TOXICITY OF WEATHERED SEDIMENT-BOUND DILBIT TO FRESHWATER FISH AND INVERTEBRATES

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DEDICATION

To all the people who facilitated my success before and during my Masters project. Thank you to my fellow students, friends, family and supervisors. This thesis is a product of your teachings and unconditional support.

ABSTRACT

Bitumen from the Alberta oil sands must be diluted to form diluted bitumen (dilbit) to facilitate transport through pipelines, yet little is known about its effects on aquatic organisms after a spill. Environmental weathering processes such as evaporation and sediment interaction manipulate spilled dilbit, which could affect its fate and toxicity in the environment. However, most studies to date that have characterized effects of dilbit to aquatic organisms have not incorporated weathering. In the present study, zebrafish (*Danio rerio*) embryos and adult freshwater amphipods (*Hyalella azteca*) were exposed to weathered sediment-bound dilbit. Sediment-bound dilbit exacerbated adverse effects associated with dilbit exposure due to oil-mineral aggregates directly interacting with amphipods and zebrafish embryos during exposure. As oil sands production expands, it is important to incorporate weathering processes when testing the toxicity of dilbit to aquatic organisms because sediment-bound dilbit can severely affect the health of freshwater fish and invertebrates.

PREFACE

The candidate is the primary author of chapters 1-4. The candidate primarily conducted the experiments and analyzed the data for chapters 2-3. Dr. Gregory Pyle and Dr. Steve Wiseman contributed to the experimental design and data interpretation of chapters 2-3 and edited chapters 1-4. Kaden Fujita participated in conducting experiments, writing and editing chapter 3.

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LIST OF ABBREVIATIONS

AChE	Acetylcholinesterase
AhR	Aryl-hydrocarbon receptor
AKR	Aldo-keto reductase
ANOVA	Analysis of variance
BCA	Bicinchoninic acid
BSD	Blue-sac disease
BTEX	Benzene, toluene, ethylbenzene, xylenes
cAMP	Cyclic adenosine monophosphate
CAT	Catalase
cDNA	Complimentary deoxyribonucleic acid
CLB	Cold Lake Blend
CYP1A	Cytochrome P4501A
ELS	Early-life stage
GCLC	Glutathione cysteine-ligase catalytic subunit
GSH	Glutathione
GST	Glutathione-*S*-transferase
GPX	Glutathione peroxidase
LPO	Lipid peroxidation
OMA	Oil-mineral aggregate
PAH	Polycyclic aromatic hydrocarbon
qPCR	Quantitative polymerase chain reaction
ROS	Reactive oxygen species
SAM-5S	Five salt reconstituted water
SDWAF	Sediment-derived water accommodated fraction
SDWSF	Sediment-derived water-soluble fraction
SOD	Superoxide dismutase
TBARS	Thiobarbituric acid reactive substances
WAF	Water-accommodated fraction
WSF	Water-soluble fraction

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1 Canadian oil sands

The Alberta oil sands contain an estimated 50 billion m^3 of recoverable bitumen, an oil reserve surpassed only by Saudi Arabia and Venezuela (National Energy Board, 2006). There are 15 deposits of bitumen located in Alberta within three major regions: Athabasca, Cold Lake and Peace River (Hein and Cotterill, 2006). The Athabasca region produces the most bitumen due to the high abundance of surface mineable material residing in the Wabiskaw-MacMurray deposit (Alberta Energy and Utilities Board, 2005). The remaining extractable bitumen in the latter two regions and other areas of the Athabasca is found at depths greater than 75 m and is removed with *in situ* mining, where steam or solvents are pumped into the deposits to decrease bitumen viscosity so that it can be pumped to the surface (Read and Whiteoak, 2003).

Canada ranks 6th in the world in oil production and produces 4.2 million barrels/day, of which oil sands comprise 63% (CAPP, 2018). Although it is more energetically costly than conventional oil production, extraction of bitumen is expected to increase from the current 2.69 million barrels/day to 4.19 million barrels/day by 2035 (Huot and Grant, 2012; CAPP, 2018). Canada is projected to become more dependent on oil sands in the future; by 2035 bitumen is expected to comprise 75% of total Canadian oil production (CAPP, 2018). The development of new pipelines such as the Keystone XL and the Kinder Morgan Transmountain expansion will facilitate export of the increasing volume of bitumen but are also in close proximity to thousands of freshwater environments, including critical salmon habitat in British Columbia (Levy *et al.,* 2009; NASEM, 2015). From 1984 – 2003, the 43,000 km of pipeline monitored by the Canadian National Energy Board had an average of 2.3 ruptures per year and it is

estimated that, on average, new pipelines rupture after 28 years (Jeglic, 2004). Although pipelines are the safest and most efficient method of transporting bitumen and the rate of pipeline ruptures decreases as new monitoring and construction technology emerges, spills from pipelines remain of environmental concern.

Bitumen is an 'unconventional' oil that is a semi-solid at atmospheric pressure and room temperature, making transport through pipelines impractical before it is diluted (Lee *et al.,* 2015). Thus, in order for bitumen to be transported via pipelines, it is diluted with natural gas condensates such as naphtha (20-30% condensate; 70-80% bitumen) after it is separated from the sand to form 'dilbit', making it suitable for transport through pipelines (Crosby *et al*., 2013). Some bitumen undergoes coking or hydrolysis and is then mixed with synthetic oil at a 1:1 ratio to create synthetic dilbit or 'synbit' (Crosby *et al.,* 2013). The chemical and physical properties of dilbits can be highly variable based on the blend (composition of dilutents), location of extraction and the season it is being transported (Crude Monitor, 2019).

1.2 History of dilbit spills in North America

The largest dilbit spill in history occurred in 2010, when Enbridge line 6b ruptured into Talmadge Creek in Marshall, Michigan and eventually flowed into the Kalamazoo River (US Fish and Wildlife, 2015). In total, 3191 m^3 of dilbit was released, resulting in $767 million in clean-up costs (as of October 2011) – the most expensive inland oil spill in history (NTSB, 2012). At the time of the spill, there was no available literature on the toxicological effects of dilbit on aquatic organisms. This spill exemplified the importance of physical weathering processes (discussed in detail later), as an estimated 10-30% of the spilled dilbit combined with suspended solids and sank, making the dilbit more accessible

to some aquatic organisms (USEPA, 2013a; US Fish and Wildlife, 2015). Smallmouth bass (*Micropterus dolomieu*) and golden redhorse (*Moxostoma erythrurum*) collected 14 km downstream of the release were found to have reduced condition factors, high hepatosomatic indices, high rates of lesions/abnormalities, spleen fibrosis and induction of cytochrome P450 1A (*cyp1a*) (Papoulias *et al.,* 2014). Dilbit-contaminated sediments from the spill were collected and found to inhibit growth and survival of freshwater amphipods (*Hyalella azteca*) and freshwater midges (*Chironomus dilutus*) (GLEC, 2012). The Enbridge line 6a ruptured two months later in Romeoville, Illinois, spilling 970 m^3 of dilbit (EPA, 2012). Enbridge paid $177 million in settlements for damages caused by these two spills (MLive, 2016).

There have been several other significant oil sands related pipeline spills in North America prior to and after the Kalamazoo River disaster (Crosby *et al.,* 2013). In 2007, an excavator struck a pipeline operated by Kinder Morgan in Burnaby, BC, spilling 234 m^3 of synthetic crude derived from bitumen (TSB, 2008). The synthetic crude then flowed into Burrard Inlet, covering 1200 m of shoreline and resulting in $15 million in clean-up costs (CBC, 2011). The TransCanada Keystone pipeline's first year of operation in 2010 resulted in 35 spills of oil sands products – 100 times the projected frequency of spills (Skinner and Sweeney, 2012). However, the largest of the 35 spills was an 80 m^3 dilbit spill in Ludden, North Dakota (Crowl, 2011). An ExxonMobil pipeline in Mayflower, Arkansas, ruptured in 2013 as the result of a construction mistake in the original pipeline, spilling an estimated 318 m^3 – 795 m^3 of dilbit (Dupre, 2013; USEPA, 2013b). The most recent spill of dilbit came from the keystone pipeline in 2017, where 795 m^3 was spilled beneath South Dakota farmland (NTSB, 2018). In many of these cases, it was unknown that the spilled oil was bitumen-derived until days after the release (Crosby *et al.,* 2013).

This lack of communication could have resulted in greater environmental damage, as the clean-up response in the first days following the spill is extremely important and should be specific to the type of spilled oil.

Evidently, pipelines that transport oil sands products fail for a number of reasons. Certain reports have speculated that dilbit pipelines pose a higher risk of failure due to the high abundance of sulphur and total acids in dilbit (Stansbury, 2011; Skinner and Sweeney, 2012). However, the most important factor in pipeline corrosion is water content, and because dilbit has similar water content to conventional oils, it therefore poses no greater corrosion risk (Zhou and Been, 2011; Dettman, 2012).

1.3 Chemical composition of dilbit

The chemical composition of crude oils often is described as "super complex". The four main chemical components of crude oils are saturates, aromatics, resins and asphaltenes (Yang *et al.,* 2014; Fingas, 2011; Lee *et al.,* 2015). Less abundant components include metals, inorganic sulphur and constituents with nitrogen, sulphur and oxygen heteroatoms (N, S, O) (Woods *et al.,* 2008; Fingas, 2011; Lee *et al.,* 2015). Saturated hydrocarbons are the most abundant constituent in conventional crude oils and are the most biodegradable (Woods *et al*., 2008, Lee *et al.,* 2015). Saturates are generally non-toxic, but some *cyclo*-alkanes can cause narcosis by accumulating in lipid membranes and disrupting membrane structure and function (Sikkema *et al.,* 1995; Adams *et al.,* 2014). The aromatic fraction includes single-ringed benzene, toluene, xylenes and ethyl benzene (BTEX) and polycyclic aromatic hydrocarbons (PAHs) (Fingas, 2011; Lee *et al.,* 2015). With the exception of some light crudes, aromatics are present in similar abundances across crude oil types (Woods *et al.,* 2008). The toxicity of

crude oils to aquatic organisms is primarily attributed to PAHs and their alkylated derivatives and is discussed in detail later (Ball and Truskewycz, 2013; Adams *et al.,* 2014). Resins and asphaltenes are non-biodegradable, water-insoluble and most prevalent in heavy crude oils (Woods *et al.,* 2008; Fingas, 2011; Lee *et al.,* 2015). Resins and asphaltenes contain N, S and O, but asphaltenes are of considerably higher molecular weight (Speight, 2002). Crude oils with high proportions of resins and asphaltenes have high densities, viscosities and adhesiveness (Speight, 2002; Akmaz *et al.,* 2011; Fingas, 2011; Lee *et al.,* 2015).

Dilbit has a different chemical composition than conventional crude oils because most of the biodegradable components were removed under anaerobic conditions present in the oil sands (Table 1-1) (NASEM, 2015). Saturates in dilbit are highly reduced compared to conventional oils and are skewed towards *cyclo*-alkanes that resist degradation (Woods *et al.,* 2008; NASEM, 2015). The aromatic fraction of dilbit favours tricyclic PAHs and high degrees of alkylation, as these resist degradation (Yang *et al.,* 2011; Environment Canada, 2013a). Indeed, dilbit contains greater abundances of dibenzothiophene, fluorene and chrysene compared to conventional oils but generally has similar or lower total PAH concentrations (Wang *et al.,* 2003; Yang *et al.,* 2011; Yang *et al.,* 2014). The BTEX content of dilbit is comparable to heavy conventional oils and is solely the result of added diluents. Dilbit contains large proportions of resins and asphaltenes, which can influence the behaviour and persistence of dilbit after a spill into an aquatic environment (Woods *et al.,* 2008; NASEM, 2015).

Table 1-1 Chemical properties of major crude oil types.

	Light Crude (Scotia Light)	Medium Crude (West Texas Intermediate)	Heavy Crude (Sockeye Sour)	Dilbit (Cold Lake Blend
Saturates (%)	92	66	38	25
Aromatics (%)	8	26	29	22
Resins (%)	1	6	20	33
Asphaltenes (%)	0	1	13	20
Sulphur (%)	0.02	0.48	4.41	3.78
BTEX (%)	0.2379	0.964	0.6748	0.7100
Total PAHs (μg/g)	3504	7947	5231	5384

(Wang *et al.,* 2003; Yang *et al.,* 2011; Yang *et al.,* 2014; Hollebone, 2015; Crude Monitor, 2019; Environment Canada, 2019)

1.4 Physical properties of dilbit

The density, adhesion and viscosity of dilbit can differ from conventional oils (NASEM, 2015). Differences in physical properties are important because they can influence the behaviour of crude oil after a spill (NASEM, 2015). Undiluted bitumen is the only crude oil that is dense enough to sink in freshwater, but due to its high viscosity it is not transported through pipelines. Dilbit has physical properties most comparable to heavy crude oils due to the high proportion of resins and asphaltenes. Physical properties of major oil types are summarized in Table 1-2.

Table 1-2 Physical properties of major crude oil types at 15°C.

	Light crude (Scotia Light)	Medium crude (West Texas Intermediate)	Heavy Crude (Sockeye Sour)	Bitumen (Cold Lake)	Dilbit (Cold Lake Blend)
Density (g/mL)	0.7655	0.8420	0.9409	1.0002	0.9172
API gravity	53.2	36.4	18.8	9.8	21.3
Viscosity (mPa•s)	1	7	821	235000	150
Adhesion (g/cm^2)	0	15	75	575	98

(Wang *et al.,* 2003; Yang *et al.,* 2011; Yang *et al.,* 2014; Hollebone, 2015; Crude Monitor, 2019; Environment Canada, 2019)

1.5 Environmental weathering

Spilled dilbit undergoes a myriad of environmental processes known as 'weathering' that influence its behaviour and toxicity to aquatic organisms (NASEM, 2015). Physical weathering processes include dispersion, emulsification, spreading, evaporation and sedimentation, whereas chemical weathering encompasses photooxidation and biodegradation (Environment Canada, 2013a; Lee *et al.,* 2015; NASEM, 2015). Weathering processes have the potential to exacerbate and/or ameliorate environmental damage caused by dilbit spills.

Dilbit can lose up to 30% of its mass due to evaporation after a spill because the added diluents are low molecular weight and extremely volatile (Environment Canada, 2013a). Evaporation increases the density, viscosity and adhesiveness of dilbit by increasing the proportion of resins and asphaltenes (Yang *et al.,* 2014). The density of evaporated dilbit can approach 1 g/mL, but this does not necessarily translate to sinking

in freshwater (Environment Canada, 2013a; Yarranton *et al.,* 2015). Heavily evaporated dilbit is extremely viscous, which limits dispersion and therefore can prevent sinking, as large masses—such as tarballs—are less likely to sink than small droplets (Zhou *et al.,* 2015). One flume tank study that simulated a spill showed that dilbit can sink in 20 practical salinity unit (psu) brackish water after six days of evaporation (King *et al.,* 2014), but other similar studies report that dilbit will remain floating or entrained in the water column with evaporation alone (SL Ross, 2012; Zhou *et al.,* 2015). Dispersion also creates smaller droplets that increase the release of water-soluble PAHs, highlighting the importance of evaporation on the physical fate of spilled dilbit (Nordtug *et al*., 2011; Hansen *et al*., 2012; Redman *et al.,* 2014).

Evaporation is an important determinant of the chemical composition, and thus the toxicity of spilled dilbit to aquatic organisms (Yang *et al.,* 2018). Weathering (20% mass loss) can attenuate the acute and chronic toxicity of the water-accommodated fraction (WAF) of dilbit to freshwater fish and invertebrates (Barron *et al*., 2018; Robidoux *et al.,* 2018). Attenuation of toxicity of weathered dilbit could be attributed to the loss of highly soluble, low molecular weight BTEX, which can be a more accurate predictor of toxicity than total concentration of PAHs in zebrafish (*Danio rerio*) embryos (Philibert *et al.,* 2016). However, weathering can also increase the potency of dilbit by increasing the proportion of tricyclic PAHs (particularly alkyl-PAHs), which are responsible for much of the cardiac impairment in fish exposed to crude oils (Carls *et al.,* 1999; Jung *et al.,* 2013).

Dilbit can combine with fine sediments to form oil-mineral aggregates (OMAs) that sink in freshwater (Environment Canada, 2013a; Waterman and Garcia, 2015; Yang *et al.,* 2018). Oil-sediment interactions have been proposed as a beneficial dispersion

process following spills into aquatic environments (Fitzpatrick *et al.,* 2015), but sunken dilbit has the potential to negatively affect benthic and pelagic fish and invertebrates (Dew *et al*., 2015). Dilbit OMAs could bind to appendages and gas exchange structures of organisms, or act as a slow release of water-soluble PAHs over time. The Kalamazoo River dilbit spill in 2010 exemplified the importance of dilbit-sediment interactions, as an estimated 21 ± 12% of the spilled dilbit remained sunken and bound to sediment as of 2013 (USEPA, 2013a; US Fish and Wildlife, 2015). Contaminated sediments collected from the spill in 2013 were acutely lethal to freshwater amphipods (*Hyalella azteca*) and freshwater midges (*Chironomus dilutus*) (GLEC, 2012).

1.6 Effects of PAHs on fish

Polycyclic aromatic hydrocarbons are more genotoxic, carcinogenic and teratogenic than other water-soluble oil-derived constituents in dilbit, such as *n*-alkanes (Nam *et al.*, 2008, Adams *et al.,* 2014; Hodson, 2017). Exposure to PAHs can result in severe adverse effects in fish that include inhibited growth, early-life stage (ELS) toxicity, blue-sac disease (BSD), oxidative stress, behavioural changes, cardiotoxicity, enzymatic imbalances and impaired reproduction. There is an abundance of information on the toxicity of individual PAHs and PAH mixtures such as conventional crude oils to fish, which will be partially drawn from for this review due to the lack of available literature on the toxic effects of dilbit.

1.6.1 Aryl-hydrocarbon mediated detoxification of PAHs

Increased expression of cytochrome P450 1A (*CYP1A)* mediated by activation of the aryl hydrocarbon receptor (AhR) precedes ELS toxicity in fish and is the

major detoxification pathway of PAHs (Brinkworth *et al.,* 2003; Hodson, 2017). Planar PAHs with three or more rings enter through the cell membrane and bind to the AhR complex in the cytoplasm, releasing heat shock protein 90 and AhR interacting protein (Basu *et al.,* 2001; Wiseman and Vijayan 2007; Jung *et al.,* 2011). The ligand bound AhR translocates to the nucleus and forms a heterodimer with the AhR nuclear translocator protein, which then binds to xenobiotic response elements, activating transcription of *CYP1A* and other xenobiotic responsive genes (Fujii-Kuriyama and Kawajiri, 2010). Cytochrome P4501A is a phase I enzyme that functionalizes PAHs via processes such as hydroxylation and oxygenation (Nebert and Dalton, 2006). Functionalized PAHs are modified further by phase II enzymes, resulting in a more water-soluble derivative that is effluxed from cells (Nebert and Dalton, 2006). While necessary for detoxification, reactive metabolic intermediates can negatively affect cellular function by forming DNA adducts, binding directly to proteins, or resulting in the formation of reactive oxygen species (ROS) (Miller and Ramos, 2001; Wills *et al.,* 2009; Sturve *et al.,* 2014). However, it is understood that greater expression and activity of CYP1A has an overall protective effect because its inhibition can lead to an exacerbation of toxicity to ELS of fish (Billiard *et al.,* 2006; Billiard *et al.,* 2008). Some PAHs are not AhR agonists and therefore are not detoxified by CYP1A and can cause adverse effects directly by non-polar narcosis in which membrane fluidity and function of membrane proteins, including Na^+/K^+ ATPase transporters, are disrupted (Sikkema *et al.,* 1995; Barron *et al.,* 2004; Kennedy and Farrell, 2006). Because dilbit contains a complex mixture of PAHs and alkyl-PAHs, there could also be interactions that exacerbate toxicity—such as non-dioxin like PAHs being oxygenated due to CYP1A enzyme activity induced by dioxin-like PAHs—but currently this is poorly understood (Hodson, 2017). Further, certain PAHs can

inhibit CYP1A activity and could therefore lead to accumulation of parent PAHs, as they will not be metabolized (Wassenberg and Di Giulio, 2004). Potential toxicity pathways following exposure to PAH mixtures such as dilbit are summarized in Fig. 1-1.

