ASSESSING THE TOXICITY OF A PETROLEUM-BASED HYDRAULIC OIL TO AQUATIC ORGANISMS AND THE PHOTO-INDUCED TOXICITY OF POLYCYCLIC AROMATIC HYDROCARBONS IN TWO AMPHIBIAN SPECIES

A Thesis Submitted to the College of

Graduate and Postdoctoral Studies

In Partial Fulfillment of the Requirements

For the Degree of Masters of Science

In the Toxicology Graduate Program

University of Saskatchewan

Saskatoon, Saskatchewan, Canada

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ABSTRACT

A hydraulic oil, UNIVIS™ HVI 13, is used extensively along pipeline networks in Canada and presents an environmental concern with reports of leaking fluid from actuator valves at compressor stations and staining of the surrounding soil. Given the thousands of stations and (in some cases) their geographic locations, there is significant potential for leaked oil to impact nearby water bodies via spring runoff or groundwater migration. The hazard of UNIVIS HVI 13 to aquatic organisms was uncertain; therefore, the acute toxicity of water-extractable constituents of both fresh (UNIVIS-F) and aged (UNIVIS-A) UNIVIS HVI 13 was assessed by exposing a range of standard test organisms to prepared water accommodated fractions (WAF). Bioassays were conducted using *Vibrio fischeri* (Microtox® test), *Daphnia magna* (invertebrate), *Lemna minor* (plant), as well as acute exposures to early life stage fish (*Pimephales promelas)*, and amphibians (*Xenopus laevis* and *Lithobates sylvaticus*). Chemical analyses of 1:1 loadings of WAF from UNIVIS-F and UNIVIS-A were performed to confirm concentrations of hydrocarbons and metals. Effective concentration (EC) values were determined for each toxicity test and expressed as g L^{-1} oil-to-water, derived from serial dilution of 1:1 (880 g L^{-1}) UNIVIS HVI 13 WAF preparation. Based on EC_{50} values, *D. magna* was the most sensitive organism to UNIVIS HVI 13 with EC_{50} values of 114 g L^{-1} for UNIVIS-F WAF and 66 µg L^{-1} for F1 hydrocarbon fraction. Results indicated that *X. laevis* is more sensitive to UNIVIS-A WAF (EC_{50} value = 372 g L^{-1}) compared to UNIVIS-F WAF (EC_{50} value > 880 g L^{-1}) but *D. magna* is more sensitive to UNIVIS-F (EC_{50} value = 114 g L^{-1}) compared to UNIVIS-F WAF (EC_{50} value > 362 g L^{-1}). Overall, the acute toxicity data indicated that the water-soluble constituents of UNIVIS HVI 13 pose minimal hazard to aquatic organisms.

Due to the presence of polycyclic aromatic hydrocarbons (PAHs) in the environmentally relevant PAH mixture UNIVIS HVI 13, a second experiment was performed to test whether ultraviolet (UV) light enhances the toxicity of UNIVIS HVI WAF. PAHs are ubiquitous environmental contaminants produced mainly from the incomplete combustion of organic materials. Generally, the acute toxicity of PAHs to aquatic organisms is low within the range of their water solubility limits; however, in the presence of ecologically relevant intensities of UV radiation, the acute toxicity of PAHs and petroleum-products containing a mixture of PAHs can increase substantially. The second experiment was designed to determine whether: (a) early-life

stage amphibians exhibit photo-induced toxicity to PAHs and a PAH mixture; (b) two amphibian species, *Xenopus laevis* and *Lithobates sylvaticus* (wood frog) demonstrate differences in sensitivity; and (c) differences in sensitivity are correlated with species-specific uptake. For each species, 96 h toxicity tests were performed where newly hatched tadpoles of each species (stage-matched) were exposed to benzo(a)pyrene, anthracene, naphthalene or the water accommodated fraction (WAF) of an environmentally relevant hydraulic oil (UNIVIS HVI 13) for 8 h, after which they were transferred to clean water and exposed to UV light for 12 h then allowed to develop without chemical or UV until the end of the 96 h test. Mortality, morphometrics, body burden and deformities were assessed and compared between species. For both larval *Xenopus* and wood frog, exposure to anthracene or benzo(a)pyrene (8 h) followed by UV light (12 h) resulted in a significant increase in mortality compared to exposure to either PAH alone. Exposure to naphthalene or WAF of UNIVIS HVI 13 and UV light did not result in increased in mortality in larval amphibians at all concentrations tested. Larval *Xenopus* exhibited decreased length and increased deformities when exposed to UV light alone regardless of chemical treatment. Wood frogs exposed to 2 and 20 $\mu g\ L^{-1}$ anthracene, and 100 $\mu g\ L^{-1}$ benzo(a)pyrene had a significantly higher body burden compared to *Xenopus* exposed to the same concentrations. The LC_{50} value for *Xenopus* exposed to anthracene and UV light was 5 $\mu g\ L^{-1}$, while the LC_{50} value for wood frogs exposed to anthracene and UV light was 124 $\mu g\ L^{-1}$ leading us to conclude that for anthracene, toxicity thresholds based on *Xenopus* would be protective for the wood frog. The LC_{50} values for benzo(a)pyrene and UV light were more comparable between species with 45 $\mu g\ L^{-1}$ for *Xenopus* and 17 $\mu g\ L^{-1}$ for wood frogs. Based on the significant mortality to low concentrations of anthracene, and the increased sensitivity to UV treatment alone, *Xenopus* allows for conservative comparisons to the wood frog when assessing the photo-induced toxicity of PAHs and complex mixtures containing PAHs.

Overall, the acute toxicity of UNIVIS HVI 13 was minimal to aquatic organisms and the low concentrations of PAHs with phototoxic potential within this environmentally relevant PAH mixture is likely the reason we did not observe photo-induced toxicity in the two amphibian species tested. However, photo-induced toxicity is still an important consideration in the hazard assessment of PAH mixtures, evidenced by the interactive effects of single PAH exposure and UV on tadpole mortality and development. This research provides new data on the acute

phototoxic effects of PAHs in two amphibian species and indicates that species differences in response are not driven by differences in PAH uptake.

ACKNOWLEDGEMENTS

I would like to thank my graduate supervisor Dr. Natacha Hogan for her continuous and immense support, encouragement, guidance and mentorship during the completion of this thesis. I would also like to extend my thanks to my other committee members, Dr. Steven Siciliano and Dr. Lynn Weber for providing me with feedback and assistance in the completion of my Masters. I thank Taylor Lane for providing fathead minnow embryos, and Dr. Mark Hanson and Dana Moore at the University of Manitoba for providing sub-cultures of *L. minor*. I thank Dr. Jeff Hudson for use of OceanOptics spectrometer, and Landon McPhee, Nicole Baldwin, Tara Stang, Dana Tkatchuk, Samantha Lundquist, and Arashpreet Natt for all assistance with animal husbandry, field work and conducting toxicity tests. I also thank McDonald Donkuru, Jenna Cantin and Chanel Yeung for all assistance with ASE and HPLC.

I would like to thank the staff and students in Toxicology and Animal and Poultry Science, including Melanie Gallant for her knowledge and support in the office, as well as housemates Larry D'Silva, Connor Maurice, and Michael Cavallaro for all the support and memories outside of graduate school.

I would like to acknowledge the funding sources that supported the research conducted as part of this thesis, including a Collaborative Research and Development (CRD) Grant from the Natural Sciences and Engineering Research Council of Canada (NSERC) with partner funding from TransCanada Pipelines. I also gratefully acknowledge support and partial stipend funding provided to me through Toxicology Centre Devolved scholarships.

Most importantly, I'd like to thank my family for their immense love and support through completion of this degree, and Kelci for her unconditional love, support, patience and positivity throughout the duration of my Masters.

DEDICATION

This dissertation is dedicated in loving memory to my beloved grandfather George Currie who passed away during the completion of this thesis. Thank you for always supporting me and encouraging me to work hard and pursue my dreams. You were an incredible role model and your words of wisdom will forever resonate.

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AHR	aryl hydrocarbon receptor
ANOVA	analysis of variance
APV	aquatic protection value
ASE	accelerated solvent extractor
ATRF	aquatic toxicology research facility
BCF	bioconcentration factor
BTEX	benzene-toluene-ethylbenzene-xylene
CCME	Canadian Council of Ministers of the Environment
COPC	chemical of potential concern
CRD	Collaborative Research and Development
CYP	cytochrome P450
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EC	effective concentration
GWQG	groundwater quality guideline
hCG	human chorionic gonadrotropin
HOMO	highest energy occupied molecular orbital
HPLC-FD	high performance liquid chromatography-fluorescence detection
HS-GC	head space-gas chromatography
ICP-MS	inductively coupled plasma mass spectrometry
LC_{50}	lethal concentration required to kill 50% of population
LL.3	three parameter log-logistic function
LOEC	lowest observed effect concentration
log K_{ow}	octanol-water partition coefficient
LSD	least significance difference
LUMO	lowest energy occupied molecular orbital
MS-222	tricaine methanesulfonate
MS/FID	mass spectrometry/flame ionization detection
NOEC	no observed effective concentration
NSERC	Natural Sciences and Engineering Research Council of Canada

OECD	Organization for Economic Co-operation and Development
PAH	polycyclic aromatic hydrocarbon
PBS	phosphate-buffered saline
ROS	reactive oxygen species
RNA	ribonucleic acid
SEM	standard error of the mean
US EPA	United States Environmental Protection Agency
UV	ultraviolet
WAF	water-accommodated fraction

PREFACE

Chapter 1 of this thesis is a general introduction and Chapter 4 is a general discussion with overall major conclusions. Chapter 2 and 3 are organized as manuscripts to be published in scientific journals. Therefore, there may be content that is repeated between the introduction and materials and methods sections across chapters. Chapter 2 is being prepared for submission to *Environmental Toxicology and Chemistry* whereas Chapter 3 is being prepared for submission to *Aquatic Toxicology*.

CHAPTER 1

GENERAL INTRODUCTION

Chapter 1 is a general introduction and literature review regarding the topics of polycyclic aromatic hydrocarbons, their toxicity to aquatic organisms, mechanisms of toxicity with emphasis on photo-induced toxicity, the concern surrounding an environmentally relevant petroleum-product (UNIVIS HVI 13) and current state of knowledge regarding PAH and UV effects in amphibians. Chapter 1 also includes the overall goals and objectives of the project and each study in particular, and includes null hypotheses.

1.1 Polycyclic aromatic hydrocarbons

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental contaminants derived primarily from the incomplete combustion of organic matter. PAHs are found naturally in the environment; however, their environmental load has increased substantially since the onset of industrialization due to the increasing use of fossil fuels (Eisler, 1987; Abdel-Shafy and Mansour, 2016). It is estimated that 230,000 metric tons of PAHs are discharged into aquatic environments per year as a result of human activities (Eisler, 1987). PAHs are a group of chemicals consisting of two or more fused aromatic benzene rings and can contain alkyl groups or incorporate nitrogen and sulfur atoms (Wojtaszek, 2000). PAHs represent some of the most persistent and detrimental substances in the aquatic environment with The United States Environmental Protection Agency (US EPA) designating 16 PAH compounds as priority pollutants (Mackay, 1991; US EPA, 2014; Salvo *et al.*, 2016). PAHs are generally characterized into being derived from petrogenic or pyrogenic sources. Petrogenic PAHs are generated from organic matter in ancient sediments/rocks under geologic conditions over time, whereas pyrogenic PAHs are generated from rapid combustion/pyrolysis of organic matter (i.e., wood, coal, petroleum, wastes) (Stout *et al.*, 2015). Petrogenic and pyrogenic PAHs are highly complex and are the main sources of PAH contamination in the environment (Stout *et al.*, 2015). Physical and chemical characteristics of PAHs vary with molecular weight. Generally, as the molecular weight of PAHs increases, their aqueous solubility decreases and their log octanol-water partition coefficient (K_{ow}) increases (Eisler, 1987; Abdel-Shafy and Mansour, 2016). Due to the lipophilic nature of PAHs, they have the ability to bioaccumulate in aquatic organisms. PAHs are usually accumulated by organisms from the water column or sediment due to their low aqueous solubilities (Eadie *et al*., 1982; Wojtaszek, 2000).

1.2 Toxicity of PAHs to aquatic organisms

The acute toxicity of PAHs to aquatic organisms is determined by PAH type, species of organism, and duration and type of exposure (Nagpal, 1994). PAHs can be characterized into low molecular weight PAHs (2-3 ring PAHs such as naphthalenes, fluorenes, phenanthrenes, and anthracenes) or high molecular weight PAHs (4 to 7 ring PAHs such as chrysenes and coronenes) (Nagpal, 1994). Generally, the low molecular weight PAHs have greater acute toxicity and lower carcinogenic potential, whereas the high molecular weight PAHs have lower acute toxicity and

greater carcinogenic potential (Eisler, 1987; Landrum *et al.,* 1987). However, the acute toxicity observed with PAHs in studies conducted in the lab is usually at concentrations greater than the water solubility of the compound. For this reason, there have been relatively few studies assessing the lethality of individual PAHs since the Canadian Water Quality Guidelines for the Protection of Aquatic Life were established for PAHs in 1999.

In general, fish are considered the most sensitive aquatic taxa to the lethal effects of PAHs with a minimum LC_{50} value of 30 μg L^{-1} reported for rainbow trout exposed to phenanthrene, a more soluble and lower molecular weight PAH (Millemann *et al.,* 1984; Nagpal, 1994). However, the 96 h LC_{50} value of 520 μg L^{-1} for the alga *Selenastrum capicornutum* exposed to acenaphthene was lower than for brown trout (LC_{50} = 580 μg L^{-1}) or fathead minnow (LC_{50} = 610 μg L^{-1}) (Nagpal, 1994). The higher molecular weight PAHs such as benz[a]anthracene and benzo[a]pyrene were acutely toxic to invertebrates at 5-10 μg L^{-1} (Trucco *et al.,* 1983; (Nagpal, 1994). Alkyl homologues of PAHs are generally more toxic to aquatic organisms than the parent compound. For example, the 48 h EC_{50} for *Daphnia pulex* exposed to anthracene was 750 μg L^{-1}, whereas the 48 h EC_{50} values for methyl anthracene and 9-methoxy anthracene were 96 μg L^{-1} and 400 μg L^{-1}, respectively (OMOE, 1990; Nagpal, 1994).

Individual PAHs exhibit a wide range of toxicity across different aquatic organisms. For freshwater fish exposed to acenaphthene, 96 h LC_{50} values range from 580 μg L^{-1} for brown trout to 1730 μg L^{-1} for juvenile fathead minnows (Holcombe *et al.,* 1983; Geiger *et al.,* 1985). Cairns and Nebeker (1982) exposed fathead minnow embryos to acenaphthene for 35 d and reported a lowest observed effective concentration (LOEC) value of 495 μg L^{-1} for growth. The 48 h LC_{50} value for *Daphnia magna* exposed to acenaphthene was 41,000 μg L^{-1} (LeBlanc, 1980), and the 96 h LC_{50} value for the snail *Aplexa hyponorum* exposed to acenaphthene was > 2040 μg L^{-1} (Holcombe *et al.,* 1983). Acute toxicity data for acridine ranges from a 48 h LC_{50} value of 1860 μg L^{-1} for *Chironomus tentans* (Millemann *et al.,* 1984) to a 48 h LC_{50} value of 2300 μg L^{-1} for *Daphnia magna* (Parkhurst *et al.,* 1981). Chronic effects of morphological abnormalities and necrosis of brain and spine have been reported in rainbow trout eggs and alevins exposed to 0.08 – 0.21 μg L^{-1} benzo(a)pyrene (Hannah *et al.,* 1982; Hose *et al.,* 1984). Invertebrates are considered very sensitive to benzo(a)pyrene with a reported 96 h LC_{50} of 5 μg L^{-1} for *Daphnia pulex*. Finger *et al.,* (1985) reported significant reductions in survival and growth of juvenile

bluegill sunfish at with exposure 500 and 250 μg L^{-1} fluorene, respectively, while 96 h LC_{50} values for rainbow trout to fluorene was 820 μg L^{-1} compared to 910 μg L^{-1} for bluegill sunfish. *Daphnia magna* exposed to fluorene levels of 125 μg L^{-1} had reduced reproduction following 14 d, and the emergence of larval midges (*Chironomus riparius*) was reduced following 30 d exposure to 600 μg L^{-1} fluorene (Finger *et al.*, 1985). Reported 96 h LC_{50} values for fathead minnows exposed to naphthalene include 7900 μg L^{-1} (DeGrave *et al.*, 1982), 6080 μg L^{-1} (Holcombe *et al.*, 1984), 1990 μg L^{-1} (Millemann *et al.*, 1984), and 6140 μg L^{-1} (Geiger *et al.*, 1985), while 48 h LC_{50} values of 3400 μg L^{-1} (Geiger and Buikema, 1981) and 4663 μg L^{-1} (Smith *et al.*, 1988) were reported for *Daphnia pulex*.

