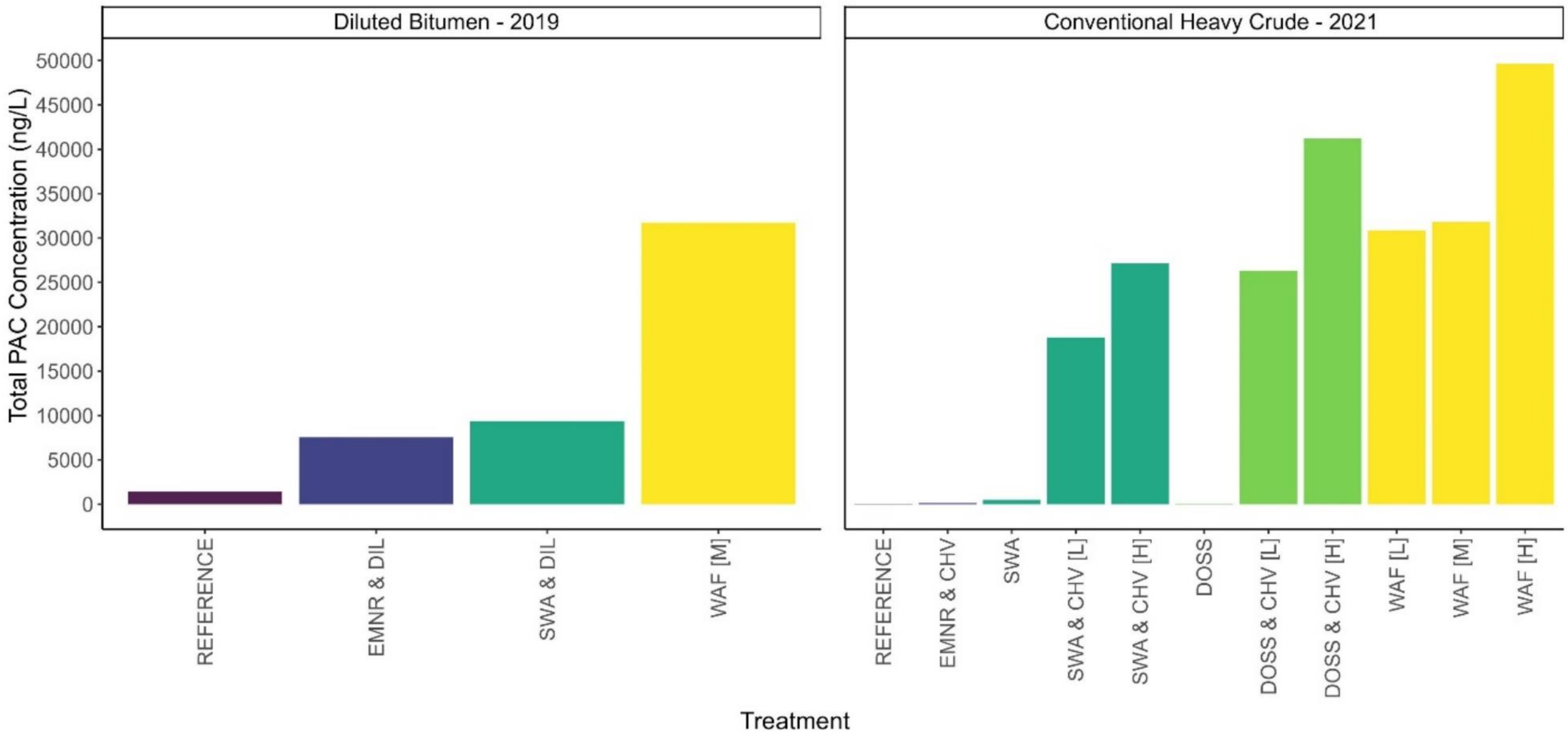

Figure 2.11. Polycyclic aromatic compound (PAC) concentrations (ng L^{-1}) used for a 5-day exposure to evaluate the response of *Hyalella azteca* to the photo-enhanced toxicity of diluted bitumen and conventional heavy crude from the Freshwater Oil Spill Remediation Study (FOReSt) and weathering experiments treated with remediation measures in combination with low or high UV exposure. Treatments include unoiled reference enclosures from the FOReSt project (Reference), nutrient additions through Enhanced Monitored Natural Recovery (EMNR), the surface washing agent (SWA) Corexit EC9580A, the active surfactant in Corexit EC9580A dioctyl sodium sulfosuccinate (DOSS), and various concentrations (L – low, M – medium, H – high) of water accommodated fraction (WAF) of conventional heavy crude (CHV).

Table 2.2. Polycyclic aromatic compound (PAC) concentrations (ng L^{-1}) and remediation treatment concentrations (mg L^{-1}) used for a 5-day exposure to evaluate the response of *Hyalella azteca* to the photo-enhanced toxicity of diluted bitumen and conventional heavy crude from the Freshwater Oil Spill Remediation Study (FOReSt) and weathering experiments in combination with low or high UV exposure. Treatments include unoiled reference enclosures from the FOReSt project (Reference), nutrient additions through Enhanced Monitored Natural Recovery (EMNR), the surface washing agent (SWA) Corexit EC9580A, the active surfactant in Corexit EC9580A dioctyl sodium sulfosuccinate (DOSS), and various concentrations (L – low, M – medium, H – high) of water accommodated fraction (WAF) of conventional heavy crude (CHV). Remediation treatment concentrations are estimates based on the weight of product added and the volume of water.

Oil	**Treatment Code**	**Year**	**PAC Concentration (ng L^{-1})**	**Remediation Treatment**	**Remediation Treatment Concentration (mg L^{-1})**
	REFERENCE	2019	1431.68		
Diluted Bitumen	EMNR & DIL	2019	7547.96	Fertilizer Addition	0.521
Diluted Bitumen	SWA & DIL	2019	9361.13	Corexit EC9580A	9.167
Diluted Bitumen	WAF [M]	2019	31,722.66		
	REFERENCE	2021	27.77		
Conventional Heavy Crude	EMNR & CHV	2021	132.81	Fertilizer Addition	0.521
	SWA	2021	501.54	Corexit EC9580A	9.167
Conventional Heavy Crude	SWA & CHV [L]	2021	18,798.48	Corexit EC9580A	9.167
Conventional Heavy Crude	SWA & CHV [H]	2021	27,145.07	Corexit EC9580A	9.167
	DOSS	2021	60.46	Dioctyl sodium sulfosuccinate	1.375
Conventional Heavy Crude	DOSS & CHV [L]	2021	26,302.64	Dioctyl sodium sulfosuccinate	1.375
Conventional Heavy Crude	DOSS & CHV [H]	2021	41,202.70	Dioctyl sodium sulfosuccinate	1.375
Conventional Heavy Crude	WAF [L]	2021	30,855.72		
Conventional Heavy Crude	WAF [M]	2021	31,802.33		
Conventional Heavy Crude	WAF [H]	2021	49,632.78		

Table 2.3. Comparison of UVA, UVB, visible light and PAR measurements of the low (~15% UV, OP3 plastic) and high (~90% UV, acrylic) UV filtering plastics used for a 5-day exposure to evaluate the response of *Hyalella azteca* to the photo-enhanced toxicity of diluted bitumen and conventional heavy crude, compared to measurements with no plastic/filter.

Group	**UV (%)**	**Plastic Type**	**Weather Conditions**	**UVA (watts/m^2)**	**UVB (watts/m^2)**	**Visible Light**	**PAR**
1	15	OP3	Cloudy	0.149	0.013	39.6	304
1	90	Acrylic	Cloudy	3.13	0.219	39.9	292
2	15	OP3	Full Sun	5.6	0.139	374	1330
2	90	Acrylic	Full Sun	22.8	1.2	365	1360
3	15	OP3	Sparse Clouds; Some Smoke	4.1	0.178	341	1461
3	90	Acrylic	Sparse Clouds; Some Smoke	19.7	0.926	340	1405
3	100	No Filter	Sparse Clouds; Some Smoke	21.8	1.03	348	1351
4	15	OP3	Full Sun; Smoke Sky	0.308	0.0368	57.7	215
4	90	Acrylic	Full Sun; Smoke Sky	1.77	0.0798	57.9	174
4	100	No Filter	Full Sun; Smoke Sky	2.08	0.0827	52.5	195
5	15	OP3	Full Sun; No Clouds; Some Smoke	1.89	0.147	264	1217
5	90	Acrylic	Full Sun; No Clouds; Some Smoke	12.26	0.69	269	1539
5	100	No Filter	Full Sun; No Clouds; Some Smoke	13.5	0.735	259	1031
6	15	OP3	Full Sun; No Clouds	4.1	0.173	372	1602
6	90	Acrylic	Full Sun; No Clouds	20.5	0.954	373	1533
6	100	No Filter	Full Sun; No Clouds	22.2	1.09	381	1556
7	15	OP3	Full Sun; Full Clouds	0.236	0.0136	37.2	169
7	90	Acrylic	Full Sun; Full Clouds	2.27	0.144	38.8	141
7	100	No Filter	Full Sun; Full Clouds	2.88	0.181	35.1	171
8	15	OP3	Full Sun; Partly Cloudy	2.37	0.206	402	1575
8	90	Acrylic	Full Sun; Partly Cloudy	20.6	0.997	403	1668
8	100	No Filter	Full Sun; Partly Cloudy	22.7	1.22	393	1629
9	15	OP3	Full Sun; Sparse Clouds	2.33	0.191	374	1617
9	90	Acrylic	Full Sun; Sparse Clouds	20.3	0.999	398	1613
9	100	No Filter	Full Sun; Sparse Clouds	22	1.2	378	1661
10	15	OP3	Cloudy; Partly Sunny				336
10	90	Acrylic	Cloudy; Partly Sunny				356
10	100	No Filter	Cloudy; Partly Sunny				396
11	15	OP3	Cloudy; Partly Sunny				407
11	90	Acrylic	Cloudy; Partly Sunny				415
11	100	No Filter	Cloudy; Partly Sunny				444
12	15	OP3	Very Cloudy; Grey				110
12	90	Acrylic	Very Cloudy; Grey				113
12	100	No Filter	Very Cloudy; Grey				123
13	15	OP3	Very Cloudy; Grey				136
13	90	Acrylic	Very Cloudy; Grey				137
13	100	No Filter	Very Cloudy; Grey				150

Table 2.4. Daily UV radiation averaged for each day of a 5-day exposure to evaluate the response of *Hyalella azteca* to the photo-enhanced toxicity of diluted bitumen and conventional heavy crude.

Oil	Exposure Day	Measurements Taken	Daily Average UV (watts/m^2)	Standard Error (SE)	Average UV (watts/m^2)	Total UV (watts/m^2)
Diluted Bitumen	1	13	4.56	0.56	10.10	40.42
Diluted Bitumen	2	13	12.75	1.58		
Diluted Bitumen	3	13	10.91	2.04		
Diluted Bitumen	4	13	12.20	0.64		
Conventional Heavy Crude	1	10	11.99	2.21	15.14	60.57
Conventional Heavy Crude	2	10	11.37	2.13		
Conventional Heavy Crude	3	10	15.57	2.30		
Conventional Heavy Crude	4	10	21.64	0.44		

Table 2.5. Daily average UV radiation across each exposure day and round of 5-day exposures to evaluate the response of *Hyalella azteca* to the photo-enhanced toxicity of diluted bitumen and conventional heavy crude.

Oil	**Round**	**Exposure Day**	**Measurements Taken**	**Daily Average UV (watts/m^2)**	**Standard Error (SE)**	**Average UV (watts/m^2)**	**Total UV (watts/m^2)**
Diluted Bitumen	1	1	13	4.56	0.56	10.10	40.42
Diluted Bitumen	1	2	13	12.75	1.58		
Diluted Bitumen	1	3	13	10.91	2.04		
Diluted Bitumen	1	4	13	12.20	0.64		
Conventional Heavy Crude	1	1	5	5.42	0.57	14.66	58.63
Conventional Heavy Crude	1	2	5	10.41	3.64		
Conventional Heavy Crude	1	3	5	22.34	0.48		
Conventional Heavy Crude	1	4	5	20.46	0.29		
Conventional Heavy Crude	2	1	5	18.56	0.36	15.63	62.51
Conventional Heavy Crude	2	2	5	12.33	2.61		
Conventional Heavy Crude	2	3	5	8.81	0.79		
Conventional Heavy Crude	2	4	5	22.81	0.34		

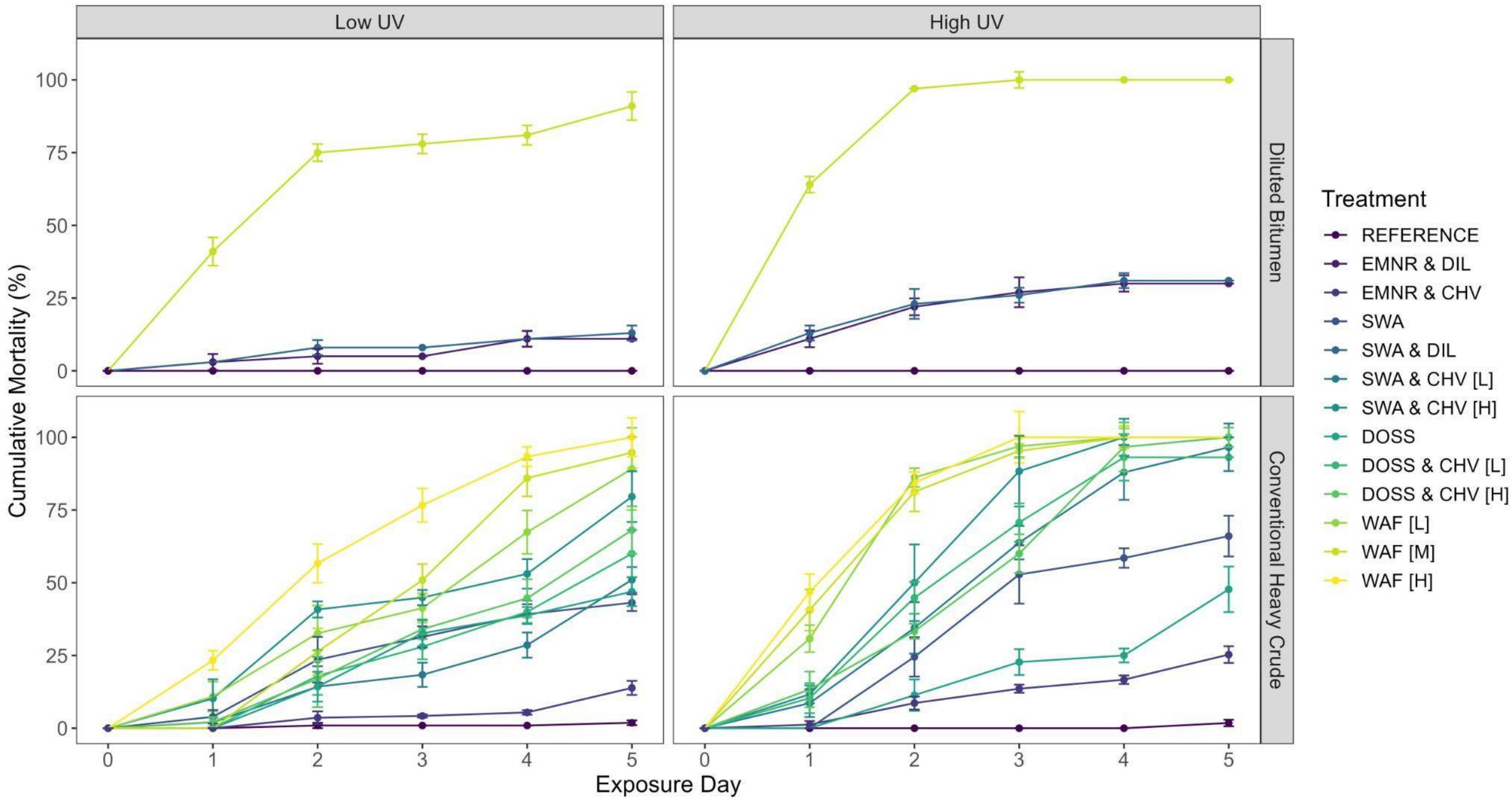

Figure 2.12. Cumulative mortality of *Hyalella azteca* over a 5-day exposure to diluted bitumen and conventional heavy crude from the Freshwater Oil Spill Remediation Study (FOReSt) and weathering experiments treated with remediation measures in combination with low or high UV exposure. Treatments include unoiled reference enclosures from the FOReSt project (Reference), nutrient additions through Enhanced Monitored Natural Recovery (EMNR), the surface washing agent (SWA) Corexit EC9580A, the active surfactant in Corexit EC9580A dioctyl sodium sulfosuccinate (DOSS), and various concentrations (L – low, M – medium, H – high) of water accommodated fraction (WAF) of the two test oils; diluted bitumen (DIL) and conventional heavy crude (CHV).

Table 2.6. Lethal concentration (LC) values with 95% confidence intervals after 5-day exposures to evaluate the response of *Hyalella azteca* to the photo-enhanced toxicity of diluted bitumen and conventional heavy crude.

Oil	Lethal Concentration Value (LC)	UV Group	Estimate	Standard Error	Lower CI	Upper CI
Diluted Bitumen	25	Low	9637.63	702.03	8073.41	11,201.85
Diluted Bitumen	50	Low	14,161.57	1145.57	11,609.09	16,714.05
Diluted Bitumen	90	Low	30,576.93	4385.10	20,806.32	40,347.55
Diluted Bitumen	25	High	6350.18	393.89	5472.54	7227.83
Diluted Bitumen	50	High	9465.99	706.94	7890.83	11,041.15
Diluted Bitumen	90	High	21,034.15	4754.86	10,439.66	31,628.64
Conventional Heavy Crude	25	Low	727.04	308.81	110.65	1343.43
Conventional Heavy Crude	50	Low	4760.83	1230.23	2305.29	7216.38
Conventional Heavy Crude	90	Low	204,141.64	75,257.60	53,926.83	354,356.44
Conventional Heavy Crude	25	High	142.48	22.79	97.01	187.94
Conventional Heavy Crude	50	High	483.57	119.02	246.13	721.01
Conventional Heavy Crude	90	High	5570.34	2773.62	9.3	11,129.98

Table 2.7. Comparison of the low UV radiation versus high UV radiation lethal concentration values to determine the toxicity ratio of low:high UV radiation of diluted bitumen and conventional heavy crude to *Hyalella azteca* after 5-day exposures to evaluate photo-enhanced toxicity.

Oil	Lethal Concentration Value (LC)	Low UV LC Value	High UV LC Value	Low:High UV Comparison
Diluted Bitumen	25	9637.63	6350.18	1.52
Diluted Bitumen	50	14,161.57	9465.99	1.50
Diluted Bitumen	90	30,576.93	21,034.15	1.45
Conventional Heavy Crude	25	727.04	142.48	5.10
Conventional Heavy Crude	50	4760.83	483.57	9.85
Conventional Heavy Crude	90	204,141.64	5570.34	36.65

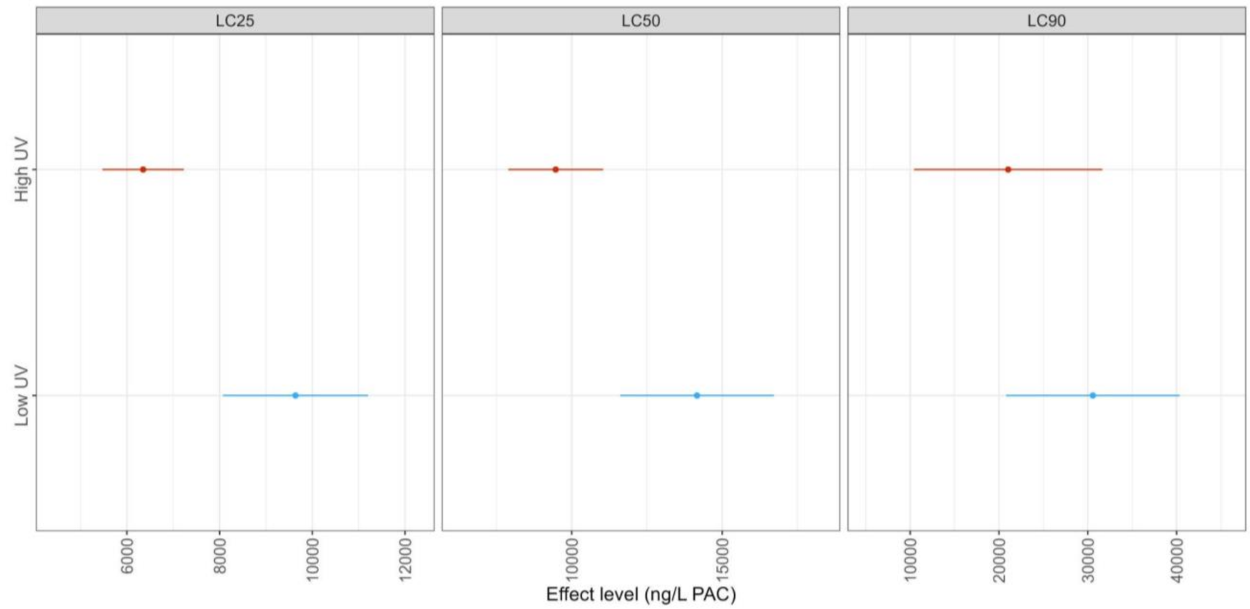

Figure 2.13. A comparison of *Hyalella azteca* lethal concentration (LC) values of the low and high UV radiation four-parameter log-logistic dose-response regression models for diluted bitumen treatments from the Freshwater Oil Spill Remediation Study (FOReSt) and weathering experiments treated with remediation measures.