Increased expression of *CYP1A* is an extensively used biomarker of exposure to crude oils because it is extremely sensitive to low concentrations of dioxin-like PAHs. In two separate studies, Japanese medaka (*Oryzias latipes*) embryos exposed to dilbit WAFs had >15-fold increases in *cyp1a* mRNA abundances, which were correlated to the number of observed developmental malformations (Madison *et al*., 2015; Madison *et al.,* 2017). Similarly, ELS of sockeye salmon (*Oncorhynchus nerka*) exposed to the water-soluble fraction (WSF) of dilbit had >30-fold increases in *cyp1a* mRNA abundance (Alderman *et al.,* 2018). Sockeye salmon parr exposed to dilbit WSF had >3-fold increases in *cyp1a* mRNA abundance (Alderman *et al.,* 2017). Fathead minnow (*Pimephales promelas*) and yellow perch (*Perca flavescens*) embryos exposed to dilbit WAF had >40-fold and 16-fold increases in *cyp1a* abundance, respectively (Alsaadi *et al.,* 2018b; McDonnell *et al.,* 2019). Fathead minnow is currently the fish species that shows the greatest induction of *cyp1a* mRNA abundance following exposure to undispersed dilbit WAF.

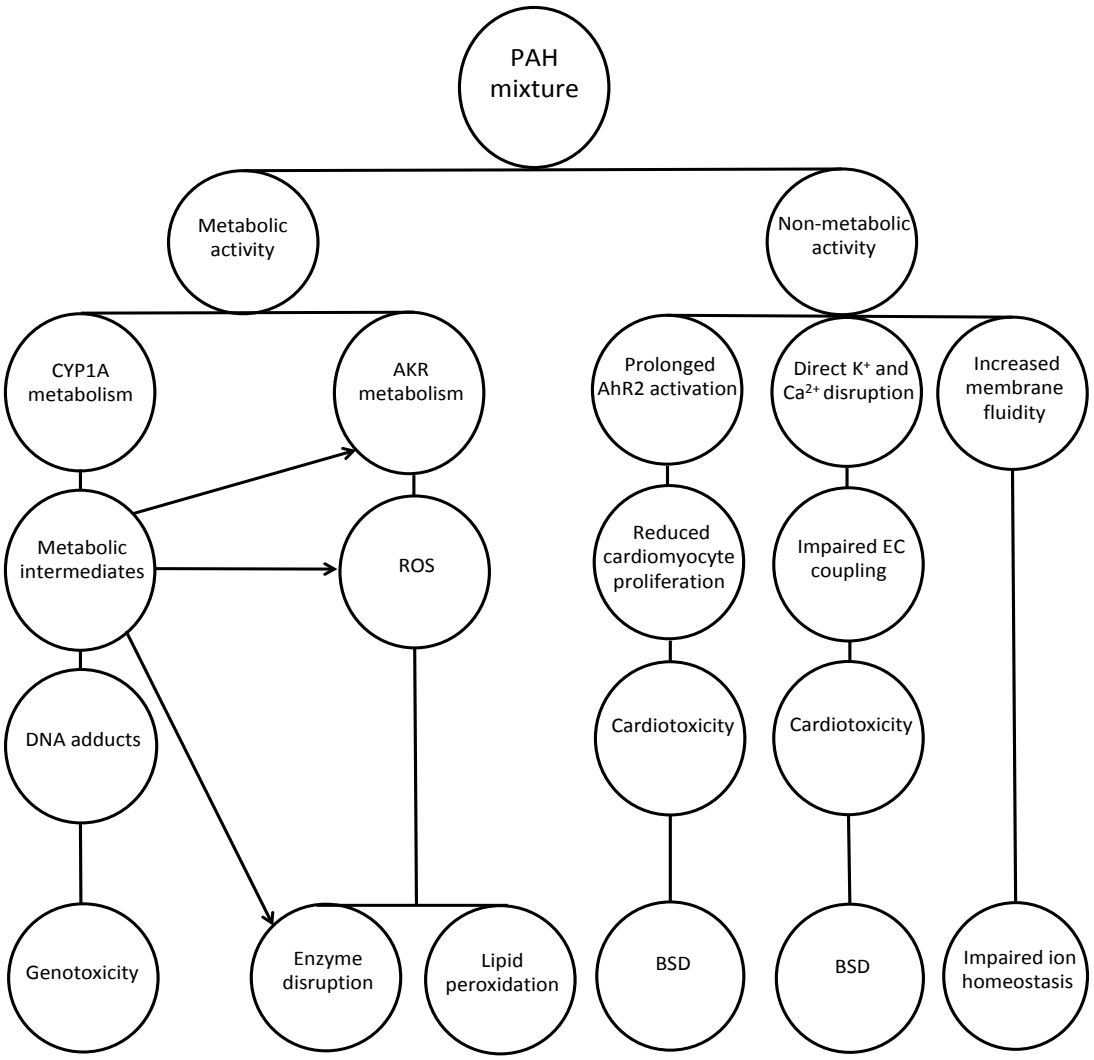

Figure 1-1 Schematic illustration of metabolic and non-metabolic mechanisms of toxicity of PAHs. Metabolic mechanisms require functionalization of PAHs to cause toxicity catalyzed by CYP1A or AKRs. Metabolic toxicity is AhR-dependent and involves metabolic intermediates generated by CYP1A forming DNA adducts or entering redox cycling that produces ROS. Alternatively, AKRs can metabolize intermediates generated by CYP1A that then enter redox cycling and produce ROS. Non-metabolic mechanisms of toxicity do not require functionalization of PAHs. Rather, PAHs can cause toxicity via a narcosis mode of action in which they directly solubilize into lipid membranes, increasing fluidity and impairing ion homeostasis. There can also be direct impairment of K^+ and Ca^{2+} transporters in cardiomyocytes that leads to cardiotoxicity. Separately, prolonged activation of AhR2 by PAHs reduces cardiomyocyte proliferation and results in cardiotoxicity. Adapted from Gauthier *et al.* (2014).

1.6.2 Embryotoxicity and blue-sac disease

The relationship between fish embryotoxicity and PAH concentration is well established, with EC50s for PAHs ranging from 0.3 – 2500 μg/L (Carls and Meador, 2009; Hodson, 2017). Fish early-life stages are particularly vulnerable to aquatic toxicants as they are sessile and cannot relocate to uncontaminated areas – which is highly relevant to events such as oil spills (Carls *et al.,* 2000). Embryos are often laid atop or within sediments, which could contain sediment-bound PAHs and serve as an additional route of exposure to water-soluble PAHs (Le Bihanic *et al.,* 2014). Embryos also have a reduced ability to metabolize PAHs compared to adults, leading to bioaccumulation during tissue differentiation (Peterson and Kristensen, 1998; Carls *et al.,* 2000; Jung *et al.,* 2015). Further, crude oil exposure can slow development, prolonging exposure and susceptibility to mechanical damage because the vitelline membrane is not fully developed (Jensen, 1997; Carls and Thedinga, 2010). Adult fish can experience latent impaired development as a result ELS exposure to PAHs (Xu *et al.,* 2017; Alderman *et al.,* 2018; Vignet *et al.,* 2019). For example, sockeye salmon embryos exposed to dilbit WAF had significantly larger brains eight months after being transferred to clean water (Alderman *et al.,* 2018). Similarly, fathead minnow embryos exposed to bituminous sediments had increased incidences of jaw malformations compared to control fish five months after being transferred to clean water (Vignet *et al.,* 2019).

Exposure to PAHs can increase the incidence of BSD in fish embryos. Blue-sac disease is the accumulation of metabolic wastes resulting in craniofacial deformities, uninflated swim bladder, spinal curvature, yolk sac and pericardial edemas (Bauder *et al.,* 2005; Lin *et al.,* 2015; Arens *et al.,* 2017). Blue-sac disease resulting from exposure to

crude oils can be explained by the cardiotoxic effects of PAHs and is discussed in detail later (Incardona *et al.,* 2004) (Fig. 1-1). Incidences of BSD are associated with high mortality, therefore there is potential for ELS exposure to crude oils to have a negative impact on fish populations (Marty *et al.,* 1997).

Embryotoxicity and BSD have been observed in several fish species exposed to crude oils, including dilbit. Pacific herring (*Clupea pallasi*) and pink salmon (*Oncorhynchus gorbushca*) exposed to Alaska North Slope crude oil in response to the Exxon Valdez spill had high incidences of BSD and slowed development, which was attributed to alkyl-PAHs such as alkyl-phenanthrene (Marty *et al.,* 1997; Carls *et al.,* 1999). Similarly, incidences of BSD have been observed in rainbow trout (*Oncorhynchus mykiss*), zebrafish (*Danio rerio*) and Japanese medaka exposed to the individual PAH retene – an alkyl-phenanthrene (Billiard *et al.,* 1999; Brinkworth *et al.,* 2003; Alharbi *et al.,* 2016). White suckers (*Catostomus commersoni*) and fathead minnows exposed to natural bitumen from the oil sands have also exhibited BSD and significant increases in *CYP1A* expression compared to controls (Colavecchia *et al.,* 2004; Colavecchia *et al.,* 2006; Vignet *et al.,* 2019). The most common malformation in fish embryos exposed to dilbit differs between species. Across several studies, pericardial edemas were the most common malformation in fathead minnows and yellow perch (Alsaadi *et al.,* 2018b; McDonnell *et al.,* 2019), yolk sac edemas were the most common malformation in sockeye salmon and zebrafish embryos (Philibert *et al.,* 2016; Alderman *et al.,* 2018), and swim bladder failure were the most common malformation in Japanese medaka (Madison *et al.,* 2015; Madison *et al.,* 2017). It is evident that BSD is a consistent effect of exposure to multiple crude oil types, although there appear to be species-specific sensitivities.

1.6.3 Oxidative stress

Oxidative stress has been proposed as a mechanism of toxicity in fish exposed to PAHs (Billiard *et al.,* 1999; Almroth *et al.,* 2005) (Fig. 1-1). Some PAHs metabolized by CYP1A enter redox cycling that can produce ROS (Burchiel *et al.,* 2007; Bravo *et al.,* 2011). Additionally, aldo-keto reductase (AKR) enzymes can metabolize PAH-dihydrodiols created via CYP1A, resulting in *o*-quinones that enter redox cycling and generate ROS (Jiang *et al.,* 2006; Zhang *et al.,* 2012). Reactive oxygen species such as O_2-•, OH• and H_2O_2 are normally scavenged by phase II enzymes, but if generated in abundance, can damage lipids, proteins and DNA, leading to cell death (Cowey *et al.,* 1985; Schlenk *et al.,* 2008). Lipid peroxidation (LPO) decreases membrane fluidity and results in the formation of unsaturated aldehydes that form DNA adducts (Schlenk *et al.,* 2008; Tai *et al.,* 2010). The cellular response to ROS includes induction and expression of several enzymes, including superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione-*S*-transferase (GST) and glutamate-cysteine ligase catalytic subunit (GCLC). Superoxide dismutase catalyzes the dismutation of O_2-• to H_2O_2 (Fridovich, 1995), which can be converted to water and oxygen by CAT (Glorieux and Calderon, 2017). The conversion of O_2-• to H_2O_2 can also be catalyzed by GPx, but unlike CAT involves converting glutathione (GSH) to glutathione disulphide (Brigelius-Flohé and Maiorino, 2013). Glutathione *S*-transferase catalyzes the conjugation of electrophilic reactive metabolic intermediates with GSH, allowing them to be excreted from the cell (Hayes and Pulford, 1995). Glutamate-cysteine ligase catalytic subunit is the rate-limiting enzyme in the formation of GSH and therefore plays an important role in many of the mechanisms that reduce cellular ROS (Lu, 2013). Altered expression of

oxidative stress response genes and cellular levels of GSH are often used as biomarkers of oxidative stress, but do not definitively determine if oxidative stress is present. For example, *GST* expression is upregulated in response to increasing cellular ROS, but is also upregulated directly by AhR and is involved in regulating cell death and proliferation (Nebert *et al.,* 2000; Laborde, 2010; Brown *et al*., 2016).

Exposure to crude oils could cause increases in cellular ROS, but this response is not consistent. Fathead minnow embryos exposed to dilbit had increased expression of *GST*, while expression of other oxidative stress response genes remained unchanged (Alsaadi *et al.,* 2018b). One study of dilbit-exposed Japanese medaka embryos reported increased expression of *SOD* and glutathione-disulphide reductase - which catalyzes the reduction of glutathione disulphide to GSH (Madison *et al.,* 2015), while another reported no change in expression for any oxidative stress response genes (Madison *et al.,* 2017). Most recently, a study reported that yellow perch did not have increased expression of any oxidative stress genes following exposure to dilbit (McDonnell *et al.,* 2019). However, no study of the ELS toxicity of dilbit has directly measured LPO. Further, retene-exposed rainbow trout embryos had unchanged concentrations of LPO, but did have lower levels of the antioxidants vitamin E and GSH compared to controls (Bauder *et al.,* 2004).

1.6.4 Cardiotoxicity

Impaired cardiac function as a result of exposure to PAHs is the main cause of ELS toxicity and BSD in fish (Incardona *et al.,* 2004) (Fig.1-1). Recent research suggests that cardiotoxicity following exposure to crude oils can also be AhR-independent, distinct

from AhR-dependent cardiotoxicity observed following exposure to organochlorines or high molecular weight PAHs (Incardona, 2017).

AhR-dependent cardiotoxicity from exposure to organochlorines is the result of activation of the AhR2 isoform. Prolonged AhR2 activation down-regulates expression of genes that are important for cardiomyocyte proliferation and cardiac development, resulting in impaired heart looping (the formation of heart chamber differentiation), eventually leading to cardiac failure (Antkiewicz *et al.,* 2005; Carney *et al.,* 2006; Lanham *et al.,* 2014). Similarly, embryos exposed to high molecular weight PAHs such as benzo(a)pyrene exhibit down-regulation of genes involved in calcium handling and cell proliferation as a result of prolonged AhR2 activation - similar to effects caused by exposure to organochlorines that are potent activators of the AhR (Prasad *et al.,* 2007; Jayasundara *et al.,* 2015). Indeed, high molecular weight PAHs induce a dioxin-like response, but these constituents are in low concentrations in crude oil WAFs (Carls *et al.,* 2008; NASEM, 2015).

The AhR-independent mechanism of cardiotoxicity is dissimilar to dioxin-like cardiotoxicity and is caused by tricyclic PAHs directly interfering with the balance of K^+ and Ca^{2+} ions in cardiomyocytes (Brette *et al.,* 2014; Brette *et al*., 2017). Phenanthrene can directly block the efflux of K^+ ions from the cell, resulting in the inability of the cell to re-polarize. Potassium imbalances in cardiomyocytes from exposure to phenanthrene resemble exposure to cardiotoxic pharmaceuticals that inhibit ether-a-go-go related gene expression: a gene that codes for formation of potassium channels (Langheinrich *et al.,* 2003). The Ca^{2+} in the sarcoplasmic reticulum is also depleted directly by tricyclic PAHs, impairing the contraction of calcium-dependent myofilaments. Ion imbalances interfere with cardiomyocyte excitation-contraction coupling, leading to cardiac arrhythmia,

reduced contractility and impaired heart looping (Incardona *et al*., 2004; Incardona *et al.,* 2009; Zhang *et al.,* 2013; Edmunds *et al.,* 2015; Incardona, 2017).

Although AhR-dependent and AhR-independent cardiotoxicity proceed via distinct modes of action, both lead to a similar phenotype. With respect to crude oil exposure, AhR activity does not exacerbate cardiotoxic effects, suggesting that AhR-independent cardiotoxicity is the dominant mechanism (Incardona *et al.,* 2005). Further, isolated myocardial cells can have no AhR activation, but the embryo can still exhibit cardiotoxicity following exposure to crude oil, whereas AhR activation is consistently present following exposure to high molecular weight PAHs (Incardona *et al.,* 2006; Jung *et al.,* 2013; Sørhus *et al.,* 2016). Lack of AhR-dependent cardiotoxicity after exposure to crude oil can be explained by the high concentrations of tricyclic PAHs and low concentrations of higher molecular weight PAHs in crude oil (NASEM, 2015). However, it cannot be ruled out that both mechanisms play a role in cardiotoxicity in ELS exposed to crude oils due to the complex mixture of PAHs present.

Juvenile fish also experience cardiotoxicity following exposure to crude oils. Sockeye salmon parr exposed to dilbit WAFs for two weeks experienced cardiac remodeling coupled with reduced swimming ability (Alderman *et al.,* 2017). Similarly, juvenile herring exposed to North Slope crude oil had lower critical swim speeds than controls and recovered slower from exhaustion (Carls *et al.,* 1999).

1.6.5 Behaviour

Exposure to PAHs causes adverse effects on fish behaviour that are pronounced in many species, at a variety of life-stages. Behavioural changes are important as they could affect fish migration, reproduction, feeding and predation (Cohen *et al.,* 2001). The sole

study that analyzed zebrafish behaviour after exposure to dilbit found that it did not impact total distance moved, but did reduce anxiety-like border seeking behaviour (thigmotaxis) at 7 dpf (Philibert *et al*., 2016). Exposure to some PAH mixtures can result in reduced activity in ELS of zebrafish (Vignet *et al.,* 2014a) and increased response to stimuli as adults (Geier *et al.,* 2018). Similarly, zebrafish fed food spiked with petrogenic fractions of crude oil had reduced exploratory behaviours two months post-hatch, and increased activity at six months post-hatch (Vignet *et al.,* 2014b). Behavioural alterations were also observed in the progeny of zebrafish adults fed a diet spiked with petrogenic fractions of crude oil (Vignet *et al.,* 2015). Zebrafish maternal and ELS exposure to crude oil WSF induced behavioural alterations, which were attributed to changes in gene expression for calcium channels and xenobiotic metabolism (Wang *et al.,* 2018). Finally, Caspian roaches (*Rutilus caspicus*) are able to avoid WSF of crude oil and when exposed to it, exhibited a change in feeding behaviour (Lari *et al.,* 2015). Although the underlying mechanisms of altered behaviour requires further attention, there is good evidence that this sensitive endpoint can be useful in detecting sublethal neurological and chemosensory abnormalities following exposure to crude oil (Vignet *et al.,* 2014a; Vignet *et al.,* 2014b; Philibert *et al.,* 2016).

1.7 PAH toxicity to invertebrates

Polycyclic aromatic hydrocarbons can impact survival and has negative developmental, biochemical and reproductive effects on invertebrates. Dibenzothiophene and phenanthrene are abundant in crude oils, and low concentrations of either have been shown to reduce mate-guarding behaviour—an important reproductive strategy—in freshwater amphipods (Satbhai *et al.,* 2017). Sediments spiked with PAH mixtures were

found to be acutely and chronically lethal to pelagic, epibenthic and benthic invertebrates and were reported to have synergistic effects compared to individual PAH exposure (Verrhiest *et al.,* 2001). However, other studies suggest that the assumption of additivity is sufficient in predicting the toxicity of PAH mixtures in invertebrates (Landrum *et al.,* 2003; Finch and Stubblefield, 2019). Parent PAHs and their metabolites can bioaccumulate in invertebrates (Obana *et al.,* 1983; Landrum *et al.,* 2003; Carrasco Navarro *et al.,* 2013). Bioaccumulation is important because metabolites of PAHs can trophically transfer, which could affect the health of predators of exposed invertebrates (Carrasco Navarro *et al.,* 2013).