Overall, the reported LC_{50} values for individual PAHs used for the Canadian Water Quality Guidelines for the Protection of Aquatic Life are generally much higher than the compounds water solubility (Nagpal, 1994). Thus, the conditions in the lab may not be achieved in the natural aquatic environment, and the responses observed in the lab may not be relevant of PAH exposure in the aquatic environment.

1.3 Mechanisms of PAH-mediated toxicity

Generally, PAHs interact with cells in two ways to cause toxic responses: they may bind reversibly to lipid-rich sites and disrupt cellular processes (narcosis), or their metabolites may bind covalently to cellular structures and macromolecules, causing long-term cellular damage (Neff, 1979). Activation of the aryl hydrocarbon receptor (AHR) and generation of reactive species are key modes of action initiated by particular PAHs, but the full extent of the molecular responses that result from exposure to PAHs is not completely understood. A number of PAHs, including benzo(a)pyrene, bind the AHR and induce expression of phase I and II metabolizing genes, such as cytochrome P450 family 1 member A1 (CYP1A1) (Nebert *et al.*, 2000). Related to this, CYP1A1 has been widely used as a biomarker for PAH exposure (Nebert *et al.*, 2000). While activation of the AHR pathway and metabolism of PAHs can have a protective effect against PAH toxicity, induction of CYPs and particularly CYP1A1 enzymatic activity within the organism can transform PAHs to genotoxic metabolites that can react with macromolecules such as DNA (Foureman, 1989; Wojtaszek, 2000; Marquis *et al.*, 2006). For example, the mechanism underlying benzo(a)pyrene toxicity is linked to its bioactivation through the CYP pathway (Ma, 2001; Uno *et al.*, 2004; Kohle and Bock, 2007). Benzo(a)pyrene diol epoxide metabolites formed

during phase I metabolism by CYP450 and epoxide hydrolase can cause oxidative stress and damage to DNA, RNA, and proteins (Foureman 1989; Wojtaszek, 2000). This oxidative stress and damage to biological macromolecules can be augmented when PAH exposure occurs together with exposure to specific wavebands of sunlight, such as ultraviolet radiation (reviewed in Roberts *et al.* 2017). Because most aquatic ecosystems receive some amount of sunlight, co-exposure to PAHs and solar radiation is likely to occur in the environment, and photo-induced toxicity of PAHs may be an important factor impacting aquatic organisms.

1.3.1 Photo-induced toxicity of PAHs in aquatic organisms

As previously mentioned, PAHs are generally not acutely toxic within their aqueous solubility limits; however, their acute toxicity can increase substantially when exposed with ultraviolet (UV) radiation. Numerous studies have shown that PAHs are acutely toxic to aquatic organisms within the range of their aqueous solubility exposure occurs simultaneously with UV radiation (Bowling *et al.,* 1983; Landrum *et al.,* 1987; Newsted *et al.,* 1987; Greenberg *et al.,* 1993*;* Ankley *et al.,* 1995). The effects of UV exposure on PAH toxicity can be drastic as demonstrated in a study by Bowling et al. (1983) in which anthracene-exposed juvenile bluegill (*Lepomis machrochirus*) maintained in the presence of sunlight experienced 100% mortality, while those held in the shade experienced <6% mortality. Increased mortality following co-exposures to PAHs and UV has been noted for a wide range of fish species including bluegill sunfish, pacific herring, medaka, and zebrafish (Bowling *et al.,* 1983; Oris and Giesy, 1985; Oris and Giesy, 1986; Barron *et al.,* 2003; Diamond *et al.,* 2006; Willis and Oris, 2014). In fact, it has been suggested that PAHs are arguably the best-studied phototoxicant in ecotoxicology (Roberts *et al.* 2017).

1.3.2 Mechanisms of PAH photo-induced toxicity

Photo-induced toxicity is dependent on a number of factors including the amount of PAH that is accumulated into an organism, the intensity of UV radiation, the amount of light penetrating the aquatic environment and organism, the amount of light absorbed by the photosensitizing molecule, the proportion of the sensitizing molecules that are transformed to a reactive species, the probability of an interaction of the excited sensitizer with a target molecule, and the duration of the co-exposure to both PAH and UV (Newsted and Giesy, 1987; Sellin Jeffries *et al.,* 2013). Photo-induced toxicity of PAHs depends on the size of the highest occupied

molecular orbital and the lowest unoccupied molecular orbital (HOMO-LUMO) gap, the total electromagnetic energy absorbed, the phosphorescence lifetime, and the triplet energy of the compound (Newsted and Giesy, 1987; Arfsten *et al*., 1996; Willis and Oris, 2014). Recent studies have used these factors to model and predict the photo-induced toxicity of a number of PAHs to aquatic organisms (Marzooghi *et al.,* 2016).

Due to their structure, PAHs can absorb UV-A (315-400 nm) and UV-B (280-315 nm) readily; however, there is only a subset of PAHs with particular structural/chemical properties that have the ability to cause photo-induced toxicity. Phototoxicity models accurately determine this subset of potentially phototoxic PAHs based on the compound's chemical and physical properties. Photo-induced toxicity occurs when PAHs absorb energy from UV radiation and become excited, resulting in the outer orbital electrons to be raised to a higher molecular energy orbital (Willis and Oris, 2014). When the excited molecule returns to the ground state, this energy is released and transferred onto oxygen molecules in the cell, which generates reactive oxygen species (ROS), free radicals, and other reactive photo-products (Arfsen *et al.,* 1996; Bjorn and Huovinen, 2007; Willis and Oris, 2014). The main targets of photo-induced PAHs within an organism are DNA, lipids, and essential cytoplasmic components (Newsted and Giesy, 1987). This can ultimately lead to cell death, DNA damage, behavioral impairment and mortality (Neff, 1979; Pelletier *et al.,* 1997; Choi and Oris, 2000; Ankley *et al.,* 2010; Willis and Oris, 2014).

Photo-induced toxicity may occur through the mechanisms of photosensitization or photomodification. Photosensitization occurs when the parent PAH molecule is firstly absorbed into the organism, and subsequently absorbs UV energy within the organism resulting in damage to biological molecules via reactive oxygen species (ROS). In contrast, photomodification occurs through the production of UV-modified photoproducts external to the organism. This occurs when the parent PAH is in the water before the photoproducts are subsequently absorbed into the organism and cause damage to biological molecules via ROS (Barron, 2000; Lampi *et al.,* 2006; Roberts *et al.,* 2017). Evidence suggests that for most compounds, the toxic products responsible for photo-induced toxicity are formed within the organism, while photoproducts formed outside seem to play a limited role, if any, in causing phototoxic injury (Arfsten *et al.,* 1996, Roberts *et al.,* 2017).

1.4 Toxicity and photo-induced toxicity of petroleum-products

1.4.1 PAH mixtures

Petroleum-based products are complex mixtures containing a wide range of chemical constituents, including PAHs. In fact, aromatic hydrocarbons, such as benzene, toluene, ethylbenzene, and xylenes (BTEX) and PAHs, are considered to be the most important drivers of oil toxicity (Boehm *et al.*, 2007). For this reason, including photo-induced toxicity in the hazard assessment of petroleum-based mixtures is warranted. Indeed, many petroleum products have also been shown to elicit photo-induced toxicity (Barron, 2017). The water-accommodated fraction (WAF) of 16/22 tested petroleum products (ranging from diesel to crude oil) exhibited photo-induced toxicity in *Daphnia magna* (Wernersson, 2003). UV exposure significantly enhanced toxicity of weathered Alaska North Slope crude oil to early life stages of Pacific herring eggs and larvae (Barron et al., 2003). Site samples of contaminated water from the Exxon Valdez oil spill were acutely toxic in the presence of sunlight and artificial UV to larval Pacific herring (Sellin *et al.,* 2013). In all cases described above, the photo-induced toxicity was attributed to the PAH constituents in the products. The limited studies that have investigated the photo-induced toxicity of petroleum products and PAH mixtures in aquatic organisms have shown similar results to individually tested PAHs. It is evident that photo-induced toxicity is the most sensitive endpoint for PAHs and the phenomenon should be taken into account when conducting risk assessments.

1.4.2 UNIVIS HVI 13

A detailed review of white oils and their toxicity is described in the introduction of Chapter 2. Briefly, hydraulic fluid lubricating oils are used to maintain the critical function of actuator valves on compressor stations located at intervals along a pipeline. Compressor stations function to pressurize oil and gas as it travels along the pipeline. Hydraulic oils are derived from the vacuum distillation of crude oils and are designed to sustain harsh environments 365 days/year. Over recent years, soil staining due to leaky actuator valves has been observed at compressor sites along a major pipeline across Canada, drawing concern from both industry and regulators. The oil used at these compressor stations is UNIVIS HVI 13, a complex petroleum-based hydraulic lubricating oil manufactured by Exxon and containing BTEX (benzene, toluene, ethylbenzene, and xylenes), metal additives, aliphatic hydrocarbons, and PAHs.

Hydraulic oils are potential environmental contaminants as they accumulate around their release point, accumulate in organisms, and exert toxicity to plants, fish and wildlife (Herdan, 1997). The fate of the hydraulic oil in soil will depend on abiotic and biotic factors, but the products of this degradation (weathered hydraulic oil) will likely differ in chemical composition from the pure product. From samples collected in the preliminary risk assessment, soil levels of F2 and F3 fractions at some sites were greater than the Canadian Council of Ministers of the Environment (CCME) soil saturation limits, indicating the potential of off-site risk to aquatic life if the free product became mobilized. The extent of vertical migration and mobility of the hydraulic fluid is currently unknown; however, aquatic environments could be exposed to the lubricating oil via groundwater infiltration as well as spring runoff via rain and snowmelt. There is currently no toxicity data on fresh or aged UNIVIS HVI 13 to aquatic organisms. PAHs were chosen as the constituent of focus in this project due to their presence in UNIVIS HVI 13, their reputation as chemicals of concern, and their known toxicity to aquatic organisms.

1.5 Exposure and effects of PAHs in amphibians

1.5.1 Amphibian decline and ecological significance

Amphibians are integral in both aquatic and terrestrial communities as they occupy diverse trophic niches, provide prey for wildlife, and play critical roles in energy and nutrient transfer through food webs (Beard *et al.,* 2002; Hopkins, 2007). Due to their ecological significance, changes in amphibian populations can affect an entire ecosystem (Hopkins, 2007).

Amphibians are highly sensitive to environmental stressors due to their permeable skin, occupancy of both aquatic and terrestrial habitats, and high rate of contaminant bioaccumulation (Dunson *et al.,* 1992, Rowe *et al.,* 2003; Hopkins, 2007, Unrine *et al.,* 2007). Over the past 40 years, there has been an alarming decline in amphibian populations around the globe, with the decline portraying the largest mass extinction of land vertebrates since the dinosaurs (Stuart *et al.,* 2004; Hopkins, 2007). There have been a number of environmental and chemical stressors identified as potential factors in this decline. These factors include opportunistic pathogens, climate change and increased levels of anthropogenic contaminants (e.g., PAHs) in the aquatic environment (Hayes *et al.,* 2010; Collins, 2010). There has not been a single underlying factor linked to the global amphibian population decline, but rather the decline is thought to be due to a number of stressors acting together.

1.5.2 Early life stage exposure to PAHs

Developing amphibian larvae can accumulate PAHs from water through dermal absorption and from sediment through dermal and dietary absorption with some of the major sources of PAH accumulation being from food, sediment, water, and fine suspended particles (Berrojalbiz *et al.*, 2009, Newman, 2010; Bilodeau, 2017). Due to their lipophilicity, PAHs tend to concentrate in sediment, permeate through biological membranes of organisms and accumulate in tissues (Lepanen, 1999). Consequently, larval amphibians that live in close proximity to sediment are particularly susceptible to PAH exposure and bioaccumulation via ingestion or direct contact (Bilodeau, 2017). Following uptake, PAHs may be biotransformed and/or incorporated into tissues, or eliminated through metabolism and/or excretion pathways (Livingstone, 1998; Newman, 2010)

Early life stages of amphibians are particularly sensitive to contaminant exposure (Hatch and Burton, 1998; Stanley, 2008, Gross *et al.*, 2009) and PAH exposure can negatively impact growth, development and survival of early life stage amphibians (Marquis *et al.*, 2006). Hersikorn and Smits (2011) reported that wood frogs raised on reclaimed wetlands near the Athabasca oil sands had delayed metamorphosis and thyroid hormone disruption, with the effects being attributed to PAH contamination in wetland sediments. Aquatic organisms collected near the Athabasca oil sands accumulate PAHs and exhibit adverse effects associated with exposure to PAHs (Hersikorn and Smits, 2011; Wayland *et al.*, 2008, Tolton *et al.*, 2012, Tetreault *et al.*, 2003, Colavecchia *et al.*, 2004). In a recent study by Bilodeau (2017), wood frog tadpoles collected near the Athabasca oil sands region had accumulated significant amounts of PAHs, and measured concentrations of some PAHs in the wetland sediment exceeded Canadians Interim Sediment Quality Guidelines (ISQG) for freshwater sediments. The dominant route of exposure for wood frog tadpoles was hypothesized to be from sediment exposure; however, surprisingly, wood frog tadpoles were efficient at eliminating PAHs as rapidly as 12 to 24 h after exposure (Bilodeau 2017). The Athabasca oil sands are an example of a major source of PAH exposure in the aquatic environment in Canada and it is apparent that amphibian species native to this region such as the wood frog may be affected by environmental exposure.

1.5.3 Photo-induced toxicity of PAHs in amphibians

The photo-induced toxicity of PAHs and petroleum products containing a mixture of PAHs has been studied in a wide range of aquatic organisms; however, there have been relatively

few studies assessing the photo-induced toxicity of PAHs to amphibians. Sensitive early life stage amphibians develop in shallow waters where they may be exposed to UV light at high intensities. Early life stage of certain amphibians (i.e., *Xenopus laevis*) have translucent skin, and the early life stages of all amphibians have permeable skin and gills, the capacity to assimilate chemicals, rapid rate of development, and lack of fully operational protective biological mechanisms. During their early life stages (tadpole stages), amphibians come into extensive contact with sediment for significant periods of time, exposing them to PAHs that adsorb onto the sediment and allow for bioaccumulation into tissues. An increased body burden of PAHs with subsequent swimming into areas of high intensities of UV could photosensitize the PAHs within the tissue and consequently lead to photo-enhanced toxicity. Limited studies have examined the effects of PAH exposure alone and in combination with UV with respect to standard acute toxicity measurements such as mortality, malformations and growth in amphibians. Similar to other aquatic organisms, UV light significantly enhanced the toxicity of certain PAHs in amphibians. Following 30 min and 5 h sunlight exposure, the LC_{50} values for anthracene in *Rana pipiens* tadpoles were 65 ug L^{-1} and 25 ug L^{-1}, respectively, highlighting a time-dependent increase in anthracene photo-induced toxicity (Kagan *et al.*, 1984). The LC_{50} values for anthracene, fluoranthene and pyrene were 110 ug L^{-1}, 90 ug L^{-1}, and 140 ug L^{-1} respectively, in late embryonic stages of *R. pipiens* following exposure to 30 min of sunlight (Devillers and Exbrayat, 1992). There was no mortality of newt embryos when exposed to 500 ug L^{-1} benzo(a)pyrene under fluorescent lighting; however, 100% mortality occurred when exposed to 12.5 ug L^{-1} benzo(a)pyrene and UV-A irradiation (Fernandez and l'haridon, 1994). Fluoranthene, in the presence of full sunlight and partial laboratory UV, enhanced mortality and malformations in *R. pipiens* and *X. laevis* (Hatch and Burton, 1998). *X. laevis* were also sensitive to UV exposure alone without fluoranthene, indicating possible interspecies differences in sensitivity to UV light.

The limited studies that assessed the acute photo-induced effects of PAHs in amphibians have reported similar results as other aquatic organisms, with an increase in mortality and malformations observed. From an ecotoxicological perspective, aquatic organisms, including early life stages of amphibians, are rarely exposed to only one stressor in the environment. Multiple stressors interact synergistically to increase the toxicity of contaminants to early life stages of amphibians (Kiesecker and Blaustein, 1995; Carey and Bryant, 1995; Zaga *et al.*, 2009;

Monson *et al.,* 2009). UV radiation is an environmental stressor of increasing concern due to the thinning of the atmospheric ozone layer (Rowland, 2006; Bais *et al.*, 2015). Specifically, increased levels of UV-B radiation have been linked to amphibian population declines (Blaustein *et al.*, 1994; Blaustein and Kiesecker, 1997; Alton and Franklin, 2017). A number of stressors have been identified as important contributors to the global amphibian population decline and recent studies have shown that these stressors can interact to enhance the toxicity of contaminants to developing amphibians. These studies highlight the importance of evaluating multiple stressors when assessing the toxicity of contaminants to amphibians and contributing to the overall studying of amphibian decline.

