UNIVERSITÉ DU QUÉBEC

INSTITUT NATIONAL DE LA RECHERCHE SCIENTIFIQUE

CENTRE EAU TERRE ENVIRONNEMENT

Embryotoxicité des produits pétroliers dans différentes conditions environnementales chez les poissons et les amphibiens

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Examen doctoral présenté pour l'obtention du grade de
Philosophiae doctor (Ph. D.) en sciences de l'eau

Jury d'évaluation

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EPIGRAPH

La science n'a pas de patrie, parce que le savoir est le patrimoine de l'humanité, le flambeau qui éclaire le monde.

- Louis Pasteur

DEDICATION

A mis queridos padres y a mi hermanita;

Y a mi pequeña sobrina Ana Sofía que llegó y se volvió la luz de mi vida.

ACKNOWLEDGEMENTS

I would like to express my gratitute to my supervisor, Dr. Langlois.

I also would like to thank the experts who were involved in this research thesis: Drs. Heather Dettman, Qin Xin, Gaëlle Triffault-Bouchet, Patrice Couture, Charles Gauthier, Géraldine Patey and Juan Manuel Gutierrez-Villagomez.

Agredezco profundamente a mi hermosa familia (Maria del Rosario Jacobo-Garcia, David Lara-Ochoa, mi hermanita Ahiko L Lara-Jacobo, mi sobrinita Ana Sofia y ami abuelita Ramona Garcia-Romero que siempre ha estado ahí para apoyarme, en todo momento. De mis padres aprendí el amor a la ciencia y a la libertad de ser uno mismo siempre. A mi familia Lara y Jacobo los cuales admiro profundamente.

Agradezco la oportunidad que la población Mexicana me ha brindado para estar aquí estudiado un posgrado con una beca completa, confiando en que los conocimientos que he adquirido aquí sirvan para el futuro de mi país.

Je remercie mon petit ami Julien Augas, qui m'a enduré dans les pires moments et a fait tant de bonheur pour moi; Je remercie également énormément les parents de cette merveilleuse personne, Chantal et Eric Augas, qui m'ont toujours soutenu et m'ont fait sentir comme faisant partie de leur belle famille.

I thank my friends who have always involved me in unconditional support and I have learned so much from them. I thank all the people I have met on this journey from whom I have learned so much (even about myself). I Thank deeply to:Sarah & Paisley; Merci beaucoup: Roxanne, Julie, Marc, Molly & Joy; Cảm ơn rất nhiều: Tuan and Muchas gracias: Diana.

RÉSUMÉ

L'attachement aux hydrocarbures n'a pas diminué ces dernières années malgré le développement de nouvelles technologies vertes. L'extraction et le transport de bitume brut et de bitume dilué continuent d'influer sur notre environnement. De nombreuses catastrophes de marées noires dans les écosystèmes aquatiques se sont produites, par exemple la marée noire de la rivière Kalamazoo (juin 2010) et du Deepwater Horizon (avril 2010) pour ne citer que quelques cas les plus célèbres. Connaître et comprendre les effets du pétrole et de ses dérivés sur les écosystèmes aquatiques est de la plus haute importance. Cela nous aidera à rechercher des alternatives stratégiques pour réduire ces risques dans les écosystèmes exposés. Les détails concernant l'écotoxicité associée au pétrole est décrite au **Chapitre 1**.

Dans cette thèse, trois différents générateurs de fraction accommodee à l'eau (WAF) ont été réalisés et comparés entre eux sur des organismes aquatiques, soit la production de WAF en laboratoire à la température ambiante, soit dans un réservoir-simulateur de vagues à 2 °C ou soit à 15 °C.

Dans l'expérience de la production de WAF en laboratoire (**Chapitre 2**), les objectifs de cette étude étaient d'étudier la toxicité du dilbit pour les embryons de grenouilles en développement et d'identifier les mécanismes d'action moléculaires impliqués. Des embryons fertilisés du xénope tropical (*Silurana tropicalis*) ont été exposés pendant 72 h à des fractions de WAF variant de 0,7 à 8,9 µg/L de Hydrocarbure aromatique polycyclique (HAP) totaux et à des WAF chimiquement améliorés (CEWAF) variant de 0,09 à 56,7 µg/L HAP totaux) préparées avec le bitume dilué (dilbit) Access Western Blend (AWB) et Cold Lake Blend (CLB). Les CEWAF des deux dilbits ont considérablement augmenté la mortalité embryonnaire et l'incidence de malformations dans les traitements les plus testés, alors que les traitements WAF n'ont entraîné aucun effet toxique observable. Des augmentations des taux d'ARNm du cytochrome P450 1A (*cyp1a*) ont été observées pour tous les traitements de dilbit suggérant que la détoxification de phase I est activée chez les larves de grenouilles exposées au dilbit. Lorsqu'elles étaient exposées à des concentrations de HAP totaux comprises entre 0,09 et 8,9 µg/L, les grenouilles ne présentaient aucune malformation observable, mais exprimaient des augmentations significatives des niveaux d'ARNm de *cyp1a* (de 2 à 25 fois plus élevés que le témoin), ce qui indique que ce

gène est un biomarqueur d'exposition approprié pour les larves de grenouilles. Également, lorsque les concentrations de dilbit étaient plus élevées, soit environ de 46,6 µg/L ou plus de HAP totaux, le phénotype de la grenouille malformée a été observé. Ces animaux exprimaient aussi de très hauts taux d'induction de l'ARNm de la *cyp1a* (plus de 250 fois celui du témoin), ce qui indique que ce gène peut également être approprié pour signaler la réponse de détoxication de l'individu au contaminant. L'expression de plusieurs gènes liés à la détoxication cellulaire et à la perturbation du système endocrinien a été mesurée, mais n'a pas été altérée de manière significative par les traitements. En résumé, le taux d'ARNm de la *cyp1a* est un paramètre très sensible pour mesurer les changements moléculaires subtils induits par l'exposition aux HAP totaux chez les larves de grenouilles. Les données suggèrent qu'une concentration de HAP supérieure à 46 µg/L serait toxique pour les embryons de *S. tropicalis* en développement.

Dans un simulateur de vagues, deux expériences ont été effectuées. La première utilisait un pétrole brut léger avec une températures de l'air et de l'eau de 14 °C et 15 °C, respectivement (**Chapitre 3**). Des échantillons d'eau ont été prélevés dans une cuve qui permet de simuler des vagues cinq fois au cours d'une expérience de vieillissement de pétrole d'une durée de 28 jours. Des fractions collectées aux jours 1, 6, 14, 21 et 28 ont été utilisé pour réaliser des expositions toxicologiques sur des embryons de tête-de-boule (*Pimephales promelas*). Pour chacune des périodes d'échantillonnage, les embryons nouvellement fécondés ont été exposés à une dilution en série de WAF, à de l'eau de rivière non contaminée (utilisée pour générer le WAF) et à de l'eau reconstituée en laboratoire. Les embryons de poissons ont été élevés jusqu'à l'éclosion et ont été utilisé pour une évaluation morphologique et une analyse de biomarqueurs, et les expositions ont été poursuivies jusqu'à l'éclosion, où le reste des larves a été récolté. Bien que la mortalité n'aie pas été significativement altérée par les traitements au pétrole brut, la sévérité des malformations observées a démontré une forte réponse en fonction de la concentration avec tous les traitements de pétrole brut. Les données suggèrent que les jours 14, 21 et 28 étaient les fractions les plus toxiques. L'analyse chimique a confirmé l'augmentation des concentrations de C10-C50 et des HAP totaux avec le temps. L'analyse de l'expression du *cyp1a* a démontré son augmentation, mais seulement au jour 14. Cette étude démontre l'importance d'étudier la toxicité des pétroles chez les espèces aquatiques lorsque les pétroles se dégradent dans l'environnement.

La deuxième expérience a permis de contraster la toxicité du dilbit CLB qui vieillit à une température de l'air et de l'eau de à 2 °C et à 15 °C (**Chapitre 4**). Des échantillons d'eau ont été prélevés dans le réservoir de déversement cinq fois au cours de l'expérience de 35 jours (périodes 1, 6, 14, 28 et 35) et ont été utilisés pour réaliser des expositions au début de la vie à l'aide des tête-de-boule, et ce, pour les deux régimes de température. Pour chaque période d'échantillonnage, les embryons nouvellement fécondés ont été exposés à une dilution en série des échantillons d'eau du réservoir d'origine, à de l'eau de rivière non contaminée (utilisée dans le réservoir) et à un système de contrôle de l'eau reconstituée. Les embryons ont été élevés jusqu'à l'éclosion. La mortalité embryonnaire et larvaire a été notée, ainsi que le temps d'éclosion, l'incidence et le type de malformations, le nombre de battements cardiaques/min et l'activité EROD *in vivo*. Les résultats ont montré que l'exposition au CLB avait des effets négatifs importants sur les poissons embryonnaires, notamment une augmentation du taux de mortalité et de malformations, ainsi que des modifications de la fréquence des battements cardiaques et de l'activité EROD. Le taux de mortalité le plus élevé a été observé aux périodes 1 et 6, tandis que l'incidence des malformations, le nombre de battements cardiaques et l'activité EROD étaient beaucoup plus affectés par les concentrations de CLB les plus élevées testées pour chacun des jours d'analyse. L'analyse en RT-PCR en temps réel d'une série de gènes liés à la détoxification des xénobiotiques et au stress oxydatif a été effectué.

Ce projet de recherche démontre l'importance de tester la toxicité inhérente associée à un plus grand nombre de conditions naturelles de vieillissement des pétroles, car l'embryotoxicité des espèces aquatiques varie en fonction du profil chimique et des concentrations des pétroles. La discussion générale de la thèse et les conclusions finales sont présentées au **Chapitre 5**.

Mots clés: Embryons de poissons, embryons de grenouilles, bitume dilué, pétrole brut, malformations, expression des gènes, *cyp1a.*

ABSTRACT

Attachment to hydrocarbons has not diminished in recent years despite the development of new green technologies. The extraction and transportation of raw and diluted bitumen (dilbit) continue to affect our environment. Numerous oil spill disasters in aquatic ecosystems have occurred, for example, the Kalamazoo River (June 2010) and the Deepwater Horizon (April 2010) oil spills to name a few. Knowing and understanding the effects of petroleum and its derivatives on aquatic ecosystems is of the utmost importance. This will help us to seek strategic alternatives to reduce these risks in exposed ecosystems. Details regarding the ecotoxicity associated with petroleum products are described in **Chapter 1**.

In this thesis, three different generators of water accommodated fraction (WAF) were produced and compared with each other on aquatic organisms, either the production of WAF in the laboratory at room temperature, or in a wave simulator tank at 2 °C or either at 15 °C.

In the experiment of producing WAF in the laboratory (**Chapter 2**), the objectives of this study were to study the toxicity of dilbit to developing frog embryos and to identify the molecular mechanisms of action involved. Fertilized Western clawed frog (*Silurana tropicalis*) embryos were exposed for 72 h to WAF fractions ranging from 0.7 to 8.9 µg/L total polycyclic aromatic hydrocarbons (or TPAH) and to chemically enhanced WAF (CEWAF) varying from 0.09 to 56.7 µg/L TPAH prepared with the Access Western Blend (AWB) and Cold Lake Blend (CLB) dilbits. CEWAFs from both dilbits significantly increased embryonic mortality and the incidence of malformations in the highest concentration, while WAF treatments had no observable toxic effects. Increases in cytochrome P450 1A (*cyp1a*) mRNA levels have been observed for all dilbit treatments suggesting that phase I detoxification is activated in frog larvae exposed to dilbit. When exposed to concentrations ranging between 0.09 and 8.9 µg/L TPAH, the frogs showed no observable malformation, but expressed significant increases in *cyp1a* mRNA levels (2 to 25 times higher than the control), indicating that this gene is an appropriate biomarker of PAH exposure for frog larvae. Also, when dilbit concentrations were higher, to approximately 46.6 µg/L TPAH or higher, the phenotype of the malformed frog was observed. These animals also expressed very high induction rates of *cyp1a* mRNA (more than 250 times that of the control), which indicates that this gene may also be suitable for signaling the detoxification response of the individual to the contaminant. The expression of several genes linked to cell detoxification and disruption of the

endocrine system was measured but was not significantly altered by the treatments. In summary, the level of *cyp1a* mRNA is a very sensitive parameter for measuring the subtle molecular changes induced by exposure to TPAHs in frog larvae. The data suggest that a PAH concentration greater than 46 µg/L TPAHs would be toxic to developing *S. tropicalis* embryos.

In a wave simulator, two experiments were carried out. The first used light crude oil with a water temperature of 15 °C (**Chapter 3**). Water samples were taken five times from a tank that simulates waves during a 28-day oil aging experiment. Fractions collected on days 1, 6, 14, 21, and 28 were used to carry out toxicological exposures on fathead minnow embryos (*Pimephales promelas*). For each of the sampling periods, the newly fertilized embryos were exposed to serial WAF dilution, uncontaminated river water (used to generate WAF), and reconstituted water in the laboratory. Fish embryos were raised until hatching, half were used for morphological evaluation and biomarker analysis, and exposures were continued until hatching, where the rest of the larvae were harvested. Although mortality was not significantly altered by crude oil treatments, the severity of the malformations observed demonstrated a strong concentration-dependent response with all crude oil treatments. The data suggest that days 14, 21, and 28 were the most toxic fractions. Chemical analysis confirmed the increase in C10-C50 and TPAH concentrations over time. Analysis of the expression of c*yp1a* demonstrated its increase, but only on day 14. This study demonstrates the importance of studying the toxicity of oils in aquatic species when oils degrade in the environment.

The second experiment contrasted the toxicity of the CLB dilbit which ages at an air and water temperature of 2 °C and 15 °C (**Chapter 4**). Water samples were taken five times from the spill tank during the 35-day experiment (periods 1, 6, 14, 28, and 35) and were used to make early life exposures to the help of *P. promelas*, for the two temperature regimes. For each sampling period, the newly fertilized embryos were exposed to serial dilution of the water samples from the original reservoir, uncontaminated river water (used in the reservoir), and a system of control of the reconstituted water. The embryos were reared until hatching. Embryonic and larval mortality was noted, as well as hatching time, incidence and type of malformations, number of heart beats/min and EROD activity in vivo. The results showed that exposure to CLB had significant negative effects on embryonic fish, including an increased rate of mortality and deformities, as well as changes in the frequency of heartbeat and EROD activity. The highest mortality rate was observed in periods 1 and 6, while the incidence of malformations, the number of heart beats, and

EROD activity were much more affected by the highest CLB concentrations tested for each of the days of analysis. Real-time RT-PCR analysis of a series of genes linked to the detoxification of xenobiotics and to oxidative stress was carried out.

This research project demonstrates the importance of testing the inherent toxicity associated with a greater number of natural aging conditions for oils since the embryotoxicity of aquatic species varies depending on the chemical profile and concentrations of oils. The general discussion of the thesis and the final conclusions are presented in **Chapter 5**.

Keywords: Fish embryos, frog embryos, diluted bitumen, crude oil, malformations, gene expression, *cyp1a.*

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ahr – aryl hydrocarbon receptor

ANSCO - Alaska North Slope crude oil

arnt – aryl hydrocarbon receptor nuclear translocator

AWB - Access Western Blend

cat - catalase

CEPA - Canadian Energy Pipeline Association

CEWAF - chemically dispersed water accommodated fraction

CG-MS - Gas chromatography-mass spectroscopy

CLB - Cold Lake Blend

CYP - Cytochrome family of enzymes

cyp1a – cytochrome P450 1a

DOR – dispersant to oil ratio

EC50 - Median effective concentration

FETAX - Frog Embryo Teratogenesis Assay-*Xenopus*

gpx - glutathione peroxidase

GSH – glutathione

gsr – glutathione-disulfide reductase

GSSG – oxidized glutathione

gst – glutathione-S-transferase

hpf – hours post fertilization

K_{ow} - Octanol-water partition coefficient

MCO - Merey crude oil

MSW – mixed sweet blend

NF – Nieuwkoop and Faber

NoRT – No-reverse transcriptase

NTC – No-template control

OCO - Oman crude oil

p53 – tumour protein

PAHs - Polycyclic aromatic hydrocarbons

ROS – reactive oxygen species

rpl8 – ribosomal protein L8

SARA - Saturates, aromatics, resins, and asphaltenes

sod - superoxide dismutase

TPAH – total polycyclic aromatic hydrocarbon

USEPA - United States Environmental Protection Agency

WAF - water accommodated fraction

WSF - Water-soluble fraction

CHAPTER 1 : INTRODUCTION

1.1 Literature Review

1.1.1 Transportation of petroleum products

With the recent increase in construction proposals for new pipeline routes (e.g., Keystone XL and Dakota Access) between Canada and the USA, some have raised concerns about the risk to flora and fauna due to the consequences of a potential spill (Lee et al., 2015). In North America, more than 40 major oil spills disasters have been recorded in freshwater and seawater since 1969 (NOAA, 2018). The pollution of crude oil and their derivatives have been studied with a lot of interest in the last decade (Perrichon et al., 2016) because of the increased oil use in developed countries, the new techniques for oil extraction, and the controversial oil spill disasters that have happened related to the pipelines operation. According to the U.S. Environmental Protection Agency (USEPA) even though freshwater oil spills are less publicized than the spills in the marine environment, the freshwater spills are more frequent and often more destructive to the environment (USEPA, 2016). The effects of an oil spill into the freshwater ecosystems varies depending of the rate of water flow and specifics to the type of ecosystem characteristics such as standing or flowing water. One of the largest inland oil spills in U.S. history is the Kalamazoo River oil spill in 2010. Ten years have passed since the pipeline operated by Endridge (Line 6B) burst and flowed into Talmadge Creek, draining into the Kalamazoo River. When this pipeline burst, 3.2 millon L of Cold Lake Blend (CLB), a diluted bitumen (dilbit) which is a mixture of crude oil and organic diluents, spilled to the river (Crosby et al., 2013). It cost more than $767 million (U.S. dollar) to clean-up and it took six years to clean the sunken oil in the river (USEIA, 2019).

Crude oil (petroleum) is a mixture of hydrocarbons that formed from biotic components that lived millions of years ago. Crude oil is a fossil fuel, and it exists in liquid form in underground pools or reservoirs, in tiny spaces within sedimentary rocks, and near the surface in oil sands deposits (USEIA, 2019). The oil sands (also known as tar sands, crude bitumen or bituminous sands) are a natural occurring mixture of bitumen-infused sands (USEIA, 2020). After bitumen is separated from the sands, it is sent to a refinery where the crude oil is separated and converted into useable petroleum products including gasoline, jet fuel, waxes, diesel fuel, heating oil, lubricating oils, feedstocks, and asphalt. For example, an U.S. 42 gallon barrel of crude oil yields about 45 gallons of petroleum products during the refinery process (Fig. 1.1). One of the most common long-distance transportation of bitumen is by pipeline, where the bitumen is diluted to lower the viscosity and facilitate the flow through the pipe system (GOC, 2019).

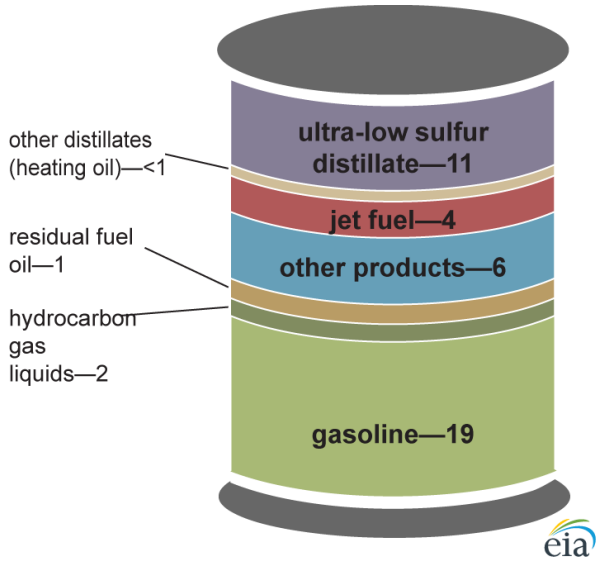

Figure 1.1 Petroleum products made from a barrel of crude oil, 2018. Source: U.S. Energy Information Administration, Petroleum Supply Monthly, April 2019, preliminary data (modified).

1.1.2 Crude oil and petroleum products (physical and chemical properties)

Bitumen is a complex chemical mixture of saturated alkanes, aromatics, terpene-based substances (resins) and asphaltenes (commonly called SARA, refers to Saturates, Aromatics, Resins, and Asphaltenes) and metals such as lead and some non-metallic compounds typically contained in organic substances, e.g. nitrogen, sulfur and oxygen are also found in a lower proportion (Dupuis and Ucán-Marín, 2015). Normally, hydrocarbons are saturated; but because they are less lipophilic, they are the least toxic (Adams et al., 2014). It also contains the aromatic compounds that are hydrophobic which include volatiles such as benzene, toluene, ethylbenzene, and xylenes (BTEX) and the polycyclic aromatic hydrocarbons (PAHs). The most commonly transported oil product is dilbit; which is a combination of bitumen and organic solvents or low viscosity hydrocarbon distillates that help decrease its viscosity. The proportion of these organic solvents change with respect to the season due to the temperatures that may affect the dilbit flow by pipeline. In this study, for example three different petroleum products were used: one a conventional light sweet crude oil from Western Canada (Mixed Sweet Blend, MSW), and two dilbits, the Access Western Blend (AWB) and Cold Lake Blend (CLB). Table 1.1 shows the chemical information of the respective batched used.

Table 1.1 Comparision of selected crude oil properties (based on crude monitor.ca)

Property	**Mixed Sweet Blend (MSW)**	**Cold Lake Blend (CLB)**	**Access Western Blend (AWB)**
Basic Analysis			
Density (kg/m3)	823.4	921.8	914.5
Gravity (oAPI)	40.2	21.9	23.1
Sulphur (wt%)	0.41	3.72	3.73
MCR (wt%)	1.7	9.8	10.3
Sediment (ppmw)	-	127	-
TAN (mgKOH/g)	-	1.05	1.66
Salt (ptb)	-	5.3	-
Nickel (mg/kg)	4.2	55.5	67
Vanadium (mg/kg)	8.4	143	167
Olefins (wt%)	-	-	-
Light Ends Summary (Volume %)			
C3-	0.66	0.07	0.08
Butanes	4.44	1.64	1.06
Pentanes	3.62	7.38	11.75
Hexanes	5.96	5.79	6.67
Heptanes	7.26	3.84	3.61
Octanes	7.27	2.64	2.19
Nonanes	5.95	1.52	1.38
Decanes	2.4	0.75	0.69
BTEX (Volume %)			
Benzene	0.2	0.21	0.19
Toluene	0.63	0.42	0.28
Ethyl Benzene	0.19	0.05	0.02
Xylenes	0.88	0.45	0.22

(Source: Crudemonitor.ca https://www.crudemonitor.ca/crudes/index.php?acr=CL)

The organic compounds derived from dilbit and other crude oils have shown to have harmful biological effects in vertebrates (Philibert et al., 2016). These organic compounds include the PAHs, which are a group of organic compounds formed of two or more benzene rings arranged in different configurations (Mumtaz et al., 1996). PAHs can originate from combustion processes or petroleum products and are widely distributed in the four environmental compartments (lithosphere, atmosphere, hydrosphere, and biota; Baek et al., 1991). More than hundreds of PAHs exist in the environment. PAHs are normally found as complex mixtures (Mumtaz et al., 1996) and Catoggio (1991) classified PAHs as the most toxic within the hydrocarbon families. PAHs are the fraction that are the most concerning in an oil spill, because their related toxicity and/or carcinogenicity to humans and wildlife is well known (Rousseau et al., 2015). Therefore, it is important to understand if oil could be detrimental to wildlife if a spill was to occur. Sixteen PAHs (Figure 1.2) have been identified as priority pollutants by the United States Environmental Protection Agency (USEPA) and World Health Organization (WHO), including benzo-(a)anthracene, chrysene, and benzo[a]pyrene. All sixteen PAHs have been considered potencial toxic in humans and other organisms.

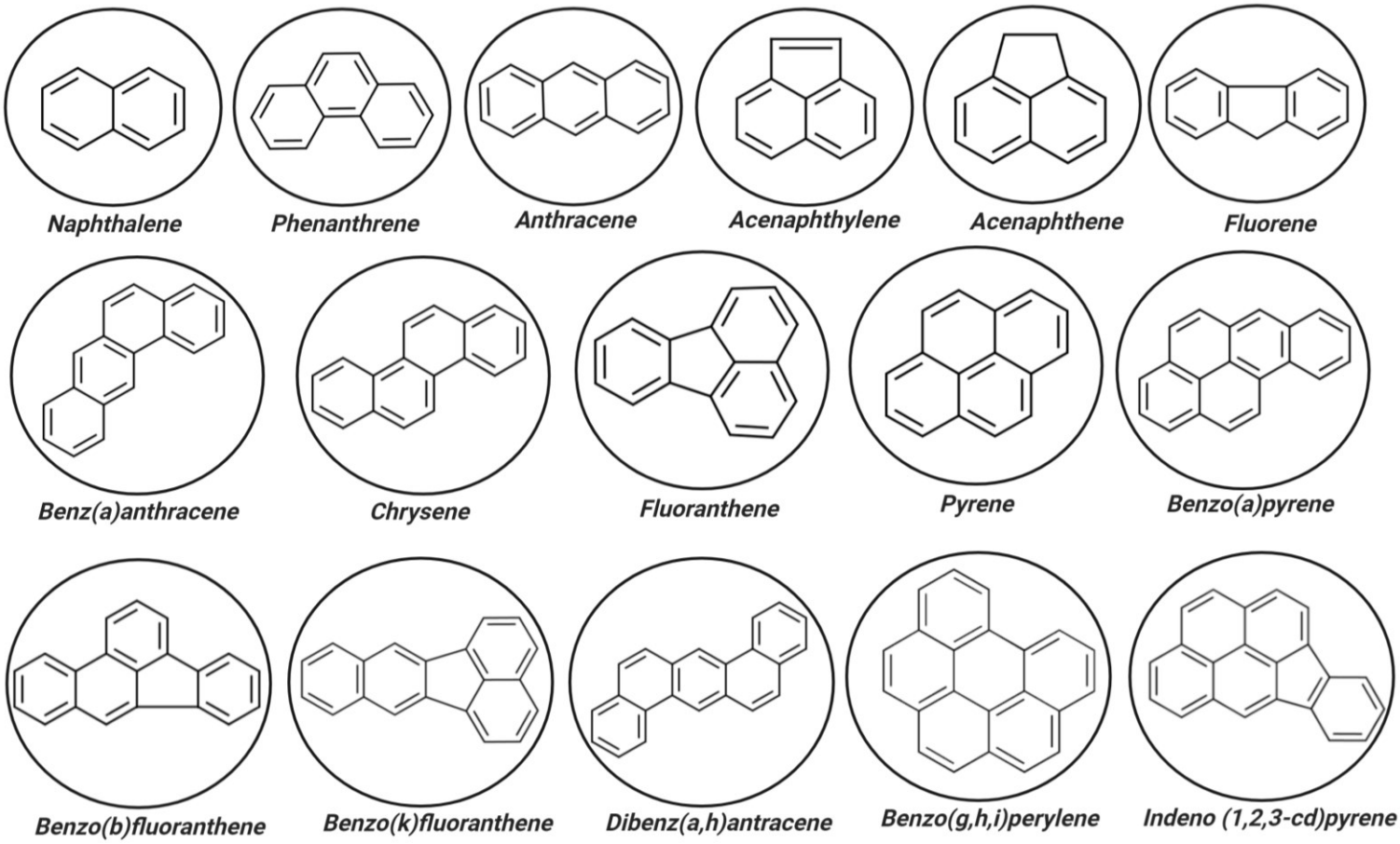

Figure 1.2 Priority polycyclic aromatic hydrocarbons pollutants per the US Environmental Protection Agency (EPA).

1.1.3 Effects of petroleum products in aquatic vertebrate species

Aquatic vertebrates can be exposed to PAHs in their aquatic ecosystems. Once absorbed, PAHs can cause toxicity through several mechanisms. Some PAHs are known to be embryotoxic to fishes undergoing early development (Hodson et al., 2007; Adams et al., 2014). There is a positive relationship between an increase in PAH concentrations and an increase in deformities in fish embryos (Billiard et al., 2008; Boudreau et al., 2009; Incardona et al., 2014). PAH exposure can also cause significant changes the fish behavior (migration, feeding, and mating), in addition to decreasing the swimming capacity, and reducing in responses to alarm (Cohen et al., 2001). PAHs can also cause toxicity following biotransformation to toxic metabolites that can bind to proteins, RNA, and DNA resulting in cell damage, teratogenesis, mutagenesis, or carcinogenesis (Payne et al., 1989). Some PAHs induce genotoxicity by binding to nucleotides which disturbs the three-dimensional structure of DNA and can lead to single or double strand breaks in DNA (Moller and Wallin, 1998; Braithwaite et al., 1999). In addition, PAHs induce blue sac disease (BSD) in fishes, a condition that consists of the accumulation of the metabolic waste and excessive amount of ammonia nitrogen (Billard et al., 2006), which is a known indicator of protein degradation resulting from a complete failure of the system.

Fishes, exposed to contaminated food, water, and sediments (Neff, 1985), readily absorb by PAHs, leading to several toxic mechanisms. For example, drug-metabolizing enzymatic reactions (e.g., oxidations, reductions, hydrolyses and conjugations catalyzed by various enzymes) and immune suppression in the flounder fish (*Pseudopleurnectes americanus*). Payne and Fancey (1989) found that during winter, the number of melanomacrophage centres (MMC) in the liver decreased in flounder exposed to sediment, contaminated with petroleum-originating PAHs.

Because of their unique physiology and habitat requirements, amphibians are often regarded as potentially more vulnerable to changes in their environment than many other vertebrates (Sparling et al., 2000, 2001). There is an increasing number of studies demonstrating the sensitivity of amphibians to pollutants such as inorganic and organic compounds. Amphibians suffer from the spread of toxic substances in the environment (Carey and Bryant, 1995; reviewed in Blaustein et al., 2003). There have been some studies where the toxicity of PAHs, and especially the effect of photo-induced toxicity on amphibian embryos and larvae (Fernandez and L'Haridon, 1992; Hatch and Burton, 1998), damage the DNA because of the ability of certain PAHs to bind nucleotides, disturbing the three-dimensional DNA structure, which can lead to single or double strand breaks in DNA (Moller and Wallin, 1998; Braithwaite et al., 1999). In addition, some studies have shown that the presence of PAHs affects the jelly protection in the eggs of different species of frogs (Marques et al., 2006).

There are several biological indicators of exposure to PAHs (Vasseur and Cossu-Leguille 2003; Downs et al., 2006). Biomarkers can be useful in determining and predicting how xenobiotic compounds affect normal physiological function. Some risk factors for certain diseases or biological lesions can be generated by correlating biochemical indicators of PAHs exposure with the onset of the disease, allowing the use of a relatively simple biomarker to predict risk. Biomarkers can also provide important information on the absorption and metabolism of PAHs, as well as the mechanisms of action of PAHs for inducing diseases. The most effective biomarker of chemical exposure are the ones related to detoxification (Gil and Pla, 2001; Johnson, 2013). One of the most studied biomarker associated to oil exposure is the cytochrome P450 enzyme that is induced by the upregulation of the gene *cyp1a*, a first step in the detoxification process. This mechanism of cellular detoxification is regulated by the aryl hydrocarbon receptor (AhR).

1.1.4 Mechanism of cellular detoxification of PAHs

1.1.5 AhR signaling pathway

The AhR is a protein that is capable of regulating gene expression (a cytoplasmic transcription factor). The inactive AhR (without a ligand) is located in the cytoplasm of the cell as an inactive protein complex belonging to the family of transcription factors with the domain bHLH-PAS (basic helix loop helix-PER ARNT-SIM) (Hoffman et al., 1991). This family of proteins plays an important role in various cellular processes such as development, hypoxia adaptation, circadian cycle control, and xenobiotic metabolism (Gu et al., 2000). Specifically, the AhR is part of a 9S heterodimeric complex. This complex is formed by a dimer of thermal shock protein 90 (Hsp90), a thermal shock protein of a weight of 23 KDa (p23) and an interaction protein with AhR known as XAP2 or Ara9. Additionally, the presence of other cytosolic proteins has, including chaperone (Cdc37) and a tyrosine kinase (c-src) (Enan et al., 1995).

AhR normally has three functional domains. A highly conserved domain bHLH consisting of 4 to 6 acidic amino acids at the amino terminal. This domain has the capability of direct interaction with DNA. The second domain are two repeats of the domains PAS (PAS A and PAS B) each one containing approximately 110 amino acids (Hao et al., 2011). Structural, biochemical and protein interaction analysis suggests that the PAS A domain is related to dimerization while PAS B functions as a signaling domain. The third domain (Q) is a poorly conserved domain located at the carboxyl terminal and is essential in transactivation (Kewley et al., 2004).

The activation of AhR occurs by the binding of a ligand. These ligands are mainly xenobiotics (for e.g., PAHs). But they also can bind to natural compounds (secondary metabolites) endogenous

compounds (bilirubin) (Adachi, J et al., 2001). With the activation of AhR, it translocates to the nucleus and the complex dissociates. Once in the nucleus, the AhR will form a heterodimer with the AhR nuclear translocator protein (ARNT). This AhR-ARNT heterodimer interacts with proteins such as histone acetyltransferases and remodeling factors of chromatin, resulting in the binding of the AhR-ARNT complex to a known DNA sequence (GCGTGA) representing a xenobiotic response element (XRE). This induces the upregulation of genes that produce the enzymes of phase I, represented by members of the CYP1 family of cytochromes P450 (CYP450; Waxman, 1999) (CYP450) and phase II which includes the glutathione S-transferases (GST) and uridine 5-diphosphate-glucuronosyltransferase (UDP-GT). The ability of AhR to bind xenobiotics and activate enzymes for the biotransformation may be exclusive to vertebrates. There have been studies that mention that the AhR in invertebrates is related more to the development process than detoxification (Hahn et al., 2002).

Previously, it was thought that the only role of AhR was to participate in the adaptive response to cellular stress, acting as an endogenous signal sensor or induced by xenobiotics. However, there is evidence that the AhR is related to various cellular processes such as cell proliferation, apoptosis, differentiation, tumor promotion, reproduction, and immune response (Kawajiri & Kuriyama, 2007). This suggests that in addition to be a xenobiotic receptor, AhR is essential in cell homeostasis. However, research about the role of AhR is not well known. On the other hand, the capacity of AhR response differs between species and cell type, which may be indicative of possible interactions with different signaling pathways and the complexity of the role. Because the AhR is involved in various cellular processes, the signaling must be strictly regulated including systems to avoid excessive signaling. The activation of AhR produces an upregulated of enzymes in phase I and II of the xenobiotic metabolism which oxidize and form ligand derivatives,

facilitating their removal through the membrane transporters dependent on ATP (Konig, 1999). Another mechanism by which the cell is able to decrease the signaling is through degradation via proteasome 26 S (Roberts & Whitelaw, 1999). Also, the activation of AhR can also be modulated by the nuclear export signal (NES) present in the AhR. This sequence allows that the activated-AhR (bind AhR), to be exported from the nucleus to the cytoplasm and in that way, avoid induction of gene transcription. On the other hand, the activation of AhR upregulates the transcription of the aryl hydrocarbon receptor repressor (AhRR). The AhRR protein dimerizes with the ARNT and compete with the AhR to bind to the xenobiotic responsive elements. This results in a negative feedback mechanism that involves downregulation of all genes regulated by the AhR transcription factor which positively regulates the expression of CYP1A (Cauchi et al., 2003) (Figure 1.3).

1.1.6 Example of the activation of the AhR by a PAHs: benzo[a]pyrene

One of the most studied xenobiotics capable of activating AhR and inducing the signaling pathway is the PAH benzo[a]pyrene (B[a]P). This compound was the first carcinogen detected in tobacco smoke and was one of the most studied carcinogens during the last century (Osborn & Crosby, 1987). The main sources of B[a]P to the environment and humans are charcoal-cooked meats, fossil fuel burning, wood burning, and tobacco smoke (Hecht, 2002).

With exposure, B[a]P can be distributed to organs such as the liver, kidney, and blood. In particular, the lipophilic nature of B[a]P favors its storage in fatty tissues including mammary glands and bone marrow (Kelman & Springer, 1982). B[a]P is also known to cross the blood-brain barrier and placenta. Although B[a]P can be metabolized in various organs, B[a]P metabolites have been reported to be primarily concentrated in the lung. The epithelial cells of the respiratory tract are the first to come into contact with the B[a]P particles present in the environment through

airborne expsure. It is estimated that a smoker may be exposed to average concentrations of 20-40 ng of B[a]P per cigarette (Aleandrov et al., 2000).

The metabolism of B[a]P is of particular importance because although it is not toxic by itself, its carcinogenic effect resides in the formation of primary and potentially toxic secondary metabolites through the AhR signaling pathway and xenobiotic metabolism (Schmidt et al., 1996). These metabolites are capable of interacting with cellular constituents and inducing cellular damage.

In the first phase, B[a]P is oxidized by CYP1A1 and produces an epoxide (B[a]PE) as shown in Figure 1.4 (Lavin et al., 1976). The second phase consists of conjugating the B[a]PE generated in phase I with polar molecules, thus avoiding their interaction and possible damage to cellular constituents such as DNA. Phase II enzymes that catalyze conjugation have different specificity because of their substrates. The level of expression depends on the type of tissue and developmental stages of the organism. On the other hand, they may be inducible or inhibited by xenobiotics (Rushmore & Kong, 2002). Several enzymes involved in phase II have been described, e.g. glutathione S-transferases (GSTs), UDP-glucuronosyltransferases (UGTs), sulfotransferases (SULTs), prostaglandin H synthase (PHS) (Weinshilboum et al., 1997).

GST is the main enzyme involved in phase II (Weng et al., 1995). The pattern of distribution and expression of the GST is complex; even the different cellular types present in the same tissue have different patterns of expression. Likewise, patterns of interindividual distribution may also vary. For example, in humans, the expression patterns of GST in the lung may vary due to the presence of polymorphisms in genes encoding enzymes (Sherratt et al., 1997). The GST catalyze the conjugation with B[a]PE, which generally increases the hydrophobicity of B[a]PE and therefore

increases its excretion through bile or urine. This mechanism is considered the most important way of inactivation for B[a]PE (Robertson et al., 1986).

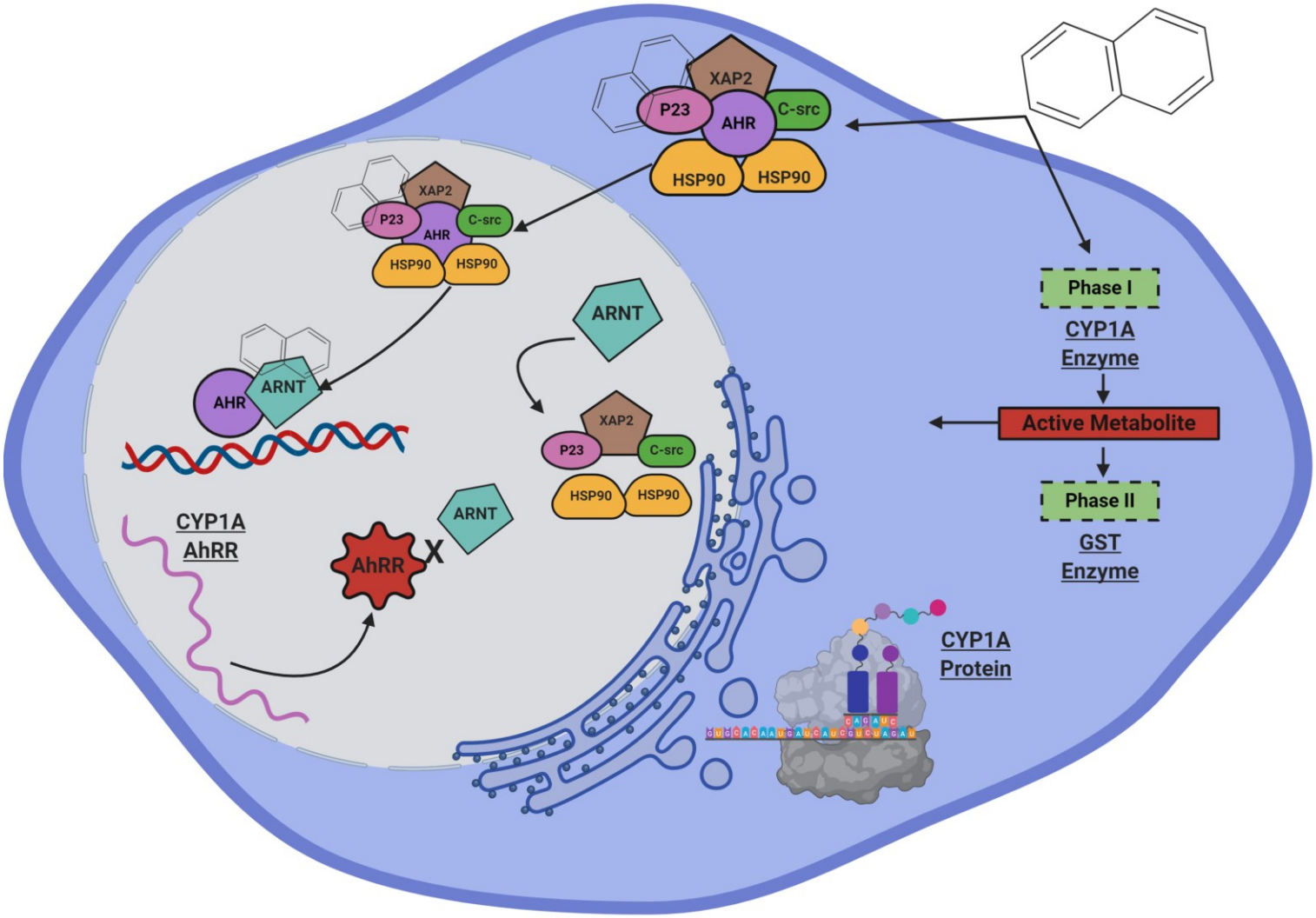

Figure 1.3 Induction of the aryl hydrocarbon receptor (AhR) pathway by a PAH, and activation of the ARNT and induction of the CYP450 enzymes. The PAH will enter the plasma membrane through passive diffusion and in the cytoplasm binds to the AhR (PAS B domain), this allows the translocation of the ligand-receptor complex into the nucleus and the dissociation of the receptor complex. In the nucleus, the complex heterodimerizes with its partner ARNT finding the DNA sequences location and bind to this region.