To date, little is known about the effects of dilbit on invertebrates. Cladocerans (*Ceriodaphnia dubia*) exposed to Cold Lake Blend (CLB) dilbit WAFs showed 8-day LC50s of 6.43 g/L and >32 g/L for fresh and weathered dilbit respectively, while both fresh and weathered dilbit WAFs severely reduced reproduction (Robidoux *et al.,* 2018). A similar study using cladocerans reported that exposure to weathered CLB dilbit resulted in similar or less mortality than exposure to fresh dilbit (Barron *et al.,* 2018). Daphniid neonates (*Daphnia magna*) exposed to dilbit WAF experienced 27% mortality after 48 h and increased immobility (Robidoux *et al.,* 2018). Both studies of dilbit weathering support the hypothesis that weathering attenuates some of the mortality caused by exposure to dilbit.

There is more research that has investigated effects of conventional crude oils on invertebrates than effects of dilbit on invertebrates. Slowed development and interrupted molting were observed in copepods exposed to crude oil (*Tigriopus japonicas*) (Han *et al.,* 2014). Crude oil WAF was not acutely toxic to juvenile mud crabs (*Rhithropanopeus harrisii*) unless chemically dispersed (Anderson *et al.,* 2014). Similarly, fresh and

weathered oils from the Deep Water Horizon spill were not acutely lethal to echinoderm and bivalve larvae unless chemically dispersed (Stefansson *et al.,* 2016). The same Deep Water Horizon study found that weathered oil was less toxic than fresh oils, agreeing with studies on the toxicity of dilbit (Stefansson *et al.,* 2016). Crude oil exposure can shape the composition of invertebrate communities, and has been demonstrated with laboratory exposed nematodes (Monteiro *et al.,* 2019) and field-sampled invertebrate communities in the oil-sands region (Gerner *et al.,* 2017).

Significant biochemical changes have been observed in invertebrates exposed to PAHs, although there is evidence that they can recover after being transferred to clean water (Ruiz *et al.,* 2014). Similar to fish, exposure of the copepod, *Tigriopus japonicas,* to crude oil resulted in increased expression of three *CYP* genes that contained xenobiotic response elements (Han *et al.,* 2014). Notably, there was increased CAT, GSR and GST activity and increased cellular GSH, suggesting there was an increase in ROS (Han *et al.,* 2014). Although this mechanism is less understood in invertebrates, it is likely that *CYP* genes play a role in the detoxification of PAHs, as they do in fish. Crude oil exposure can also affect lipid metabolism and protein expression in prawns (*Macrobrachium borellii*) (Lavarías *et al.,* 2007; Pasquevich *et al.,* 2013). Expression of lipocalin-like crustacyanin—an exoskeleton pigment—was increased following exposure to crude oil, with up to an 8-fold increase in gene expression and up to a 1.9-fold increase in protein expression (Pasquevich *et al.,* 2013). Unexpectedly, the expression of the detoxification proteins GST and fatty aldehyde dehydrogenase were both down regulated (Pasquevich *et al.,* 2013). Individual PAHs can also negatively affect the biochemistry of invertebrates. Gauthier *et al.* (2016) found that phenanthrene can significantly reduce

acetylcholinesterase (AChE) activity in freshwater amphipods, leading to irregular behaviour and increased respiration as a result of uncontrolled appendage movement (Gauthier *et al.,* 2016). This phenotype was remarkably similar to AChE inhibition caused by organophosphates, but the exact mechanism requires further attention (Gauthier *et al.,* 2016). Explaining PAH toxicity becomes more complex when other contaminants—notably metals—are also present; because co-exposure of PAHs and metals can elicit more than additive toxicity in freshwater amphipods (Gauthier *et al.,* 2015).

1.8 Research rationale and objectives

Effects of conventional crude oils on aquatic organisms are well established, but the effects of dilbit on aquatic organisms are only beginning to be characterized. Lack of understanding of the effects of dilbit on aquatic organisms is of environmental concern due to the growing volume of dilbit being transported in close proximity to freshwater throughout North America, and the potential for spills into these systems.

Despite the rapid increase in the number of studies of toxicity of dilbit to aquatic organisms that has occurred in recent years, only two have incorporated weathering and none have incorporated sediment interactions (Madison *et al.,* 2015; Philibert *et al.,* 2016; Alderman *et al.,* 2017; Madison *et al.,* 2017; Alderman *et al.,* 2018; Alsaadi *et al.,* 2018a; Barron *et al.,* 2018; Robidoux *et al.,* 2018; McDonnell *et al.,* 2019). Because dilbit has unique physical and chemical properties compared to conventional crude oils, it is important to understand how physical processes, such as weathering and interaction with sediments, alter the toxicity of dilbit. Further, only two publications have quantified the

toxicity of dilbit to invertebrates, which are a vital component of freshwater ecosystems (Barron *et al.,* 2018; Robidoux *et al.,* 2018).

In response to the lack of knowledge surrounding dilbit toxicity, this thesis will explore the effects of weathered sediment-bound dilbit (WSD) on the health of freshwater amphipods (*Hyalella azteca*) and ELS of zebrafish (*Danio rerio*). Freshwater amphipods are abundant in freshwater ecosystems in North America and are indicators of aquatic health (Environment Canada, 2013b). Freshwater amphipods are model organisms used in toxicology and their responses to toxicants such as PAHs are well documented, therefore there is opportunity for comparison of this work to previous studies (Environment Canada, 2013b; Gauthier *et al.,* 2016). Although not native to North America, zebrafish are a model species used extensively in toxicology, and developmental, molecular and biochemical responses of ELS of zebrafish to a variety of contaminants, including PAHs, have been described in numerous publications (Bambino and Chu, 2017). Using zebrafish allows for an in-depth investigation of the underlying biochemical mechanisms of toxicity that can then be adapted to native species (Bambino and Chu, 2017).

The overall objective of this research is to better understand how spills of dilbit affect freshwater organisms. To this end, the research will address three specific objectives:

1. Determine if WSD affects adult freshwater amphipods behaviourally and biochemically.
2. Determine if WSD adversely affects zebrafish ELS behaviourally and biochemically.
3. Determine if physical contact with sunken oil-mineral aggregates alters toxicity of dilbit to zebrafish and freshwater amphipods.

CHAPTER 2: EFFECTS OF WEATHERED SEDIMENT-BOUND DILBIT ON FRESHWATER AMPHIPODS (*HYALELLA AZTECA*)

Abstract

The Alberta oil sands contain over 165 billion barrels of bitumen. Bitumen is transported as diluted bitumen (dilbit) after the addition of natural gas condensates. Dilbit is transported predominantly through pipelines, which come in close proximity to freshwater ecosystems. If dilbit is spilled into or near an aquatic environment, environmental weathering processes such as sediment interaction and evaporation influence the fate and toxicity of dilbit to aquatic organisms. To date, most studies of the effects of dilbit on the health of aquatic organisms have not considered weathering processes. Thus, the goal of this study was to assess the toxicity of weathered sediment-bound dilbit (WSD) to an aquatic organism. Adult freshwater amphipods (*Hyalella azteca*) were exposed to WSD directly or to the water-soluble fraction (WSF) of WSD. Direct exposure to WSD resulted in oil-mineral aggregates adhering to the appendages and gas exchange structures of amphipods, causing acute lethality. After a 10 min exposure, amphipods consumed half as much oxygen and their appendage movement was impaired. Exposure to the WSF did not cause acute lethality but did cause small increases in respiration and acetylcholinesterase (AChE) activity after 96 h exposure. Results of the present study indicate that physical interaction with WSD after a spill of dilbit is a threat to benthic invertebrates. As the production of dilbit in North America increases, it is imperative that studies incorporate environmental weathering processes when determining effects of dilbit on aquatic organisms.

2.1 Introduction

The Alberta oil sands contain an estimated 165 billion barrels of bitumen, which is surpassed only by Saudi Arabia and Venezuela (National Energy Board, 2006). Bitumen is an 'unconventional' oil with a high viscosity and density, making transport through pipelines (the safest and most efficient mode of transport) impractical (Lee *et al.,* 2015). To overcome this, bitumen is diluted with natural gas condensates (20-30% condensate; 70-80% bitumen) after it is separated from the sand to form 'dilbit', making it suitable for transport through pipelines (Crosby *et al*., 2013). Although mining and upgrading of bitumen is more energetically costly than conventional oil, mining of dilbit is expected to increase from the current 2.69 million barrels/day to 4.19 million barrels/day by 2035 (Huot and Grant, 2012; CAPP, 2018). New pipelines such as the Keystone XL and the Kinder Morgan Transmountain expansion will increase the volume of dilbit transported in pipelines. However, sections of these pipelines will run in close proximity to thousands of freshwater environments, including critical salmon habitat in British Columbia (Levy, 2009; NASEM, 2015). Despite the threat of spills of dilbit into freshwater ecosystems, little is known about the effects of dilbit on aquatic organisms compared to conventional oils, particularly invertebrates (Dew *et al*., 2015; Alsaadi *et al*., 2018a).

The toxicity of dilbit has been assessed in aquatic organisms via exposure to the water-accommodated fraction (WAF) of dilbit. Dilbit WAFs contain most notably polycyclic aromatic hydrocarbons (PAHs), their alkylated relatives and the monoaromatics benzene, toluene, ethylbenzene and xylene (BTEX). Cladocerans (*Ceriodaphnia dubia*) exposed to fresh Cold Lake Blend (CLB) dilbit WAFs had an 8-day LC50 of 6.43 g/L, and exhibited severely impaired reproduction (Robidoux *et al.,* 2018). A similar study using cladocerans reported a 48 h LC50 of 70.7% for fresh dilbit

WAFs with oil loading of 25 g/L (Barron *et al.,* 2018). Daphniid neonates (*Daphnia magna*) exposed to dilbit WAF experienced 27% mortality after 48 h and increased immobility (Robidoux *et al.,* 2018).

Diluents used to enhance the flow of dilbit in pipelines cause dilbit to behave differently than conventional oils after a spill (Environment Canada, 2013a; Lee *et al.,* 2015; NASEM, 2015). Once exposed to the atmosphere, low-molecular weight components will evaporate rapidly (Hua *et al.,* 2018), meaning weathering processes have the potential to influence the behavior of dilbit in aquatic systems and to influence toxicity to aquatic organisms (Dew *et al.,* 2015). Effects of weathering on toxicity of dilbit have not been extensively characterized, but studies suggest that weathered dilbit is less acutely and chronically toxic to aquatic invertebrates, compared to unweathered dilbit (Barron *et al*., 2018; Robidoux *et al.,* 2018). Attenuation of toxicity as a result of weathering has been attributed to the loss of highly soluble, low molecular weight BTEX, which can be a more accurate predictor of toxicity than total PAHs in zebrafish (*Danio rerio*) exposed to dilbit (Philibert *et al.,* 2016). However, weathering can also increase the potency of dilbit by increasing the proportion of tricyclic PAHs and alkyl-PAHs, which are responsible for much of the cardiac impairment in fish following exposure to dilbit (Carls *et al.,* 1999; Jung *et al.,* 2013).

Spilled dilbit can combine with fine sediments either on land or suspended in the water-column and form oil-mineral aggregates (OMAs) that are dense enough to sink in freshwater (Environment Canada, 2013a; Waterman and Garcia, 2015; Hua *et al*., 2018). The Kalamazoo River dilbit spill in 2010 exemplified the importance of dilbit-sediment interactions, as an estimated 21 ± 12% of the spilled dilbit remained sunken and bound to sediment as of 2013 (USEPA, 2013a; US Fish and Wildlife, 2015). Oil-sediment

interactions have been proposed as a beneficial dispersion process following spills into aquatic environments (Fitzpatrick *et al.,* 2015), but sunken dilbit has the potential to negatively affect benthic and pelagic fish and invertebrates (Dew *et al*., 2015). For example, contaminated sediments collected two years after the Kalamazoo River dilbit spill were acutely toxic to freshwater amphipods (*Hyalella azteca*) and freshwater midges (*Chironomus dilutus*) (GLEC, 2012). However, because of an absence of controlled laboratory studies, little is known about effects of sediment bound dilbit on aquatic organisms. It is known that PAHs can significantly reduce acetylcholinesterase (AChE) activity in freshwater amphipods, leading to irregular behaviour and increased respiration as a result of uncontrolled appendage movement (Gauthier *et al.,* 2016). Therefore, the toxicity of sediment-bound dilbit could be twofold; it could physically impair benthic invertebrates by binding to gas exchange structures or appendages and it could be a constant source of hydrocarbons released into the water.

The purpose of this study is to differentiate the physical and chemical effects of weathered sediment-bound dilbit (WSD) on the health of the freshwater amphipod, *Hyalella azteca*. Freshwater amphipods were exposed to WSD either directly as a substrate or to the water-soluble fraction (WSF) of WSD. This is the first laboratory study to assess the toxicity of WSD to an invertebrate and is an important step in determining how benthic invertebrates would be affected by sinking dilbit after a spill.

2.2 Methods

2.2.1 Preparing weathered sediment-bound dilbit

Dilbit was weathered according to methods described by Fieldhouse *et al.* (2010) and bound to sediment following methods adapted from Environment Canada (2013a)

and Waterman and Garcia (2015). The preparation of WSD is outlined in a flowchart in Appendix 1 (Fig. A5). In short, 200 mL of fresh Cold Lake Blend (CLB) dilbit was weathered on a Buchi-121 rotary evaporator at 135 rpm in an 80ºC water bath for 10 minutes to produce a 10% mass loss. At the top of the condenser an 8 mm airline was inserted, attached to a 9 mm plastic pipette that extended to the top of the evaporation flask. The tubing and pipette were attached to a vacuum pump giving positive airflow at a rate of 13 L/min. Next, 30 mL aliquots of the weathered dilbit were dispensed into 1 L glass bottles with 600 mL of five-salt reconstituted water (SAM-5S) water (Borgmann, 1996) and 12 g of kaolin (Sigma, Oakville, ON) and allowed to thermally equilibrate for 4 h in the dark. Sediment loading was double what has previously been used by Environment Canada (2013a) because the objective of this study was to create the maximum amount of sediment-bound dilbit per volume of dilbit used. Preliminary tests showed that doubling the sediment load did not impair the formation of OMAs (Appendix 1). Bottles were placed horizontally in a culture table shaker and mixed for 16 h in the dark at 160 rpm and 23.0ºC. Next, the entire contents of each bottle were transferred to one 3 L beaker, the beaker was covered with tin foil, and the contents were allowed to settle for 24 h at 23.0°C in the dark. After the OMAs had settled, the overlaying water was slowly siphoned and the WSD was collected in amber bottles fitted with Teflon™ caps.

2.2.2 Amphipod culture

Amphipod cultures were maintained in 50 L tanks with 30 L of SAM-5S water at 23.0°C on a 16 h light:8 h dark schedule. Amphipods were fed 0.1 - 0.12 g of ground

Tetramin™ three times per week. Cotton gauze bandages were placed at the bottom of the tanks to serve as a substrate. One 50% water change and one 80% water change was performed weekly. Two 122 cm fluorescent bulbs, emitting 2000 lux at the water surface, provided lighting.

Adult amphipods were sorted with a 700 μm nylon mesh and used for all toxicity tests. Adults were used to ensure video tracking and behavioural analysis would be possible. Amphipods were kept in 1 L beakers with 800 mL of SAM-5S water (100 amphipods/beaker), gauze bandage and 0.05 g of ground Tetramin™ for 48 h prior to exposure. Amphipods were selected two at a time and randomly assigned to a treatment. All exposures were conducted at 23.0°C with a light intensity of 2000 lux. For 96 h exposures, daily temperature and dissolved oxygen were monitored, while alkalinity, hardness, pH and ammonia were recorded at 0 and 96 h only.

2.2.3 Effect of weathered sediment-bound dilbit on acute lethality

Adult amphipods were exposed for 96 h to WSD as a substrate with methods adapted from Environment Canada (2013b). Exposures were performed in 300 mL tall form beakers, with 200 mL of SAM-5S water and 30 mL of sediment. Treatments were made by serially diluting WSD with uncontaminated kaolin. Treatments were 100%, 50%, 25%, 12.5%, 6.25%, 3.13%, 1.56%, 0.78% and 0% WSD by volume with three replicates for each treatment. Prepared sediments were added to the exposure beakers and allowed to settle for 24 h before adding 10 amphipods to each vessel. Beakers were covered but not sealed to facilitate gas exchange. At 96 h, survival was assessed.

2.2.4 Physical interaction with weathered sediment-bound dilbit

To assess how amphipods were affected immediately after physically interacting with WSD, they were exposed directly to WSD for 10 minutes. In a glass petri dish, 50 mL of SAM-5S water and 1 mL of WSD was added, gently mixed, and WSD particles were allowed to settle for 1 h. For a control, 50 mL of SAM-5S water and 1 mL of kaolin was used. Next, 10 amphipods were added to each petri dish and exposed for 10 minutes. This amount of time was determined in preliminary tests to ensure amphipods sufficiently interacted with the sediment but a short enough duration that did not cause lethality. After 10 minutes, pleopod beating frequency, behaviour and respiration were assessed. All exposures were replicated eight times.

2.2.5 Pleopod beating frequency

Two amphipods per replicate were randomly assessed for pleopod beating frequency assessment following methods from Gauthier *et al.* (2016). Pleopod movement was measured because pleopods direct water over the gills primarily for respiration. Amphipods were placed in a welled slide with a drop of SAM-5S water void of particles to facilitate video recording and a coverslip was gently placed on top of the amphipod to restrict body movement but allow pleopod movement. Video was recorded for 1 min, then a computer-generated random 10 s segment was slowed down and pleopod beats were counted and expressed in beats per minute (bpm).

2.2.6 Exposure to the water-soluble fraction of weathered sediment-bound dilbit

Methods for formulating the water-soluble fraction of WSD using the slow stir method were adapted from previous studies and is outlined in Appendix 1 (Singer *et al.,*

2000; Philibert *et al.,* 2016) (Fig. A5). In a sealed 2 L Erlenmeyer flask, 270 mL of WSD and 1530 mL of SAM-5S freshwater (15% WSD) was mixed at 23.0°C in the dark, leaving 20% headspace in the bottle. The contents were mixed for 20 h with a 45 mm stir bar spinning at approximately 200 rpm such that no vortex was created. After mixing, the contents were allowed to settle for 4 h after which the overlying water was filtered through a 0.45 μm filter to ensure that no WSD particles or emulsified oils remained in the solution. The 100% WSF was diluted with SAM-5S water such that treatments were 100%, 50% and 25%.

Adult amphipods were exposed to the WSF for 96 h, with 10 amphipods in each beaker and four replicates per treatment. Beakers were covered but not sealed to facilitate gas exchange. Water quality was monitored once daily, and no water changes were implemented. At 96 h, respiration, behaviour, AChE activity and oxidative stress were assessed.

2.2.7 Behaviour

Behaviour trials were conducted immediately after the respective exposure period following methods outlined by Gauthier *et al.* (2016). Three amphipods per replicate and 50 mL of treatment water was transferred to glass petri dishes. The amphipods were allowed to acclimate for 1 min and then recorded for 3 min. Activity was measured manually and expressed as percent of the time spent swimming.

2.2.8 Respiration

Respiration trials were conducted in glass scintillation vials, measured with a Presens Fibox 3 (Regensburg, Germany) fiber optic oxygen transmitter. Vials were fitted with rubber stoppers and fiber optic oxygen spot sensors to allow for oxygen measurements to be made while the vials were sealed. Vials were filled with 7.55 mL of aerated, uncontaminated SAM-5S culture water and seven amphipods per replicate. Vials were sealed and the sensor spots were given 5 min to equilibrate. Initial oxygen concentrations (mg/L) were recorded immediately and after 1 h. Oxygen consumption was normalized to dry weight for direct exposure (2.2.3) and normalized to wet weight for exposures to the WSF (2.2.6). Wet weight was used for section 2.2.6 to ensure the animals could be used for AChE activity analysis, as drying the organisms would denature the enzyme. Oxygen consumption was recorded as $mgO_2/h/g$ for both experiments.