1.5.4 Species-specific differences in toxicity

To date, a large proportion of studies examining the effects of contaminants on amphibians has been performed under laboratory conditions with model species, such as *Xenopus laevis* (African clawed frog). It is reasonable to hypothesize that there may be important species differences in sensitivity to contaminants such as PAHs. Two amphibian species, *Xenopus laevis (Xenopus)* and *Lithobates sylvaticus* (wood frog) were chosen to evaluate the photo-induced toxicity of individual PAHs and an environmentally relevant PAH mixture (UNIVIS HVI 13). *Xenopus* was chosen as a test organism due to its availability, ease of use in the laboratory, and abundance in the literature as the model amphibian species. The wood frog was chosen as a test organism because it's widely distributed across Canada and is an ecologically relevant vertebrate species to be exposed to UNIVIS HVI 13. Early life stages of amphibian species in the aquatic environment may become exposed to the hydraulic oil through spring runoff or contaminated groundwater. The timing of exposure to the hydraulic oil via spring runoff may pose a significant risk to sensitive embryonic and larval stages of amphibians, especially early breeding species. Life stage can be an important factor when assessing photo-induced toxicity, especially organisms with translucent early-life stages such as *Xenopus* larvae. Despite previous beliefs, it has been found that in clear, shallow aquatic systems, all wavelengths of solar radiation can penetrate to the bottom of the water column (Landrum *et al.,* 1987). This is ecologically significant, as embryo and larval amphibians develop at various stages in shallow water and may be simultaneously exposed to both PAH and UV light.

Stage matching between *Xenopus* and the wood frog has been described to allow for direct comparisons of endpoints at specific stages of development during respective tests

(McDiarmid and Altig, 1999). Compared to native species such as the wood frog, *Xenopus* have significant differences in their genetics, life history, physical characteristics, and relative tolerance to environmental contaminants (McDiarmid and Mitchell, 2000). Compared with *R. pipiens*, *X. laevis* were sensitive to UV exposure with and without PAHs (Hatch and Burton, 1998), indicating *X. laevis* may be more sensitive to UV than other amphibians. PAH body burden is also an important consideration for photo-induced toxicity as PAH photo-induced toxicity depends on the amount of PAH accumulated into the organism. PAHs are nonpolar lipophilic compounds that concentrate in lipid-rich regions within organisms, and their lipophilicity increases with increasing molecular weight. Body burden is defined as the amount of a chemical present in an organism at a given point (Willis and Oris, 2014). Photo-induced toxicity within an organism is dependent on how much PAH is accumulated by that organism; therefore, PAH body burden is a useful endpoint for predicting and assessing the photo-induced toxicity of PAHs to aquatic organisms. A decrease in photo-induced toxicity was directly related to *Lepomis macrochirus* body burden of parent anthracene (Oris and Giesy, 1986), and the rapid elimination rate of anthracene by *Daphnia pulex* dramatically decreased the acute toxicity (Leversee *et al.*, 1981; Newsted and Giesy, 1987). To date, there are no studies examining the species-specific differences in toxicokinetics of PAHs in amphibians and influence on photo-induced toxicity. Recent research indicates that wood frog tadpoles rapidly uptake parent and alkylated PAHs and are efficient at metabolizing and eliminating these compounds (Bilodeau, 2017) while other studies demonstrate that *Xenopus* tadpoles rapidly uptake, metabolize and eliminate a wide range of chemical contaminants (Edginton and Rouleau, 2005, Fini Jean-Baptiste *et al.*, 2011; Ose *et al.*, 2017). Species-specific differences in uptake may lead to differences in photo-induced toxicity between larval *Xenopus* and wood frog.

1.6 Purpose of research

Individual PAHs and complex mixtures containing PAHs pose significant risk to aquatic organisms, particularly if exposure is coupled with other environmental stressors such as UV light. One complex oil-based mixture, UNIVIS HVI 13 hydraulic oil, is leaking from actuator valves at compressor stations along a major pipeline in Canada. Due to the concern of oil reaching nearby aquatic environments, the first goal of this research was to assess the hazard of the water-soluble constituents of UNIVIS HVI 13 to a wide range of aquatic organisms, and to

compare the toxicities of fresh (UNIVIS-F) and aged (UNIVIS-A) oil in the model organisms *X. laevis* and *D. magna.* The second goal of this research was to further examine the potential photo-induced toxicity of UNIVIS HVI 13 as well as individual PAHs using early life stage exposures to two amphibian species, *X. laevis* and the wood frog. If petroleum mixtures are phototoxic due to PAH constituents, risk assessments may be underestimating adverse effects resulting from oil spill contamination. There is also limited data concerning the photo-induced toxicity of PAHs and petroleum products in amphibian species, and it is unknown whether the model species *X. laevis* is a good predictor of the photo-induced toxicity of PAHs for a native species of anuran, the wood frog. To this end, the relationship between PAH body burden and photo-induced toxicity in amphibians was assessed to determine if differences in uptake between species resulted in differences in toxicity.

1.7 Objectives of research and associated hypotheses

1) Assess and compare the toxicity of fresh UNIVIS HVI 13 WAF (UNIVIS-F) by calculating effective concentration (EC) values for *Vibrio fischeri* (Microtox® test), *Daphnia magna* (invertebrate), *Lemna minor* (plant), *Pimephales promelas* (fish), *Xenopus laevis* and *Lithobates sylvaticus* (amphibians) (Chapter 2).

   H_0: There are no differences in toxicity across trophic levels of tested aquatic organisms.

2) Determine if aged oil (UNIVIS-A) differs in toxicity to fresh oil (UNIVIS-F) (Chapter 2).

   H_0: There are no differences in toxicity between UNIVIS-A and UNIVIS-F.

3) Determine if UV light enhances the toxicity of benzo(a)pyrene, anthracene, naphthalene or UNIVIS HVI 13 WAF in larval *Xenopus* and wood frog using mortality, morphometrics, body burden and deformities (Chapter 3).

   H_0: UV light does not enhance the toxicity of benzo(a)pyrene, anthracene, naphthalene or UNIVIS HVI 13 WAF in larval Xenopus and wood frog.

   H_0: There are no differences in sensitivity to the photo-induced toxicity of PAHs between Xenopus and wood frog.

4) Determine if PAH body burden at 8 h correlates with *Xenopus* and wood frog mortality at 96 h (Chapter 3).

   H_0: There is no correlation between PAH body burden at 8 h and Xenopus and wood frog mortality at 96 h.

5) Determine if species-specific differences in body burden correlate with species-specific differences in photo-induced toxicity in *Xenopus* and wood frog (Chapter 3).

   H_0: There is no correlation between species-specific differences in body burden and species-specific differences in photo-induced toxicity in Xenopus and wood frog.

6) Compare results of *Xenopus* and wood frog toxicity tests to determine if the model amphibian species *Xenopus* is a good predictor of PAH photo-induced toxicity in a non-model native species of amphibian, the wood frog (Chapter 3).

   H_0: The model amphibian species *Xenopus* is not a good predictor of PAH photo-induced toxicity in non-model native species of amphibian, the wood frog,

CHAPTER 2

TOXICITY OF THE WATER ACCOMMODATED FRACTION OF A PETROLEUM-BASED HYDRAULIC OIL TO AQUATIC ORGANISMS

This study assessed the acute toxicity of an environmentally relevant petroleum-based hydraulic oil (UNIVIS HVI 13) to aquatic organisms. UNIVIS hydraulic oil was identified to be leaking from actuator valves along pipelines and staining the surrounding soil. The toxicity of UNIVIS HVI 13 is uncertain and with concern of UNIVIS HVI 13 reaching aquatic ecosystems, the purpose of this study was to assess the hazard of UNIVIS HVI 13 to aquatic organisms. Due to the known concentrations of PAHs in the oil, the acute toxicity assessment of UNIVIS HVI 13 continued into Chapter 3, where a UV radiation component was added to assess the photo-induced toxicity of the oil mixture and three individual PAHs to two amphibian species. This chapter is being prepared as a manuscript for submission to *Environmental Toxicology and Chemistry* under joint authorship with Landon McPhee, Laura Halyk, Tara Stang, Dana Tkatchuk, Steven D. Siciliano and Natacha S. Hogan. Zac Currie conducted 80 % of the animal husbandry, exposure assays and related data collection (*Daphnia*, duckweed, and fathead minnow bioassays with UNIVIS-F, and *Daphnia* and *Xenopus* bioassays with UNIVIS-A) while Landon McPhee conducted the Microtox®, wood frog and *Xenopus* exposures with UNIVIS-F. Laura Halyk, Tara Stang, and Dana Tkatchuk aided with exposures and data collection. Zac Currie conducted 100 % of the data analysis and manuscript preparation. Dr. Steven D. Siciliano contributed by providing expertise, commented on and edited the manuscript. Dr. Natacha Hogan provided scientific input and guidance, commented on and edited the manuscript, and provided funding for the research.

2.1 Introduction

The growing global demand for oil and gas requires vast pipeline networks to transport the material across diverse ecological regions. The environmental impact of pipelines is of increasing concern with potential for leaks, ruptures and spills associated with both the pipe itself and the above-ground infrastructure used to regulate and maintain flow (e.g., compressor stations) (Herdan, 1997). Compressor stations are located at intervals along a pipeline and function to pressurize oil and gas as it travels along the pipeline. Actuator valves found within compressor stations are automated to perform several open-close cycles daily, 365 days/year. In order to maintain their critical function, actuator valves use hydraulic oils as a lubricant and there are often multiple valves at one compressor station site (Intrinsik, 2011).

Hydraulic oils are derived from the vacuum distillation of crude oils. The carbon number of hydraulic oils usually ranges between C15-C50, with the viscosity of the fluid increasing as the number of C atoms increases. Base oils are produced through multiple distillation processes of crude oil to produce base oil fractions that subsequently undergo a series of processes to improve performance. The toxicity of the hydrocarbons that form the basis of these hydraulic oils is influenced by the degree of refining and extracting (American Petroleum Institute, 2011), however, these base stocks are further modified by mixing with 10-25% chemical additives (e.g., barium and zinc phenolates, chromium salts, selenides, borax, etc.) to produce commercial hydraulic oils. These additives are necessary to impart desired properties for specific applications including extreme pressure additives, corrosion and oxidation inhibitors, viscosity index improvers, and demulsifying agents (Speight and Arjoon, 2012). A type of hydraulic oil commonly used on actuator valves at compressor stations along pipelines is UNIVIS HVI 13, a petroleum-based hydraulic oil manufactured by Exxon with petroleum distillates, hydro-treated light naphthenics and proprietary additives. There are reports of UNIVIS HVI 13 leaking from actuator valves and staining the surrounding soil, which could potentially impact nearby water bodies via spring runoff or groundwater migration.

Hydraulic oils such as UNIVIS HVI 13 are complex mixtures, and through their use and discharge onto landscapes, have become potential environmental contaminants due to their tendency to bioaccumulate and have adverse effects on plants, fish and wildlife (Herdan, 1997). The environmental hazard of hydraulic oils is not determined solely by their base fluids; the additives used may also contribute to toxicity (Herdan, 1997).

The critical exposure pathways for hydraulic oil that may lead to aquatic risk include spring runoff or high rain events that will wash hydraulic oil into nearby aquatic ecosystems, or groundwater migration that will move hydraulic oil into nearby aquatic ecosystems. Melt water runoff can carry oil that accumulated in the snowpack over winter, as well as erode contaminated surficial soil into waterways. Concurrent rainfall could also magnify the volume and force of a spring runoff event resulting in extreme pollutant loads (Oberts, 1994). The timing of hydraulic oil exposure via this pathway may pose a significant risk to sensitive embryonic and larval stages of aquatic organisms, especially early breeding species. Similarly, the water-soluble fraction of hydraulic oil, containing hydrocarbons, could migrate downward through the soil by percolation of water, resulting in contaminated ground water that could ultimately feed into surface water.

There is currently no published toxicological data on UNIVIS HVI 13 to aquatic organisms, and there are limited comparative studies on the acute toxicity of petroleum-based hydraulic oils to aquatic organisms. It is also unknown whether commercial fresh UNIVIS HVI 13 differs in toxicity to aged UNIVIS HVI 13 found leaking from actuator valves at pipeline compressor stations. It is likely that aged oil will differ in toxic potency compared to fresh oil due to increased concentration of constituents (i.e., sulfur, zinc, PAHs) due to oxidative degradation and mechano-chemical reactions associated with aged oils.

Due to the concern of UNIVIS HVI 13 hydraulic oil reaching aquatic ecosystems, the purpose of this study was to assess the hazard of UNIVIS HVI 13 to aquatic organisms, and compare fresh and aged UNIVIS HVI 13 to determine if differences in oil type results in differences in toxicity. Acute toxicity of fresh and aged UNIVIS HVI 13 was assessed for protists (Microtox® test), invertebrates (*Daphnia magna*), plants (*Lemna minor*), as well as early life stages of fish (*Pimephales promelas*), and amphibians (*Xenopus laevis* and *Lithobates sylvaticus*). Test species were selected to represent a wide range of trophic levels and were chosen on the basis of their ecological relevance and availability of standard tests with known sensitivity and reproducibility.

2.2 Materials and Methods

2.2.1 Test substance and WAF preparation

Exxon Mobil UNIVIS HVI 13 hydraulic oil (Product code: 201560109720, 431007-00, 97Q043) was purchased from Marsollier Petroleum, Ltd. (Saskatoon, SK). This product is fresh

oil and is hereafter referred to as fresh UNIVIS (UNIVIS-F). UNIVIS HVI 13 was also taken from a compressor station site in Winnipeg, MB during a routine maintenance and oil change, collected in a 20 L opaque pail and shipped immediately to the University of Saskatchewan. This product is aged oil and is hereafter referred to as aged UNIVIS (UNIVIS-A).

Toxicity tests in which aquatic organisms are exposed to complex mixtures containing petroleum hydrocarbons are often conducted using water accommodated fractions (WAF) as this exposes the organisms to the water-soluble constituents representative of an exposure in an aquatic environment. The WAF of UNIVIS-F and UNIVIS-A were prepared according to established guidelines (Singer *et al.,* 2000) with some modifications. Briefly, 1 L of UNIVIS HVI 13 and 1 L of water were added to a 2 L glass aspirator bottle to achieve oil to water ratio of 1:1 and then sealed with aluminum foil to prevent evaporation. For WAF preparation, Hutner's negative growth media was used for the *Lemna minor* toxicity test while Microtox® reagent was used for the Microtox® test. For all other WAF preparations, exposure solutions were made with aerated de-chlorinated facility water from the Aquatic Toxicology Research Facility (ATRF), Toxicology Centre, University of Saskatchewan. The WAF mixture was gently stirred at low energy (no vortex) using a magnetic stirrer for 24 h in the dark and allowed to settle for one hour (Singer *et al.,* 2000). The resulting water-soluble fraction was then decanted into a 1 L glass bottle and serial dilutions were performed on the 1:1 (880 g L^{-1}) soluble fraction to achieve test concentrations of 440, 220, 110, 55, and 28 g L^{-1}. Nominal WAF loading test concentrations were calculated based on the density of 0.88 for UNIVIS HVI 13. For all toxicity tests, UNIVIS-F and UNIVIS-A were prepared fresh to coincide with respective exposure. Water quality values for pH, dissolved oxygen, conductivity, and temperature for the test solutions were measured at the completion of each toxicity test using a HI9829 multi-parameter water quality meter (Canadawide Scientific Ltd, Ottawa, ON). Ammonia levels were also measured at the completion of each toxicity test using API ammonia test strips (API Fishcare).

2.2.2 Chemical analysis

Samples (500 mL) were taken from 1:1 loadings of UNIVIS-F and UNIVIS-A WAF and shipped on ice and in the dark to Maxxam Analytics (Calgary, AB) for chemical analysis. Polycyclic aromatic hydrocarbons (PAHs), benzene, toluene, ethylbenzene and xylene (BTEX), F1-F4 hydrocarbon fractions and Canadian Council of Ministers of the Environment (CCME)-regulated metals were analyzed from WAF loadings of UNIVIS-F and UNIVIS-A. PAHs were

analyzed in water by gas chromatography/mass spectrometry (GC/MS), BTEX and F1-F4 hydrocarbons were analyzed in water by headspace gas chromatography/mass spectrometry with flame ionization detector (HS GC/MS/FID), and metals were analyzed by inductively coupled plasma mass spectrometry (ICP-MS). Limit of detection for each compound is reported in Appendices A and B.

2.2.3 Toxicity tests

Toxicity values were expressed as g L^{-1} derived from serial dilution of 1:1 (880 g L^{-1}) UNIVIS-F and UNIVIS-A WAF preparation. Nominal test concentrations for all toxicity tests were 880, 440, 220, 110, 55 and 28 g L^{-1} WAF respectively and aerated de-chlorinated municipal water was used as the control for each test. Toxicity tests for *V. fischeri, D. magna, P. promelas, X. laevis* and *L. sylvaticus* were conducted at the Toxicology Centre at the University of Saskatchewan, Saskatoon, Saskatchewan, Canada using standardized protocols as described by the Organisation for Economic Cooperation (OECD). Toxicity tests for *L. minor* were conducted in the Controlled Environmental Facility (Phytotron) of the Agriculture Building, University of Saskatchewan, Saskatoon, Saskatchewan, Canada.