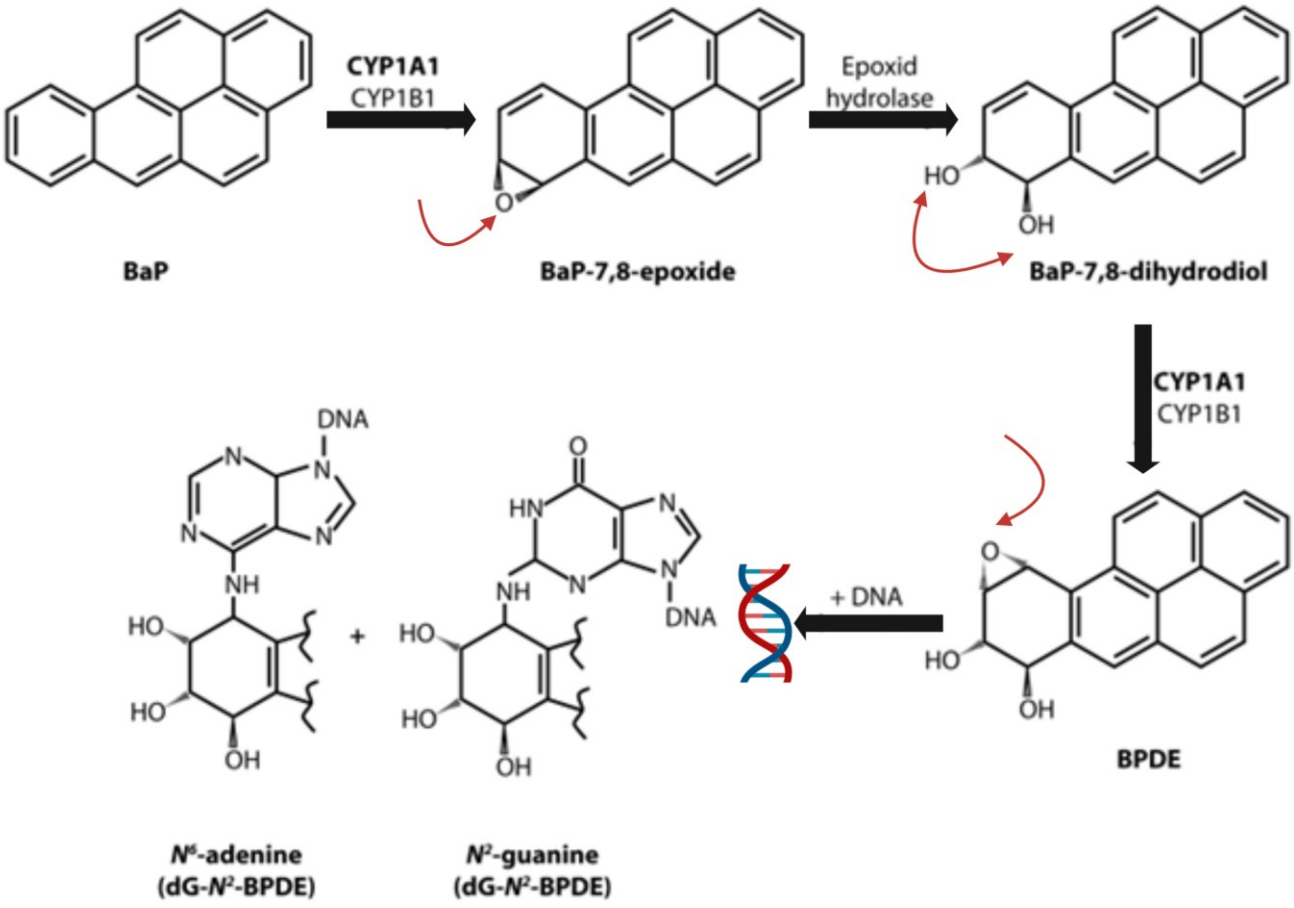

Figure 1.4 Metabolic pathway of benzo[a]pyrene (B[a]P), benzo[a]pyrene-7, 8-dihydrodiol-9,10-epoxide (BPDE), glutathione S-transferases (GSTs), UDP-glucuronosyltransferases (UGTs), sulfotransferases (SULTs), prostaglandin H synthase (PHS) (figure adapted from Lee & Yang, 2008).

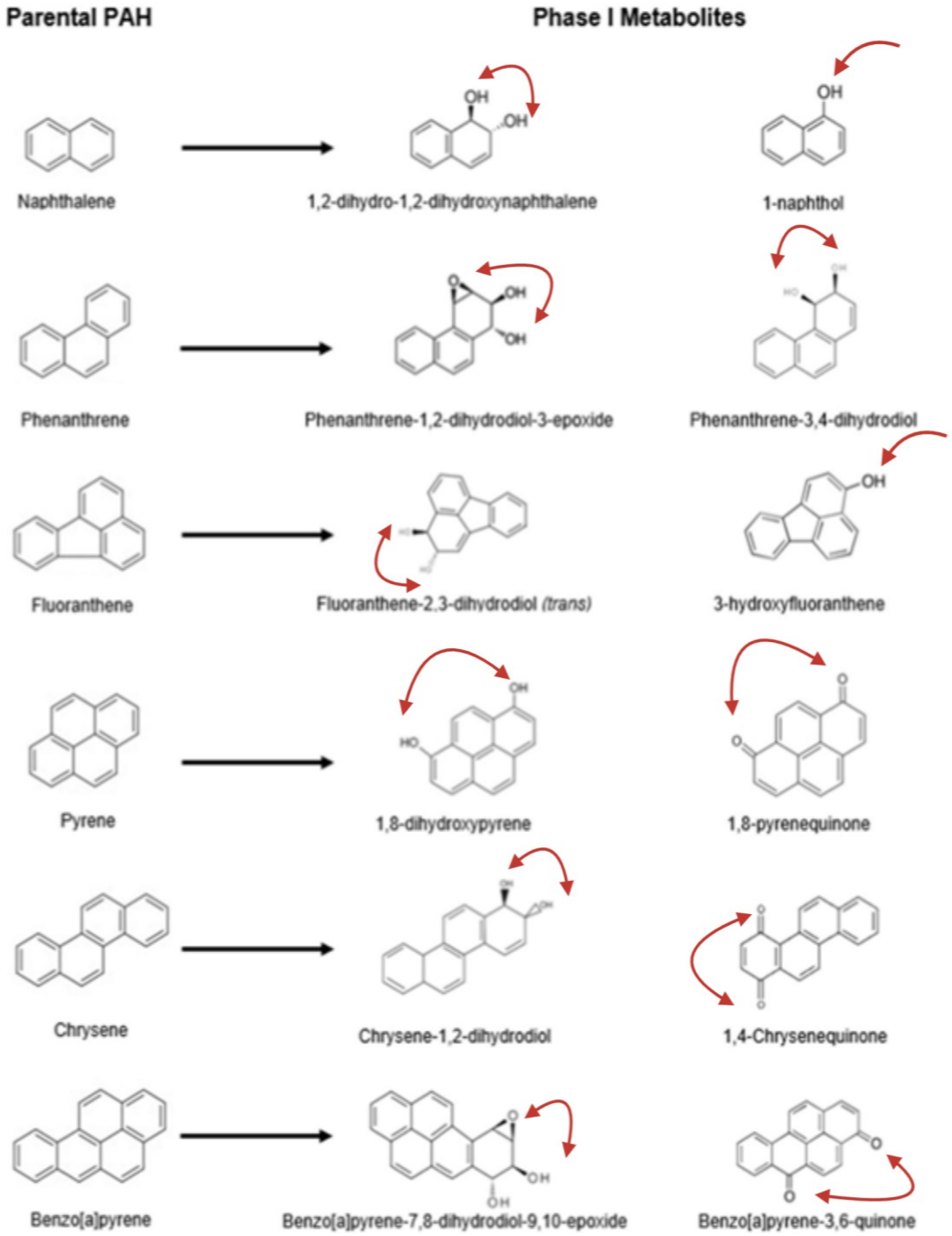

Figure 1.5 Examples of six polycyclic aromatic hydrocarbons (PAHs) enlisted by the U.S Environmental Protection Agency as priority pollutants, and representative metabolites formed during phase I (CYP1A-mediated) biotransformation (figure adapted from Franco & Lavado, 2019).

1.2 Model organisms

1.2.1 Amphibian model: *Silurana tropicalis*

Amphibians are phylogenetically well positioned for comparasions to other vertebrates like mammals, birds and reptiles. The genus Xenopus (meaning “strange foot”) include more than 20 species of frogs all of them native from the African continent (Hopkins, 2007). The Western African clawed frog (*Silurana tropicalis*), belongs to the Pipidae family and is also know as the tropical clawed frog (Gray, 1864). It has been recognized as a model animal, because of its advantage for molecular studies. Has a small genome, about 1.7 Gbp on 10 chromosomes, more than 20,000 protein-coding genes express homology with many human genes (Hellsten et al., 2010) for example the protein Shq1p, that is a protein involved in the rRNA processing pathway (Yang et al., 2002). *S. tropicalis* has a shorter generation time, mature in only 4 months (Mitsui et al., 2005). Moreover, the genome of *S. tropicalis* was published in 2010. Because of their unique physiology and habitat requirements, amphibians are often regarded as potentially more vulnerable to changes in their environment than many other vertebrates (Sparling et al., 2001). This vulnerability related to the ecological aspect; because in the early 1990’s was the first time that amphibians population were recognized in declined (Wake, 1991).

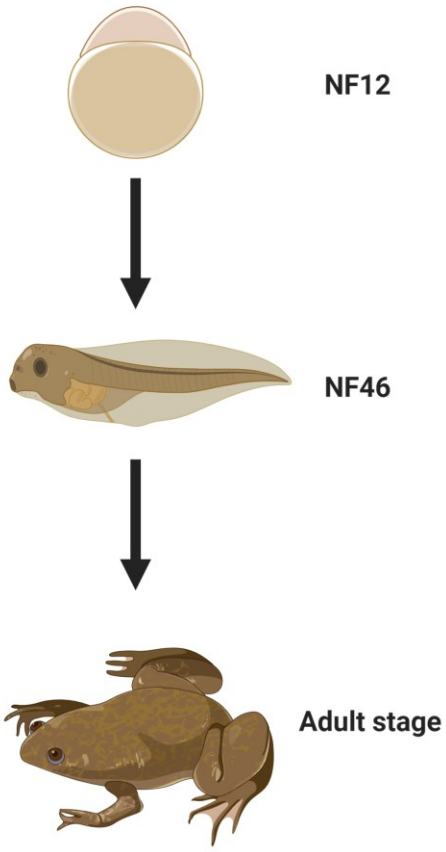

Figure 1.6 Photographs of the different stages of the *Silurana tropicalis* based on Nieuwkoop & Faber (1994). Stages Nieuwkoop & Faber (NF) NF12 and NF46 as well as the adult stage are shown.

1.2.2 Fish model: *Pimephales promelas*

Fathead minnow (*Pimephales promelas*) belongs to the family Cyprinidae, the carps and minnows. Is a dominant freshwater family with 44 species of the family that are endemic of Canada (EC BTM, 2011). FHM show sexual dimorphism in adults where the males fish length approximately 7 cm would weight about 3.5 to 5 g and female fish of 5 to 6 cm would weight about 1.5 to 2 g (Benoit and Carlson, 1977; Korver and Sprague, 1989), also the males exhibit tubercles on the snout (Alderton, 2005). FHM has been used in ecotoxicological studies to test chemical compounds and mixtures toxicity to fish egg, larvae, and during its full life cycle (Ankley and Villeneuve, 2006; Belanger et al., 2013; Marentette et al., 2014). In the last decade, there has been a focus on embryonic studies for toxicology studies (Bolser et al., 2018). Because of its tolerance to a wide range of water characteristics (pH, hardness, and temperature), *P. promelas* is a useful model in aquatic toxicology research (Mount, 1973). This species has a well-defined reproductive and developmental (larval and adult) cycle and genomic resources are also available for this species (Devlin et al., 1996). In February 1992, Environment Canada published a 7-day test for measuring the tocix effects of environmental contaminants on the growth and survival of larval fathead minnows (EC, 1992; EC BTM, 2011). Lastly, the natural geographic range extends throught North America (from central Canada to north of Mexico) (Crissman and Scott, 1998).

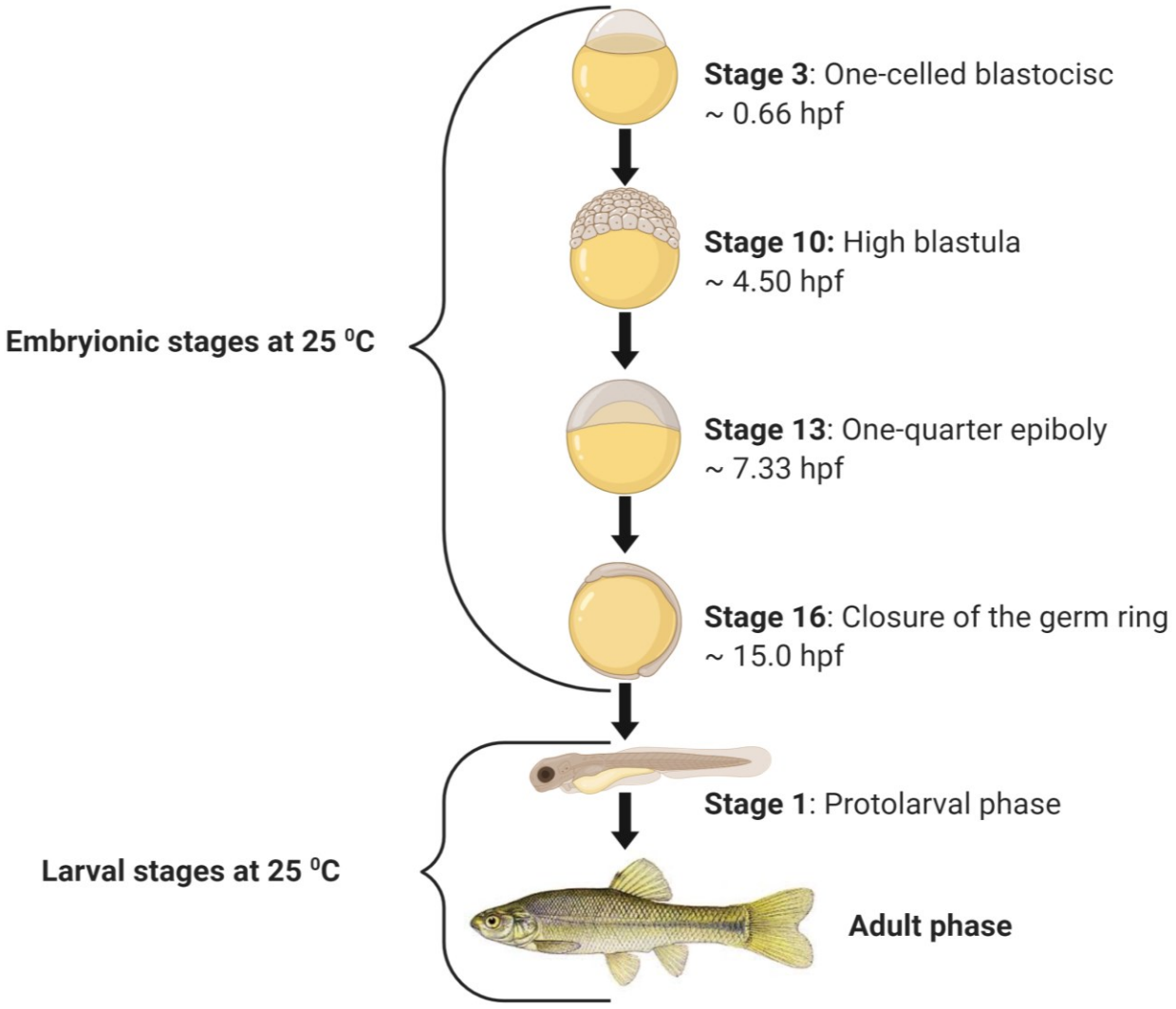

Figure 1.7 Development of fathead minnow (*Pimephales promelas*) based in Devlin et al., (1996).

1.3 Research objectives, hypotheses, and chapter presentation

1.3.1 Overarching research objectives

This thesis encompasses four overaching research objectives that will help to understand the effects of unweathered and/or weathered crude oil and dilbit on two aquatic species.

1. Determine the effects of two unweathered dilbits (AWB and CLB) prepared in the laboratory as WAF and as CEWAF in the frog embryos of *S. tropicalis*.
2. Characterize the effects of weathered crude oil (MSW) prepared in a wave tank at 15 °C in the fish embryos of *P. promelas.*
3. Analyze the effects of weathered CLB prepared in a wave tank at 2 °C and 15 °C in the fish embryos of *P. promelas*.
4. Compare the embryotoxicity of weathered crude oil vs dilbit in fish embryos.

This doctoral thesis aims to understand the differential toxicity of weathered vs unweathered oil to aquatic species. Simulated weathering of oil was accomplished by adding oil to a wave tank, which mimics limnic conditions. The comparative toxicity of the oil fraction in the water was then evaluated at different points (day points). In assessing these results, the observation that environmental conditions may influence oil toxicity raised further questions.

1.3.2 Specific research objectives, hypotheses, and chapters presentation

As there is a lack of data on the effects of PAHs in amphibians, in **Chapter 2**, we studied the embryotoxicity of two unweathered dilbits, AWB and CLB, in frog larvae. The specific research objectives were to compare the acute toxicity of AWB and CLB in *S. tropicalis* and to assess the effects of frog exposure to AWB and CLB on the mutagenic response and detoxification pathways. The hypotheses were that exposures of *S. tropicalis* embryos to WAF and CEWAF of AWB and

CLB dilbits will increase the prevalence of malformations, that CLB will yield a higher rate of malformed frog larvae than in AWB due to the higher concentration of alkyl-PAHs in CLB; and that frog embryos will express high *cyp1a* mRNA levels once in presence of PAHs.

For **Chapter 3**, using a lake wave simulator tank, we tested differential toxicity associated with crude oil as its weathers in fish. The specific research objectives were to compare fish embryotoxicity of weathered crude oil with time and to assess the effects of weathered crude oil on fish mutagenic and detoxification responses. The hypotheses were that exposure of *P. promelas* embryos to MSW will increase the prevalence of malformations (including blue sac disease); will yield a lesser rate of malformations with time; and will significantly increase mRNA levels in the fish embryos.

In **Chapter 4**, we analyzed if different environmental conditions (i.e., different air and water temperatures) would alter the intrinsect toxicity associated to weathered CLB dilbit in fish. The specific research objectives were to compare the toxicity of weathered CLB between two environmental condition settings (simulation of summer and winter temperature) in fish embryos and to compare weathered conventional crude oil toxicity (from Ch. 3) to weathered dilbit toxicity (from Ch. 4). The hypotheses were that exposure of *P. promelas* embryos to CLB summer condition will increase the prevalence of malformations in comparasion to winter conditions and that weathered crude oil and dilbit will yield comparable toxicity to fish.

Finally, I wrote my general discussions and final conclusions in **Chapter 5**.

CHAPTER 2: CYTOCHROME P4501A TRANSCRIPT A SUITABLE BIOMARKER FOR BOTH EXPOSURE AND RESPONSE TO DILUTED BITUMEN IN THE DEVELOPING FROG EMBRYO

LES TRANSCRITS DU CYTOCHROME P4501A SONT UN BIOMARQUEUR APPROPRIÉ À LA FOIS POUR L'EXPOSITION ET LA RÉPONSE AU BITUME DILUÉ CHEZ L'EMBRYON DE LA GRENOUILLE

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Article published in **Environmental Pollution**, 246, 501-508 (2019)

https://doi.org/10.1016/j.envpol.2018.12.039

Contributions:

Writing: LRL-J, BW, SJW, VSL

Exposure and molecular analyses: LRL-J, BW, SJW

Data analysis: LRL-J

Original ideas and experimental design: LRL-J, SJW, VSL

2.1 Abstract

In order for Alberta's thick bitumen to be transported through pipelines, condensates are added creating a diluted bitumen (dilbit) mixture. Recent pipeline expansion projects have generated concern about potential dilbit spills on aquatic wildlife health. Studies have suggested that polycyclic aromatic hydrocarbons (PAHs) are toxic to aquatic vertebrates and could potentially also interfere with their endocrine system. The research objectives of this study were to investigate the toxicity of dilbit to the developing frog embryos and to identify the molecular mechanisms of action involved. Fertilized embryos of Western clawed frog (*Silurana tropicalis)* were exposed for 72 h to water-accommodated fractions (WAF; 0.7 to 8.9 µg/L TPAHs) and chemically-enhanced WAFs (CEWAF; 0.09 to 56.7 µg/L TPAHs) of Access Western Blend (AWB) and Cold Lake Blend (CLB) dilbits. Both dilbit's CEWAFs significantly increased embryonic mortality and malformation incidence in the highest treatments tested, while WAF treatments led to no observable toxic effects. Increases of the cytochrome P450 1A (*cyp1a*) mRNA levels were observed for all dilbit treatments suggesting that phase I detoxification is activated in the dilbit-exposed larvae. When exposed to PAH concentrations ranging from 0.09 to 8.9 µg/L, the frogs displayed no observable malformation, but expressed significant increases of *cyp1a* mRNA levels (2- to 25-fold; suitable biomarker of exposure), while when concentrations were of 46.6 µg/L or higher, both malformed frog phenotype and induction *cyp1a* mRNA level (over 250-fold) were measured (suitable biomarker of response). The expression of several genes related to cellular detoxification and endocrine disruption were measured, but were not significantly altered by the treatments. In sum, *cyp1a* mRNA level is a highly sensitive endpoint to measure subtle molecular changes induced by PAH exposure in the frog embryos and larvae, and data suggest that PAH concentration higher than 46 µg/L would be toxic to the developing *S. tropicalis* embryos.

2.2 Résumé

Pour que le bitume de l'Alberta soit transporté par pipelines, des condensats de gaz sont ajoutés, créant ainsi un mélange de bitume dilué (dilbit). De récents projets d'expansion de pipelines ont suscité des inquiétudes quant aux déversements potentiels de dilbit sur la santé de la faune aquatique. Des études ont suggéré que les hydrocarbures aromatiques polycycliques (HAP) sont toxiques pour les vertébrés aquatiques en plus d'interférer sur leur système endocrinien. Les objectifs de recherche de cette étude étaient d'étudier la toxicité du dilbit pour les embryons de grenouilles en développement et d'identifier les mécanismes d'action moléculaires sous-jacents Des embryons fertilisés du xénope tropical (*Silurana tropicalis*) ont été exposés pendant 72 h à des fraction accommodee à l'eau (WAF; 0,7 à 8,9 µg/L HAP totaux) et à des WAF chimiquement améliorés (CEWAF; 0,09 à 56,7 µg/L HAP totaux) constituées du dilbit Access Western Blend (AWB) et Cold Lake Blend (CLB). Les deux traitements CEWAF des deux dilbits ont augmenté de manière significative la mortalité embryonnaire et l'incidence des malformations dans les traitements les plus élevés, tandis que les traitements WAF n'ont entraîné aucun effet toxique observable. Des augmentations des niveaux d'ARNm du cytochrome P450 1A (*cyp1a*) ont été observées pour tous les traitements de dilbits suggérant que la détoxification de phase I est activée chez les larves de grenouilles exposées au dilbit. Lorsqu'elles sont exposées à des concentrations de 0,09 à 8,9 µg/L HAP totaux, les embryons de grenouilles ne présentent aucune malformation observable, mais expriment des augmentations significatives des niveaux d'ARNm de *cyp1a* (de 2 à 25 fois plus élevés que le témoin), ce qui indique que ce gène est un biomarqueur d'exposition approprié pour les larves de grenouilles. Également, lorsque les concentrations de dilbits étaient plus élevées, soit environ de 46,6 µg/L ou plus de HAP totaux, le phénotype de la grenouille malformée ont été observé. Ces animaux exprimaient aussi de très hauts taux d'induction de

l'ARNm de *cyp1a* (plus de 250 fois celui du témoin), ce qui indique que ce gène peut également être approprié pour indiquer la réponse de l'individu au contaminant. L'expression de plusieurs gènes liés à la détoxification cellulaire et à la perturbation endocrinienne a été mesurée, mais n'a pas été significativement altérée par les traitements. En somme, le niveau d'ARNm de la *cyp1a* est un paramètre très sensible pour mesurer les changements moléculaires subtils induits par l'exposition aux HAP dans les embryons et les larves de grenouilles, et les données suggèrent qu'une concentration de HAP supérieure à 46 µg/L serait toxique pour les embryons de *S. tropicalis* en développement.

2.3 Introduction

Following Saudi Arabia and Venezuela, Canada has the largest oil reserves, and distributes about three million barrels of oil via pipeline every day (Dew et al., 2015). Canada also has the largest bitumen deposits worldwide (Yang et al 2011). This oil needs to be transported, either by tanker truck, railway, marine tanker, or pipeline. As of 2016, the Canadian Energy Pipeline Association (CEPA) managed 119,000 km of transmission pipelines, which carry diluted bitumen (or dilbit), crude oil, and natural gas (CEPA, 2016); these pipelines make up an extensive network that traverses freshwater, marine, and terrestrial ecosystems. It is therefore crucial to further our limited understanding the implications of a pipeline oil spill to freshwater environments (reviewed in Alsaadi et al., 2018). Bitumen's high viscosity prevents it from being transported via pipeline unless it is diluted (GOC, 2013) to a composition of about 20-30% diluent and 70-80% bitumen (Crosby et al., 2013). Diluents used to produce dilbit from bitumen include natural gas condensate and naphtha, although condensates are more commonly used (Lee et al., 2015). Alberta's dilbits contain many polycyclic aromatic hydrocarbons (PAHs), alkyl-polycyclic aromatic hydrocarbons (alkyl-PAHs), and some n-alkanes (Yang et al., 2011).

It is well established that PAHs, especially 3-5 ringed alkyl-PAHs, are embryotoxic to fish species (reviewed in Hodson 2017). For instance, retene, a labile alkylated phenanthrene PAH, has been found to be toxic to larval zebrafish (*Danio rerio*) and rainbow trout (*Oncorhynchus mykiss*; Billiard et al., 1999). After 14 days of exposure, zebrafish exhibited stunted growth, edema, and mortality. Similarly, rainbow trout were exposed to retene for 42 days and showed symptoms of edema, stunted growth, craniofacial malformations, increased activity of cytochrome P450 1A (CYP1A) enzymes, and blue sac disease. Recent studies have investigated the effects of dilbit in fish species. Madison and colleagues (2015; 2017) exposed Japanese medaka (*Oryzias latipes*)

embryos to WAF and CEWAF of Access Western Blend (AWB) and Cold Lake Blend (CLB) dilbits from fertilization to hatch (maximum of 17 days). They found the greatest increase in the expression of *cyp1a,* as well as upregulation of genes such as the tumour protein (*p53*), superoxide dismutase (*sod*), glutathione reductase (*gsr*), heat shock protein 70 (*hsp70*), and/or nuclear factor erythroid-2 (*nfe2*). Similarly, significant cues of *cyp1a* transcript levels were found in other fish species following dilbit exposure, including yellow perch (*Perca flavescens*; McDonnell et al., 2019), fathead minnow (*Pimephales promelas*; Alsaadi et al., 2018), and sockeye salmon (*Oncorhynchus nerka*; Alderman et al., 2017).

Although the effects of dilbit have been studied in fish, nothing is known about its effects in frogs, and only few studies have investigated the effects of PAHs or related compounds in amphibians. Stabenau and colleagues (2006) found that Northern leopard frogs (*Lithobates pipiens*) exposed to naphthalenes experienced disruption in normal gas exchange. Benzo[a]pyrene (BaP) increased the frequency of erythrocyte micronuclei in two South America frogs (*Physalaemus cuvieri* and *Leptodactylus fuscus*; Fanali et al., 2018). Some studies have showed an additive toxicity of photo-induced PAHs in amphibian larvae (Fernandez and L'Haridon, 1992; Hatch and Burton, 1998). In addition to toxicity, a transcriptomic analysis in liver of adult Western clawed frogs (*Xenopus (Silurana) tropicalis*) exposed to 10 µg/L benzo[a]pyrene resulted in disrupted gene regulation of steroid biosynthesis pathways among other metabolic disorder related pathways (Regnault et al., 2014). Similarly, Truter et al., (2016) showed that weathered bunker and unweathered refinery crude oil water accommodated fractions (WAFs) could disrupt frog's normal endocrinology. The expression of thyroid hormone receptor beta and adipogenesis-linked peroxisome proliferator–activated receptor gamma were significantly down-regulated in African clawed frogs (*Xenopus laevis*) exposed to 1% crude oil WAF (0.25 g/L; Truter et al., 2016).

Together, data provide evidence that components of oil may lead to adverse health effects in amphibians as a result of increased genotoxicity and altered endocrine signaling.

This study aimed to understand the mechanisms of toxicity of dilbit in early frog development. Water accommodated fractions (WAFs) and chemically-enhanced WAFs (CEWAFs) were performed using the most transported dilbits in Canada, AWB and CLB. Although dispersants are not permitted for use in freshwater environments, Corexit® 9500A was used in this study to improve PAH's dispersion to the water column allowing for testing of higher PAH concentrations for LC50 and EC50 determination. At the individual level, survival and malformation endpoints were assessed, while the expression of several genes involved in cellular detoxification (*cyp1a*, *gsr*, *gpx*, *gst, sod, cat, ahr,* and *arnt*), mutation/cell cycle (*p53*), and in reproduction and development (*erα, ar, srd5α1, srd5α2, srd5α3, dio1, dio2, dio3, thrα, and thrβ*) were measured. Understanding the effects of dilbit in amphibians will assist in risk management and response of frog populations in case of oil spills to freshwater ecosystems.

2.4 Materials and methods

2.4.1 Animal husbandry

Western clawed frog (*S. tropicalis*) embryos were collected from our colony kept at Queen's University, Kingston ON, Canada according to the animal care protocol Langlois 2015-1584. All animal care procedures were performed in adherence with Queen's University's Animal Care Committee and the guidelines of the Canadian Council on Animal Care. Embryos were obtained following an established breeding protocol (Langlois et al., 2010). Briefly, six pairs of frogs from the colony were injected with 40 µL human chorionic gonadotropin (hCG; Sigma-Aldrich,

Oakville, ON) 24 h prior to mating and 160 µL hCG on the mating day. Approximately 5,000 fertilized eggs were carefully collected. When the eggs reached the stage NF8 (Nieuwkoop and Faber 1994) at 5 h post-fertilization, they were dejellied in 2% w/v L-cysteine (Sigma-Aldrich Canada, Oakville, ON), then rinsed with a rearing solution of the Frog Embryo Teratogenesis Assay-Xenopus (FETAX; ASTM International, 2004) and assigned randomly to each treatment.

2.4.2 Preparation of solutions

Access Western Blend (AWB) dilbit, Cold Lake Blend (CLB) dilbit, and the dispersant Corexit® EC9500a were obtained from Thomas King, Department of Fisheries and Oceans Canada (Centre for Off-shore Oil, Gas and Energy Research, Dartmouth, NS). The stock was stored in airtight containers at 4 °C. For the exposures, stock fractions of AWB and CLB WAF and CEWAF were modified following methods of Madison et al., (2017) and Singer et al., (2000), using a 1:9 ratio of dilbit to FETAX (ASTM International, 2004; per liter of deionized water: 625 mg NaCl, 96 mg $NaHCO_3$, 30 mg KCl, 15 mg $CaCl_2$, 60 mg $CaSO_4 \cdot 2H_2O$, and 75 mg $MgSO_4$ at pH 7.6-7.9). Stock fractions were mixed via a magnetic stirrer for 18 h. Corexit® 9500a (DOR 1:10) was added to the CEWAFs and stirred for an additional hour; all were left to settle 1 h prior to taking fractions. The fractions were extracted from beneath the top oil layer with two 60 mL syringes per jar of stirred dilbit and FETAX. The fractions were further diluted with FETAX for the following nominal dilutions: WAF (10, 30, and 50% v/v) and CEWAF (0.01, 0.1, 1, 5, and 10% v/v) from the two dilbit blends. The diluted fractions were made in 200 mL and divided equally into four glass jars each containing 50 randomly embryos, with four replicates for each concentration. Fractions were prepared fresh daily, with the addition of 0.1% v/v of the antibiotic gentamicin (0.04 mg/L; Sigma-

Aldrich, Oakville, ON). The embryos were exposed for 72 h from stage NF12 to stage NF46 (Nieuwkoop and Faber 1994) at the onset of feeding.

A negative control (FETAX only) and a 1% dispersant control with non-toxic mineral oil (Nujol, Sigma-Aldrich, Oakville, ON) were included to the experimental design. To prepare the dispersant control, Nujol was mixed with FETAX for 18 h (1:9 FETAX to Nujol) and dispersed with Corexit® EC9500a at a dispersant to oil ratio (DOR) of 1:10 for 1 h, using the same procedure as above. Two additional controls were added to the experimental design, which were two dilutions (0.1% and 1% v/v) of the dispersant alone (without Nujol) also prepared by adding Corexit® EC9500a to FETAX and stirred for 1 h followed by a settling period of 1 h.

2.4.3 Embryo collection and phenotypical measurements

Jars were checked for mortality at 24, 48, and 72 h at the time of static daily renewal of the fractions. Dead embryos were recorded. When the embryos reached NF46, the exposure was completed. Embryos were randomly selected from the four replicate jars for either malformation or gene expression analysis. For malformation analysis, embryos were pooled into 3 jars of 20 embryos (60 embryos per treatment) with formalin (10% v/v, Fischer Scientific, Ottawa, ON). For gene expression analysis, embryos were pooled into 10 samples of 10 embryos each per treatment (80 embryos per treatment) and kept on dry ice before storing at -80 °C for further analysis.

Malformation analysis and embryo full body length measurement were performed using a Nikon SMZ18 stereomicroscope (Nikon Canada, Mississauga, ON). Embryos were photographed and scored for presence of malformations of the gut, head, spine, eye, and for optic and abdominal edemas via a double-blind analysis, using guidelines from The Atlas of Abnormalities (Bantle et al., 1998). Embryos were considered “malformed” if they exhibited at least one malformation.

2.4.4 Analyses of test solutions

Test solutions were prepared independently from the exposure fractions due to equipment limitations. The test solutions were analyzed for total polycyclic aromatic hydrocarbons (TPAHs) by gas chromatography-mass spectrometry (GC-MS) including nine classes of PAHs, totaling 49 PAHs in WAF 10, 30, and 50% and CEWAF 0.1, 1, and 5% water samples (AGAT Laboratories, Edmonton, AB) for both AWB and CLB. In addition, these same test solutions were analyzed for total petroleum hydrocarbons via fluorescence spectroscopy (TPH-F) with a QMI Fluorescence Spectrometer (Photon Technologies International, Kingston, ON) following methods of Madison et al., (2017). The linear relationship between the TPAH concentration obtained by GC-MS and the TPH-F area obtained from fluorescence analysis of the test solutions (Fig. 2.1) was used to estimate the PAH concentration from the TPH-F measured daily from samples of all the treatments (for details, refer to Supplementary Information, Table S2.1).

2.4.5 Gene expression analysis

RNA was isolated from embryo tissue (n = 10 pooled frog larvae per sample; 10 samples per treatment) using the e.Z.N.A Total RNA mini kit (Omega Bio-Tek, Norcross, GA, USA) following the manufacturer's protocol. Samples were homogenized using a Retch MM400 mixer mill (Roche, Mississauga, ON) for 1 min at 20 Hz. The Ambion Turbo DNA-free Kit (Fisher Scientific, Ottawa, ON) was used to eliminate any DNA from the isolated RNA following manufacturer's protocol. The purity and concentration of isolated RNA was assessed using a spectrophotometer (Nanodrop-2000, Fisher Scientific, Ottawa, ON). GoScript Reverse Transcriptase System (Promega, Madison, WI, USA) was used to convert the RNA to complementary DNA (cDNA) with 1 µg RNA input and random primers. A no-reverse transcriptase control was also prepared.

Each cDNA sample was diluted to 1:40 and 1:80 for optimized quantitative polymerase chain reaction (qPCR) analysis.

Relative mRNA levels of target genes (Table 2.1) were assessed using a CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories Inc., Mississauga, Ontario) following MIQE guidelines (Bustin et al., 2009). GoTaq qPCR Mastermix (Promega, Madison, WI, USA) containing Taq polymerase, nucleotides, double-stranded binding Bryt green fluorescent dye, $MgCl_2$, and reaction buffer was mixed with the appropriate forward and reverse primers for each target gene (Table 2.1). A standard curve was prepared in duplicate on each plate with serial-diluted pooled cDNA to create a calibration curve with a dilution factor of four (50 - 0.0048 ng). Each plate contained a no-template control and a no reverse transcriptase control to assess contamination levels. Each sample was run in duplicate, with six to eight replicates per treatment that were divided evenly across four plates per target gene. The thermocycler profile consisted of 35 cycles of heating and cooling: 95 °C to activate Taq polymerize and denature cDNA strands, and a gene-dependent optimized temperature for primer annealing and elongation (Table 2.1). Standard curve efficiencies of 90.1-109.9% and R^2 values of 0.985-0.999 were considered optimized. Gene expression analysis was performed for two normalizing genes (ribosomal protein L8 (*rpl8*) and ornithine decarboxylase (*odc*)). Target genes cytochrome P450 1A (*cyp1a*), aryl hydrocarbon receptor (*ahr*), aryl hydrocarbon receptor nuclear translocator (*arnt*), catalase (*cat*), superoxide dismutase (*sod*), glutathione S-reductase (*gsr*), glutathione S-transferase (*gst*), glutathione peroxidase (*gpx*), tumor protein P53 (*p53*), androgen receptor (*ar*), estrogen receptor alpha (*erα*), deiodinase iodothyronine type I (*dio1*), deiodinase iodothyronine type II (*dio2*), deiodinase iodothyronine type III (*dio3*), steroid-5-alpha-reductase alpha polypeptide 1 (3-oxo-5 alpha-steroid delta 4-dehydrogenase alpha 1) (*srd5α1*), steroid-5-alpha-reductase alpha

polypeptide 2 (3-oxo-5 alpha-steroid delta 4-dehydrogenase alpha 2) (*srd5α2*), steroid-5-alpha-reductase alpha polypeptide 3 (3-oxo-5 alpha-steroid delta 4-dehydrogenase alpha 3) (*srd5α3*), thyroid hormone receptor alpha (*thrα*), and thyroid hormone receptor beta (*thrβ*) were normalized to the average transcript level of *rpl8* and *odc* to determine the relative fold change.

2.4.6 Statistical analyses

Normalized fold change values were considered outliers if they were 1.5 times outside of the interquartile range and removed from the analysis. All statistical analyses were performed in Prism (v.7, GraphPad). mRNA levels were transformed using the logarithm base 10 for all genes of interest (except *gsr*, *arnt*, *sod*, *dio2,* and *srd5α1* with square root transformation) to adhere by the assumptions of normality (Shapiro-Wilk normality test) and equal variances (Brown-Forsythe test). Two-way ANOVA was used to determine significant differences in mean mortality, mean malformation percentage, full body length, and gene expression fold change for each treatment by each oil blend. Since there were no differences in the percent mortality or malformed embryos between oil blends, embryos exposed to AWB and CLB were combined for regression analysis of WAF and CEWAF treatments to increase statistical power for the malformation and gene expression analysis.

Table 2.1 A list of *S. tropicalis* primer conditions for qPCR, providing the following: target genes, their primer sequences (F, forward; R, reverse), annealing temperatures (°C), amplicon size (bp), and primer concentrations (μM).