2.2.9 Acetlycholinesterase activity

Methods for assessing AChE activity in *Hyalella azteca* were followed as described by Ellman *et al.* (1961) and Bartlett *et al.* (2016). Tris buffer (0.05 M) was used to mix solutions for the AChE assay. Homogenizing buffer was created by mixing Tris buffer at pH 8 with 1% v/v Triton X-100. Ellman's reagent (5,5'-dithiobis[2-nitrobenzoic acid]) (0.25 mM) was mixed with Tris buffer at pH 7.4. Acetylthiocholine iodide solutions (0.156 M) were prepared in distilled water. Electric eel cholinesterase (0.2 units/mL) was prepared in pH 8 homogenizing buffer. Whole amphipods were homogenized in 500 μL homogenizing buffer and centrifuged at 10,000 g for 10 min at

4ºC. Next, 40 µL of either homogenizing buffer (blank), electric eel cholinesterase (enzyme standard) or supernatant from homogenized samples were added to a 96 well plate with 250 µL Ellman's reagent and 10 µL of acetylthiocholine iodide. Absorbance was measured at 405 nm in two-minute intervals for 30 min using a spectrophotometer at room temperature. AChE activity was calculated using equation 1 (Fairbrother *et al.,* 1991):

$$\text{Specific activity} = \frac{(\text{A x VolR x } 1000)}{(\text{E x PL x VolH x PR})} \quad (1)$$

where specific activity was in mmol/min/g protein, A was the change in absorbance per minute (slope of the linear portion of the curve), Vol_R was the reaction volume (300 µL), 1000 was a unit conversion factor (g to mg), E was the extinction coefficient for Ellman's reagent ($1.36\ 10^4\ M^{-1}\ cm^{-1}$), PL was the pathlength (1 cm), Vol_H was the homogenate volume (500 µL), and PR was the concentration of protein in the homogenate (mg/mL). AChE activity was expressed as percent of the control.

2.2.10 Oxidative stress

Lipid peroxidation as an indicator of oxidative stress was quantified by measuring the concentration of thiobarbituric acid reactive substances (TBARS) in the adult amphipods (R&D systems, USA). Briefly, 10 amphipods were homogenized in 500 µL of tris buffer (0.05 M, pH 7.4) with 1% Triton X-100 (v/v). The homogenate was centrifuged at 10,000 g for 10 min at room temperature and the supernatant was collected. The supernatants were acidified with 0.6 N trichloroacetic acid at a 1:1 ratio, incubated at

room temperature for 15 min and centrifuged at 12,000 g for 4 min at room temperature to remove interfering proteins. A TBARS standard solution was made by combining 100 μL of 500 μM 1,1,3,3,-tetramethoxypropane with 200 μL of 0.6 N trichloroacetic acid and serially diluting to make final TBARS concentrations of 0.26 μM, 0.52 μM, 1.04 μM, 2.09 μM, 4.18 μM, 8.35 μM and 16.7 μM to be used as a standard curve. Next, 150 μL of the acidified samples or TBARS standard was combined with 75 μL of thiobarbituric acid in a 96-well plate. An initial spectrophotometer reading was taken at 532 nm, the plates were then incubated for 2 h at 50°C and then read again at 532 nm. The difference between the first and second reading was calculated and the concentrations of TBARS were then interpolated from the standard curve and multiplied by two to account for dilution during the acidification step, and normalized to the amount of protein in the assay.

2.2.11 Bicinchoninic acid protein assay

Concentrations of proteins were determined by use of the bicinchoninic acid (BCA) assay, with bovine serum albumin as the standard, according to the manufacturer's protocol. To form a BCA working reagent, bicinchoninic acid and 4% w/v copper(II) sulfate pentahydrate were mixed at a volume ratio of 50:1 respectively. Protein standards of bovine serum albumin were prepared at concentrations of 0 μg/mL, 100 μg/mL, 250 μg/mL, 500 μg/mL, and 1000 μg/mL in tris buffer. In a 96-well plate, 20 μL of either tris buffer, bovine albumin protein standards or homogenate supernatant were added with 200 μL of the BCA working reagent. The plate was incubated for 30 min at 28°C and read at

562 nm with a spectrophotometer. Protein concentrations in each sample were calculated by interpolating from the BCA standard curve.

2.2.12 Statistical analysis

Data were tested for normality using the Shapiro-Wilk test and homogeneity of variances among treatments was determined using a Bartlett test. All data met these assumptions and therefore parametric tests were used. Two-sample t-test was used for experiments with two treatments and one-way ANOVA was used for experiments with >2 treatments. All statistics were performed using R 3.6.0 (R Core Team, 2019) base package. Alpha level of 0.05 was used to determine significance for all tests.

2.3 Results

2.3.1 Weathered sediment-bound dilbit as a substrate

In all concentrations of WSD as a substrate, there was 100% mortality after 96 h exposure. There was 0% mortality in the kaolin controls. Amphipods exposed to WSD were visibly coated in dilbit, particularly on the ventral side (Fig. 2-1).

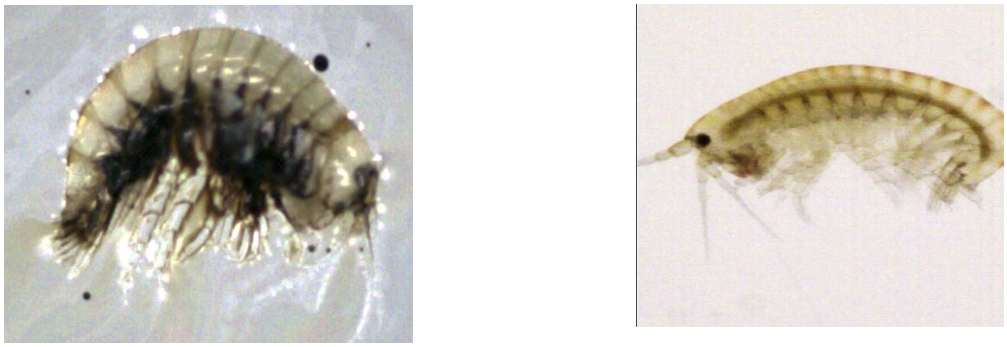

Figure 2-1 Amphipods after 96 h exposure to weathered sediment-bound dilbit (WSD) as a substrate (left) compared to controls exposed to uncontaminated kaolin (right).

2.3.2 Sub-lethal effects of physical interaction with weathered sediment-bound dilbit

Compared to controls, amphipods exposed directly to WSD for 10 min had significantly lower oxygen consumption, pleopod beating frequency and activity (Fig. 2-2). Exposed amphipods consumed 47% less oxygen compared to controls (Fig. 2-2a). The average pleopod beating frequency was reduced by 78% in exposed amphipods compared to controls (Fig. 2-2b). Activity was most severely affected by direct exposure to WSD. Direct exposure reduced average time spent swimming from 63% for controls to 0% for exposed amphipods (Fig. 2-2c)

2.3.3 Effects of exposure to the water-soluble fraction of weathered sediment-bound dilbit

There was no significant mortality at any concentrations of WSF after 96 h. Further, there were no statistically significant changes in any of the sublethal effects measured (Fig. 2-3). However, there were trends of increased respiration and increased AChE activity with increasing concentration of WSF. Respiration increased by 46% in amphipods exposed to WSF100 compared to controls. Amphipods exposed to WSF25, WSF50 and WSF100 had AChE activities that increased to 139%, 141%, and 146% of the control respectively (Fig. 2-3c). There was no change in the average time spent swimming or amount of lipid peroxidation (data not shown) in amphipods exposed to any concentration of WSF compared to the control (Fig. 2-3b).

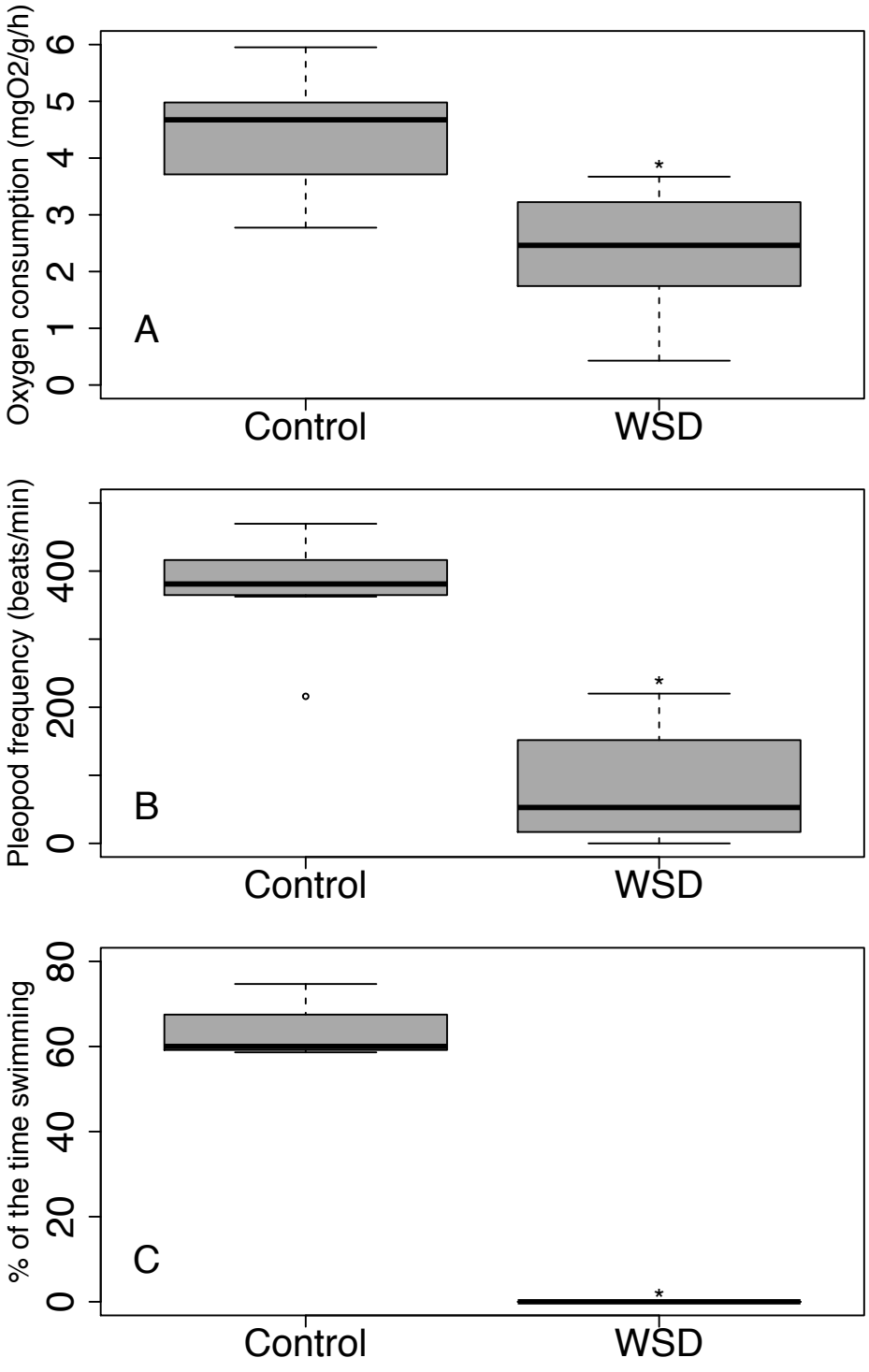

Figure 2-2 Oxygen consumption (per g dry weight) (a) pleopod beating frequency (b) and behaviour (c) of amphipods following 10 min physical interaction with weathered sediment-bound dilbit compared to controls. Replication for pleopod beating frequency and oxygen consumption were n=8 and replication for behaviour was n=4. Significant differences were determined by two sample t-test and are indicated with an asterisk at α=0.05.

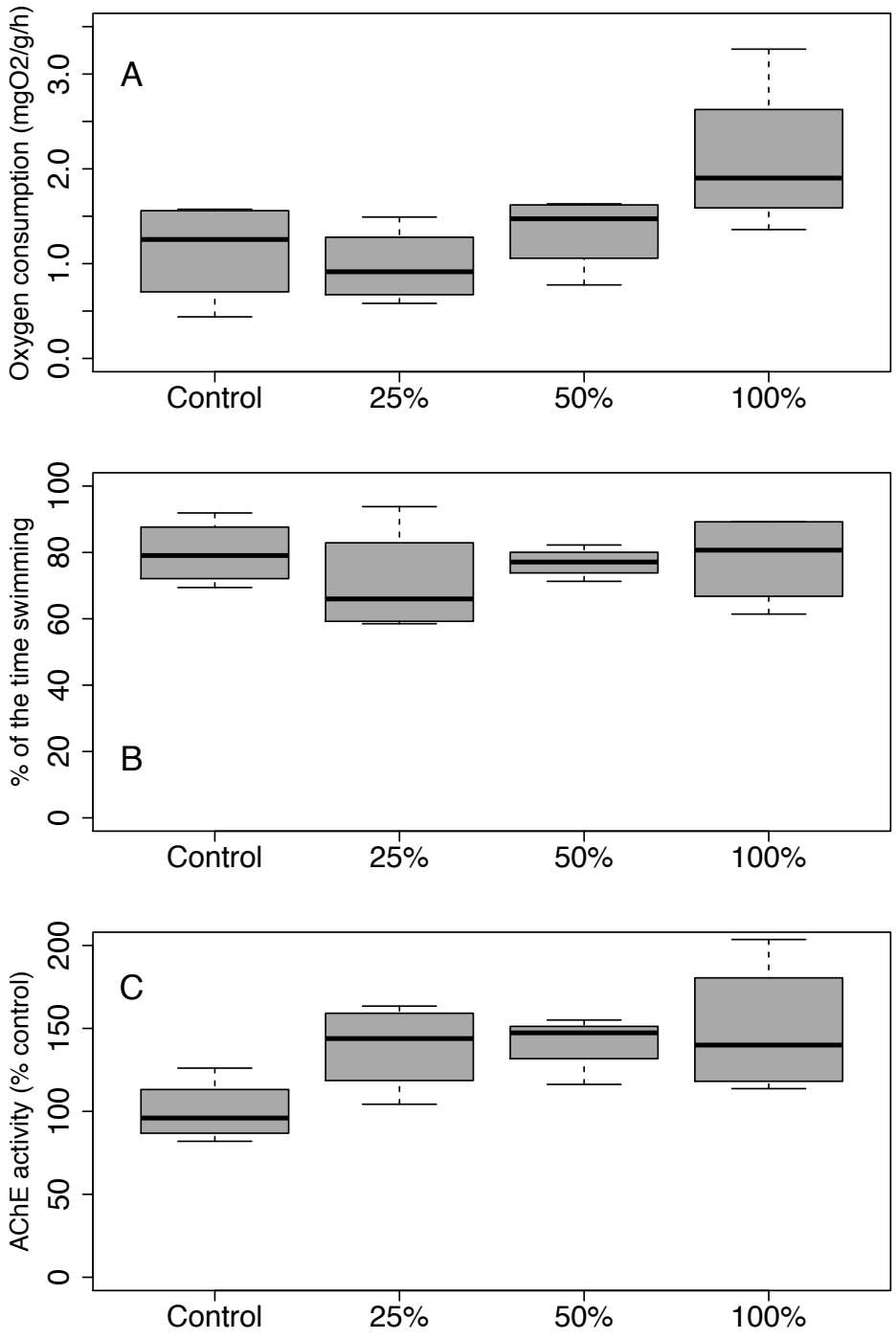

Figure 2-3 Oxygen consumption (per g wet weight) (a) behaviour (b) and acetylcholinesterase (AChE) activity (c) of amphipods following 96 h exposure to the water-soluble fraction of weathered sediment-bound dilbit compared to controls. Controls were SAM-5S culture water and treatments were dilutions of 100% WSF. Replication for all endpoints was n=4.

2.4 Discussion

2.4.1 Physical interaction with weathered sediment-bound dilbit

In the present study, OMAs impacted appendage movement and respiration after 10 min exposure. Inhibition of appendage movement might have been caused by particles making appendages too heavy to move, or causing appendages to stick to the rest of the body. Weathering of the dilbit could have exacerbated this, as it made the dilbit more adhesive and viscous. Respiration might have been impaired for two reasons; particles adhered directly onto the gills blocking gas exchange or because the pleopods were not able to move fresh oxygenated water over the gills. The decreased respiration could have been overestimated because the amphipods were coated in dilbit, thus overestimating their dry weight, but this could not be accounted for because measuring wet weight prior to exposure could have caused stress to the amphipods. Taken together, these data suggest that acute lethality of amphipods exposed to OMA of weathered dilbit might have been due to suffocation.

Mechanical dispersion into droplets in the absence of sediment could exacerbate the toxicity of dilbit to some fish and invertebrates. Dispersed droplets promote solubilization of hydrocarbons that replenish lost constituents during exposure, but are not known to exacerbate toxic effects as a result of physical contact with aquatic organisms (Nordtug *et al*., 2011; Hansen *et al*., 2012; Redman *et al.,* 2014). However, in the present study, physical impairment was evident when sediment and dilbit were combined with mechanical dispersion (mixing). Dilbit can form irregularly shaped aggregates with sediments (compared to round droplets without sediment), increasing the surface area and possibly promoting more physical interaction with organisms, particularly if the dilbit is weathered (Lee *et al.,* 2012; Waterman and Garcia, 2015). Further, OMAs will sink in

freshwater and therefore have a high potential to interact with benthic and pelagic aquatic organisms (Fitzpatrick *et al.,* 2015; Hua *et al.,* 2018). A recent study found that in turbulent rivers with high concentrations of suspended fine sediment, 80% of a dilbit spill could sink in two hours (Perez *et al.,* 2016). Results of the current study suggest dilbit OMAs could cause severe reductions in the benthic invertebrate population.

2.4.2 Exposure to the water-soluble fraction of weathered sediment-bound dilbit

Combining weathered dilbit with sediment in freshwater creates a complex mosaic of sinks and sources of water-soluble hydrocarbons. Weathering removes BTEX and some dicyclic PAHs (which are the most water-soluble), but much of the tricyclic and larger PAHs and alkyl-PAHs will remain (Yang *et al.,* 2018). Further, mechanical dispersion promotes the formation of smaller dilbit droplets, facilitating release of hydrocarbons into the water (Nordtug *et al*., 2011; Hansen *et al*., 2012; Redman *et al.,* 2014). Finally, dilbit binding to sediment will significantly reduce the abundance of water-soluble PAHs (Yang *et al.,* 2018), but it is still possible that OMAs might release bound PAHs at a later time. In the context of a river, all of these processes occur simultaneously, meaning there could be areas that differ in the abundance of water-soluble hydrocarbons.

The WSF tested did not cause lethality of adult amphipods during a 96 h exposure. In a previous study, cladocerans (*Ceriodaphnia dubia*) and daphniids (*Daphnia magna*) were exposed to WAFs of CLB dilbit (Robidoux *et al.,* 2018). They found that fresh dilbit WAF killed 27% of daphniid neonates after 48 h with a dilbit loading of 32 g/L (Robidoux *et al.,* 2018). The authors also reported LC50s for cladocerans after 8-day exposure of 6.43 g/L for fresh dilbit and >32 g/L for weathered dilbit (Robidoux *et al.,*

2018). A similar study using cladocerans reported that exposure to weathered dilbit resulted in similar or less mortality than exposure to fresh dilbit (Barron *et al.,* 2018). Comparing studies using varying methods of WAF preparation, dilbit loading and weathering states is difficult, but given the results of the present study, it appears adult *Hyalella azteca* and *Ceriodaphnia dubia* neonates could have similar sensitivity to weathered dilbit. Oil loadings in this study were greater than in previous studies (Robidoux *et al*., 2018, Barron *et al*., 2018), but this might not translate to greater concentrations of hydrocarbons due to solubility constraints, particularly for weathered dilbit, because the lighter constituents that evaporate are generally the most soluble.

The WSF did not cause AChE inhibition and hyperstimulation of appendages in adult amphipods. Previous work has shown these effects at 195 μg/L of phenanthrene (Gauthier *et al.,* 2016), whereas the WAF of weathered sediment-bound dilbit in a separate study prepared with similar methods as the present study contained <2 μg/L phenanthrene (Yang *et al.,* 2018). It is possible that there was simply not a high enough concentration of phenanthrene in the WSF to inhibit AChE and the other PAHs present may not inhibit AChE. The statistically insignificant increases in respiration and AChE activity that were observed in the present study may still affect amphipods and other invertebrates over time. Higher metabolic demands could require the affected organism to forage more often, reducing the time the organism allocates to other essential tasks such as reproduction.