The Microtox test for acute toxicity is based on inhibition of luminescence generated by the marine, gram-negative bacterium *Vibrio fischeri*. The Microtox analysis was performed on a M500 Toxicity Analyzer (Modern Water Inc, DE, USA) and using the Basic Test procedure provided by Azur Environmental (Azur Environmental, 1992). A six-concentration serial dilution with three replicates was set up for each type of WAF (fresh and aged), and two exposures were conducted per WAF. Effective concentrations resulting in half maximal response (EC_{50}) values were calculated as the concentration of WAF required to reduce bioluminescence by 50% after 5 min exposure, relative to the control. Positive control tests were conducted using phenanthrene, with an EC_{50} of 203.9 μg L^{-1} (Azur Environmental, 1992). Data were analyzed with Microtox Omni software.

Two independent *D. magna* (daphnia) acute immobilization tests (48 h) were conducted with the WAF of UNIVIS HVI 13. During the preliminary tests with UNIVIS-F and UNIVIS-A, the *D. magna* appeared to become trapped in an oily film that was present on the surface of the water. The *D. magna* had decreased mobility and were clumping together. This was observed in all treatment groups, but not control groups at 24 and 48-h. For this reason, the test was repeated using a WAF that underwent two decants during the WAF preparation. Following the

standardized WAF preparation as described above, the WAF was allowed to settle again for 1 h before a second decant was performed. During subsequent exposures, no surface-trapping effect was observed. The *D. magna* 48 h acute immobilization tests were performed with neonates <24 h old. Static exposures were carried out in 100 mL beakers consisting of 80 mL test solution, ten neonates per beaker and three replicates per treatment and six treatments of descending WAF concentrations as outlined above. Experimental units were placed in an environmental chamber at 20 ± 2°C with a 16/8 light/dark photoperiod. Mortality was observed at 24 and 48 h following test initiation.

P. promelas (fathead minnow) embryos were obtained from breeding of an on-going healthy adult culture in the Aquatic Toxicology Research Facility (ATRF) at the Toxicology Centre, University of Saskatchewan. Culture tanks were maintained at 25 ± 1°C with a 16/8 h light/dark photoperiod. Minnows were fed frozen bloodworms to satiety twice daily. Exposures of embryos to WAF concentrations were conducted according to He *et al.*, (2012). Briefly, exposures were performed in 6 well polystyrene microplates (Fisher Scientific) consisting of 4 mL test solution, four embryos per well, and four replicates per treatment. *P. promelas* embryos were exposed to WAF test concentrations < 24 h post fertilization for 96 h and mortality was observed at 24, 48, 72 and 96 h. Exposures were performed at 25 °C with a 16/8 day/night photoperiod, and 50% renewal of test solution was performed daily.

A 7-day growth inhibition test using *L. minor* (duckweed) was conducted as described by OECD (2002), with a daily static renewal procedure as described by Brain and Solomon (2007). Disposable polystyrene petri dishes (60 x 15 mm) (Fisherbrand, Ottawa, Canada) were used as experimental units. Seven days before test initiation, 20-40 healthy plants were transferred from the stock culture into a 2,800 mL flask containing 1000 mL Hutner's negative growth media, which was prepared as described by Brain and Solomon (2007). The test culture was decanted into a sterile tray in a laminar flow hood. A 20 mL aliquot of each test solution, containing Hutner's media spiked with the appropriate test concentration, was pipetted into each petri dish. Using a sterilized fork, two plants were transferred into each experimental unit. There were three replicates per treatment for a total of 21 experimental units. The experimental units were placed in an environmental growth chamber at 25 ± 0.2°C with an average 7-day light intensity of 625 ± 8 μmol m^{-2} s^{-1}. Exposure media was renewed daily with all procedures carried out in a laminar

flow hood to avoid contamination. Frond counts were performed daily and following the 7-day exposure, growth rate and fresh weight were evaluated.

Amphibian species for early life stage amphibian toxicity testing were chosen to include a standard test species (*Xenopus laevis*, African clawed frog) as well as an ecologically relevant species (*Lithobates sylvaticus*, wood frog*). X. laevis* embryos were obtained through breeding of male and female pairs from an adult *X. laevis* colony established in the ATRF at the University of Saskatchewan. Adults were maintained in a Min-O-Cool tank (48" L X 24" W X 20" D) at a 12:12 photoperiod and 16 ± 1 °C. They were fed frog brittle (Nasco Frog Brittle (Medium Nuggets)) ad libitum three times weekly. Use of these animals was approved by University Committee on Animal Care and Supply Animal Research Ethics Board at the University of Saskatchewan and followed Canadian Council of Animal Care guidelines. Breeding pairs were acclimated (males and females separately) to 21 ± l °C by increasing the temperature by 1 °C per day and then injected with a 25 IU priming dose of human chorionic gonadotropin (hCG) dissolved in sterilized phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4). A second dose of hCG was administered approximately 24 hours later (males 250 IU hCG; females d500 IU (ASTM, 2012). Breeding pairs were placed together into aquaria with water temperature maintained at 21 ± 1 °C, and left to spawn overnight. Adults were removed, embryos collected, and normally cleaving embryos selected for use in the experiment. *L. sylvaticus* egg masses were collected from a local breeding site located at Cranberry Flats Conservation Area flood plain (52.0311° N, 106.7004° W) near Saskatoon, SK. Cranberry Flats is a marshy wetland that connects to the South Saskatchewan River during the spring snowmelt runoff season. Eggs were transported to the University of Saskatchewan and maintained in multiple 20 L glass stock aquaria filled with facility water and raised to Gosner stage 26 (Gosner, 1960). *L. sylvaticus* tadpoles were randomly selected for exposures at stage 26 while exposures with *X. laevis* were initiated within 24 h of fertilization (Nieuwkoop and Faber, 1994). For both species, ten individuals were assigned to each beaker containing 100 mL WAF exposure solution with four replicates per test concentration. Exposures were conducted for 96 h at 17 ± 0.5 °C *(L. sylvaticus)* or 21 ± 0.5 °C *(X. laevis)* and 16:8 h light–dark cycle with a 75 % daily renewal of test solutions. Mortality was recorded daily and malformations and morphometrics (total length, tail length, snout-vent length) were evaluated at 96 h. Surviving individuals were euthanized with buffered MS-222 (ethyl 3-aminobenzoate methanesulfonate;

700 mg L^{-1}). Total length, snout-vent length and malformations were evaluated under using an Olympus model S261 dissecting microscope with Image-Pro Discovery Software.

2.2.4 Analysis of data

Effective median concentration (EC_{50}), as well as the concentrations required for a 10, 25 and 90% reduction in the each of the measured endpoints were calculated in R v3.1.2 (R Core Team, 2017) via non-linear regression. The estimated toxicity values were derived using nominal values from serial dilution of the 1:1 WAF as well as for actual measured concentrations of specific hydrocarbon fractions (i.e., F1-F3 hydrocarbons). Non-linear regression was applied to each end point using a three-parameter log-logistic model (LL.3) with the *drc* package in R (Ritz and Streibig, 2005). Maximum and minimum were fixed to specific values for the data (i.e., 0 and 100 for percent mortality) in order to simplify the model. The 5 minute EC_{50} value for the Microtox assay was calculated using probit analysis in SPSS Statistics v19.0 (IBM Corporation, Armonk, NY). No Observed Effect Concentration (NOEC) and Lowest Observed Effect Concentration were estimated via ANOVA (α=0.05) in SPSS. One-way ANOVA with Tukey's post hoc test was used to determine significance between the treatment and control groups (α = 0.05). Levene's test was used to test for equality of error variances and residual plots were assessed to test for goodness of fit.

2.3 Results

2.3.1 Water quality

Mean values ± standard deviation for pH, dissolved oxygen (%), dissolved oxygen (mg L^{-1}), conductivity (µS/cm), and temperature (°C) of WAF test solutions at completion of toxicity tests were 7.27 ± 0.43, 90.30 ± 4.00, 7.73 ± 0.52, 440.00 ± 15.40, and 20.40 ± 1.69 respectively. Ammonia levels in toxicity tests were <0.25 ppm for all tests conducted.

2.3.2 Chemical analysis

Results for chemical analysis of petroleum hydrocarbon fractions, PAHs, and total metals in 1:1 UNIVIS-F WAF and 1:1 UNIVIS-A WAF are outlined in Table 2.1 and Appendices A-B respectively. Concentrations indicated with "<" represent concentrations below the reportable detection limits for each respective analysis. UNIVIS-A WAF had 1063, 28,700, 21,700 and 260

μg L^{-1} of F1, F2, F3, and F4 total hydrocarbon fractions respectively, while UNIVIS-F had 507, 2,180, 3,000, and <200 μg L^{-1} of F1, F2, F3, and F4 total hydrocarbon fractions respectively (Table 2.1). UNIVIS-A had increased concentrations of semi-volatile organics including acenaphthylene, acridine, fluorene and phenanthrene (Appendix A), and increased concentrations of metals including sodium, sulphur and zinc (Appendix B) compared to UNIVIS-F.

Table 2.1. Average chemical analysis (n=3) of total petroleum hydrocarbon fractions (F1-F4) in 1:1 loadings of fresh and aged UNIVIS HVI 13 WAF.

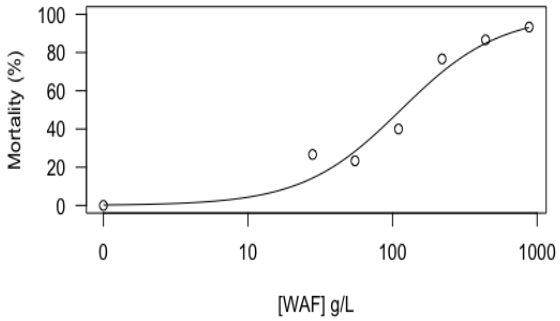
Petroleum hydrocarbon fraction (concentration)	Oil type	
	1:1 Fresh	**1:1 Aged**
F1 ($\mu g\ L^{-1}$)	507	1063
F2 ($\mu g\ L^{-1}$)	2,180	28,700
F3 ($\mu g\ L^{-1}$)	3,000	21,700
F4 ($\mu g\ L^{-1}$)	<200	260

2.3.3 Toxicity tests

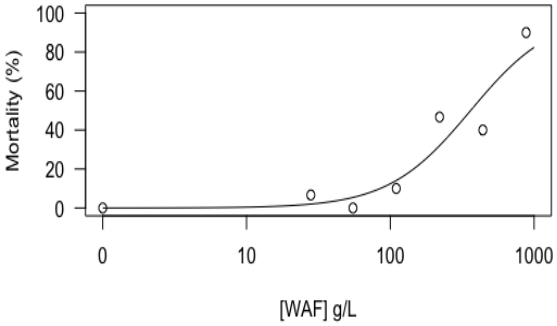
Dose response curves for *D. magna, V. fischeri, L. minor, and P. promelas* exposed to UNIVIS-F WAF and *D. magna* and *X. laevis* exposed UNIVIS-A WAF are shown in Figure 2.1. Estimated toxicity values for UNIVIS-F WAF and UNIVIS-A WAF for all tested organisms are summarized in Table 2.2. Fitting parameters and associated EC standard errors for each endpoint are shown in Appendix C. Estimated toxicity values for hydrocarbon fractions (F1-F3) are summarized in Table 2.3 and were calculated based on respective toxicity values from Table 2.2, total WAF concentration (i.e., 880), and measured analytical concentrations of hydrocarbon fractions in the respective UNIVIS-F and UNIVIS-A WAF. Based on EC_{50} values, the most sensitive species was *D. magna* with an EC_{50} value for mortality of 114 g L^{-1} UNIVIS-F WAF and 66 μg L^{-1} F1 hydrocarbon fraction. Based on NOEC and LOEC values, the most sensitive species was *L. minor* with a NOEC value of 0 g L^{-1} and a LOEC value of 28 g L^{-1}. The EC_{50} values of UNIVIS-F WAF in *X. laevis, P. promelas* and *L. sylvaticus* were greater than the highest tested concentration used in the associated test (Table 2.2). The lowest EC_{10} values estimated for measured F1, F2, and F3 hydrocarbon fractions were 1, 13, and 10 μg L^{-1} UNIVIS-A WAF respectively for *X. laevis* (Table 2.3).

Acute toxicity was assessed for UNIVIS-A in order to compare differences in toxicity of water-soluble constituents in UNIVIS-F and UNIVIS-A. Standardized toxicity tests using *X. laevis* and *D. magna* were chosen for this comparison. The EC_{50} value for *D. magna* exposed to UNIVIS-A was 362 g L^{-1} while the EC_{50} value for *D. magna* exposed to UNIVIS-F was 114 g L^{-1} (Table 2.2). The EC_{50} value for *X. laevis* exposed to UNIVIS-A was 373 g L^{-1} while the EC_{50} value for *X. laevis* exposed to UNIVIS-F was > 880 g L^{-1} (Table 2.2). Total length, tail length and snout-vent length were significantly decreased in *X. laevis* embryo-larvae exposed to 220, 440, and 880 g L^{-1} UNIVIS-F WAF, while exposure to UNIVIS-A WAF significantly decreased *X. laevis* total length and tail length, as well as significantly increased the incidence of malformations at the highest concentration tested (880 g L^{-1}).

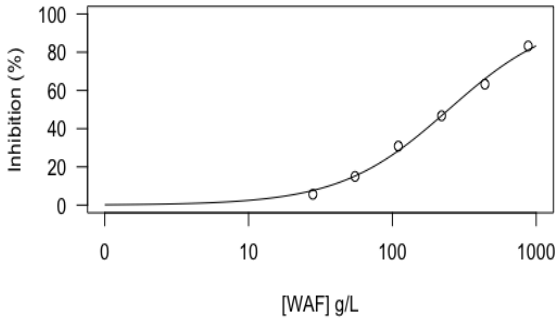
A) *D. magna* (UNIVIS-F)



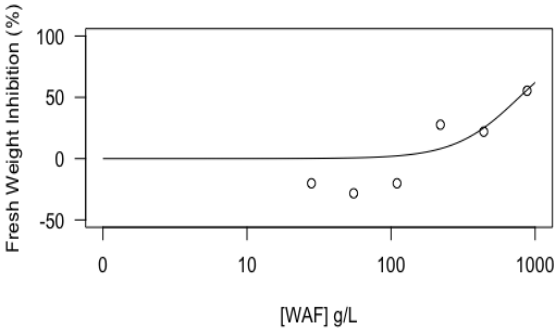
B) *D. magna* (UNIVIS-A)



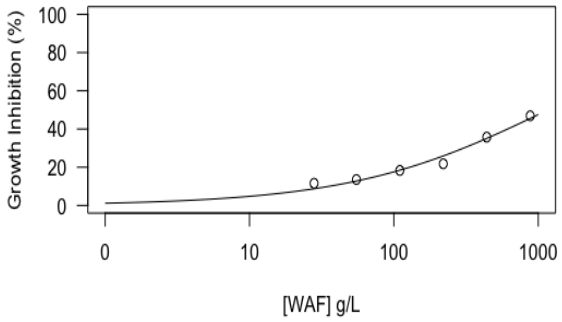
C) *V. fischeri* (UNIVIS-F)



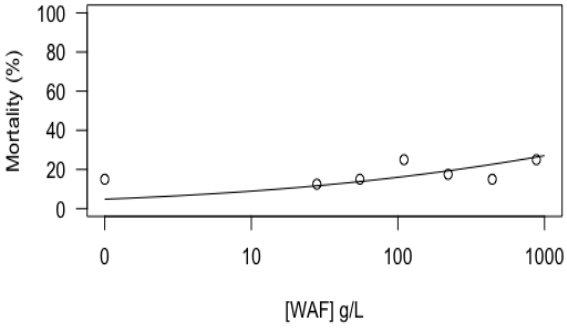
D) *L. minor* (UNIVIS-F)



E) *L. minor* (UNIVIS-F)



F) *P. promelas* (UNIVIS-F)



G) *X. laevis* (UNIVIS-A)

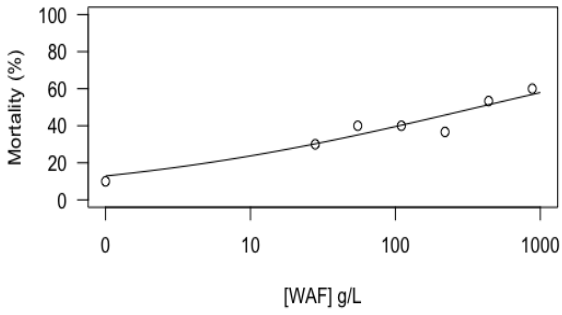


Figure 2.1. Dose response curves for UNIVIS HVI 13 toxicity tests.

Table 2.2. Summary of the estimated toxicity values including no observed effect concentration (NOEC) and lowest observed effect concentration (LOEC) values for the water-accommodated fraction (WAF) of UNIVIS HVI 13 (fresh and aged specific for each test) based on serial dilution of the 1:1 (880 g L^{-1}) WAF. The ">" indicates toxicity tests in which effective concentrations (EC) were unable to be calculated due to the lack of response (i.e., lack of mortality).