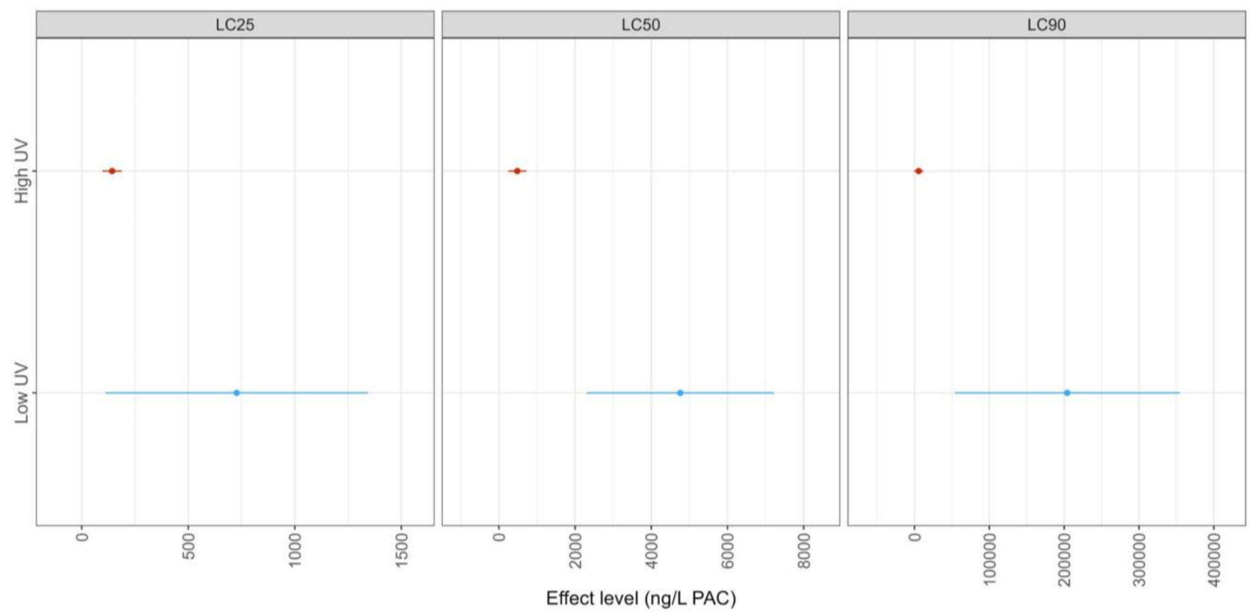

Figure 2.14. A comparison of *Hyalella azteca* lethal concentration (LC) values of the low and high UV radiation four-parameter log-logistic dose-response regression models for conventional heavy crude treatments from the Freshwater Oil Spill Remediation Study (FOReSt) and weathering experiments treated with remediation measures.

Table 2.8. Average length of *Hyalella azteca* at the end of a 5-day exposure to diluted bitumen and conventional heavy crude from the Freshwater Oil Spill Remediation Study (FOReSt) and weathering experiments treated with remediation measures in combination with low or high UV exposure. Treatments include unoiled reference enclosures from the FOReSt project (Reference), nutrient additions through Enhanced Monitored Natural Recovery (EMNR), the surface washing agent (SWA) Corexit EC9580A, the active surfactant in Corexit EC9580A dioctyl sodium sulfosuccinate (DOSS), and various concentrations (L – low, M – medium, H – high) of water accommodated fraction (WAF) of the two test oils; diluted bitumen (DIL) and conventional heavy crude (CHV).

Oil	Treatment Code	UV Exposure	Measured Individuals	Length (mm)	Standard Error
Diluted Bitumen	Reference	Low (~15%)	36	1.99	0.04
Diluted Bitumen	Reference	High (~90%)	36	1.97	0.03
Diluted Bitumen	EMNR & DIL	Low (~15%)	31	1.86	0.05
Diluted Bitumen	EMNR & DIL	High (~90%)	26	1.67	0.05
Diluted Bitumen	SWA & DIL	Low (~15%)	32	1.80	0.04
Diluted Bitumen	SWA & DIL	High (~90%)	26	1.62	0.04
Diluted Bitumen	WAF [M]	Low (~15%)	1	1.12	NA
Conventional Heavy Crude	Reference	Low (~15%)	108	2.01	0.02
Conventional Heavy Crude	Reference	High (~90%)	108	1.90	0.02
Conventional Heavy Crude	EMNR & CHV	Low (~15%)	141	1.81	0.01
Conventional Heavy Crude	EMNR & CHV	High (~90%)	128	1.73	0.01
Conventional Heavy Crude	SWA	Low (~15%)	23	1.89	0.03
Conventional Heavy Crude	SWA	High (~90%)	9	1.67	0.05
Conventional Heavy Crude	SWA & CHV [L]	Low (~15%)	25	1.87	0.02
Conventional Heavy Crude	SWA & CHV [L]	High (~90%)	2	1.50	0.06
Conventional Heavy Crude	SWA & CHV [H]	Low (~15%)	4	1.66	0.07
Conventional Heavy Crude	DOSS	Low (~15%)	24	1.78	0.03
Conventional Heavy Crude	DOSS	High (~90%)	28	1.74	0.03
Conventional Heavy Crude	DOSS & CHV [L]	Low (~15%)	14	1.79	0.04
Conventional Heavy Crude	DOSS & CHV [H]	Low (~15%)	17	1.80	0.04
Conventional Heavy Crude	WAF [L]	Low (~15%)	21	1.78	0.02
Conventional Heavy Crude	WAF [M]	Low (~15%)	3	1.65	0.03
Conventional Heavy Crude	WAF [H]	Low (~15%)	1	1.53	NA

Table 2.9. Bubble severity ranking* of *Hyalella azteca* at the end of a 5-day exposure of diluted bitumen and conventional heavy crude from the Freshwater Oil Spill Remediation Study (FOReSt) and weathering experiments treated with surface washing agents or surfactants remediation measures in combination with low or high UV exposure. Treatments include the surface washing agent Corexit EC9580A, the active surfactant in Corexit EC9580A dioctyl sodium sulfosuccinate (DOSS), and various concentrations (L – low, H – high) of the two test oils; diluted bitumen (DIL) and conventional heavy crude (CHV). *Normal: 0% body area affected by bubbles, Low: < 5%, Medium: 5-30%, High: > 30%.

Oil	Treatment	UV	Bubble Severity Category	Average Affected Body Area (%)	Frequency (%)	Count
Diluted Bitumen	Corexit EC9580A	High	Normal	0	65.38	17
			Low	1.93	11.54	3
			Medium	11.75	3.85	1
			High	39.46	19.23	5
Conventional Heavy Crude [L]	Corexit EC9580A	Low	Normal	0	32	8
			Low	2.09	64	16
			High	28.32	4	1
Conventional Heavy Crude [H]	Corexit EC9580A	Low	Normal	0	50	2
			Low	2.05	50	2
Conventional Heavy Crude [L]	Corexit EC9580A	High	Low	2.63	50	1
			Medium	10.03	50	1
Conventional Heavy Crude [L]	DOSS	Low	Normal	0	28.57	4
			Low	2.15	57.14	8
			Medium	11.88	14.29	2
Conventional Heavy Crude [H]	DOSS	Low	Normal	0	47.06	8
			Low	2.49	41.18	7
			Medium	6.78	11.76	2
NA	Corexit EC9580A	Low	Normal	0	52.17	12
			Low	0.90	47.83	11
NA	Corexit EC9580A	High	Normal	0	11.11	1
			Low	1.55	77.78	7
			High	28.76	11.11	1
NA	DOSS	Low	Normal	0	58.33	14
			Low	1.52	25	3
			Medium	13.85	12.5	1
			High	46.36	4.17	5
NA	DOSS	High	Normal	0	25	7
			Low	1.49	64.29	18
			Medium	13.07	10.71	3

Table 2.10. Number of bubbles and attempts* to remove those bubbles by *Hyalella azteca* at the end of a 3-day exposure to the active surfactant in Corexit EC9580A, dioctyl sodium sulfosuccinate (DOSS), with the air-water interfaced blocked off to determine the mode-of-action of bubble physical effects after exposure to Corexit EC9580A and DOSS. *Attempts are defined as a body movement or convulsion in a direction that would push or remove the bubble.

Jar	Individual	Bubbles	Attempts to Remove Bubbles	Successful Bubble Removals
2	4	11	5	2
2	5	18	1	0
2	6	16	0	0
2	7	9	0	0
2	8	14	0	0
2	9	5	4	0
2	10	5	0	0
3	7	0	0	0
3	8	7	2	0
3	9	2	0	0
3	10	7	2	0
3	11	7	5	0
3	12	2	1	0
3	13	3	12	0
3	14	34	0	0
4	4	7	7	0
4	5	37	3	0
4	6	10	6	0
4	7	3	10	0
4	8	1	12	0
4	10	1	0	0
4	11	31	6	0
4	12	17	16	0
4	13	31	8	0
4	14	3	0	0

Chapter 3: Examining the photo-enhanced toxicity and sub-lethal effects of diluted bitumen to early-life stages of wild fathead minnows (*Pimephales promelas*).[2]

Abstract

Diluted bitumen is one of the major products of the Canadian oil and natural gas industry. Spills into aquatic environments pose risks to biota, particularly in freshwater environments where there are still knowledge gaps on the effects of oil and remediation strategies. Oil spill remediation methods applicable to low energy freshwater environments are limited, but for this work we investigated the efficacy of the surface washing agent Corexit EC9580A, nutrient additions, and engineered floating wetlands in freshwater shoreline, littoral environments. Previous studies have evaluated the direct toxicity of dilbit to biota, but many overlook potential interactive effects of photo-enhanced toxicity. To examine the photo-enhanced toxicity of dilbit, we exposed early-life stages of wild fathead minnows to varying water accommodated fractions of dilbit resulting from various remediation treatments. Exposures were performed under low (~15%) and high (~90%) UV exposure at three timepoints; 12-, 20-, and 38-days post oil spill. Mortality was similar among all treatments, with a slight increase in high UV treatments at 38-days post oil spill, meaning there were no significant differences in calculated LC_{25} values. Early-life stage fathead minnows upregulated *cyp1A* when exposed to oil, and oil and UV in later rounds, but there were no significant trends in the expression of thyroid and development related genes. We also evaluated sub-lethal yolk sac, spinal, craniofacial, and cardiac malformations, and the lack of swim bladder inflation. Individuals were more likely to have higher malformation scores when exposed to oil, or oil and high UV, with an increased probability of malformations in yolk sac, cardiac, and swim bladder malformation categories. We highlight the need to evaluate photo-enhanced toxicity, wild populations, and sub-lethal effects when examining the impacts of oil spills and spill mitigation strategies.

3.1 Introduction

Canada has some of the largest bitumen reserves, with one of the biggest products being Cold Lake Blend (CLB) diluted bitumen (Alsaadi et al. 2018b; Lee et al. 2015). Canada is also one of the top five oil producers globally, producing over 1.5 times its domestic demand (CAPP 2019; Daisy et al. 2022; Lee et al. 2015). The most common product of the Canadian oil sands, bitumen, has a high viscosity and density, necessitating mixing with natural gas condensates (in the case of diluted bitumen (dilbit), approximately 20-30% condensate and 70-80% bitumen) to aid in transport (Lee et al. 2015). Most oil is transported through pipelines in North America, due to their ability to transport large quantities of oil with statistically the lowest accident rate, with some transport also occurring via rail (CAPP 2019; Crosby et al. 2013). Spill occurrences from Canadian crude pipelines are down by approximately 85% since the 1970s, with spill volume from this time being down by 92% (Etkin 2023). However, aquatic oil spills, particularly in fresh water, are still a major

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concern because the impacts of oil spills in freshwater systems are relatively unknown (Lee et al. 2015; Crosby et al. 2013). Many pipelines and rail networks are located near accessible fresh water, which can pose increased risks to these vital ecosystems (Alsaadi et al. 2018a; Dew et al. 2015; Levy 2009).

Polycyclic aromatic compounds (PACs) are a class of chemicals that occur naturally in coal, crude oil, and gasoline and are associated with acute and chronic toxicity, mortality, narcosis, reduced reproduction, and various pericardial, craniofacial, and spinal malformations in aquatic organisms (Lee et al. 2015; Barron 2017; Incardona et al. 2014; Madison et al. 2015, 2017). PACs exert their effects via several pathways, including through the aryl hydrocarbon receptor (AhR), which causes developmental toxicity and oxidative stress (Alsaadi et al. 2018b). This pathway includes the cytochrome P450 genes that regulate phase I xenobiotic metabolism, including c*yp1A* (Alsaadi et al. 2018b; Holth et al. 2014; R. O. Kim et al. 2013; Olsvik et al. 2012). The expression of *cyp1A* has been shown to be strongly associated with the exposure of fish to dilbit, and therefore is a useful and sensitive biomarker of embryotoxicity after oil exposure and PAC accumulation (Alsaadi et al. 2018a; Carls et al. 2005; Madison et al. 2015, 2017). PAC exposure and associated increases in *cyp1A* expression have been shown to impair cardiac function and cause yolk sac malformations (Billiard et al. 1999; Madison et al. 2017).

PAC exposure can impact the thyroid gland, which is essential for regulating development, growth, metabolism, reproduction, and behavior in fishes (Movahedinia et al. 2018; Johnson & Lema 2011; Porazzi et al. 2009). During normal thyroid function, the hypothalamus induces the pituitary gland to secrete thyroid-stimulating hormones, which activates synthesis of thyroxine (T4) and triiodothyronine (T3) in the thyroid gland (Movahedinia et al. 2018). PACs can significantly decrease plasma T4 levels and the circulation of thyroid hormones, leading to a change in the structure of the thyroid gland and hormone synthesis disruption (Movahedinia et al. 2018; Teles et al. 2005). In teleost fish, there are 3 iodothyronine deiodinase enzymes that regulate TH levels; type I (*dio1*), type II (*dio2*), and type III (*dio3*) (Köhrle 1999, 2000; Orozco & Valverde-R 2005). The thyroid receptors (TRs) *thrα* and *thrβ* are also important to thyroid signalling and mediation of T3 and T4 (Power et al. 2001; Mortensen & Arukwe 2006). These are present in tissues at varying times due to their roles in development, *thrα* being predominant in heart and brain development, whereas *thrβ* is more predominant in muscle, kidney, and liver development (Deal & Volkoff 2020). PACs can cause early-life stage fish to have reduced *thrα* and *thrβ* transcription, which has the potential to disrupt the function of thyroid hormones (Sun et al. 2008). Exposure to varying pollutants has been shown to cause impacts to thyroid hormone signaling and development systems through *dio* and *thr* genes (He et al. 2012; Johnson & Lema 2011; Morgado et al. 2009; Mortensen & Arukwe 2006; Mousavi et al. 2022; Picard-Aitken et al. 2007; Truter et al. 2017).

Another potential concern surrounding oil spills is photo-enhanced toxicity (PET). PET is the synergistic interaction between a contaminant and ultraviolet (UV) radiation that can increase the toxic response by 2- and 1000-fold in aquatic organisms (Barron 2007; Barron et al. 2018; Little et al. 2000). UV radiation in freshwater environments can interact with PACs in individuals that do not have UV protections (e.g. pigmentation, armouring, behavioural avoidances), increasing the toxicity of those compounds *in vivo* (Barron 2007). Certain 3-5 ring PACs, including anthracenes, fluoranthenes, pyrenes, and benzopyrenes, cause PET due to their ability to interact with UV radiation, causing oxidative damage, lipid peroxidation, cell death, and ultimately, with prolonged exposure, mortality (Barron 2017; Barron et al. 2018). Typical laboratory toxicity tests occur in isolation and do not evaluate the impact of UV radiation or PET. PET is important to consider since contaminants in natural environments would have unavoidable UV exposure. Conducting environmentally-relevant interaction studies will develop accurate and reliable toxicity information, which will help regulatory agencies recognize the complexity of nature and contribute to the development of appropriate environmental policy.

We conducted toxicity tests incorporating the effects of environmentally-relevant model dilbit spills, UV radiation, and PET on sensitive early-life stage wild fathead minnows (FHM; *Pimephales promelas*). We evaluated various effects in early-life stage wild FHM arising from dilbit exposures after model spills were treated with different remediation treatments. Lethal and sublethal endpoints, including gene expression (*cyp1A*, *dio2*, *dio3*, *thrα*, and *thrβ*) and malformations were examined. To our knowledge, this is the first laboratory study to assess the PET of dilbit to wild early-life stage FHMs under a realistic model spill exposure scenario. Results from this study will support risk assessments focused on early-life stages of fish in near shore freshwater environments.

3.2 Methods

3.2.1 Freshwater Oil Spill Remediation Study (FOReSt) Project

This experiment was conducted at the IISD-Experimental Lakes Area (IISD-ELA), located in Northwestern Ontario in the boreal forest region of Canada (49°40′N, 93°44′W). This study was part of the Oil Spill Remediation Study (FOReSt) that was conducted on Lake 260 (49°42′N, 93°46′W; Palace et al. 2021a). Lake 260 is an oligotrophic boreal lake that has an area of 330,000 m^2, a volume of 2,000,000 m^3, an average depth of 7 m, and a maximum depth of 16 m (Brunskill & Schindler 1971). The FOReSt project was created to study the effects of oil spills and compare the efficiency of minimally invasive methods to remediate freshwater shorelines after oil spills. Full methods of the experimental oil spill, primary and secondary remediation are outlined in Palace et al. (2021). Briefly, model oil spills were conducted in 5 m wide × 10 m long shoreline enclosures that contained approximately 20,000-25,000 L of littoral aquatic habitats that were then exposed to ~1.25 kg of Cold Lake Blend (CLB) dilbit weathered for 36 h prior to being applied. The weathered oil was left to interact with the shoreline in the FOReSt enclosures for 96 h following the spills to simulate conservative response times for spills in remote locations. After this interaction, period primary cleanup began. Free floating oil was removed from the surface of the water inside the FOReSt enclosures using polypropylene sorbent pads (Spill Ninja, MEP Brothers, Winnipeg, Canada), followed by 1200 L of flushing with fresh water using a low-pressure manifold, followed by another round of removal of floating product using sorbent pads. No manual removal of oil from the shoreline substrates or vegetation was attempted. After primary clean up, secondary remediation methods were applied to address residual oil remaining in the FOReSt enclosures. The secondary remediation methods included Enhanced Monitored Natural Recovery (EMNR), the SWA Corexit EC9580A, and Engineered Floating Wetlands (EFWs). The EMNR refers to the addition of nutrients, specifically nitrogen and phosphorus, which are added to approximate the Redfield ratio (106:16:1 C:N:P) with the carbon coming from oil. Nutrient addition is intended to stimulate microbial activity to assimilate carbon by degrading oil. In the FOReSt enclosures, approximately 50-60 g of fertilizer (Scotts-Osmocote, Everris, NA INC., Fusion 19-6-9, Nitrogen 19%, Phosphate 6%, Potassium 9%) was introduced into the near shore area ~50 cm from the shoreline. Corexit EC9580A is a petroleum-based product that contains a surfactant and coagulating agent to remove oil from the shoreline so it can be rinsed into the aquatic environment and collected from the surface. Corexit EC9580A was applied to the oiled area of the shoreline in each enclosure using a garden chemical sprayer according to manufacturer recommendations with an application rate of 265 g to an area of ~2.5 m^2. After a contact time of 30 minutes, shorelines were rinsed as noted above and any liberated oil was collected. Finally, one Engineered Floating Wetland (EFW) platform containing cattails (*Typha spp.)* and other wetland plants (*Carex spp.*) that have vast root systems was augmented in the EFW enclosure. These root systems, collectively referred to as the rhizosphere, provide a large surface area that hosts rich microbial communities that can consume carbon from the oil. Reference enclosures, to which had no oil applied, also underwent fresh water flushing.

3.2.2 Oil Weathering & Collection

Dilbit was weathered according to the methods described in (Palace et al. 2021). Briefly, 7 kg of Cold Lake Blend dilbit was weathered in 1.2 m diameter stainless steel evaporation pans containing 25 cm of Lake 260 water for 36 h. After weathering, the oil was collected from the surface of the water using stainless steel spoons. The weathered oil was applied at a rate of ~1.25 kg (approximately 1.25 L) to each of the FOReSt enclosures. Water samples were collected from the FOReSt enclosures at 12-, 20-, and 38- days post oil addition (8-, 16-, 34- days post recovery). Surface water (approximately 10 cm depth) was collected in 4 L amber glass bottles from each FOReSt enclosure using a peristaltic pump, avoiding any active surface slicks.