Increased AChE activity has been reported in *Hyalella azteca, Claassenia* spp., and *Procambarus clarkii* after exposure to AChE inhibitors such as organophosphate pesticides (Repetto *et al.,* 1988; Day and Scott, 1990). In all cases, increased AChE

activity was observed at the beginning of the exposure, followed by an eventual decrease in activity, or the observed decrease was only at low concentrations of organophosphate. Because the present study ended at 96 h, it cannot be determined if chronic exposure would eventually result in inhibition of AChE activity following exposure to dilbit. There are two plausible explanations for the observed increase in AChE activity following exposure to dilbit based on available literature: (1) AChE is upregulated by increased concentrations of intracellular cyclic adenosine monophosphate (cAMP) (2) AChE is upregulated in cells undergoing apoptosis. Elevated concentrations of cAMP have been observed in mussels (*Mytilus galloprovincialis*) collected near wastewater effluent (Dailianis *et al.,* 2003). Increased cAMP can be a general response to multiple environmental contaminants and could result from a change in cAMP phosphodiesterase or adenylyl cyclase activity (Pareschi *et al.,* 1997). Inducers of cAMP are linked to upregulation of AChE in mouse neuroblastoma cells and therefore increased cAMP could be an explanation for the increased AChE activity observed in the present study (Curtin *et al.,* 2006). Activity of AChE is upregulated in human and mouse cell lines undergoing apoptosis, and could be a regulator for apoptosis (Zhang *et al.,* 2002). If exposure to the WSF induced apoptosis in certain cell types in amphipods, it could have altered the measured whole-body AChE activity.

In a freshwater environment, the constant release of PAHs from WSD over time could affect the health of benthic invertebrates. The Athabasca River contains natural bitumen seeps that are a source of PAHs that shape the invertebrate community towards species with shorter generation times and lower sensitivities to toxicants (Gerner *et al.,* 2017). Phenanthrene inhibits the reproduction of *Hyalella azteca* (Satbhai *et al.,* 2017),

and weathered and fresh dilbit both inhibit *Ceriodaphnia dubia* reproduction (Robidoux *et al.,* 2018).

2.5 Conclusion

There was a difference between effects of exposure to the WSF and exposure to WSD directly as a substrate on *Hyalela azteca*. WSD bound to the body of amphipods immediately after initiation of the exposure, inhibiting movement and respiration, resulting in acute lethality. Exposure to the WSF for 96 h did not result in lethality and did not significantly change AChE activity, respiration or behaviour. These results suggest that physical interaction with WSD likely is the primary threat to benthic invertebrates immediately after a spill of dilbit. Over time, the constant release of water-soluble hydrocarbons, including PAHs, from WSD could affect the health of the benthic invertebrate community. Further research on the toxicity of dilbit WSF to amphipods should evaluate chronic effects on sublethal endpoints such as reproduction, growth and behaviour. Amphipods are a keystone species in freshwater ecosystems near dilbit pipelines, therefore understanding their response to dilbit is important for understating the ecological costs of spills. It is imperative that future studies of the toxicity of dilbit to aquatic organisms incorporate weathering processes into the experimental methods to generate a more complete understanding of the effects of dilbit spills in freshwater ecosystems.

CHAPTER 3: TOXICITY OF WEATHERED SEDIMENT-BOUND DILBIT TO THE EARLY-LIFE STAGES ZEBRAFISH (*DANIO RERIO*)

Abstract

Due to the high viscosity of bitumen extracted from the Alberta oil sands, it must be diluted with natural gas condensates to form diluted bitumen (dilbit) to facilitate transport through pipelines. Pipelines stemming from the oil sands span thousands of freshwater environments and pose a risk to aquatic organisms. If dilbit is spilled into or near a waterbody, environmental weathering processes such as evaporation, and interaction with sediments can alter the fate of dilbit and its toxicity to aquatic organisms. To date, most studies of dilbit have focused on effects of the water-accommodated fraction of fresh dilbit to aquatic organisms, primarily fish. Here, we present the first study that assesses the toxicity of weathered sediment-bound dilbit (WSD) to a fish species. Zebrafish (*Danio rerio*) embryos were exposed to the water-soluble fraction (WSF) or water-accommodated fraction (WAF) of weathered sediment-bound dilbit from 30 min post-fertilization to five days post-fertilization. Exposed embryos showed increases of pericardial edema, yolk sac edema and incidences of uninflated swim bladder. The presence of oil-mineral aggregates in the WAF severely increased the toxicity of dilbit across all endpoints. There was no change in the abundance of transcripts related to the response to oxidative stress (*sod, gpx, gst, gclc)* or swim bladder formation (*lef1, axin2, pbx1a, pbx1b*), but there was induction of *cyp1a*, suggesting the embryos were exposed to polycyclic aromatic hydrocarbons that may have been responsible for the deformities. These results show that despite weathering and

combination with sediment, water-soluble compounds remain bioavailable and negatively affect the early development of zebrafish.

3.1 Introduction

The Alberta oil sands are among the largest deposits of oil on earth, containing an estimated 165 billion barrels of bitumen: an 'unconventional' heavy oil (Lee *et al.,* 2015; NASEM, 2015; CAPP, 2018). Bitumen is too viscous to flow through pipelines and must be diluted with natural gas condensates to create diluted bitumen or 'dilbit' (Crosby *et al*., 2013). Despite the projected increase in oil sands development and dilbit production (Huot and Grant, 2012; CAPP, 2018), the toxicity of dilbit is largely undocumented compared to that of conventional oils. (Dew *et al*., 2015; Alsaadi *et al*., 2018a).

Effects of the water-accommodated fraction (WAF) of dilbit have been explored in fish and invertebrates (Madison *et al.,* 2015; Alderman *et al.,* 2017; Madison *et al.,* 2017; Alderman *et al.,* 2018; Alsaadi *et al.,* 2018; Barron *et al.,* 2018; Robidoux *et al.,* 2018; McDonnell *et al.,* 2019). The WAF of multiple blends of dilbit is acutely toxic to fish and invertebrates (Barron *et al.,* 2018; Robidoux *et al.,* 2018) and can cause a variety of cardiotoxic sublethal effects in juvenile and adult fish. When exposed to dilbit, embryos of a variety of species of fishes consistently show a high prevalence of blue sac disease (BSD) that is coupled with a significant increase in cytochrome P450 1A (*cyp1a)* mRNA abundance and in some studies, an increase in the mRNA abundance for genes related to oxidative stress, such as glutathione-S-transferase (*gst*) (Madison *et* al., 2015; Madison *et al*., 2017; Alderman *et al.,* 2018; Alsaadi *et al*., 2018b; McDonnell *et al*., 2019). Therefore, adverse effects of exposure to dilbit during development have been attributed to polycyclic aromatic hydrocarbons (PAHs) (Madison *et al.,* 2015; Madison *et*

al., 2017; Alderman *et al.,* 2018; Alsaadi *et al*., 2018b; McDonnell *et al*., 2019). Because dilbit contains a complex mixture of PAHs and alkyl-PAHs, there could also be interactions that exacerbate toxicity - such as non-dioxin like PAHs being oxygenated due to CYP1A enzyme activity induced by dioxin-like PAHs - but currently this is poorly understood (Hodson, 2017).

Dilbit has the potential to behave differently than conventional oils after a spill due to rapid evaporation of the added diluents and subsequent interaction with sediments (Environment Canada, 2013a; Lee *et al.,* 2015; NASEM 2015). Evaporation can remove some chemicals that are toxic to aquatic organisms and can increase the density of the remaining weathered dilbit to the point that it approaches the density of freshwater (SL Ross, 2012; King *et al.,* 2014; Hua *et al.,* 2018; Yang *et al.,* 2018). Studies that have compared the effects of fresh and weathered dilbit to fathead minnow (*Pimephales promelas*), rainbow trout (*Oncorhynchus mykiss*), daphniids (*Daphnia magna*), cladocerans *(Ceriodaphnia dubia*), mysids (*Americamysis bahia*) and inland silver side (*Menidia beryllina*) found that exposure to fresh dilbit results in greater mortality than weathered dilbit, but both weathered and fresh dilbit inhibited reproduction in cladocerans and increased immobility in daphniids (Barron *et al.,* 2018; Robidoux *et al.,* 2018). Dilbit can combine with fine sediment in the water column, forming oil-mineral aggregates (OMAs) that sink in freshwater and saltwater (Lee *et al*., 2012; Environment Canada, 2013a; Hua *et al*., 2018). Aggregation of dilbit with sediment can reduce the bioavailability of compounds that could adversely affect the health of aquatic organisms (Yang *et al.,* 2018). However, OMAs that are deposited on the benthos could negatively affect benthic fish and invertebrates (Environment Canada, 2013a; Dew *et al*., 2015). Samples of contaminated sediment-bound dilbit from the Kalamazoo River dilbit spill

that were collected in 2012, two years after the spill, were acutely toxic to freshwater amphipods (*Hyalella azteca*) and freshwater midges (*Chironomus dilutus*) (GLEC, 2012). However, because sediment-bound dilbit has not yet been formulated and used for toxicity tests in the laboratory, little is known about effects on aquatic organisms.

The objective of this study was to characterize the effects of weathered sediment-bound dilbit (WSD) on the health of zebrafish embryos (*Danio rerio*). Zebrafish embryos were exposed to the water-soluble fraction (WSF) or the water-accommodated fraction (WAF) of WSD from within 0.5 hours post-fertilization (hpf) to 120 hpf, and lethality, occurrences of malformations, and molecular indicators of toxicity were quantified. This was the first study to assess the effects of sediment-bound dilbit to fish, which is necessary to understand the totality of the threat that spills of dilbit pose to freshwater aquatic organisms and create effective guidelines for cleanup of spills of dilbit.

3.2 Methods

3.2.1 Preparing water-soluble and water-accommodated fractions of weathered sediment-bound dilbit

Dilbit was weathered according to Fieldhouse *et al.* (2010) and bound to sediment following methods adapted from Environment Canada (2013a) and Waterman and Garcia (2015). The preparation of WSD and the various fractions is outlined in a flowchart in Appendix 1 (Fig. A5). In short, 200 mL of fresh Cold Lake Blend (CLB) dilbit was weathered on a Buchi-121 rotary evaporator at 135 rpm in an 80ºC water bath for 10 minutes, producing approximately 10% mass loss. At the top of the condenser an 8 mm airline was inserted, attached to a 9 mm plastic pipette that extended to the top of the evaporation flask. The tubing and pipette were attached to a vacuum pump giving positive

airflow at a rate of 13 L/min. Next, 30 mL aliquots of the weathered dilbit were dispensed into 1 L glass bottles, containing 600 mL of dechlorinated City of Lethbridge water and 12 g of kaolin (Sigma, Oakville, ON), and the mixture was allowed to thermally equilibrate for 4 h at 26.0°C. Sediment loading was double what has previously been used by Environment Canada (2013a) because the objective of this study was to create the maximum amount of sediment-bound dilbit per volume of dilbit used. Preliminary tests showed that doubling the sediment load did not impair the formation of OMAs (Appendix 1). Bottles were placed horizontally on a culture table shaker and mixed for 16 h in the dark at 160 rpm and 26.0ºC. Next, the entire contents of each bottle were poured into one 3 L beaker, the beaker was covered with aluminum foil, and the contents were allowed to settle for 24 h at 26.0°C in the dark. After the OMAs had settled, the overlaying water was siphoned into amber bottles fitted with Teflon™ caps.

Two water-soluble fractions (WSFs) and two water-accommodated fractions (WAFs) were prepared for exposures of zebrafish embryos. The overlaying water in the 3 L beaker during the settling step comprised the first WAF, which represented a high-energy mixing environment and had a nominal oil load of 50 mL oil/L. The second WAF (sediment-derived WAF – SDWAF) was derived from the collected WSD by modification of standard methods for preparation of WAF (Singer *et al.,* 2000; Philibert *et al.,* 2016). Briefly, in a 2 L Erlenmeyer flask, 270 mL of WSD and 1530 mL of filtered dechlorinated city water was mixed (15% WSD), leaving 20% headspace in the bottle. The contents were mixed for 20 h in the dark at 26.0°C with a 45 mm Teflon™-coated stir bar set to approximately 200 rpm such that no vortex was created, and then allowed to settle for 4 h. The overlaying water was slowly siphoned and collected. This fraction

represented a low-energy mixing environment and had a nominal loading of 36 mL oil/L. The WSF and SDWSF were created with identical methods as the WAFs, except they were vacuum filtered through a 0.45 μm cellulose filter, thereby excluding the neutrally buoyant OMAs in the solution and leaving only water-soluble contaminants. The WSFs and WAFs were stored at 4°C in amber bottles fitted with Teflon^{TM} caps with no headspace. Serial dilutions of both WSFs and WAFs were prepared with filtered dechlorinated city water such that treatments were 100%, 50% and 10% (v/v).

3.2.2 Exposure and embryotoxicity

Zebrafish (TL strain) were cultured in vertical flow-through racks (Tecniplast, Toronto, ON) supplied with dechlorinated city water (average water quality: conductivity 378 μS/cm, alkalinity 128 mg as $CaCO_3$/L, hardness 165 mg as $CaCO_3$/L, >90% oxygen saturation, pH 8.01) at 26°C and kept on a 16:8 light:dark photoperiod. Adults were fed to satiety with a 1:1 mixture of Gemma Micro 300 (Skretting, Vancouver, BC) and Ziegler (Ziegler, Gardners, PA) 1 mm pellets twice a day.

The night prior to a breeding event, two adult females and three adult males were placed into an aerated sloped breeding tank (Tecniplast) with a false bottom and a divider separating females and males. Fish were left in the tank overnight at 26°C. Within 30 minutes of the light turning on the next morning, the divider was removed and the fish were left undisturbed for 30 minutes. Fertilized eggs were collected and placed immediately into their respective exposure solutions

Embryos were exposed from approximately 0.5 hpf to 120 hpf in glass petri dishes (50 embryos/dish) containing 40 mL of either dechlorinated city water (control), WSF,

SDWSF, WAF or SDWAF at concentrations of 10%, 50% and 100% (v/v). At 2 hpf, all unfertilized embryos were removed and not counted towards total mortality or hatch success. Each day, 50% of the volume of the exposure solution was replaced. Mortality and hatch success were recorded immediately after each water renewal. At 48 hpf, the heart rate, recorded as number of beats/min, of five embryos per dish were recorded using a StrREO Discovery V12 Stereo microscope (Zeiss, North York, ON). At 120 hpf, all surviving embryos were assessed for pericardial edema, yolk-sac edema, presence of an inflated swim bladder, and spinal curvature, then flash frozen and stored at -80°C until required for analysis.

3.2.3 Gene expression using real-time qPCR

Semi-quantitative real-time PCR (qPCR) was used to assess mRNA abundances of genes involved in swim bladder development, AhR activation, cardiogenesis and the response to oxidative stress (Table 3-1). Total RNA was isolated from 10 larvae per replicate in treatments with adequate survival, collected at 120 hpf by use of TRIzol™ Reagent (Invitrogen, Carlsbad, CA) according to the protocol provided by the manufacturer and the concentration of RNA was quantified using a NanoDrop One spectrophotometer (ThermoFisher Scientific, Ottawa, ON). Complimentary DNA (cDNA) was synthesized from RNA (1500 ng/μL) using Superscript IV VILO Master Mix containing ezDNAse (Invitrogen). Real-time qPCR was run at 95°C for 2 min for initial denaturation, followed by 40 cycles of denaturation at 95°C for 5 s and annealing at 60°C for 10 s. A melt curve was generated to ensure amplification of a single PCR product. Efficiencies of primer sets for qPCR were established using serial dilutions of

cDNA template (Table 4-1). mRNA abundance of glutathione S-transferase (*gst*), superoxide dismutase (*sod*), glutamate cysteine ligase catalytic subunit (*gclc*) and glutathione peroxidase (*gpx*) genes were quantified as they are involved in the oxidative stress response (Di Giulio et al., 1989). mRNA abundance of *cyp1a* gene was quantified as an indicator of aryl hydrocarbon receptor (AhR) activation. mRNA abundance of *lef1*, *β –catenin*, *axin2, pbx1b* and *pbx1b* were assessed due to their role in swim bladder development. Abundance of mRNA from target genes was then normalized to the abundance of *β-actin*, *18s rRNA* and *rpl8.*

Table 3-1 Sequences and efficiencies of primers used for real-time qPCR.

Target Gene	Function	Primer Sequence	Efficiency
β-actin	Housekeeping	F: CGAGCTGTCTTCCCATCCA R: TCACCAACGTAGCTGTCTTTCTG	100%
18s rRNA	Housekeeping	F: CCACTCCCGAGATCCAACTA R: CAAATTACCCATTCCCGACA	105.0%
rpl8	Housekeeping	F: CTCCGTCTTCAAAGCCCATGT R: TGTTCCTCGCAGTCTGCCAG	99.0%
sod	Oxidative stress response	F: CGTCTATTTCAATCAAGAGGGTG R: GATTGCAGCCGTTTGTGTTGTC	107.0%
gst	Oxidative stress response	F: TGGTGCTTTGAAGATCATGC R: CTGAAACAGCACCAGGTCAC	101.0%
gpx	Oxidative stress response	F: GAAATACGTCCGTCCTGGAA R: CATAAGGGACACAGGGTCGT	105.5%
gclc	Oxidative stress response	F: AACCGACACCCAAAGATTCAGCACT R: CCATCATCCTCTGGAAACACCTCC	106.5%
cyp1a	AhR activation	F: GCATTACGATACGTTCGATAAGGAC R: GCTCCGAATAGGTCATTGACGAT	101.9%
axin2	Wnt signalling	F: GGACACTTCAAGGAACAACTAC R: CCTCATACATTGGCAGAACTG	97.34%
lef1	Wnt signalling	F: GAGGGAAAAGATCCAGGAAC R: AGGTTGAGAAGTCTAGCAGG	106.4%
β-catenin	Wnt signalling	F: GACAGGACGACCCAAGCTAC R: GCCGTCTACGGGGTAATCAG	92.45%
pbx1a	Surfactant production	F: ACGAAAAAGGAGAAACTTCAACAAG R: AACCAGTTGGATACCTGTGAG	102.94%
pbx1b	Surfactant production	F: GAAAACATGCGCTCAACTGCC R: GAGCTCCACGGATACTCAACA	99.1%

3.2.4 Oxidative stress

Lipid peroxidation was quantified as an indicator of oxidative stress by measuring the concentration of thiobarbituric acid reactive substances (TBARS) in the surviving embryos of the highest concentration of each fraction at 120 hpf (R&D systems, Minneapolis, MN). Briefly, 10 embryos per replicate were homogenized in 500 μL of tris buffer (0.05 M, pH 7.4) with 1% Triton X-100 (v/v). Homogenate was centrifuged at 10,000 g for 10 min at 4°C and the supernatant was collected. Supernatants were acidified with 0.6 N trichloroacetic acid at a 1:1 ratio, incubated for 15 min and centrifuged at 12,000 g for 4 min to remove interfering proteins. A standard curve of TBARS solution was made by combining 100 μL of 500 μM 1,1,3,3,-tetramethoxypropane with 200 μL of 0.6 N trichloroacetic acid and diluting this stock to final concentrations of 0.26 μM, 0.52 μM, 1.04 μM, 2.09 μM, 4.18 μM, 8.35 μM and 16.7 μM to be used as a standard curve. Next, 150 μL of the acidified samples or TBARS standards were combined with 75 μL of thiobarbituric acid in a 96-well plate. An initial spectrophotometer reading was taken at 532 nm, then plates were incubated for 2 h at 50°C and then read again at 532 nm. The difference between the first and second reading was calculated and concentrations of TBARS were interpolated from the standard curve and multiplied by two to account for dilution during the acidification step. Concentrations of TBARS were normalized to the amount of protein in the supernatant. Concentrations of proteins were determined by use of the bicinchoninic acid (BCA) assay (Sigma), with bovine serum albumin (BSA) as a standard, according to the protocol provided by the manufacturer. A BCA working reagent was made by mixing bicinchoninic acid and 4% w/v copper(II) sulfate pentahydrate at a volume ratio of 50:1 respectively. In a 96-well plate, 20 μL of either tris

buffer, bovine albumin protein standards or homogenate supernatant were added with 200 μL of the BCA working reagent. The plate was incubated for 30 min at 28°C and read at 562 nm with a spectrophotometer. Protein standards of bovine serum albumin were prepared at concentrations of 0 μg/mL, 100 μg/mL, 250 μg/mL, 500 μg/mL, and 1000 μg/mL to form a standard curve. Protein concentrations in each sample were calculated by interpolating from the BCA standard curve.