Species and duration of test	Oil type	Endpoint	NOEC	LOEC	EC values in g L^{-1} (upper and lower 95% CI)			
					EC10	EC25	EC50	EC90
D. magna (48 h)	Fresh	Mortality	55	110	20 (3.3 – 37)	48 (23 – 72)	114 (78 – 150)	646 (175 – 880)
D. magna (48 h)	Aged	Mortality	110	220	85 (40 – 130)	175 (117 – 234)	362 (274 – 450)	>880
V. fischeri (5 min)	Fresh	Bacterial counts	28	55	36 (26 – 47)	94 (77 – 110)	246 (216 – 276)	>880
L. minor (7 d)	Fresh	Growth rate	0	28	35 (18 – 52)	203 (162 – 245)	>880	>880
L. minor (7 d)	Fresh	Fresh weight	440	880	247 (0 – 502)	438 (158 – 720)	775 (323 – 880)	>880
P. promelas (96h)	Fresh	Mortality	880	>880	15 (0 – 45)	692 (19 – 880)	>880	>880
L. sylvaticus. (96 h)	Fresh	Mortality	880	>880	>880	>880	>880	>880
X. laevis (96 h)	Fresh	Mortality	880	>880	>880	>880	>880	>880
X. laevis (96 h)	Aged	Mortality	440	880	0.40 (0 – 2.6)	12 (0 – 42)	372 (0 – 809)	>880

Table 2.3. Summary of the estimated toxicity values for the hydrocarbon fractions of the WAF of UNIVIS HVI 13 (fresh and aged specific for each test) based on measured concentrations of petroleum hydrocarbon fractions (F1-F3) and serial dilution of the 1:1 (880 g L^{-1}) WAF. Upper and lower 95% confidence intervals are indicated in brackets.

Species and duration of test	Oil type	Endpoint	Effective concentration	Petroleum hydrocarbon fraction		
				F1 (µg L^{-1})	F2 (µg L^{-1})	F3 (µg L^{-1})
D. magna (48 h)	Fresh	Mortality	EC10	11 (2 – 21)	50 (8 – 92)	68 (11 – 126)
			EC25	28 (13 – 41)	119 (57 – 178)	164 (78 – 245)
			EC50	66 (45 – 86)	282 (193 – 372)	389 (266 – 511)
D. magna (48 h)	Aged	Mortality	EC10	103 (48 - 157)	2772 (1305 – 4240)	2096 (986 – 3206)
			EC25	211 (141 - 283)	5707 (3816 – 7632)	4315 (2885 – 5770)
			EC50	437 (331 - 544)	11806 (8936 – 14676)	8927 (6757 – 11097)
V. fischeri (5 min)	Fresh	Bacterial counts	EC10	21 (15 – 27)	89 (64 – 116)	123 (89 – 160)
			EC25	54 (44 – 63)	233 (191 – 273)	320 (263 – 375)
			EC50	142 (124 – 159)	609 (535 – 684)	839 (736 – 941)
L. minor (7 d)	Fresh	Fresh weight	EC10	142 (0 – 289)	612 (0 – 1244)	842 (0 – 1711)
			EC25	252 (91 – 415)	1085 (391 – 1784)	1493 (539 – 2455)
			EC50	447 (186 – 507)	1920 (800 – 2180)	2642 (1101 – 3000)
X. laevis (96 h)	Aged	Mortality	EC10	1 (0 – 3)	13 (0 – 85)	10 (0 – 64)
			EC25	15 (0 – 51)	391 (0 – 1370)	296 (0 – 1036)
			EC50	449 (0 – 977)	12132 (0 – 26384)	9173 (0 – 19949)

Example calculation: (EC value from respective toxicity test in table 2.2 / total WAF concentration (880 g/L) x measured fraction concentration in table 2.1). For *D. magna* fresh WAF F1 EC_{50} = (114 / 880) x 507 = 66 µg L^{-1}.

2.4 Discussion

In this study, we evaluated the acute toxicity of the hydraulic oil UNIVIS HVI 13 to Microtox, *D. magna, L. minor,* and early life stages of *P. promelas, X. laevis* and *L. sylvaticus,* and compared the toxicity of WAFs prepared with fresh (UNIVIS-F) and aged (UNIVIS-A) oil. We also attempted to determine if differences in chemical concentrations between WAFs were responsible for toxicity. In general, toxicity of UNIVIS-F decreased from bacteria to vertebrates with minimal toxicity observed in larval amphibians and fish embryos. *X. laevis* embryos were more sensitive to UNIVIS-A compared to UNIVIS-F; however, *D. magna* were more sensitive to UNIVIS-F compared to UNIVIS-A.

The estimated EC values from the bioassays demonstrate that UNIVIS HVI 13 WAF is practically non-toxic (United Nations, 2011) to aquatic organisms, with all EC values > 0.1 g L^{-1}. The lowest estimated EC value was an EC_{10} of 0.4 g L^{-1} for UNIVIS-A in *X. laevis,* which is greater than the generic water quality guidelines (GWQG) of 0.0098 and 0.0013 g L^{-1} for protection of freshwater life from F1 and F2 in coarse soil from agricultural land use and commercial and industrial land use (Pott, 2012). To our knowledge, these are the first aquatic toxicity data for UNIVIS HVI 13 and demonstrate that the water-soluble constituents in the hydraulic oil, derived from a 1:1 loading of oil-to-water, exert minimal toxicity to aquatic organisms even at high loadings of WAF.

The difference in *X. laevis* sensitivity to oil type may be attributed to differences in constituents between UNIVIS-F and UNIVIS-A. UNIVIS-A was determined to have higher concentrations of F1-F4 hydrocarbon fractions, metals (i.e., zinc) and sulphur. The toxic potency of UNIVIS-A to *X. laevis* may be a result of increased concentrations of constituents (i.e., hydrocarbons, sulfur, zinc,) due to oxidative degradation and mechano-chemical reactions associated with aged oils. Specifically, UNIVIS-A contained approximately two times the amount of F1, approximately thirteen times the amount of F2, and approximately seven times the amount of F3 compared to UNIVIS-F. Although UNIVIS-A had higher concentrations of F2 and F3, the toxicity of UNIVIS-A to *X. laevis* is likely driven by the F1 fraction, as this fraction had the lowest estimated EC_{50} value of 449 μg L^{-1}. In addition, if we express the toxicity of both UNIVIS-F and UNIVIS-A to *X. laevis* on an F1 basis using the same equation used for Table 2.3, they have approximately the same toxicity (507 vs 449 μg L^{-1}). Conversely, the WAF of UNIVIS-F was more toxic to *D. magna* than the WAF of UNIVIS-A with an estimated EC_{50}

value of 114 g L^{-1} compared to 362 g L^{-1}. Interestingly, the increased sensitivity of *D. magna* to UNIVIS-F compared to UNIVIS-A cannot be attributed to higher concentrations of WAF constituents as UNIVIS-A had higher concentrations of F1-F4 hydrocarbons, metals and sulphur. This was surprising, as *D. magna* are known to be one of the most sensitive aquatic organisms to metals (Bianchini and Wood, 2008). However, total petroleum hydrocarbon concentrations have shown weak correlation to overall WAF toxicity to aquatic species, including *D. magna* (Blinova *et al.*, 2015), and some studies have observed similar or higher toxicities in WAFs with lower total petroleum hydrocarbons compared to WAFs with higher total petroleum hydrocarbons (Barron *et al.*, 1999). It is also possible that the observed toxicity of UNIVIS-F to *D. magna* was due to an unmeasured constituent (i.e., phenol) in the WAF. A more complete chemical analysis of both UNIVIS-F and UNIVIS-A may be more indicative of additional constituents to attribute observed toxicity. Overall, the increased toxicity of aged oil to the amphibian *X. laevis* does not appear to be reflective for the invertebrate *D. magna*, and the increased toxicity of fresh oil to *D. magna* does not appear to be reflective for the amphibian *X. laevis*. Further toxicity tests with UNIVIS-A in other aquatic organisms are required to fully examine the differences in toxicity between UNIVIS-F and UNIVIS-A.

The EC values for the WAF of UNIVIS HVI 13 were estimated to be > 0.1 g L^{-1}; however, the estimated EC values of the measured hydrocarbon fractions indicate that the F1, F2, F3 fractions range from very toxic (< 1 mg L^{-1}) to moderately toxic (1 mg L^{-1} to 10 mg L^{-1}) (United Nations, 2011). The F1-F3 hydrocarbon constituents of UNIVIS HVI 13 may pose a risk to aquatic organisms if hydrocarbons from contaminated soil on site reached groundwater and migrated to off-site surface water. The environmental fate of UNIVIS HVI 13 in contaminated soil is currently unknown. The probability that oil in contaminated soil reaches groundwater and migrates to off-site surface water depends on a number of factors including vertical distance to groundwater, concentrations of chemicals of potential concern (COPC) in contaminated soil, degradation of COPC by ultraviolet radiation and soil microorganisms, and physical and chemical properties of the oil (Intrinsik, 2011; OMOE, 2011; Pott, 2012). Although the EC values for the specific hydrocarbon fractions are low and of concern if the hydraulic oil were to reach aquatic organisms via groundwater, it is important to note that the water solubility of UNIVIS HVI 13 is very low (i.e., < 0.1% in water), and the oil has a short half-life (EXXON, 1991). Therefore, based on the physical and chemical properties of UNIVIS HVI 13 the

probability of groundwater migration is expected to be low (Intrinsik, 2011). Further research into the environmental fate of the oil in soil will provide a better understanding of the likelihood that the oil will reach off-site surface waters with potential to impact aquatic biota.

This study examined the acute toxicity of UNIVIS HVI 13 and did not consider the chronic effects of the oil on aquatic organisms. The increase in toxicity of UNIVIS-A compared to UNIVIS-F in *X. laevis* is of concern since the aged oil is continually released in pulses from actuator valves at compressor stations. Future research is required to quantify the chronic toxicity of UNIVIS HVI 13 in aquatic organisms; however, due to the low acute toxicity of hydraulic oil observed in this study, the physical and chemical properties of the oil combined with the low probability of oil reaching surface waters via groundwater, the chronic toxicity of hydraulic oil is expected to be low.

Estimated EC_{50} values were used to compare sensitivities of species to UNIVIS HVI 13 in this study rather than NOEC and LOEC values because EC_{50} values were estimated from a dose response curve (i.e., seven test concentrations) rather than only being compared to the control. Therefore, based on EC_{50} values, *D. magna* was the most sensitive organism to UNIVIS HVI 13 with a EC_{50} value of 114 g L^{-1} UNIVIS-F WAF and results indicate that *X. laevis* is more sensitive to UNIVIS-A compared to UNIVIS-F, likely due to increased concentrations of hydrocarbons, metals and sulphur in UNIVIS-A. Estimated EC values for measured hydrocarbon fractions were lower than estimated EC values for UNIVIS-F WAF and UNIVIS-A WAF; however, due to the chemical and physical properties of UNIVIS HVI 13, the off-site risk to aquatic organisms is expected to be low. The toxicity of UNIVIS HVI 13 WAF varied across test organisms; however, based off the minimal toxicity of UNIVIS HVI 13 WAF to early life stages of fish and amphibians and the high WAF loading used to elicit toxicity in *Daphnia* and Microtox, we conclude that the water-soluble constituents of UNIVIS HVI 13 pose minimal hazard to aquatic organisms.

CHAPTER 3

PHOTO-INDUCED TOXICITY OF POLYCYCLIC AROMATIC HYDROCARBONS AND A PETROLEUM-BASED HYRAULIC OIL IN TWO AMPHIBIAN SPECIES

The goal of the research presented in Chapter 3 was to assess and compare the photo-induced toxicity of three individual PAHs and an environmentally relevant mixture of PAHs (UNIVIS HVI 13) in two amphibian species. The acute toxicity of UNIVIS HVI 13 to aquatic organisms was determined to be minimal in Chapter 2; however, UV radiation is known to substantially increase the acute toxicity of PAHs and petroleum-products containing PAHs. For this reason, a UV radiation component was included in thesis Chapter 3. This chapter is being prepared as a manuscript for submission to *Aquatic Toxicology* under joint authorship with Samantha Lundquist, Tara Stang, Steven D. Siciliano and Natacha S. Hogan. Zac Currie conducted 100 % of the animal husbandry, exposure assays and related data while Tara Stang and Samantha Lundquist aided with exposures and data collection. Zac Currie conducted 100 % of the data analysis and manuscript preparation. Dr. Steven D. Siciliano contributed by providing expertise on HPLC analyses of PAH body burden, commented on and edited the manuscript. Dr. Natacha Hogan provided scientific input and guidance, commented on and edited the manuscript, and provided funding for the research.

3.1 Introduction

The global amphibian population decline has been linked to a number of stressors including climate change and increased levels of contaminants in the aquatic environment (Hayes *et al.,* 2010). Aquatic organisms such as larval amphibians are rarely exposed to only one stressor in the environment, and chemical and environmental stressors can interact synergistically to increase the toxicity of contaminants in developing amphibians (Kiesecker and Blaustein, 1995; Carey and Bryant, 1995; Zaga *et al.,* 2009; Monson *et al.,* 2009). Ultraviolet (UV) radiation is an important environmental stressor of increasing concern due to the thinning of the atmospheric ozone layer (Rowland, 2006; Bais *et al.,* 2015). Increased levels of UV-B radiation in particular have been linked to amphibian population declines (Blaustein *et al.,* 1994; Blaustein and Kiesecker, 1997; Alton and Franklin, 2017). A number of stressors have contributed to the global amphibian decline and these stressors can interact within the aquatic environment to enhance the toxicity of contaminants to developing amphibians.

Polycyclic aromatic hydrocarbons (PAHs) are a class of organic molecules composed of multiple benzene rings that readily bioaccumulate in aquatic organisms due to their lipophilic nature. PAHs are ubiquitous environmental contaminants derived primarily from the incomplete combustion of organic matter. Although PAHs are found naturally in the environment, their environmental load has increased substantially since the onset of industrialization due to the increasing use of fossil fuels (Eisler, 1987). PAHs represent some of the most persistent and detrimental substances in the aquatic environment, and the United States Environmental Protection Agency (US EPA) has designated 16 PAH compounds as priority pollutants (US EPA, 2014). Generally, low molecular weight PAHs (2-3 rings), have greater acute toxicity and lower carcinogenic potential, whereas high molecular weight PAHs (4-7 rings) have lower acute toxicity and greater carcinogenic potential (Eisler, 1987; Landrum *et al.,* 1987). However, the acute toxicity observed with low molecular weight PAHs is generally at concentrations greater than the water solubility of the compound, presenting less of a risk in the aquatic environment due to low bioavailability (Nagpal, 1994).

Although PAHs are generally not acutely toxic within their aqueous solubility limits, their acute toxicity can increase substantially when exposed with ultraviolet (UV) radiation. Previous studies have shown that PAHs are acutely toxic to a wide range of aquatic organisms within the range of their aqueous solubility when they are simultaneously exposed to UV radiation

(Bowling *et al.,* 1983; Landrum *et al.,* 1987; Newsted *et al.,* 1987; Greenberg *et al.,* 1993*;* Ankley *et al.,* 1995). Due to their aromatic structure, PAHs can absorb UV-A (315-400 nm) and UV-B (280-315 nm) readily; however, there is only a subset of PAHs with particular structural and chemical properties that have the ability to cause photo-induced toxicity. The photo-induced toxicity of PAHs is dependent on several factors including the amount of PAH that is accumulated by an organism, the intensity of UV radiation, the amount of light penetrating the aquatic environment and organism, and the duration of exposure to both PAH and UV (Newsted and Giesy, 1987; Sellin Jeffries *et al.,* 2013). Photo-induced toxicity may occur through two mechanisms: photosensitization or photomodification. Photosensitization occurs through the absorption of UV energy by the chemical within the organism causing damage to biological molecules via reactive oxygen species (ROS), while photomodification occurs through the production of UV-modified chemicals in water and subsequent absorption into the organism (Barron, 2000; Lampi *et al.,* 2006; Roberts *et al.,* 2017).

Petroleum-based products are complex mixtures containing a wide range of chemical constituents including PAHs. Petroleum products have been shown to elicit photo-induced toxicity in aquatic organisms due to their PAH constituents (Barron, 2017). The water soluble constituents of 16 out of 22 tested petroleum products (ranging from diesel to crude oil) were phototoxic in *Daphnia magna* (Wernersson, 2003). UV exposure significantly enhanced the toxicity of weathered Alaska North Slope crude oil to early life stages of Pacific herring eggs and larvae (Barron *et al*., 2003). Site samples of contaminated water from the Exxon Valdez oil spill were acutely toxic in the presence of sunlight and artificial UV in larval Pacific herring (Sellin Jeffries *et al.,* 2013). In all cases described above, the photo-induced toxicity was attributed to the PAH constituents in the petroleum-products.