3.2.3 Embryo Fathead Minnows

At the IISD-ELA, FHMs are abundant in a small Lake 114 (49°40′30”N, 93°45′W). Lake 114 has a surface area of 21,000 m^2, an average depth of 4-5 m, a maximum depth of 5 m, and remains well mixed throughout the summer (Malley et al. 1988). In the past, this lake has been used for acid addition, aluminium, selenium, and metformin studies (Cruikshank 1986; Malley et al. 1988; Graves et al. 2019; Nielsen et al. 2022), however, fish populations are considered healthy and stable within the lake. In observations at the IISD-ELA, FHMs typically spawn in shallow waters under fallen debris (i.e. logs). After viability trials, we designed artificial spawning substrate (i.e. wood with PVC piping cut in half) and deployed it in high spawning areas. For egg collection, clean tiles were deployed 24 h prior to collection to ensure only recently fertilized eggs were collected. Egg clutches on the spawning tile were gently covered in a 1.5% sodium sulfite solution for 1-2 minutes to facilitate removal from the tiles. The clutches were then rinsed with lake water to transfer the eggs into a single collection vessel prefilled with lake water. Collecting multiple eggs clutches into the same vessel ensured individual variability and randomness. Eggs were immediately transported back to the laboratory, where unfertilized and low-quality eggs were removed (e.g. opaque, misshaped, or clumped eggs).

3.2.4 Experimental Setup

Individual early-life stage FHMs were randomly selected and placed in jars until n=20 individuals per jar was reached. Each of the triplicate exposure vessels was 250 mL and contained 150 mL of water from the appropriate treatment. Individual vessels were incubated at 22°C (± 2°C) for 24 h before the first UV exposure. An 80% static water renewal was completed every second day. Once individuals began hatching, each jar received approximately 5 mg of powdered TetraMin fish food every 2 days. Individuals were inspected for mortality (lack of movement and opaque colour) once daily for a 7-day exposure. Mortalities were documented and removed daily.

The exposure vessels were kept in a mobile cart that held a circulating water bath to maintain temperatures (Figure 3.1). They were also stored in a climate-controlled room when not being exposed to UV radiation and had constant low level of aeration in the vessels to ensure sufficient oxygen (DO > 95%). HOBO® temperature and light loggers were placed on both sides of the exposure system to monitor and ensure the exposure cooling system maintained water temperatures at 22°C (± 2°C). The mobile system allowed all the exposure vessels to be simultaneously transported outside once every day for an hour during solar noon (approximately 13:00-14:00 during July 2019).

We altered UV radiation exposure by resting acrylic sheets over the corresponding treatment vessels. The acrylic sheets blocked either ~15% UV or allowed ~90% UV radiation to reach the exposure vessels (Figure 3.1). Space was left between the exposure vessels and the acrylic sheets to allow for sufficient air exchange. High UV exposures (~90% UV) were achieved with a thin acrylic plastic and were chosen to represent full UV radiation and sunlight exposure that may occur in shallow, littoral zones. Low UV exposures (~15% UV) were achieved with an OP3 plastic and represent minimal UV exposure that may occur in deeper waters or during standard toxicological testing protocols.

Differing UV radiation exposures were accomplished by placing acrylic sheets over the corresponding treatment vessels that either reduced penetrating UV to ~15% or allowed ~90% UV radiation (Figure 3.1). Space was left between the exposure vessels and the acrylic sheets to allow for air exchange. High UV exposures (~90% UV) represent full UV radiation and sunlight exposure that may occur in shallow, littoral zones. Low UV exposures (~15% UV) represent attenuated UV exposure in deeper water or in waters with lower UV penetration. Test vessels were distributed randomly to eliminate potential edge-shading differences and were labeled with codes to ensure endpoint assessments were conducted blindly. To reduce the potential for different jars to be exposed to different shadowing or shading, the exposure system was lined with non-specular aluminum (Figure 3.1).

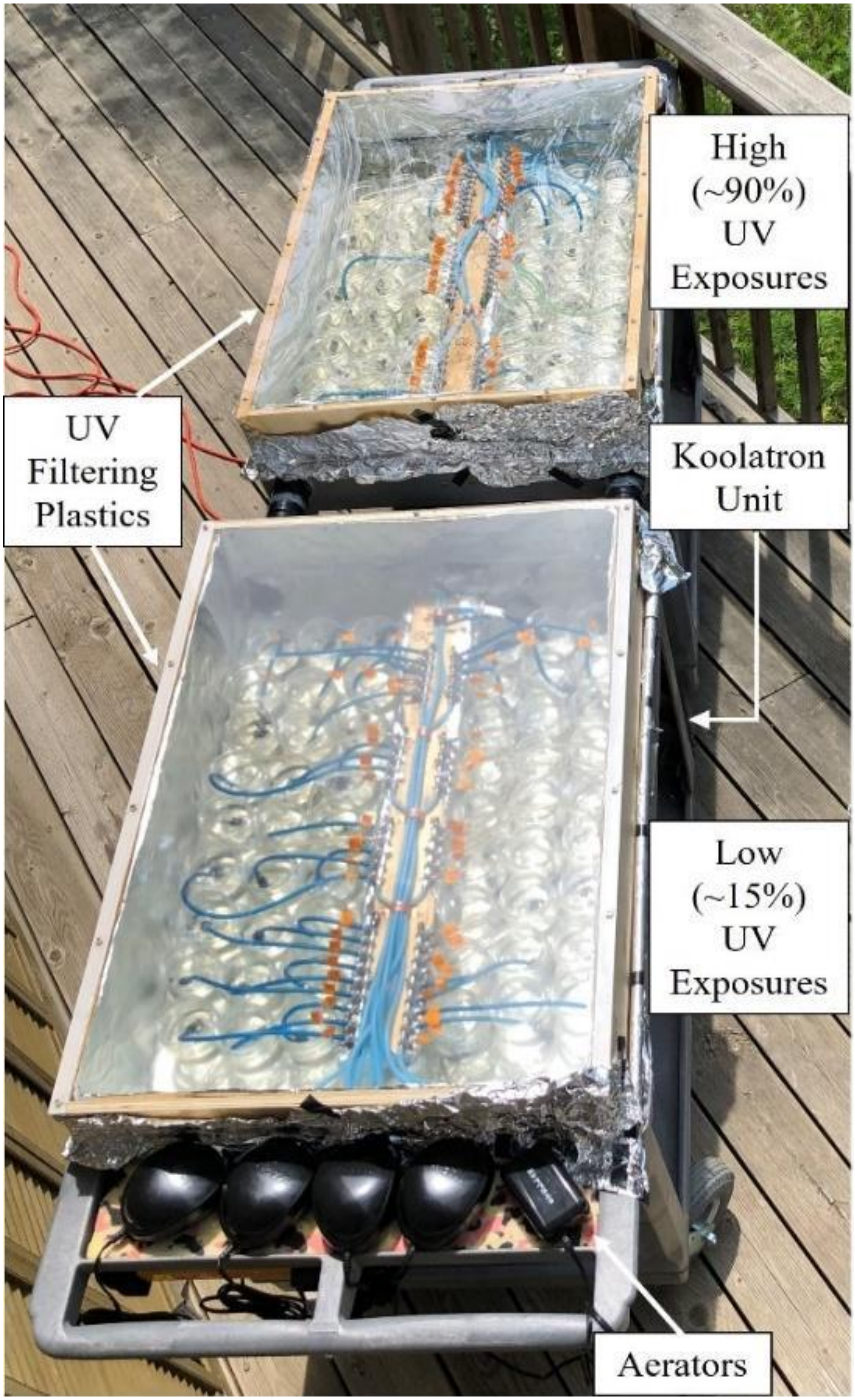

Figure 3.1. Experimental setup for the exposure of early-life stages of fathead minnows to diluted bitumen from Freshwater Oil Spill Remediation Study (FOReSt) enclosures and oil weathering experiments treated with remediation measures in combination with low or high UV exposure.

3.2.5 Photo Analysis

At the end of the 7-day exposures, early-life stage FHMs were sedated within their respective vessel with a 4 µg/L concentration of tricaine methanesulfonate (MS222). All individuals were photographed for future malformation analysis in a consistent orientation. The early-life stage FHMs were photographed individually using a Leica WILD M10 microscope fitted with a Leica MC170 HD camera. After all individuals from the vessel were photographed, they were promptly euthanized in a 25 µg/L lethal dose of MS222 and flash frozen on dry ice to preserve them for future gene expression analysis. Photos of individual early-life stage FHMs had their malformations evaluated using two scoring rubrics (Table 3.1 & 3.2). Malformations evaluated included yolk sac edemas, cardiac malformations (cardiac edemas, tube hearts, hemorrhaging), lack of swim bladder inflation, spinal malformations (kyphosis, lordosis, scoliosis, bent tails), necrosis, bubbles, a failure to hatch, and craniofacial malformations (craniofacial & eye malformations). Malformations were evaluated using both a binomial (Table 3.2) and an additive ordinal scoring approach that included four possible scores for each malformation: 0 - normal, 1 - mild, 2 - moderate, or 3 - severe (Table 3.1). The scores of individual malformations were summed to determine a final score for each individual. Total malformations were reported as the ordinal scores plus the binomial scores of those malformations that were only evaluated binomially (Table 3.3). The evaluated malformations were also split into five categories, yolk sac, cardiac, swim bladder, spinal & body, and craniofacial, based on target malformations previously associated with oil exposure. The summed malformation scores for each category, along with the total of all malformations, were compared to the null model (score~1) to determine model validity, and then were modeled to determine if there were significant differences (no overlap of the 95% confidence intervals) between the predicted values of the severity rankings in each round and UV group.

Table 3.1. Ordinal malformation analysis rubric used for evaluating various categories of malformations in early-life stage fathead minnows following a 7-day exposure to evaluate the photo-enhanced toxicity of diluted bitumen from the Freshwater Oil Spill Remediation Study (FOReSt).

Malformation Category	***Malformation***	***Malformation Score***			
		0 - Normal	**1 - Mild**	**2 - Moderate**	**3 - Severe**
General	**General**	No malformation present.	Mild presence of the malformation.	Moderate presence of the malformation.	Severe presence of the malformation.
Yolk Sac	**Yolk Sac Edema**	No swelling or lack of reabsorption of the yolk sac.	Mild swelling and lack of reabsorption that does not impact movement or development and will not enhance other malformations.	Swelling and lack of reabsorption strong enough to potentially impact movement or development, cause lethargy, and enhance other malformations.	Swelling and inflammation strong enough to cause lethargy, difficulty moving, and other malformations are enhanced.
Cardiac	**Cardiac Edema**	No swelling or inflammation around the pericardial area.	Mild swelling and inflammation that does not impact movement or development and will not enhance other malformations.	Swelling and inflammation strong enough to potentially impact movement or development, cause lethargy, and enhance other malformations.	Swelling and inflammation strong enough to cause lethargy, difficulty moving, and other malformations are enhanced.
Swim Bladder	**Lack of Swim Bladder Inflation**	A fully inflated swim bladder.	A slightly smaller than average swim bladder.	A swim bladder that appears like a thin line, likely impacting development.	A total lack of a swim bladder, hindering development.
Spinal & Body	**Kyphosis**	A straight spine that lacks any curve.	Downwards curvature that does not affect swimming and movement.	Downwards curvature that impacts swimming, but movement is still possible.	Downwards curvature so strong that swimming and movement is impossible or unsuccessful.
	Lordosis	A straight spine that lacks any curve.	Upwards curvature that does not affect swimming and movement.	Upwards curvature that impacts swimming, but movement is still possible.	Upwards curvature so strong that swimming and movement is impossible or unsuccessful.
	Scoliosis	A straight spine that lacks any curve.	Sidewards curvature that does affect swimming and movement.	Sidewards curvature that impacts swimming, but movement is still possible.	Sidewards curvature so strong that swimming and movement is impossible or unsuccessful.
	Necrosis	No appearance of necrosis.	Slight appearance of necrosis.	Significant portion of its body impacted by necrosis.	Necrosis impacting its entire body.
	Bubbles	No appearance of bubbles.	Appearance of < 3 small sized bubbles.	Appearance of < 3 medium sized bubbles.	Appearance of < 3 large sized bubbles or 3+ of any size of bubbles.
Craniofacial	**Craniofacial**	No facial malformations.	Slight facial malformation that may slightly affect development (e.g. slight opening of the jaw, slightly misshapen head).	Facial malformation strong enough that it may affect development, sight and/or feeding (e.g. opening of jaw, underdeveloped head).	Facial malformation so strong that development, sight, and feeding are severely hindered (e.g. fusing of jaw, short jaw, abnormal head development).

Table 3.2. Binomial malformation analysis rubric used for evaluating various categories of malformations in early-life stage fathead minnows following a 7-day exposure to evaluate the photo-enhanced toxicity of diluted bitumen from the Freshwater Oil Spill Remediation Study (FOReSt).

Malformation Category	***Malformation***	***Malformation Score***	
		0 - Normal	**1 - Malformed**
General	**General**	No malformation present.	Malformation presence in any severity.
Cardiac	**Tube Heart**	No abnormal blood vessels.	Any wrapping of blood vessels around a swelling. This only occurs in the presence of an edema.
	Hemorrhage	No pooling of blood.	Any pooling of blood anywhere in the body.
Spinal & Body	**Bent Tail**	A straight tail that lacks any curve.	A tail that is crooked or not entirely straight.
	Hatch	A fully hatched individual.	An individual that is not fully hatched.
Craniofacial	**Eye Malformations**	Normal shaped, fully pigmented eye.	Any presence of misshapen, ruptured, or damaged eyes, or a lack of pigment.

Table 3.3. Total malformation analysis rubric used for evaluating various categories of malformations in early-life stage fathead minnows following a 7-day exposure to evaluate the photo-enhanced toxicity of diluted bitumen from the Freshwater Oil Spill Remediation Study (FOReSt).

Malformation Category	**Malformation**	**Scale**	**Total Possible Score**	**Total Possible Category Score**
Yolk Sac	Yolk Sac Edema	Ordinal	3	3
Cardiac	Cardiac Edema	Ordinal	3	5
	Tube Heart	Binomial	1	
	Hemorrhage	Binomial	1	
Swim Bladder	Lack of Swim Bladder Inflation	Ordinal	3	3
Spinal & Body	Kyphosis	Ordinal	3	17
	Lordosis	Ordinal	3	
	Scoliosis	Ordinal	3	
	Bent Tail	Binomial	1	
	Necrosis	Ordinal	3	
	Bubbles	Ordinal	3	
	Hatch	Binomial	1	
Craniofacial	Craniofacial	Ordinal	3	4
	Eye Malformations	Binomial	1	
Total			**32**	**32**

3.2.6 Chemical Analysis of the Water Accommodated Fraction of Oil

Water samples were taken directly from the FOReSt enclosures that were treated with oil and secondary remediation measures. The samples were analyzed for PACs and alkylated-PACs as described by (Dearnley 2022). For enclosure samples, surface water was pumped from a depth of approximately 10 cm and approximately three meters from the spill-impacted shoreline. Samples were collected into and stored in glass amber bottles with minimal headspace at 4°C prior to transport. Water was filtered within 24 h and extractions occurred offsite within seven days of the sample collection. Samples were filtered by Whatman GF/C filters, added to a separatory funnel, spiked with 20 µL of a 5 ng/µL a recovery internal standard (d8-naphthalene, d8-acenaphthylene, d10-acenaphthene, d10-fluorene, d10-phenanthrene, d10-pyrene, d12-Benz(a)anthracene, d12-chrysene, d12-benzo(b)fluoranthene, d12-benzo(k)fluoranthene, d12-benzo(a)pyrene, d12-indeno(1,2,3-c,d)pyrene, d14-dibenzo(a,h)anthracene, and d14-benzo(g,h,i)perylene), and had 10 g sodium chloride added to aid in extraction. The samples were then double extracted using 50 mL (100 mL total) of DCM and added to a round bottom flask. The volume of DCM was then reduced using rotary evaporation. The excess water was removed with sodium sulfate and the extract volume was further reduced to approximately 1 mL. Reduced extracts were fortified with an internal standard of d10-anthracene (20 µL of 5 ng/µL) and stored at 4°C in amber GC vials until instrumental analyses.

Native and d-labelled PAH and alkyl PAH quantification for the extracts was completed on an Agilent 7890 gas chromatograph coupled to a 7000C triple quadrupole mass spectrophotometer fitted with an electron ionization (EI) source. Helium was used as the carrier gas at a flow rate of 1.2 mL/min with an Agilent J&W HP-5ms ultra inert column (30 m × 0.25 mm, 0.25 µm film thickness). Sample volumes of 1 µl were injected by a PAL RSI 85 auto sampler at a temperature of 60°C. Final concentrations were corrected for internal standard recoveries. Gas chromatograph conditions and details of the product ion transitions for the 16 priority PAHs (identified by the US EPA as priority pollutants harmful to human health (Hussar et al. 2012)) and alkylated PAHs that were analyzed in the samples can be found in (Idowu et al. 2018). Treatment PAC concentration values can be found in the Appendix (Table 3.5, Figure 3.13).

3.2.7 RNA Extraction and cDNA Synthesis

Total RNA was isolated from the early-life stage FHMs using the RNeasy Micro Kit (Qiagen) as described in the manufacturer's protocol. The RNA integrity was assessed by the presence of two defined bands on an agarose gel (Aranda et al. 2012; Gutierrez-Villagomez et al. 2019; Lebordais et al. 2021). One sample out of 60 was not observed in the agarose gel and therefore not included in the gene expression analysis. The concentration and purity of all samples were assessed using a NanoDrop ND-2000 spectrophotometer (Thermo Fisher Scientific) and 3800 ng of total RNA was used for complementary DNA (cDNA) synthesis. The cDNA was prepared using Maxima™ H Minus cDNA Synthesis Master Mix with dsDNase (Thermo Fisher Scientific) as described in the manufacturer's protocol. The cDNA samples were synthesized in parallel in a Mastercycler® nexus gradient (Eppendorf) and then stored at -20°C. A no reverse transcriptase (NRT) and no template controls (NTC) were included during the cDNA synthesis.

3.2.8 Real-Time Quantitative Polymerase Chain Reaction

We targeted the expression of genes related to xenobiotic exposure (i.e. cytochrome P450 1A; *cyp1A*), and the thyroid hormone axis, including thyroid hormone receptor alpha (*thrα*), thyroid hormone receptor beta (*thrβ*), deiodonase-2 (*dio2*) and deiodonase-3 (*dio3b*). We used the expression of elongation factor 1-α (*ef1α*), myogenic differentiation (*myod*) and ribosomal protein L8 (*rpl8*) as reference genes.

Real-time quantitative polymerase chain reaction (RT-qPCR) with SYBR green dye technology was used to estimate relative gene expression. Gene-specific primers based on the FHM genome were designed using Primer-Blast and synthesized by Sigma Aldrich (Table 3.11, Appendix). The primers for *ef1α*, *myod* and *rpl8* have also been reported (Table 3.11, Appendix). The Maxima SYBR Green qPCR Master Mix (Thermo Fisher Scientific) and CFX96 Real-time PCR Detection System (Bio-Rad®) were used to amplify and detect the transcripts of interest. The qPCR thermal cycling parameters were as suggested by the manufacturer; an activation step at 95°C for 10 minutes, followed by 40 cycles of 95°C denaturation step for 15 seconds and one primer annealing/extension temperature depending on the primer set for 1 minute (Table 3.11, Appendix). After 40 cycles, a melt curve was performed over a range of 60–95°C with increments of 1°C to ensure a single amplified product. To confirm the specificity of the primers the RT-qPCR products were observed in a 2% agarose gel and one single band was observed in the gels (Figure 3.16, Appendix). The concentration of each primer in all the RT-qPCR reactions was 0.3 µM. The final volume in all the reactions was 20 µL. Progene® thin-wall PCR strip tubes and PCR 8-Strip flat optically clear caps for qPCR were used for the reactions. The efficiency of all RT-qPCR reactions was 92.8 ± 2.1% and the coefficient of determination (R2) was 0.994 (± 0.003). The relative standard curve method was used to calculate relative mRNA abundance between samples (Gutierrez-Villagomez et al. 2019; Lebordais et al. 2021). The signal was normalized using the reference genes *ef1α*, *myod* and *rpl8* and then presented as a mean fold change of mRNA transcript abundance for each group (n = 9-10; assayed in duplicate).

3.2.9 Statistical Analysis

R Statistical Software (V4.1.2) and RStudio (version 2023.03.0) were used for all data and statistical analysis and visualization (R Core Team 2021; Posit Team 2022). No transformations were applied to the data prior to analysis. A 2-way analysis of variance test (ANOVA) with an interaction and a Tukey Kramer HSD test were used to examine the significance between groups. The percent final mortality (the total mortality of each treatment at the end of the 5-day exposure) and relative gene expression was evaluated in a linear regression model to assess how the interaction between UV and treatment variables impacted these variables. Across all tests, differences were considered significant if $p < 0.05$.

The lethal concentrations of dilbit and CHV were modeled using a four-parameter log-logistic dose-response function to define the sigmoidal concentration response, using the following formula:

$$f(x) = c + \frac{d - c}{1 + exp(b(log(x) - log(e)))} \qquad \textit{(Equation 1)}$$

The upper and lower asymptotes were fixed to the observed mortality values of 0% and 100% (parameter c and d). This results in the abbreviated formula:

$$f(x) = \frac{100}{1 + exp(b(log(x) - log(e)))} \qquad \textit{(Equation 2)}$$

This four-parameter log-logistic dose-response function is called LL.4 in the drc package in R (Ritz et al. 2015). The four-parameter log-logistic dose-response function was chosen so the limits (0 & 100%) could be fixed as the limits in the model.