3.2.5 Behaviour

At 120 hpf three larvae per treatment per replicate were randomly chosen and assessed for effects on behaviour following standard methods (Selderslaghs *et al.,* 2010; Phillibert *et al.,* 2016). Larvae exposed to the WAF were excluded from behavioural analysis due to the dark particles interfering with movement tracking. Severely deformed larvae were not used in the assay. One larva was placed into each well of a polystyrene 24-well plate with 2 mL of their respective exposure solution. Plates were placed in a DanioVision observation chamber (Noldus, NL) at 26.0°C and allowed to acclimate in the dark for 10 min. Behaviour was tracked for 10 min in the dark, followed by 10 min in the light, then this cycle was repeated once. Light intensity was set to 5% (approximately 500 lux) as this matched the light intensity of the incubator the embryos were raised in. Behaviour was tracked using Ethovision XT (Noldus, Ottawa, ON). Thigmotaxis was determined by quantifying the amount of time larvae spent in the 3 mm perimeter of the well as this is a known anxiety response (Richendrfer *et al.,* 2012; Kalueff, *et al.,* 2013) and total distance swam was measured.

3.2.6 Statistical analysis

Data were tested for normality using the Shapiro-wilk test and homogeneity of variances between treatments was determined using a Bartlett test. For all data that were parametric, one-way analysis of variance (ANOVA) followed by a Dunnett's test was used to determine significant effects of treatments compared to the control. For data that were non-parametric, a Kruskall-Wallis test followed by a Dunn test coupled with a Bonferroni correction was used to assess effects of treatments. All statistics were performed using R 3.6.0 base package (R Core Team, 2019) with the additional "dunn.test" (Dinno, 2017) and "multcomp" (Hothorn *et al.,* 2008) packages. Alpha level of 0.05 was used to determine significance for all tests.

3.3 Results

3.3.1 Embryotoxicity

Fractions of the WSD were acutely toxic to embryos (Table 3-2). Both WAFs caused greater five-day mortality than the WSFs. Mortality of embryos exposed to WAF50, WAF100 and SDWAF100 treatments was 100% and therefore sublethal effects of these concentrations could not be assessed. No significant mortality was observed for embryos exposed to either WSF. Across all treatments, the greatest concentration that did not cause 100% mortality (WSF100, SDWSF100, WAF10, SDWAF50) caused significantly greater incidences of pericardial edema, yolk sac edema and uninflated swim bladder compared to controls (Fig. 3-1). Pericardial edema was the most common malformation at lower concentrations, with 15% and 23% of embryos exposed to WSF50 and SDWSF50, respectively, exhibiting this malformation. Neither incidences of spinal curvature or changes in heart rate were observed after exposure to either fraction of WSD.

Table 3-2 Embryotoxicity after 120 h exposure to water-soluble and water-accommodated fractions of weathered sediment-bound dilbit showing total mortality, presence of malformations at 120 hpf and heart rate at 48 hpf. Values are presented as averages ± one SE. PE: pericardial edema, YSE: yolk-sac edema, USB: uninflated swim bladder. An asterisk indicates statistical significance compared to control (n=5-6).

Fraction	Mortality (%)	PE (%)	YSE (%)	USB (%)	Heart rate (bpm)
Control	13.0 (± 5.3)	2.1 (± 0.8)	0.4 (± 0.4)	0.4 (± 0.4)	195.6 (± 11.1)
WSF10	11.0 (± 2.8)	3.6 (± 1.2)	0.4 (± 0.4)	1.6 (± 0.8)	206.8 (± 9.2)
WSF50	8.7 (± 3.3)	15.3 (± 2.8)*	1.6 (± 1.2)	3.9 (± 2.5)	212.9 (± 10.4)
WSF100	21.3 (± 4.9)	90.4 (± 2.4)*	66.3 (± 6.5)*	98.0 (± 4.2)*	206.8 (± 10.3)
Control	13.0 (± 5.3)	2.1 (± 0.8)	0.4 (± 0.4)	0.4 (± 0.4)	195.6 (± 11.1)
SDWSF10	9.7 (± 3.5)	3.8 (± 1.5)	0.4 (± 0.4)	2.6 (± 1.0)	200.4 (± 8.7)
SDWSF50	9.7 (± 4.0)	22.5 (± 2.6)*	2.2 (± 1.4)	5.7 (± 2.5)	219.7 (± 6.2)
SDWSF100	15.6 (± 6.3)	81.6 (± 4.1)*	40.2 (± 4.9)*	86.2 (± 4.6)*	223.0 (± 10.7)
Control	13.7 (± 5.8)	0	0	0.6 (± 0.6)	186.7 (± 11.6)
WAF10	19.9 (± 5.7)	63.0 (± 10.5)*	33.9 (± 13.5)*	75.2 (± 10.8)*	195.8 (± 7.3)
WAF50	100*	NA	NA	NA	180.9 (± 11.8)
WAF100	100*	NA	NA	NA	175.9 (± 11.6)
Control	13.7 (± 5.8)	0	0	0.6 (± 0.6)	186.7 (± 11.6)
SDWAF10	34.1 (± 5.7)	3.5 (± 1.8)	2.2 (± 1.1)	17.0 (± 6.7)*	201.3 (± 7.0)
SDWAF50	24.1 (± 8.2)	96.2 (± 1.5)*	86.7 (± 6.4)*	100*	189.1 (± 7.7)
SDWAF100	99.0 (± 1.0)*	NA	NA	NA	175.9 (± 15.4)

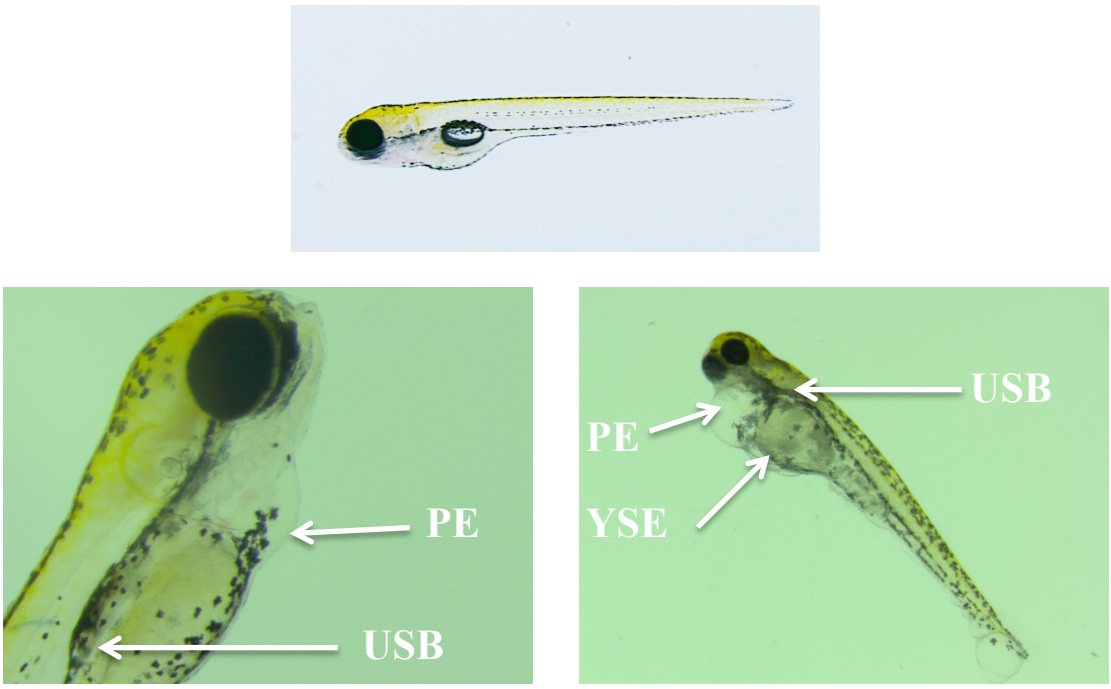

Figure 3-1 Representative control (top), moderately malformed (left) and severely malformed (right) zebrafish larvae after 120 h exposure to fractions of weathered sediment-bound dilbit. Note PE: pericardial edema, YSE: yolk sac edema, USB: uninflated swim bladder.

3.3.2 Gene expression

The mRNA abundance of *cyp1a* increased in a concentration-dependent manner in embryos exposed to all fractions of the WSD. Abundance was greater by up to 17-fold in larvae exposed to the WSF and the SDWSF (Fig. 3-2), whereas abundance was 150-fold and 164-fold greater in embryos exposed to the WAF and SDWAF, respectively (Fig. 3-2). There were no statistically significant changes in mRNA abundances of genes important for the response to oxidative stress in embryos exposed to either fraction of the WSD (Fig. 3-3). Although not statistically significant, mRNA abundances of *sod* and *gpx* were up to 2.0-fold lower in larvae exposed to increasing concentrations of WSF and SDWSF (Fig. 3-3). Similarly, the mRNA abundance of *gst* was 2-fold greater in larvae

exposed to the WSF and SDWSF and was 3-fold and 4-fold greater in larvae exposed to WAF and SDWAF respectively (Fig. 3-3).

Exposure to fractions of the WSD did not affect mRNA abundances of a subset of genes involved in development of the swim bladder. mRNA abundances of *axin2, lef1* and *β-catenin* were unaffected by exposure to either fraction of the WSD (Fig. 3-4). mRNA abundances of *pbx1a* and *pbx1b* were not different in larvae exposed to WSD (Fig. 3-4).

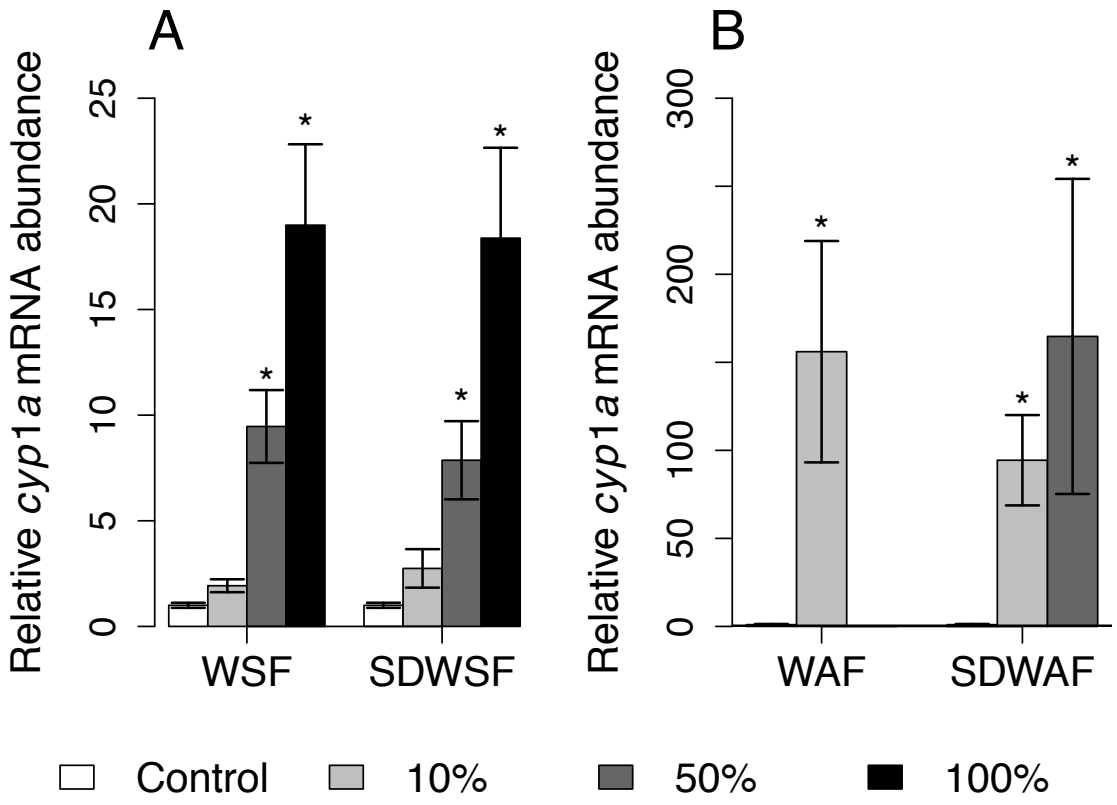

Figure 3-2 Relative *cyp1a* mRNA abundances in zebrafish larvae following120 h exposure to water-soluble (a) and water-accommodated (b) fractions of weathered sediment-bound dilbit. Expression was normalized to *18s RNA, β-actin* and *rpl8.* Asterisk indicates statistical significance compared to control (n=5-6). Error bars are +/- one SE

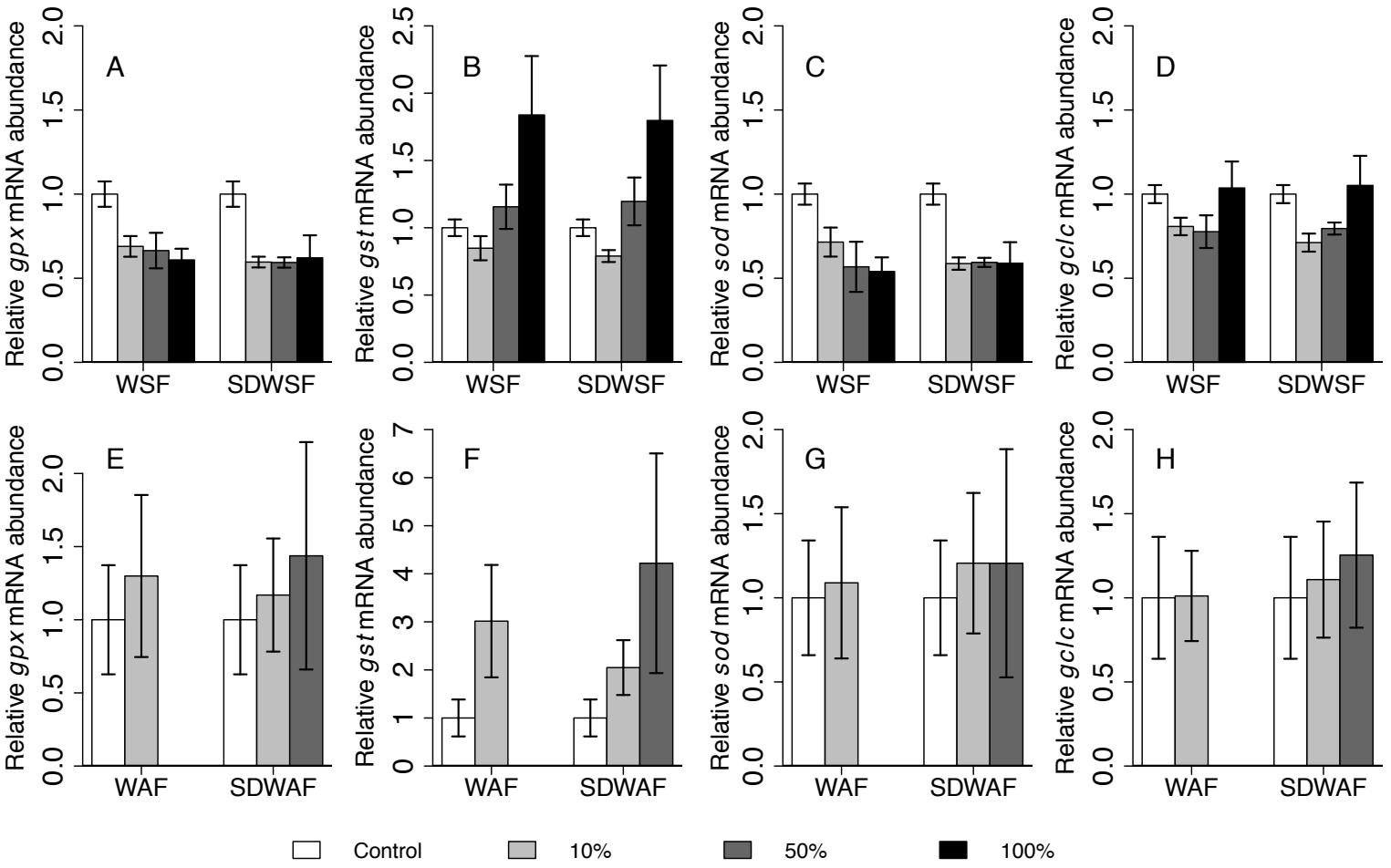

Figure 3-3 Relative mRNA abundances (fold change from control) of genes related to the oxidative stress response in zebrafish larvae following 120 h exposure to water-soluble (a-d) and water-accommodated (e-h) fractions of weathered sediment-bound dilbit. (a,e) *gpx* (b,f) *gst* (c,g) *sod* (d,h) *gclc.* Expression was normalized to *18s RNA, β-actin* and *rpl8.* Error bars are +/- one SE (n=5-6).

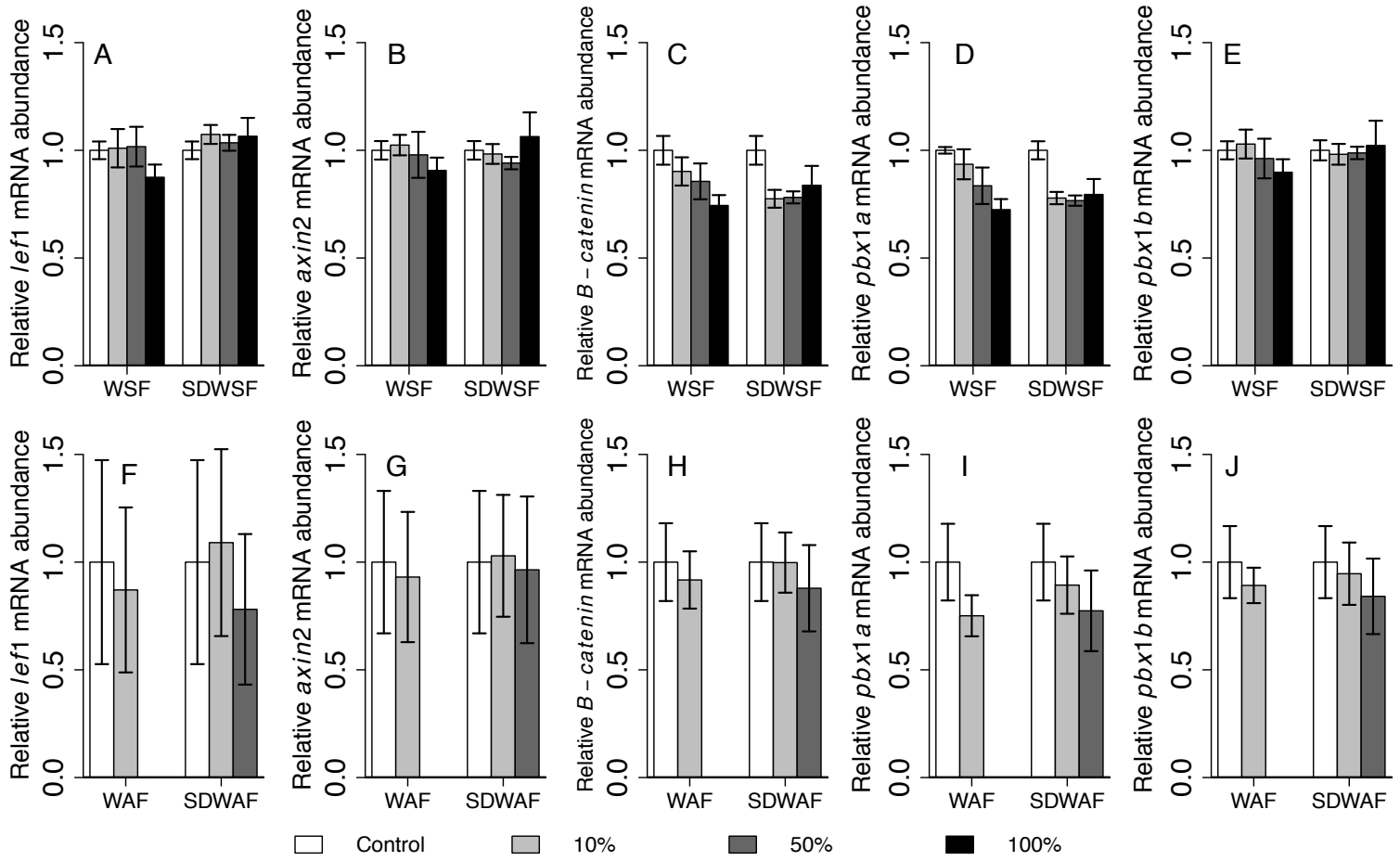

Figure 3-4 Relative mRNA abundances (fold change from control) of genes related to swim bladder formation in zebrafish larvae following 120 h exposure to water-soluble (a-e) and water-accommodated (f-j) fractions of weathered sediment-bound dilbit. (a,f) *lef1* (b,g) *axin2* (c,h) *β-catenin* (d,i) *pbx1a* (e,j) *pbx1b.* Expression was normalized to *18s RNA, β-actin* and *rpl8.* Error bars are +/- one SE (n=5-6)

3.3.3 Lipid peroxidation

Embryos exposed to SDWAF50 had 25% greater abundances of peroxidized lipids than controls (Fig. 3-5). However, there was no significant change in lipid peroxidation in embryos exposed to other fractions (Fig. 3-5).