The photo-induced toxicity of PAHs has been studied extensively in a wide range of species; however, there have been relatively few studies assessing the photo-induced toxicity of PAHs in amphibians. During their early life stages, amphibians have permeable skin, the capacity to assimilate chemicals, rapid rate of development, and lack of fully operational metabolism and oxidative stress defensive mechanisms. In addition, the early life stages of specific amphibian species have translucent skin (e.g. *Xenopus laevis*). These characteristics increase the susceptibility of embryonic and larval stage amphibians to the photo-enhanced toxicity of PAHs in the aquatic environment. The limited studies with amphibians report similar results as in other

aquatic organisms, with UV light significantly enhancing the toxicity of specific PAHs (Kagan *et al.*, 1984, Devillers and Exbrayat, 1992, Fernandez and l'haridon, 1994, Hatch and Burton, 1998; Monson *et al.,* 2009). Early life stages of amphibian species may be exposed to individual PAHs or mixtures of PAHs through oil spills, spring runoff or migration of contaminated groundwater. Timing of exposure to PAHs is important, as embryonic and larval stages of amphibians develop in shallow vernal pools where wavelengths of solar radiation can penetrate to the bottom of the water column (Landrum *et al.,* 1987).

The photo-induced toxicity of PAHs is dependent on many factors including internal PAH concentration and intensity of UV radiation. Therefore, differences in species body burden (i.e., bioaccumulation) and differences in physical characteristics (i.e., skin pigmentation) may lead to differences in photo-induced toxicity. These differences are important when comparing toxicity data for the model amphibian species such as *Xenopus laevis* with non-model, ecologically relevant species for risk assessments. To our knowledge, the relevance of *Xenopus* for predicting thresholds for the photo-induced toxicity of PAHs in non-model anurans is unknown.

There is limited data concerning the photo-induced toxicity of PAHs in amphibian species, and it is unknown whether the model species *Xenopus* is a good predictor of PAH photo-induced toxicity for native species of anurans. Given extensive evidence of photo-induced toxicity in early life-stage fish, I hypothesized that early life stage amphibians would be at risk to photo-induced toxicity following exposure to PAHs and a petroleum-based mixture known to contain PAHs (UNIVIS HVI 13®) and that relative differences in sensitivity between species would be related to accumulated levels of specific PAHs. To test this hypothesis, *Lithobates sylvaticus* (wood frog) and *Xenopus laevis* (African clawed frog) tadpoles were exposed to three individual PAHs (benzo(a)pyrene, anthracene, and naphthalene) as well as the water accommodated fractions (WAF) of a petroleum-based mixture known to contain PAHs (UNIVIS HVI 13®). Tadpoles were allowed to accumulate chemicals over 8 h and then transferred to clean water under UV exposure for 12 h in order to specifically test whether larval *Xenopus* and wood frog exhibit photo-induced toxicity by mechanism of photo-sensitization. Mortality, morphometrics, body burden and deformities were assessed and compared between the two species.

3.2 Materials and Methods

3.2.1 Obtaining and maintaining embryos of amphibian species

Amphibian species chosen for early life stage toxicity testing include a standard test species *Xenopus laevis* (*Xenopus*, African clawed frog) as well as the ecologically relevant species *Lithobates sylvaticus* (wood frog). The amphibian embryo life stage develops in a thick jelly layer, which protects the developing embryo from direct exposure to contaminants and UV radiation (Marquis *et al.*, 2006; Blaustein and Belden, 2002). Thus, larval amphibians were chosen as the life stage of study for PAH photo-induced toxicity due to their translucent bodies, permeable skin and free-swimming nature in shallow vernal pools.

Xenopus embryos were obtained through breeding of male and female pairs from an adult *Xenopus* colony established in the ATRF at the University of Saskatchewan. Adults were maintained in a Min-O-Cool tank (48" L X 24" W X 20" D) at a 12:12 photoperiod and 16 ± 1 °C. They were fed frog brittle (Nasco Frog Brittle (Medium Nuggets)) ad libitum three times weekly. Use of these animals was approved by University Committee on Animal Care and Supply Animal Research Ethics Board at the University of Saskatchewan and followed Canadian Council of Animal Care guidelines. Breeding pairs were acclimated (males and females separately) from 16 °C to 21 ± l °C over the span of six days by increasing the temperature by 1 °C per day and then injected with a 25 IU priming dose of human chorionic gonadotropin (hCG) dissolved in sterilized PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4). A second dose of hCG was administered approximately 24 hours later (males 250 IU hCG; females d500 IU (ASTM, 2012). Breeding pairs were placed together into aquaria with water temperature maintained at 21 ± 1 °C and left to spawn overnight. Adults were removed, embryos collected, and normally cleaving embryos selected for use in the experiment. Embryos were transferred to environmental chamber in Toxicology Centre and reared in multiple 20L glass stock aquaria filled with facility water and raised to appropriate larval stage corresponding to respective toxicity test. *Xenopus* tadpoles at Nieuwkoop and Faber (NF) developmental stage 46-49 were used for all toxicity tests (Nieuwkoop and Faber, 1994). Specifically, NF stage 46 tadpoles were used for the anthracene and UNIVIS HVI 13 WAF toxicity tests, NF stage 48 *Xenopus* tadpoles were used for the benzo(a)pyrene toxicity test, and NF stage 49 *Xenopus* tadpoles were used for the naphthalene toxicity test.

Wood frog egg masses were collected from a local breeding site located at Cranberry Flats Conservation Area flood plain (52.0311° N, 106.7004° W) near Saskatoon, SK. Eggs were transported to environmental chamber at the Toxicology Centre, University of Saskatchewan and maintained in multiple 20 L glass stock aquaria filled with facility water and raised to appropriate larval stage corresponding to respective toxicity test. Wood frog tadpoles at developmental Gosner stage 25-27 were used for all toxicity tests (Gosner 1960). Specifically, Gosner stage 25 tadpoles were used for the anthracene and UNIVIS HVI 13 WAF toxicity tests, Gosner stage 26 tadpoles were used for the benzo(a)pyrene toxicity test, and Gosner stage 26 tadpoles were used for the naphthalene toxicity test. Stage matching was performed between *Xenopus* and wood frogs in order to conduct direct species comparison for specific chemical exposures (McDiarmid and Altig 1999).

3.2.2 Experimental procedure

3.2.2.1 Test substances and WAF preparation

Benzo(a)pyrene, anthracene and naphthalene were purchased from Sigma Aldrich (Product code: B1760, A89200, and 147141, respectively). Benzo(a)pyrene was dissolved in dimethyl sulfoxide (DMSO) while anthracene and naphthalene were dissolved in acetone to achieve stock solutions concentrations of 1 g L^{-1}. Stock solutions were kept in the dark at 4°C until toxicity tests commenced at which time test dishes were spiked with stock solutions to achieve nominal exposures concentrations. Carrier solvents did not exceed 0.2 % v/v in any of the exposure concentrations.

Exxon Mobil UNIVIS HVI 13 hydraulic oil (Product code: 201560109720, 431007-00, 97Q043) was purchased from Marsollier Petroleum, Ltd. (Saskatoon, SK). Aquatic exposures to complex mixtures containing petroleum hydrocarbons are often conducted using water-accommodated fractions (WAF) as this exposes the organisms to the water-soluble constituents representative of an exposure in an aquatic environment. The WAF of UNIVIS HVI 13 was prepared according to established guidelines (Singer *et al.*, 2000) with some modifications. The WAF test loadings (1:100, 1:10, and 1:1) were made independent of each other. For the highest test loading, 1 L of UNIVIS HVI 13 and 1 L of water were added to a 2 L glass aspirator bottle to achieve oil to water ratio of 1:1 and then sealed with aluminum foil to prevent evaporation. For the medium test loading, 200 mL of UNIVIS HVI 13 and 1800 mL of water were added to a 2 L

glass aspirator bottle to achieve oil to water ratio of 1:10 and then sealed. For the lowest test loading, 20 mL of UNIVIS HVI 13 and 1980 mL of water were added to a 2L glass aspirator bottle to achieve oil to water ratio of 1:100 and then sealed. Exposure solutions were made with aerated de-chlorinated facility water from the ATRF, Toxicology Centre, University of Saskatchewan. For each of the WAF loadings, the mixture was gently stirred at low energy (no vortex) using a magnetic stirrer at room temperature for 24 h in the dark and then allowed to settle for one hour to confirm the phase separation (Singer *et al.,* 2000). The resulting WAF was then carefully decanted from the bottom of the bottle into a 1 L glass bottle and covered in aluminum foil to prevent photo-degradation. For all exposures, UNIVIS HVI 13 was prepared fresh to coincide with the start of a specific exposure. No dispersants were used.

3.2.2.2 UV exposure set-up

Toxicity tests were conducted under a light fixture of four 48'' T8 ReptiSun® 10.0 UVB fluorescent bulbs (40W; Zoo Med Laboratories Inc.®) suspended approximately 15 cm above test dishes. Irradiance for UVB (280-315 nm), UVA (315-400 nm) and visible (400-700 nm) light spectrums during exposures was quantified with an OceanOptics USB2000+UV-VIS spectrometer with cosine correction (OceanOptics). UVB and UVA irradiance were used and quantified for exposures because both anthracene and benzo(a)pyrene are known to absorb significant amounts of electromagnetic radiation from these wavelength regions (Newsted and Giesy, 1987). Average natural sunlight irradiance measurements ± SEM taken on a sunny clear day at ground level of a local wood frog breeding site (Cranberry flats, Saskatoon, SK) on April 29th, 2016 were 82.6 ± 7.75 μW/cm² (UVB), 3015 ± 6.29 μW/cm² (UVA) and 28, 279 ± 59.1 μW/cm² (visible) wavelength ranges, respectively. Due to the concern of increasing global UVB radiation playing a role in amphibian population declines, lab UVB irradiance was chosen to simulate ~ 100% UVB in the field, while UVA irradiance was chosen to simulate ~10-20 % field UVA irradiance. Average irradiance measurements ± SEM during 12 h UV light exposure in toxicity tests were 104 ± 18.9 μW/cm² (UVB), 632 ± 98.3 μW/cm² (UVA) and 1689 ± 275 μW/cm² (visible) wavelength ranges, respectively. Overall, lab irradiance values were on average 125 %, 20 %, and 6 % of field UVB, UVA, and visible light, respectively. Irradiance measurements for UVC (100-280 nm) wavelengths were 0 throughout all toxicity tests. Ultraviolet dose (cumulative irradiance) (kJ/m^2) was calculated by multiplying irradiance

(μW/cm²) by exposure time (seconds). Average ultraviolet dose (cumulative irradiance) after 1 h of UV light treatment was calculated to be 3.74 and 23 kJ/m^2 for UVB and UVA respectively for all toxicity tests. Average ultraviolet dose after 12 h of UV light treatment was calculated to be 44.9 and 276 kJ/m^2 for UVB and UVA respectively for all toxicity tests.

3.2.2.3 Tadpole exposures

The experimental design for the 96 h exposure set up is shown in Figure 3.1. For both amphibian species, 20 individuals were randomly assigned to 750 mL crystallizing test dishes containing 200 mL exposure water with three replicates per test concentration. Tadpoles were exposed to test concentrations for 8 h in the dark. Nominal exposure concentrations for PAHs were: (1) benzo(a)pyrene at 1 μg L^{-1}, 10 μg L^{-1} and 100 μg L^{-1}, (2) anthracene at 2 μg L^{-1}, 20 μg L^{-1} and 200 μg L^{-1}, (3) and naphthalene at 20 μg L^{-1}, and 200 μg L^{-1}. Exposure concentrations for UNIVIS HVI 13 WAF were derived from the preparation of 1:1 (880 g L^{-1}), 1:10 (88 g L^{-1}) and 1:100 (8.8 g L^{-1}) WAF loadings. Controls for each exposure included a water control (de-chlorinated ATRF water) and solvent control (DMSO or acetone). At 8 h, three tadpoles per replicate for the benzo(a)pyrene and anthracene exposures were sampled for body burden analysis. Tadpoles were euthanized in buffered MS-222 (ethyl 3-aminobenzoate methanesulfonate; 700 mg L^{-1}), flash frozen and stored at -80 °C until extraction and analysis. Remaining tadpoles (n = 17 tadpoles per replicate were transferred to clean facility water and each treatment was equally divided into "No UV" and "UV" treated groups and the latter were exposed to UV light for 12 h during their regular 16 h light ambient light conditions. The UV and No UV treatment test dishes were arranged on separate shelves of the shelving unit in a completely randomized design. The UV treatment shelf was covered with black garbage bags during 12 h UV exposure to prevent UV treatment within the No UV treatment test dishes. At 20 h, UV light was turned off and remaining tadpoles (n = 11 per replicate) were fed *ad libitum* daily for the remaining 76 h. Total duration of exposure, UV treatment and monitoring was 96 h and was carried out at 19 ± 0.5 °C (wood frog) or 21 ± 0.5 °C (*Xenopus*) with a 16:8 h light–dark cycle and 75 % daily renewal of exposure water. Water quality measurements (pH, dissolved oxygen, conductivity, and temperature) were taken daily following water changes using a HI9829 multi-parameter water quality meter (Canadawide Scientific ltd, Ottawa, ON). Ammonia levels were also measured daily using API ammonia test strips (API Fishcare).

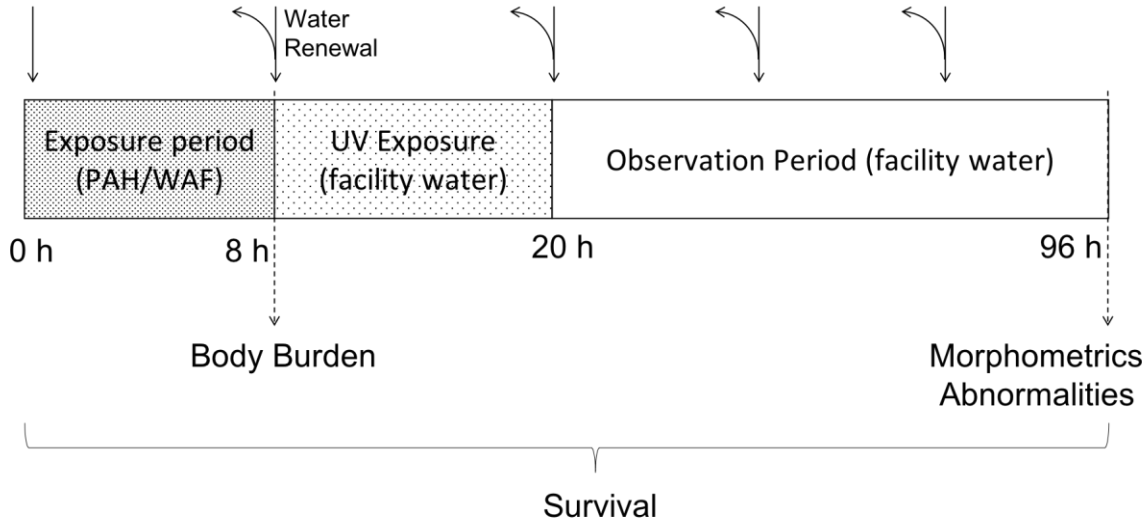


Figure 3.1. Experimental design of early larval amphibian 96 h exposure assay. During the exposure period, tadpoles were exposed to PAH/WAF for 8 h in the dark. At 8 h, a subset (n=3) of tadpoles were sampled for body burden analysis. Remaining tadpoles (n=11) were transferred to clean water and exposed to UV light for 12 h. At 20 h, UV light was turned off and tadpoles were monitored for mortality for 76 h during observation period. At 96 h surviving tadpoles were euthanized and imaged for morphometric and deformity analysis.

3.2.3 Chemical Analysis

3.2.3.1 Analysis of constituents in WAF

Samples (500 mL) were taken from 1:10 and 1:1 WAF preparations of UNIVIS HVI 13 and shipped on ice and in the dark to Maxxam Analytics (Calgary, AB) for chemical analysis (outlined in Chapter 2 of this thesis). Briefly, polycyclic aromatic hydrocarbons (PAHs), benzene, toluene, ethylbenzene and xylene (BTEX), F1-F4 hydrocarbon fractions and Canadian Council of Ministers of the Environment (CCME)-regulated metals were analyzed. PAHs were analyzed in water by gas chromatography/mass spectrometry (GC/MS), BTEX and F1-F4 hydrocarbons were analyzed in water by headspace gas chromatography/mass spectrometry with flame ionization detector (HS GC/MS/FID), and metals were analyzed by inductively coupled plasma mass spectrometry (ICP-MS). Limit of detection for each compound are reported in Appendices E and F.

3.2.3.2 Tissue benzo(a)pyrene and anthracene analysis in tadpoles

Whole-body amphibian tissue samples were flash frozen on dry ice, stored at -80° C, freeze dried using a Virtis Genesis 25ES freeze drier, and stored at -20° C until extraction. Extractions were carried out using a Dionex ASE 200 accelerated solvent extractor (ASE). Samples were weighed and placed into methanol-rinsed 1 mL stainless-steel cells. Cells were fit with a cellulose filter at both ends before being sealed and then situated in the carousel of the ASE 200 system. The conditions were as follows: oven temperature of 125 ° C with a 6 min heat–up time with a pressure of 1500 psi and two static cycles with a static time of 5 min. Samples were purged using pressurized nitrogen for 1 min with a flush volume of 10%. The solvent mixture used consisted of 1:1 dichloromethane:acetone.