Function	Target gene		Sequence (5'-3')	Length (bp)	Temp. (°C)	[Primer] (μM)	Reference
Xenobiotic metabolism	*cyp1a*	F	CAACACTCCCAGGAGAAGAGAG	103	62	0.15	Jonsson *et al.,* (2011)
		R	TATCAAACCCAGCACCGAAG				
	ahr	F	TGGGCTTTTGACCCATCTCATCCC	243	60	0.35	Bissegger *et al.,* (2018)
		R	TGCCCCGTCTTTCCCTTTCTTATTC				
	arnt	F	GGCGGAGAAACAAGATGACTGCCT	228	60	0.35	Bissegger *et al.,* (2018)
		R	GGAACCCATCTGCTGCCTCCAAAA				
Phase II detoxification	*cat*	F	ACAATGCCAAGTGCCCAAAGAAGGA	116	60	0.35	Bissegger *et al.,* (2018)
		R	AGGCGTAGGGTTAATGAAGCGGAGA				
	sod	F	CTGGCTTGTGGCGTGATTG	99	60	0.35	Bissegger *et al.,* (2018)
		R	AACAGGGAGACCAACCAACA				
	gsr	F	CGCCGCTACTCTAACAACAA	98	60	0.35	Bissegger *et al.,* (2018)
		R	GGACTGAGGAGTAAAGGGCA				
	gst	F	ATTGCGTGGGAGATGAGGTG	99	60	0.35	Langlois *et al.,* (2010)
		R	ATTGTGGGATAGGGGGCAAG				
	gpx	F	CGAACCCAACTTCCCCTTGT	94	60	0.35	Langlois *et al.,* (2010)
		R	TAGGATACGGAAGTTGCCCC				
	p53	F	GCTGCTTTGAGGTTCGTGTG	99	58	0.35	Soriano *et al.,* (2014)
		R	TCTTCCCGTTGGGTTTCAGG				
Male and female reproduction	*ar*	F	GCTGCTTTGAGGTTCGTGTG	99	58	0.60	Langlois *et al.,* (2010)
		R	TCTTCCCGTTGGGTTTCAGG				
	erα	F	CCCAACATTTTACAGGTCAAGTTC	80	62	0.20	Langlois *et al.,* (2010)
		R	ATTGATCTCCACATTAGTCCCATC				
	srd5α1	F	GTTGAATGGTCTGGCTTTGC	96	62	0.35	Langlois *et al.,* (2010)
		R	CTGTTGTGCCCTGGAAGTC				
	srd5α2	F	ACCAGAAGGGAAGCACACAA	120	62	0.65	Langlois *et al.,* (2010)
		R	CCATAAGCAGCAGGATAAGTGA				
	srd5α3	F	GCTGGTCTGAGGAAAAGTGC	90	58	0.45	Langlois *et al.,* (2010)
		R	AGGGCAGGACACTCTCTCAA				
Thyroid hormone axis	*dio1*	F	GTAAGGACACCAACTGAGCAA	171	58	0.31	Langlois *et al.,* (2010)
		R	GCTGCAACCGTCACTAACAA				
	dio2	F	GTGTTGCCGACTTTGTGTTG	112	60	0.27	Langlois *et al.,* (2010)
		R	CGTTCTTCTTGGTTTCTGTGCT				
	dio3	F	TCGGAACTGAGGATGTGGT	199	60	0.17	Langlois *et al.,* (2010)
		R	ATGCCCAAGGAGATGAGTG				
	thrα	F	TAAGTTCTCTGTTCCCCTTTCCG	77	62	0.30	Langlois *et al.,* (2010)
		R	TAAGTTCTCTGTTCCCCTTTCCG				
	thrβ	F	ATCCAACACAGCAAAGGTATTTTC	106	62	0.30	Langlois *et al.,* (2010)
		R	GTAATGACTGCCCCACATTGC				
Housekeeping	**odc*	F	TGAATGATGGCGTGTATGGA	120	62	0.15	Langlois *et al.,* (2010)
		R	GTCCCCAAATGCTGCTTG				
	**rpl8*	F	CCCTCAACCATCAGGAGAGA	117	62	0.45	Langlois *et al.,* (2010)
		R	TCTTTGTACCACGCAGACGA				

*(*odc*) ornithine decarboxylase 1 (housekeeping gene); **(*rpl8*) ribosomal protein L8 (housekeeping gene); (*cyp1a*) cytochrome p450-1a; (*ahr*) aryl hydrocarbon receptor; (*arnt*) aryl hydrocarbon receptor nuclear translocator; (*cat*) catalase; (*sod*) superoxide dismutase; (*gsr*) glutathione reductase; (*gst*) glutathione transferase; (*gpx*) glutathione peroxidase; (*p53*) tumor protein P53; (*ar*) androgen receptor; (*erα*) estrogen receptor alpha; (*dio1*) deiodinase, iodothyronine, type I; (*dio2*) deiodinase, iodothyronine, type II; (*dio3*) deiodinase, iodothyronine, type III; (*Srd5α1)* steroid-5-alpha-reductase, alpha polypeptide 1 (3-oxo-5 alpha-steroid delta 4-dehydrogenase alpha 1); (*Srd5α2*) steroid-5-alpha-reductase, alpha polypeptide 2 (3-oxo-5 alpha-steroid delta 4-dehydrogenase alpha 2); (*Srd5α3*) steroid-5-alpha-reductase, alpha polypeptide 3 (3-oxo-5 alpha-steroid delta 4-dehydrogenase alpha 3); (*thrα*) thyroid hormone receptor alpha; (*thrβ)* thyroid hormone receptor beta.

2.5 Results

2.5.1 TPAH concentration is similar between the two blends

The estimated TPAH concentrations of the nominal dilutions spanned 0.80 – 8.90 µg/L in AWB WAF and 0.70 – 8.20 µg/L in CLB WAF (Table 2.2; 1st column). For CEWAF, the TPAH concentrations range from 0.09 – 46.6 µg/L in AWB CEWAF and 0.18 – 56.7 µg/L in CLB CEWAF (Table 2.2; 1st column).

2.5.2 Lethal and sub-lethal effects

After 72 h exposure, mean mortality in the controls was less than 10% (Table 2.2), thus not exceeding the FETAX guidelines threshold (ASTM International, 2004). The two highest dispersed dilbit treatments (AWB and CLB CEWAFs; 152 – 247 µg/L TPAHs) resulted in significantly higher mortalities (100%) compared to the negative control ($p < 0.0001$) and the 1% dispersed mineral oil control ($p < 0.0001$). Therefore, exposed animals from these higher levels of CEWAF treatments could not be further evaluated for malformation and gene expression analysis as no individuals remained.

Despite the percent of embryos with at least one malformation in the negative and dispersant controls was 12% exceeding the 10% FETAX threshold (ASTM International, 2004), a

significantly higher number (more than 75%) of embryos exposed to over 46.6 µg/L TPAHs were malformed in the CEWAF dilbit treatments compared to all of the controls ($p < 0.0001$; Figs. 2.2-2.3). For the combined AWB and CLB CEWAF treatments, the non-linear regression of log(agonist) vs response (3 parameters), with a top constraint of 100%, estimated an EC50 of 17.63 µg/L TPAHs [95% CI: 6.67 – 48.16] (Fig. 2.3). Altogether the experimental animals exhibiting malformations showed gut malformations (38.5%) followed by craniofacial (22.5%), axial (16.3%), edema (14.7%), eye (6.4%), and profoundly malformed embryos (1.1%) (Fig. 2.3). In addition, the growth of embryos was negatively affected by dilbit exposure where embryo full body length was negatively correlated with TPAH concentration and percentage of malformed embryos. Embryos exposed to 46.6 µg/L AWB and 56.7 µg/L CLB CEWAF treatments exhibited approximatively 10% shorter full body lengths to the negative control (AWB: $p < 0.0001$; CLB: $p = 0.0002$) and the 1% dispersed mineral oil control ($p < 0.0001$).

2.5.3 *cyp1a* and other candidate biomarkers

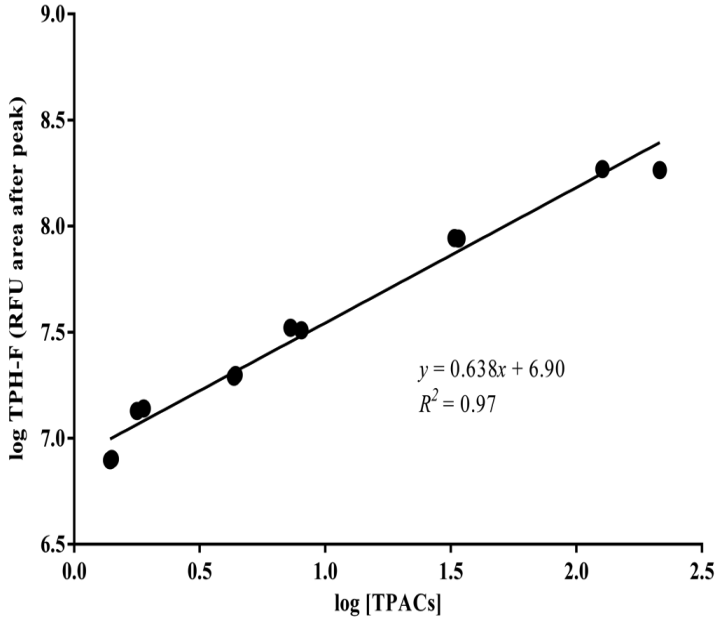
Transcript levels of *cyp1a* in all oil treatments were significantly increased compared to the negative control ($p < 0.05$; Fig. 2.4). The expression of *cyp1a* increased with increased TPAH concentrations in a curvilinear manner for CEWAF (log(agonist) vs response, 3 parameters, $R^2 = 0.85$; Fig. 2.4a) and a linear increase for WAF ($y = 9.16x + 4.23$, $R^2 = 0.69$; Fig. 2.4b) for both AWB and CLB data altogether. Most noteworthy, embryos exposed to CEWAF treatments of 46.6 - 56.7 µg/L TPAH had over 200-fold increase in *cyp1a* levels (Fig. 2.4a). The levels of mRNA of the other 18 genes of interest did not differ significantly from the controls (Figs. S2.1, S2.2, S2.3, S2.4). However, the TPAH concentration change in the WAF and CEWAF treatments contributed

significantly to some of the variation seen in *gst*, *gsr*, *cat*, *sod*, *srd5α2*, *srd5α3*, *dio1*, *dio3*, and *thrα* transcript profiles (Table 2.2).

Table 2.2 Mortality Mortality, total length, and malformations observed in *Silurana tropicalis* larvae exposed to water accommodated fraction (WAF) and chemically-enhanced WAF (CEWAF) of Access Western Blend (AWB) and Cold Lake Blend (CLB) diluted bitumens. Malformation types include axial, blistering, edema, eye, head, face, gut, and profound abnormalities. Asterisks (*) represent statistically different than controls.

Treatments	% Mortality ± SD	Length (mm) ± SD	% Malformed individuals (*n*)	Malformations observed (*n*)					
				Axial	Blistering/ edema	Eye	Head/ face	Failure of gut to coil	Profound abnormalities
Controls									
FETAX only	2.5 ± 1.6	5.0 ± 0.1	12.2 (9)	3	1	0	1	8	0
Mineral oil + dispersant 1%	6.5 ± 0.5	5.1 ± 0.1	35.6 (26)	8	2	4	8	17	1
Dispersant 0.1%	5 ± 1.3	5.3 ± 0.1	13.7 (10)	5	4	2	5	7	0
Dispersant 1%	8 ± 2.2	5.0 ± 0.1	33.3 (25)	11	4	6	13	20	1
Water-accommodated fractions (WAFs)									
AWB 0.8 µg/L	4 ± 1.4	4.8 ± 0.1	49.3 (37)	7	10	4	21	28	1
4.3 µg/L	8 ± 2.2	4.9 ± 0.1	25.7 (19)	4	4	4	7	15	1
8.9 µg/L	6.5 ± 2.6	5.0 ± 0.1	26.7 (20)	10	5	2	8	13	0
CLB 0.7 µg/L	6 ± 2.2	5.1 ± 0.1	29.7 (22)	11	4	3	6	15	0
3.8 µg/L	8 ± 2.9	5.0 ± 0.2	21.3 (15)	7	8	2	4	12	1
8.2 µg/L	8.5 ± 2.9	5.2 ± 0.1	17.3 (13)	4	7	6	9	9	0
Chemically Enhanced WAFs (CEWAFs)									
AWB 0.09 µg/L	7.5 ± 1.0	4.9 ± 0.1	41.3 (31)	13	13	6	10	27	0
2.7 µg/L	6 ± 1.4	5.0 ± 0.1	18.7 (14)	4	4	3	7	10	3
46.6 µg/L	12 ± 1.2	4.4 ± 0.2*	97.2 (70)*	29	31	7	31	63	1
152.5 µg/L	100 ± 0.0*	-	-	-	-	-	-	-	-
190.1 µg/L	100 ± 0.0*	-	-	-	-	-	-	-	-
CLB 0.18 µg/L	9 ± 3.3	5.0 ± 0.1	25.3 (19)	10	6	1	11	14	0
3.3 µg/L	6.5 ± 2.2	5.1 ± 0.1	26.7 (20)	8	6	2	10	10	0
56.7 µg/L	12 ± 2.3	4.6 ± 0.1*	78.7 (59)*	17	12	1	36	52	0
193.0 µg/L	100 ± 0.0*	-	-	-	-	-	-	-	-
247.4 µg/L	100 ± 0.0*	-	-	-	-	-	-	-	-

a)



b)

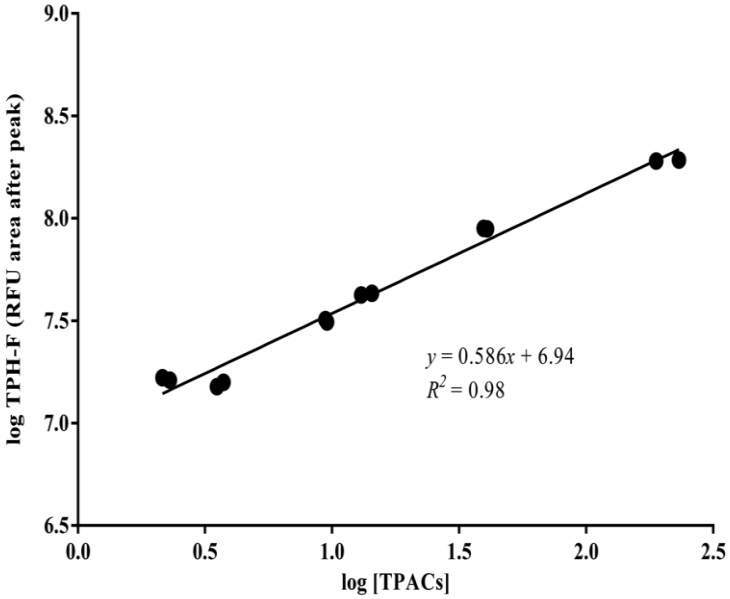


Figure 2.1 A relationship of the total polycyclic aromatic hydrocarbons (TPAHs) measured by GC-MS (n = 12) and the total petroleum hydrocarbons (TPH-F) measured by fluorescence using a QMI Fluorescence Spectrometer (Photon Technologies International, Birmingham, New Jersey, USA; Felix software Ver 1.4) (TPH-F; n = 12) in (a) Access Western Blend (AWB) and (b) Cold Lake Blend (CLB) diluted bitumen (dilbit) test samples. Dilbit concentrations in solution are related to relative fluorescence units (RFU, background corrected) and show the measured total log area under peak (excitation 302 nm; emission range: 341-460 nm, 1 mm slit). The equations of the lines were used to estimate the TPAH content of the exposure solutions from the measured fluorescence.

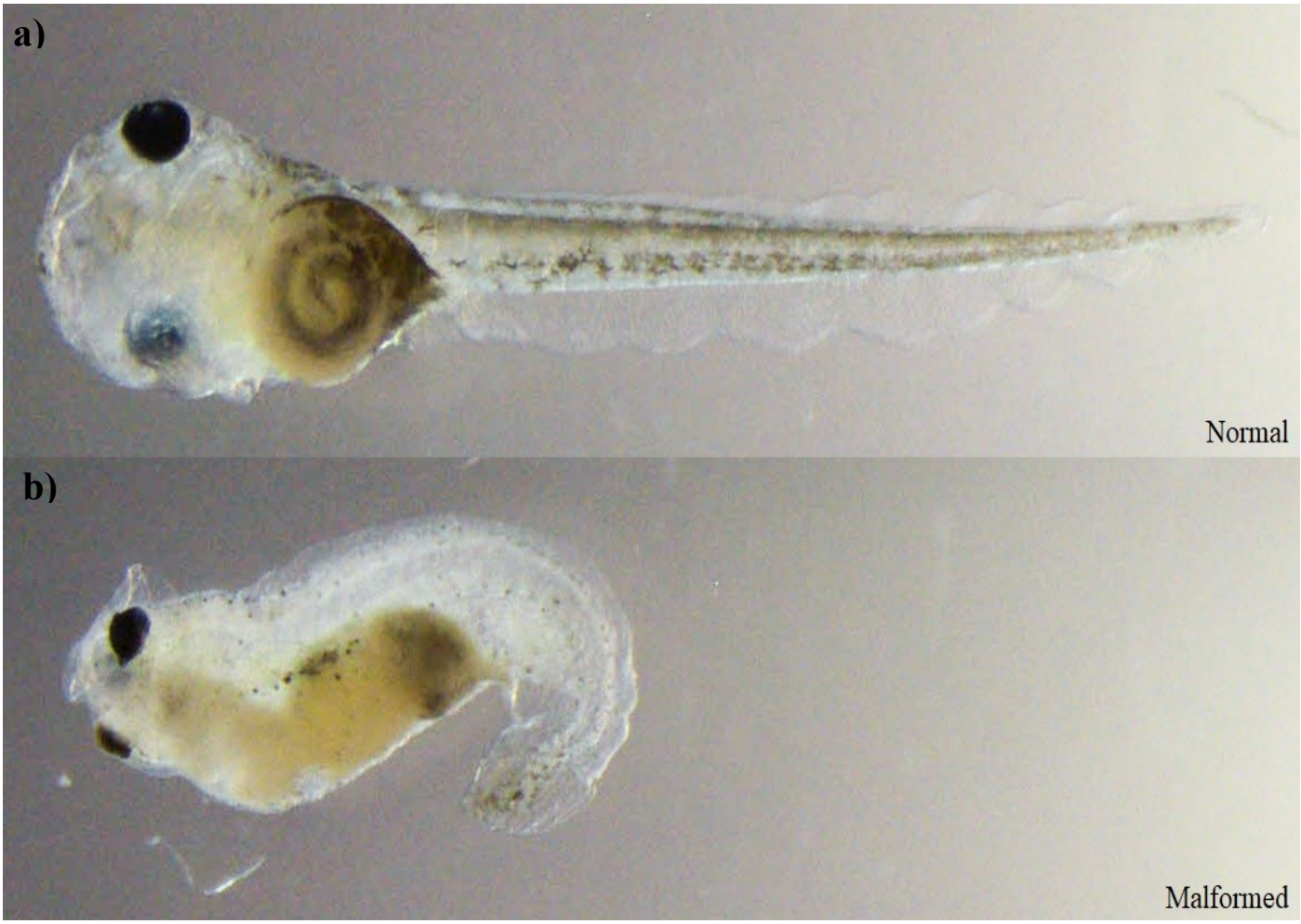


Figure 2.2 Examples of normal and malformed tadpoles. (a*) Silurana tropicalis* control larvae and (b) *S. tropicalis* malformed larvae exposed in AWB CEWAF (46.6 µg/L TPAH).

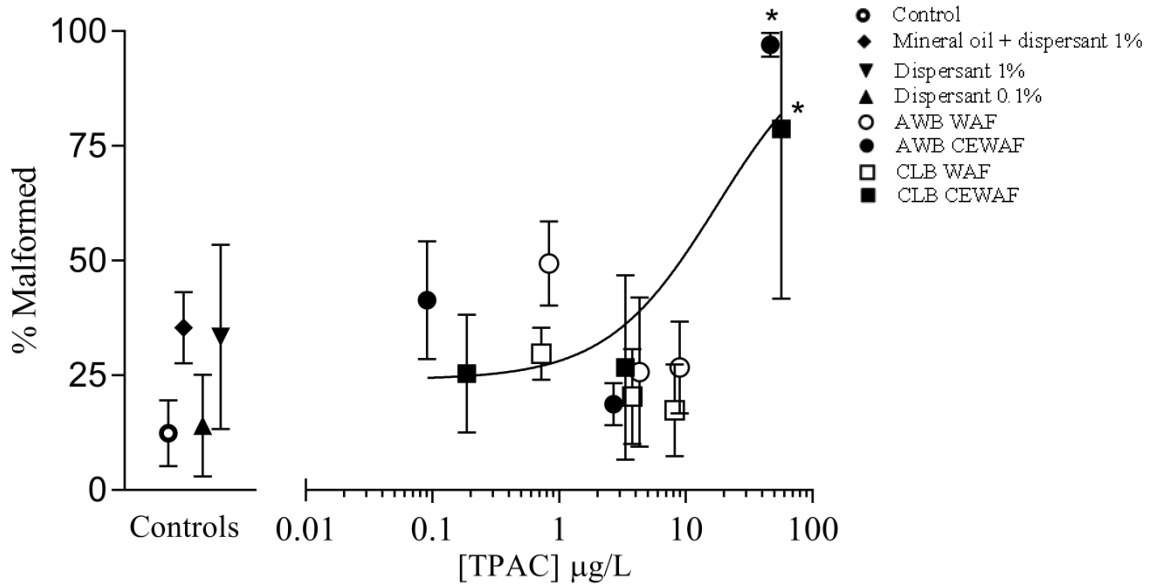


Figure 2.3 Prevalence of malformations (% malformed) in *Silurana tropicalis* embryos after exposure to water accommodated fractions (WAFs) and chemically-enhanced WAFs (CEWAFs) of two diluted bitumens (dilbits): Access Western Blend (AWB) and Cold Lake Blend (CLB). Data are presented as mean ± SEM. The independent axis represents the mean logarithmic total polycyclic aromatic hydrocarbon (TPAH) content of treatments as estimated with the relationship to total petroleum hydrocarbons by fluorescence (TPH-F). Asterisks (*) represent statistically different than controls.

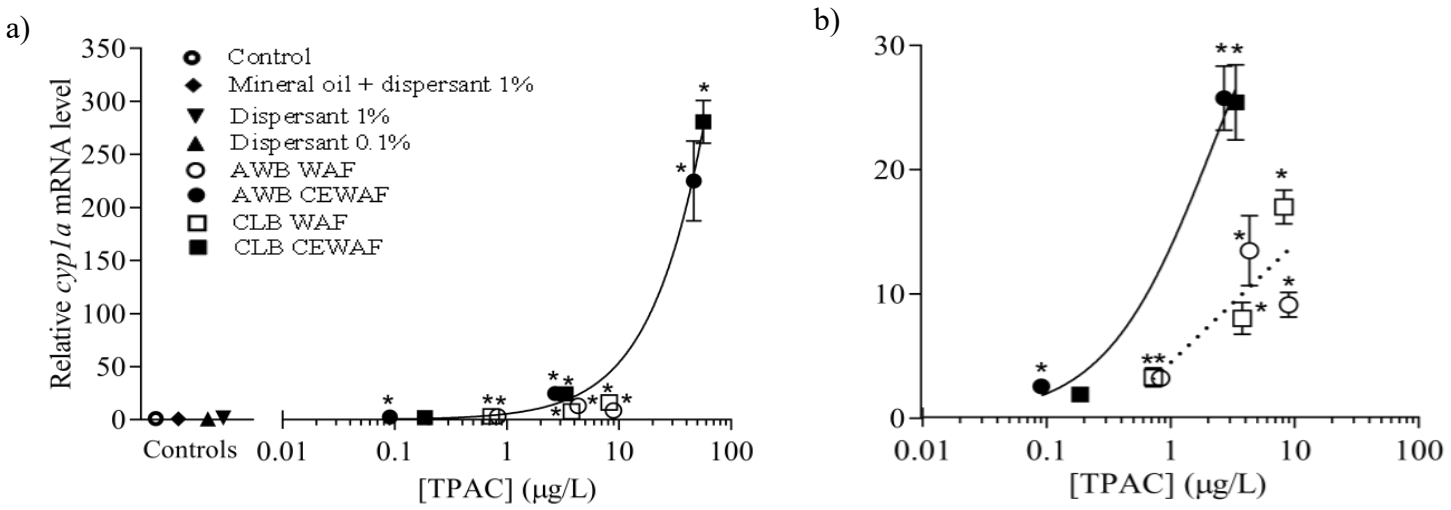


Figure 2.4 Levels of cytochrome P450 (*cyp1a*) transcripts in *Silurana tropicalis* embryos exposed to ranging concentrations of the Access Western Blend (AWB) and Cold Lake Blend (CLB) Water Accommodated Fractions (WAFs) and Chemically Enhanced WAFs (CEWAFs; panel a). A close-up of the effects of WAFs and CEWAFs on *cyp1a* mRNA level at PAH concentrations ranging between 0.01 and 10 µg/L is pictured on panel b. Regression lines include AWB and CLB data as no difference between treatments was found. Fold changes of *cyp1a* transcripts are normalized to the mean of *odc* and *rpl8* ± SEM. Statistical differences between the treatments and the water control are denoted with an asterisk (*; two-way ANOVA; $p < 0.05$).

2.6 Discussion

Embryos of *S. tropicalis* exposed to a range of AWB and CLB dilbits from NF stages 12 to 46 exhibited signs of toxicity through increased mortality, increased malformation prevalence, decreased total body length, and increased *cyp1a* transcript levels. Both AWB and CLB CEWAF dilbits yielded 100% mortality at concentrations reaching 152 µg/L TPAHs or more, with a LC50 of 53.1 µg/L TPAHs. This was likely due to the higher amount of dissolved PAHs bioavailable to the frog embryos when using the CEWAF oil dispersion methodology; however, none of the WAF treatments (environmentally realistic conditions) induced embryo lethality. In larval fish, dilbits' PAH concentrations of approximatively 40 µg/L TPAHs were sufficient to induce LC50 (Barron et al., 2018); thus, this present study highlights the increased resilience of frog embryos to survive in higher levels of TPAH-contaminated water as compared to some species of fish.

In addition, *S. tropicalis* embryos exposed to approximatively 45 µg/L TPAHs had significantly higher prevalence of malformations and shorter body length compared to embryos in all of the controls, yielding an EC50 of 17 µg/L TPAHs. Similarly to the mortality data, this suggests that despite the same loadings of dispersant, dispersed-dilbits induced malformations independently of the dispersant added to the treatments. Likewise, Atlantic herring (*Clupea harengus*) embryos exposed to CEWAFs of Medium South American crude oil had higher chronic toxicity compared to those exposed to WAFs, but only due to increased exposure to PAHs through dispersion (Adams et al., 2014). Here how these findings compare with results from others that have explored the toxicity of dispersed dilbit to fish expressed through PAH concentration and have reported EC50s ranging from 0.1 – 1.6 µg/L TPAHs in Japanese medaka (Madison et al., 2015; Madison et al., 2017) and 11.0 – 24.1 µg/L TPAHs in yellow perch (McDonnell et al., 2019).

Developmental abnormalities in frogs may lead to poor reproductive success, competitive ability, disease resistance, and predator avoidance; all threats to amphibian survival. As 38.5% of the malformed embryos exhibited failure to coil gut and a 10% decrease in body length, high level of TPAHs may have caused developmental delays in some frog larvae. African clawed frog (*Xenopus laevis*) embryos exposed to 30 ppm TPAH from coal tar were smaller and took a longer time to develop to metamorphosis (Bryer et al., 2006). A longer developmental time with a longer exposure to benzo[a]pyrene correlated with increased DNA adducts and micronuclei in *X. laevis* larvae (Sadinski et al., 1995). In addition, stunted growth or reduced growth rate has been associated with fish exposure to PAHs (Billiard et al., 1999; Brewton et al., 2013). While blue-sac disease (BSD) has generally been associated with fish exposed to PAHs (Adams et al., 2014; Madison et al., 2015; Martin et al., 2014), *S. tropicalis* demonstrated similar effects to those reported in the above studies, including edema, craniofacial malformations, stunted growth, and mortality. Although frog larvae are resilient to higher environmental PAH concentrations, data suggest that once they reach homeostasic imbalance, frogs will exhibit similar toxicity signs than fishes.

S. tropicalis embryos exposed to dilbit yieded significant changes in *cyp1a* expression, a known biomarker of exposure to AHR agonists in frogs (Jönsson et al., 2011). Increased induction of cyp1a as a biomarker of exposure has been documented in developing fish exposed to dilbit (Madison et al., 2015; Philibert et al., 2016; Alderman et al 2017; Madison et al., 2017). For example, *cyp1a* transcripts were increased compared to controls before and after the malformation prevalence EC50 in Japanese medaka exposed to increasing PAH concentration in dilbit, suggesting that the *cyp1a* mRNA response is a valuable biomarker of PAH exposure (Madison et al., 2017). Similarly, spotted seatrout (*Cynoscion nebulosa*) exposed to high energy WAFs and

CEWAFs of source oil from the Deepwater Horizon oil spill that had the highest *cyp1a* induction as had the greatest reduction in growth, supporting *cyp1a* as a useful biomarker of effect (Brewton et al., 2013). For similar PAH concentrations, *S. tropicalis* embryos had similar induction levels of *cyp1a* (this study) compared to medaka eleuthroembryos exposed to dilbit (Madison et al., 2015, 2017). These findings are distinguished from previous studies that have observed lower sensitivity of *cyp* genes in developing frogs compared to fish when exposed to halogenated AHR agonists (Jönsson et al., 2011). However, despite the *cyp1a* induction of nearly 300-fold, genes involved in phase II detoxification (e.g., *gsr*, *sod*) and cell cycling/mutation response (*p53*) did not change in the frog embryos as they did in Japanese medaka embryos exposed to dilbit (Madison et al., 2015, 2017). Furthermore, despite evidence that benzo[a]pyrene (Regnault et al., 2014) and crude oil (Truter et al., 2016) could interact with the endocrine axis in amphibians, none of the genes tested involved in reproduction or development were significantly affected following dilbit treatments. However, TPAH concentration did significantly contribute to about 14 – 23% of the variation in the expression of the endocrine-related genes. Therefore, future studies should conduct chronic studies to investigate if dilbit would delay frog metamorphosis and alter reproduction later on.

In conclusion, AWB and CLB dilbits can be toxic to the developing *S. tropicalis* once cellular homeostasis does no longer cope with the environmental concentrations of exposure. Frog larvae will then exhibit similar molecular, morphological, and physiological responses to those seen in fish. And most notably, the expression of *cyp1a* transcript can be used as both a biomarker of exposure and response to PAHs in amphibian toxicity assessments.

2.7 Acknowledgments

The authors would like to acknowledge Lucie Baillon, Christina Emerton, and Alex Leclerc for their help with the exposure. Funding was provided by a Canada Research Chair (to VSL) and a doctoral scholarship from CONACYT-México (to LRLJ).

TRANSITION FROM CHAPTER 2 TO CHAPTER 3

After determining that *cyp1a* mRNA level was a good biomarker for frogs exposed to unweathered oil, and observing that there was no significant change in genes related to the endocrine system in **Chapter 2**, we were wondering if we could use *cyp1a* mRNA level to assess the differential toxicity responses of aquatic organisms as oil weathered.

So the next **Chapter** (**3**) deals precisely with how unweathered oil changes chemical profile while weathering and how this changes its toxicity over time. For this work, we chose to use a fish species revelant to North America's environment and we investigated a conventional crude oil.

CHAPTER 3: FATE AND EMBRYOTOXICITY OF WEATHERING CRUDE OIL IN A PILOT-SCALE SPILL TANK

DEVENIR ET EMBRYOTOXICITÉ DU PÉTROLE BRUT ALTÉRÉ DANS UN RÉSERVOIR DE DÉVERSEMENT À L'ÉCHELLE PILOTE

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The article will be submitted to **Environmental Toxicology & Chemistry.**

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Chemical analysis: QX, FD, GT-B, HDD

Data analysis: LRL-J

Original ideas and experimental design: LRL-J, CG, QX, PC, HDD, VSL

3.1 Abstract

For several years now, the Natural Resources Canada (NRCan) research facility at CanmetENERGY Devon has been performing experiments in a pilot-scale spill tank using 1,200 L river water examining the physical and chemical behaviors of various crude oil/water mixtures under varying temperature regimes. As oil toxicity can be modulated by weathering of petroleum products, this study aims to assess changes in fish embryotoxicity to mixed sweet blend (MSW) crude oil as it weathered at air and water temperatures of 14 °C and 15 °C respectively for 28 days. The physicochemical behavior of the oil was also monitored. Water samples were taken from the spill tank five times during the 28-day experiment at Days 1, 6, 14, 21, and 28 and were used to perform toxicity exposures using fathead minnow embryos (*Pimephales promelas*). For each water sampling day, newly fertilized embryos were exposed to a serial dilution of the spill tank water, non-contaminated river water (used in the spill tank), and a reconstituted water laboratory control. Embryos were raised until hatching. While mortality was not significantly altered by the oil contamination over the time period, malformation occurrence and severity showed concentration-dependent responses to all contaminated water collected. Data suggest that Days 14, 21, and 28 were the most toxic time periods for the fish embryos, which corresponded to increasing concentrations of unidentified oxidized organic compounds detected by Q-TOF. This study highlights a novel area for oil research, which could help to better understand the toxicity associated with oil weathering for aquatic species.

3.2 Résumé

Depuis plusieurs années, le Centre de recherche CanmetÉNERGIE de Ressources naturelles Canada (RNCan) effectue des expériences dans un réservoir de déversement à l'échelle pilote utilisant 1200 L d'eau de rivière pour examiner les comportements physiques et chimiques de divers mélanges pétrole brut/eau sous différents régimes de température. La toxicité du pétrole pouvant être modulée par la dégradation de ce dernier, cette étude vise à évaluer les changements dans l'embryotoxicité des poissons du Mixed Sweet Blend (MSW) altéré à des températures de l'air et de l'eau de 14 ° C et 15 ° C, respectivement, pendant 28 jours. De plus, cette étude caractérise le devenir et le comportement physico-chimiques du MSW pendant sa dégradation. Des échantillons d'eau ont été prélevés cinq fois dans le réservoir de déversement au cours de l'expérience soit aux jours 1, 6, 14, 21 et 28 et ont été utilisés pour effectuer des tests d'embryotoxicité chez les mené à grosse tête (*Pimephales promelas*). Pour chaque jour d'échantillonnage de l'eau, des embryons nouvellement fécondés ont été exposés à une dilution en série de soit l'eau du réservoir de déversement, soit de l'eau de rivière non contaminée (utilisée dans le réservoir de déversement) ou soit à un contrôle d'eau reconstituée. Les embryons ont été exposés jusqu'à l'éclosion. Bien que la mortalité n'ait pas été significativement modifiée par les traitements au MSW, la fréquence des malformations observées était liée à l'augmentation des concentrations de MSW. Les données suggèrent que les fractions testées aux jours 14, 21 et 28 étaient les fractions les plus toxiques pour les embryons de poissons, ce qui correspondait à l'augmentation de la concentration de composés oxydés inconnus identifiés par Q-TOF. Cette étude met en évidence un nouveau domaine de recherche sur le pétrole, qui pourrait aider à mieux comprendre la toxicité associée au vieillissement du pétrole pour les espèces aquatiques.

3.3 Introduction

The chemical complexity of crude oil increases the difficulty of environmental risk assessments for the ecosystems that may be exposed to an oil spill (Wang et al,. 2006). One of the major crude oil disasters in subarctic environments occurred on March 24th, 1989 when the Exxon Valdez spilled 35,000 metric tonnes of crude oil in the waters of Prince William Sound, Alaska, USA (Brown et al., 1996). Until this event, there were few studies related to the toxicity of petroleum products to fish embryos. Currently, the literature on fish toxicity to crude oils has increased and we now better understand the correlation between the observed toxicity to fish embryos with the amount of dissolved and bioavailable oil (Beyer et al., 2016). Pericardial and yolk sac edemas, spinal and craniofacial deformities, and cardiac or circulatory failure are among the most frequent embryotoxicity responses observed following crude oil exposure (Jung et al., 2011). It is now well known that the complex crude oil mixture does weather with time, which changes its chemical profile (Adams et al., 2014; Bornstein et al., 2014; Carls et al., 1999; Heintz et al., 1999) and so could modulate its toxicity.

Crude oil is made up of a wide range of organic compounds, from very volatile hydrocarbons, such as propane, butane, and benzene to larger, more complex compounds such as asphaltenes, resins, and waxes (Wang et al,. 2003). Once oil is spilled in a water environment, it is immediately subject to a variety of abiotic and biotic processes, including spreading, drifting, dispersion, stranding, evaporation, dissolution, biodegradation, photo-oxidation, and emulsification (Dew et al., 2015). Waves provide mixing energy that can break surface films of oil and distribute oil droplets into the water column (Lee et al., 2015). Polar compounds in the oil have relatively high-water solubility so with time leave the oil phase and dissolve into the water (Wallace et al., 2020). Given that the dissolved fraction of oil can diffuse across biological

membranes (e.g., gills), solubility is arguably the most important factor in determining toxicity to aquatic biota (Cherr et al., 2017). Differences in oil composition due to origins between different crudes can cause significant differences in the fate and behavior of the oils in water (Dew et al., 2015). Overall, light aromatics in crude oil, such as benzene/toluene/ethylbenzene/xylene (BTEX), have acute toxicity to organisms, but tend to evaporate or biodegrade rapidly. Intermediate-sized components, such as polycyclic aromatic hydrocarbons (PAHs) and alkylated PAHs, are less volatile and contribute less to acute toxicity because they are taken up more slowly than lower molecular weight compounds (Dupuis & Ucán-Marín, 2015). However, PAHs and alkylated PAHs are responsible for more chronic, mutagenic and carcinogenic impacts to a higher extent than n-alkanes and other petroleum biomarkers (Meador & Nahrgang, 2019; Wallace et al., 2020). It is also clear that 2-to-3-ringed PAHs have higher bioavailability than those with more rings (4-to-5) (Wu et al., 2012). This is consistent with PAHs that have higher octanol-water partition coefficient (Kow) values being less polar and, so less water-soluble (Lee et al., 2015). Strong adsorption of PAHs to sediment particles may also lead to their low bioavailability and biodegradation rate, preserving them in sediment for an extended period (Wang et al., 2004). The very heavy components, such as asphaltenes and resins, are thought not to be toxic because they do not readily cross biological membranes in the environment. However, they are the most persistent oil compounds due to their extremely low solubility in water (Lee et al., 2015).

Several studies have assessed the impact of crude oil on fish. Adams and colleagues (2013) exposed Atlantic herring (*Clupea harengus*) to artificially weathered Middle America (MESA) crude oil using laboratory-prepared water accommodated fractions (WAFs) and chemically enhanced WAF (CEWAF) daily. Malformations, developmental delays, pericardial edema, decreased heart rate, and blue sac disease were observed. Nakayama et al. (2008) exposed Japanese

flounder (*Paralichthys olivaceus*) to unweathered Bunker C heavy fuel oil to study the immunotoxic effects of heavy oil and the biological responses underlying toxic effects. They found that exposure to heavy oils decreases pathogen resistance through negative regulation of genes related to the immune system. Rainbow trout (*Oncorhynchus mykiss*) larvae showed a constant toxicity when treated with heavy fuel oil stranded on gravel (allowing for a continual exposure of weathering oil) and were particularly sensitive to 3- and 4-ringed alkylated-PAHs (Martin et al., 2014). However, it remains unclear what other compounds could have explained a constant toxicity when most of the PAHs and alkylated-PAHs are known to degrade over time as the oil mixture weathered. Therefore, the purpose of this study was to mimic a crude oil spill in a freshwater lake to study its chemical weathering profile along with corresponding changes in fathead minnow (*Pimephales promelas*) embryotoxicity with time, a fish that is native to freshwater lakes in North America from the Rocky Mountains in Canada south to Texas and northern Mexico (Danylchuk & Paszkowski, 2012).

3.4 Materials and methods

3.4.1 Chemicals and water source

Mixed Sweet Blend (MSW; a conventional crude oil) was obtained directly from a pipeline in Edmonton, Alberta, Canada. North Saskatchewan River water (Alberta, Canada) was provided by the municipal water facility from Edmonton before the potable water treatment process was performed. River sediment was collected from a floodplain on the bank of the North Saskatchewan River. All other chemicals and solvents were purchased from Sigma Aldrich as “reagent grade” and were used as received.

3.4.2 Sediment analyses

Sediment was added to increase the sediment loading of the test water. It was homogenized prior to use and analysis. The particle size distribution of the sediment was determined by the following in-house procedure. Approximately 40 grams of material was added to a 400 mL beaker. The beaker was half filled with tap water and stirred on a hot plate at 400 rpm and 60°C for 30 minutes. The beaker was then transferred to a sonic bath where it was sonicated for 30 minutes. A pre-weighed stack of sieves (no. 60, 100, 140, 200 and 325) was placed on a Ro-Tap and secured in place. The mesh in these sieves corresponds to 250 µm, 150 µm, 106 µm, 75 µm and 45 µm. Each sieve was wetted with tap water in turn to ensure that hydrostatic pressure was not a factor in the sieving. The sample was then transferred into the top most sieve quantitatively. A strike plate was placed on top and water inlets secured. The sample was agitated for 15 minutes on the Ro-tap while ~8 L of tap water flowed through the sieves and was collected along with the fines in a 10L pail. After 15 minutes, each sieve was examined to see if any more material was forth coming. When all the sieves expelled clean water, the sieves were allowed to dry in an oven at 110 ^{o}C for 16 hours. The pail with the fines was allowed to settle for 24 hours. After 16 hours, the sieves were weighed again to determine the amount of solids captured. After 24 hours, the water cap was removed from the fines pail and the fines were transferred to a pre-weighed 500 mL aluminium pan for drying in an oven. The next day, the fines were weighed, collected out of the pan, crushed, and homogenized before further analysis. A quantity of 5.5 grams was removed for sedigraph analysis. This material was put into a beaker along with 40 mL of 0.05% Calgon solution. The sample was then stirred on a hot plate at 400 rpm and 60^{o}C for 30 minutes. The beaker was then transferred to a sonic bath where it was sonicated for a further 30 minutes. Once homogenized, it was transferred to a plastic container quantitatively. Two drops of photo flow

were added to the solution and it was agitated for one minute with a sonic probe. The sample was run using an autosampler and a 5120 Sedigraph III particle analyzer. The Sedigraph can determine percent passing between 55 μm and 0.2 μm.