Malformation scores were modeled using ordinal regression (cumulative link model, clm function) from the ordinal package in R (Christensen 2022). The malformations were split into five target categories: yolk sac malformations, cardiac malformations, swim bladder malformations, spinal and body malformations, and craniofacial malformations. These categories have between 1-7 individual malformations assessed either ordinally or binomially, that were then evaluated cumulatively within their respective category (outlined in Tables 1-3). Each of these groups used a consistent scale of malformation ranking, including normal, mild, moderate, and severe, with cumulative scores of 0, 1, 2, and ≥ 3 respectively. Total scores were also evaluated, using this same normal, mild, moderate, and severe scale, but used cumulative scores of 0, ≤ 2, 3-6, and ≥ 7 respectively. Likelihood ratio test results between the respective model (score~treatment) and a null model (score~1) were conducted, where models with p-values > 0.05 are considered to have significant treatment effects.

3.3 Results

3.3.1 Ultraviolet Radiation

UV radiation exposures were achieved by moving the experimental setup outdoors to be exposed to natural sunlight during solar noon, approximately 13:00-14:00 during July 2019. Measurements of UVA, UVB, visible light, and photosynthetic active radiation (PAR) were recorded above and below the two plastics using a radiometer and HOBO loggers. The low UV (~15% UV radiation) exposure let 15.3% of UVA and 15.4% of UVB (15.3% total UV) penetrate, while the high UV (~90% UV radiation) exposure let 89.5% of UVA and 87.2% of UVB (89.4% total UV) through. The low UV group maintained 95.3% of visible light and 97.2% of PAR compared to the high UV exposures of 96.2% visible light and 99.6% PAR (Table 3.2, Appendix). There were no significant differences between visible light and PAR in the low and high UV plastics compared to without plastic.

In these experiments, natural sunlight was used as the UV radiation exposure, and therefore did not remain consistent day to day or between the different rounds (Figure 3.2). The average UV radiation (including both UVA and UVB) of each round was 14.81 watts/m^2, 15.43 watts/m^2, and 13.85 watts/m^2 for rounds 1, 2, and 3 respectively (Table 3.7, Appendix). The overall average UV radiation did not significantly differ, following similar trends throughout the exposure (Figure 3.2).

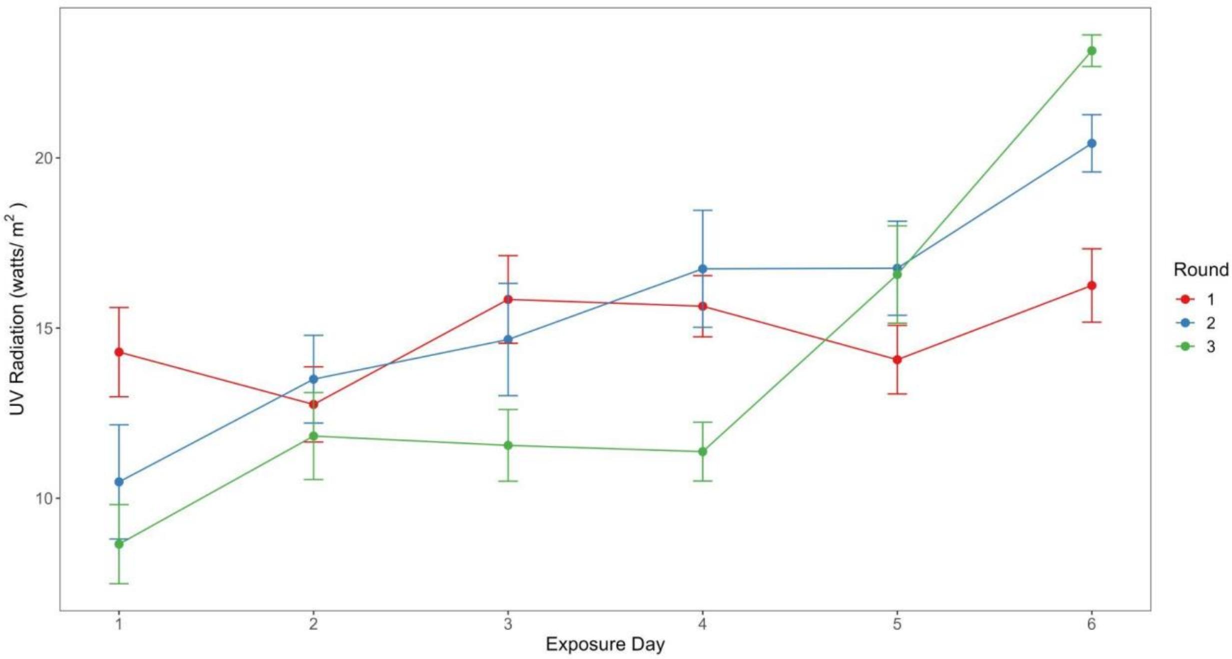

Figure 3.2. Daily solar noon ultraviolet (UV) radiation (watts/m^2) from exposures of diluted bitumen from Freshwater Oil Spill Remediation Study (FOReSt) enclosures and weathering experiments treated with remediation measures to early-life stage fathead minnows. Points represent the average (with standard error) UV radiation over 1 h centered over solar noon (n=26-52).

3.3.2 Mortality

Photo-toxic responses for the mortality of early-life stages of FHMs exposed to dilbit were identified by modelling the interaction between the treatments and UV radiation in the statistical analysis (Tukey Kramer HSD, Mortality ~ UV*Exposure + Round). The exposure variable refers to individual FOReSt enclosures of the same treatments. Full cumulative mortality is displayed in Figure 3.14 in the Appendix. At 12 days post oil (Round 1), there was a significant increase in mortality between the references and low UV EMNR 3 exposure (Tukey Kramer HSD, p-value < 0.05, Figure 3.3A). At 20 days post oil (Round 2), the EFW, EMNR 1, EMNR 3, SWA 1, and SWA 2 exposures had significantly more mortality than the references (Tukey Kramer HSD, p-value < 0.05, Figure 3.3B). Finally, at 38 days post oil (Round 3), the high UV EFW, SWA 1, and all EMNR exposures had significantly more mortality than the references, with significant differences between the UV groups in the EFW and EMNR 2 exposures (Tukey Kramer HSD, p-value < 0.05, Figure 3.3C).

Mortality was also evaluated at the treatment level, combining FOReSt enclosures of the same remedial treatments (Tukey Kramer HSD, Mortality ~ UV*Treatment + Round). There were no significant differences in mortality at 12 days post oil (Figure 3.3D). At 20 days post oil, the low UV oil treatments had significantly more mortality compared to the high UV reference (Tukey Kramer HSD, p-value < 0.05, Figure 3.3E). Finally, at 38 days post oil, the high UV oil treatments had significantly more mortality compared to the low UV reference, and the EFW and EMNR had significantly more mortality in the high UV group compared to the low UV group (Tukey Kramer HSD, p-value < 0.05, Figure 3.3F).

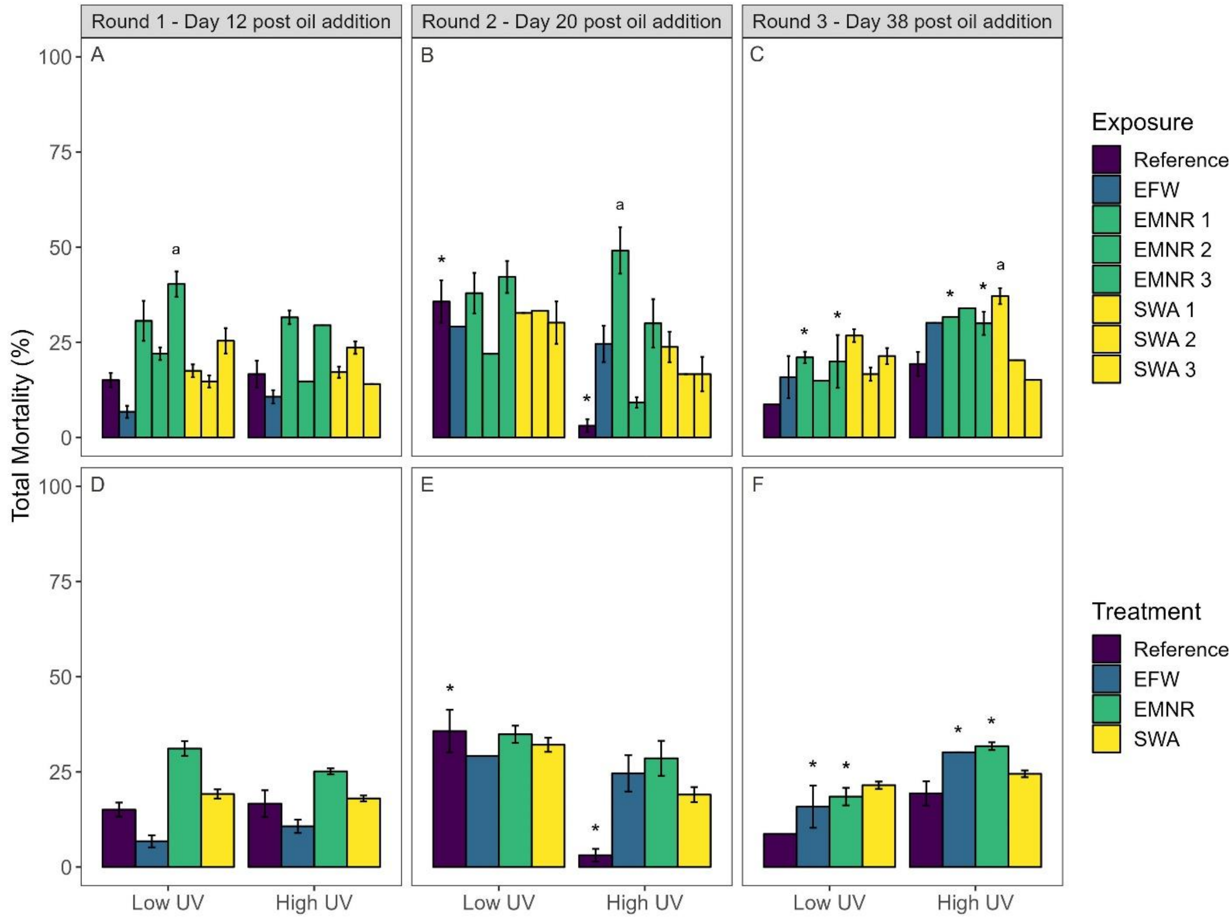

Figure 3.3. Mortality of early-life stages of fathead minnows at the end of a 7-day exposure to diluted bitumen from the Freshwater Oil Spill Remediation Study (FOReSt) in combination with low or high UV exposure. Treatments include unoiled reference enclosures from the FOReSt project (Reference), engineered floating wetlands (EFW), nutrient additions through Enhanced Monitored Natural Recovery (EMNR), and the surface washing agent (SWA) Corexit EC9580A. **a** indicates a significant difference from the reference, and ***** indicates a significant difference between the low and high UV groups of the same treatment.

3.3.3 Lethal Concentrations

The lethal concentration (LC) values were modeled using a four-parameter log-logistic dose-response function. The dilbit exposures at low UV concentrations resulted in the following model (Figure 3.4):

$$y = \frac{100}{1 + exp(-0.368(log(x) - log(e)))} \qquad \textit{(Equation 3)}$$

For the high UV model, the model was described by (Figure 3.4):

$$y = \frac{100}{1 + exp(-0.368(log(x) - log(e)))} \qquad \textit{(Equation 4)}$$

None of the LC values were significantly different between the low and high UV groups (Figure 3.15, Table 3.9 Appendix). However, the high UV group had a lower LC_{25} of 130 ng L^{-1} [2.33, 333 ng L^{-1}] compared to the 269 ng L^{-1} [23.4, 763 ng L^{-1}] LC_{25} of the low UV group, which is about 2.07 times greater (Figure 3.4, Table 3.10 Appendix).

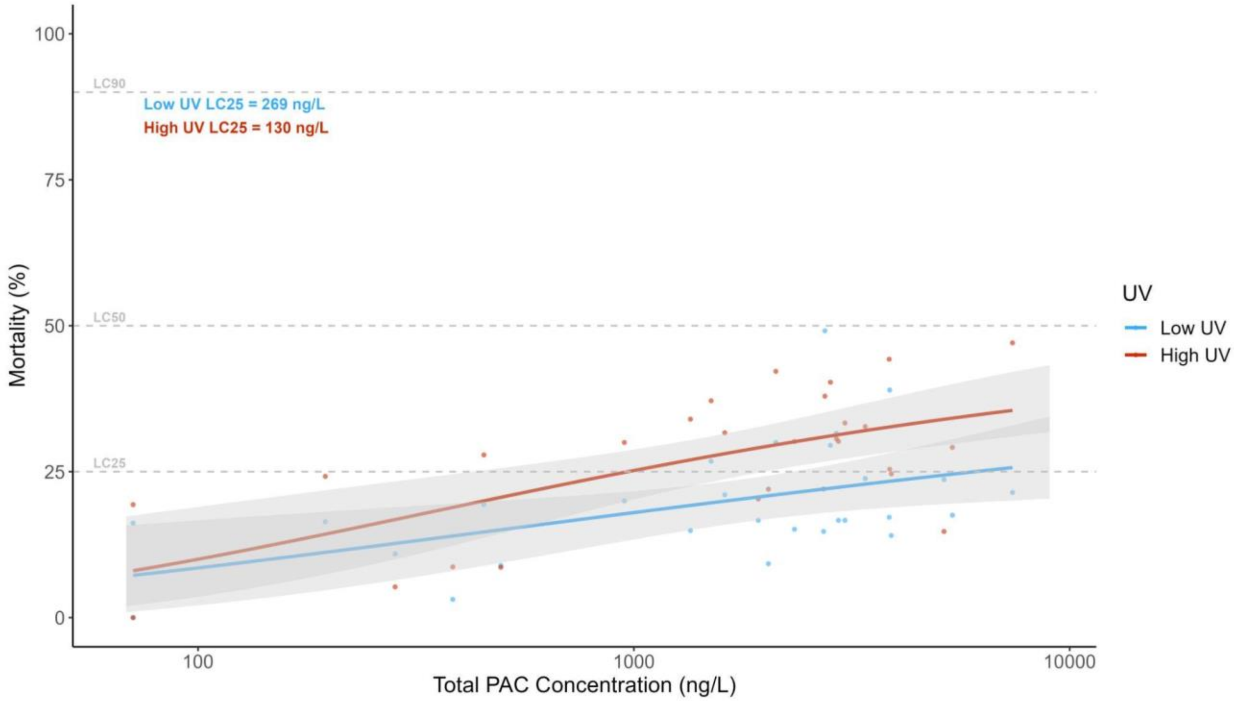

Figure 3.4. Regression of the 7-day cumulative mortality of early-life stage fathead minnows against the total PAC concentration of diluted bitumen from the Freshwater Oil Spill Remediation Study (FOReSt), seperated by low and high UV exposure. The 25% lethal concentration (LC_{25}) values for the low (blue) and high (red) UV exposures are provided at the top left. Total PAC concentration (ng L^{-1}) on the x-axis is presented on a logarithmic scale.

3.3.4 cyp1A Expression

The expression of *cyp1A* was measured in early-life stage FHMs to determine if up-regulation occurred among embryos exposed to dilbit. The *cyp1A* gene is a sensitive biomarker of PAC exposure in early-life stage FHMs (Alsaadi et al. 2018b). Significance and photo-toxic responses for *cyp1A* expression in early-life stage FHMs exposed to dilbit were identified by modelling the interaction between the treatments and UV radiation across rounds (Tukey Kramer HSD, Mortality ~ UV*Exposure + Round). The expression of *cyp1A* was significantly upregulated in treatments exposed to dilbit (Tukey Kramer HSD, p-value < 0.005, Figure 3.5). At 12 days post oil (Round 1), the EMNR was significantly upregulated in both UV groups, and the SWA in the high UV group (Tukey Kramer HSD, p-value < 0.005, Figure 3.5). At 20 days post oil (Round 2), all three dilbit treatments had significant upregulation on *cyp1A* in both UV groups (Tukey Kramer HSD, p-value < 0.005, Figure 3.5). At 38 days post oil (Round 3), all three dilbit treatments has significant upregulation on *cyp1A* in the high UV group, with only the EFW treatment being significantly upregulated in the low UV group (Tukey Kramer HSD, p-value < 0.005, Figure 3.5). There are also three cases of increased upregulation of *cyp1A* in the high UV group of a treatment compared to a low UV group, the EMNR treatment at 20 days post oil, and the EFW and SWA treatments at 38 days post oil (Tukey Kramer HSD, p-value < 0.005, Figure 3.5). There is also a trend of increasing upregulation of *cyp1A* in later days post oil addition, however, this trend is not significant (Figure 3.5).

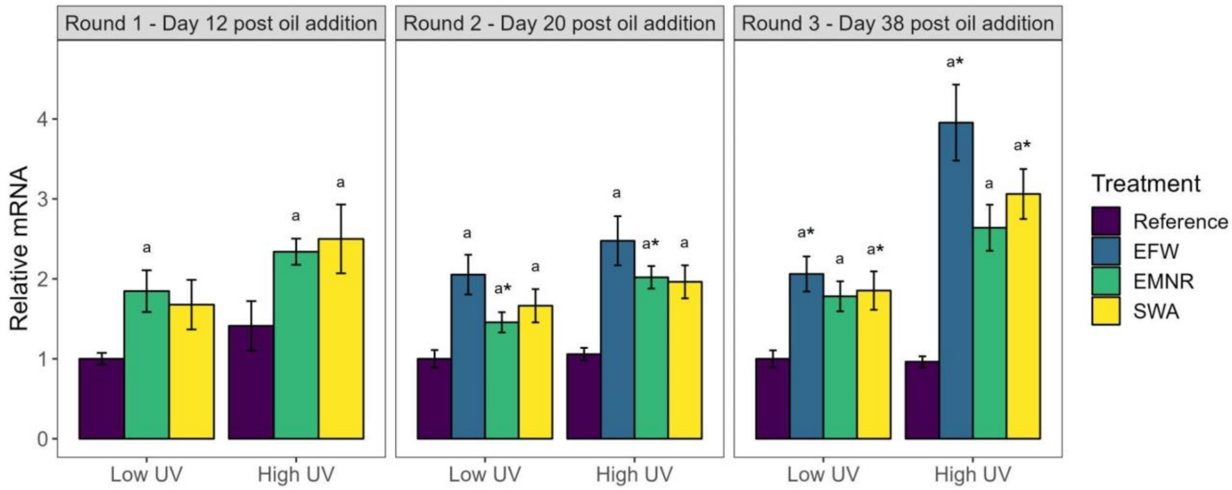

Figure 3.5. Relative mRNA *cyp1A* expression of early-life stages of fathead minnows following a 7-day exposure to diluted bitumen from the Freshwater Oil Spill Remediation Study (FOReSt) in combination with low or high UV exposure. Treatments include unoiled reference enclosures from the FOReSt project (Reference), engineered floating wetlands (EFW), nutrient additions through Enhanced Monitored Natural Recovery (EMNR), and the surface washing agent (SWA) Corexit EC9580A. **a** indicates a significant difference from the reference, and ***** indicates a significant difference between the low and high UV groups of the same treatment.

3.3.5 Thyroid Related Gene Expression

The expression of thyroid related genes, including *dio2*, *dio3b*, *thrα* and *thrβ*, were measured in early-life stage FHMs to determine if there was an impact to thyroid axis regulation following exposure to dilbit. These genes are present in different tissues at differing times due to their roles in development; *dio2* works to convert T4 to T3 through outer ring activation, *dio3* converts T4 and T3 though inner ring inactivation (Johnson & Lema 2011), *thrα* is predominant in heart and brain development, and *thrβ* is predominant in muscle, kidney, and liver development (Deal & Volkoff 2020). Significance and photo-toxic responses for the thyroid related expression in early-life stage FHMs exposed to dilbit were identified by modelling the interaction between the treatments and UV radiation across rounds for each gene (Tukey Kramer HSD, Mortality ~ UV*Exposure + Round). Overall, there was little significance in the expression of these genes compared to the reference, with the only observation of this being in the SWA treatment from day 38 post oil where *dio2* was significantly upregulated (Figure 3.6A). There were observations of high UV groups being more upregulated compared to the low UV groups, as seen at 38 days post oil in *thrα* where all oil treatments were more upregulated in the high UV group, and *thrβ* where EMNR was more upregulated (Figure 3.6C & D). The opposite trend was also seen at 38 days post oil in *dio3b* where the SWA treatment was significantly upregulated in the low UV group compared to the high UV group (Figure 3.6B). There is also a trend of increasing upregulation of all these genes in later days post oil addition, as 38 days post oil is the only round that has significant upregulation, however, this trend is not significant (Figure 3.6).