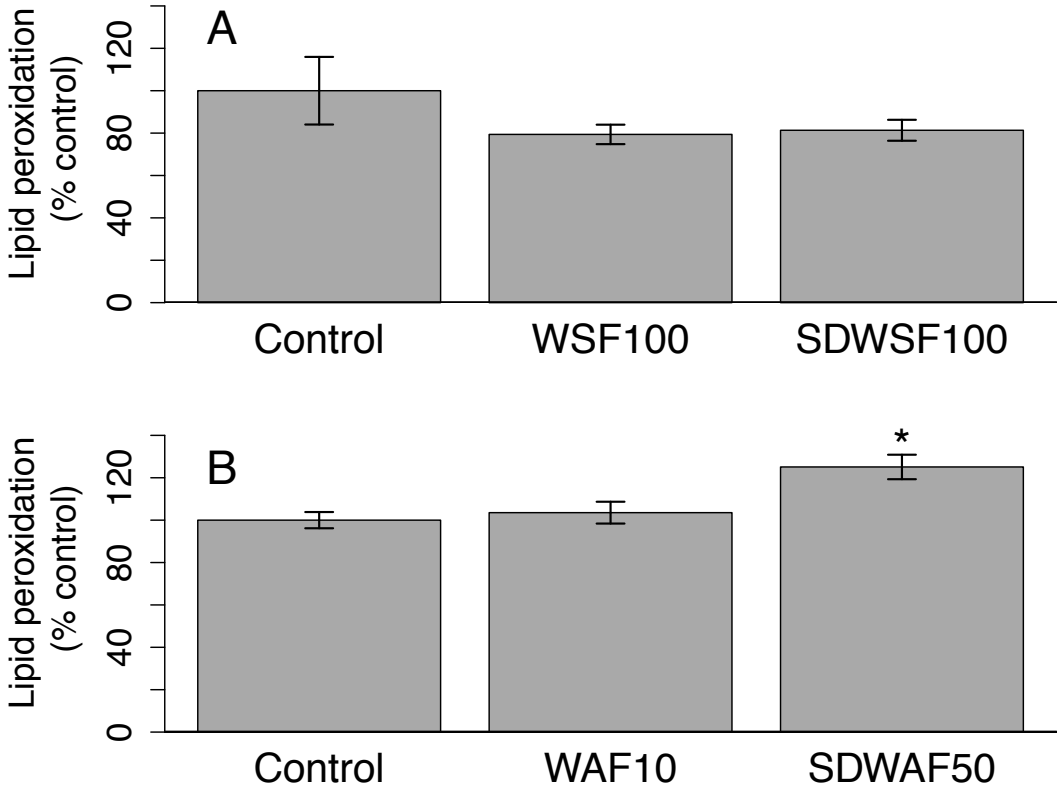

Figure 3-5 Amount of lipid peroxidation in zebrafish larvae after 120 h exposure to water-soluble (a) and water-accommodated (b) fractions of weathered sediment-bound dilbit. Only the highest concentration with adequate survival was tested for each fraction. Lipid peroxidation was quantified as μM TBARS/mg protein and expressed as percent of the control. Asterisk indicates statistical significance compared to control. Error bars are +/- one SE (n=5-6).

3.3.4 Behaviour

There were distinct differences in total distance travelled but not thigmotaxis between the light and dark periods across all treatments. Distance travelled during light periods was less than half than that of the dark periods (Fig. 4-6), but thigmotaxis was similar between light and dark periods (Fig. 4-7). However, no significant differences in total distance travelled between concentrations of either fraction within any light or dark period was observed (Fig. 4-6). In the second dark period, larvae exposed to WSF100 travelled half the distance of controls, but this was not seen in larvae exposed to other fractions of WSD and was not statistically significant (Fig. 4-6). Thigmotaxis behaviour increased in the larvae exposed to WSF100 compared to controls across all light and dark periods, although changes during the light period were not statistically significant (Fig. 4-7). Control larvae spent 66% and 65% of the time in the border of the well and WSF100 exposed larvae spent 93% and 91% of the time in the border during the first and second dark period respectively (Fig. 4-7). Changes in thigmotaxis were less pronounced in the SDWSF exposed larvae. However, in the second dark period, time spent in the border increased to 85% for SDWSF100 exposed larvae from 65% for controls (Fig. 4-7).

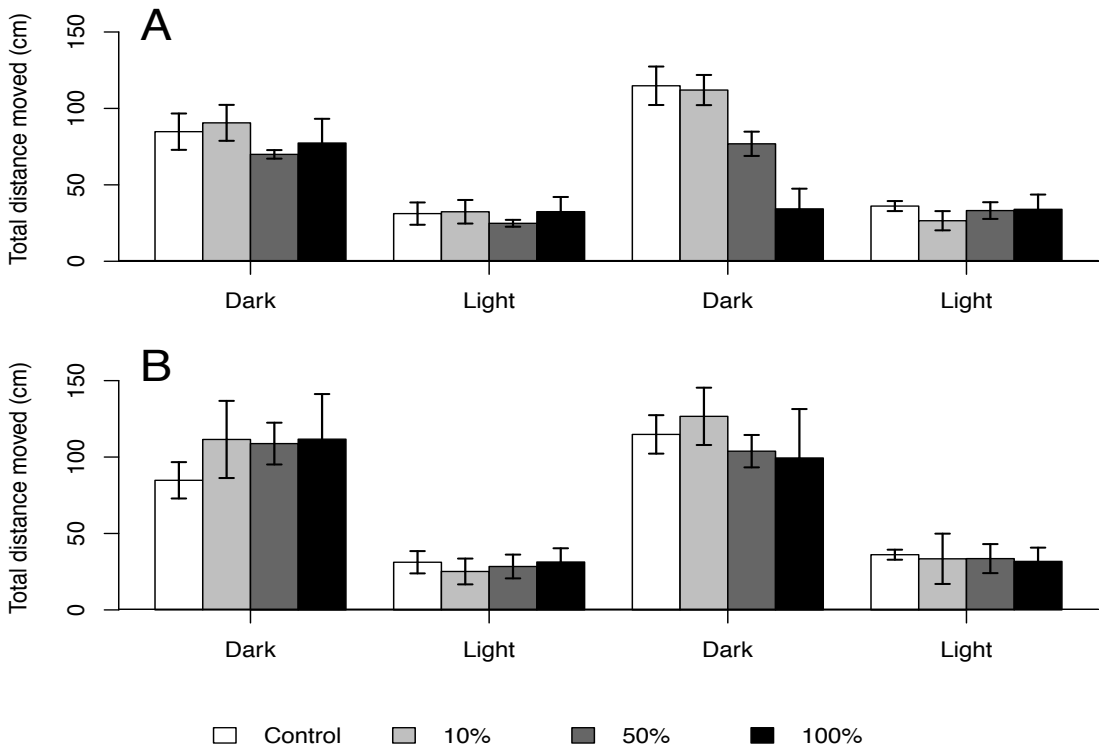

Figure 3-6 Total distances travelled by zebrafish larvae following 120 h exposure to two water-soluble fractions of weathered sediment-bound dilbit. Each group of bars represents a 10 min period under dark or light conditions. (a) WSF fraction (b) SDWSF fraction. Error bars are +/- one SE (n=5).

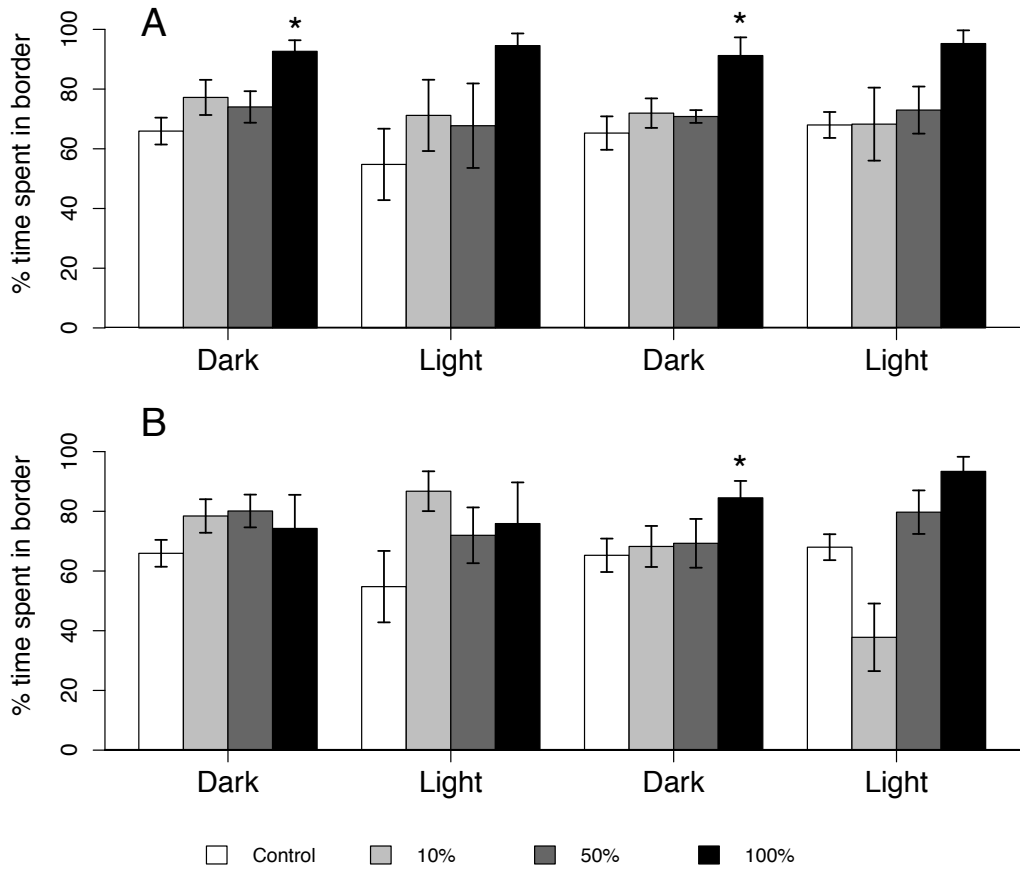

Figure 3-7 Percent of the time spent in the 3 mm border of the well (thigmotaxis) by zebrafish larvae after 120 h exposure to two water-soluble fractions of weathered sediment-bound dilbit. Each group of bars represents a 10 min period under dark or light conditions. (a) WSF fraction (b) SDWSF fraction. Asterisk indicates significant difference from control within the respective light/dark period. Error bars are +/- one SE (n=5).

3.4 Discussion

3.4.1 Dilbit weathering

Although this study lacks an unweathered control fraction and therefore it is impossible to quantify the degree to which weathering and interaction with sediment attenuated the toxicity of dilbit to zebrafish embryos, these processes can reduce the bioavailability and presence of PAHs released from dilbit and therefore attenuate toxicity to aquatic organisms (Environment Canada, 2013a; Barron *et al.,* 2018; Yang *et al.,* 2018; Robidoux *et al.,* 2018). Weathering has been shown to reduce the abundance of low molecular weight PAHs such as naphthalenes and biphenyls, and almost completely eliminate benzene, toluene, xylene and ethylbenzene (BTEX) (Barron *et al.,* 2018; Yang *et al.,* 2018). Although concentrations of BTEX are lower in dilbit compared to some conventional oils (Zhou *et al.,* 2015), a recent study found that compared to PAHs, BTEX is a more accurate predictor of effects of dilbit on zebrafish embryos (Philibert *et al.,* 2016). Despite these losses of chemicals, weathering could increase the potency of dilbit as the proportion of alkylated and non-alkylated tricyclic PAHs, which are responsible for much of the adverse effects of conventional crude oils–including cardiotoxicity—has been shown to increase with weathering (Carls *et al.,* 1999; Jung *et al.,* 2013).

3.4.2 Water-accommodated and water-soluble fractions

Two different methods were used for preparation of both the WSF (WSF, SDWSF) and WAF (WAF, SDWAF) respectively. Across all endpoints, WSF and SDWSF were remarkably similar in their effects on development and changes in gene expression. While the nominal oil loadings were different between the WSF and SDWSF, previous work has shown no difference in total concentrations of PAHs between WAFs

prepared with 10 g/L or 32 g/L dilbit (Robidoux *et al.,* 2018). This suggests the WSF and SDWSF likely were at or approaching the limit of solubility of PAHs. The WAF and SDWAF were more variable in the effects they caused, which could be attributed to the variable amount of OMAs between the two fractions, as this was not controlled for.

The WSF and WAF were assumed to contain the same amount of water-soluble dilbit-derived compounds, with the only difference being the presence of neutrally buoyant OMAs in the WAF. Exposure to the WAFs caused greater mortality, prevalence of malformations and induction of *cyp1a* than exposure to the WSFs, at their respective nominal concentrations (Table 3-1, Fig. 3-2). The presence of OMAs could have increased mortality and the prevalence of malformations by interacting directly with the chorion, increasing the uptake of PAHs and other dilbit-derived compounds. This increase in uptake was supported by the significantly greater *cyp1a* mRNA abundance in embryos exposed to the WAFs. In contrast to findings in the current study, another study with zebrafish embryos showed that dissolved hydrocarbons caused ELS toxicity and crude oil droplets did not exacerbate toxicity (Carls *et al.,* 2008). However, some studies have found that direct contact of oil droplets with the chorion exacerbated toxicity by either creating a greater concentration of dissolved hydrocarbons surrounding the embryo or by directly increasing their uptake due to the physical interaction of the oil droplets with the chorion (Gonz *et al.,* 2008; Elin *et al.,* 2015). In the current study, it was observed that in exposures to either the WAF or SDWAF, a large number of OMAs adhered to the chorion, which could be due to the WAFs containing large amounts of high-density particles that sank and came into contact with the embryos. Weathering also increases the adhesiveness of the dilbit (Environment Canada, 2013a; Zhou *et al.,* 2015), which could promote more particle-chorion interactions. Finally, the concentration of oil-

derived contaminants in WAFs of crude oils is known to decrease over exposure time due to volatilization, adhesion to glassware and degradation (Redman and Parkerton, 2015). Therefore, compared to the WSF and SDWSF, the OMAs in the WAF and SDWAF might have maintained a higher concentration of PAHs by releasing them over time.

3.4.3 Oxidative stress as a mechanism of toxicity

Results of the current study suggest that the main mechanism of toxicity in zebrafish embryos exposed to fractions of WSD was not oxidative stress. There was a 25% increase lipid peroxidation in SDWAF50 exposed embryos, but this would have been observed in other fractions if it were the main mechanism of toxicity (Fig. 3-5). It is possible, however, that at high concentrations of WAF, oxidative stress could have contributed to the adverse effects caused by exposure to dilbit. The presence of OMAs could expose zebrafish embryos to less soluble PAHs that were not present in the WSF and SDWSF, and possibly PAHs that induce oxidative stress. Expression of *SOD*, *GPX* or *GCLC* was not significantly increased in embryos exposed to either fraction of WSD (Fig. 3-3). Although not statistically significant, expression of *GST* was greater in embryos exposed to higher concentrations of WAF and WSF (Fig. 3-3). Expression of GST has been used as a biomarker for the cellular oxidative stress response because it catalyzes the conjugation of electrophilic reactive metabolic intermediates with glutathione, allowing them to be excreted from the cell (Hayes and Pulford, 1995; Lu *et al.,* 2009). However, expression of GST is known to be regulated by activation of the AhR (Nebert *et al.,* 2000; Laborde, 2010; Brown *et al*., 2016). Previous studies have proposed oxidative stress as a mechanism of toxicity in ELS of fishes exposed to dilbit (Madison *et al.,* 2015), but the findings of the current study agree with other studies of dilbit that found little evidence of

oxidative stress in ELS of fishes following exposure to dilbit (Madison *et al*., 2017; Alsaadi *et al.,* 2018; McDonnell *et al*., 2019). Additionally, rainbow trout and Japanese medaka exposed to the tricyclic PAH retene displayed BSD, but no indications of oxidative stress (Bauder *et al.,* 2005; Alharbi *et al.,* 2016).

3.4.4 Cardiac impairment as a mechanism of toxicity

Effects of fractions of WSD on zebrafish embryos are consistent with cardiotoxicity as a mechanism of toxicity. Polycyclic aromatic hydrocarbons were most likely responsible for the observed effects as they are significantly more genotoxic, carcinogenic and teratogenic than other water-soluble oil-derived constituents (Nam *et al*, 2008, Hodson, 2017; Yang *et al*., 2018). Further, three to four-ringed alkyl-PAHs have been shown to be embryotoxic to rainbow trout whereas other constituents in heavy oils, such as naphthalenes, alkanes, resins and asphaltenes have been shown to be relatively benign (Adams *et al.,* 2014). Both water-soluble and water-accommodated fractions of WSD showed effects indicative of ELS exposure to PAHs, comparable to those seen in other studies that exposed ELS of fish to dilbit (Phillibert *et al.,* 2016; Madison *et al.,* 2017; McDonnell *et al.,* 2019). Cardiotoxicity is a pronounced effect in ELS of fish exposed to crude oils. Although reductions in heart rate, which is a well described effect of crude oil on fish embryos (Incardona *et al.,* 2009; Philibert *et al.,* 2016), was not evident in embryos exposed to the fractions of WSD (Table 1), incidences of pericardial edema were increased. Previous studies suggest that the developing fish heart is the organ most severely affected by exposure to crude oils, and that other effects commonly associated with exposure to crude oil, including edemas, craniofacial malformations, and

failure of the swim bladder to inflate, are due to poor circulation as a result of cardiotoxicity (Incardona *et al*., 2014).