The mineral composition of the sediment was determined using the following in-house method. Approximately 1.5 g of the mineral solids were weighed and transferred to a micronizing mill tube loaded with quartz pellets. About 5 mL of methanol was added, and the sample was micronized for six minutes to reduce the particle size to less than 5 microns. The sample was then transferred to a beaker and heated at 100°C for two hours to evaporate the methanol. The dried mineral was then transferred into a mixing mill vessel and eight drops of Vertrel XF fluid were added to the sample and mixed for ten minutes to obtain spherical particles with an average of 2 to 5 micron in diameter. X-ray diffraction analysis was carried out on a Rigaku D/MAX Rapid-II rotating anode powder diffractometer equipped with an imaging plate detector, using CrKα radiation. Diffraction data were obtained at 35 kV and 25 mA, scanning from 5° to 150° 2θ with a scan step of 0.045° 2θ for 0.2 s. Quantification of the mineral species in the randomly oriented specimen was carried out using the Rietveld least square refinement program, AUTOQUAN™.

To measure the content of oil in the final recovered sediment slurry, an in-house extraction method using methylene chloride (CH_2Cl_2) as a solvent was used. Briefly, oil contaminated sediments was subjected to two sequential extractions with CH_2Cl_2 to determine oil content and capture the low boiling point range of the oil. The first extraction used a small volume (approximately 5 to 10 mL) of CH_2Cl_2, while the second extraction used as much CH_2Cl_2 as was required to extract the remaining oil. Water and particulates were removed from the extracts by freezing followed by filtration through 2.5 um pore size filters. The concentration of oil dissolved in the first CH_2Cl_2 extract was determined gravimetrically by an in-house method. In order to

capture the low boiling components of the oil (<200°C), high temperature simulated distillation was performed directly on the extract using CH_2Cl_2 as the carrier solvent. Solvent and any residual water content was evaporated from the second CH_2Cl_2 extract by a dynamic flow of N_2 gas, followed by a vacuum oven at 70°C and 2.5 mm Hg for 3 hours. The mass of oil extracted by the second CH_2Cl_2 extract was then determined gravimetrically. The total oil content of sediments was calculated by the addition of the masses of oil extracted in the first and second CH_2Cl_2 extractions.

3.4.3 Water analyses

The following water analyses were performed. Inorganic anions were measured by ion chromatography according to EPA Method 300.0 (Pfaff,1993). A five point calibration curve was generated for each analyte of interest and validated with two different quality control/quality assurance samples. Sample alkalinity was measured with a PC-titrate supplied by Mantech Associates. The sample was titrated with 0.02N hydrochloric acid until the pH of the sample reached 4.5. The endpoint was determined using a pH electrode. A standardization protocol was followed for determining the concentration of the titration acid and buffer solutions were used as calibration standards. Quality control samples with known concentrations of bicarbonate and carbonate were inserted into the sample sequence to ensure that the instrument was operating correctly. The conductivity of each sample was measured with a handheld Exstix conductivity total dissolved solids (TDS) meter. The meter was calibrated daily with three calibration standards; 84 µS, 1413 µS, and 12,880 µS. Samples and standards were allowed to reach room temperature before measurements were taken. Samples were analyzed for trace metals in solution according to EPA Method 6010B. Analysis was completed using an Agilent 8800 Inductively Coupled Plasma Tandem Mass Spectrometer (ICP MS/MS). Calcium, sodium and potassium in the samples were

measured using a Radial Varian Vista-Pro inductively coupled plasma spectrometer (ICP-OES). Four point calibration curves were used for the analysis and calibration standards were made from certified stock solutions. The matrix for the aqueous introduction was a 5% Nitric Acid solution. Samples were not diluted or digested for the analysis.

Contents of different types of organic compounds in the contaminated water were measured. Benzene-toluene-ethylbenzene-xylene (BTEX) contents in the sampled waters were measured using EPA 5021/8015&8260 GC-MS & FID. Total concentrations of polycyclic aromatic hydrocarbons (PAHs) and alkylated PAHs in selected water samples were analyzed using EPA 3510C/8270-GC/MS. Total organic carbon (TOC) of water samples collected during the test was determined using a Shimadzu TOC V CPH instrument according to a modified version of ASTM D7573-09 (2009).

In addition to the above analyses of organic compounds in undiluted contaminated water samples, the diluted contaminated water samples used for toxicity tests were also analyzed at each time point for PAHs and alkylated PAHs, volatile organic compounds (VOCs), and for molecular profiling of oxidized compounds. For PAH analyses, about 800 mL of pH 2 acidified water sample was extracted by liquid–liquid extraction with DCM to a final volume of 100 mL of DCM. A 10-mL aliquot of this extract was solvent-exchanged to hexane, treated with silica gel and concentrated down to 1 mL. The prepared sample was analyzed without further purification using GC/MSD (Agilent Technologies 7890B GC and 5977A MSD) for nine classes of PAHs, totaling 126 alkylated PAHs (CEAEQ 2016) and the VOCs (CEAEQ 2015). The same extract used for alkylated PAHs/VOC analysis was also used for the molecular profiling of unidentified oxidized compounds by GC/QTOF (Agilent Technologies 7890B GC and 7200B QTOF mass spectrometer).

3.4.4 Preparation of water-accommodated fraction (WAF) in the spill test tank

The spill tank measuring 3 m x 1 m x 1.5 m (L x W x H) and made of 316-stainless steel is shown in Fig. S3.1-a. To start the tank test, approximately 1,200 L of fresh river water was added in the tank to a height of 0.7 m. One end of the tank was built at an incline to simulate a shoreline situation. The tank had a paddle-style wave-generating flap to create waves during the test where a motor setting of 105 rpm generated breaking waves with a wavelength of 60 cm, a wave amplitude of approximately 8 cm, and a wave period of 0.5 s. Three temperature transmitters (TT-1001, TT-1002, and TT-1003) were located at different heights to provide a temperature profile in the tank. TT-1001 was above the water surface and recorded the ambient air temperature throughout the test period, while TT-1002 and TT-1003 recorded the water temperature. For this test, an external heater unit was used to maintain the water temperature at 15°C ± 1°C using heat-exchange coils located along the bottom and sides of the spill tank; the ambient air temperature an average of 14°C ± 2°C. No light source was available to simulate the effects of photo-oxidation. The intrinsically safe LED-based lights installed were chosen to meet the electrical code for the potentially explosive environment above the tank. As LED lights tend to have low emissions in the ultraviolet wavelength range, it is expected that photo-oxidation contributions to weathering processes in the tank were also low.

At the start of the test, 2.4 kg of North Saskatchewan River sedimentary deposit was added to the water and mixed with waves for 5 min to achieve a content of 2,000 ppm of suspended sediment. Immediately afterwards, 8.255 kg of fresh MSW (approximately 10 L) was poured onto the surface of the water through a pouring device attached to the edge of the tank (Fig. S3.2-b), and time was recorded as the zero hour (T0). The pouring device was designed to direct the oil out

sideways to prevent oil splashing down into the water column. Waves were applied continuously for the first 2 days then turned off for the next two days, where the on-off cycle was repeated over the duration of the test. When the change over from off to on, or on to off occurred on a weekend, the state was maintained until the Monday for a third day in the state. Over the entire 28-day period, the waves were on half the time and off half the time. The evaporation of water during the test was made up using reverse osmosis water to maintain a constant water height of 70 cm.

Water samples were removed in intervals during the 28 days from the tank sample port, labelled with a red "X" in Fig. S3.1-a for analyses and toxicity testing. At the end of the test, weathered oil samples from different locations in the tank were recovered for mass balance purposes and analyses. Finally, the majority of the sediment was collected from the bottom of the tank. The oil content of the sediment was determined by the in-house extraction method described in the sediment analyses section. Pictures were taken to document the tank contents at the various sampling time points (Fig. S3.2). For toxicity testing five 20-L pails of water were collected on each of the sample days (Day 1, Day 6, Day 14, Day 21, and Day 28) and shipped overnight to the *Institut national de la recherche scientifique* (INRS), Quebec City, QC, Canada for embryotoxicity testing.

3.4.5 Physical and chemical analyses of the oil

Fresh MSW was tested as it was received. At the end of the test (Day 28), after collecting the required water samples for analyses, weathered oils from the water surface (floating oil) and shoreline (shoreline oil) were recovered and weighed. Both fresh and weathered oils were analyzed using the following methods. Density was measured using a densitometer at 20, 25 and 30°C as per American Standards for Testing and Materials (ASTM) method D4052 (2016). Density at 15°C

was calculated from the linear relationship between the measured densities and temperature. Viscosity was measured using a Brookfield viscometer at 20, 25 and 30°C as per ASTM method D7042 (2016). Kinematic viscosity at 15°C was estimated from the logarithmic relationship between the experimentally determined kinematic viscosities and the inverse of absolute temperature (K^{-1}). In both cases, the R^2 values of MSW data were close to 100 % for the three measured values. The R^2 values for the floating oil data were lower (> 94 %) due to the heterogeneity of the water present in the floating oil emulsions.

The boiling point distributions of fresh MSW and the weathered oils by high temperature simulated distillation (HTSD) were determined using method (ASTM D7169, 2016) on an Agilent gas chromatograph with Analytical Control software. As well, both fresh MSW and floating oil were distilled to a cut point of 204°C to separate the light oil fraction [initial boiling point to 204°C (IBP–204°C)] from the heavier portion of the oil (BP>204°C) using ASTM D1160 (2015). The basic sediment and water content (BS&W) was measured using ASTM D7829 (2018) to quantify water in the fresh MSW and weathered shoreline oil. For the weathered floating oil, water content was measured by freezing the water in its IBP–204°C fraction and then pouring the little oil present off the ice and recording their weights. The 1 wt% oil recovered in the IBP–204°C fraction was found to actually be BP>204°C material removed by the imprecision of the distillation method.

Elemental analysis was performed to determine the contents of carbon (C), hydrogen (H), nitrogen (N), and sulfur (S) using an Elementar Analyzer (ASTM D5291). Oxygen (O) content was determined with an Elementar Oxygen Analyzer using an in-house method. Reported elemental data are normalized with respect to total CHNSO content. Separations of the fresh and weathered oil samples into saturates, aromatics, resins, and asphaltenes (SARA) subfractions were

performed according to an in-house modification of ASTM methods D2007 (2019) and D2549 (2017).

3.4.6 Embryotoxicity assays

Fathead minnow (FHM; *Pimephales promelas*) eggs were collected from the colony held at INRS. Eggs were obtained following an established breeding protocol based on the INRS Animal Care Committee and the Canadian Council for Animal Care guidelines. Eggs were collected from approximately 30 couples of FHM, and randomized before use. For each of the time points of interest (Day 1, Day 6, Day 14, Day 21, and Day 28), MSW-contaminated water stock was stored at 4°C. For each assay, dilutions were prepared for the following nominal dilutions 12.5, 25, 50, and 100% v/v from the MSW-contaminated water stock. The fractions were diluted with North Saskatchewan River water and the experimental design included a river water control (0% oil). A second control was included to ensure that the river water did not affect the tests. It was a reconstituted water control prepared according to a standard protocol (ASTM International, 2004; per liter of deionized water: 0.55 mg NaBr, 51.2 mg $NaHCO_3$, 2.1 mg KCl, 35.3 mg $CaCl_2$, 3.4 mg $CaSO_4 \cdot 2H_2O$, and 32.8 mg $MgSO_4$ at pH 7.6-7.9). The dilution fractions were made in 1-L glass jars and 150 mL of the dilutions were divided equally into 250 mL glass jars each containing 25 embryos, with five replicates for each concentration and control. Dilutions were prepared fresh daily. The embryos were exposed from the fertilized egg stage (stage 1) to hatching time (stage 32; approx. 5 days).

For each bioassay, jars were checked daily for mortality at the time of static daily renewal. Dead embryos were recorded and removed to prevent the spread of pathogens. When all of the embryos hatched, the exposure was completed. Embryos were randomly selected from the five

replicate jars for either malformation or gene expression analysis. For malformation analysis, embryos were analyzed individually (40 embryos per treatment). For gene expression analysis, embryos were collected by pools of 6 for 6 replicates (n = 36) and flash frozen in liquid nitrogen before storing at -80°C before further analysis. Malformation analysis and embryo full body length measurement were performed using a Nikon SMZ18 stereomicroscope (Nikon Canada, Mississauga, ON). Embryos were analyzed and scored for presence of malformations of the heart tube, craniofacial, spinal deformity, pericardial, and yolk sac edema. Embryos were considered "malformed" if they exhibited at least one malformation.

3.4.7 Gene expression analysis

RNA was isolated from embryo tissue (n = 6) using the RNeasy Micro kit (Qiagen) following the manufacturer's protocol. The purity and concentration of isolated RNA was assessed using a spectrophotometer (Nanodrop-2000, Fisher Scientific, Ottawa, ON). QuantiTect Reverse Transcription Kit (Qiagen) was used to convert the RNA to complementary DNA (cDNA) with 1 µg RNA input and random primers. A no-reverse transcriptase (NoRT) control was also prepared. Each cDNA sample was diluted to 1:40 and 1:80 for optimized quantitative polymerase chain reaction (qPCR) analysis.

Relative mRNA levels of target genes were assessed using a CFX96 Touch Real-Day PCR Detection System (Bio-Rad Laboratories Inc., Mississauga, Ontario) following MIQE guidelines (Bustin, et al., 2009). GoTaq qPCR Mastermix (Promega, Madison, WI, USA) containing Taq polymerase, nucleotides, double-stranded binding fluorescent dye, $MgCl_2$, and reaction buffer was mixed with the appropriate forward and reverse primers for each target gene (Table S3.5). A standard curve was prepared in duplicate on each plate with serial-diluted pooled cDNA to create

a calibration curve with a dilution factor of four (0.0048-50 ng). Each plate contained a no-template control to ensure nuclease-free water was not contaminated with DNA and a no reverse transcriptase control to ensure RNA samples were not contaminated with DNA. Each sample was run in duplicate, with six to eight replicates per treatment that were divided evenly across four plates per target gene. The thermocycler profile consisted of 35 cycles of heating and cooling: 95°C to activate Taq polymerize and denature cDNA strands, and a gene-dependent optimized temperature for primer annealing and elongation 58°C (Table S3.1). Standard curve efficiencies of 90.0-111.5%, and R^2 values of 0.978-0.999 were considered optimized. Gene expression analysis was performed for two normalizing genes (ribosomal protein L8 (*rpl8*) and elongation factor 1 alpha (*ef1a*)). Target genes cytochrome p450-1a; (*cyp1a)* and glutathione transferase (*gst),* known to be highly responsive to oil exposure, were chosen as biomarkers of exposure (Alsaadi et al., 2018). Target genes were normalized to the average transcript level of *rpl8* and *ef1a* to determine the relative fold change.

3.4.8 Statistical analyses

Normalized fold change values were considered outliers if they were 1.5 time outside of the interquartile range and removed from the analysis. All statistical analyses were performed in Prism (v.7, GraphPad). Transcript levels were transformed to respect the assumptions of normality (Shapiro-Wilk normality test) and equal variances (Brown-Forsythe test). One-way ANOVA was used to determine significant difference between standard deviations of treatments. If significant, Tukey's honest significance test was used for post-hoc analysis. Two-way ANOVA was used to determine significant differences in mortality and malformation for each treatment. Regression analysis was performed when there were five or more data points to analyze. Linear regression

was performed to determine the median lethal dose (LC50) of total polycyclic aromatic hydrocarbons (TPAH) concentration range was presented on a $\log_{10}$ x-axis.

3.5 Results

3.5.1 Preparation of WAF in the Spill Tank

The water-accommodated fraction (WAF) samples were prepared in the spill tank over time as described in the Methods Section. North Saskatchewan River water and sediment from its flood plain were used. River water was analyzed before and after the 28-day spill test and selected results for pH, conductivity, and total dissolved solid (TDS) are shown in Table S3.2. After adding sediment to the river water, a slight increase of water pH was observed, while the addition of oil had a negligible effect on pH (T0). The water pH decreased slightly by the end of the test. The conductivity was not affected by either the addition of sediment or oil, but also dropped slightly by the end of the spill test. TDS values did not change significantly from start to the end of the test. The size distribution and composition of the sediment added to the tank are shown in Fig. S3.3 and Table S3.3, respectively.

Water samples were collected on days 1, 6, 14, 21 and 28 for analyses and toxicity testing. On Day 28 after the spill, the floating oil mass and oil from the shoreline were recovered. The oil samples together with water and sediment samples were analyzed.

3.5.2 Oil Behavior – Mass Balance Determination

To understand the behavior of the oil in the fresh water, it was important to determine the mass balance (by weight) distribution of the original fresh oil into weathered oil as floating oil and

oil deposited on the shoreline, as well as lost to the air, aqueous phase and sediment. The masses of floating and shoreline oil were determined by weights of oil recovered after correction for water trapped in the oil as emulsions. The water contents of the recovered oils compared to the fresh oil are shown in Table 3.1 The floating oil was highly emulsified containing 73% by weightt in water; the shoreline oil had lower water content with 16%. Table 3.1 also gives the contents of oil fractions that boil below 204°C (initial boiling point [IBP] to 204°C) and above 204°C (BP>204°C). Further analyses of the BP>204°C fractions are described in "Oil Behavior – Changes in Properties and Composition" below.

To quantify the loss of light ends of the oil to the air by evaporation and biodegradation, the boiling point distributions of the recovered oils were compared to that of the fresh oil (Fig. 3.1). The initial boiling points (IBPs) of the weathered oils were close to 230°C compared to the fresh oil that had an IBP below the detection limit for the analyses (36°C). Consequently, over the 28-day test, close to 42% of the fresh crude was lost from the oil phase. As these low-boiling, organic compounds are smaller than 15 carbons in size, it is assumed that they were lost from the oil phase to the air due to a combination of evaporation and complete biodegradation to carbon dioxide.

Over the 28-day spill test, on average the waves were on half of the time to promote surface mixing. It was observed that the tank water color changed with time where Day 1 had the blackest water column compared to that observed on days 6, 14, 21, and 28. This visual evidence of oil in the aqueous phase as either oil droplets or dissolved organic compounds correlated well with chemical analyses results shown in Figs. 3.2 to 3.4 where the highest BTEX, TOC, and PAH contents in the aqueous phase were found on the first day. This likely resulted from the highly-fluid (low viscosity) oil being mixed into the water with the wave action on Day 1 while by Day

6, the weathered oil was less fluid (higher viscosity) and so resisted mixing by the waves and so remained on the surface. The BTEX content diminished to close to the detection limit after about 24 h (Fig. 3.2). The total PAHs and alkylated-PAHs in the water column dropped rapidly over the first few hours as well, and remained relatively constant and low after the Day 6 time point (120 h) (Fig. 3.3). The time course of TOC in the aqueous phase in Fig. 3.4 also shows an initial decrease until Day 5 (96 h). Interestingly, in contrast to the BTEX and PAH results, after Day 5 the TOC values increased steadily until the end of the test. The trend of increasing TOC values does not agree with BTEX and PAH contents but correlates well with the increasing contents of oxidized organic species found in the water column during the later time period shown in Fig 3.5.

After the floating oil and shoreline oil samples were removed from the tank, the slurry of bottom sediment and water was collected. The average oil content in the mixture was 138 ± 57 g/kg sediment, accounting for approximately 10.4% of the spilled oil. By difference, the remaining 3.4% of oil was lost in the water column as dissolved or dispersed oil. The chart showing the distribution of oil between the different environmental phases is shown in Fig. 3.6.

3.5.3 Oil Behavior – Properties and Composition Changes

The fresh MSW is a conventional crude with a medium density of 0.8233 g/mL, and a relatively low viscosity of 5.9 cSt at the water temperature of 15°C (Table 3.2). After weathering for 28 days in the spill tank, Table 3.2 also shows that the density and viscosity of the floating oil emulsion had increased to 0.9286 g/mL and 1.14×10^5 cSt at 15°C, respectively. Although the density of the floating oil confirms that it is expected to float, efficient recovery of the oil with its 73% of content found in water would be hampered by its high viscosity and increased volume.

The BP>204°C fractions of the fresh crude and floating oil described in Table 3.1 were subjected to further compositional analyses. Both were separated into subfractions based on polarity resulting in their contents of saturates, aromatics, resins and asphaltenes. As well, the elemental (CHNSO) analyses of the BP>204°C fractions were determined. Both sets of results are shown in Table 3.3. Saturates content in the BP>204°C fraction of the fresh oil was significantly higher than that of the weathered oil. The loss of saturates in the weathered oil appears to be countered by similar increases in contents of all three polar sub-fractions. The elemental contents data support the SARA results in that the hydrogen to carbon ratio of the fresh BP>204°C fraction at 1.79 is higher than that of the weathered product at 1.76, suggesting higher aromatic carbon content. The BP>204°C fraction from the floating oil also contained higher nitrogen and oxygen contents that contributed to its higher polarity. The increased oxygen content in the oil would be due to the same oxidation activities that resulted in the increasing contents of oxidized organic compounds with time in the water column, shown in Fig. 3.5.

3.5.4 Morphological and physiological effects in fish

There were no significant differences in mortalities, hatching time, or larvae weight compared with contaminated water treatments over time (Figs. S3.4-S3.6). For malformations shown in Fig. 3.7, the occurrence of malformed embryo initially reflected TPAH concentrations, then at later times increased with time despite low TPAH concentrations. Data were clustered into two groups. In the first cluster, Day 1 and Day 6 induced malformations at higher TPAH concentrations than in the second cluster (Day 14, Day 21, and Day 28). These data suggest that the contaminated water samples generated from Day 14 to Day 28 were more toxic than those

generated earlier. The most common malformations observed included pericardial edema, followed by heart tube, spinal and craniofacial deformities, and yolk sac edema (Table S3.4).

3.5.5 Transcriptomic analysis

Following the results of malformations (i.e., two clusters, see Section 3.5.4), transcriptomic analysis was performed on the most toxic-related cluster (Day 14 to Day 28) in order to determine if the animals were differentially coping with the range of concentrations of MSW contaminated water. Transcript levels of *cyp1a* significantly differed between negative control and the low (p = 0.023), medium ($p < 0.0001$), and high ($p < 0.0001$) MSW contaminated water treatments (Fig. 3.8). Levels of *cyp1a* transcripts increased with TPAH concentrations in a curvilinear manner. Regression analysis was performed and *cyp1a* transcript level increased in a concentration-dependent manner. A similar profile was observed for *gst* in which Day 14 was the only time point exhibiting a concentration response.

Table 3.1 Contents of distillation fractions and water in fresh and recovered (floating and shoreline) oil.

Fraction, % of mass	Fresh MSW	Floating oil	Shoreline oil
IBP-204°C	37.4	0.0	0.0
BP>204°C	62.5	26.7	84.0
Water	0.1	73.3	16.0

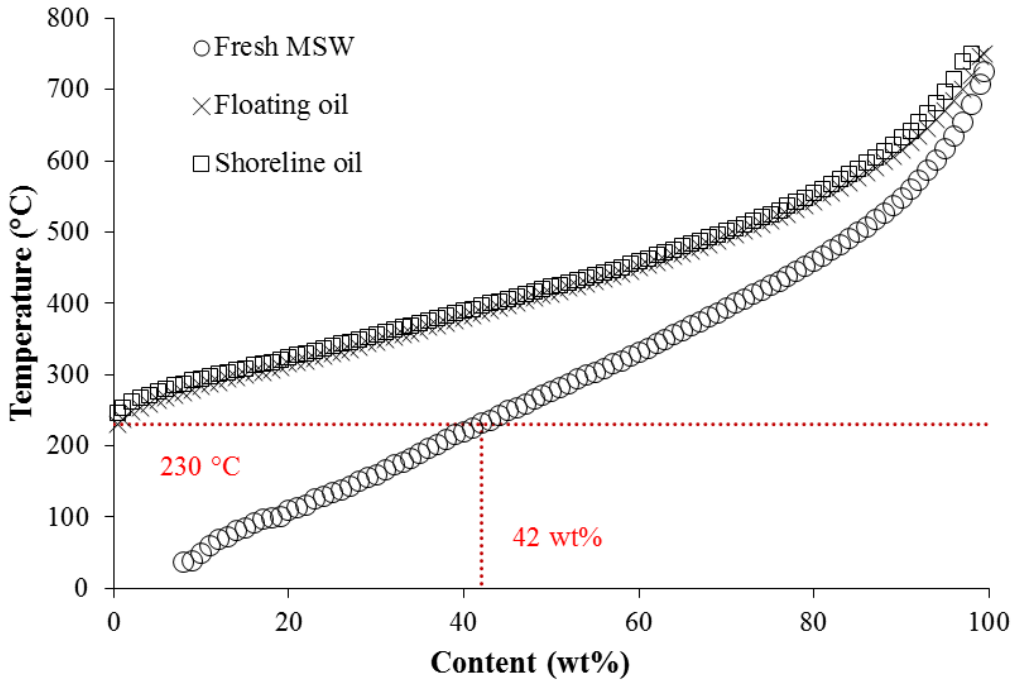
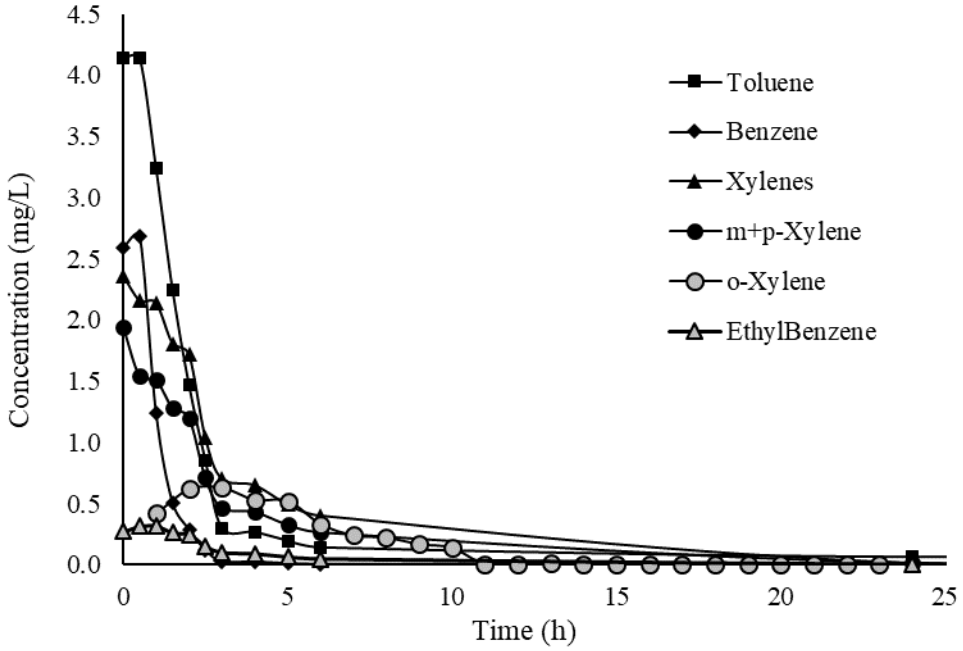


Figure 3.1 Boiling point distributions of fresh MSW and its weathered oils (either floating or from shoreline) after 28 days (648 hours).

a)



b)

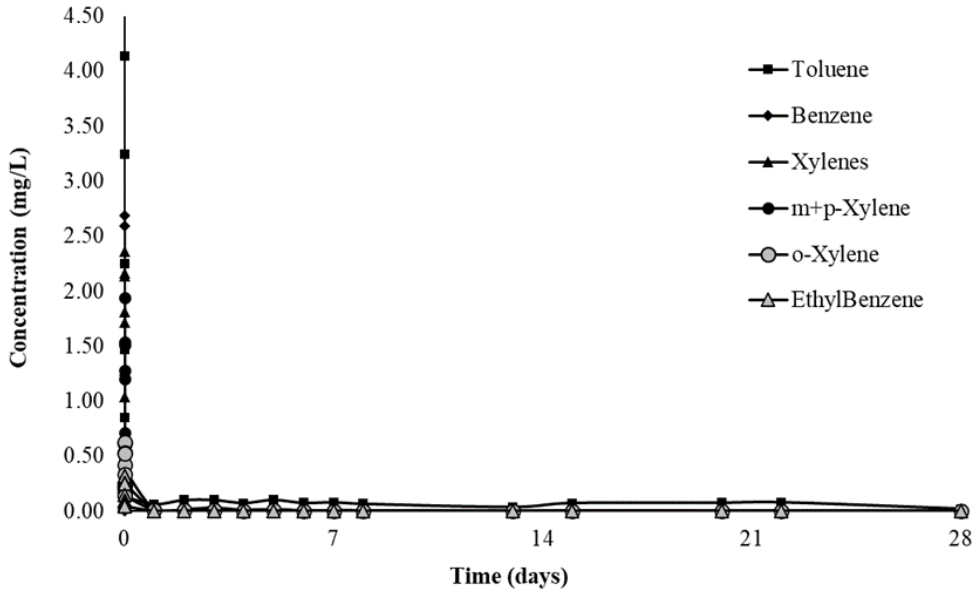


Figure 3.2 BTEX concentration in water sampled at different times over 24 h (a) and 648 h (b).

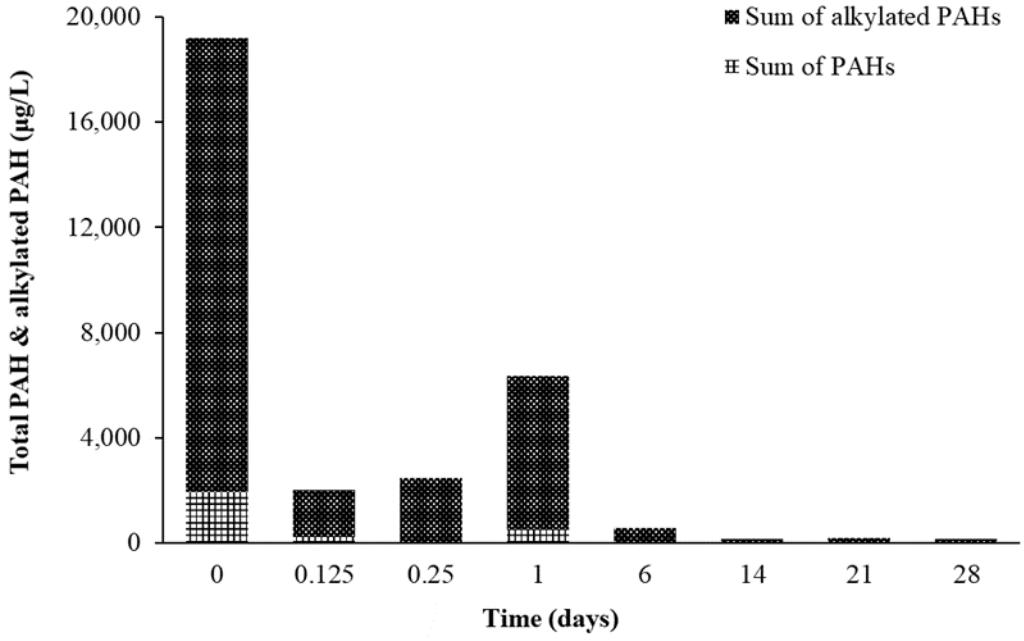


Figure 3.3 Total PAHs and alkylated-PAHs concentration in water at different time points over 648 h.

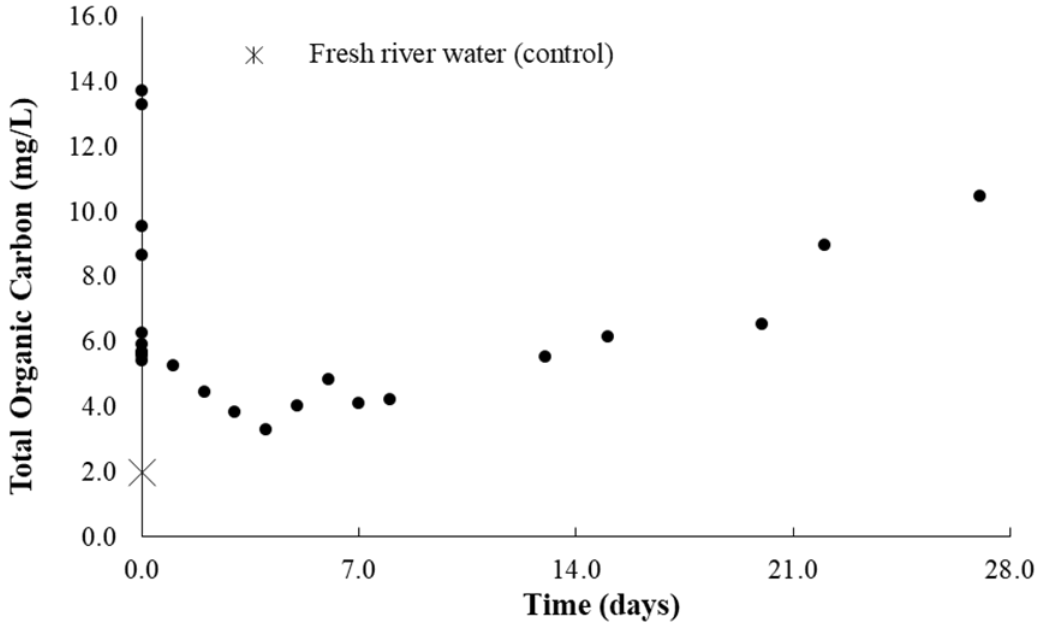


Figure 3.4 Total organic carbon in water at different time points over 28 days (648 hours).

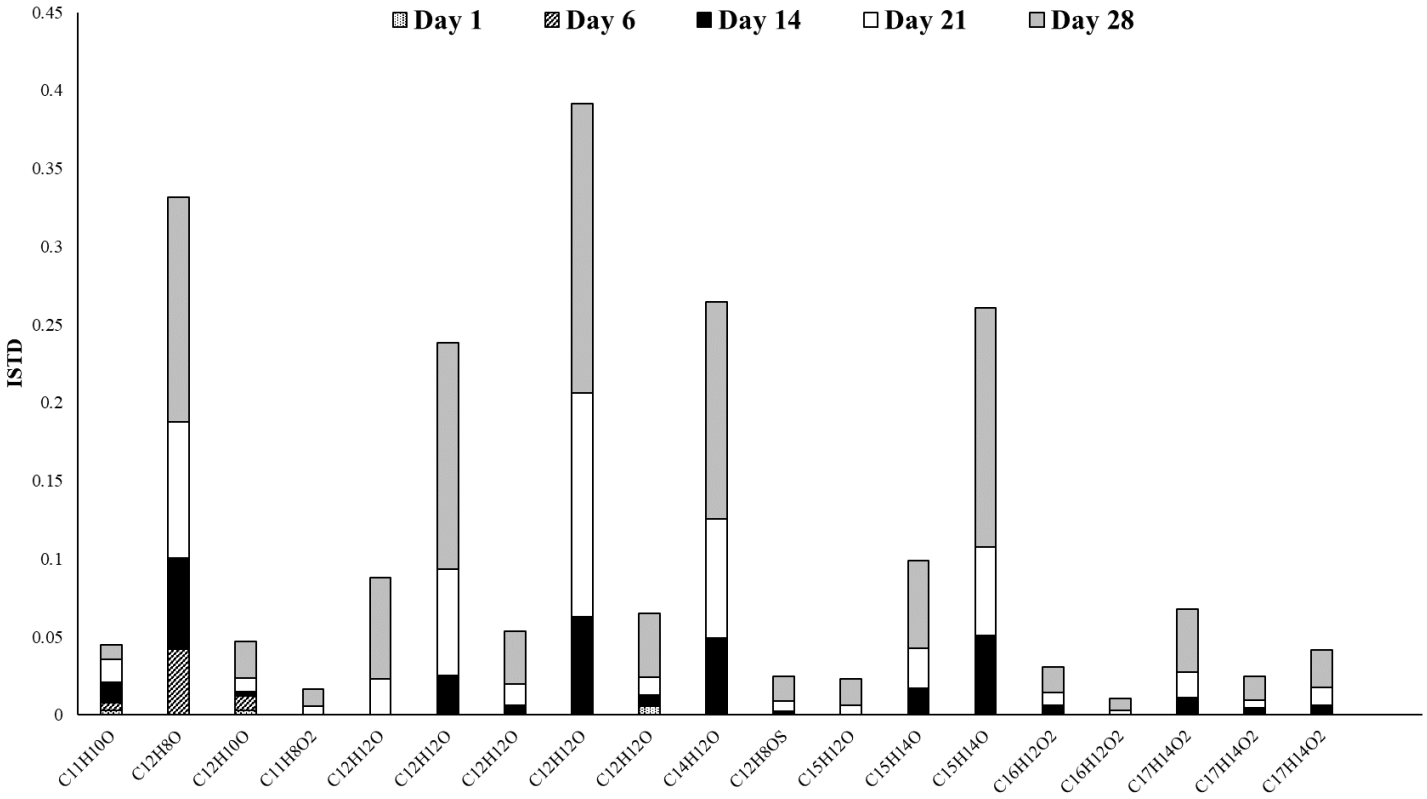


Figure 3.5 Relative contents of possible oxidized organic compounds found in the contaminated water dilutions over time normalized against an internal standard (ISTD), Acenaphthene-D10. The y-axis is the ISTD area ratio (ISTD peak area / unidentified compound detected peak area).

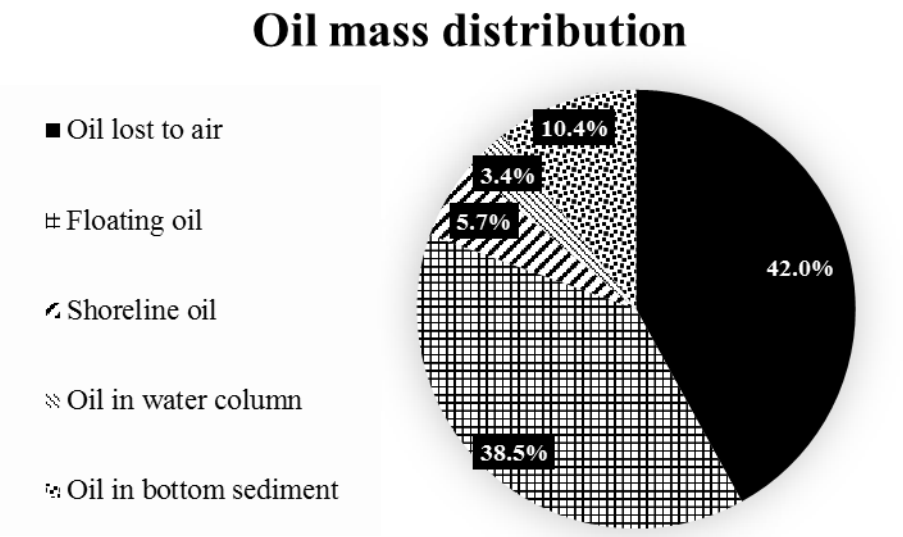


Figure 3.6 Distribution of total oil mass (by weight %) 28 days after the spill.

Table 3.2 Density and viscosity of fresh MSW and floating oil.

Property	Fresh MSW	Floating oil
Density, g/mL		
at 15°C *	0.8233	0.9286
at 20°C	0.8197	0.9269
at 25°C	0.816	0.9245
at 30°C	0.8124	0.9234
R^2	0.9999	0.9560
Viscosity, cSt		
at 15°C *	5.9	1.14×10^5
at 20°C	4.7	2.28×10^4
at 25°C	3.8	1.76×10^3
at 30°C	3.0	672
R^2	0.9984	0.9405

* Calculated based on the measured data at 20, 25 and 30°C as explained in the text.

Table 3.3 Contents of SARA subfractions and elements (CHNSO) in the BP>204°C boiling fractions of the fresh crude and weathered floating oil.