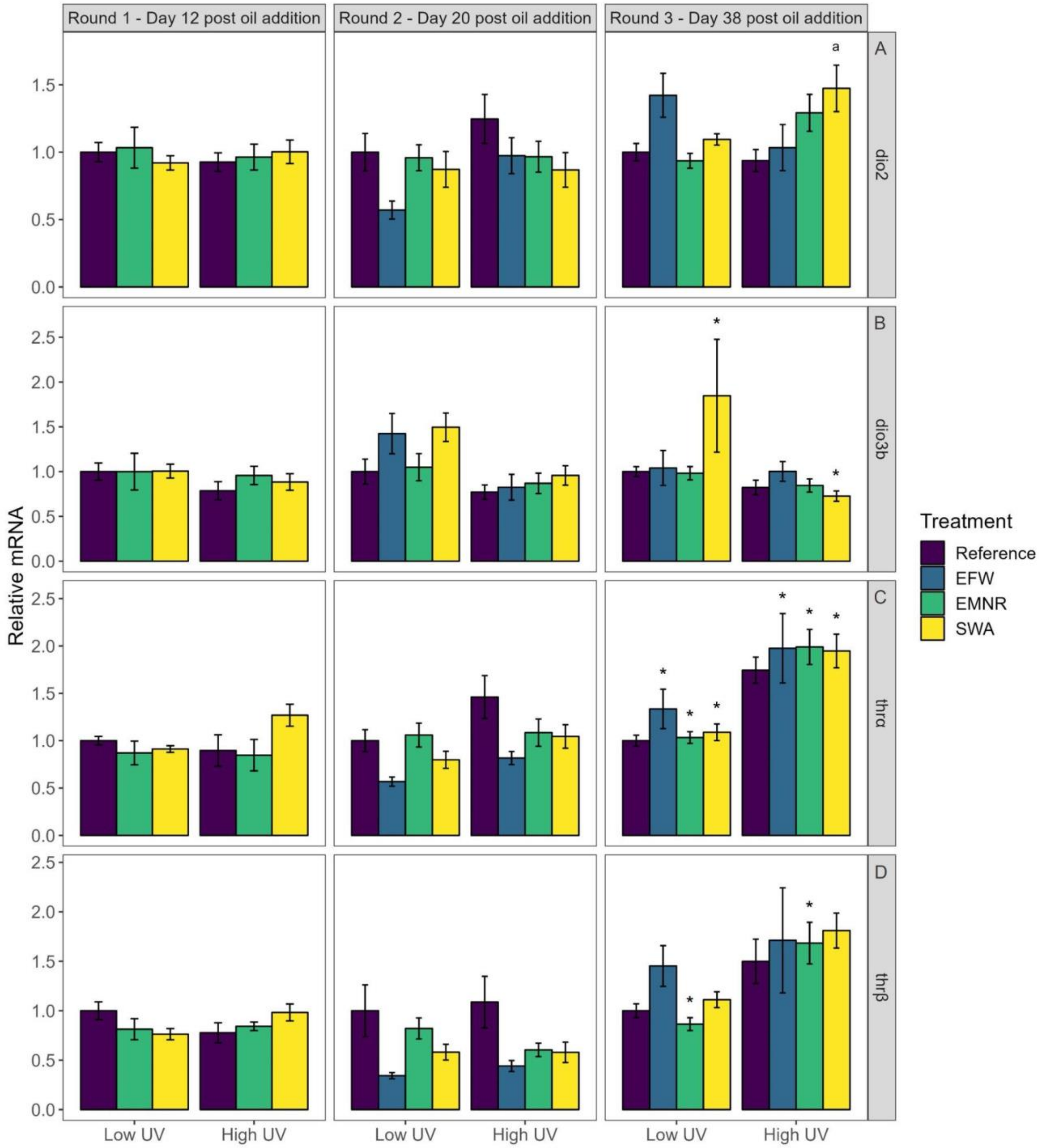

Figure 3.6. Thyroid related relative mRNA expression of *dio2* (A), *dio3b* (B), *thrα* (C), and *thrβ* (D) in early-life stages of fathead minnows following a 7-day exposure to diluted bitumen from the Freshwater Oil Spill Remediation Study (FOReSt) in combination with low or high UV exposure. Treatments include unoiled reference enclosures from the FOReSt project (Reference), engineered floating wetlands (EFW), nutrient additions through Enhanced Monitored Natural Recovery (EMNR), and the surface washing agent (SWA) Corexit EC9580A. **a** indicates a significant difference from the reference, and ***** indicates a significant difference between the low and high UV groups of the same treatment.

3.6 Malformations

Each model was compared to the null model (score~1) to determine the significance of the treatment variables, where models with p-values < 0.05 are considered to have significant treatment effects. All total, yolk sac, cardiac and swim bladder models in both UV groups were significant, along with the spinal and body (Round 1 & 2) and craniofacial models in the high UV group, and the low UV spinal and body model from Round 2 (Table 3.4). The remaining spinal and body and craniofacial models were not significant.

Table 3.4. Likelihood ratio test results [model result (score~treatment) versus the null model (score~1)] between ordinal logistic malformation models of early-life stages of fathead minnows exposed to diluted bitumen from the Freshwater Oil Spill Remediation Study (FOReSt) to evaluate its photo-enhanced toxicity. Models with p-values < 0.05 are considered to have significant treatment effects. Non-significant models are highlighted in grey.

Deformity Category	*Round*	*Low UV P-Value*	*High UV P-Value*
Total Score	1	0.04	3.65×10^{-12}
Yolk Sac	1	2.95×10^{-5}	2.04×10^{-8}
Cardiac	1	1.45×10^{-5}	2.97×10^{-12}
Swim Bladder	1	5.05×10^{-8}	2.43×10^{-9}
Spinal & Body	1	0.08	2.10×10^{-4}
Craniofacial	1	0.08	0.02
Total Score	2	8.28×10^{-15}	2.20×10^{-16}
Yolk Sac	2	1.19×10^{-14}	2.20×10^{-16}
Cardiac	2	1.42×10^{-11}	1.50×10^{-14}
Swim Bladder	2	7.91×10^{-16}	2.20×10^{-16}
Spinal & Body	2	3.55×10^{-5}	5.72×10^{-5}
Craniofacial	2	0.15	6.19×10^{-6}
Total Score	3	7.57×10^{-4}	3.75×10^{-14}
Yolk Sac	3	1.49×10^{-4}	2.20×10^{-16}
Cardiac	3	4.34×10^{-11}	9.73×10^{-5}
Swim Bladder	3	7.11×10^{-3}	5.30×10^{-4}
Spinal & Body	3	0.78	0.12
Craniofacial	3	0.57	0.05

3.3.6.1 Total Malformations

The total malformations include all malformations across all five categories (outlined in Tables 1-3). Total malformation scores were modeled to determine if there were significant differences (no overlap of the 95% confidence intervals) between severity rankings in each round and UV group. The overall trend was that oiled treatments had a decreased probability of having a normal malformation score compared to the reference, with the average normal score of the oil treatments in both UV groups being 18% lower in round 1, 46% lower in round 2, and 31% lower in round 3 (Figure 3.7). Individuals also exhibited increasing photo-toxic responses in later rounds, with the average high UV normal score of the oil treatments being 9% lower compared to the low UV in round 1, 13% lower in round 2, and 43% lower in round 3 (Figure 3.7). At 12 days post oil, early-life stage FHMs at low UV were significantly more likely to have a normal malformation score in every treatment, followed by a mild score (not significant in EFW; Figure 3.7). In the high UV group, the EFW treatment had a significantly increased probability of having no malformations compared to other scores, while the EMNR and SWA treatments had 49% and 43% lower probability of having a normal malformation score compared to the reference (Figure 3.7). The EMNR treatment in the high UV group was 31% less likely to have a normal score and 26% more likely to have a severe score compared to the low UV group (Figure 3.7). At 20 days post oil, the EMNR and SWA treatments in both UV groups had a significantly decreased probability of having a normal score compared to the reference (Figure 3.7). The EMNR treatment was 39-47% more likely to be severely malformed in the low UV group compared to other scores and 52% more likely compared to the reference (Figure 3.7). The EMNR and SWA treatments in the high UV group had a significantly increased probability of being severely or moderately malformed, with the severe malformation probability being 73% and 59% (respectively) more likely than the reference (Figure 3.7). The EMNR and SWA treatments were also between 8-20% less likely to have a normal or mild malformation score in the high UV group compared to the low UV group (Figure 3.7). Finally, at 38 days post spill, the EMNR and SWA treatments had a significantly increased probability of being normal compared to other malformation scores in the low UV group, with the EMNR probability significantly reduced by 29% compared to the reference (Figure 3.7). In the high UV group, the EMNR and SWA treatments had a significantly increased probability of being severely or moderately malformed compared to other scores (Figure 3.7). The EFW treatment in the high UV group was 39% less likely of having a normal score and 39% more likely of having a moderate score, the EMNR treatment was 42% less likely of having a normal score, 20% less likely of having a mild score, and 42% more likely of having a severe score, and the SWA treatment was 46% less likely of having a normal score, 17% less likely of having a mild score, 31% more likely of having a moderate score, and 33% more likely of having a severe score all compared to their low UV group counterpart, which exhibits a photo-toxic effect (Figure 3.7).

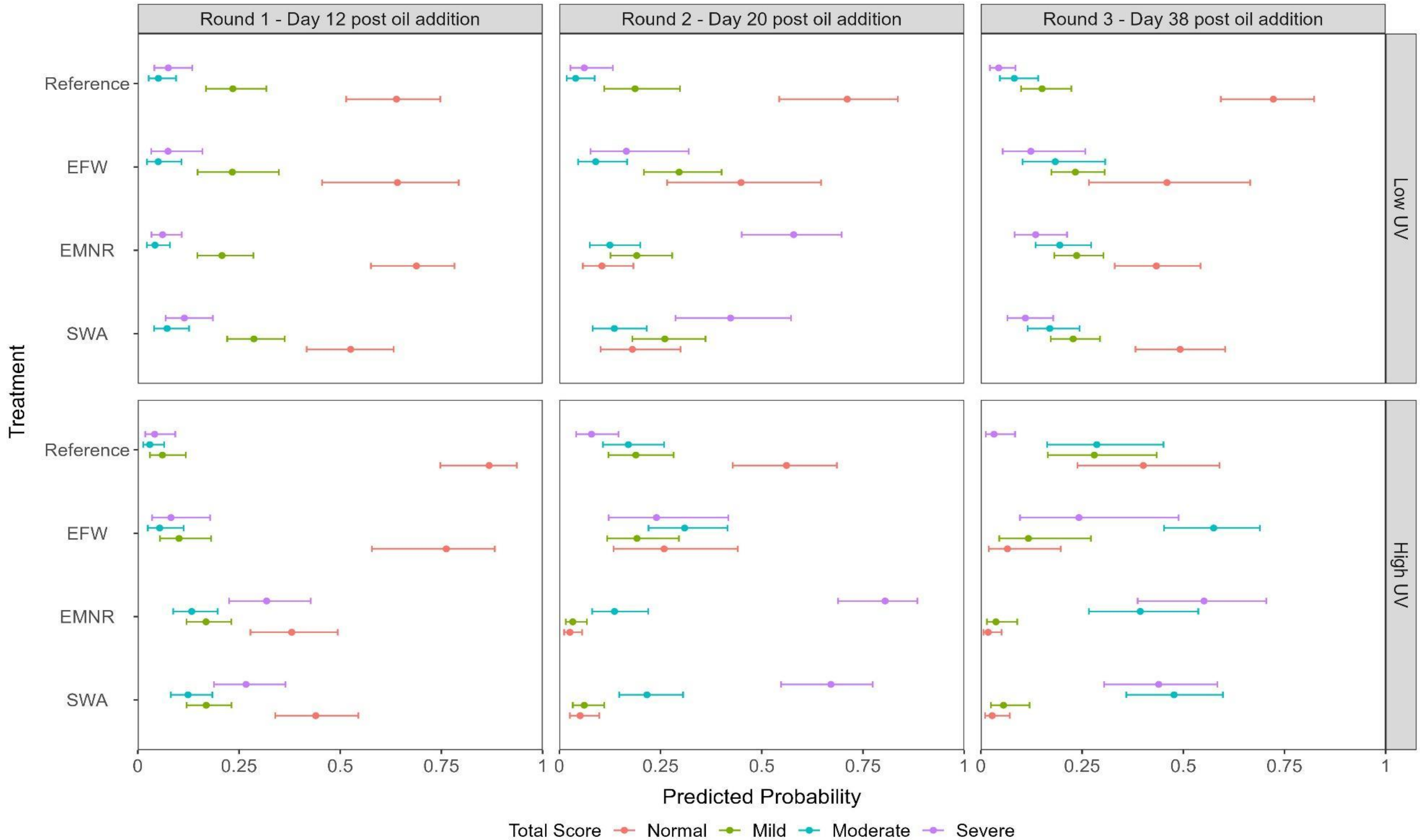

Figure 3.7. Probability of scored severity rankings* of the total malformation scores split by exposure round from early-life stage fathead minnows at the end of a 7-day exposure to diluted bitumen from the Freshwater Oil Spill Remediation Study (FOReSt) in combination with low or high UV exposure. Treatments include unoiled reference enclosures from the FOReSt project (Reference), engineered floating wetlands (EFW), nutrient additions through Enhanced Monitored Natural Recovery (EMNR), and the surface washing agent (SWA) Corexit EC9580A. *Normal: 0 malformations, Mild: ≤ 2 malformations, Moderate: 3-6 malformations, Severe: ≥ 7 malformations.

3.3.6.2 Yolk Sac Malformations

The yolk sac malformation scores (outlined in Tables 1-3) were modeled to determine if there were significant differences (no overlap of the 95% confidence intervals) between severity rankings in each round and UV group. The overall trend is that oiled treatments had a decreased probability of having a normal malformation score compared to the reference, with the average normal score of the oil treatments in both UV groups being 18% lower in round 1, 33% lower in round 2, and 27% lower in round 3 (Figure 3.7). Individuals also exhibited photo-toxic responses, with the average high UV normal score of the oil treatments being 23% lower compared to the low UV in round 1, 5% lower in round 2, and 34% lower in round 3 (Figure 3.7). At 12 days post oil in the low UV group, the EMNR treatment was 37% less likely to have a normal score, 22% more likely to have a mild score, and 15% more likely to have a moderate score in the high UV group compared to the low UV (Figure 3.8). The SWA treatment was 26% less likely in the low UV group and 35% less likely in the high UV group to have no yolk sac malformations compared to the reference and had a significantly increased probability of having a mild or moderate malformation score compared to the reference in the high UV group (Figure 3.8). At 20 days post oil, the EMNR and SWA treatments had an increased probability of being mildly or moderately malformed in both UV groups, with the high UV SWA treatment being 23% more likely to be mildly malformed over moderately malformed (Figure 3.8). The EFW treatment was 24% more likely in the low UV group and 40% more likely in the high UV group to be mildly malformed compared to normal (Figure 3.8). The EMNR and SWA treatments were 37-40% less likely to have no malformations compared to the reference in both UV groups (Figure 3.8). At 38 days post oil, in the low UV group, the EMNR and SWA treatments were 27% and 20% (respectively) more likely to have a mild over normal malformation score, with the normal malformation score being 26% and 22% (respectively) less likely and the mild malformation score being 23% and 19% (respectively) more likely compared to the reference (Figure 3.8). The EFW treatment in the low UV group was more likely to have a mild or normal score compared to a moderate or severe score (Figure 3.8). In the high UV group, all treatments had a significantly increased probability of being mildly malformed compared to the low group, and the oiled treatments had a 28-31% decreased probability of having a normal malformation score compared to the reference (Figure 3.8).

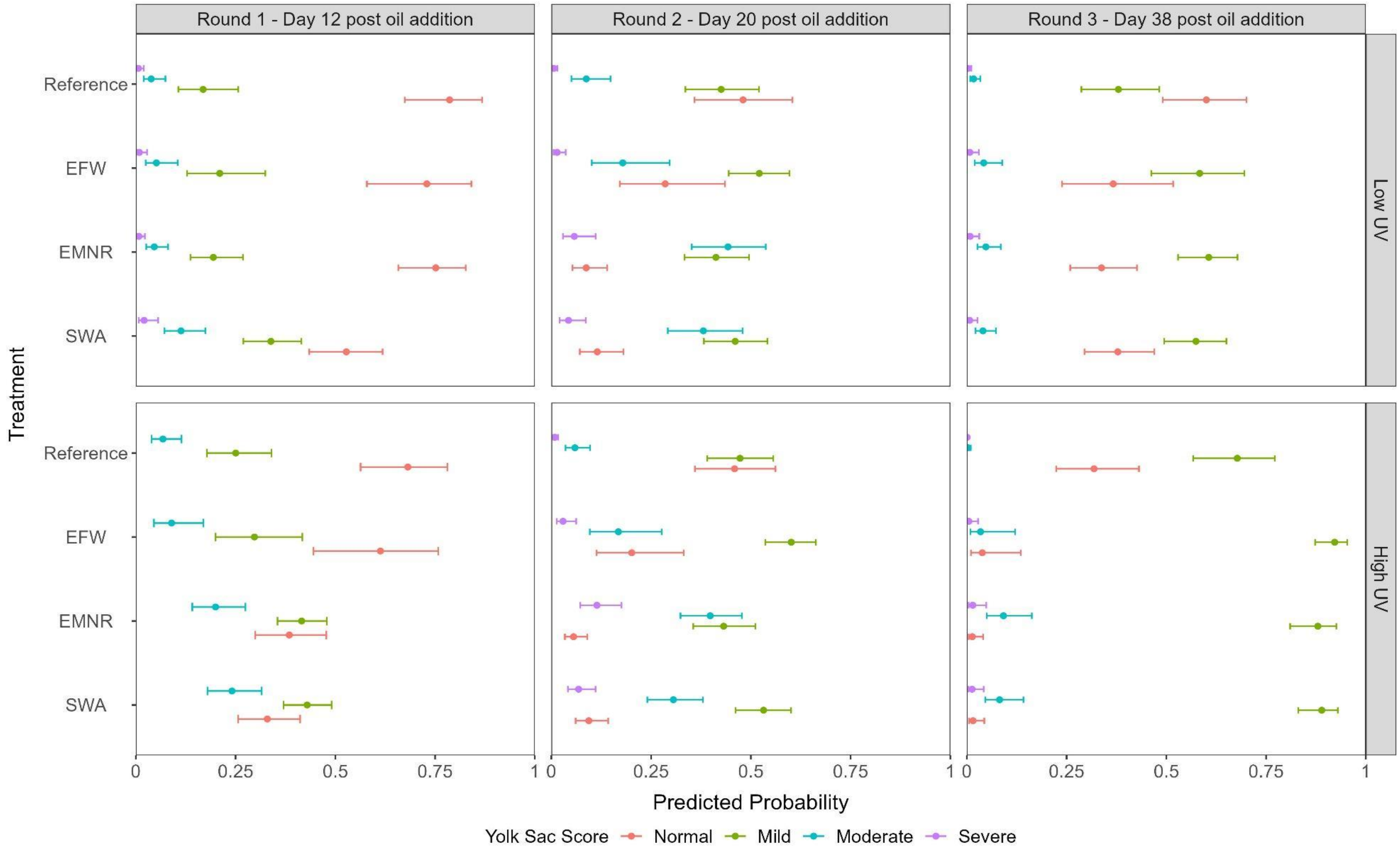

Figure 3.8. Probability of scored severity rankings* of the yolk sac malformation scores split by exposure round from early-life stage fathead minnows at the end of a 7-day exposure to diluted bitumen from the Freshwater Oil Spill Remediation Study (FOReSt) in combination with low or high UV exposure. Treatments include unoiled reference enclosures from the FOReSt project (Reference), engineered floating wetlands (EFW), nutrient additions through Enhanced Monitored Natural Recovery (EMNR), and the surface washing agent (SWA) Corexit EC9580A. *Normal: 0 malformations, Mild: 1 malformation, Moderate: 2 malformations, Severe: ≥ 3 malformations.

3.3.6.3 Cardiac Malformations

The cardiac malformation category includes cardiac edemas, tube hearts, and hemorrhaging (outlined in Tables 1-3). The summed malformation scores were modeled to determine if there were significant differences (no overlap of the 95% confidence intervals) between severity rankings in each round and UV group. The overall trend is that oiled treatments had a decreased probability of having a normal cardiac malformation score compared to the reference, with the average normal score of the oil treatments in both UV groups being 19% lower in round 1, 34% lower in round 2, and 25% lower in round 3 (Figure 3.9). Individuals also exhibited increasing photo-toxic responses in later rounds, with the average high UV normal score of the oil treatments being 8% lower compared to the low UV in round 1, 25% lower in round 2, and 31% lower in round 3 (Figure 3.9). Across the rounds, the SWA treatment in the high UV group also has significantly increased malformation scores in later rounds (Figure 3.9). At 12 days post oil, the EMNR treatment was 33% more likely to have a normal malformation score and 19% less likely to have a moderate malformation score in the low UV group compared to the high UV group (Figure 3.9). All oiled treatments had a significantly lower probability of having a normal malformation score compared to the reference in the high UV group, with the EMNR and SWA treatments also having an increasing risk of being mildly or moderately malformed compared to the reference (Figure 3.9). At 20 days post oil, the EFW treatment was 38% more likely to have a normal malformation score in the low UV group and compared to the high UV group (Figure 3.9). The EMNR and SWA treatments in the high UV group had an increased probability of being moderately malformed compared to other scores (Figure 3.9). The EMNR and SWA treatments also were on average 41% less likely to have a normal malformation score and 28% more likely to have a moderate malformation score compared to the reference in both UV groups (Figure 3.9). At 38 days post oil, the EMNR treatment in the low UV group had a significantly increased probability of having a normal malformation score compared to other scores (Figure 3.9). The SWA treatment was 27% more likely to have a moderate malformation score in the high UV group compared to the low UV group (Figure 3.9). The EFW treatment was 18% more likely to have a mild malformation score, and the EMNR treatment was 14% more likely to have a mild score and 26% more likely to have a moderate score in the high UV group compared to the low UV group (Figure 3.9). All oil treatments were on average 38% less likely to have no malformations, 12% more likely to be mildly malformed, and 25% more likely to be moderately malformed compared to the reference in the low UV group (Figure 3.9). In the high UV group, the SWA was 15% less likely to have no malformations and 25% more likely to be moderately malformed compared to the reference (Figure 3.9).