Polycyclic aromatic hydrocarbons are known to cause cardiotoxicity by AhR-dependent and AhR-independent mechanisms. Activation of the AhR by planar PAHs causes dysregulation of expression of genes that regulate cell proliferation in cardiomyocytes (Incardona, 2017). Tricyclic PAHs can cause cardiotoxicity independent of AhR by interfering with the balance of K^+ and Ca^{2+} ions in the cell (Brette *et al.,* 2014; Brette *et al*., 2017). The efflux of K^+ ions from the cell is blocked directly by tricyclic PAHs, resulting in the inability of the cell to re-polarize properly. The Ca^{2+} in the sarcoplasmic reticulum is also depleted and the influx of new Ca^{2+} through L-type calcium channels is blocked, impairing the contraction of calcium-dependent myofilaments. Both AhR-dependent and AhR-independent mechanisms of cardiotoxicity affect development of the heart in fish embryos, resulting in BSD (Incardona *et al*., 2004; Incardona *et al.,* 2009; Zhang *et al.,* 2013; Edmunds *et al.,* 2015; Incardona, 2017). The concentration-dependent increase in *cyp1a* mRNA abundance suggests there could be AhR-dependent cardiotoxicity following exposure to dilbit fractions. Further, there could also be AhR-independent cardiotoxicity due to the high abundance tricyclic PAHs present in dilbit. Results of the current study are consistent with previous studies of conventional crude oils and therefore fish ELS toxicity following exposure to dilbit could be resulting from cardiotoxicity as well.

3.4.5 Swim bladder formation

In the present study, incidences of uninflated swim bladders were common malformations in zebrafish embryos exposed to WSD. Early development of the swim

bladder occurs from 36 hpf –120 hpf in three stages: epithelial budding, followed by the formation of the mesenchymal and outer mesothelial tissue layers, inflation of the swim bladder (Winata et al., 2009). During these stages, expression of various genes including those involved in Wnt signalling are required for proper development of the swim bladder development (Yin et al., 2011). Early in development, Wnt signaling is vital for formation of the epithelial bud, whose correct organization is necessary for proper formation of mesenchyme and mesothelium (Yin et al, 2011). The classical Wnt signalling pathway involves many genes including *β-CATENIN*, *LEF1* and *AXIN2* (MacDonald et al., 2009). Expression of *AXIN2* and *LEF1* occurs at 36 hpf – 72 hpf (Yin et al., 2011). In addition to genes in the Wnt signaling pathway, *PBX1A* and *PBX1B* are expressed in the swim bladder from 28 hpf – 6 dpf, and play an important role in surfactant production that is vital to inflation of the swim bladder (Teoh et al., 2010).

The absence of any effects of fractions of the WSD on expression of *β-catenin*, *LEF1*, *AXIN2*, *PBX1A* and *PBX1B* suggests that dysregulation of expression of these genes might not be the mechanism of impaired development of the swim bladder. This indicates that a change in gene expression was at an earlier time during development or that the mechanism of improper development of the swim bladder was via another mechanism such as cardiotoxicity, behavioural alteration or thyroid disruption.

3.4.6 Behaviour

Larvae exposed to WSF and SDWSF exhibited greater thigmotaxis behaviour than controls (Fig. 4-7). Thigmotaxis is an anxiety response and is also seen in zebrafish larvae exposed to caffeine (Richendrfer *et al.,* 2012). Although the mechanism of the altered behaviour is unclear, it could reduce the amount of time larvae spend foraging or

performing swim-up behaviour, as they will not leave shelter. The sole other study that analyzed zebrafish behaviour after exposure to dilbit found that it did not impact total distance travelled, but did reduce thigmotaxis (Philibert *et al*., 2016). The discrepancy in the total distance travelled could be due to the presence of malformations that inhibit proper movement in the larvae selected for behavioural testing. Severely malformed fish were excluded, but it is possible that WSF100 exposed larvae chosen were inhibited from moving due to edemas or the absence of a swim bladder. Reduced swimming could also have been due to cardiotoxicity, as juvenile fish can have reduced swimming performance following exposure to crude oil (Carls *et al.,* 1999). However, if this were the case, we would expect to see similar behavioural changes in the SDWSF treatment, because for all other endpoints in the present study the effects of exposure to fractions of WSD were remarkably similar. The opposite response in the thigmotaxis behaviour compared to Philibert *et al.* (2016) could be because WAFs used in that study were unweathered and contained BTEX, possibly producing a different behavioural response. Behavioural differences can also be explained because their test was performed with embryos at 7 dpf, not 5 dpf, and different stages of development can result in pronounced differences in behaviour (Kalueff, *et al.,* 2013).

There was no inflation of the swim bladder in many larvae, but it remains a possibility that the tissues and structure of the swim bladder were properly developed but the subsequent inflation never occurred. Inflation of the swim bladder occurs when zebrafish perform a swim-up behavior, swallowing air which is moved into the swim bladder, thereby inflating it (Goolish & Okutake, 1999; Lindsey et al, 2010; Woolley and Qin, 2010). Although this behavior was not assessed, it is hypothesized that larvae may not have performed the swim up behaviour due to malformations or the swim-up

behaviour could have been inhibited due to hydrocarbons in the WSD fractions forming a film on the surface of the water (Marty *et al.,* 1995; Madison *et al.,* 2017). To further investigate the mechanism of improper swim bladder development, behavioural analysis could be performed during the inflation stage to assess if the larvae are performing the swim-up behavior to inflate the swim bladder.

3.5 Conclusion

The fate and behaviour of dilbit after a spill is important to consider when developing post-spill cleanup practices. Similar to mechanical dispersion, combining with sediment (particularly fine-grained) is effective at dispersing dilbit, which can increase the release of dilbit-derived contaminants, especially when there are no solubility constraints (large water-bodies) as is seen with chemically dispersed crude oils. Oil-mineral aggregates will also facilitate transport of dilbit to the pelagic and benthic zones of aquatic ecosystems, increasing the potential for a spill to affect a broader range of organisms. This study used novel methods to expose early-life stages of fish to dilbit while incorporating ecologically relevant environmental factors that can influence the toxicity of dilbit. Given the results of this study, weathering and combination with sediment does not attenuate the toxicity dilbit and still affects the health of developing fish embryos. Similar to conventional crude oil, cardiotoxicity rather than oxidative stress appears to be the critical mechanism of toxicity of WSD to ELS of fishes. The presence of OMAs appears to significantly exacerbate toxicity, perhaps by facilitating increased uptake of PAHs by the embryos. Ecologically, this is important because OMAs can affect benthic and pelagic organisms for long periods of time. Future studies should consider how weathering and interactions with sediments influences the effects of dilbit on aquatic

organisms in order to generate a more comprehensive understanding of potential effects that any spills of dilbit might have on aquatic systems.

CHAPTER 4: GENERAL DISCUSSION AND CONCLUSIONS

In the present study, exposure to WSD affected the health of adult amphipods and ELS of zebrafish. Weathering and interaction with sediment is not adequate to completely attenuate toxicity of dilbit to either species. In fact, the presence of OMAs significantly exacerbated toxicity of WSD to both species. Oil-mineral aggregates adhered to freshwater amphipods, inhibiting respiration and proper appendage movement, resulting in acute lethality. Zebrafish embryos exposed directly to OMAs were subject to greater concentrations of PAHs, had greater mortality and greater rates of malformations than if OMAs were removed. Effects of WSD on ELS of zebrafish were comparable to other studies of dilbit and conventional oils in multiple fish species and are likely the result of cardiotoxicity.

The findings in this thesis are relevant to spills of dilbit into freshwater environments because weathering and sediment interaction are inevitable environmental processes that evidently influence the fate and toxicity of spilled dilbit. Dilbit spills could be a greater threat to aquatic organisms in freshwater bodies that have high sediment loads and turbulence compared to slow-flowing freshwater bodies with low sediment loads. Aquatic organisms such as fish embryos and benthic invertebrates are particularly vulnerable to WSD because of their potential to interact with contaminated sediments. Weathered sediment-bound dilbit can affect the health of aquatic organisms by physically impairing gas exchange structures and appendage movements or serve as a constant source of water-soluble PAHs. Although PAHs bind to sediment and interaction with sediment disperses dilbit, it is evident that sediment interaction is not an exclusively beneficial process following a spill of dilbit.

Future research on the effects of dilbit to aquatic organisms should also incorporate environmental weathering processes to provide a more complete assessment of the risk dilbit spills pose to aquatic organisms. Studies investigating the long-term effects of dilbit spills on aquatic organisms are required, because a large proportion of dilbit is non-biodegradable and could persist for long periods of time following a spill. Finally, underlying mechanisms of toxicity—such as cardiotoxicity—in aquatic organisms exposed to dilbit should be further investigated and compared to conventional oils.

There were shortcomings in the present study. First, there was no quantitative assessment of how weathering and sediment interaction affected the concentration of water-soluble PAHs in the WSFs used for exposures of zebrafish embryos. Without pre and post weathering PAH measurements, the only conclusion that can be made is that weathering and sediment interaction does not completely attenuate toxicity of dilbit WSF to ELS of zebrafish. If pre and post weathering and sediment interaction PAH measurements were made, it would be possible to determine to what degree weathering processes attenuate toxicity – if at all. Further, it would be useful to determine the concentration of OMAs in the WAFs used for exposure of zebrafish embryos. Because OMA quantification was not performed, it is difficult to determine to what degree OMAs exacerbate toxicity compared to the filtered WSFs. It is also possible that the filtration process removed some soluble PAHs, thus underestimating toxicity of the WSFs. Finally, effects of dilbit WSF to amphipods could have been assessed at an earlier life stage, or for a longer period of time. Amphipod neonates are generally more sensitive to aquatic contaminants and therefore could have a different response to dilbit WSF than adults. A

chronic study of the effects of dilbit WSF on amphipods would have allowed for assessments of growth and reproduction to be made.

Due to the rapid expansion of the oil sands, it is important that the effects of dilbit on aquatic organisms receive further attention in a broad range of species and exposure conditions to adequately understand the ecological and economic costs associated with dilbit spills. Toxicity of dilbit will not only be impacted by sediment interaction, but also by dissolved organic carbon, which would ultimately be dependent on pH, alkalinity and conductivity. Studies that incorporate pH, alkalinity and conductivity changes allow for the application of the findings to a variety of freshwater systems. Further, it is necessary for future experiments to include additional weathering processes such photomodification and biodegradation. Our novel research is an essential step to fully understanding immensely complex dilbit spills in freshwater environments.

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APENDIX 1: PRELIMINARY CHARACTERIZATION OF COLD LAKE BLEND DILBIT

The composition of dilbits can vary greatly between different blends and the time of year it was formulated. Therefore, prior to performing toxicity tests that incorporated weathering and combination with sediments, it was important to characterize the Cold Lake Blend (CLB) dilbit that was acquired for this project. Dilbit was weathered according to Fieldhouse *et al.* (2010) and bound to sediment following methods adapted from Environment Canada (2013a) and Waterman and Garcia (2015). In short, 100 mL of fresh dilbit was weathered in 500 mL pear-shaped evaporation flask attached to a Buchi-121 rotary evaporator at 135 rpm in an 80ºC water bath for 48 h (Fig. A1a). Between weathering events, the evaporation flask was sealed and stored in the fridge. A Lauda WK 300 circulation chiller set to 4°C was attached to the condenser (Fig. A1b). The top of the condenser was open to the atmosphere, where an 8 mm airline was inserted, attached to a 9 mm plastic pipet that extended to the top of the evaporation flask (Fig. A1a). The tubing and pipet were attached to a vacuum pump giving positive airflow at a rate of 13 L/min (Fig. A1b).

Three weathering states were then chosen to assess for sediment combination based on mass loss over 48 h: W1 (9.7%), W2 (19.3%), and W3 (29.0%). 30 mL portions of the weathered dilbits were dispensed into 1 L glass bottles with 600 mL of freshwater and 12 g of kaolin and allowed to thermally equilibrate for 4 h (Fig. A2a). Bottles were placed horizontally in a culture table shaker and mixed for 16 h in the dark at 160 rpm and 21.0ºC. Immediately after mixing (Fig. A2b), bottles were poured into 1 L graduated cylinders to observe the settling process over 24 h (Fig. A3a, Fig. A3b).

Figure A1 Lauda WK 300 circulation chiller and vacuum pump. Chiller set at 4°C and vacuum pump set to 13 L/min (left). Buchi-121 Rotary evaporator. Entering at the top of the condenser is an 8mm airline tube connected to a 9mm plastic pipet tube that extends to the beginning of the evaporation flask (right).

Figure A2 One-liter glass bottles filled with 30 mL weathered dilbit, 12 g kaolin and 600 mL freshwater. Prior to mixing (left) and after 16 h mixing on table shaker (right). For both pictures the left bottle is W1 (9.7% mass loss), the middle bottle is W2 (19.3% mass loss), and the right bottle is W3 (29.0% mass loss).

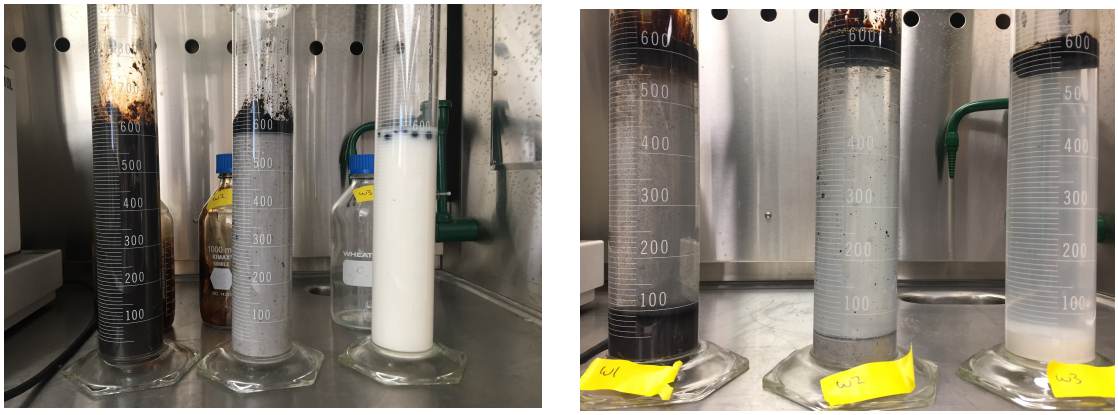

Figure A3 One-liter graduated cylinders filled with a mixture of 30 mL of dilbit weathered to varying degrees, 12 g kaolin and 600 mL freshwater that were mixed on a table shaker for 16 h. Immediately after addition to the cylinder (a) after 24 h settling (b). For both pictures, the left cylinder is W1 (9.7% mass loss), the middle cylinder is W2 (19.3% mass loss), and the right cylinder is W3 (29.0% mass loss).

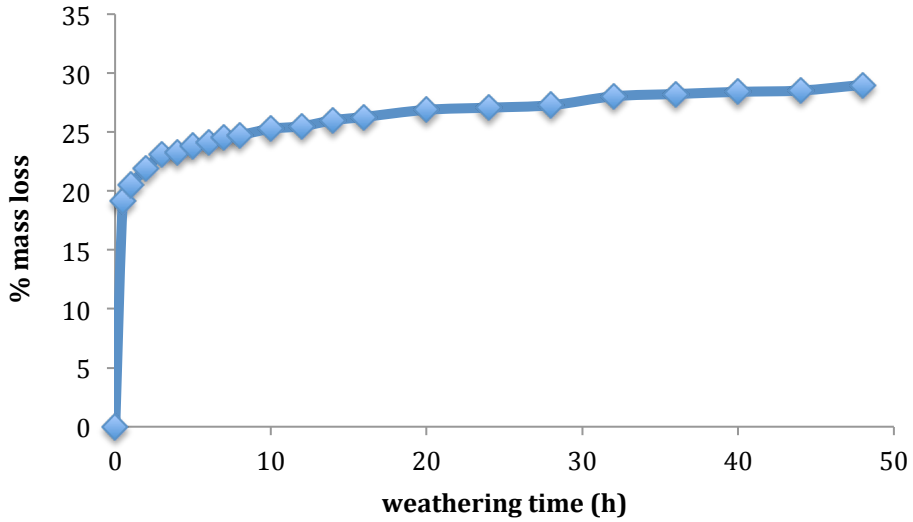

Figure A4 Evaporation of Cold Lake Blend dilbit over 48 h. Weathering was done on a rotary evaporator at 80°C with a positive airflow of 13 L/min.

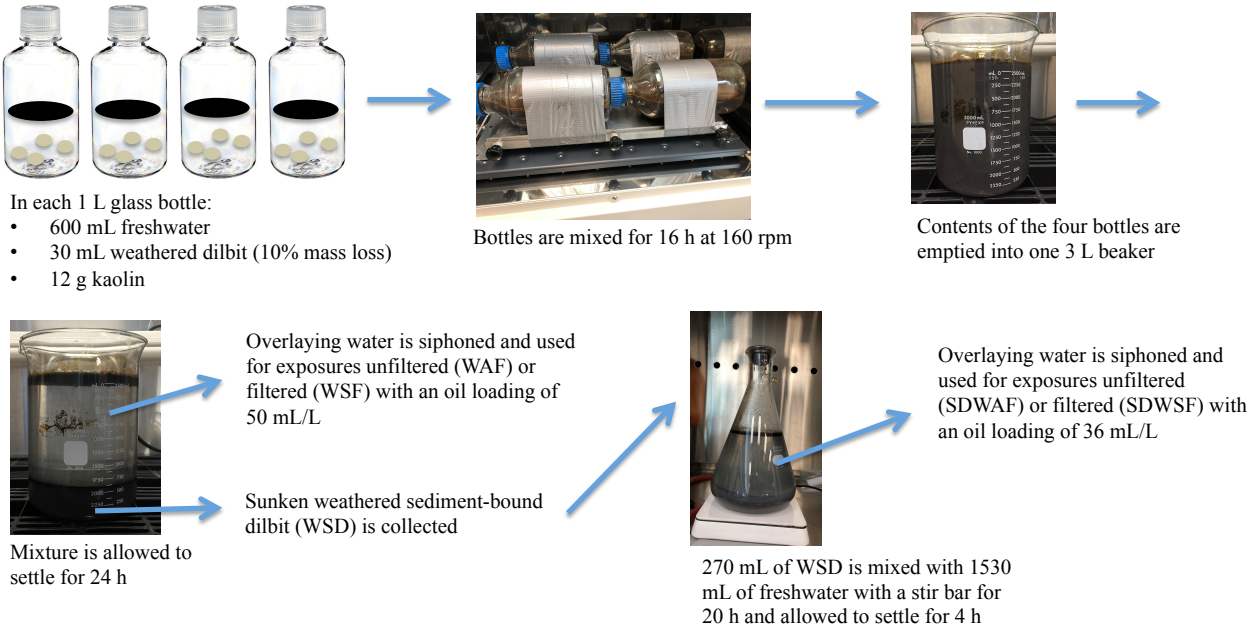

Figure A5 Flow chart depicting the preparation of weathered sediment-bound dilbit, water-accommodated fractions and water-soluble fractions.

The primary purpose of this preliminary experiment was to characterize the weathering and sediment interaction characteristics of the acquired dilbit. The maximum amount of weathering achievable was found to be 29.0%, comparable to previous findings with CLB dilbit (Environment Canada, 2013a). Weathering states of 9.7% (W1), 19.3% (W2) and 29.0% (W3) mass loss were chosen to combine with sediment. There was a negative relationship with the degree of weathering and efficiency of OMA formation. W1 dilbit combined well with kaolin, as there were no kaolin particles that were un-bound to dilbit (Fig. A3b). The dilbit in W2 formed a viscous matte on the surface of the water and there were few kaolin particles bound to dilbit (Fig. A3b). Tarballs in W3 were prevalent and there was no visible combination with sediment (Fig. A3b). These results were comparable to previous studies that explored dilbit weathering and sediment interaction (a, 2013; Hua *et al.,* 2018). As dilbit weathers and loses low-molecular weight components, it becomes more viscous and dense. Although the increased density should facilitate sinking of dilbit, the increased viscosity prevents emulsification into small droplets, which is required for the formation of OMA (Environment Canada, 2013a; Zhou *et al.,* 2015; Hua *et al.,* 2018). Without the formation of OMAs, spilled dilbit will remain floating or become neutrally buoyant (SL Ross, 2012; Zhou *et al.,* 2015).

Based on these preliminary results, a weathering state of 10% mass loss was chosen for all subsequent toxicity tests. This degree of weathering represents approximately 1.5 h open-pan evaporation at 15°C with no agitation and is a realistic degree of weathering that would occur during a spill before the dilbit interacted with sediment (Environment Canada, 2013a).