Fraction, wt%	Fresh MSW	Floating oil
SARA		
Saturates	57.0	49.9
Aromatics	27.5	30.5
Resins	13.7	16.4
Asphaltenes	1.8	3.2
Elemental		
Carbon	86.2	86.3
Hydrogen	13.0	12.7
Nitrogen	0.03	0.14
Sulfur	0.7	0.6
Oxygen	<0.1	0.2

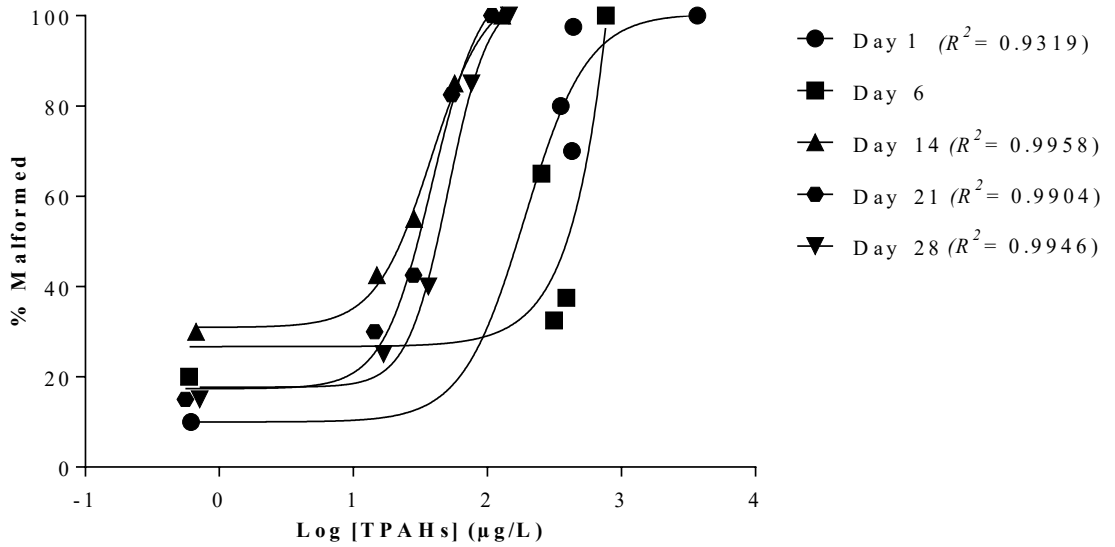
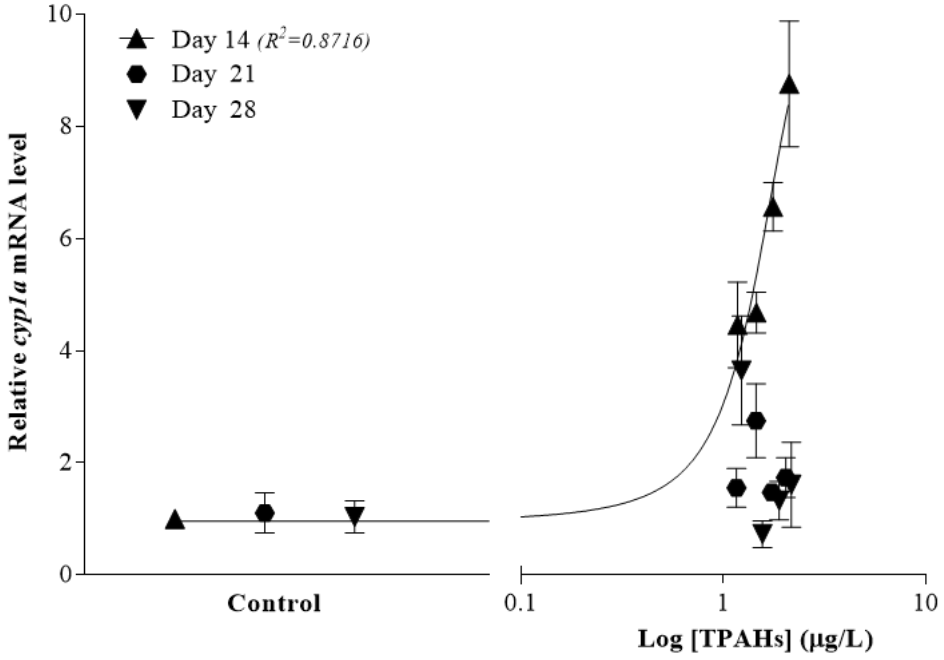


Figure 3.7 Prevalence of malformations (% malformed) in *Pimephales promelas* embryos after exposure to weathering oil water dilutions of mixed sweet blend (MSW). The independent axis represents the mean logarithmic total polycyclic aromatic hydrocarbons (TPAHs). Data clustered into two groups, cluster 1: Day 1 & Day 6, compared to cluster 2: Day 14 to 28.

a)



b)

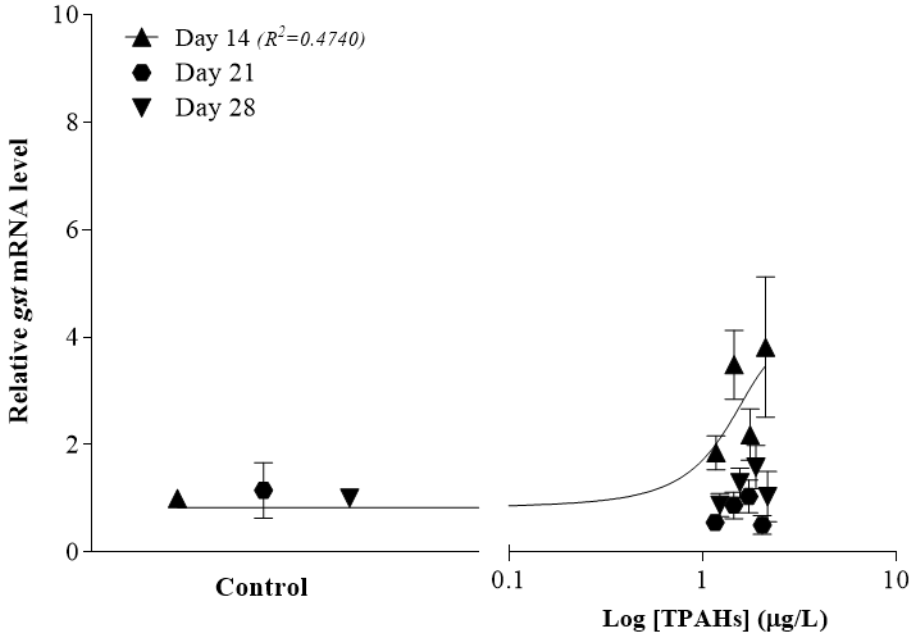


Figure 3.8 Levels of cytochrome P450 (*cyp1a*) and *gst* transcripts in *Pimephales promelas* embryos exposed to varying concentrations of MSW contaminated water. a) The effects of contaminated water dilutions on *cyp1a* mRNA level at TPAH concentrations ranging between 0.1 and 10 µg/L; b) Changes of *gst* transcripts are normalized to the mean of *odc* and *rpl8* ± SEM.

3.6 Discussion

Increased knowledge for the fate and behavior of oil after a spill in water is needed to help responders make decisions on how to mitigate environmental damage from spills. An intrinsically-safe oil spill test facility has been constructed at the Natural Resources Canada laboratory in Devon, Alberta that is large enough to be able to weather oil in the presence of waves but small enough that the collection of water and sediment samples contain detectable quantities of contaminating organic compounds. During spill tank tests, the composition of the organic compounds originating from the crude oil changes with time due to weathering processes such as dispersion, evaporation, dissolution, emulsion formation, and biodegradation. Little photo-oxidation is expected during weathering in this system due to the lighting sources used. Given that this is a stagnant system, concentrations of contaminants are high relative to any large-volume or flowing-water environment. However, by being able to measure the evolution of types of contaminants present with time, and doing dilutions to determine concentration dependencies of the toxic effects detected, insight is revealed as to how toxicity in low-flow and smaller-volume environments that can include oil trapped in sediment may evolve with time.

For this work, a spill test was conducted in the tank for 28 days to simulate an accidental release of MSW crude oil into a subarctic freshwater river during late summer-type conditions (an average water temperature of 15°C and an average air temperature of 14°C). Analyses of the water, weathered oil, and sediment collected at the end of the test together with water analyses and fish toxicity testing throughout the test period has provided detailed information for the fate and behavior of the spilled oil over the given time.

In terms of oil behavior, MSW crude is a conventional crude oil and so, having a medium density of 0.8233 g/mL at the temperature of the water, may be expected to “float” and obtain high

recovery. However, the high fluidity (low viscosity; Table 3.2) of the fresh oil allowed the breaking waves employed to mix the oil into the water as small droplets. As sediment had just been added to the water, this facilitated sedimentation of oil droplets, resulting in 10.4 wt% of the oil being caught in the sunken sediment at the end of the test. As well, evaporation, and possibly biodegradation processes started soon after the oil was spilled. By the end of the 28-day test, 42.0 wt% of the fresh oil had evaporated or was biodegraded to carbon dioxide so that the initial boiling point of the floating recovered oil had increased to 230°C. Most of the recovered oil was floating (0.9286 g/mL density; Table 3.2) but was highly emulsified, containing 73% of total mass Iin the water (Table 3.1) and was a relatively viscous material at 15°C (114,000 cSt; Table 3.2); a smaller portion of recovered oil was stuck on the shoreline of the tank. Together, the oil mass distribution of the fresh oil into weathered oil in the different environmental compartments after 28 days is shown in Fig. 3.6. This figure demonstrates that the maximum recovery from the water surface and shoreline that could have been achieved for this oil from a spill site would have been 44.2 wt%. However, its high viscosity and water content would make recovery difficult. As well, even though the oil mat was still floating at the end of the test, 13.8 wt% was still lost to the water and sediment.

With regards to ecotoxicity, the impacts of oil contaminants of biota in the water environments depends not only on the toxicity of the organic compound but also on its water solubility to become bioavailable. Analyses of the both recovered oil and oxygenated organic compounds isolated from the water column confirm that the oil was becoming oxidized with time. Biodegradation of the oil at the oil-water interface resulted in oxidized oil products, some of which were water-soluble and dissolved in the aqueous phase. Toxicity testing with fathead minnows showed that these new organic compounds are more toxic than PAHs.

The study design aimed to examine how oil weathering processes affect the water hydrocarbon profiles and its toxicity to fish with time. Figs. 3.2-3.4 show that the oil concentration in water was highest at T0 due to the initial dispersion of the fresh oil. The monoaromatics (measured as BTEX) have the highest water solubility in petroleum and their concentrations in water were also the highest at T0 (Fig. 3.2). Consequently, BTEX compounds would have contributed significantly to the total organic carbon (TOC) measured in the water over the first 24 h (Fig 3.4). Apart from BTEX, some short-chain alkanes, and small PAHs (e.g. naphthalene) or polar compounds may have contributed to the TOC in water (Lee et al., 2015). While dissolution of volatile saturates and aromatics is a competitive process with evaporation immediately after a spill, the quick decrease of BTEX in the water column over the first 24 h (Day 2) to below detection limits also resulted in the significant decrease of TOC in the aqueous phase. For TPAH concentrations, since most of the PAHs and alkylated PAHs analyzed have very low water solubility and high boiling points (> 218°C), the high total PAHs and alkylated PAHs measured at T0 in Fig. 3.3 indicate that oil droplets were also present in the water column. The rapid reduction of TPAH concentrations after the first 3 h demonstrated that the oil droplets were relatively quick to either resurface or be pushed to the bottom by sedimentation pr ocesses. The fluctuation between 3 h and 24 h reflected certain small PAHs (e.g. naphthalene) taking time to partition into water. The continuous decrease of TOC concentration in water through this time period was consistent with the decrease of TPAH concentrations between 24 h (Day 2) to 120 h (Day 6), suggesting their removal by evaporation of smaller polyaromatic compounds (e.g. naphthalene), and biodegradation processes.

Beyond 120 h (Day 6), the values of the BTEX and TPAH concentrations were low for the remainder of the test period. This was expected, particularly for the BTEX concentrations. Fig. 3.5

suggests that the increasing TOC values reflect increasing concentrations of oxidized organic compounds. A major source of oxidized organic compounds in the aqueous phase would be from incomplete biodegradation of the oil where partially degraded, oxidized products would accumulate with time. Oxidized organic compounds will have increased water solubility compared to their solubility before the addition of oxygen. For instance, oxidized PAHs (like, ketone- or quinone-substituted) partition better into the aqueous phase, showing an increase in water solubility and a decrease in lipophilicity (Idowu et al., 2019).

In terms of ecotoxicity, the increased water solubility by oxidation would increase the bioavailability of toxic compounds. However, the decrease in lipophilicity would be expected to decrease the uptake of the compounds by biota. In this study, the oxidized compounds are more toxic than the identified PAHs and alkylated PAHs (Fig. 3.7). The malformation pattern matched the oxidized organic compound profile where the fish embryos had higher proportions of malformations at lower TPAH concentrations for Day 14 to Day 28. These data suggest that the increased occurrence of fish malformations at the later times resulted from the increasing concentration of the unidentified oxidized compounds as the oil weathered. Several studies have reported increases in malformations with PAH concentrations on fish embryos (e.g., Carls et al., 2008; Incardona et al., 2013; Dubansky et al., 2013; Incardona et al., 2014; Mager et al., 2014), but few have studied the embryotoxicity of weathered oil, which would be a more realistic indicator of the impact of spilled oil into aquatic ecosystems. The weathered oil is a complex mixture that changes with time and environmental conditions (Hodson et al., *Under review*). Hansen and colleagues (2018) evaluated the toxicity of weathered crude oil (from 0 to 21 days) in a marine environment for Atlantic cod (*Gadus morhua)* and concluded that the metabolites produced from biodegradation, called the “unresolved complex mixture” fraction, were equally

toxic as the parent compounds. Robidoux et al. (2018) compared fathead minnow toxicity of weathered oil and fresh oil in minnows for seven days and observed that lethality was higher for fresh oil than for weathered oil, presumably due to the higher BTEX content in the fresh oil. Based on the present study, a proportion of the resulting "unresolved complex mixture" fraction would be the presence of unidentified oxidized compounds. Few studies have addressed the toxicity of oxidized PAHs (or oxy-PAHs) to aquatic species. In zebrafish (*Danio rerio*) embryos, exposure to 0.1 – 2 μM oxy-PAHs during early development yielded higher DNA damage than for embryos exposed to 1 μM benzo[a]pyrene (McCarrick et al., 2019). Knecht and colleagues (2013) observed similar physiological and molecular effects to those reported in the present study (e.g., yolk sac edema, spinal curvatures, and change in the expression of genes related to oxidative stress) in zebrafish embryos exposed to 38 oxy-PAHs.

Although the three last time points (Day 14 to Day 28) yielded similar malformation patterns and TPAH concentrations, the increase in *cyp1a* mRNA levels was different among the time points. The induction of *cyp1a* is well known to be responsive to several PAHs (reviewed in Wallace et al., 2020); however, only the fraction at Day 14 exhibited an increase in *cyp1a* mRNA levels. The lack of inductions observed at times Day 21 and Day 28 could be explained by an absence of aryl hydrocarbon receptor (AhR) agonists (e.g., 4-ring PAHs; Incardona et al., 2006), the presence of inhibitors, or the general disruption to the integrity of membranes, proteins and/or nucleic acids. For example, in an exposure of 4,340 – 12,200 ng/L PAHs through oil gravel effluent for 48 h (Day 3), olive flounder (*Paralichthys olivaceus*) embryos accumulated high PAH concentrations, but had a low *cyp1a* mRNA level-response (Jung et al., 2015). It was noteworthy that the reduction of *cyp1a* expression increased the prevalence of pericardial edema and poor cardiac looping in zebrafish embryos compared to normal embryos exposed to 9 ppb PAHs. This

suggests that *cyp1a* induction protects the embryo (Hicken et al., 2011). However, despite lacking a *cyp1a* response, other effects could occur in the fish embryos through AHR-independent pathways (Incardona et al., 2005). Similarly, the mRNA profile of *gst* also involved in the detoxification pathway was only significantly expressed at Day 14. Together, these data highlight the importance to continue studying side-by-side the chemistry and toxicity profiles of oil complex mixtures as they weather.

In conclusion, in the course of the 28-day spill test, the highest concentrations of total PAHs and alkylated PAHs in the water column were observed immediately after the oil was added to the tank. These concentrations decreased to 500 µg/L at 120 h (Day 6), and then to less than 200 µg/L at the end of the spill test. The BTEX concentrations in the water column were all below the detection limit after 24 h (Day 2). The TOC concentrations in the water column showed a continuous decrease from the first hour until 120 h (Day 6) due to the loss of water-soluble volatile compounds, and a subsequent increase apparently due to increasing concentrations of oxidized organic compounds accumulating in the water at later time points. This study suggests that WAFs from Day 14 to Day 28 are the most toxic fractions to the fish embryos, which corresponded to increasing concentrations of unidentified oxidized compounds. This study highlights a novel area for oil research, which could help to better understand the toxicity associated with oil weathering for aquatic species.

3.7 Acknowledgments

This project was funded by the Government of Canada's Oceans Protection Plan Program (Natural Resources Canada), Canada's Research Chair Program (to VSL), and the CONACYT-México (to LRLJ). Part of the analyses was funded by a program of the Quebec Government, the

Stratégie maritime du Gouvernement du Québec, Plan d'action 2015-2020. The authors would like to acknowledge that ALS Global performed the BTEX and PAH analyses of the undiluted water samples, and would like to thank Hena Farooqi leading the tank test operations. Analyses conducted at CanmetENERGY were performed by both the Standard Analytical Laboratory, and Upstream and Environment Team in Devon.

TRANSITION FROM CHAPTER 3 TO CHAPTER 4

After evaluating the toxicity of weathered conventional crude oil (i.e., MSW) in **Chapter 3** and discovering the increase of "unknown organic compounds" with time, we were wondering if the same was occurring during dilbit weathering. In addition, we were questioning if environmental conditions, such as temperature changes, could also alter the toxicity response of aquatic species. Therefore, we ran two consecutive experiments: one where the simulation of the oil spill in the experimental tank occurred at a temperature of 2 °C and the other at 15 °C. To do these, we used the same endemic fish species (*P. promelas)* as **Chapter 3** and the same dilbit (CLB) as **Chapter 2**. This way we will be able to also compare the embryotoxicity associated to crude oil weathered at 15 °C (**Chapter 3**) and dilbit weathered at 15 °C (this upcoming **Chapter (4)**).

CHAPTER 4: WEATHERING OF DILUTED BITUMEN LEADS TO A DIFFERENTIAL FISH EMBRYOTOXICITY

LE VIEILLISSEMENT DU BITUME DILUÉ CONDUIT À UNE EMBRYOTOXICITÉ DIFFÉRENTIELLE DU POISSON

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Fish exposure and molecular analyses: LRL-J

EROD analysis: LRL-J, GP

Chemical analysis: QX, GT-B, HD

Data analysis: LRL-J, JMV-G

Original ideas and experimental design: LRL-J, CG, QX, DH, VSL

4.1 Abstract

As the collaboration work continues between the Natural Resources Canada (NRCan) research facility at CanmetENERGY in Devon, AB and our research group at INRS. The aim of this particular chapter is to understand how the natural weathering processes, such as temperature changes, affect the fate of spilled diluted bitumen (dilbit) Cold Lake Blend (CLB) in freshwater, as well as its biological impact to embryonic fish with time. During these simulated spill scenarios, circa 10-L CLB was spilled into 1,200-L river water with premixed 2000 ppm sediment and had allowed natural weathering for 35 days with an average of air and water temperatures at either 2 °C or 15 °C in a controlled pilot-scale spill tank. Water samples were taken from the spill tank five times during the 35-day experiments (days 1, 6, 14, 28, & 35) and were used to perform early life stage exposures using fathead minnows (*Pimephales promelas*). For each sampling time, newly fertilized embryos were exposed to a serial dilution of the original tank water samples, to non-contaminated river water (used in the tank), and to reconstituted water control. Embryos were exposed for four days. Fish mortality, hatching time, incidence and type of malformations, heartbeat count, and *in vivo* EROD activity were recorded. Results showed that CLB exposure yielded significant negative impacts on embryonic fish, including an increased rate of mortality and malformations, and alterations of the heartbeat frequency and EROD activity. The highest mortality rate was found on Day 1 and 6 in both temperatures, while malformation incidence, heartbeat count, and EROD activity were much more affected in the highest CLB concentrations tested for each of the time points. Real-time RT-PCR analysis of *cyp1a* and *gst* transcription showed a similar tendency of dose-response and only a significant difference in the highest concentration of all the time points. This chapter demonstrates the importance of testing inherent

toxicity associated with different temperature environments in oil weathering, because even if there was a changing oil chemical profiling with time, the fish embryotoxicity was still maintained.

4.2 Résumé

Alors que le travail de collaboration se poursuit entre CanmetÉNERGIE des Ressources naturelles Canada (RNCan) situé à Devon, AB et notre groupe de recherche à l'INRS, l'objectif de ce chapitre est de comprendre comment les processus d'altération naturelle, tels que les changements de température, affectent le devenir du bitume dilué (dilbit) Cold Lake Blend (CLB) déversé dans l'eau douce, ainsi que son impact sur les embryons de poissons avec le temps. Au cours de ces scénarios de déversement simulés, environ 10 L de CLB ont été déversés dans 1 200 L d'eau de rivière avec des sédiments prémélangés de 2000 ppm suivi d'une altération naturelle pendant 35 jours avec une température moyenne de l'air et de l'eau soit à 2 °C ou soit à 15 °C dans un réservoir de déversement contrôlé à l'échelle pilote. Des échantillons d'eau ont été prélevés cinq fois dans le réservoir de déversement au cours des expériences de 35 jours (jours 1, 6, 14, 28 et 35) et ont été utilisés pour effectuer des expositions au stade précoce de la vie à l'aide de tête-de-boule (*Pimephales promelas*). Pour chaque période d'échantillonnage, les embryons nouvellement fécondés ont été exposés à une dilution en série des échantillons d'eau du réservoir d'origine, à de l'eau de rivière non contaminée (utilisée dans le réservoir) et à un contrôle de l'eau reconstituée. Les embryons ont été exposés pendant quatre jours. La mortalité des poissons, le temps d'éclosion, l'incidence et le type de malformations, le nombre de battements cardiaques et l'activité EROD *in vivo* ont été enregistrés. Les résultats ont montré que l'exposition au CLB avait des impacts négatifs significatifs sur les embryons de poisssons, y compris une augmentation du taux de mortalité et de malformations, et des altérations de la fréquence du rythme cardiaque et de l'activité EROD. Le taux de mortalité le plus élevé a été observé aux jours 1 et 6 pour les deux températures, tandis que l'incidence des malformations, le nombre de battements cardiaques et l'activité EROD étaient beaucoup plus affectés aux concentrations de CLB les plus élevées testées pour chacun des

périodes d’échantillonnage. Une analyse en RT-PCR à temps réel du *cyp1a* et de la *gst* a montré une tendance similaire de dose-réponse et seulement une différence significative dans la concentration la plus élevée de tous les temps d’échantillonnage a été mesurée. Ce chapitre démontre l'importance de tester la toxicité associée à différentes températures lors de l'altération du pétrole, parce que même en cas de modification du profil chimique du pétrole avec le temps, l'embryotoxicité du poisson est toujours maintenue.

4.3 Introduction

During this century, it is undeniable that petroleum products are an essential part of daily life. Just in 2018 the world production was estimated to 98.3 MMb/d (NRCan, 2020). After Saudi Arabia and Venezuela, Canada has the largest oil reserves and distributes around three million barrels of oil per pipeline per day (Dew et al., 2015). Also, Canada has the largest bitumen deposits in the world (Yang et al., 2011). The oil sand accounted for 64% of Canada's oil production in 2018 or 2.9 million barrels per day (NRCan, 2020). This oil must be transported either by truck, rail, or pipeline. In 2016, the Canadian Energy Pipeline Association (CEPA) managed 119,000 km of transmission pipelines, which transported diluted bitumen (dilbit), crude oil and natural gas (CEPA, 2016). These pipelines are an extensive network that crosses freshwater, marine, and terrestrial ecosystems. Therefore, it is crucial to understand the consequences of a spill on the Canadian environment and the living organisms it contains.

Bitumen is a complex crude oil composed of approximately 6,000 different high molecular weight compounds (approximately 200 to 800 daltons) (Strausz et al., 2011). Bitumen in the Athabascan region of Alberta (Canada) contains high levels of aromatics, heteroatoms, aromatics, and metals (Strausz et al., 2011). The high viscosity of the bitumen prevents it from being transported by pipeline unless diluted (GOC, 2013) to form a composition of approximately 20-30% diluent and 70-80% bitumen (Crosby et al., 2013). Diluents used to produce dilbit from bitumen include natural gas condensate and naphtha, although condensates are more commonly used (Lee et al., 2015). Yang and his colleagues (2011) performed GC-FID and GC-MS on oil sands bitumen in Alberta to determine their chemical composition. Examples include, but are not limited to, naphthalene, dibenzothiophene, fluorene, chrysene, benzo[a]pyrene, and their alkylated equivalents.

In general, the study of dilbit (e.g., its chemical composition, behavior and fate during a spill and its effects on organisms) is complex since the diluent and bitumen used in mixture have a variable composition. Factors that may contribute to the behavior of an oil spill include the type of oil, weather conditions at the time of the spill, and environmental factors post-spill. These chemical and environmental conditions affect the rate of evaporation, photo-oxidation, mixing, and biodegradation (Lee et al., 2015). For example, a major spill occurred in July 2010, where approximately 877,000 gallons of dilbit was discharged from an Enbridge (Line 6B) pipeline to the Kalamazoo River, Marshall, Michigan, United States (Crosby et al., 2013). The spill cleanup took over three years because unconventional oils such as dilbit have an unpredictable behavior in the environment.

It is well known that polycyclic aromatic hydrocarbons (PAHs), particularly alkylated-PAH, are toxic to aquatic species (**Chapters 2 and 3**; Fallahtafti et al., 2011; Scott et al., 2011; Alsaadi et al., 2018; McDonnell et al., 2019). For example, it has been shown that retene, a labile PAH, was toxic to larval zebrafish (*Danio rerio*) and rainbow trout (*Oncorhynchus mykiss*; Billiard et al., 1999). After 14 days of exposure, the *D. rerio* showed growth retardation, edema, and mortality. Similarly, rainbow trout exposed to retene for 42 days, showed symptoms of edema, growth retardation, craniofacial malformations, increased activity of cytochrome P4501A (CYP1A) enzymes, and blue sac disease. These symptoms were similar to those obtained in mumminchog embryos exposed to the carcinogenic compound TCDD (2,3,7,8-tetrechlorodibenzo-p-dioxin), since induced prolonged activity of mixed function oxidases (Billiard et al., 1999).

Numerous studies have investigated the effects of dilbit on several aquatic species. Lara-Jacobo et al. (**Chapter 2**) have shown that water accommodation fraction (WAF) and chemical

enhanced WAF (CEWAF) from the dilbit Cold Lake Blend (CLB) yieled high incidence of malformations in a model frog *Silurana tropicalis* as well as high level of *cyp1a* (gene related to phase I detoxification). Similarly in fish, Madison and colleagues (2017) exposed Japanese medaka embryos (*Oryzias latipes*) to CLB WAF and CEWAF for 42 days. They found significant increase in the expression of *cyp1a*, as well as positive upregulation of genes related to cell cycle (tumor protein p53), detoxification *(ahr, arnt*) and oxidative stress in phase II, such as superoxide dismutase (*sod*), and catalase (*cat*).

Dilbit weathered through physical (dispersion, spreading, and emulsification), chemical (dissolution and UV-oxidation) and biological (microbial degradation) processes (Faksness et al., 2008; Bagi et al., 2013). Degraded dilbit chemical profile often ended up containing more hydrophilic molecules, thus polar PAHs such as oxygenated PAHs (oxy-PAHs), nitrated PAHs (N-PAHs), and N/S/O-heterocyclic PAHs (Idowu et al., 2019). However, few studies have used weathered dilbit when conducting their experiment. Robidoux and colleagues (2018) compared acute and chronic toxicity of the unweathered and weathered dilbit Access Western Blend (AWB) dilbit on *P. promelas* and CLB dilbit on rainbow trout (*Oncorhynchus mykiss*). *P. promelas* yielded a higher mortality in unweathered AWB in the chronic exposure, while *O. mykiss* showed a higher toxicity in the unweathered CLB dilbit. In contrast, Hansen and colleagues (2018) studied the effects of biodegradated Troll oil on cod (*Gadus morhua*) and suggested that the oil metabolites formed during biodegradation were likely contributing to fish larvae toxicity.

Therefore, more studies are needed to understand which factors contribute to weathered oil's toxicity. The objective of this study is to compare the embryotoxicity (measuring physiological, morphological, and molecular parameters) of the dilbit CLB in two seasonal settings (fall vs.

winter) and comparing the toxicity of degraded dilbit over time to determine if the oxidized PAHs could contribute to embryotoxicity.

4.4 Materials and methods

4.4.1 Chemicals and water source

Cold Lake Winter Blend (CLB) dilbit was obtained directly from a pipeline in Edmonton, (AB, Canada). North Saskatchewan River water was collected from the city (Edmonton, AB, Canada) water facility before the potable water treatment process on September 25th, 2018, for the "warm-temperature spill run" and on January 21st , 2019, for the "cold-temperature spill run" to simulate the North Saskatchewan River (NSR) temperature in fall and winter seasons, respectively. Same sediment source has been used as described in Lara-Jacobo et al. (**Chapter 3**).

4.4.2 Controlled oil spill in the spill tank

The experiment setup was the same as the previous 28-day tank test used in **Chapter 3**, except these two tests had to run for 35 days (Figure S4.1).

4.4.3 Physical and chemical analyses of the oil

After collecting the required water samples for analyses, weathered oil emulsions from the water surface (floating oil) and shoreline (shoreline oil) were recovered, weighed, and analyzed. Both weathered oils were analyzed by high temperature simulated distillation (HTSD). The floating oil was distilled to a cut point of 204 °C to separate the initial boiling point (IBP) to 204 °C-oil fraction

from the heavier portion of the oil that has boiling points >204 °C using ASTM D1160. The IBP–204 °C fraction was placed in a freezer to freeze the water in the oil. The oil was poured off the ice and its weight measured to determine the water content in the original emulsion. The density and viscosity, as well as other characteristics of fresh CLB and the floating oil was determinated. Density was measured using a densitometer at 20.0, 25.0, and 30.0 °C (ASTM D4052), viscosity was measured using a Brookfield viscometer at the same temperatures using ASTM (D7042). The basic sediment and water content (BS&W) in fresh CLB was measured using ASTM D7829. Boiling point distribution was determined by HTSD analyses (ASTM D7169) on an Agilent gas chromatograph with Analytical Control software. Elemental analysis was performed to determine the contents of carbon (C), hydrogen (H), nitrogen (N), and sulfur (S) using an Elementar Analyzer (ASTM D5291). Oxygen (O) content was determined with an Elementar Oxygen Analyzer using an in-house method. Reported elemental data are normalized with respect to total CHNSO content. Separations of the fresh and weathered oil samples into saturates, aromatics, resins, and asphaltenes (SARA) subfractions were performed according to an in-house modification of ASTM methods D2007 and D2549.

4.4.4 Embryotoxicity assays

Fathead minnow (FHM; *Pimephales promelas*) eggs were collected from the colony held at INRS, Quebec City, QC, Canada. Eggs were obtained following an established breeding protocol based on the INRS' Animal Care Committee and the Canadian Council for Animal Care guidelines. Eggs were collected from approximately 30 couples of FHM, randomized and scooped before use. For each of the time points of interest (Day 1, Day 6, Day 14, Day 28, and Day 35), in two different environment conditions (2 °C and 15 °C) with CLB-contaminated water stock was stored at 4 °C.

For each assay, dilutions were prepared for the following nominal dilutions 12.5, 25, 50, and 100% v/v from the CLB-contaminated water stock. The fractions were diluted with North Saskatchewan River water and the experimental design included a river water control (0% oil). A second control was included to ensure that the river water did not affect the tests, a reconstituted water control prepared according to a standard protocol (ASTM International, 2004; per liter of deionized water: 0.55 mg NaBr, 51.2 mg $NaHCO_3$, 2.1 mg KCl, 35.3 mg $CaCl_2$, 3.4 mg $CaSO_4 \cdot 2H_2O$, and 32.8 mg $MgSO_4$ at pH 7.6-7.9). The dilution fractions were made in 1-L glass jars and 150 mL of the dilutions were divided equally into 250 mL glass jars each containing 25 embryos, with five replicates for each concentration and control. Dilutions were prepared fresh daily. The embryos were exposed from the fertilized egg stage (stage 1) to hatching time (stage 32; approx. 5 days). All animal work was conducted in accordance with the INRS' Animal Care Committee and the Canadian Council for Animal Care guidelines.

For each bioassay, jars were checked daily for mortality at the time of static daily renewal. Dead embryos were recorded and removed to prevent the spread of pathogens. When all of the embryos hatched, the exposure was completed. Embryos were randomly selected from the five replicate jars for either malformation, gene expression analysis, heartbeat rate, and/or EROD *in vivo*. For malformation analysis and heartbeat rate, embryos were analyzed individually (40 and 15 embryos per treatment, respectively), whereas for EROD *in vivo* analysis, 36 embryos per treatment were analyzed. For gene expression analysis, embryos were collected by pools of 6 and 6 replicates were prepared (n = 36 embryos total were used) and then, they were flash frozen in liquid nitrogen before storing at -80 $^{\circ}$C before further analysis. Malformation analysis and heartbeat rate were performed using a Nikon SMZ18 stereomicroscope (Nikon Canada, Mississauga, ON). Embryos were analyzed and scored for presence of malformations of the heart

tube, craniofacial, spinal deformity, pericardial, and yolk sac edema. Embryos were considered "malformed" if they exhibited at least one malformation. For the heartbeat rate embryos were placed in a clear plate of 24-well individually and recorded the number of heart beats for 60 sec. For EROD *in vivo* analysis, embryos were collected by pools of 6 for 6 replicates (n = 36) and immediately analyzed.

4.4.5 EROD *in vivo* and gene expression analysis

Embryos were transferred after the exposure to a 2-ml Eppendorf tubes. A 600-µL aliquot of 7-ethoxyresorufin (7-ER) was added to each tube. After 4 h of incubation at 25 °C, 100-µL aliquots were transferred from each tube to a Thermo Scientific Microwell 96-well microplate. A standard curve with resorufin was prepared (0 nM - 10 nM) diluted in FHM's aquaria water. On each plate there were duplicates of both the standard and the aliquots. The fluorescence was determined using a FilterMax F5 Multi-Mode Microplate Reader (Molecular Devices) at the wavelengths 544 nm (ex) and 590 nm (em). EROD activity was calculated and expressed as picomole of resorufin per protein milligram per min. At the end of the assay embryos were sacrificed and storing at -80 °C.

RNA was isolated from embryo tissue (n = 6) using the RNeasy Micro kit (Qiagen) following the manufacturer's protocol. The purity and concentration of isolated RNA was assessed using a spectrophotometer (Nanodrop-2000, Fisher Scientific, Ottawa, ON). The QuantiTect Reverse Transcription kit (Qiagen) was used to convert the RNA to complementary DNA (cDNA) with 1 µg RNA input and random primers. A no-reverse transcriptase (NoRT) control was also prepared. Each cDNA sample was diluted to 1:40 and 1:80 for optimized quantitative polymerase chain reaction (qPCR) analysis.

Relative mRNA levels of target genes were assessed using a CFX96 Touch Real-Day PCR Detection System (Bio-Rad Laboratories Inc., Mississauga, Ontario) following MIQE guidelines (Bustin et al., 2009). GoTaq qPCR Mastermix (Promega, Madison, WI, USA) containing Taq polymerase, nucleotides, double-stranded binding fluorescent dye, $MgCl_2$, and reaction buffer was mixed with the appropriate forward and reverse primers for each target gene (Table S4.1). A standard curve was prepared in duplicate on each plate with serial-diluted pooled cDNA to create a calibration curve with a dilution factor of four (0.0048 - 50 ng). Each plate contained a no-template control to ensure nuclease-free water was not contaminated with DNA and a no reverse transcriptase control to ensure RNA samples were not contaminated with DNA. Each sample was run in duplicate, with 6 to 8 replicates per treatment that were divided evenly across four plates per target gene. The thermocycler profile consisted of 35 cycles of heating and cooling: 95 °C to activate Taq polymerize and denature cDNA strands, and a gene-dependent optimized temperature for primer annealing and elongation 58 °C (Table S4.1). Standard curve efficiencies of 97.3-110.7%, and R^2 values of 0.989-0.999 were considered optimized. Gene expression analysis was performed for two normalizing genes (ribosomal protein L8 (*rpl8*) and elongation factor 1 alpha (*ef1a*). Target genes cytochrome p4501a; (*cyp1a)* and glutathione transferase (*gst),* known to be highly responsive to oil exposure, were chosen as biomarkers of exposure (Alsaadi et al., 2018). Target genes were normalized to the average transcript level of *rpl8* and *ef1a* to determine the relative fold change.

4.4.6 Statistical analyses

Normalized fold change values were considered outliers if they were 1.5 time outside of the interquartile range and removed from the analysis. All statistical analyses were performed in Prism

(v.7, GraphPad). Transcript levels were transformed to respect the assumptions of normality (Shapiro-Wilk normality test) and equal variances (Brown-Forsythe test). One-way ANOVA was used to determine significant difference between standard deviations of treatments. If significant, Tukey's honest significance test was used for post-hoc analysis. Two-way ANOVA was used to determine significant differences in mortality and malformation for each treatment. Regression analysis was performed when there were five or more data points to analyze. Non-linear regression was performed to determine the effective concentration median dose (EC50) of total polycyclic aromatic hydrocarbons (TPAH) concentration range was presented on a $\log_{10}$ x-axis. In addition, Principal Components Analysis (PCA) and clustering data analysis were performed to identify covariance among the samples. The methodology has been previously reported (Gutierrez-Villagomez et al., 2020); however some changes were made. Briefly, the concentration of total PAHs, C10-C50 concentration, alkyl PAHs, VOCs, mortality, hatching time, malformation, heart beats rate, and EROD *in vivo* assay were used for pattern analysis. The software R (R Core Team, 2013) and R Studio as the interface (RStudio Team, 2016) were used for PCA and clustering analysis. The components that explained more than 10% of the variance were chosen for the plots. Clustering and bootstrapping analysis were performed using the package pvclust in R using Ward's algorithm based on the Euclidean distance (Legendre and Legendre, 1998; Suzuki and Shimodaira, 2006; Ward, 1963). Bootstrapping was used to assess the uncertainty in hierarchical cluster analysis. Statistical support for each node in the dendrogram was obtained by the bootstrap method with 1000 bootstraps. The R package pvclust (Suzuki and Shimodaira, 2006), provides AU (approximately unbiased) p-values and BP (bootstrap probability) p-values. Multiscale bootstrap resampling was used for the calculation of AU p-values, reducing bias over BP values calculated by the ordinary bootstrap resampling (Shimodaira, 2002). In the bootstrapping analysis, the null

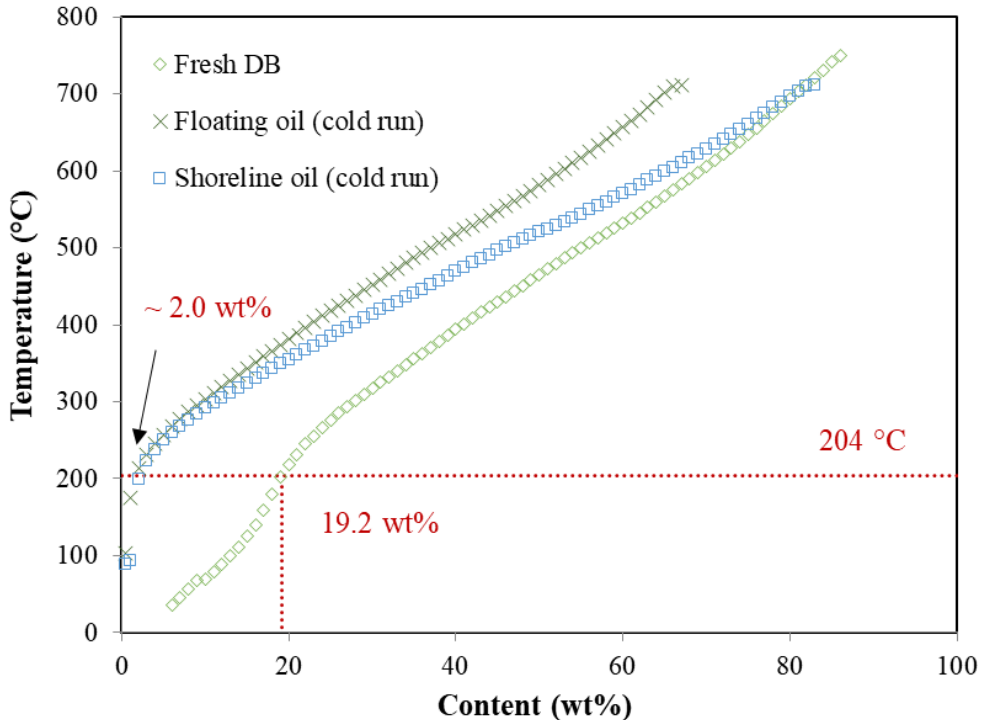
hypothesis we tested was "the cluster does not exist". Thus, nodes with AU p-values > 95 were considered supported with a significance level 0.05 (Suzuki and Shimodaira, 2006). The R package Scatterplot3d was used to visualize the PCA (Ligges and Maechler, 2003). The R package rgl was used to visualize the PCA loadings (Adler et al., 2020). The R package RcolorBrewer was used to assign color blind-friendly colors to the PCA plots (Neuwirth, 2014). The R package dendextend was used to improve the visualization of the dendrograms (Galili, 2015).