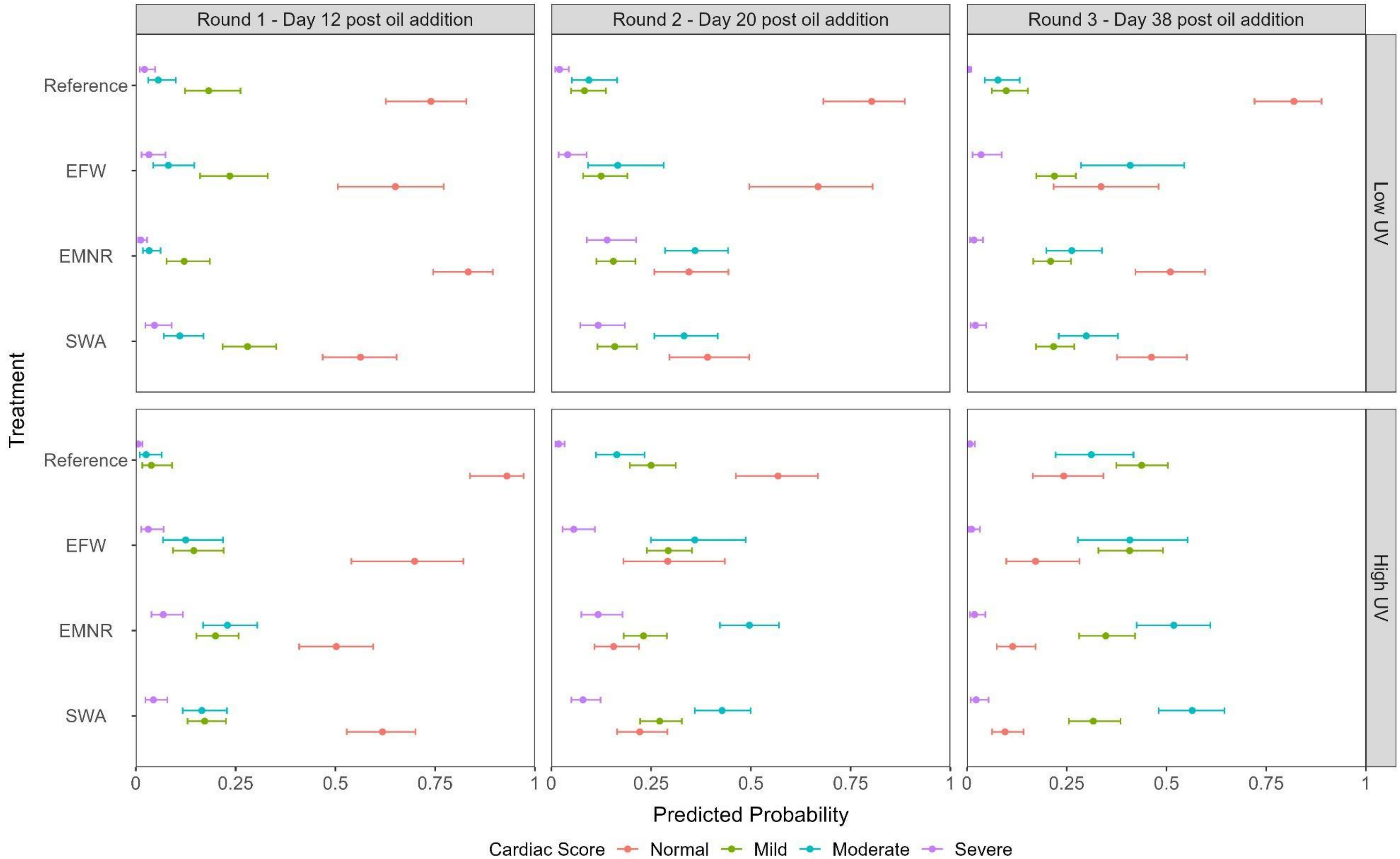

Figure 3.9. Probability of scored severity rankings* of the cardiac malformation scores split by exposure round from early-life stage fathead minnows at the end of a 7-day exposure to diluted bitumen from the Freshwater Oil Spill Remediation Study (FOReSt) in combination with low or high UV exposure. Treatments include unoiled reference enclosures from the FOReSt project (Reference), engineered floating wetlands (EFW), nutrient additions through Enhanced Monitored Natural Recovery (EMNR), and the surface washing agent (SWA) Corexit EC9580A. *Normal: 0 malformations, Mild: 1 malformation, Moderate: 2 malformations, Severe: ≥ 3 malformations.

3.3.6.4 Swim Bladder Malformations

The swim bladder malformation category includes lack of swim bladder inflation (outlined in Tables 1-3), and malformation scores were modeled to determine if there were significant differences (no overlap of the 95% confidence intervals) between severity rankings in each round and UV group. Overall, swim bladder inflation seems to worsen in later rounds for all treatments, with the oiled treatments having a decreased probability of having a normal cardiac malformation score compared to the reference, with the average normal score of the oil treatments in both UV groups being 20% lower in round 1, 30% lower in round 2, and 12% lower in round 3 (Figure 3.10). Individuals also exhibited photo-toxic responses, with the average high UV normal score of the oil treatments being 27% lower compared to the low UV in round 1, 21% lower in round 2, and 44% lower in round 3 (Figure 3.10). At 12 days post oil, the SWA treatment in the low UV group was 25% less likely and SWA and EMNR treatments in the high UV group were 32% and 38% more likely (respectively) to have normal malformation scores compared to the reference (Figure 3.10). The EMNR treatment was also 21% more likely to be moderately malformed and 19% more likely to be severely malformed in the high UV group compared to the low UV group (Figure 3.10). At 20 days post oil in the low UV group, the EFW treatment had a significantly increased probability of having a normal malformation score compared to other scores (Figure 3.10). The EMNR and SWA treatments on average were 41% less likely to have no malformations compared to the reference in both UV groups, 22% more likely to have a severe malformation score compared to the reference, and 40% more likely to have a severe malformation score in the high UV group compared to the low UV group (Figure 3.10). At 38 days post oil in the low UV group, all treatments had a significantly increased probability of having no malformations (Figure 3.10). The EMNR and SWA treatments had an increased probability of having a severe or moderate malformation score compared to other scores in the high UV group and were 34% and 35% (respectively) more likely to be severely malformed in the high UV group compared to the low UV group (Figure 3.10). The probability of having a normal malformation score was on average 44% less likely for all treatments, and the probability of having a moderate malformation score was on average 19% more likely for all treatments when comparing the high to low UV groups (Figure 3.10).

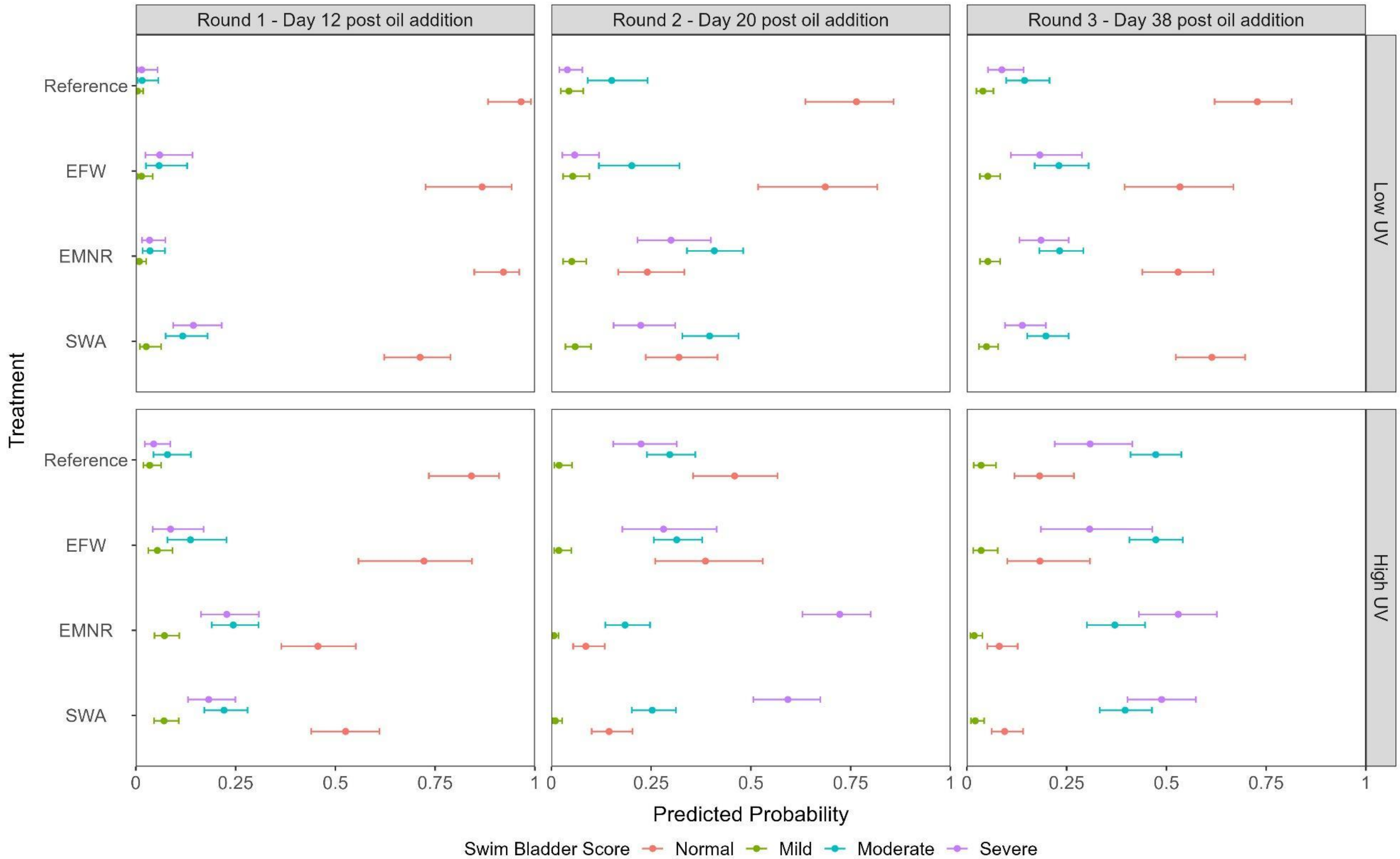

Figure 3.10. Probability of scored severity rankings* of the swim bladder malformation scores split by exposure round from early-life stage fathead minnows at the end of a 7-day exposure to diluted bitumen from the Freshwater Oil Spill Remediation Study (FOReSt) in combination with low or high UV exposure. Treatments include unoiled reference enclosures from the FOReSt project (Reference), engineered floating wetlands (EFW), nutrient additions through Enhanced Monitored Natural Recovery (EMNR), and the surface washing agent (SWA) Corexit EC9580A. *Normal: 0 malformations, Mild: 1 malformation, Moderate: 2 malformations, Severe: ≥ 3 malformations.

3.3.6.5 Spinal & Body Malformations

The spinal and body malformation category includes spinal bends (kyphosis, lordosis, scoliosis), bent tails, necrosis, bubbles, and failures to hatch (outlined in Tables 1-3). The summed malformation scores were modeled to determine if there were significant differences (no overlap of the 95% confidence intervals) between severity rankings in each round and UV group. The spinal and body models that are significant/valid include the 20 days post oil models, and the high UV model at 12 days post oil (non-significant models are greyed out; Table 3.4). There were no significant differences on 12 days post oil in the high UV group. At 20 days post oil, the EMNR treatment in the low UV group was 21% less likely, and the EMNR and SWA treatments in the high UV group were 20% and 16% (respectively) less likely to have a normal malformation score compared to the reference (Figure 3.11). The EMNR and SWA treatments in the high UV group were also 15% and 12% (respectively) more likely to have a mild malformation score compared to the reference (Figure 3.11). This is showing that overall, the EMNR and SWA are slightly more likely to have spinal and body malformations (Figure 3.11).

3.3.6.6 Craniofacial Malformations

The craniofacial malformation category includes craniofacial and eye malformations (outlined in Tables 1-3). The summed malformation scores were modeled to determine if there were significant differences (no overlap of the 95% confidence intervals) between severity rankings in each round and UV group. Across all rounds and both UV groups, all treatments had a significantly increased probability of having a normal malformation score (Figure 3.12). The only craniofacial models that are significant/valid include the high UV groups (non-significant models are greyed out; Table 3.4). There were no significant differences on 12- and 38-days post oil in the high UV group. At 20 days post oil, the EMNR and SWA treatments were 22% and 19% (respectively) less likely to have a normal malformation score, 15% and 14% more likely to have a mild malformation score, and 6% and 5% more likely to have a moderate malformation score compared to the reference (Figure 3.12). This is showing that overall, the EMNR and SWA are slightly more likely to have craniofacial malformations (Figure 3.12).

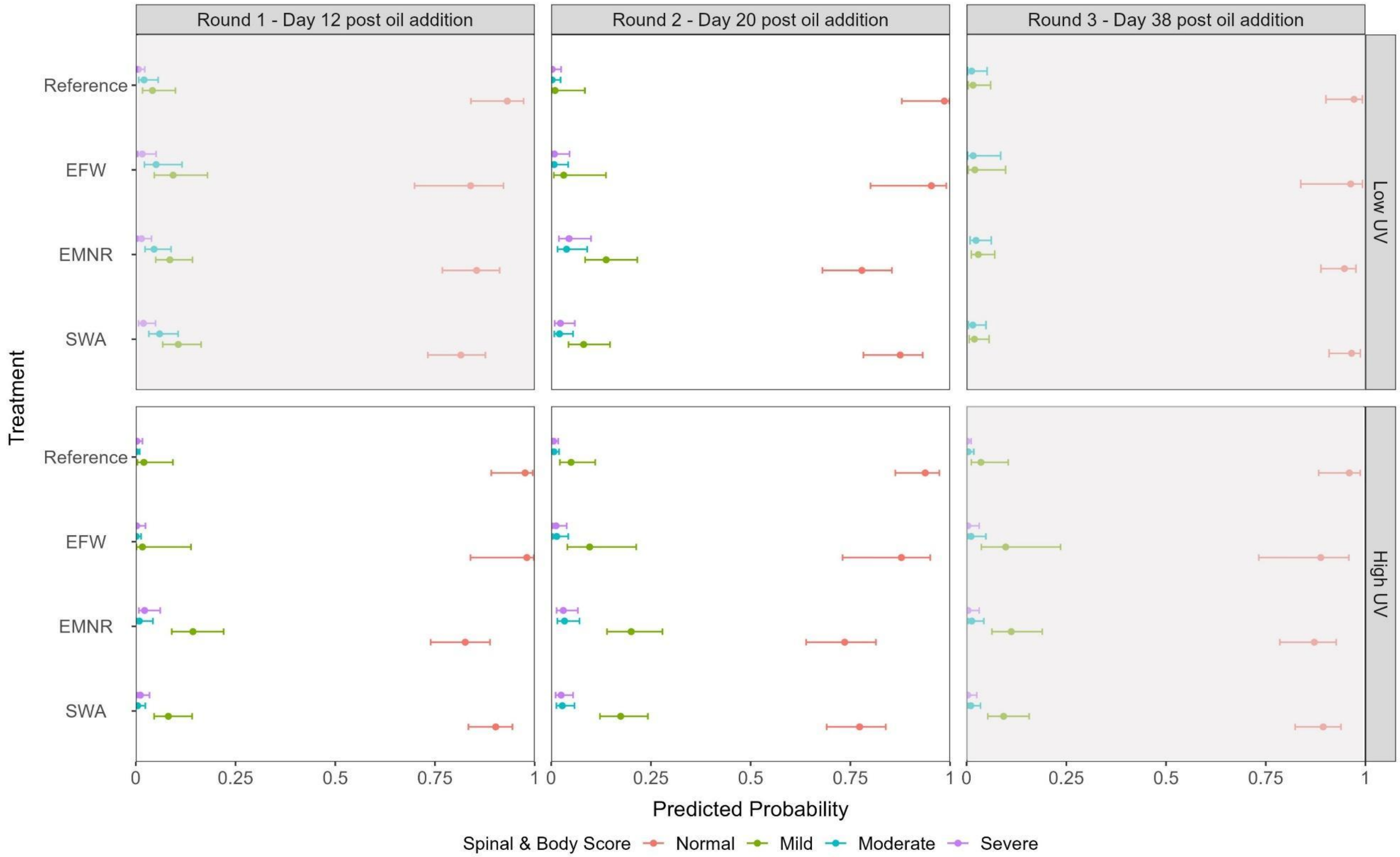

Figure 3.11. Probability of scored severity rankings* of the spinal & body malformation scores split by exposure round from early-life stage fathead minnows at the end of a 7-day exposure to diluted bitumen from the Freshwater Oil Spill Remediation Study (FOReSt) in combination with low or high UV exposure. Treatments include unoiled reference enclosures from the FOReSt project (Reference), engineered floating wetlands (EFW), nutrient additions through Enhanced Monitored Natural Recovery (EMNR), and the surface washing agent (SWA) Corexit EC9580A. *Normal: 0 malformations, Mild: 1 malformation, Moderate: 2 malformations, Severe: ≥ 3 malformations. Non-significant models are greyed out.

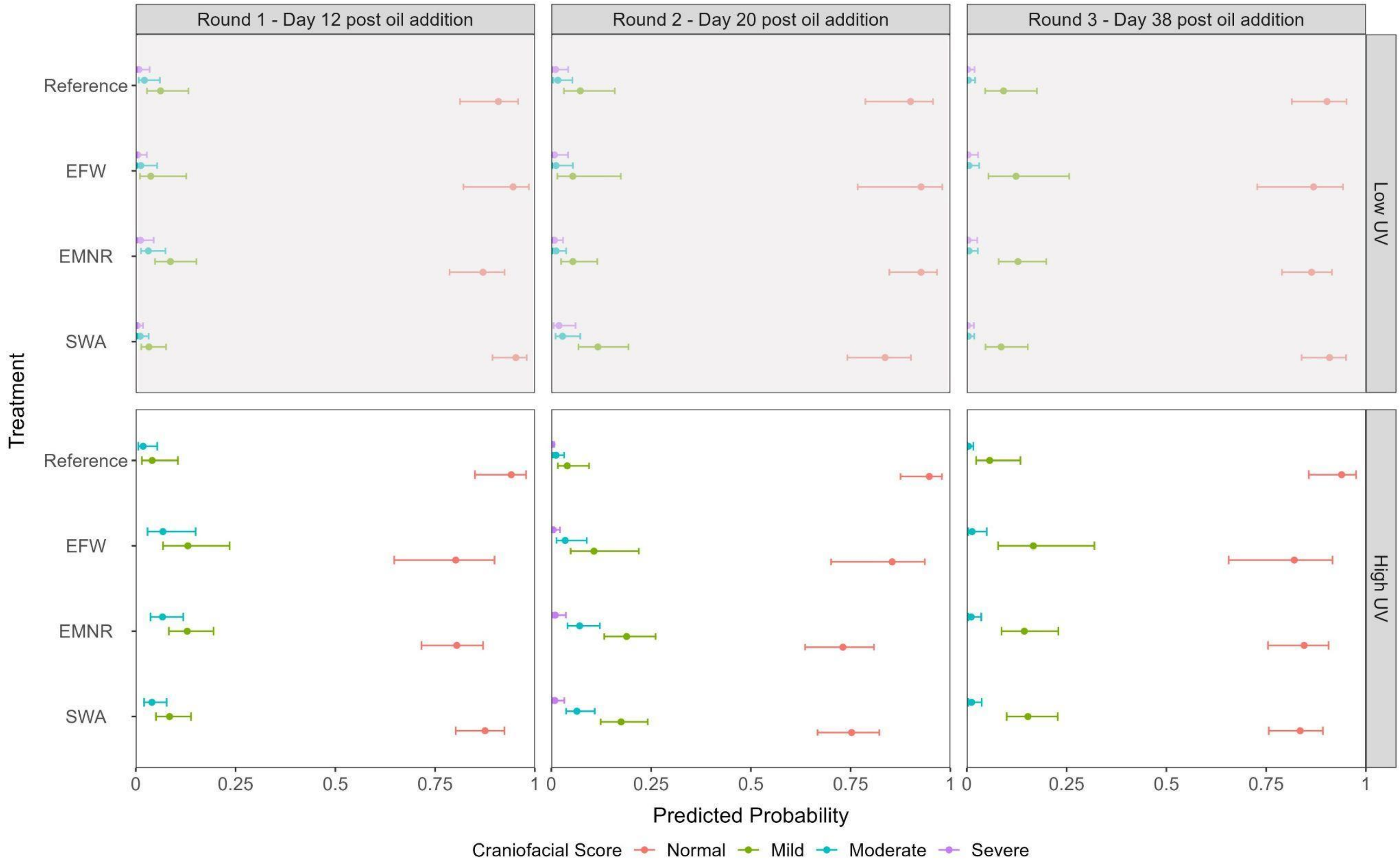

Figure 3.12. Probability of scored severity rankings* of the craniofacial malformation scores split by exposure round from early-life stage fathead minnows at the end of a 7-day exposure to diluted bitumen from the Freshwater Oil Spill Remediation Study (FOReSt) in combination with low or high UV exposure. Treatments include unoiled reference enclosures from the FOReSt project (Reference), engineered floating wetlands (EFW), nutrient additions through Enhanced Monitored Natural Recovery (EMNR), and the surface washing agent (SWA) Corexit EC9580A. *Normal: 0 malformations, Mild: 1 malformation, Moderate: 2 malformations, Severe: ≥ 3 malformations. Non-significant models are greyed out.