Following ASE extraction, samples were evaporated to near dryness under a stream of nitrogen and then re-suspended in 1 mL acetonitrile. Samples were then glass syringe-filtered through Whatman GMF 0.45 μM filters into labeled 2 mL amber vials. An agilent 1260 infinity high pressure liquid chromatography system coupled with fluorescence detection (HPLC-FD) was then used to analyze benzo(a)pyrene and anthracene concentrations in the prepared samples according to the Agilent method for PAH analysis in soil (Volk and Gratzfeld-Huesgen, 2011) with some modifications. An aliquot of 10 μL was injected into an Agilent PAH pursuit column maintained at 25 °C. The run time was set for 25 min with a solvent flow rate of 1.5 mL/min

starting at a gradient of 60:40 acetonitrile:water and gradually increasing to 95:5 acetonitrile:water by 20 min. Anthracene eluted at ~ 5-7 min with a constant excitation wavelength of 260 nm and an emission wavelength of 350 nm. Benzo(a)pyrene eluted at ~ 12-15 min with a constant excitation wavelength of 260 nm and an emission wavelength of 420 nm. Recovery of benzo(a)pyrene and anthracene from blank tadpole samples spiked with a known amount was 113 ± 7% and 86 ± 5% respectively.

Total benzo(a)pyrene and anthracene (ng PAH) was calculated by PAH concentration in extraction (μg PAH/L, measured via HPLC using standard curve equation and experimental peak height) multiplied by total volume of extraction fluid (mL). Body burden (ng PAH/g tadpole) was then calculated by total PAH divided by the weight of the tadpole (g tadpole). Total naphthalene was not measured in this study because it was a negative control and was not expected to cause photo-induced toxicity. Naphthalene is also difficult to analyze via HPLC due to its high volatility.

3.2.4 Lethal and morphological analysis

Mortality was monitored and recorded daily. Malformations and morphometrics (total length, tail length, snout-vent length) were evaluated at 96 h. Surviving individuals were euthanized in buffered MS-222 (ethyl 3-aminobenzoate methanesulfonate; 700 mg L^{-1}). Total length, snout-vent length, tail length and deformities were evaluated using an Olympus model S261 dissecting microscope with Image-Pro Discovery Software. Deformities were assessed using established reference criteria (Bantle, 1991).

3.2.5 Statistical analysis

Statistical analyses for mortality, morphometric, body burden and deformity data were conducted in SPSS Statistics v19.0 (IBM Corporation, Armonk, NY). For all data analyses, Levene's test was used to test for equality of error variances ($p > 0.05$), and residual plots were assessed to test for goodness of fit. Prior to ANOVA analysis, student's t-test was performed to determine significant differences between control and solvent treatment groups. There were no significant differences between control and solvent control groups for any of the exposures ($p > 0.05$); therefore, these groups were combined for all data analyses. Two-way ANOVA was used to test for a significant interaction between respective PAH or WAF treatment and UV light treatment for mortality, morphometrics and deformities. Two-way ANOVA was performed with

respective PAH or WAF exposure and UV light as main factors. If a significant interaction was found ($p < 0.05$), main factors were split and t-test were performed to determine significant differences between no UV and UV groups at each exposure concentration ($p < 0.025$). If only main effects were indicated by two-way ANOVA, Fisher's LSD or Dunn-Bonferroni post hoc tests were performed to determine significant differences between PAH/WAF treatment concentrations ($p = 0.05$). For body burden data, t-test was performed to determine significant differences between wood frog and *Xenopus* groups at each exposure concentration. If mortality exceeded 50% in treatment groups, Probit analysis was used to calculate estimated LC_{50} values for larval tadpoles exposed to respective treatment. Pearson's correlation analysis ($p < 0.05$) was performed to determine significant correlation between body burden at 8 h and mortality at 96 h.

3.3 Results

3.3.1 Water quality

During the wood frog exposures, water quality parameters (mean ± standard deviation) were measured as: pH = 7.12 ± 0.41, dissolved oxygen = 92.00 ± 3.05 %, dissolved oxygen = 8.01 ± 0.20 mg L^{-1}, conductivity = 438.00 ± 17.40 µS/cm, and temperature = 19.20 ± 0.82 °C. During the *Xenopus* exposures, water quality parameters were measured as: pH = 7.70 ± 0.07, dissolved oxygen = 85.20 ± 0.95 %, dissolved oxygen = 6.90 ± 0.07 mg L^{-1}, conductivity = 429.00 ± 0.74 µS/cm, and temperature = 22.90 ± 0.06 °C. Ammonia levels in all toxicity tests were <0.25 ppm for all tests conducted.

3.3.2 Chemical analysis of WAF

Results for chemical analysis of petroleum hydrocarbon fractions, PAHs, and total metals for 1:10 and 1:1 loadings of UNIVIS HVI 13 WAF are shown in Appendices D-F, respectively. Concentrations indicated with "<" represent concentrations below the reportable detection limits for each respective analysis. The 1:10 loading of WAF had 0.35 mg L^{-1} (F1), 1043 mg L^{-1} (F2), 1033 mg L^{-1} (F3) and 5.60 mg L^{-1} (F4), while the 1:1 loading of WAF had 0.51 mg L^{-1} (F1), 2.18 mg L^{-1} (F2), 3.0 mg L^{-1} (F3), and < 0.20 mg L^{-1} (F4) total hydrocarbon fractions, respectively (Appendix D). The 1:10 loading of WAF had increased concentrations of semi-volatile organics including acenaphthene, acridine, anthracene, benzo(c)phenanthrene, fluorine, 2-

methylnaphthalene, naphthalene and pyrene (Appendix E), while concentrations of metals were similar between WAF loadings (Appendix F).

3.3.3 Toxicity tests

3.3.3.1 Mortality

Mortality (96 h) of *Xenopus* and wood frogs exposed to anthracene, benzo(a)pyrene, naphthalene and UNIVIS HVI 13 WAF with and without UV light are shown in Fig 3.2. In wood frogs exposed to anthracene and UV light, there was a significant interaction ($F = 21.242$, $p < 0.001$) whereby the presence of UV light significantly increased mortality at 200 μg L^{-1} compared to anthracene alone (82 % vs 6%, $F = 49.368$, $p = 0.002$; Fig 3.2A). The estimated LC_{50} value with 95% confidence intervals (95% CI) for wood frogs exposed to anthracene and UV light was 124 (99 – 157) μg L^{-1}, while the estimated LC_{50} for anthracene alone was >200 μg L^{-1}. For *Xenopus*, there was a significant interaction ($F = 24.684$, $p < 0.001$) whereby exposure to anthracene in presence of UV light significantly increased mortality at 2 μg L^{-1} (30 % vs 0 %, $F = 14.286$, $p = 0.019$), 20 μg L^{-1} (100 % vs 9 %, $F = 306.704$, $p < 0.001$) and 200 μg L^{-1} (100 % vs 9 %, $F = 306.704$, $p < 0.001$) compared to anthracene alone (Fig 3.2B). The estimated LC_{50} (95% CI) for *Xenopus* exposed to anthracene and UV light was 5 (3 – 13) μg L^{-1}, while the estimated LC_{50} for anthracene alone was >200 μg L^{-1}.

For wood frogs exposed to benzo(a)pyrene, there was a significant interaction ($F = 15.126$, $p < 0.001$) whereby exposure to benzo(a)pyrene in presence of UV light significantly increased mortality at 10 μg L^{-1} (36 % vs 9 %, $F = 13.500$, $p = 0.021$), and 100 μg L^{-1} (100 % vs 12 %, $F = 122.921$, $p < 0.001$) compared to benzo(a)pyrene alone (Fig 3.2C). The estimated LC_{50} (95% CI) for wood frogs exposed to benzo(a)pyrene was 17 (9 – 54) μg L^{-1}, while the estimated LC_{50} value for benzo(a)pyrene alone was >100 μg L^{-1}. Similarly, in *Xenopus* exposed to benzo(a)pyrene and UV light, there was a significant interaction ($F = 79.681$, $p < 0.001$) whereby the presence of UV light increased mortality at 100 μg L^{-1} compared to benzo(a)pyrene alone (97 % vs 3 %, $F = 490.889$, $p < 0.001$; Fig 3.2D). The estimated LC_{50} (95% CI) for *Xenopus* exposed

to benzo(a)pyrene and UV light was 45 (34 – 60) μg L^{-1}, while the estimated LC_{50} for benzo(a)pyrene alone was > 100 μg L^{-1}.

In contrast to anthracene and benzo(a)pyrene, there was no significant interaction of naphthalene exposure and UV on mortality for wood frog and *Xenopus* ($F = 0.612$, $p = 0.553$; $F = 0.341$, $p = 0.716$, respectively) whereby the presence of UV light did not significantly increase mortality when compared with naphthalene treatment alone (Fig 3.2E, F). There were no significant main effects of naphthalene treatment or UV light on wood frog or *Xenopus* mortality, respectively.

For wood frog and *Xenopus* exposed to UNIVIS HVI 13 WAF, there was no significant interaction on mortality ($F = 0.698$, $p = 0.567$; $F = 0.583$, $p = 0.635$ respectively) whereby the presence of UV light did not significantly increase mortality when compared with WAF treatment alone (Fig 3.2G, H). There were no significant WAF or UV light main effects on wood frog mortality and no significant UV light main effect on *Xenopus* mortality. However, WAF treatment alone significantly increased *Xenopus* mortality at the highest loading (1:1) compared to the other WAF loadings ($F = 13.544$, $p < 0.001$; Fig 3.2H).

Time to mortality over the 96 h toxicity tests for *Xenopus* and wood frogs exposed to anthracene or benzo(a)pyrene is depicted in Figure 3.3. After only 2 h of UV light treatment, there was 82% mortality of wood frogs exposed to 20 μg L^{-1} anthracene and 100% mortality of *Xenopus* exposed to 20 μg L^{-1} and 200 μg L^{-1} anthracene (Fig 3.3A, B). Similarly, after 2 h of UV light treatment, there was 100% mortality of wood frog and 97% mortality of *Xenopus* exposed to 100 μg L^{-1} benzo(a)pyrene (Fig 3.3C, D).

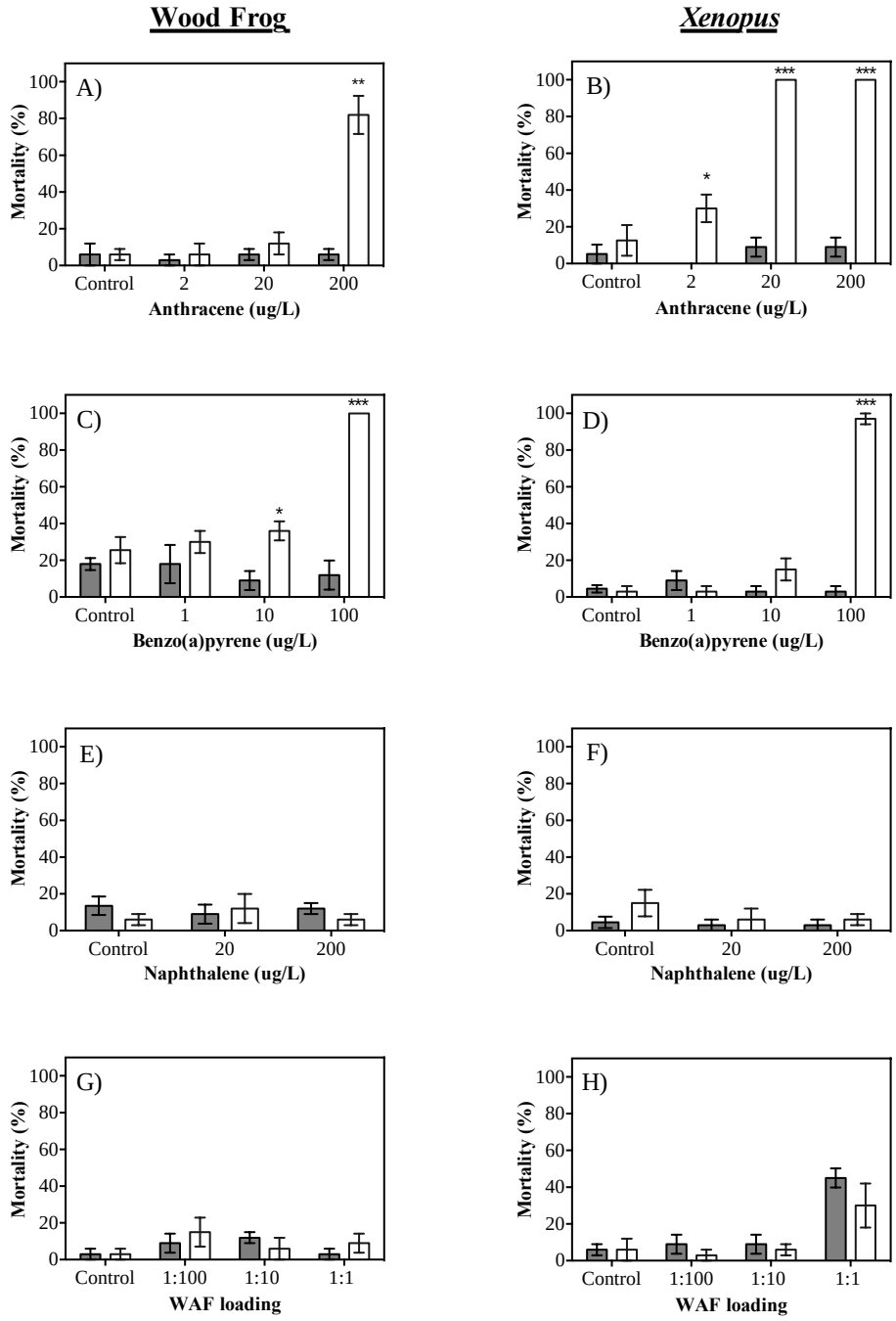


Figure 3.2. Mortality of larval wood frogs (left panels) and *X. laevis* (right panels) at 96 h after aqueous exposure to anthracene (A, B), benzo(a)pyrene (C, D), naphthalene (E, F), WAF of UNIVIS HVI 13 (G, H) or solvent control for 8 h followed by UV light treatment (white bars) for 12 h, or no UV light treatment (grey bars). Data is mean ± SEM with $n = 3$ for all analyses. Percent mortality was calculated as the percent per exposure unit ($n = 11$ tadpole per unit). * $p < 0.025$, ** $p < 0.01$, *** $p < 0.001$ indicate significant UV light treatment effect ($p < 0.025$, t-test) within respective PAH or WAF treatment group following significant two-way ANOVA interaction ($p < 0.05$).

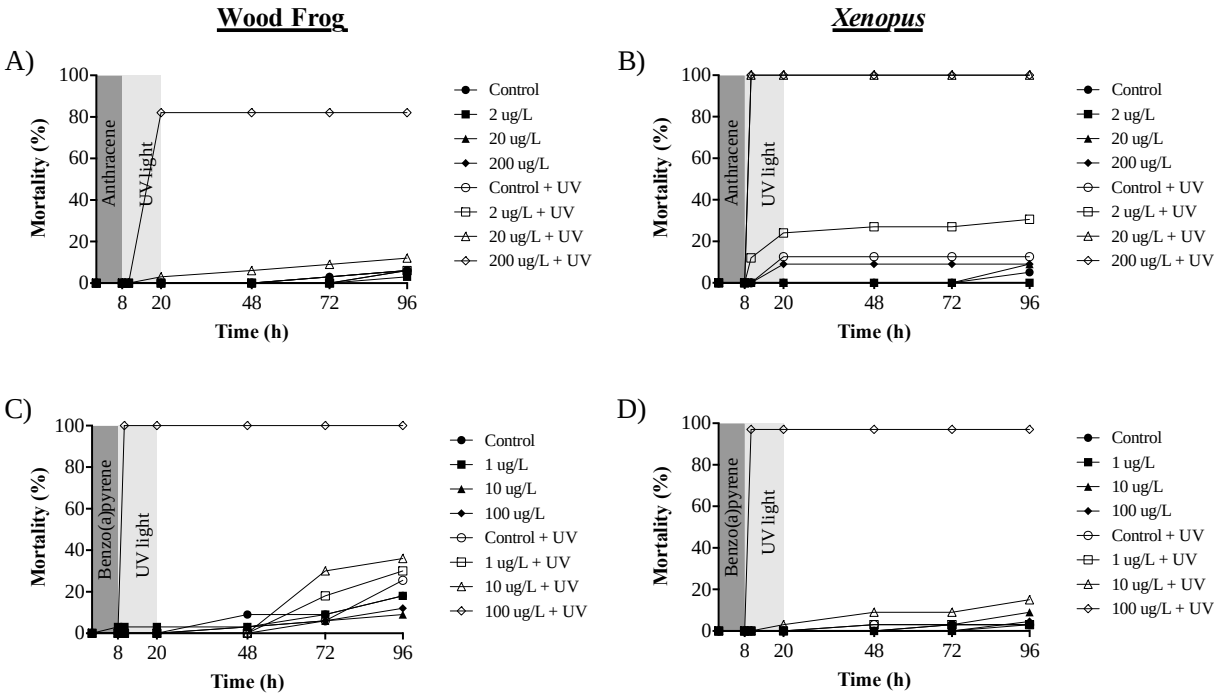


Figure 3.3. Cumulative percent mortality for larval wood frogs (left panels) and *X. laevis* (right panels) exposed to anthracene (A, B) or benzo(a)pyrene (C, D) with or without UV. The dark grey bar indicated the 8 h PAH exposure while the light grey bar indicates the subsequent 12 h (8-20 h) UV light exposure.