4.5 Results

4.5.1 Oil and water analyses

Tables S4.2 and S4.3 summarize the oil physicochemical properties of fresh CLB and floating oil that went through natural weathering processes on the water surface for 35 days. The fresh CLB before the spill had a density of 0.9292 g/mL and a viscosity of 531.6 cSt at 15 °C (Table S4.2). By the end of the tests (day 35), the density and viscosity of the floating oils recovered from the water surface increased significantly to close to 1.0 g/mL, and over 1 x 10^5 cSt at 15 °C, respectively (Table S4.2). It appears that these changes were mainly due to (i) the loss of IBP-204 °C fraction from 17.06 wt% to 1-2 %, as shown from the distillation results and (ii) the emulsification process that increased the water content from 0.3 wt% to above 30 wt % (Table S4.3). Consistently, the boiling point distributions of fresh diluted bitumen (dilbit) and weathered oils in Figure 4.1 showed that almost all the light hydrocarbons with boiling points of less than 204 °C were disappeared in the warm run, while the weathered oil from the cold run had about 2.0 wt% IBP-204 °C fraction.

a)



b)

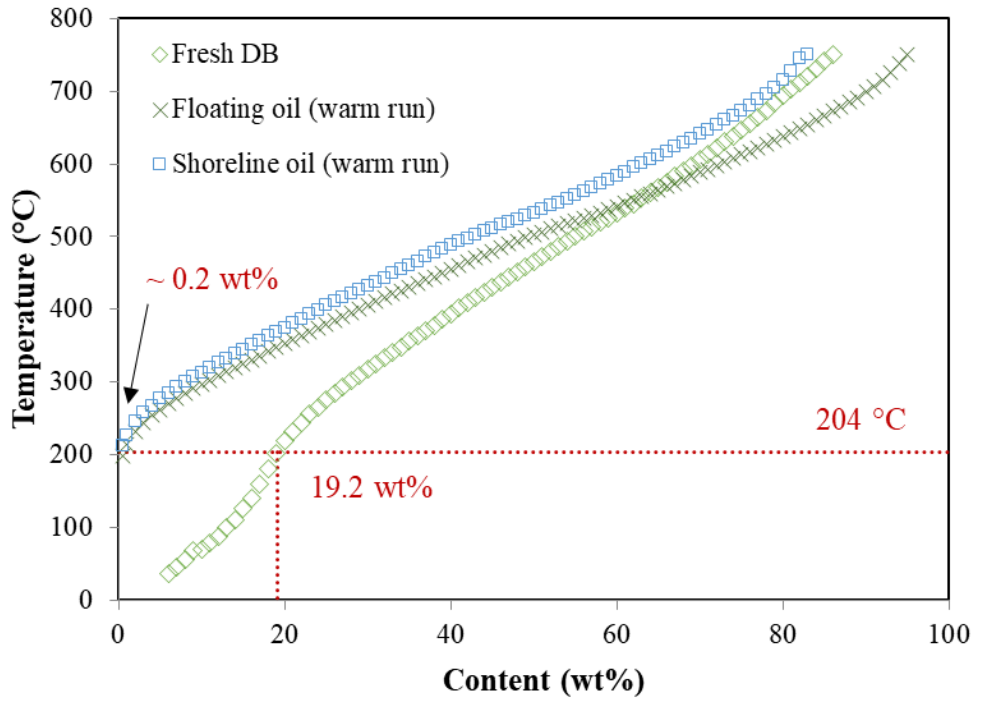


Figure 4.1 Boiling point distributions of fresh CLB and its weathered oils (either floating or from shoreline) after 35 days (816 hours): (a) at 2 °C (b) and at 15 °C.

A comparison of the elemental contents of the BP > 204 °C fraction of the fresh CLB and floating oils showed that there were no significant differences within these fractions (Table S4.3). The SARA data from the same fractions showed that the aromatics content of oil had decreased greatly (> 20 wt%) when compared to that of the fresh CLB, which resulted in the increases in the proportions of the saturates, resins and asphaltenes. Overall, the saturates, resins and asphaltenes in the 204 °C-IBP fraction have stayed relatively constant (Table S4.3). Collectively, these results suggest that the changes in the physicochemical properties of the CLB during the cold and warm tests were mainly due to the evaporation of the light hydrocarbons (IBP-204 °C fraction), emulsification process, and/or biodegradation process in the removal of some aromatics.

The BTEX concentration in water diminished to close to the detectable limit after 24 h in both temperatures (2 °C and 15 °C) (Fig. 4.2). Fig. 4.3 presents the two time courses of total organic carbon (TOC) in water during the two 35-day spill tests, which both showed a quick decrease in earlier hours and stayed constant afterwards. Total PAH and alkylated-PAH concentration in water at different time points are presented in Fig. 4.4. Noteworthy, both the total PAHs and alkylated PAHs concentrations were highest at T3 but the value in the 15 °C trial was two times higher than that from the 2 °C trial.

Moreover, after draining of the tank water, the oil associated with the bottom sediment in tank was recovered and assessed using in-house solvent extraction method to determine the degree of oil that was sunken. It was found that around 30 ± 2 mg oil per gram dry sediment were ended up in the bottom sediments at both tests, regardless of the operating temperatures. Oil sedimentation was accounted for around 1.0 % of the total initially added dilbit under the testing conditions, probably due to the high viscosity of dilbit and its less tendency to disperse. Table 4.1 summarizes the overall mass balance of distributed oil in the spill tank after 35 days.

Table 4.1 Oil mass balance at the end of 35 days in the warm and cold trials.

	Trial at 2 °C	**Trial at 15 °C**
Operation air/water temperature, °C	6/2	14/15
Oil distribution (wt %)		
(1) Lost to air or biodegradation*	19.0 ± 0.2	17.2 ± 0.2
(2) 1200 L water column**	0.1 ± 0.05	0.1 ± 0.05
(3) Floating water-in-oil emulsion	76.7 ± 0.6	82.7 ± 0.6
(4) Oil on shoreline	1.8 ± 0.2	0.4 ± 0.2
(5) Bottom wet sediment	1.2 ± 0.2	0.8 ± 0.2
Total oil from (1)-(5), %	98.8 ± 1.25	101.2 ± 1.25

*Calculated based on the distillation results of fresh dilbit and floating emulsions in Figure S4.3.

**Calculated based on the differences in the TOC of water column before and after the run.

a)

b)

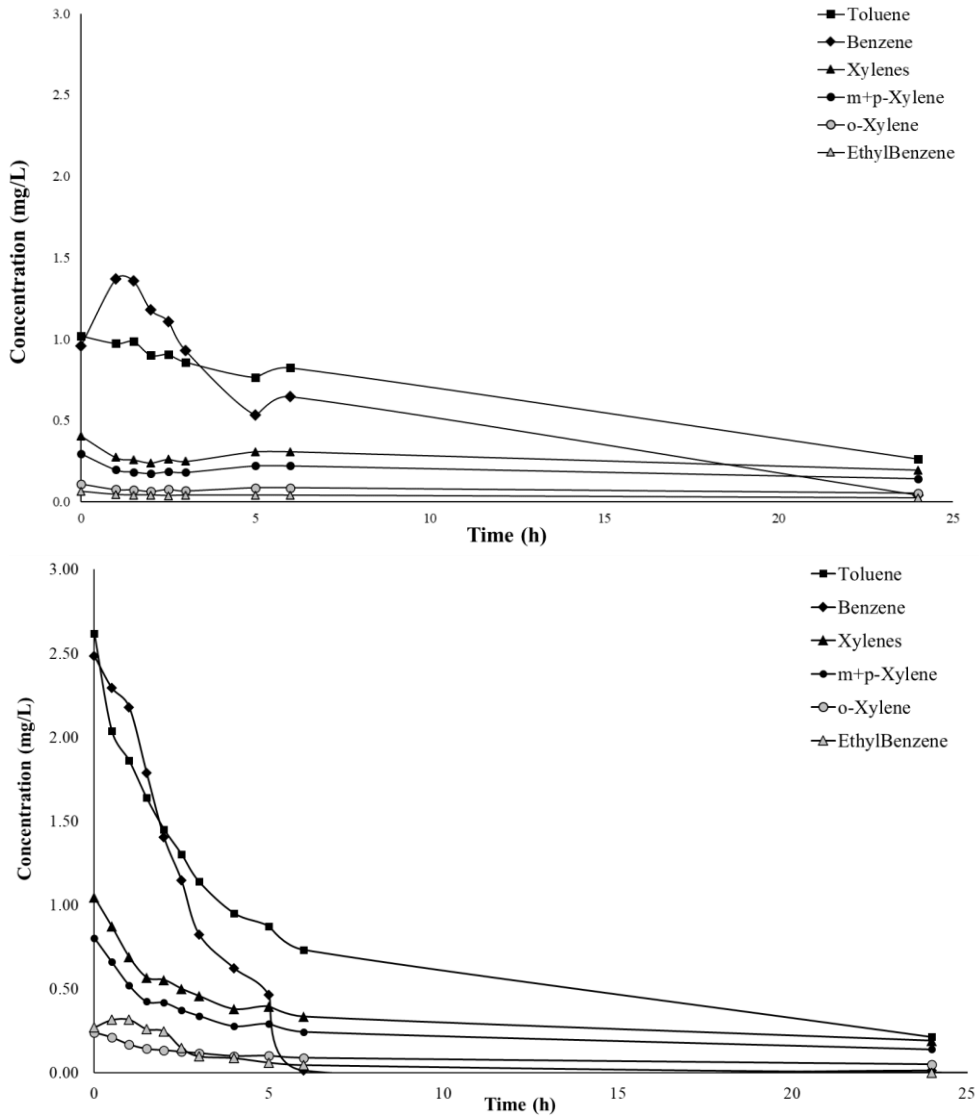
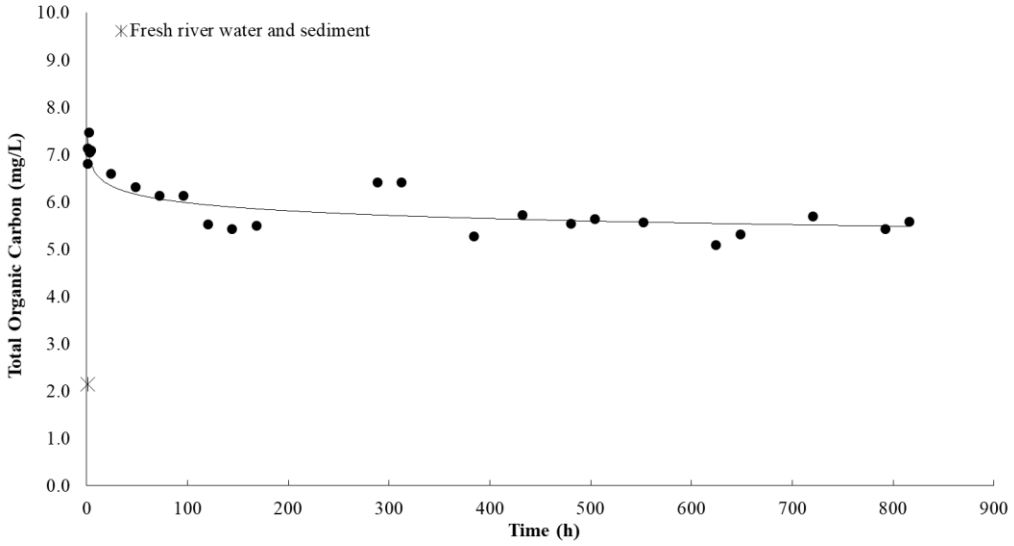


Figure 4.2 BTEX concentrations in water sampled at different times over 24 h: (a) at 2 °C and (b) at 15 °C.

a)



b)

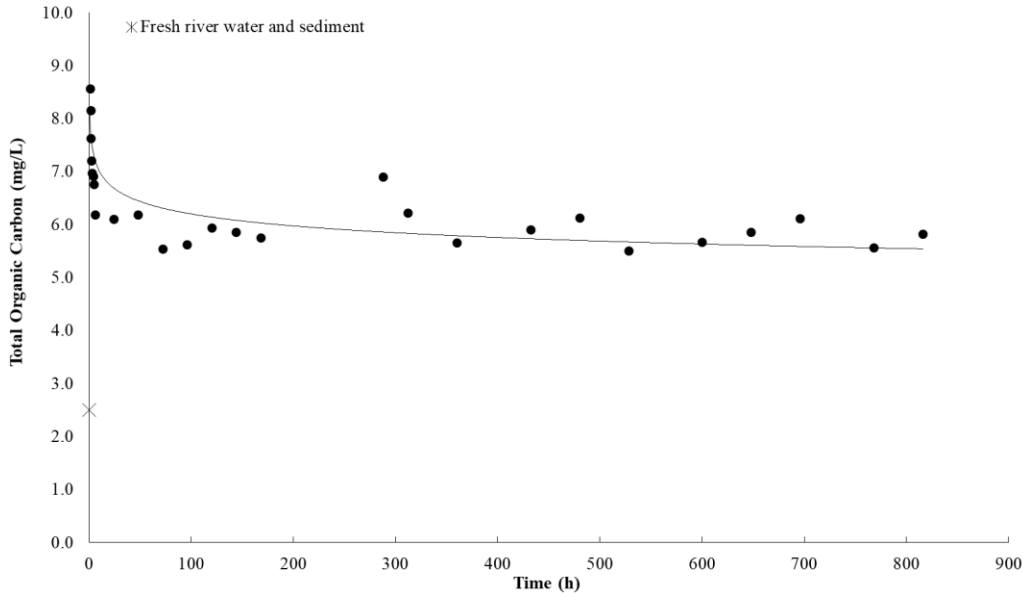
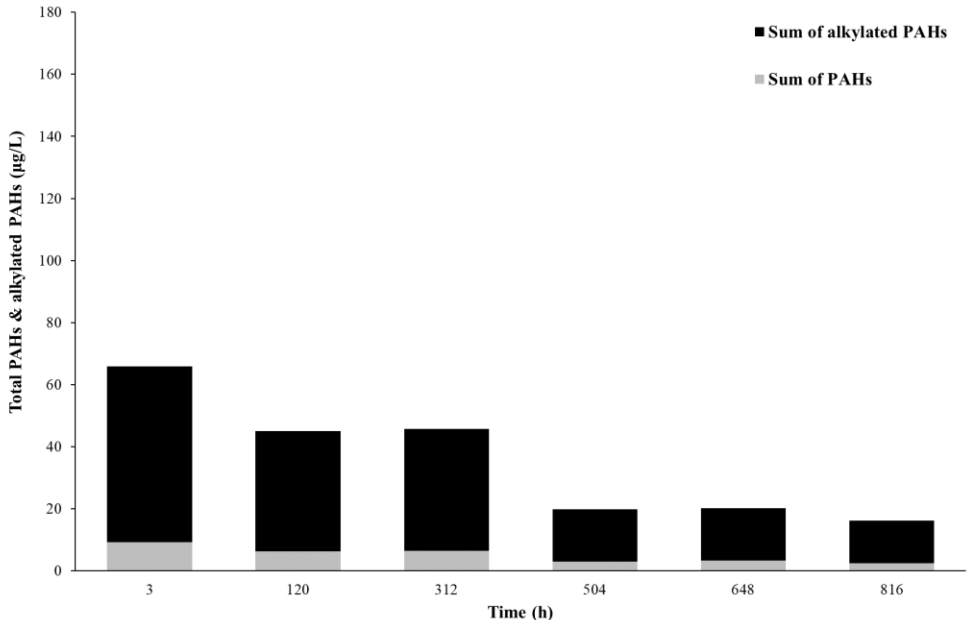


Figure 4.3 Total organic carbon in water at different time points over 35 days (816 hours): (a) at 2 °C and (b) at 15 °C.

a)



b)

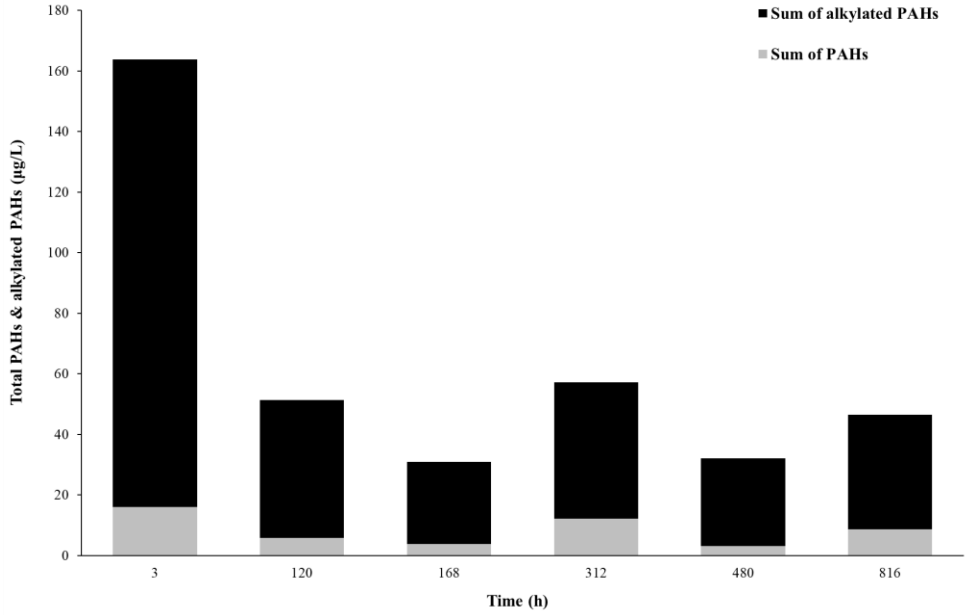


Figure 4.4 Total PAH and alkylated-PAH concentrations in water at different time points over 816 h: (a) at 2 °C and (b) at 15 °C.

4.5.2 Morphological and physiological effects in fish

There were no significant differences in mortalities or hatching time, compared with contaminated water treatments over time in any of the two temperatures (Fig. S4.2 and S4.3). For malformation analysis, in both temperature regimes, the occurrence of malformed embryos initially reflected TPAH concentrations, then at later times, the toxicity is maintained with time despite low PAH concentrations (Fig. 4.5).

Data clustered into two groups at 2 °C and at 15 °C. At 2 °C, the first cluster (i.e., Day 1, 6 and 35) induced malformations at higher TPAHs concentrations than in the second cluster (Day 14 and 28) even if the TPAH concentrations was low the highest dilution showed 100% malformation for all timepoints and both temperatures, suggesting that saturation was reached. Besides the significant differences between dilutions concentrations, it was observed between times only in the 25% nominal dilution. Where Day 1 was a significant difference with Day 6, Day 14, and Day 28.

In contrast to the 15 °C trial, the first cluster (Day 1, 6 and 14) showed the highest rate of malformation followed by the second cluster (Day 28 and 35). There were significant changes between the WAF concentrations and the controls for each time point and only the 25% WAF was statistically different between Day 1, and both days 28 & 35. In both figures (Fig. 4.5 a-b), the same sigomoidal trend was observed. The most common type of malformations noted included pericardial edema, followed by heart tube, spinal and craniofacial deformities, and yolk sac edema (Table S4.4).

The heartbeat rate data showed the same pattern at Day 1 for both environmental conditions (2 °C and 15 °C) yielding the lowest heartbeat rate of all the other time points (Fig. 4.6). For the

trial ran at 2 °C, there were significant differences between WAF concentrations and between time points. Similar to the malformation data, the heart rate was significantly different at 25% WAF, but this time the difference was between days 1 and 6 versus days 14 and 35. Finally, for the trial performed at 15 °C, significant differences were observed at 12.5% WAF between Day 1 versus Day 6.

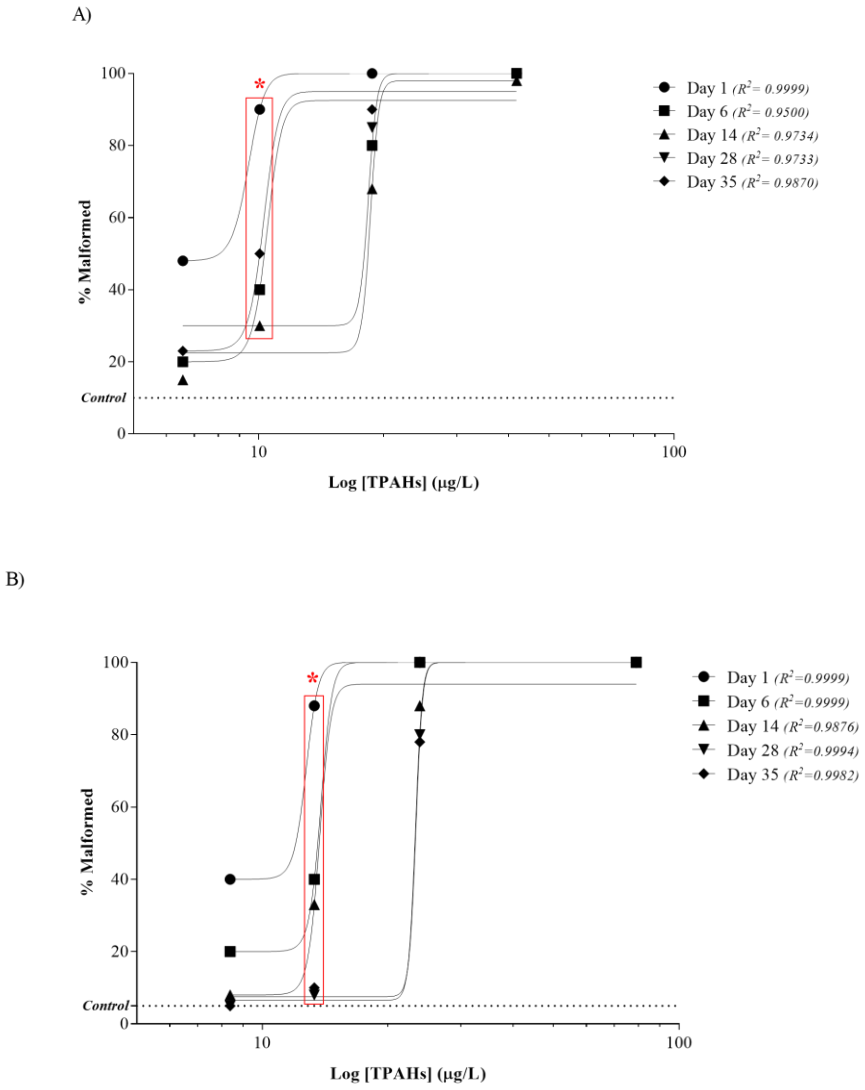


Figure 4.5 Prevalence of malformations (% malformed) in *Pimephales promelas* embryos after exposure to weathering oil water dilutions of the dilbit Cold Lake Blend (CLB). The independent axis represents the mean logarithmic total polycyclic aromatic hydrocarbons (TPAHs). The asterisk (* Turkey's multiple comparisons, $p < 0.05$) represents significant differences and the red box the significant difference between (A) Day 1 versus Day 6, 14 and 28 (at 4 °C) and (B) Day 1 versus Day 28 and 35 (at 15 °C)

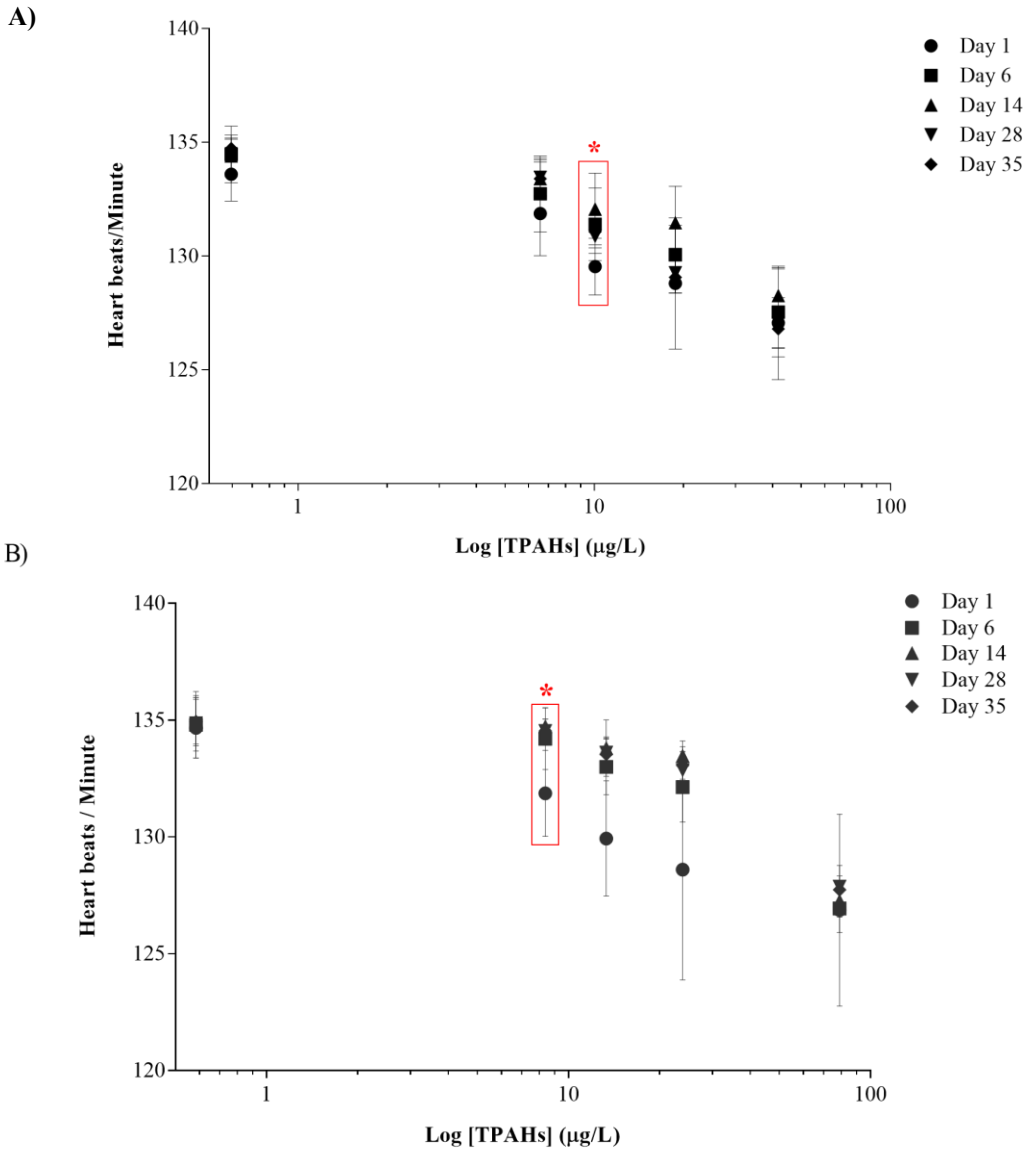


Figure 4.6 Effects on the heart beats rate of recently hatched *P. promelas,* exposed to weathered CLB dilutions ($n = 15$: analysis of variance with Dunnett's test). Error bars represent the standard deviation: asterisks indicate a statistically significant ($\alpha = 0.05$) the red box the significant difference between (A) Day 1 versus Day 6, 14 and 34 (at 4 °C) and (B) Day 1 versus Day 6 (at 15 °C)

4.5.3 Activity of EROD *in vivo* and transcriptomic analysis

The EROD activity showed a different pattern between the 2 °C and 15 °C trials. For the 2 °C, the highest induction of EROD activity was measured at Day 1 (0.5 pmol/mgprotein/min) compared to the control but data are not significant. Noteworthy, Day 14 showed the lowest induction of EROD activity of all the other time points, but between concentrations there was a significant difference ($p < 0.05$) between the control and the two highest concentrations. At 15 °C, all the time points showed a similar profile with the highest increase of EROD induction in the medium-range concentration and a decrease in the highest concentration. At Day 28, there was a significant difference between the medium-range concentration and the control (Fig. 4.7).

Transcript levels of *cyp1a* showed differences between 100% WAF and the control, but data are only significant for Day 6 for both temperatures (Fig. 4.8). A regression analysis showed that *cyp1a* transcript level increased in a concentration-dependent manner to TPAH levels. A similar profile was observed for *gst* in which Day 6 also showed a significant difference compared to the control (Fig. 4.9).

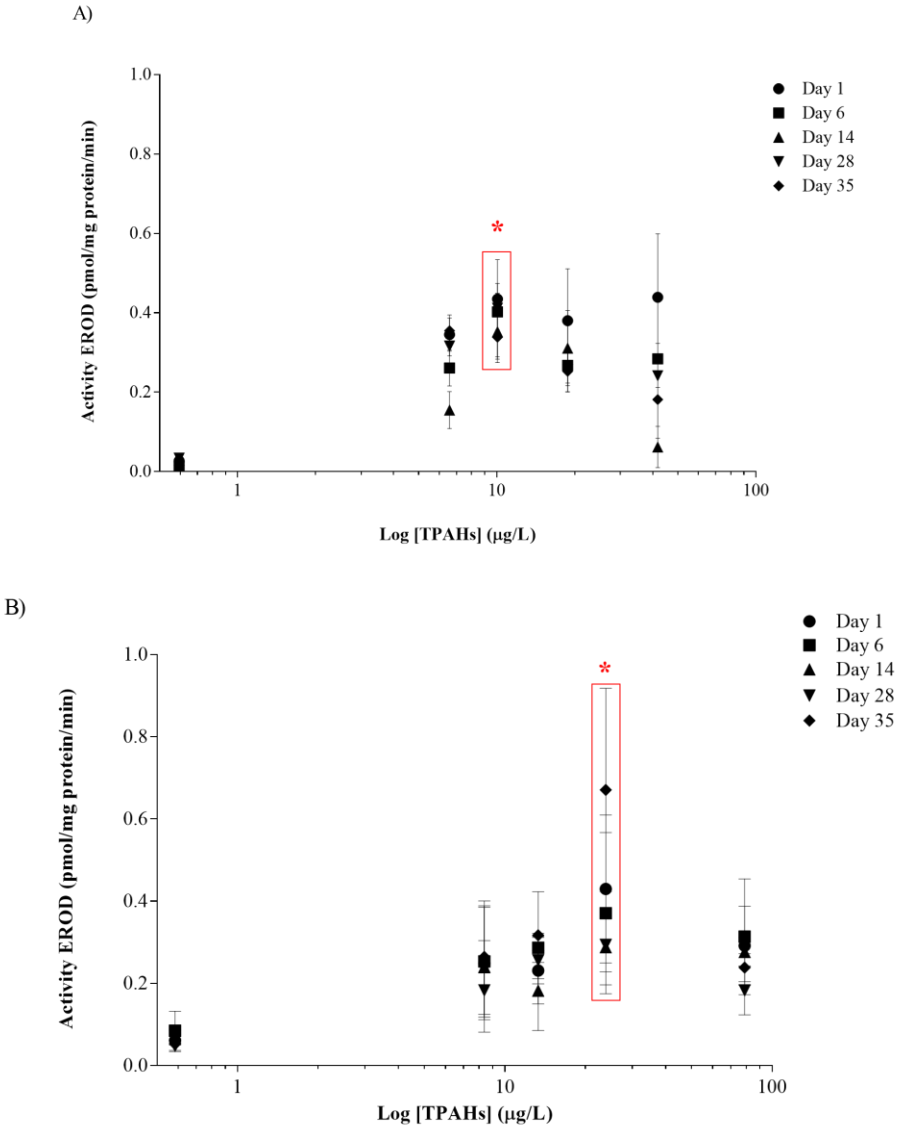


Figure 4.7 Activity Ethoxyresorufin-O-deethylase (EROD) *in vivo* (pmol/mgprotein/min). Values represent mean ± standard error. The asterisk (*Turkey's multiple comparisons, $p < 0.05$) represents significant differences and the red box the significant difference between (A) Day 1 versus Day 35 (at 4 °C) and (B) Day 14 versus Day 35 (at 15 °C)

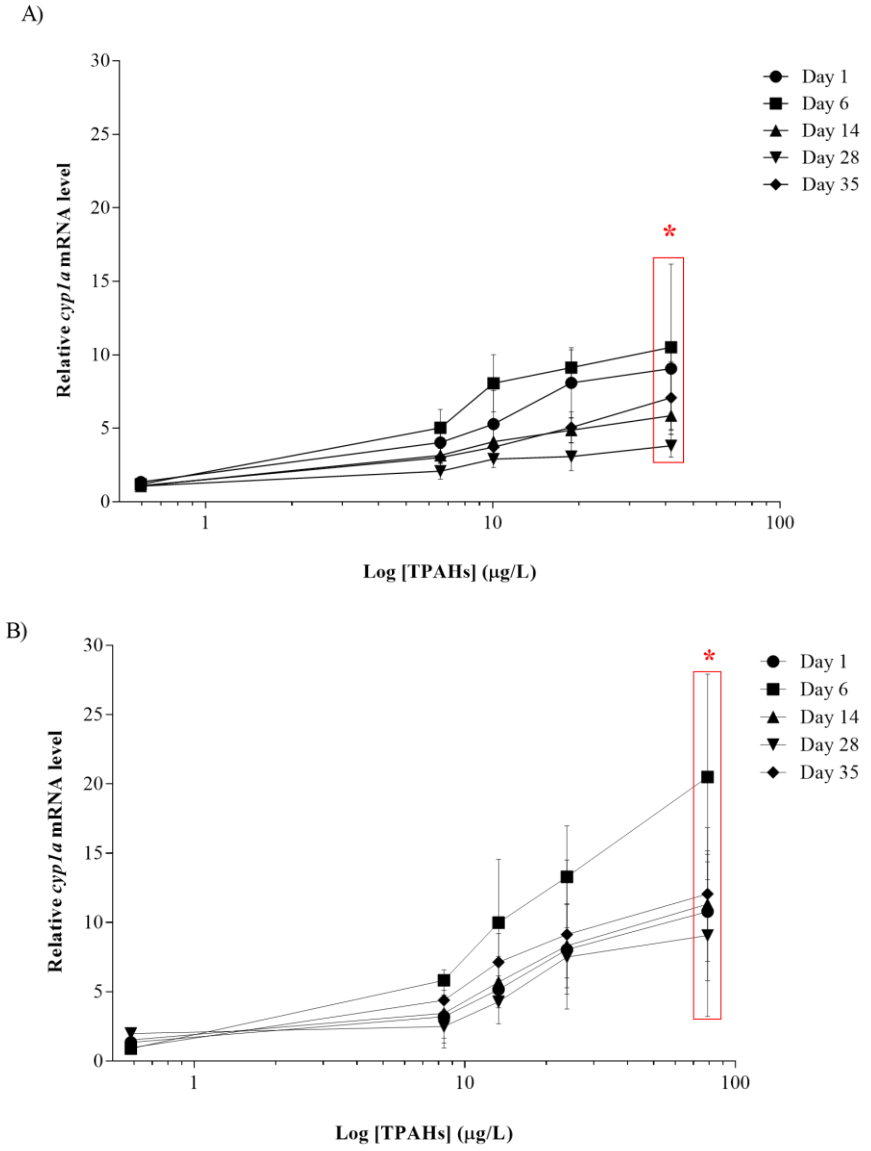


Figure 4.8 Levels of cytochrome P450 (*cyp1a*) transcripts in *Pimephales promelas* embryos exposed to varying concentrations of CLB contaminated water. Values represent mean ± standard error. The asterisk (*Turkey's multiple comparisons, $p < 0.05$) represents significant differences and the red box the significant difference between (A) Days 1 and 6 vs Day 35 (at 4 °C) and (B) Day 6 compared to the other time points (at 15 °C)

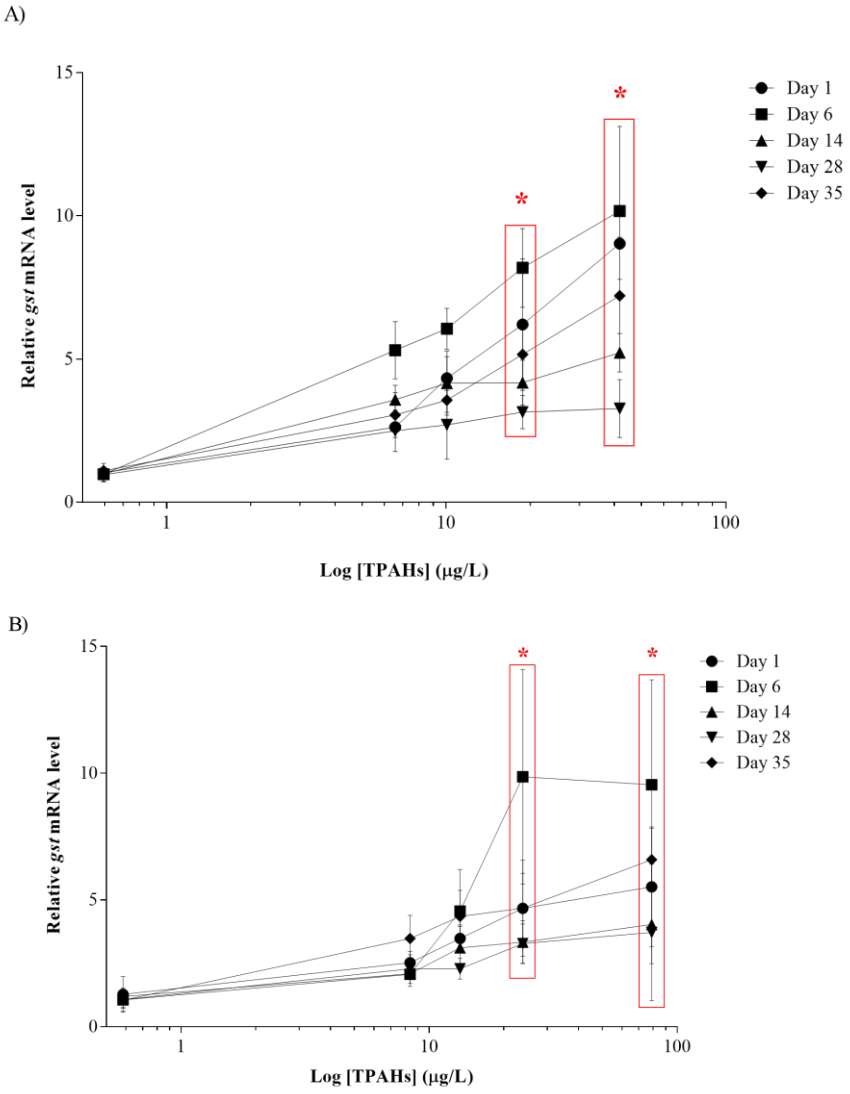


Figure 4.9 Changes of *gst* transcripts are normalized to the mean of *odc* and *rpl8*. Levels of in *Pimephales promelas* embryos exposed to varying concentrations of CLB contaminated water. Values represent mean ± standard error. The asterisk (*Turkey's multiple comparisons, $p < 0.05$) represents significant differences and the red box the significant difference between (A) Days 1 and 6 compared to Day 35 (at 4 °C) and (B) Day 6 compared to the other time points (at 15 °C).

4.5.4 PCA analysis

The relationship between the chemical and biological profiles of the different WAF dilutions and temperatures was analyzed using PCA and clustering analysis. The PCA is a multivariate method for dimensionality reduction (closely located in space) (Cordella, 2012). PCA was performed independenly for each experiment at 2 °C and 15 °C, in both cases the % variance include three number of components. For 2 °C, the component 1 (54.14%), component 2 (18.82%), and component 3 (11.90%) were obtained, while for 15 °C, the component 1 (56.64%), component 2 (17.71%), and component 3 (13.19%) were calculated (Fig. S4.4). In the 2 °C experiment, the PCA plot showed three clusters, the first one represents the heart beats separated from mortality and hatching time and the third one the rest of the variables (malformation, EROD assay, C10-C50 , TPAHs and VOC). The cluster analysis showed two main clades (Fig. 4.10) that marks the separation between the controls and the WAF dilutions.

Based on the PCA coefficient data, the correlation between variables was determined. A positive correlation was observed for component 1 of the 2 °C experiment between C10-C50, TPAHs, alkylated-PAHs, VOC, mortality, malformation, and hearts beats (0.4082, 0.4103, 0.4099, and 0.3285, respectively) and a negative correlation was obtained between heart beats with the rest (-0.3342). For component 2, a positive correlation among mortality and malformation was measured (0.3820 and 0.3633) and a negative correlation between VOCs and hatching time was assessed (-0.3297 and -0.6605). Lastly, for component 3, a negative correlation between VOCs, hatching time and EROD activity was observed (-0.3027, -0.3006 and -0.8462).

For the 15 °C experiment, a correlation in component 1 was measured between C10-C50, TPAHs, alkyl-PAHs, VOCs, and malformations (0.3718, 0.4372, 0.4367, 0.3299 and 0.3776). For the component 2, a positive correlation between malformations and EROD activity was shown

(0.3132 and 0.5615) and a negative correlation between C10-C50, VOCs, mortality, and heart beats were calculated (-0.3148, -0.3166, -0.4999 and -0.3155). Finally, for component 3, no correlation was observed.