3.4 Discussion

We used natural sunlight as our UV radiation exposure in this experiment, meaning the absolute UV exposure was not consistent each day. This resulted in varying, but not statistically different UV radiation exposure between the three rounds (Figure 3.2, Table 3.7). Natural sunlight was chosen as the UV exposure due to environmental relevance as freshwater environments have daily variability, along with geographical and seasonal variability. While this environmental variability is important to note, the outcome of this experiment was to compare a low UV exposure to a high UV exposure. The low and high UV exposures that were compared occurred simultaneously, and therefore the daily fluctuations in levels of UV radiation were consistent between the experimental UV treatments.

The mortality in some reference treatments is high for what is considered acceptable mortality in a reference treatment, which is typically < 10% (Parrott 2005). However, because these were wild collected eggs, we anticipated higher mortality due to natural die-off, stress in captivity, and increased sensitivity to handling. We selected eggs in good visual condition, meaning eggs that were opaque, misshaped, or clumped together were not selected to increase the chances of having high quality eggs for the experiments. However, some eggs that appeared in good condition may not have been high quality, causing higher natural mortality. Up to 30% of FHM nests can have unsuccessful hatchings, even with proper guarding and tending by males (Divino & Tonn 2008).

This experiment evaluated eggs over a period of approximately 33 days, meaning wild eggs were collected at different times during the spawning season. It is possible that egg quality potentially declined later in the season as the spawning season approached its end. When evaluating the data, we did see an increase in sub-lethal effects – both gene expression and malformations – at 38 days post oil addition compared to early time points. Using wild collected eggs from later in the spawning season could have potentially exacerbated this trend. Although FHMs are fractional spawners and will continue to spawn under ideal temperature and photoperiod conditions, spawning frequency decreases over time and with more spawns, as adults can become sterile or spawned-out (US EPA 2006). Biotic and abiotic factors change through a spawning season, therefore seasonal reproductive timing can affect hatching success (Divino & Tonn 2008). This is evident at different incubation times as temperatures decrease later in the spawning season or from year-to-year as fish adjust to optimal spawning times, meaning success of early and late clutches may be impacted (Mills 1991; Divino & Tonn 2008). We did, however, observe an increase in the photo-toxic responses of these sub-lethal parameters at 38 days post oil addition. While egg condition may contribute, increased photodegradation of oil in the FOReSt enclosures could also be a factor. Since oil had weathered in-situ in the FOReSt enclosures for longer by the third round, it is possible more photodegradation products were present compared to earlier rounds. This trend may also be supported by the mortality at 38 days post oil addition, where the EFW and EMNR treatments exhibit a slight photo-toxic trend, as opposed to just having overall higher mortality.

Examining the effects of contaminants on wild populations is important, albeit difficult, as they can be more sensitive than laboratory cultures. This can be an unintentional difficulty of using wild populations, particularly outside their native settings. While using more resilient laboratory cultures may yield more consistent data with less confounding factors, using wild populations may provide more relevant insight of toxicological effects, including the effects of multiple stressors (Wendelaar Bonga 1997). In studies such as this, conducting *in situ* experiments or designing fish housing to mimic more natural conditions may reduce the stress imposed on wild populations and could be incorporated into future experiments.

The oil exposures used in this experiment were designed to examine long term effects of residual oil after cleanup. As a result, overall PAC concentrations are low, particularly at 38 days oil post addition. However, at 20 days post oil addition, there are higher concentrations compared to at 12 days (Figure 3.13, Appendix). This was caused by a large rain event in the days prior to the beginning of round two, which caused remobilization of oil from sequestered sites on the shoreline substrates into the aquatic environment of the FOReSt enclosures. This increased concentration may have contributed to slightly higher mortalities in round two. The lower PAC concentrations also impacted the LC analysis. Lower PAC concentrations and lower mortality that did not reach 50% resulted in the inability to calculate LC_{50} values (Figure 3.4). Examining higher concentrations for future exposures may allow more accurate LC values to be calculated for early-life stages of FHMs exposed to dilbit.

The results of the gene expression analysis show that *cyp1A* was upregulated in oil treatments compared to the reference treatment, and on some instances in the high UV group compared to the low UV rale group (Figure 3.5). While there is no definitive trend that *cyp1A* is upregulated with increased UV radiation (in combination with oil), the expected upregulation of *cyp1A* with PAC exposure is present (Figure 3.5). There were no consistent trends with the thyroid related genes (Figure 3.6). The genes we analyzed, *dio2*, *dio3b*, *thrα*, and *thrβ*, start to be expressed at 2 days post-fertilization and prior to the development of the thyroid gland (Vergauwen et al. 2018; Liu et al. 2000; Power et al. 2001; Rurale et al. 2022). This means these genes are expressed earlier in the development, specifically in the case of *thrα* and *thrβ* which are expressed earlier in higher levels, then decrease after hatch (Liu et al. 2000; Power et al. 2001). This means an effect could possibly be occurring but may be missed due to development timing. For example, *dio2*, *dio3*, *thrα* and *thrβ* have all displayed normal expression when exposed to PACs and WAFs (Rurale et al. 2022; Truter et al. 2017). Thyroid hormones are also passed maternally and are stored in the yolk sac to be reabsorbed by the embryo, meaning this store of thyroid hormones could potentially be masking the impact of PACs (Rurale et al. 2022). Although there are no clear impacts to the thyroid axis, there are indications of other development failures in the malformation analysis.

The malformation analysis was broken into target groups to evaluate known malformations of dilbit to early-life stages of FHMs, such as yolk sac, cardiac, craniofacial, and spinal malformations (Alsaadi et al. 2018b; Colavecchia et al. 2004, 2007; Lara-Jacobo et al. 2021). The failure of swim bladder inflation was also included as a category due to PACs causing this in other species (Incardona et al. 2004; Madison et al. 2015, 2017; Price & Mager 2020). We did not see any significant increase in craniofacial or spinal malformations in early-life stages of FHMs but did see trends that suggest an increased probability of yolk sac, cardiac and swim bladder malformations in the oil treatments (Figures 7-12). Individuals in the high UV group also had an increased probability of having yolk sac, cardiac and swim bladder malformations compared to the low UV group, suggesting a photo-toxic response. Yolk sac and cardiac edemas have been shown with increased PAC exposure and *cyp1A* upregulation and can cause increased stress on an individuals cardiac and development system (Alsaadi et al. 2018b). This, along with the failure of swim bladder inflation, is concerning for future development, swim performance, overall fitness, and survival.

Although we did not observe definitive evidence that dilbit exhibits PET to early-life stage FHMs, this highlights a gap of literature surrounding environmentally-relevant test scenarios, such as with UV radiation and wild organisms. Standard toxicity tests do not include UV radiation and are typically completed with laboratory-reared cultures, which does not reflect natural conditions organisms may face in the natural environment. PET is also becoming an increasingly relevant mechanism of toxicity, especially when considering climate change. Climate change is altering water clarity through changes in precipitation, dissolved organic carbon inputs, and pH, which can change the intensity of UV radiation in the aquatic environment (Hooper et al. 2013). An increase in UV intensity associated with climate change can potentially increase the effects of photosensitive pollutants to aquatic organisms (Hooper et al. 2013; J. Kim et al. 2010). This may result in lower toxicological tolerance, increased sensitivity, and higher mortalities when pollutant exposure occurs (Hooper et al. 2013). This may be particularly relevant for early-life stages of fish that live in shallow riparian areas that may or may not have areas to escape UV radiation. Even if early-life stages of fish do have shade to evade UV in, during feeding and avoiding predation, they may be more exposed to UV radiation, which could exacerbate pollutant effects.

The gene expression and malformation analyses exhibit trends that suggest PET may be occurring when early-life stages of FHMs are exposed to dilbit. However, this trend was not clearly observed in mortality. The use of wild populations and lower concentrations of dilbit may convolute this, and concentrations more indicative of immediate post spill conditions should be examined. In future work, evaluating wild populations in more natural conditions, reducing handling and confinement stress, and using a larger range of oil concentrations may help determine the PET of dilbit to FHMs.

3.5 Conclusion

We saw an upregulation in *cyp1A* and increase in malformations when early-life stages of wild FHMs were exposed to dilbit alone and in combination with UV radiation. Mortality and LC values did not significantly differ from standard toxicity testing conducted under low UV conditions. These results have important ramifications for oil spill remediation in freshwater environments emphasizing the need to consider the photic environment and the sensitivity of wild populations. We also highlighted the importance of considering sub-lethal physical toxicity endpoints for affected species. The physical malformations affecting early-life stages of FHMs, specifically yolk sac, cardiac, and swim bladder malformations, could be an important factor in long term fitness and survival. Additional studies to examine the potential for PET of other oils and SWAs to wild populations of FHMs are warranted.

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3.8 Appendix

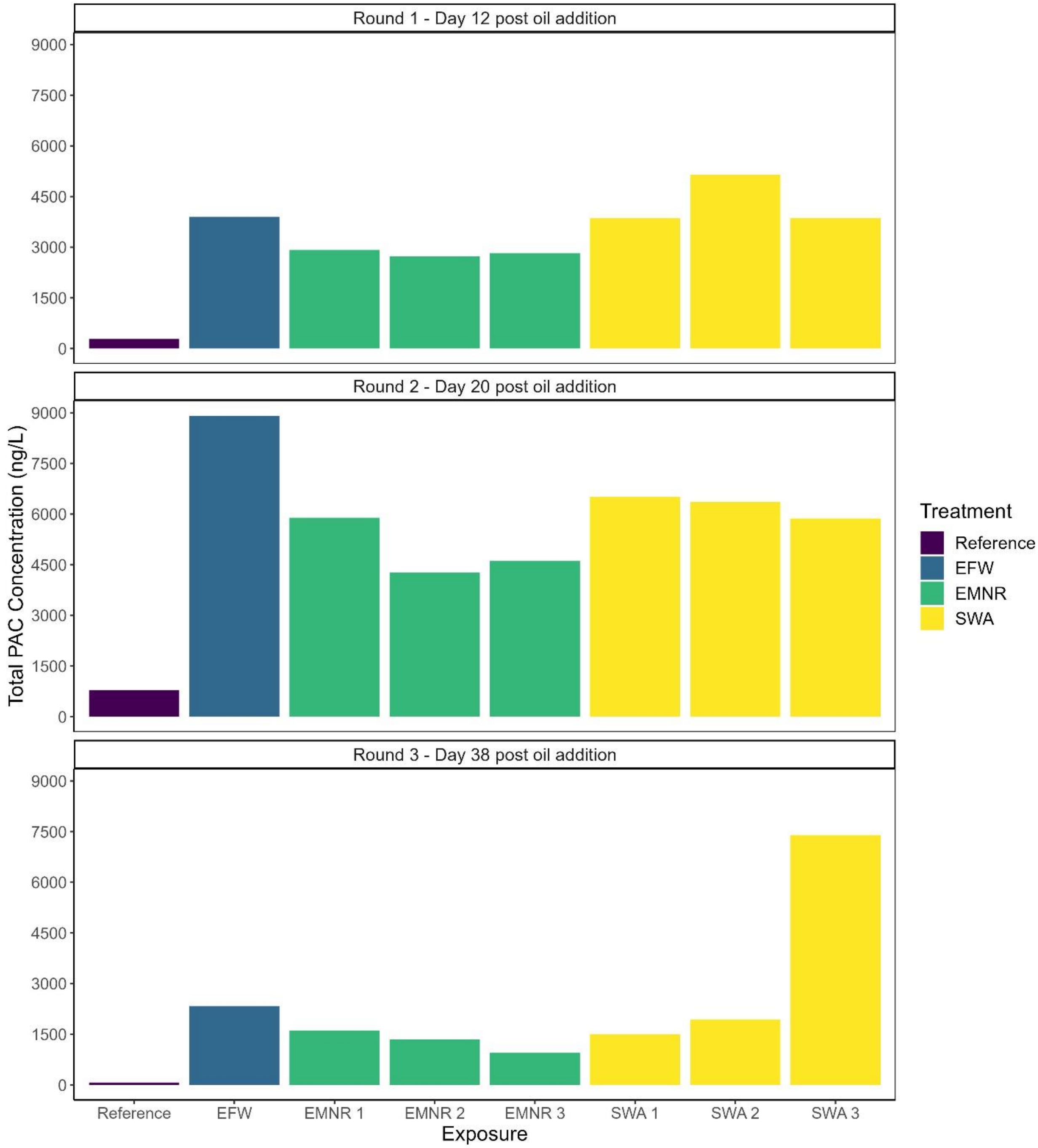

Figure 3.13. Polycyclic aromatic compound (PAC) concentrations (ng L^{-1}) used for a 7-day exposure to evaluate the response of early-life stages of fathead minnows to the photo-enhanced toxicity of diluted bitumen from the Freshwater Oil Spill Remediation Study (FOReSt) in combination with low or high UV exposure. Treatments include unoiled reference enclosures from the FOReSt project (Reference), engineered floating wetlands (EFW), nutrient additions through Enhanced Monitored Natural Recovery (EMNR), and the surface washing agent (SWA) Corexit EC9580A. Concentrations are seperated by enclosures (1-3 enclosures per treatment).

Table 3.5. Polycyclic aromatic compound (PAC) concentrations (ng L^{-1}) and remediation treatment concentrations (mg L^{-1}) used for a 7-day exposure to evaluate the response of early-life stages of fathead minnows to the photo-enhanced toxicity of diluted bitumen from the Freshwater Oil Spill Remediation Study (FOReSt) in combination with low or high UV exposure. Treatments include unoiled reference enclosures from the FOReSt project (Reference), engineered floating wetlands (EFW), nutrient additions through Enhanced Monitored Natural Recovery (EMNR), and the surface washing agent (SWA) Corexit EC9580A. Remediation treatment concentrations are estimates based on the weight of product added and the volume of water.

Round	**Enclosure**	**Treatment Code**	**PAC Concentration (ng L^{-1})**	**Remediation Treatment**	**Remediation Treatment Concentration (mg L^{-1})**
1	WL1	REFERENCE			
1	WL7	REFERENCE		Floating Wetland	
1	WL2	EMNR		Fertilizer Addition	0.521
1	WL5	EMNR		Fertilizer Addition	0.521
1	WL8	EMNR		Fertilizer Addition	0.521
1	WL3	SWA		Corexit EC9580A	9.167
1	WL4	SWA		Corexit EC9580A	9.167
1	WL9	SWA		Corexit EC9580A	9.167
1	WL6	EFW		Floating Wetland	
2	WL1	REFERENCE	1196.21		
2	WL7	REFERENCE	7107.77	Floating Wetland	
2	WL2	EMNR	9670.32	Fertilizer Addition	0.521
2	WL5	EMNR	8788.74	Fertilizer Addition	0.521
2	WL8	EMNR	6499.82	Fertilizer Addition	0.521
2	WL3	SWA	12440.81	Corexit EC9580A	9.167
2	WL4	SWA	1667.16	Corexit EC9580A	9.167
2	WL9	SWA	9036.29	Corexit EC9580A	9.167
2	WL6	EFW	9624.33	Floating Wetland	
3	WL1	REFERENCE			
3	WL7	REFERENCE		Floating Wetland	
3	WL2	EMNR		Fertilizer Addition	0.521
3	WL5	EMNR		Fertilizer Addition	0.521
3	WL8	EMNR		Fertilizer Addition	0.521
3	WL3	SWA		Corexit EC9580A	9.167
3	WL4	SWA		Corexit EC9580A	9.167
3	WL9	SWA		Corexit EC9580A	9.167
3	WL6	EFW		Floating Wetland	

Table 3.6. Comparison of UVA, UVB, visible light and PAR measurements of the low (~15% UV, OP3 plastic) and high (~90% UV, acrylic) UV filtering plastics used for a 7-day exposure to evaluate the response of early-life stages of fathead minnows to the photo-enhanced toxicity of diluted bitumen, compared to measurements with no plastic/filter.

Group	UV (%)	Plastic Type	Weather Conditions	UVA (watts/m^2)	UVB (watts/m^2)	Visible Light	PAR
1	15	OP3	Cloudy	0.149	0.013	39.6	304
1	90	Acrylic	Cloudy	3.13	0.219	39.9	292
2	15	OP3	Full Sun	5.6	0.139	374	1330
2	90	Acrylic	Full Sun	22.8	1.2	365	1360
3	15	OP3	Sparse Clouds; Some Smoke	4.1	0.178	341	1461
3	90	Acrylic	Sparse Clouds; Some Smoke	19.7	0.926	340	1405
3	100	No Filter	Sparse Clouds; Some Smoke	21.8	1.03	348	1351
4	15	OP3	Full Sun; Smoke Sky	0.308	0.0368	57.7	215
4	90	Acrylic	Full Sun; Smoke Sky	1.77	0.0798	57.9	174
4	100	No Filter	Full Sun; Smoke Sky	2.08	0.0827	52.5	195
5	15	OP3	Full Sun; No Clouds; Some Smoke	1.89	0.147	264	1217
5	90	Acrylic	Full Sun; No Clouds; Some Smoke	12.26	0.69	269	1539
5	100	No Filter	Full Sun; No Clouds; Some Smoke	13.5	0.735	259	1031
6	15	OP3	Full Sun; No Clouds	4.1	0.173	372	1602
6	90	Acrylic	Full Sun; No Clouds	20.5	0.954	373	1533
6	100	No Filter	Full Sun; No Clouds	22.2	1.09	381	1556
7	15	OP3	Full Sun; Full Clouds	0.236	0.0136	37.2	169
7	90	Acrylic	Full Sun; Full Clouds	2.27	0.144	38.8	141
7	100	No Filter	Full Sun; Full Clouds	2.88	0.181	35.1	171
8	15	OP3	Full Sun; Partly Cloudy	2.37	0.206	402	1575
8	90	Acrylic	Full Sun; Partly Cloudy	20.6	0.997	403	1668
8	100	No Filter	Full Sun; Partly Cloudy	22.7	1.22	393	1629
9	15	OP3	Full Sun; Sparse Clouds	2.33	0.191	374	1617
9	90	Acrylic	Full Sun; Sparse Clouds	20.3	0.999	398	1613
9	100	No Filter	Full Sun; Sparse Clouds	22	1.2	378	1661

Table 3.7. Daily UV radiation averaged for each day of a 7-day exposure to evaluate the response of early-life stages of fathead minnows to the photo-enhanced toxicity of diluted bitumen.

Round	Exposure Day	Measurements Taken	Daily Average UV (watts/m^2)	Standard Error (SE)	Average UV (watts/m^2)	Total UV (watts/m^2)
1	1	52	14.29	1.31	14.81	88.85
1	2	52	12.76	1.10		
1	3	52	15.84	1.29		
1	4	52	15.64	0.90		
1	5	52	14.07	1.01		
1	6	52	16.25	1.08		
2	1	26	10.48	1.68	15.43	92.57
2	2	26	13.50	1.29		
2	3	26	14.66	1.65		
2	4	26	16.74	1.72		
2	5	26	16.76	1.38		
2	6	26	20.43	0.84		
3	1	26	8.65	1.16	13.85	83.12
3	2	26	11.83	1.28		
3	3	26	11.55	1.05		
3	4	26	11.37	0.86		
3	5	26	16.57	1.43		
3	6	26	23.15	0.46		

Table 3.8. Daily average UV radiation across each exposure day and round of 7-day exposures to evaluate the response of early-life stages of fathead minnows to the photo-enhanced toxicity of diluted bitumen.