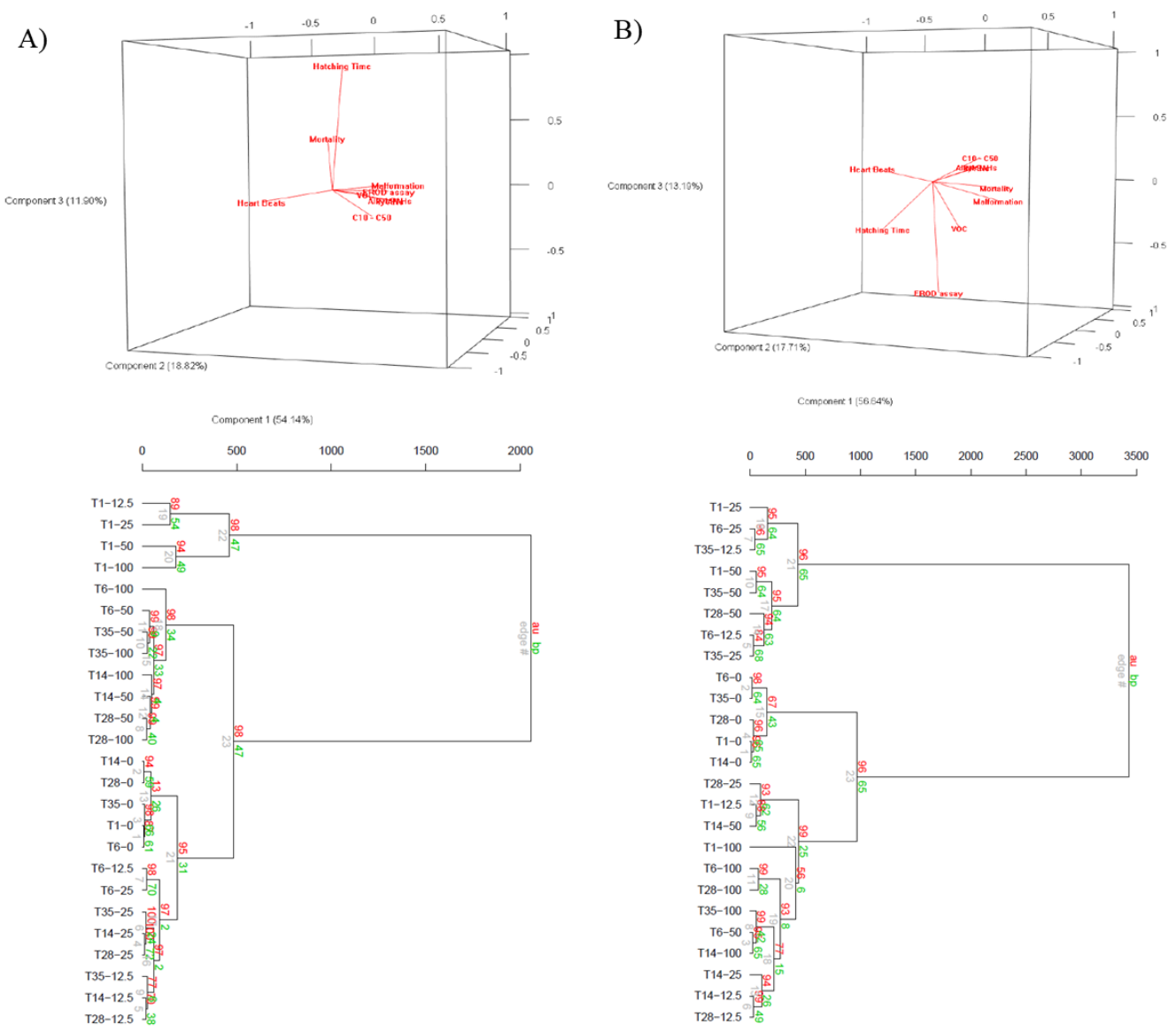


Figure 4.10 Score plot for PCA of the chemical-biological variables in (A) Cluster dendogram of the chemical biological variables in the 2 °C experiment and the score plot for the PCA of the chemical-biological variables. (B) Cluster dendogram of the chemical biological variables in the 15 °C experiment and the score plots for PCA of the chemical-biological variables. The percentages along with component 1, component 2, and component 3 indicate their contribution to the total variance in the PCA. The bootstrap support for cluster provide as AU (Approximately Unbiased) *p*-values in red and BP (Bootstrap Probability) value in green.

4.6 Discussion

Two 35-day spill tank tests were carried out using CLB in North Saskatchewan River (NSR) water and NSR sediment mixtures to examine the fate and behavior of a potential dilbit spill in simulated fall and winter seasons (warm test and cold test, respectively). Natural weathering processes, which include evaporation, dispersion, and dissolution, happened immediately once the dilbit spilled into the water. The early evaporation of volatile compounds in fresh dilbit resulted in quick decreases of TOC and BTEX in water columns, while the dispersion contributed to the highest of TPAHs in water columns in the early hours. Followed, the dissolution, sedimentation and biological process have further changed the TOC and TPAHs contents in the water columns. The overall oil mass balance in both tests followed a similar pattern with minor effect from the seasonal/temperature effect, where the majority of spilled oils (between 77 % - 83%) recovered as floating water-in-oil emulsion. A slightly higher portion of the oil was lost due to the evaporation and/or biodegradation processes in the warm test (19.0 ± 0.2 %) as compared to the cold test (17.2 ± 0.2 %), where the majority of hydrocarbons had boiling points lower than 204 °C. Followed by the loss of aromatics in fresh dilbit (over 20 wt%) based on the evidence from the SARA results and the active PAH and alkylated PAH biodegradation happened in both water columns. It appeared that the resins and asphaltenes contents of dilbit were more persistent. Only 1.0 % of the spilled dilbit has resulted in the sedimentation in both tests under the tested conditions of 2000 ppm sediment loading in freshwater, which indicate that dilbit spill would pose a relatively low risk to the sediment-dwelling organisms within the tested spilled duration. Overall, the residual dilbit after weathering 35 days in both conditions were recoverable as a floating oil in our simulated freshwater environment.

There have been some studies comparing the oil weathering at different temperatures in sea water like Payne and colleges (1991), that evaluated temporal changes in the physical and chemical properties of crude oil (Exxon Valdez) in ice-free subarctic marine environments, a series of summer and winter outdoor flow-through seawater wave- tank experiments conducted at the National Oceanic and Atmospheric Administration (NOAA) and found different chemical profiles in the different environmental temperatures (Payne et al., 1983).

Also some oil degradation studies have investigated the role of temperature on the rate of biodegradation of PAHs relating the chemical changes of PAHs to a temperature-dependent biodegradation. Delille and colleagues (2009) studied the effects of temperature on degradation of petroleum hydrocarbons (Arabian light crude oil) in sub-Antarctic coastal seawater at three temperatures (4, 10 and 20 °C) and they concluded that water temperature had little effects on biodegradation efficiency of PAHs. The latter data are similar to the data obtained in the present study, as both 2 and 15 °C trials exhibited a similar PAH concentration with time.

As for the toxicity assays, a similar malformation pattern was observed in both temperature regimes over time, with fish embryos yielding high proportions of malformations at the beginning of the trials, which also yielded the highest PAH concentrations. Noteworthy, the proportion of malformed animals stayed stable over the remaining time points, lasting until the end of the trial at day 35. These data suggest that the continous occurrence of fish malformations observed with time could be explained by the increased presence of the oxidized compounds as dilbit weathered, mainly as oxy-PAHs (**Chapter 3**). The observation that fish malformations increase with PAH concentrations has been reported in several studies (e.g. Carls et al., 2008; Dubansky et al., 2013; Incardona et al., 2013; Incardona et al., 2014; Mager et al., 2014; Madison et al., 2015; Madison et al., 2017;), but there is still lack of data on the embryotoxicity of weathered oil and the associated

chemical changes that oil undergoes in different environmental temperatures and how these chemical changes could modify toxicity.

Although the malformation patterns were similar for both temperatures indicating that the toxicity barely change with time, the induction of *cyp1a* seems to respond to the dose-response pattern. The gene *cyp1a* is well known to be activated via the AhR-pathway by several PAHs (reviewed in Wallace et al., 2020); and in this study, *cyp1a* mRNA levels were induced at all the time points in a dose-response manner. However, *cyp1a* fold-change's magnitude decreased with time. This could be explained either by the decrease of the PAHs capable of binding to the AhR with time or by the increased presence of oxidized compounds (oxy-PAHs, S-PAHs and/or S-PAHs), which are known weak AhR activators (Machala et al., 2001; Misaki et al., 2007; **Chapter 3**). Indeed, Wincent and colleges (2015) exposed embryonic zebrafish (*Danio rerio*) to PAHs and oxy-PAHs and demonstrated that oxy-PAHs were weak inductors of the AhR signaling, but still capable of inducing the expression of several genes involved in AhR pathway, such as *cyp1a*. However, another study exposed zebrafish embryos until 5 days post-fertilization to 38 oxy-PAHs, where the majority of the oxy-PAHs tested did not induce *cyp1a* expression (Knecht et al., 2013). In the present study, we observed a significantly induction of *cyp1a* mRNA levels at the highest concentrations tested, but the magnitude of the induction decreased with time. Similarly, the mRNA profile of *gst* (also involved in the detoxification pathway) showed the same trends as *cyp1a* transcripts with concentrations and with time. These data highlight the importance of studying side-by-side the chemistry and toxicity profiles of complex oil mixtures as they weather in different environmental conditions.

EROD induction, which measured CYP1A enzymatic activity, has been well documented in fish exposed to some PAHs such as B[a]P (Schnitz and O'Connor, 1992). EROD assays are

often performed on the S9 microsomal fraction of larvae homogenate or directly in the liver of the fish (Peters and Livingstone, 1995; Koponen et al., 1998; Chen and Cooper, 1999; Billiard et al., 2004; Gagnon and Rawson, 2017), but because of the small size of the *P. promelas* larvae, we have optimized an *in vivo* EROD assay for this species. Fin et al. (2020) exposed juvenile sockeye salmon (*Oncorhynchus nerka*) to unweathered CLB dilbit and showed a concentration-dependent induction of EROD activity. Similar resuts were observed by Alderman and colleges (2017) where juvenile sockeye salmon was exposed to a summer blend dilbit from the Cold Lake region and found a significant interaction between time and concentration. Avey and colleagues (2020) exposed a juveniles Atlantic salmon to the dilbit Cold Lake Summer Blend and compared the gene expression of *cyp1a* versus EROD activity in liver noticing similar patterns, increasing both at higher concentrations. This contrasts with the present study where the induction of EROD was not significantly altered and no similar pattern between *cyp1a* expression and EROD activity was observed. This could be explained by the aryl hydrocarbon receptor repressor (AhRR) that produces a negative feedback mechanism in the AhR pathway reducing the binding of AhR-ARNT to XRE sequence, thus decreasing transcription of *cyp1a* (Zhou et al., 2010; Calo et al., 2014).

Some PAHs have been identified to cause cardiotoxicity in fish (Incardona et al., 2006; Incardona et al., 2011; Scott et al., 2011) through the AhR activation (Sestak et al., 2018). Previous studies have found that cardiotoxicity can yield irregular heart beats like bradycardia (Linden, 1976; Middaugh et al., 1996; Incardona et al., 2009; Shen et al., 2010; Incardona et al., 2012; Zhang and Yan, 2014; Tissier et al., 2015) or tachycardia (Philibert et al., 2016). Incardona et al. (2009) found cardiac abnormalities of embryonic Pacific Herring (*Clupea pallasi*) exposed to the ANSCO during weathering. Another study conducted by Li et al. (2019) exposed zebrafish to the OCO and the MCO oil and showed a concentration-dependent cardiac toxicity, such as severe

bradycardia, cardiac defects, and poor blood circulation. This is similar to the results obtained in the present study as the high concentrations of dilbit tested yielded significant decreases of the heart rate of dilbit treated embryos compared to the controls and this for all time points. Complementary PCA analysis showed a negative correlation between the chemical profiles and heart beats suggesting that when PAH levels are decreasing, the heart beat rates are increasing.

In conclusion, over the 35-day spill test, the highest concentrations of TPAHs in the water column were observed immediately after the oil was added to the tank. These concentrations decreased with time from Day 1 to Day 35, to concentrations 4.5X less at 2 °C and approximately to 9X less in 15 °C. This study suggests that the toxicity is maintained during the 35 days exposure even though the chemical composition change in each temperature. This study highlights the importance of well understanding the contribution of environmental factors like temperature (fall and winter seasons) to the toxicity associated with oil weathering for aquatic species.

4.7 Acknowledgments

This project was funding by the Government of Canada's Oceans Protection Plan Program (Natural Resource Canada), Canada's Research Chair Program (to VSL), and the CONACYT-México (to LRLJ). Part of the analyses was funded by a program of the Quebec Government, the Stratégie maritime du Gouvernement du Québec, Plan d'action 2015-2020. The authors would also like to thank Hena Farooqi and Behnam Namsechi for conducting the tank test, and the assistances from Standard Analytical Laboratory, and Upstream and Environment Team at CanmetENERGY Devon, for their assistances with the performance of the oil physical and chemical analyses of oil, and water analyses, respectively.

CHAPTER 5 : GENERAL DISCUSSION AND FINAL CONCLUSION

Oil spills can happen for many reasons. They can occur during the extraction of oil. Oil will then be spilled on land or sea. They can also occur during transportation. Transportation routes include vial rail cars, trucks, tanker vessels, and through pipelines. Several petroleum products can be transported such as conventional crude oil, refined oil, diluted bitumen (dilbit), etc. Furthermore, there are several examples where large oil spill disasters have occurred in North America, affecting the fauna of aquatic ecosystems. Knowing the effects of these disasters on wildlife will help us understand the impact and repercussions of oil spills in the ecosystems and help to prevent these disasters. This thesis assessed the effects of oil on model organisms and validated biomarkers, which can now be used if an oil spill would occur.

The present thesis also provided one of the first assessments of toxicity of two dilbits (CLB and AWB) to amphibians and showed that exposure at early stages of development to dilbit increase mortality, teratogenic effects (e.g., edemas, cranial, heart, gut and ocular abnormalities), reduced body size, and a significant induction of *cyp1a* as shown in **Chapter 2**.

Then, I was interested to know what could happen in a real oil spill; what would be the chemical changes of the oil in the environment (weathering), and therefore, how these changes could affect the toxicity in aquatic organisms. As crude oil weathered during a real oil spill, knowing the toxicity effects of the weathered crude oil over time is of outmost importance. So in **Chapter 3**, the embryos of the fish *P. promelas* exposed to weathered crude oil (MSW) during embryonic stages showed an increased mortality, teratogenic effects (e.g., pericardial and yolk sac edema, heart tube, craniofacial and spinal deformity), and an absence of response on *cyp1a* and *gst,* the more weathered the oil.

In both aquatic species (frog and fish), teratogenesis was present when exposed to oil in the early stages of development. Regarding *cyp1a* induction, there was a notable difference between the two species. Although *cyp1a* mRNA levels have been recognized as a good biomarker for PAHs in fishes, noteworthy in **Chapter 3**, no such response was observed. This could be explained by the presence of oxidized compounds in which the PAHs were transformed into as the oil weathered and to which *cyp1a* induction does not respond, and therefore, explains the lack of response of *cyp1a*. On the other hand, the frogs responded to *cyp1a* in the exposure of unweathered oil, where the concentration of PAHs was maintained, which could indicate that *cyp1a* is a good biomarker for frogs when exposed to PAHs.

The differential responses in toxicity for both species can also be explained by the differences inherent of the two experimental designs. In **Chapter 2**, frog larvae's toxicity was determined by the concentration of total PAHs in the water and two unweathered dilbits and two types of dilbit preparations (WAF and CEWAF) were used. On the other hand, in **Chapter 3**, fish embryo's toxicity was determined not only during the first days of the exposure using the total concentration of PAHs, but also as the days went by and as the oil became more weathered. Then, the toxicity changed depending on the amount of oxidized PAHs being generated in the water column. Leaving me a question, what role does temperature play in oil weathering? And how it could affect toxicity in aquatic species?

As we know several factors can affect the weathering of crude oil in aquatic systems, which could lead to a change in the chemical composition of the oil. In **Chapter 4**, I shown that the embryos of the fish *P. promelas* yielded as weathered the CLB an increased mortality, teratogenic effects (e.g., pericardial and yolk sac edema, heart tube, craniofacial, and spinal deformity), bradycardia syndrome, and a small response of *cyp1a* and *gst.* When comparing the fish embryo

malformations noted between crude oil (MSW; **Chapter 3**) and dilbit (CLB; **Chapter 4**) at 15 °C, both present pericardial edema as the most recurrent malformation. In both dose-response graphs (% malformation vs. concentration of PAHs in $\log_{10}$), a sigmoidal trend was observed. In the MSW experiment, data showed that the greater the weathering of crude oil was, the more toxicity increased, leaving days 1 and 6 as the least toxic. On the other hand, the CLB data showed that even if the oil has weathered, embryotoxicity did not decrease significantly.

With respect to the biomarkers *cyp1a* and *gst*: In the MSW trial (**Chapter 3**), only on day 14, there was a gene induction compared to the CLB experiment (**Chapter 4**), where a response was measured for every time points tested, and interesting, both *cyp1a* and *gst* mRNA levels decreased with time. In both cases, the lack of response (**Chapter 3**) or decreased response (**Chapter 4**) might be related to the biomarker *per se*, showing that biomarkers related to oxidized PAHs need to be found.

In **Chapter 4**, the study of CLB at different temperatures (2 °C and 15 °C) showed a similar sigmoidal trend in the occurrence of malformations, with the main malformations observed included pericardial edema and malformed cardiac tube. When comparing the other biological parameters measured in **Chapter 4**, such as heart beats rate, EROD activity, and *cyp1a* and *gst* mRNA levels, there were no significant differences observed between both temperature experiments. This suggests that similar toxicity is maintained over the 35-day duration of the trials. This finding is interesting since, although the toxicity appeared to have decreased slightly over time, it is not sufficient to remove the induced teratogenic damage in the exposed larvae. Altogether, this study showed that temperature range between 2 to 15 °C was not sufficient to alter fish embryotoxicity associated to dilbit exposure. It is also possible that over time, toxicity is

maintained even though PAHs are decreasing, because the fraction of oxidized compounds is increasing, which would also be participating in fish teratogenesis.

For future research, there is a need to find biomarkers for oxidized PAHs, since as observed in my thesis, *cyp1a* mRNA level does not seem to be a good biomarker for these oxidized compounds (**Chapter 3**). In addition, measuring the presence of oxidized compounds in weathered dilbit could be also studied and would address some of the hypotheses generated in **Chapter 4**. Future studies should also analyze the genotoxicity associated to these oxidized PAHs in fish and frogs because of the capacity of some PAHs of producing DNA adducts (Dasgupta et al., 2014). Also, studies for longer period of time could be interesting. For example, oil spill simulations for at least two seasons could be performed to observe the changes of oil fate, behavior, toxicity under these natural conditions. This would give us a more realistic picture of what could happen in real life. In conclusions, more research is necessary to examine the chronic effects associated with weathered oil during the life cycle of aquatic species in order to understand how these transformed PAHs will affect fish and wildlife in long term (e.g., Deepwater horizon oil spill) (Fig. 5.1).

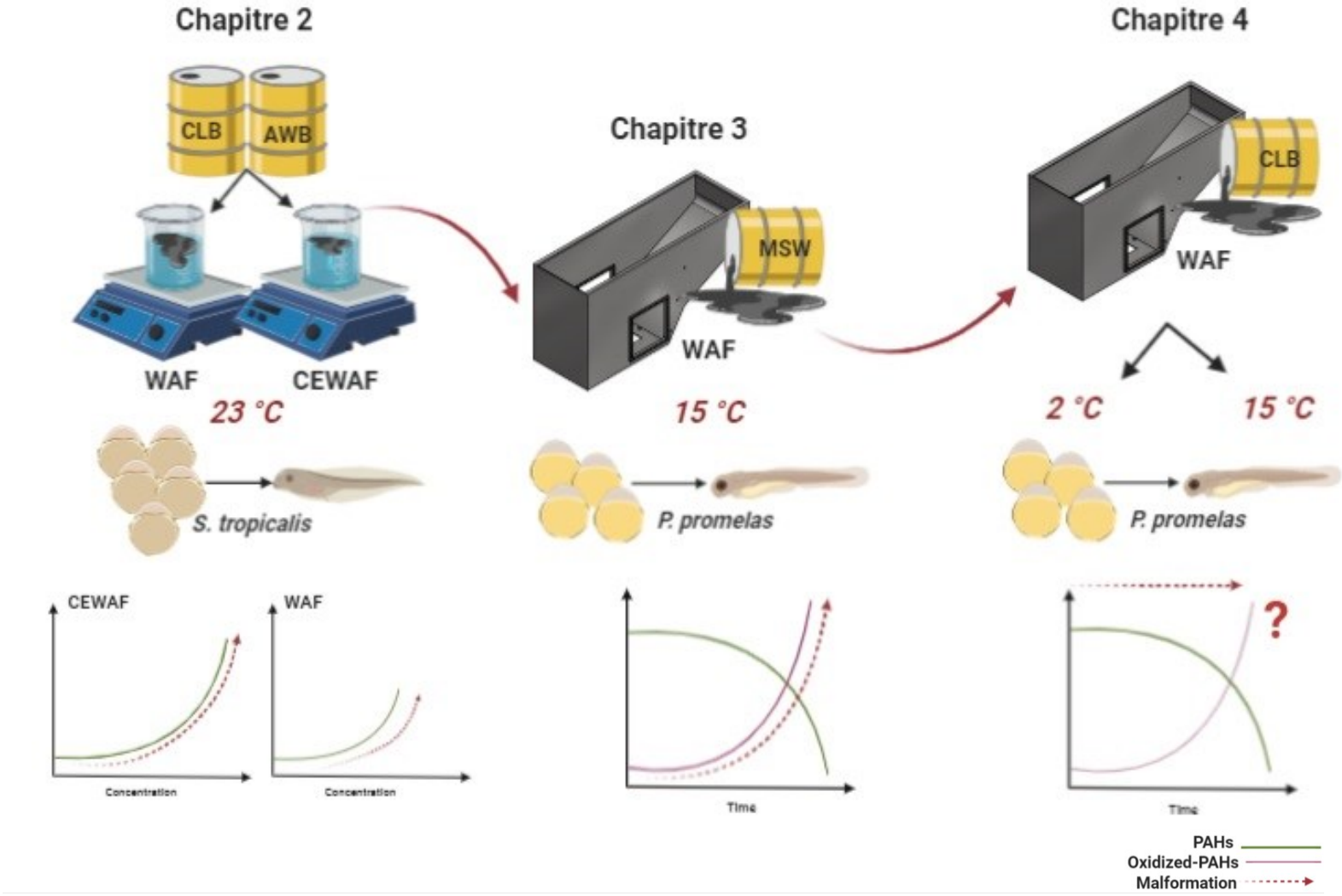


Figure 5.1 Diagram showing the research conducted on this theses. *S. tropicalis*; *Silurana (Xenopus) tropicalis*. WAF; water accommodated fraction. CEWAF; chemically-enhanced WAF. CLB; cold lake blend. AWB; access western blend. MSW; mixed sweet blend. PAHs; polycyclic aromatic hydrocarbons.

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APPENDICES

Appendix I. Supplemental Information from Chapter 2: Cytochrome *p4501a* transcript a suitable biomarker for both exposure and response to diluted bitumen in the developing from embryo

Table S2.1 The total petroleum hydrocarbons as measured by fluorescence (TPH-F) and the total polycyclic aromatic hydrocarbons as measured by GCMS (TPAH) for test (n = 2 per concentration analyzed) and exposure solutions (n = 2 for 4 days per concentration). TPAH for exposure solutions was estimated by using the average TPH-F for exposure solutions measured over 4 days in a dilbit blend specific linear regression of the log TPH-F vs log TPAH of test solutions.

			Test solutions		Exposure solutions			
		Nominal	TPH-F	TPAH	TPH-F		TPAH	
Dilbit		(% v/v)	(RFU)	(µg/L)	(RFU)	± SD	(µg/L)	± SD
AWB	WAF	10	7931730	1.40	7139375	2164099	0.83	0.09
	WAF	30	19673835	4.36	20422427	5339019	4.31	0.42
	WAF	50	32830899	7.66	32567511	7000283	8.95	0.66
	CEWAF	0.01			1737473	1339082	0.09	0.04
	CEWAF	0.1	13739058	1.84	15120175	4736133	2.69	0.34
	CEWAF	1	87555859	33.34	93308283	17002305	46.57	3.02
	CEWAF	5	184951822	170.99	198986686	23804268	152.51	5.37
	CEWAF	10			229042078	18659033	190.10	3.54
CLB	WAF	10	15435521	3.63	7362348	2654637	0.72	0.13
	WAF	30	31630016	9.49	19375004	5246473	3.78	0.41
	WAF	50	42647118	13.69	30486052	7197597	8.19	0.70
	CEWAF	0.01			3323818	1950254	0.19	0.08
	CEWAF	0.1	16429237	2.23	18047654	3622864	3.35	0.22
	CEWAF	1	89122148	40.14	94710182	19166965	56.65	3.71
	CEWAF	5	191480755	210.33	194222197	7200355	192.97	0.70
	CEWAF	10			224678904	4671525	247.43	0.33

Table S2.2 The amount of variation explained by dilbit blend (AWB and CLB), by TPAH concentration (and WAF/CEWAF treatment) and by the interaction of the two factors. Significant contribution to variation is indicated by an asterisk (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

	Percent of total variation in two-way ANOVA		
Target gene	**Dilbit blend**	**TPAH concentration**	**Interaction**
cyp1α	0.003	92.08****	0.74
ahr	0.14	9.32	3.72
arnt	2.60	10.58	1.97
cat	0.09	11.58*	5.91
sod	0.13	12.15*	3.48
gsr	1.68	13.22*	2.70
gst	0.10	22.98****	1.07
gpx	0.42	11.85	1.33
p53	1.61	7.37	2.85
ar	0.29	11.14	2.21
erα	0.20	9.05	0.98
srd5α1	0.14	5.46	1.77
srd5α2	0.21	13.70*	3.50
srd5α3	0.11	16.56**	2.51
dio1	0.33	17.39*	1.41
dio2	1.29	10.60	4.22
dio3	0.004	17.00**	1.05
thrα	0.24	23.18****	3.59
thrβ	0.10	7.59	1.52

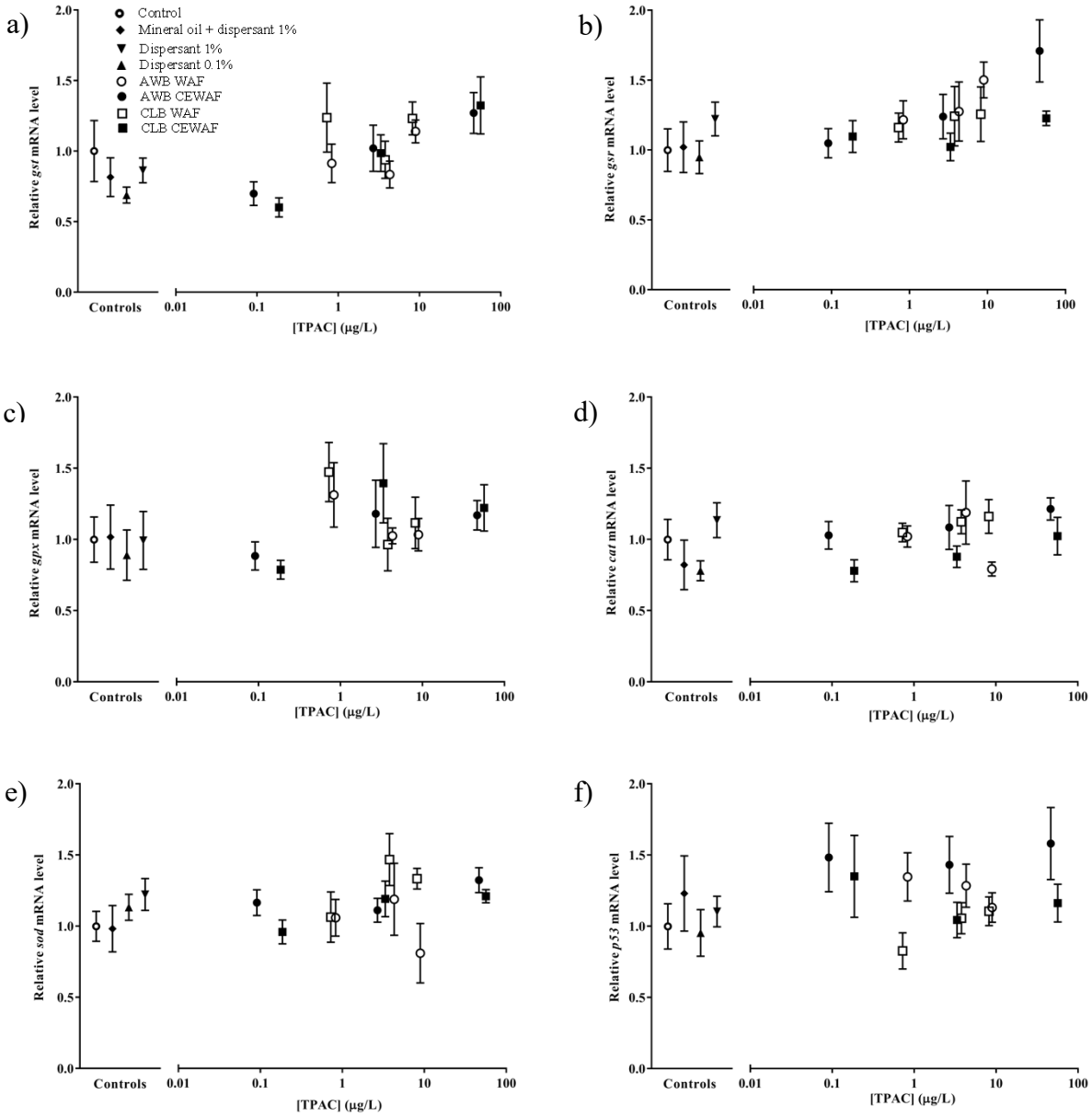


Figure S2.1 The relative transcript levels of a) *gst,* b) *gsr,* c) *gpx,* d) *cat,* e) *sod*, and e) *p53* in *Silurana tropicalis* embryos exposed to the Access Western Blend (AWB) and Cold Lake Blend (CLB) Water Accommodated Fractions (WAFs) and Chemically Enhanced WAFs (CEWAFs). These genes are involved in phase II detoxification pathways and tumour suppression (*p53*). Transcripts were normalized to the mean of *odc* and *rpl8* ± SEM. No significant results were found (two-way ANOVA; $p > 0.05$).

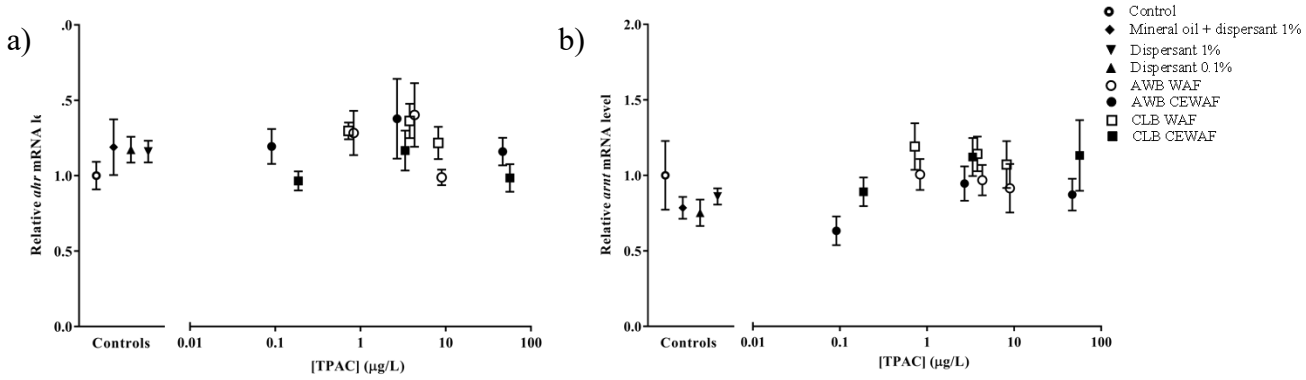


Figure S2.2 The relative fold change in transcript level of a) *ahr*, and b) *arnt* in embryos exposed to Access Western Blend (AWB) and Cold Lake Blend (CLB) Water Accommodated Fractions (WAFs) and Chemically Enhanced WAFs (CEWAFs). These genes are involved in Phase I detoxification. Transcripts were normalized to the mean of *odc* and *rpl8* ± SEM. No significant results were found between treatments (two-way ANOVA; $p > 0.05$).

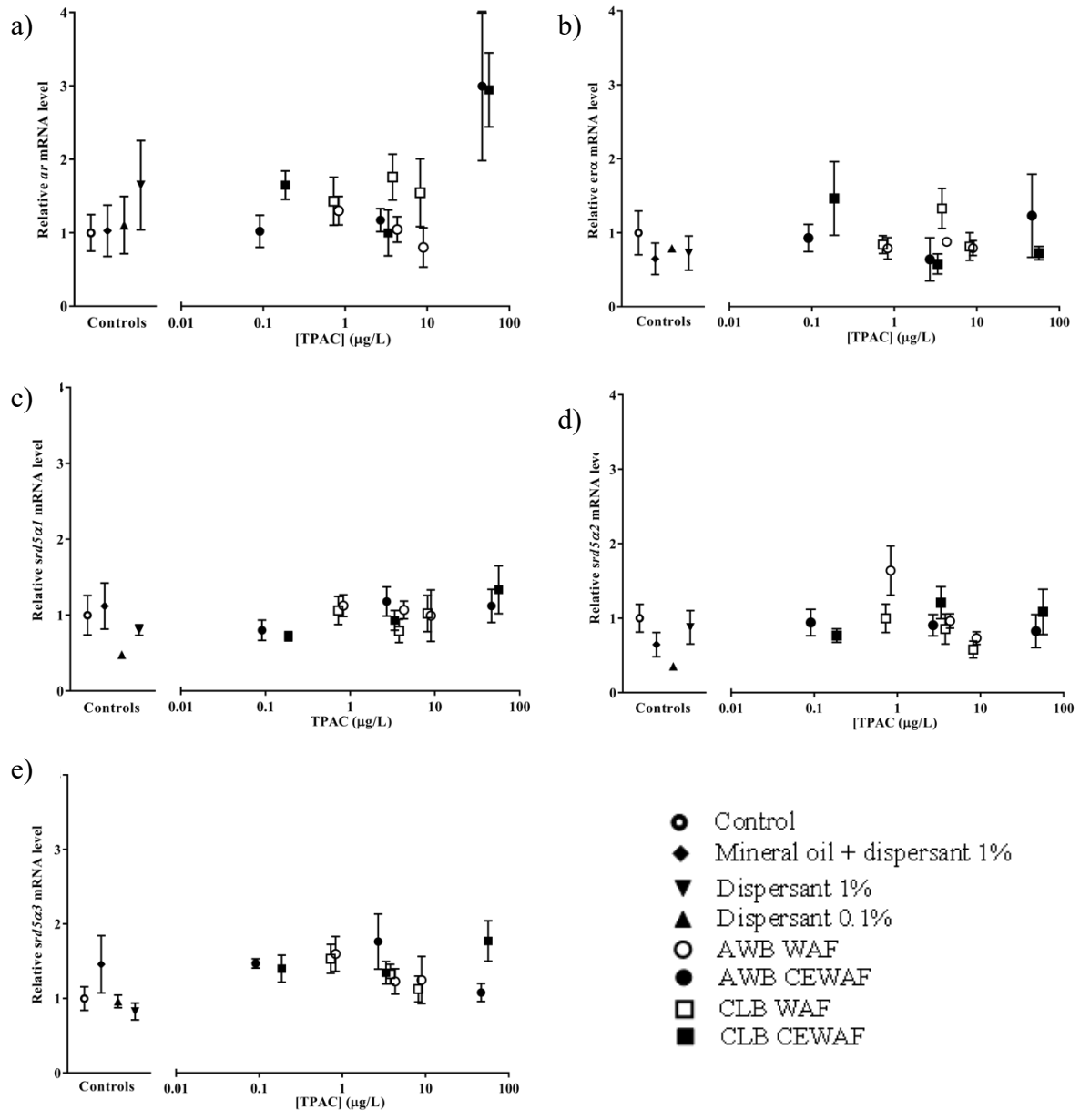


Figure S2.3 The relative fold change in transcript level of a) *ar,* b) *erα,* c) *srd5α1,* d) *srd5α2,* and e) *srd5α3* in embryos exposed to the Access Western Blend (AWB) and Cold Lake Blend (CLB) Water Accommodated Fractions (WAFs) and Chemically Enhanced WAFs (CEWAFs). These genes are involved in male and female reproduction. Transcripts were normalized to the mean of *odc* and *rpl8* ± SEM. No significant results were found between treatments (two-way ANOVA; $p > 0.05$).

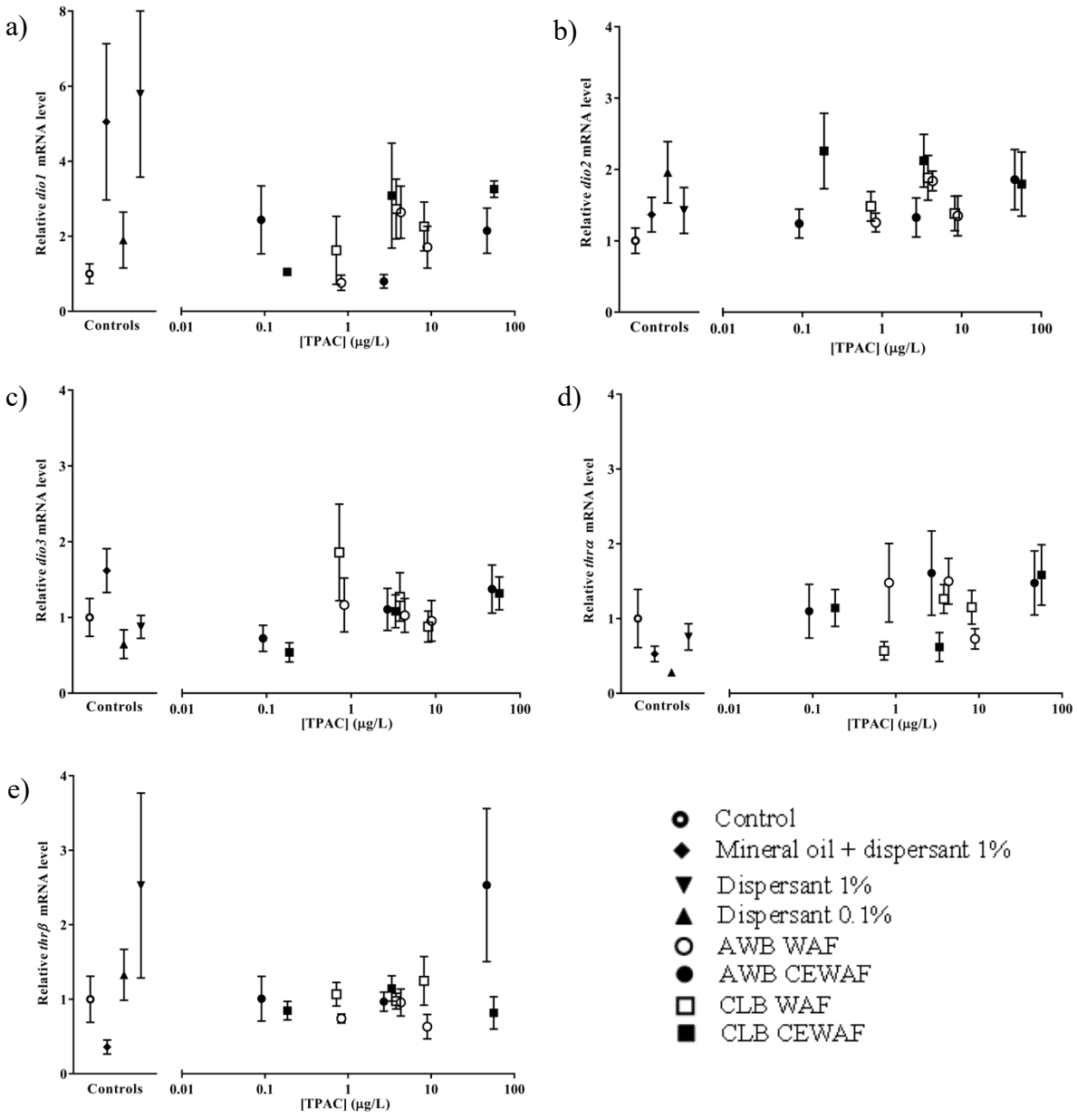


Figure S2.4 The relative fold change in transcript level of a) *dio1,* b) *dio2,* c) *dio3,* d) *trα,* and e) *trβ* in embryos exposed to WAF and CEWAF of AWB and CLB. These genes are involved in the thyroid hormone axis. Transcripts were normalized to the mean of *odc* and *rpl8* ± SEM. No significant results were found between treatments (two-way ANOVA; $p > 0.05$).

Appendix II. Supplemental Information from Chapter 3: Fate and embryotoxicity of weathering crude oil in a pilot-scale spill tank

Table S3.1 A list of *Pimephales promelas* primers conditions for qPCR, including target genes, their primers sequences (F, forward; R, reverse), annealing temperatures (°C), amplicon size (bp), and primer concentrations (μM).

Target gene	Gene name	Forward and reverse primers	Amplicon size (bp)	Primer conc. (nM)	Annealing temp. (^{0}C)	Reference
*ef1a**	elongation factor 1 alpha	F: AGCGTAAGGAGGGAAATGCT R: CTCAGAAAGGGACTCGTGGT	254	350	58	(Martyniuk et al., 2012)
*rp18**	ribosomal protein L8	F: TTGTTGGTGTTGTTGCTGGT R: TCCTGATTGTTGAGGGCTTG	186	300	58	(Alsaadi et al., 2018)
cyp1a	cytochrome P450, family 1, subfamily A, polypeptide 1 elongation factor 1 alpha	F: TCCTGGGCTGTCGTCTATCT R: CTGGGAATGAAGTATCCGTTG	230	300	58	(Alsaadi et al., 2018)
gst	glutathione-s-transferase	F: GACGTTCATCTTCTGGAAGC R: GAGGCTTTCTCGCACTGC	151	300	58	(Mager et al., 2008)

Gene full names in order to appearance: *(*ef1a*) elongation factor 1 alpha (housekeeping gene); *(*rpl8*) ribosomal protein L8 (housekeeping gene). And (*cyp1a*) cytochrome p450-1a; (*gsr*) glutathione reductase. Alsaadi F, Madison BN, Brown RS1, PV Hodson1 and VS Langlois. 2018. Morphological and molecular effects of two diluted bitumens on developing fathead minnow (*Pimephales promelas*) Aq Tox. 204:107-116. Martyniuk, C.J., Alvarez, S., Lo, B.P., Elphick, J.R., Marlatt, V.L., 2012. Hepatic Protein Expression Networks Associated with Masculinization in the Female Fathead Minnow (Pimephales promelas). J. Proteome Res. 11, 4147–4161; Mager, E.M., Wintz, H., Vulpe, C.D., Brix, K.V., Grosell, M., 2008. Toxicogenomics of water chemistry influence on chronic lead exposure to the fathead minnow (Pimephales promelas). Aquat. Toxicol. 87, 200–209.

Table S3.2. River water properties after the addition of sediment to the water (Start), after fresh MSW was spilled (T0), and at the end of the spill test (End).

River water property	Blank	Start	T0	End
pH	7.9	8.1	8.2	8.0
Conductivity (mS/cm)	0.35	0.35	0.35	0.32
TDS Calculated (g/L)	0.23	0.22	0.22	0.22

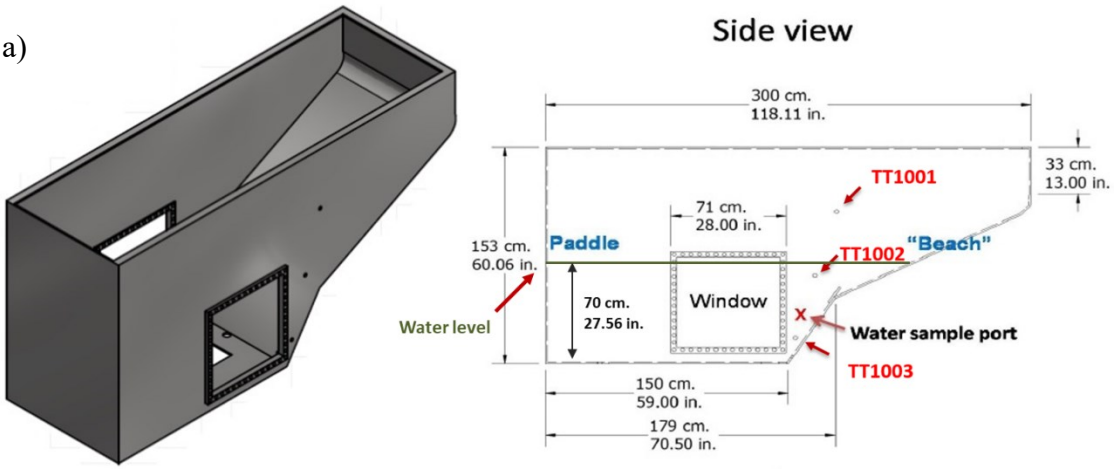
Table S3.3. X-ray diffraction analyses of North Saskatchewan River sediments.

Sediment Type		North Saskatchewan River sediments	% Uncertainty
Clays	Kaolin	11.0	3.0
	Illite	5.1	1.6
	Chlorite	4.1	1.1
Carbonates	Calcite ($CaCO_3$)	7.6	0.8
	Dolomite $Ca,Mg(CO_3)_2$	12.5	1.1
Feldspars	Microcline	3.3	0.8
	Plagioclase Albite	4.9	0.8
	Quartz (SiO_2)	51.5	2.0

Table S3.4 Mortality, hatching time and malformations observed in *Pimephales promelas* larvae exposed to water accommodated fractions (WAFs) of mixed sweet blend (MSW) crude. Malformation types include pericardiac and yolk sac edema and heart tube, craniofacial and spinal deformity.

Nominal concentrations	% Mortality ± SD	% Hatching time	Weight (mg) ± SD	% Malformed individuals (*n*)	Pericardial edema	Heart Tube	Craniofacial	Yolk sac edema	Spinal deformity
				Day 1					
Reconstituted water	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
River water	16.70 ± 2.4	4.01	1.04 ± 0.28	10 (4)	4	4	1	0	1
12.50%	16.02 ± 3.1	4.17	1.15 ± 0.36	70 (28)	28	27	2	1	1
25%	22.68 ± 5.9	4.2	1.34 ± 0.43	80 (32)	32	32	0	0	6
50%	26.10 ± 1.5	3.83	1.69 ± 1.35	97.5 (39)	39	39	0	0	8
100%	30.50 ± 5.7	4.33	1.69 ± 0.88	100 (28)	28	28	1	0	10
				Day 6					
Reconstituted water	1 ± 1.66	4.19	1.34 ± 0.39	5 (2)	2	0	0	0	0
River water	1.54 ± 0.5	4.44	1.20 ± 0.29	20 (8)	7	8	0	0	0
12.50%	32.16 ± 0.5	4.42	1.11 ± 0.20	32.5 (13)	13	13	0	0	0
25%	5.24 ± 0.5	4.68	1.21 ± 0.22	37.5 (15)	15	15	0	0	0
50%	0	4.1	1.41 ± 0.75	65 (26)	26	23	0	0	2
100%	11.42 ± 0.5	4.64	1.11 ± 0.18	100 (40)	40	40	0	0	8
				Day 14					
Reconstituted water	1.40 ± 1.6	4	1.39 ± 0.33	5 (2)	2	2	0	0	0
River water	23.74 ± 15.30	4	1.09 ± 0.20	30 (12)	12	11	0	0	1
12.50%	25.72 ± 13.58	4.16	1.07 ± 0.20	42.5 (17)	17	17	0	0	2
25%	33.68 ± 31.85	4.59	1.11 ± 0.49	55 (22)	22	22	0	0	3
50%	32.76 ± 35.63	4.81	1.19 ± 0.29	85 (34)	34	34	0	0	5
100%	17.56 ± 31.14	4.24	1.12 ± 0.29	100 (40)	40	40	0	0	10
				Day 21					
Reconstituted water	17.57 ± 1.6	4	1.20 ± 0.28	5.56 (1)	1	1	0	0	0
River water	7.52 ± 15.30	4.33	1.23 ± 0.87	15 (6)	6	6	0	0	0
12.50%	5.06 ± 13.58	4.46	1.18 ± 0.30	30 (12)	12	12	0	0	0
25%	17.50 ± 31.85	4.21	1.03 ± 0.33	42.5 (17)	17	17	0	0	1
50%	58.78 ± 35.63	4.22	1.09 ± 0.18	82.5 (33)	33	32	0	0	3
100%	50 ± 31.14	3.55	1.04 ± 0.17	100 (19)	19	19	0	0	12
				Day 28					
Reconstituted water	1 ± 1.6	4	1.53 ± 0.86	15 (6)	5	5	0	0	1
River water	1.12 ± 15.30	4.4	1.28 ± 0.40	15 (6)	6	6	0	0	0
12.50%	0	3.78	1.05 ± 0.22	25 (10)	10	10	0	0	0
25%	18.26 ± 31.85	3.99	1.07 ± 0.22	40 (16)	16	16	1	1	2
50%	15 ± 35.63	4.24	1.09 ± 0.21	85 (34)	34	34	0	0	7
100%	28.36 ± 31.14	3.95	1.16 ± 0.27	100 (24)	24	24	0	0	11

a)



b)

Figure S3.1. Spill test tank schematic (a); and, picture including pouring device attached to tank wall on left-side (b).

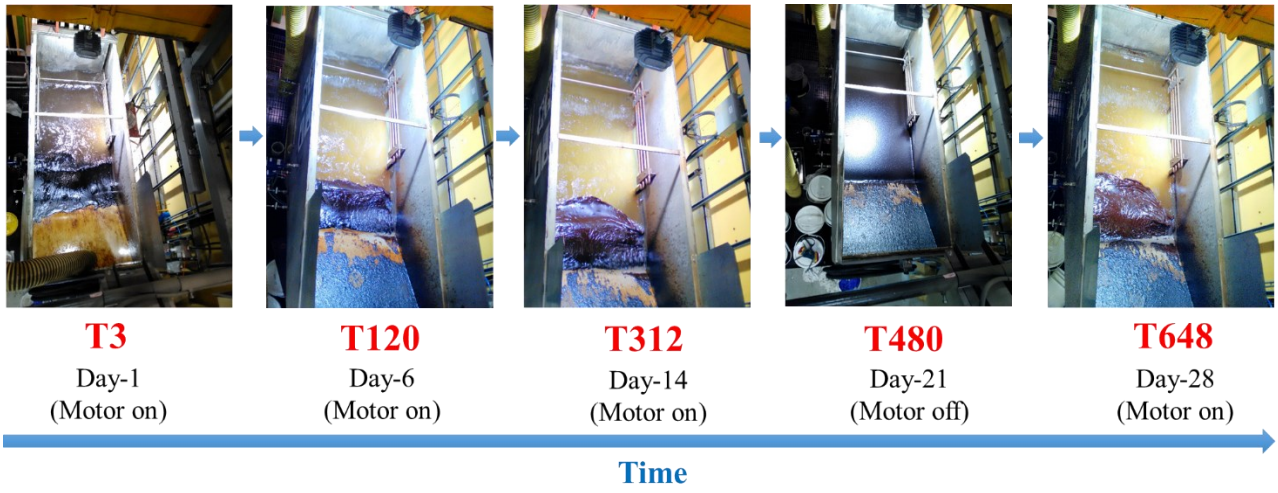


Figure S3.2 Time evolution of oil appearance on the water surface.

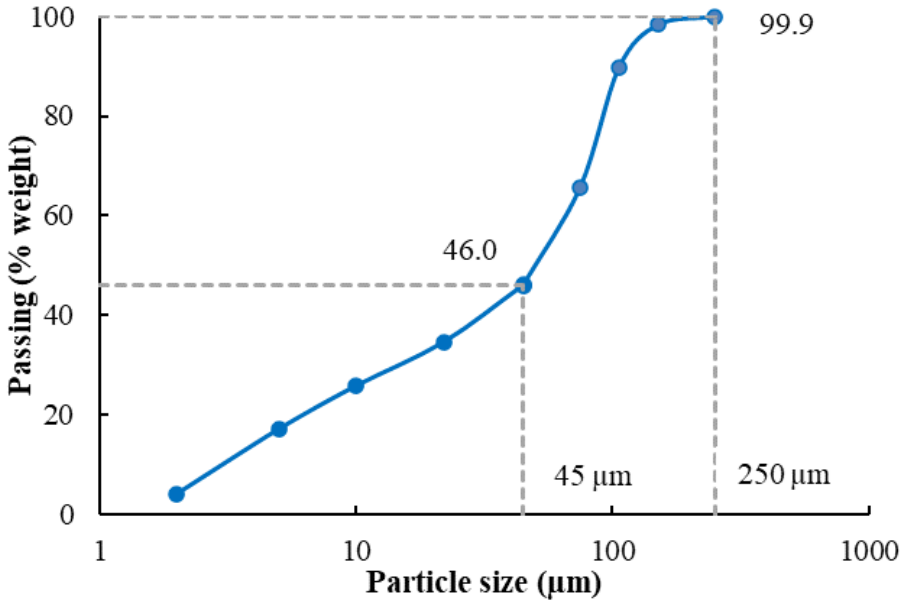


Figure S3.3 Size distribution of the North Saskatchewan River sediment.

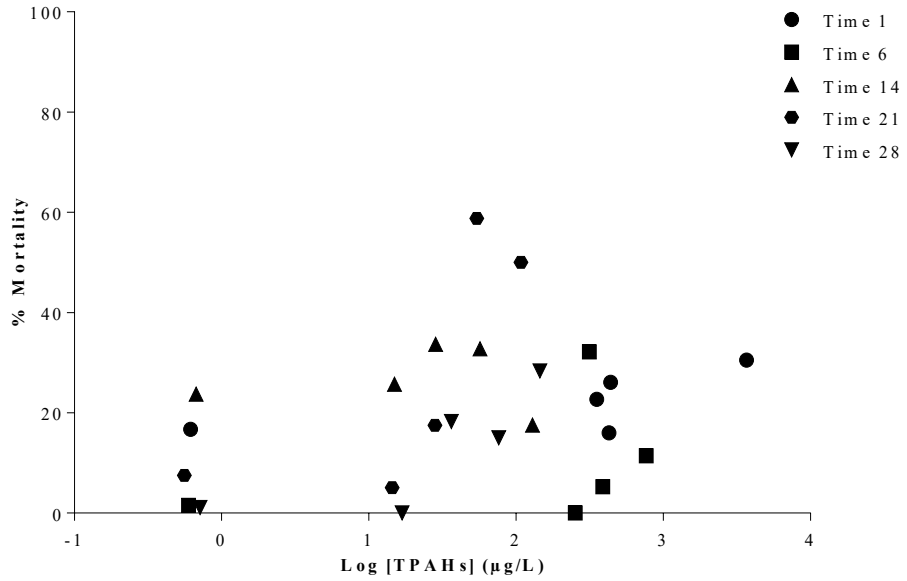


Figure S3.4 Mean of the percentage of mortality calculated from 2 days post-fertilization (eyed stage of development) of *Pimephales promelas* exposed to weathering oil water dilutions of MSW.

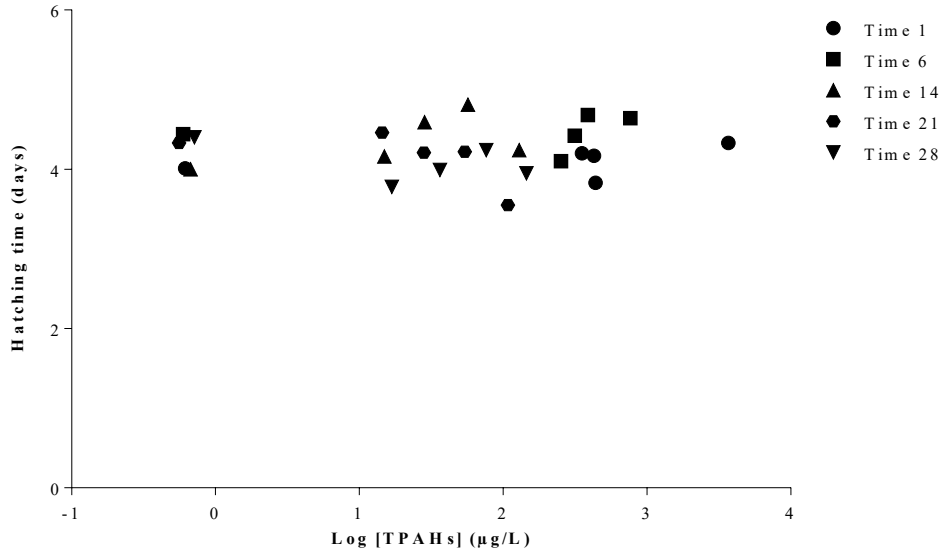


Figure S3.5 Calculated hatched time in days the majority hatched at four days of experiment of *Pimephales promelas* exposed to weathering oil water dilutions of MSW.

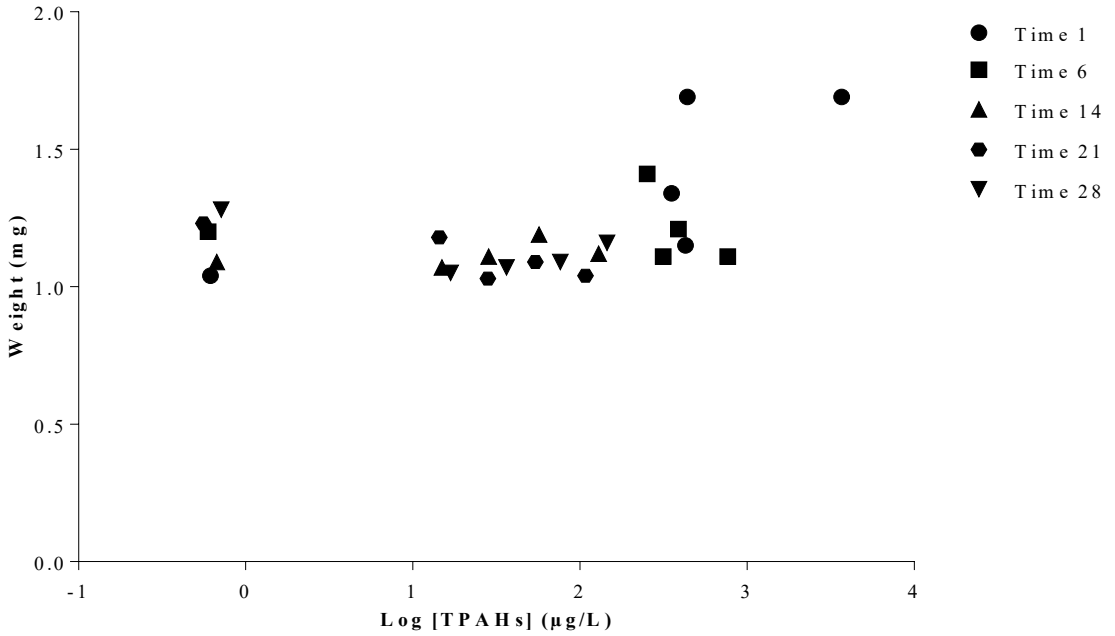


Figure S3.6 Weight of the larvae in milligrams (measured at hatching time) of *Pimephales promelas* exposed to weathering oil water dilutions of MSW.

Appendix III. Supplemental Information from Chapter 4: Weathering of diluted bitumen leads to a differential fish embryotoxicity

Table S4.1 A list of *Pimephales promelas* primers conditions for qPCR, including target genes, their primers sequences (F, forward; R, reverse), annealing temperatures (°C), amplicon size (bp), and primer concentrations (μM).

Target gene	Gene name	Forward and reverse primers	Amplicon size (bp)	Primer conc. (nM)	Annealing temp. (^{0}C)	Reference
*ef1a**	elongation factor 1 alpha	F: AGCGTAAGGAGGGAAATGCT R: CTCAGAAAGGGACTCGTGGT	254	350	58	(Martyniuk et al., 2012)
*rp18**	ribosomal protein L8	F: TTGTTGGTGTTGTTGCTGGT R: TCCTGATTGTTGAGGGCTTG	186	300	58	(Alsaadi et al., 2018)
cyp1a	cytochrome P450, family 1, subfamily A, polypeptide 1 elongation factor 1 alpha	F: TCCTGGGCTGTCGTCTATCT R: CTGGGAATGAAGTATCCGTTG	230	300	58	(Alsaadi et al., 2018)
gst	glutathione-s-transferase	F: GACGTTCATCTTCTGGAAGC R: GAGGCTTTCTCGCACTGC	151	300	58	(Mager et al., 2008)

Gene full names in order to appearance: *(*ef1a*) elongation factor 1 alpha (housekeeping gene); *(*rpl8*) ribosomal protein L8 (housekeeping gene). And (*cyp1a*) cytochrome p450-1a; (*gsr*) glutathione reductase.

Table S4.2 Density and viscosity of fresh CLB and floating oils in both tests.

Property	Fresh CLB	Floating oil (15 °C)	Floating oil (4 °C)
Density, g/mL			
at 4 °C*	0.9358	0.9940	1.002
at 15 °C*	0.9292	0.9885	0.9963
at 20 °C	0.9290	0.9940	0.9922
at 25 °C	0.9264	0.9910	0.9890
at 30 °C	0.9231	0.9890	0.9874
R^2	0.9953	0.9643	0.9868
Viscosity, cSt			
at 4 °C*	1186.0	8.13×10^5	5.43×10^5
at 15 °C*	531.6	1.97×10^5	1.31×10^5
at 20 °C	377.5	1.07×10^5	7.08×10^4
at 25 °C	268.4	5.94×10^4	3.93×10^4
at 30 °C	196.0	3.36×10^4	2.21×10^4
R^2	0.9998	1	1

* Calculated based on the measured data at 20, 25 and 30°C as explained in the text.

Table S4.3 Characteristics of the fresh CLB and floating oils at 2 °C and 15 °C trials.

Fraction, wt%	Fresh CLB	Floating oil (15 °C)	Floating oil (4 °C)
IBP - 204 °C	17.06	1.06	2.54
BP>204 °C	82.64	66.05	58.56
Water	0.30	32.89	38.90
Fraction BP>204 °C			
Elemental content			
Carbon	83.84	83.51	83.99
Hydrogen	11.11	10.92	11.11
Nitrogen	0.37	0.46	0.43
Oxygen	0.50	0.51	0.54
Sulphur	4.30	4.24	4.12
SARA			
Saturates	20.88	22.90	26.00
Aromatics	46.86	37.17	37.12
Resins	16.06	19.67	17.58
Asphaltenes	16.20	20.26	19.32

Table S4.4 Mortality and malformations observed in *Pimephales promelas* larvae exposed to water accommodated fractions (WAFs) of Cold Lake Blend (CLB) dilbit at 2 °C and 15 °C trials. Malformation types include pericardial and yolk sac edema and heart tube, craniofacial and spinal deformity.

At 4 °C							
Nominal concentrations	**% Mortality ± SD**	**% Malformed individuals *(n)***	**Pericardial edema**	**Heart Tube**	**Craniofacial**	**Yolk sac edema**	**Spinal deformity**
			Day 1				
Reconstituted water	13 ± 11.51	10 (4)	4	4	0	4	0
River water	18 ± 2.74	12.5 (4)	5	5	0	3	0
12.50%	24 ± 11.94	47.5 (19)	19	19	0	10	1
25%	27 ± 26.36	90 (36)	36	36	0	19	3
50%	46 ± 15.17	100 (40)	40	40	0	39	5
100%	52 ± 2.74	100 (40)	40	40	40	40	23
			Day 6				
Reconstituted water	15 ± 10.61	10 (4)	4	4	0	0	0
River water	24.53 ± 14.82	12.5 (5)	5	5	0	0	0
12.50%	13 ± 8.37	20 (8)	8	8	0	3	4
25%	18 ± 8.37	40 (16)	16	16	0	10	6
50%	12 ± 9.08	80 (32)	32	32	0	18	5
100%	25 ± 13.04	100 (40)	40	40	0	40	21
			Day 14				
Reconstituted water	46 ± 16.36	2.5 (1)	1	1	0	0	0
River water	48 ± 35.64	5 (3)	3	3	0	0	0
12.50%	48 ± 17.89	15 (6)	6	6	0	0	0
25%	33 ± 12.04	30 (12)	12	12	0	8	0
50%	29 ± 21.62	67.5 (27)	27	27	0	15	1
100%	30 ± 23.45	97.5 (39)	39	39	0	19	8
			Day 28				
Reconstituted water	41.25 ± 19.31	7.5 (3)	3	3	0	0	0
River water	41 ± 24.85	5 (2)	2	2	0	0	0
12.50%	48 ± 31.14	20 (8)	8	8	0	8	0
25%	30 ± 16.96	40 (16)	16	16	0	16	0
50%	47 ± 13.04	85 (34)	34	34	0	20	5
100%	48 ± 28.64	100 (40)	40	40	0	40	10
			Day 35				
Reconstituted water	27.5 ± 35.64	2.5 (1)	1	1	0	1	0
River water	20 ± 26.69	5 (1)	1	1	0	1	0
12.50%	30 ± 38.89	22.5 (9)	9	9	0	9	0
25%	33 ± 33.28	50 (20)	20	20	0	20	0
50%	19 ± 15.97	90 (36)	36	36	0	20	12
100%	11 ± 17.46	100 (40)	40	40	0	40	24

At 15 °C							
Nominal concentrations	**% Mortality ± SD**	**% Malformed individuals *(n)***	**Pericardial edema**	**Heart Tube**	**Craniofacial**	**Yolk sac edema**	**Spinal deformity**
			Day 1				
Reconstituted water	10 ± 6.67	3 (1)	1	1	0	0	0
River water	19.33 ± 4.35	5 (2)	2	2	0	0	0
12.50%	27.33 ± 6.41	40 (16)	16	16	1	1	1
25%	29.33 ± 7.60	87.5 (35)	35	35	12	17	6
50%	39.53 ± 19.59	100 (40)	40	39	6	17	15
100%	53.33 ± 24.46	100 (40)	40	40	40	40	18
			Day 6				
Reconstituted water	17.42 ± 7.62	0 (0)	0	0	0	0	0
River water	16.15 ± 9.12	5 (2)	2	2	0	0	0
12.50%	24.16 ± 13.31	20 (8)	8	8	0	1	1
25%	30.18 ± 10.50	40 (16)	16	16	2	5	2
50%	53.91 ± 8.61	100 (35)	35	35	0	35	9
100%	57.49 ± 22.61	100 (40)	40	40	0	40	14
			Day 14				
Reconstituted water	11.38 ±0.27	2.5 (1)	1	1	0	0	0
River water	11.25 ± 2.07	2.5 (1)	1	1	0	0	0
12.50%	13.60 ± 9.21	7.5 (3)	3	3	0	0	0
25%	29.19 ± 4.76	32.5 (13)	12	12	1	8	2
50%	46.58 ± 9.22	87.5 (35)	35	35	0	16	4
100%	50.68 ± 19.67	100 (40)	40	40	0	23	11
			Day 28				
Reconstituted water	8.67 ± 5.01	2.5 (1)	1	1	0	0	0
River water	3.62 ± 5.10	2.5 (1)	1	1	0	0	0
12.50%	27.46 ± 12.49	5 (2)	2	2	0	2	0
25%	45.69 ± 16.44	7.5 (3)	3	3	0	3	0
50%	42.58 ± 14.32	80 (32)	32	32	0	19	5
100%	49.76 ± 13.89	100 (40)	40	40	0	40	15
			Day 35				
Reconstituted water	8.23 ± 8.01	0 (0)	0	0	0	0	0
River water	20 ± 5.66	0 (0)	0	0	0	0	0
12.50%	25.60 ± 7.27	5 (2)	2	2	0	2	0
25%	38 ± 22.03	10 (4)	4	4	0	4	0
50%	30.38 ± 21.77	77.5 (31)	31	31	0	15	8
100%	32.73 ± 26.23	100 (40)	40	40	0	40	17

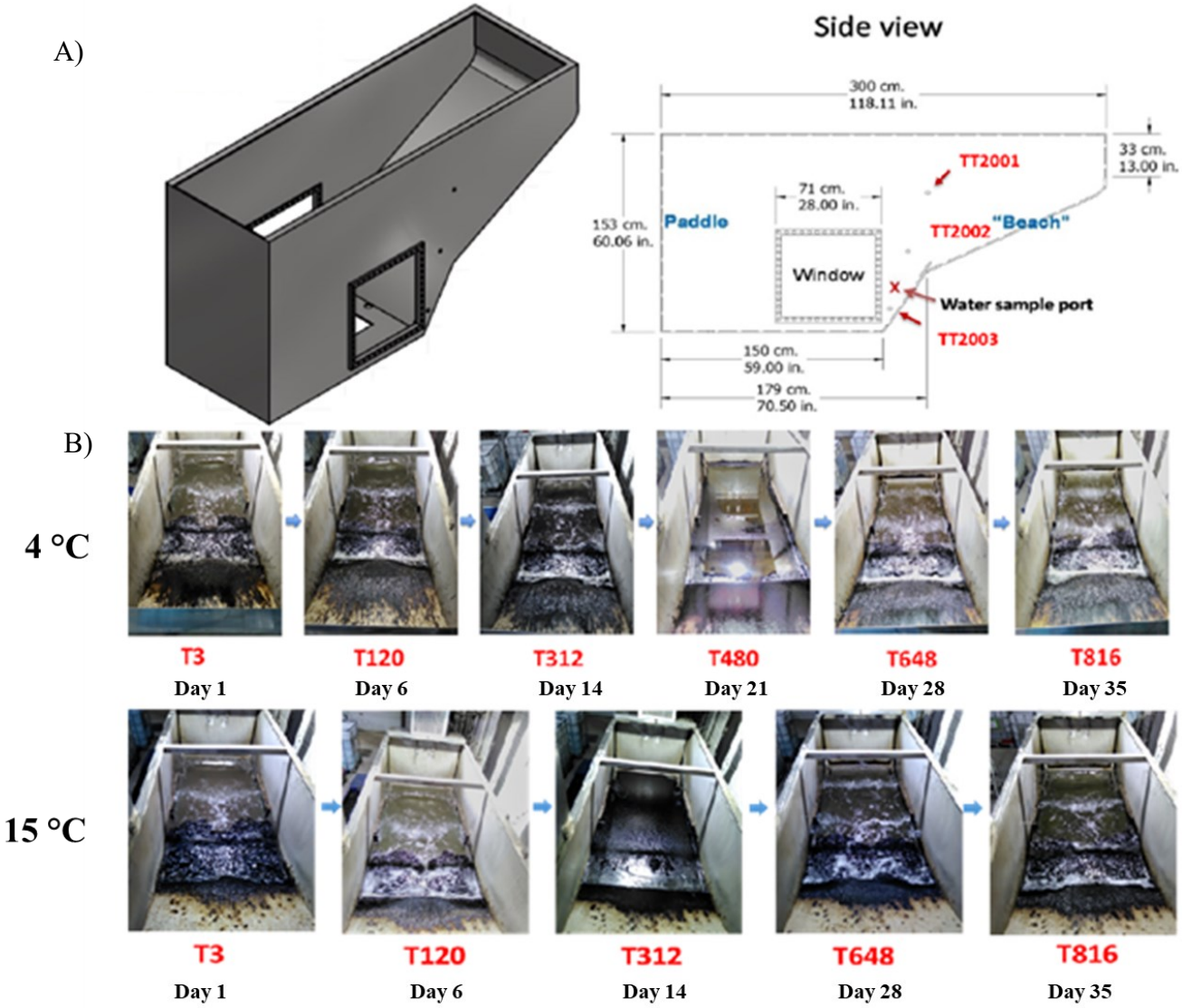


Figure S4.1 (A) Schematics of the spill tank (B) Time evolution of oil appearance on the water surface.

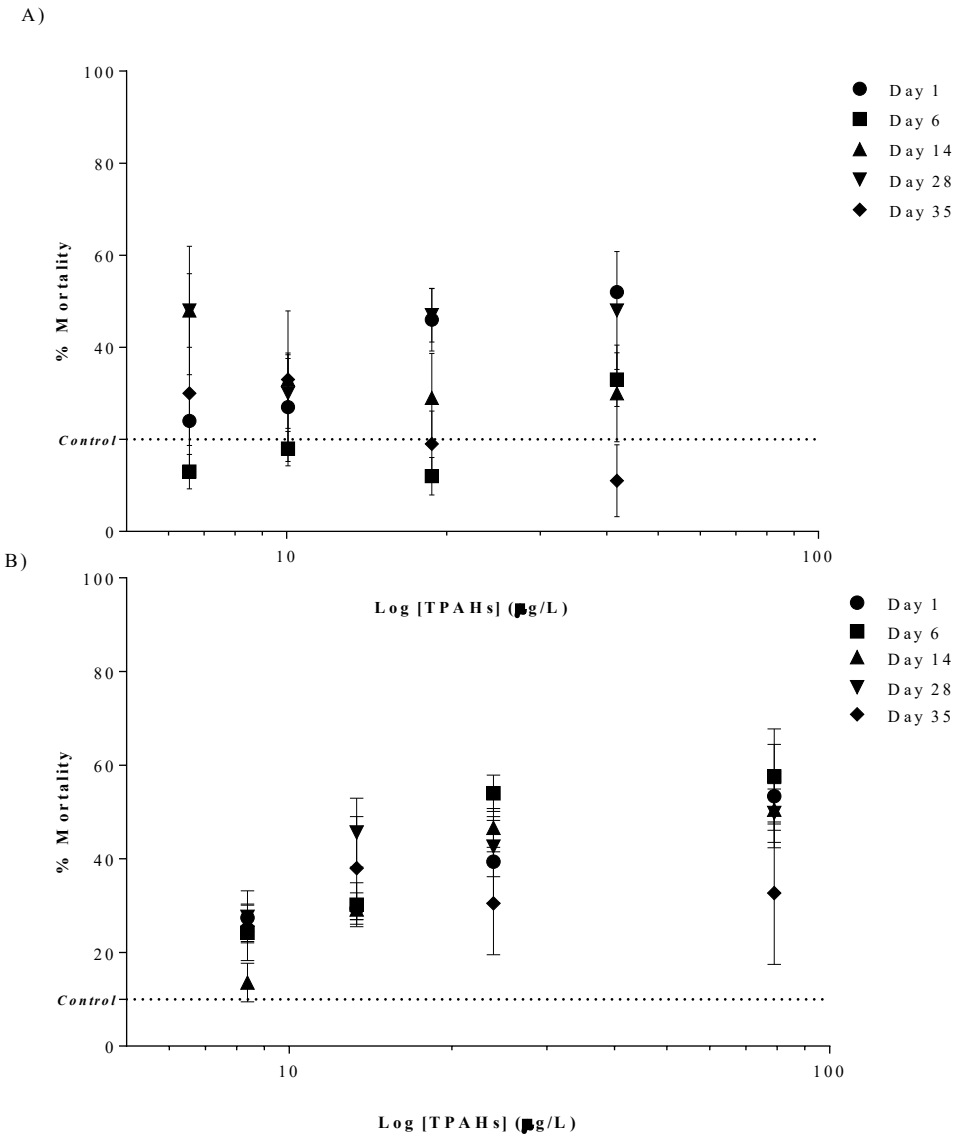


Figure S4.2 Mean of the percentage of mortality calculated from 2 days post-fertilization (eyed stage of development) of *Pimephales promelas* exposed to different dilutions of CLB contaminated water. (A) at 2 °C and (B) at 15 °C.

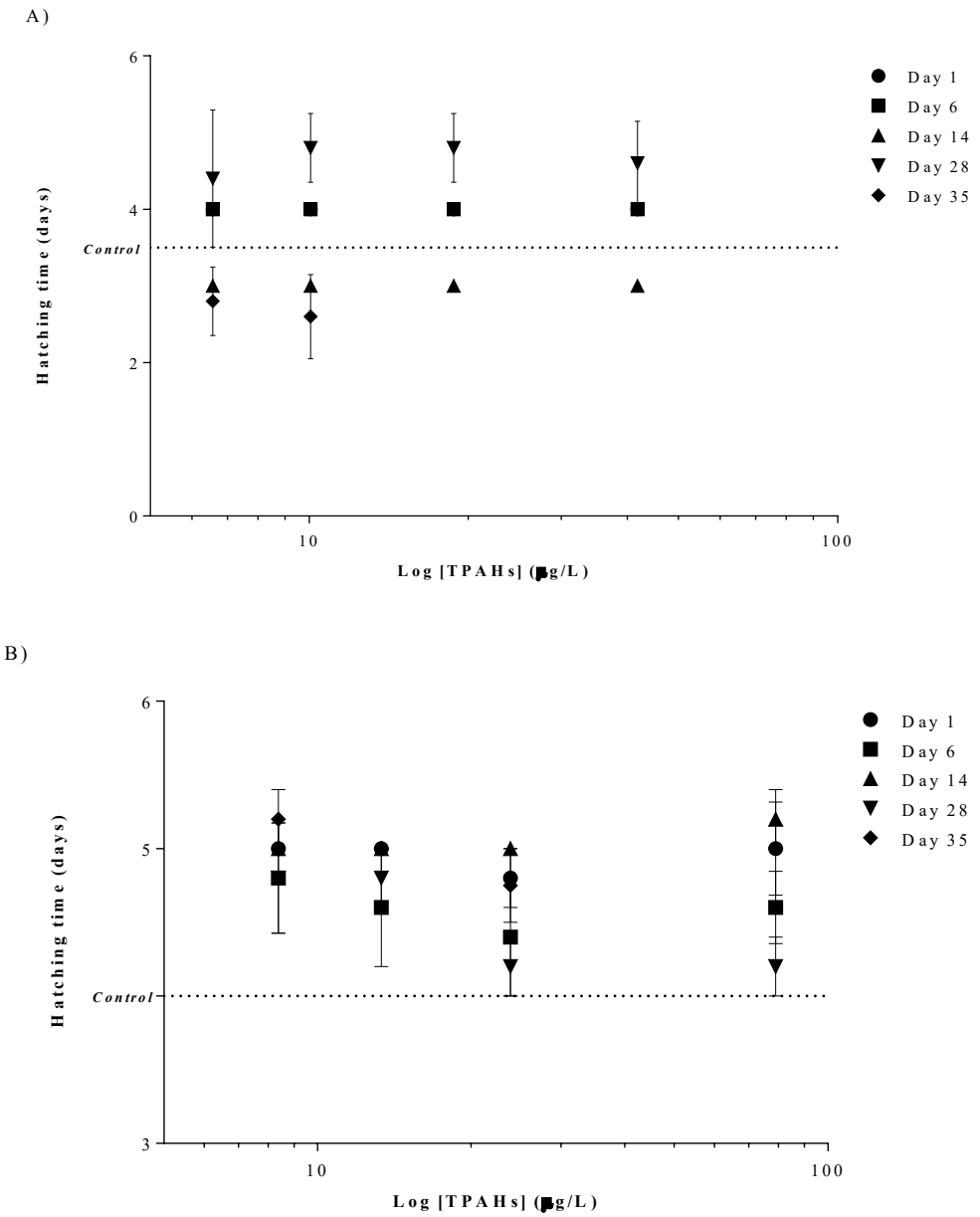
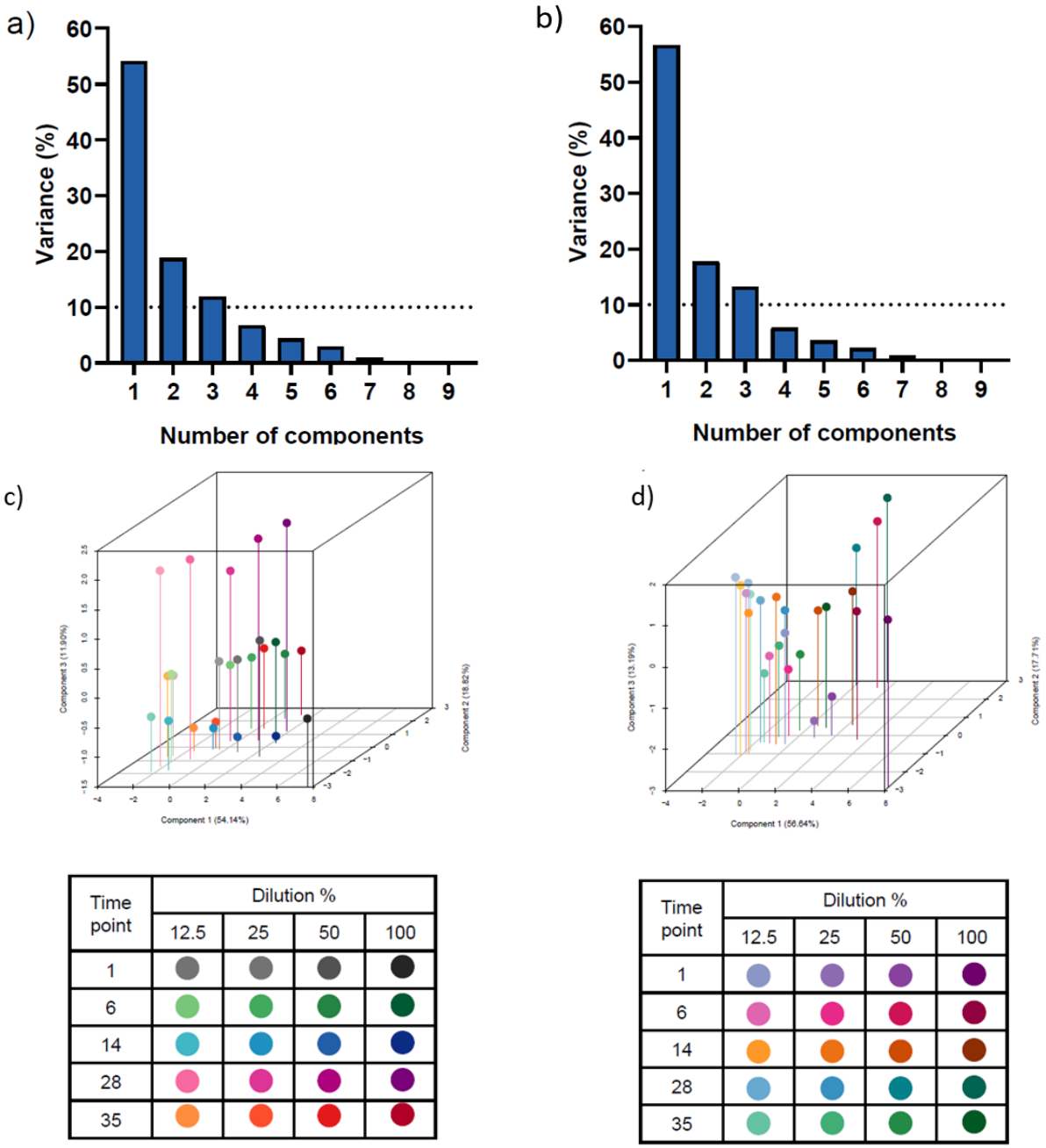


Figure S4.3 Calculated hatched time in days the majority hatched at four days of experiment of *Pimephales promelas* exposed to weathering oil water dilutions of CLB. (A) at 2 °C and (B) at 15 °C.



Time point	Dilution %			
	12.5	25	50	100
1				
6				
14				
28				
35				

Time point	Dilution %			
	12.5	25	50	100
1				
6				
14				
28				
35				

Figure S4.4 (a) Number of components greater than 10% variance at 2 °C (b) and at 15 °C. Component 1, 2 and 3 with the respectively (%) variance (c) at 2 °C (d) and at 15 °C