Round	Trial	Exposure Day	Measurements Taken	Daily Average UV (watts/m^2)	Standard Error (SE)	Average UV (watts/m^2)	Total UV (watts/ m^2)
1	A	1	13	24.09	0.86	15.40	92.39
1	A	2	13	21.17	1.32		
1	A	3	13	2.20	0.46		
1	A	4	13	18.03	0.12		
1	A	5	13	17.18	1.80		
1	A	6	13	9.72	0.81		
1	B	1	13	21.17	1.32	12.99	77.94
1	B	2	13	2.20	0.46		
1	B	3	13	18.03	0.12		
1	B	4	13	17.18	1.80		
1	B	5	13	9.72	0.81		
1	B	6	13	9.64	0.68		
1	C	1	13	2.20	0.46	13.79	82.74
1	C	2	13	18.03	0.12		
1	C	3	13	17.18	1.80		
1	C	4	13	9.72	0.81		
1	C	5	13	9.64	0.68		
1	C	6	13	25.97	0.28		
1	D	1	13	9.72	0.81	17.07	102.39
1	D	2	13	9.64	0.68		
1	D	3	13	25.97	0.28		
1	D	4	13	17.64	2.43		
1	D	5	13	19.75	2.49		
1	D	6	13	19.67	1.59		
2	A	1	13	3.84	0.12	13.97	83.81
2	A	2	13	17.12	2.09		
2	A	3	13	9.87	0.61		
2	A	4	13	19.46	2.67		
2	A	5	13	14.03	1.98		
2	A	6	13	19.49	1.67		
2	B	1	13	17.12	2.09	16.89	101.34
2	B	2	13	9.87	0.61		
2	B	3	13	19.46	2.67		
2	B	4	13	14.03	1.98		
2	B	5	13	19.49	1.67		
2	B	6	13	21.37	0.15		
3	A	1	13	4.56	0.56	12.20	72.29
3	A	2	13	12.75	1.58		
3	A	3	13	10.91	2.04		
3	A	4	13	12.20	0.64		
3	A	5	13	9.67	1.67		
3	A	6	13	22.20	0.10		
3	B	1	13	12.75	1.58	15.50	91.82
3	B	2	13	10.91	2.04		
3	B	3	13	12.20	0.64		
3	B	4	13	9.67	1.67		
3	B	5	13	22.20	0.10		
3	B	6	13	24.09	0.86		

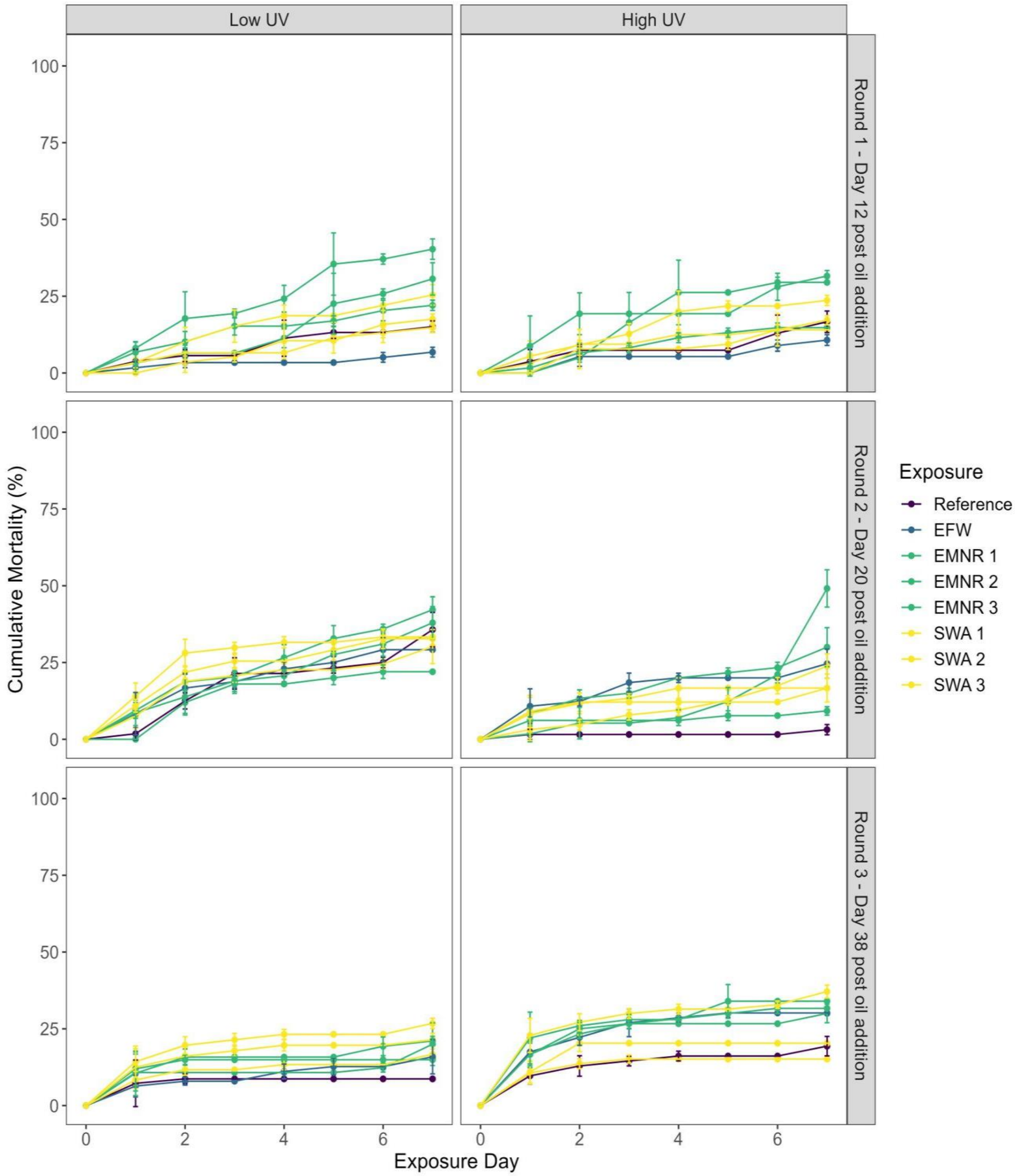

Figure 3.14. Cumulative mortality of early-life stages of fathead minnows over a 7-day exposure to diluted bitumen from the Freshwater Oil Spill Remediation Study (FOReSt) in combination with low or high UV exposure. Treatments include unoiled reference enclosures from the FOReSt project (Reference), engineered floating wetlands (EFW), nutrient additions through Enhanced Monitored Natural Recovery (EMNR), and the surface washing agent (SWA) Corexit EC9580A. Concentrations are seperated by enclosures (1-3 enclosures per treatment).

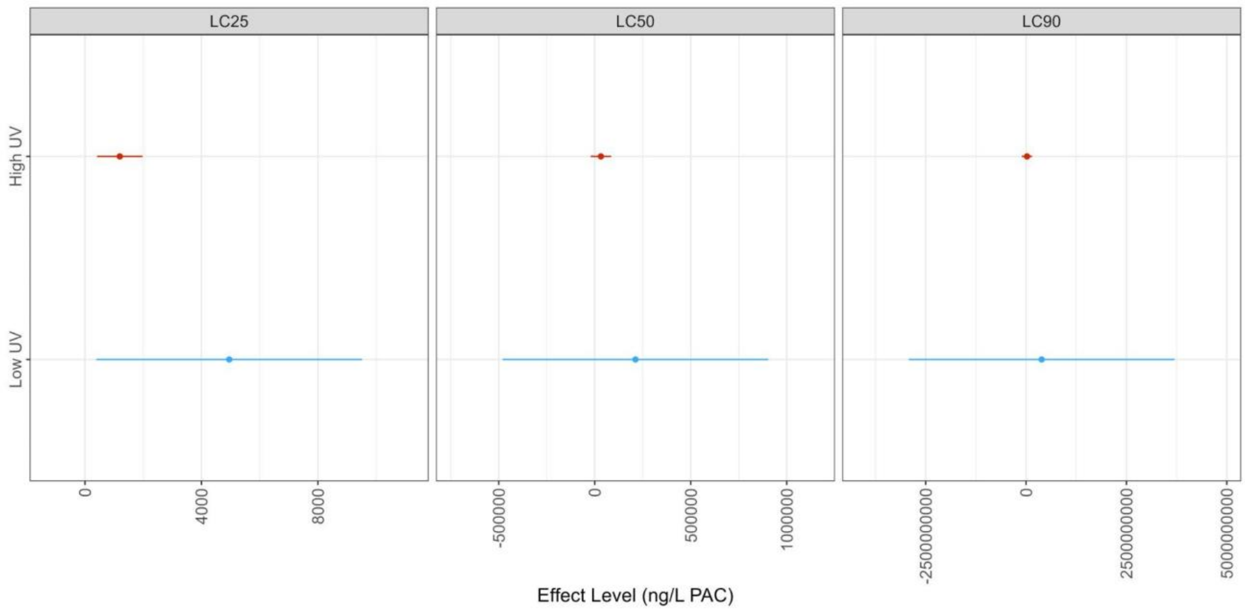

Figure 3.15. A comparison of early-life stages of fathead minnows lethal concentration (LC) values of the low and high UV radiation four-parameter log-logistic dose-response regression models for diluted bitumen treatments from the Freshwater Oil Spill Remediation Study (FOReSt).

Table 3.9. Lethal concentration (LC) values with 95% confidence intervals after 7-day exposures to evaluate the response of early-life stages of fathead minnows to the photo-enhanced toxicity of diluted bitumen.

Oil	Lethal Concentration Value (LC)	UV Group	Estimate	Standard Error	Lower CI	Upper CI
Diluted Bitumen	25	Low	269.99	240.04	23.43	763.40
Diluted Bitumen	50	Low	5330.00	3094.30	-1030.40	11,690
Diluted Bitumen	90	Low	2,077,300	5,956,800	-10,167,000	14,322,000
Diluted Bitumen	25	High	130.21	98.53	2.33	332.75
Diluted Bitumen	50	High	1079.14	336.79	-387.85	1771.43
Diluted Bitumen	90	High	74,119	83,068	-96,630	244,148

Table 3.10. Comparison of the low UV radiation versus high UV radiation lethal concentration values to determine the toxicity ratio of low:high UV radiation of diluted bitumen to early-life stages of fathead minnows after 7-day exposures to evaluate photo-enhanced toxicity.

Oil	Lethal Concentration Value (LC)	Low UV LC Value	High UV LC Value	Low:High UV Comparison
Diluted Bitumen	25	269.99	130.21	2.07
Diluted Bitumen	50	5330.00	1079.14	4.94
Diluted Bitumen	90	2,077,300	74,119	28.03

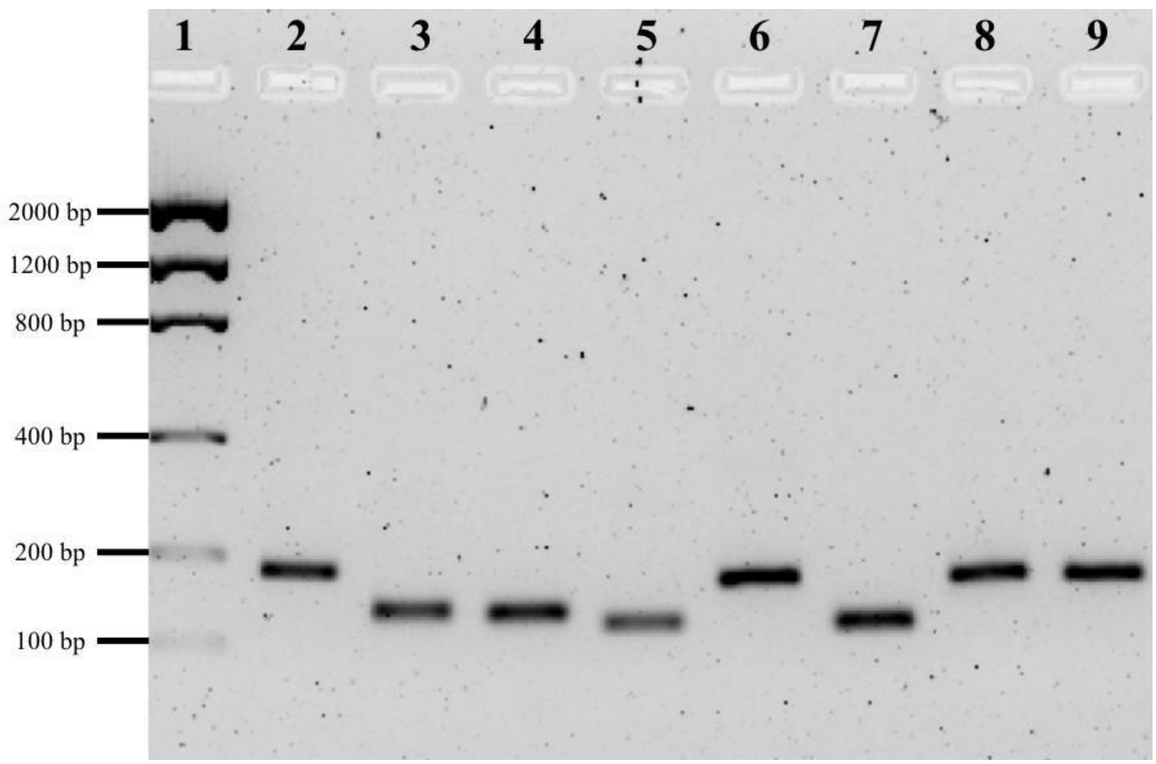

Figure 3.16. Agarose gel electrophoresis of RT-qPCR amplicons to validate the amplification of a single product. The 2% agarose gel was stained with SYBR™ Safe DNA Gel Stain and was visualized under UV. Lanes: 1, low DNA mass ladder (Thermo Fisher Scientific); 2, *thrα* amplicon; 3, *thrβ* amplicon; 4, *dio2* amplicon; 5, *dio3b* amplicon; 6, *cyp1A* amplicon; 7, *ef1α* amplicon; 8, *myod* amplicon; and 9, *rpl8* amplicon.

Table 3.11. Primer sets validated and used for gene expression analysis of early-life stages of fathead minnows.

Gene name	Gene symbol	Forward primer (5'-3')	Reverse primer (5'-3')	Product size (bp)	Temperature (°C)	Accession number
Thyroid hormone receptor alpha a	*thrα*	CCGTTGTTTGGGAAAGTGTTGT	GCAGGGATGAGGGGTAAGTG	191	63	XM_039686189.1
Thyroid hormone receptor beta	*thrβ*	TGGGGGTGAAGGAAGCAAAG	TGGCAAAATCTACGACACGA	144	62	XM_039651457.1
Deiodinase 2	*dio2*	GCCCTTTTCGTTTGAAGTGAGG	TAGGCTGCGTTGGCGTTGT	141	60	XM_039671292.1
Deiodinase 3	*dio3b*	TCTCCACTTCTCTCGTGTCCT	CAAATATGACCAAACCCGACC	132	62	XM_039690067.1
Cytochrome P450 Family 1 Subfamily A	*cyp1A*	GGGCTGTCGTCTATCTGGTG	GATGGTGAACGGGAGGAAGG	176	63	AF232749.1
Elongation factor 1-α	*ef1a*	CATCAAGAGCGTTGAGAAGAAA	TTGGGCAGAACATACCACAAC	127	60	XM_039680833.1
Myogenic differentiation	*myod*	CGCTGCTTAGGGGTCAAGAG	GGCGTGTCGTTGAAGTAAGA	175	61	NA
Ribosomal Protein L8	*rpl8*	CTGGGAGAGAAGCCTGGAGA	CCACCAGCAACAACACCAAC	170	61	AY919670.1

Chapter 4: Conclusion

4.1 Conclusion

In Canada and North America, there has been an increasing amount of economic investment and reliance on pipelines for transporting oil, and with this the concern surrounding accidental oil spills into fresh water has also increased (Lee et al. 2015). The majority of our current understanding of oil spill impacts comes from marine environments and species, and it has been highlighted in the literature that there is not enough research into the effects of petroleum products in freshwater environments (Lee et al. 2015). This lack of knowledge includes impacts to aquatic organisms, oil weathering and behaviour, and ways to best remediate these environments without causing further harm (Lee et al. 2015). On top of these concerns, toxicity studies evaluating the effects of oil typically evaluate it under controlled conditions using laboratory-based cultures. While this does provide standardized, reproducible results, it fails to incorporate other elements that are present in natural environments, such as environmental weathering and UV radiation. Understanding the impacts of oil under environmentally-relevant conditions that are present in a natural environment, along with testing wild populations from these environments, is important for our understanding of the effects of petroleum products in freshwater environments, which in turn dictates spill response timelines and protocols. This thesis helps address knowledge gaps in our overall understanding of the effects of dilbit, CHV, and UV radiation to both laboratory-cultured and wild-collected populations of at-risk early-life stages and lower trophic level species, while highlighting the need for the inclusion of UV radiation and photo-enhanced toxicity (PET) in oil and environmental contaminant related toxicity testing.

The study in Chapter 2 was designed to evaluate the environmentally-relevant scenario of UV radiation in combination with dilbit and CHV to *Hyalella azteca*. These experiments were initially designed to determine if these oil products did exhibit PET, and then to explore the impacts to other species and wild populations. In these experiments, we saw statistically significant increases in mortality and decreases in growth in a range of concentrations of dilbit and CHV, which agree with the literature surrounding the effects PET of oil and PACs to amphipods and lower-trophic level organisms (Barron et al. 2018; Barron et al. 2021; Cleveland et al. 2000; Hatch and Burton 1999a; 1999b; Lampi et al. 2006). The lethal concentrations on these oils were 1.5-10 times lower under high UV radiation, which significantly differs from standard toxicity testing (low UV) and has important ramifications on oil spill response in freshwater environments. We also highlighted the importance of considering physical toxicity endpoints, as well as PET and chemical toxicity. The SWA Corexit EC9580A caused individuals to develop internal and external bubbles, resulting in disruptions to buoyancy, ultimately leading to increased mortality. When examined further, this occurred in both Corexit EC9580A and its active surfactant, DOSS, suggesting SWA and surfactants pose risk to aquatic organisms on their own, and should not be considered for freshwater remediation following an accidental oil release. Corexit EC9580A on its own also exhibited slight photo-toxic effects through increased mortality and decreased growth, suggesting this petroleum-based SWA might also exhibit PET. These results support the need to include physical effects and PET in standard toxicity testing, as they are often overlooked. Further research could include evaluating various oil mixtures and products, a wider range of concentrations, a wider range of UV radiation to determine UV exposure thresholds, and behaviour modification in the presence of UV radiation. To understand how the impacts of PET may change with climate change, modeling UV exposure through dissolved organic carbon (DOC) and other variables could also be conducted. Evaluating more species, especially sensitive species, keystone species, and wild populations, will also contribute to a better understanding of PET.

The study in Chapter 3 was designed to expand on the knowledge learned from Chapter 2 and evaluate whether dilbit also exhibited PET at lower, post-remediation concentrations to a wild population of FHMs. In these experiments, we did not observe statistically significant differences in mortality or lethal concentrations as observed in *Hyalella azteca*. The wild population of FHMs did have higher reference mortality compared to what we might expect to see from a lab population, which is a sign of increased sensitivity among wild fathead minnows that may have masked potential PET effects. Instead, sub-lethal impacts, such as the upregulation of *cyp1A* and an increase in malformations, particularly yolk sac edemas, cardiac malformations, and deflated swim bladders were evident. These increases in sub-lethal impacts appeared in oil-exposed individuals with both low and high UV radiation and can lead to decreased fitness and survival. Individuals in the oil treatments exposed to high UV radiation also had increased malformations and *cyp1A* regulation compared to their low UV counterparts. This suggests dilbit likely had a mild photo-toxic effect at these lower concentrations, just not at a lethal level. While in the future this study could be repeated with a laboratory culture to confirm the findings, the environmental relevance of these results is paramount to the effects populations would see after an accidental oil spill in fresh water. Instead, future research could focus on evaluating a larger range of concentrations to determine specific lethal concentrations to these wild populations and impacts to the thyroid axis at more time points during early development. For example, assessing 3-5 days post fertilization instead of 7 days, because it is a more critical window when thyroid development is more rapidly occurring. Photodegradation and its impacts on PET through time should also be examined by conducting these experiments under conditions that minimize stress and non-contaminant based mortality.

Numerous groups have identified the knowledge gaps surrounding the effects of oil spills on freshwater communities and the need for more efficient spill response following accidental releases (CSAS 2017; Lee et al. 2015; NASEM 2016). The inclusion of environmentally-relevant populations and conditions into oil spill research can assist in developing a better understanding of the potential impacts in these systems and provide information to improve spill response and remediation strategies for accidental releases in fresh water. My thesis helps to address some of these knowledge gaps by evaluating oil toxicology under environmentally-relevant scenarios, including evaluating the effects to wild fish populations and under UV exposure. Wild fish have increased sensitivity, seen through consistent mortality even across reference treatments. This sensitivity justifies the need to evaluate wild species that would be impacted by real spill scenarios. Including UV exposure and the effects of photo-enhanced toxicity, particularly with wild populations, provides realistic lethal concentrations, impacts to organism health, and the change of impacts through time. It also highlights the importance of accurately reflecting multiple stressors individuals face during accidental spills, which might increase the relative impact these wild populations face compared to laboratory cultures. The results of this thesis will help direct future research by identifying the potential thresholds and sub-lethal impacts to both laboratory and wild populations exposed to oil under environmentally-relevant conditions of UV radiation, as well as highlight the need to include UV in toxicological testing to accurately understand multiple stressors.

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