3.3.3.2 Morphometrics

Morphometric data for *Xenopus* and wood frogs exposed to anthracene, benzo(a)pyrene, naphthalene or UNIVIS HVI 13 WAF and UV light at 96 h are shown in Table 3.1. For wood frogs exposed to benzo(a)pyrene, there was no significant interaction ($F = 0.612$, $p = 0.543$) whereby the presence of UV light with benzo(a)pyrene did not significantly affect whole-body length compared to benzo(a)pyrene alone. There were no main effects of benzo(a)pyrene ($F = 0.544$, $p = 0.582$) or UV light ($F = 2.020$, $p = 0.157$) treatment on wood frog whole-body length. Similarly, for *Xenopus* exposed to benzo(a)pyrene, there was no significant interaction ($F = 2.434$, $p = 0.090$) whereby the presence of UV light did not significantly decrease whole-body length compared to benzo(a)pyrene alone. There was no main effect of benzo(a)pyrene exposure on *Xenopus* whole-body length ($F = 0.117$, $p = 0.890$); however, UV light treatment alone significantly decreased *Xenopus* whole-body length ($F = 19.264$, $p < 0.001$).

For wood frogs exposed to anthracene, there was a significant interaction ($F = 7.378$, $p = 0.001$) whereby the presence of UV light significantly decreased whole-body length at 20 $\mu g\ L^{-1}$ ($F = 8.651$, $p = 0.005$) and 200 $\mu g\ L^{-1}$ ($F = 9.005$, $p = 0.005$) compared to anthracene alone. Conversely, for *Xenopus* exposed to anthracene, there was no significant interaction ($F = 0.062$, $p = 0.804$) whereby the presence of UV light did not significantly decrease *Xenopus* whole-body length compared to anthracene alone. There was no significant main effect of anthracene on *Xenopus* whole-body length ($F = 0.992$, $p = 0.321$); however, UV light treatment alone significantly decreased *Xenopus* whole-body length ($F = 18.096$, $p < 0.001$).

For wood frogs exposed to naphthalene, there was no significant interaction ($F = 0.469$, $p = 0.626$) whereby the presence of UV light did not significantly decrease whole-body length compared to naphthalene alone. There were no significant main effects of naphthalene ($F = 2.138$, $p = 0.120$) or UV light ($F = 0.182$, $p = 0.670$) treatment on wood frog whole-body length. Similarly, for *Xenopus* exposed to naphthalene, there was no significant interaction ($F = 1.169$, $p = 0.312$) whereby the presence of UV light did not significantly decrease whole-body length compared to naphthalene alone. There was no main effect of naphthalene ($F = 2.877$, $p = 0.058$) treatment on *Xenopus* whole-body length; however, UV light ($F = 146.353$, $p < 0.001$) treatment significantly decreased *Xenopus* whole-body length.

For wood frogs exposed to UNIVIS HVI 13 WAF, there was no significant interaction ($F = 0.600$, $p = 0.615$) whereby the presence of UV light did not significantly decrease whole-body

length compared to WAF alone. However, both WAF ($F = 4.486$, $p = 0.004$) and UV light ($F = 15.322$, $p < 0.001$) treatment significantly decreased wood frog whole-body length. There were significant differences in wood frog whole-body length between control and 1:100 WAF treatment groups ($p < 0.001$). Similarly, for *Xenopus* exposed to UNIVIS HVI 13 WAF, there was no significant interaction ($F = 2.366$, $p = 0.072$) whereby the presence of UV light did not significantly decrease whole-body length. There was no main effect of WAF treatment ($F = 1.500$, $p = 0.215$) on *Xenopus* whole body length; however, UV light treatment alone significantly decreased *Xenopus* whole-body length ($F = 26.880$, $p < 0.001$).

Table 3.1. Whole-body length (cm) of larval wood frogs and *Xenopus* at 96 h following exposure to benzo(a)pyrene, anthracene, naphthalene, WAF of UNIVIS HVI 13 for 8 h followed by UV light treatment for 12 h.

Treatment	Nominal dose (μg/L)	Wood frog		*Xenopus*	
		No UV	UV	No UV	UV
Benzo(a)pyrene	Control	1.30 ± .01	1.31 ± .01	1.32 ± .01	1.29 ± .01[b]
	1	1.31 ± .01	1.33 ± .01	1.34 ± .01	1.26 ± .01[b]
	10	1.28 ± .01	1.32 ± .01	1.33 ± .02	1.27 ± .02[b]
Anthracene	Control	1.21 ± .01	1.22 ± .01	1.19 ± .01	1.11 ± .01[b]
	2	1.24 ± .01	1.23 ± .01	1.17 ± .01	1.09 ± .01[b]
	20	1.26 ± .01	1.19 ± .01[**]	-	-
	200	1.21 ± .01	1.10 ± .01[**]	-	-
Naphthalene	Control	1.41 ± .02	1.42 ± .02	1.60 ± .02	1.36 ± .02[b]
	20	1.45 ± .02	1.43 ± .02	1.64 ± .02	1.42 ± .02[b]
	200	1.42 ± .02	1.42 ± .02	1.59 ± .02	1.42 ± .02[b]
WAF	Control	1.24 ± .01[c]	1.21 ± .02[bc]	1.22 ± .01	1.10 ± .01[b]
	1:100[a]	1.20 ± .01[c]	1.16 ± .01[bc]	1.22 ± .01	1.19 ± .01[b]
	1:10[a]	1.21 ± .01[c]	1.20 ± .01[bc]	1.21 ± .01	1.16 ± .01[b]
	1:1[a]	1.23 ± .01[c]	1.19 ± .02[bc]	1.22 ± .01	1.13 ± .01[b]

Data is mean ± SEM (n = 6-33 individuals).
** Indicates significant UV light effect within respective PAH or WAF exposure level ($p < 0.025$, t-test) following significant two-way ANOVA interaction ($p < 0.05$).
[a] WAF loading (oil-to-water ratio) of UNIVIS HVI 13
[b] significant main effect of UV light in two-way ANOVA ($p < 0.05$)
[c] significant main effect of PAH/WAF in two-way ANOVA ($p < 0.05$)

3.3.3.3 Deformities

Visual deformity analyses for *Xenopus* and wood frogs exposed to PAH or UNIVIS (with and without UV light) included the following deformities: optic and abdominal edema, axial shortening, bent tail, and spinal curvature. No deformities were observed in wood frogs for all exposures; therefore, only deformity incidence (%) for *Xenopus* are shown in Fig 3.4. For all exposures, there were no interactions between exposure level and UV ($p > 0.05$) but UV light treatment alone significantly increased the incidence of deformities in *Xenopus* for the benzo(a)pyrene exposure (25 % vs 1 %, $F = 22.969$, $p < 0.001$), naphthalene exposure (10 % vs 0 %, $F = 7.465$, $p = 0.006$), and UNIVIS HVI 13 WAF exposure (34 % vs 2 %, $F = 16.107$, $p < 0.001$). There was no main effect of UV treatment on incidence of deformities in *Xenopus* in the anthracene exposure ($p = 0.811$).

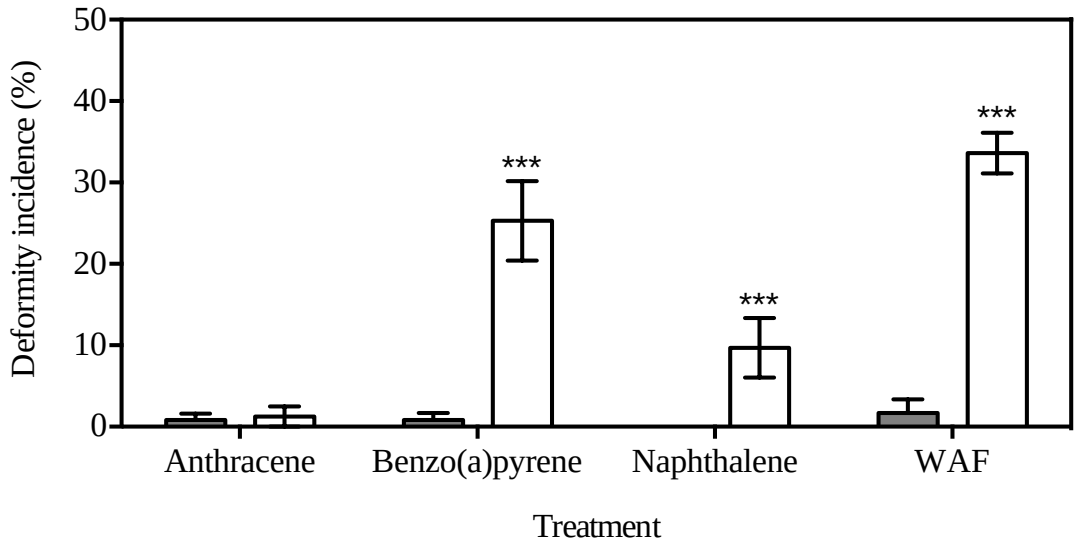


Figure 3.4. Incidence of deformities in larval *Xenopus* at 96 h following PAH or WAF exposure for 8 h followed by 12 h exposure to UV light (white bars) or no UV light (grey bars). Data is mean ± SEM percent incidence of deformities (n = 12 replicates with 6-33 tadpoles per replicate). *** indicates significant difference between no UV and UV treatment for each PAH or WAF exposure ($p < 0.001$, t-test).

3.3.3.4 Body burden

Body burden data for *Xenopus* and wood frogs following 8 h exposure to anthracene or benzo(a)pyrene are shown in Fig 3.5. Exposure of wood frogs to nominal concentrations of 0, 2, 20, and 200 μg L^{-1} anthracene resulted in body burden levels (mean ± SEM) of 14.4 ± 6.4, 91.9 ± 20.3, 118.9 ± 22.0, and 105.3 ± 15.9 ng g^{-1}, respectively (Fig 3.5A). Exposure of *Xenopus* to nominal concentrations of 0, 2, 20 and 200 μg L^{-1} anthracene resulted in body burden levels (mean ± SEM) of 4.1 ± 1.5, 22.0 ± 8.5, 34.0 ± 12.0, and 133.1 ± 46.8 ng g^{-1}, respectively (Fig 3.5A). Wood frogs exposed to 2 and 20 μg L^{-1} anthracene had a significantly higher body burden compared to *Xenopus* exposed to the same concentrations of anthracene (p = 0.017, p = 0.015, respectively).

Exposure of wood frogs to nominal concentrations of 0, 1, 10 and 100 μg L^{-1} benzo(a)pyrene resulted in body burden levels (mean ± SEM) of 19.2 ± 2.27, 24.0 ± 5.98, 88.0 ± 12.0, and 182.9 ± 29.6 ng g^{-1}, respectively (Fig. 3.5B). Exposure of *Xenopus* to nominal concentration of 0, 1, 10 and 100 μg L^{-1} benzo(a)pyrene resulted in body burden levels (mean ± SEM) of 5.55 ± 0.14, 24.7 ± 1.82, 13.2 ± 0.77, 47.0 ± 17.7 ng g^{-1}, respectively (Fig 3.5B). Wood frogs exposed to 100 μg L^{-1} benzo(a)pyrene had a significantly higher body burden compared to *Xenopus* exposed to the same concentration of benzo(a)pyrene (p = 0.015).

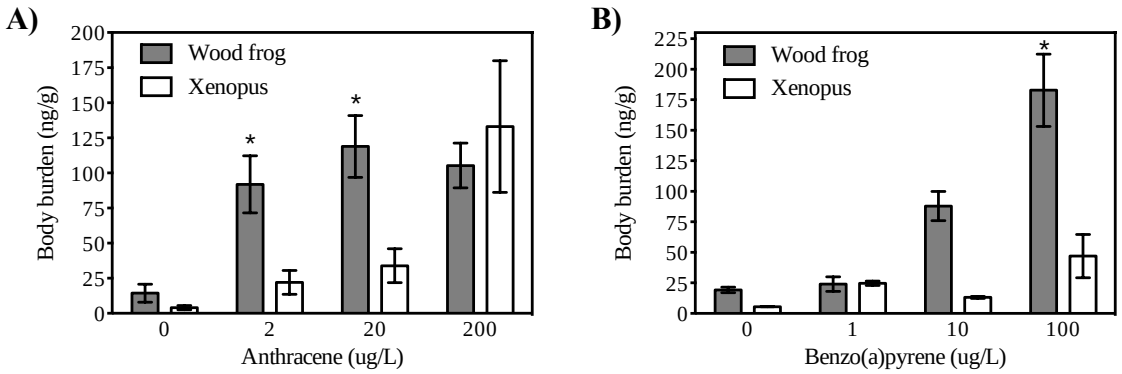


Figure 3.5. Body burden (ng/g, dry mass) of anthracene (A) and benzo(a)pyrene (B) in larval wood frogs and *Xenopus* following aqueous exposure for 8 h prior to UV exposure. Data is expressed as mean ± SEM (n = 3-5). Body burden was calculated as the amount of PAH per replicate (n = 8-10 whole-body tadpoles per replicate) divided by the dry mass of the sample (0.1 g). * indicates significant difference between species at each exposure concentration ($p < 0.05$, t-test).

3.3.3.5 Body burden and mortality correlations

In *Xenopus* exposed to anthracene, there was a significant positive correlation between anthracene body burden at 8 h and mortality at 96 h ($r = 0.583$, $p = 0.029$), while for wood frog exposed to anthracene, there was no significant correlation between anthracene body burden at 8 h and mortality at 96 h ($r = 0.286$, $p = 0.368$). In *Xenopus* exposed to benzo(a)pyrene, there was a significant positive correlation between benzo(a)pyrene body burden at 8 h and mortality at 96 h ($r = 0.863$, $p < 0.001$), and in wood frog exposed to benzo(a)pyrene there was a significant positive correlation between benzo(a)pyrene body burden at 8 h and mortality at 96 h ($r = 0.847$, $p = 0.001$).

3.4 Discussion

In this study, we evaluated and compared the photo-induced toxicity of anthracene, benzo(a)pyrene, naphthalene and the WAF of the hydraulic oil UNIVIS HVI 13 following exposure in early life stage *Xenopus* and wood frog. Anthacene and benzo(a)pyrene exhibited photo-induced toxicity in both amphibian species evident by the rapid and significant increase in mortality following UV light treatment. In contrast, naphthalene and the WAF of an environmentally relevant mixture of PAHs (UNIVIS HVI 13) did not exhibit photo-induced toxicity in either amphibian species. Wood frog tadpoles accumulated higher levels of anthracene and benzo(a)pyrene compared to *Xenopus*; however, based on mortality, morphometric and deformity data, early life stages of *Xenopus* were more sensitive to the photo-induced toxicity of PAHs and UV light treatment alone compared to wood frog.

Naphthalene and loadings of UNIVIS HVI 13 WAF were not phototoxic to larval amphibians at any concentrations tested. Naphthalene was used as a negative control in the present study and tadpole responses were as expected; naphthalene is known to be a non-phototoxic compound due to its physical and chemical properties (i.e., inability to absorb significant amounts of UVB and UVA light) (Newsted and Giesy, 1987). In contrast, the WAF of UNIVIS HVI 13 did not exhibit photo-induced toxicity in either *Xenopus* or wood frog tadpoles, as UV light did not enhance the toxicity of UNIVIS HVI 13 WAF at any loadings tested. Other studies have reported photo-induced toxicity in aquatic organisms exposed to the WAF of oil products (Wernersson, 2003; Barron *et al.*, 2003; Sellin Jeffries *et al.*, 2013), and analyses of the

WAF of UNIVIS HVI 13 confirmed that it contained a mixture of PAHs including anthracene, benzo(a)pyrene and other phototoxic compounds. The lack of observed photo-induced toxicity with exposure to this WAF is likely due to the low concentrations of phototoxic compounds within the WAF. Pelletier *et al.,* (1997) found that increasing PAH concentration in WAF correlated with increased phototoxicity. The concentration of phototoxic constituents in the WAF depends on preparation methodology, water solubility of compound, presence of suspended particles and the viscosity of product (Lee *et al.,* 1992; Ortiz *et al.,* 1999; Wernersson, 2003). Study-to-study variability in dilution water, light intensity, nominal load concentrations, WAF preparation methodology, and analyzed medium can produce differences in phototoxicity of petroleum products (Wernersson, 2003). It was also suggested that if the concentration-addition of eight phototoxic parent PAHs (anthracene, benzo[a]anthracene, benzo[g,h,i]perylene, indeno[1,2,3,c,d]pyrene, benzo[a]pyrene, fluoranthene, pyrene and dibenzo[a,h]anthracene) were above a threshold of 21 mg kg^{-1}, one could accurately predict that the product would exhibit phototoxicity to *Daphnia magna* (Wernersson, 2003). Similarly, the phototoxicity of creosote could be accurately predicted by measuring four PAHs (fluoranthene, pyrene, anthracene, and benzo[a]anthracene) in a WAF preparation (Schirmer *et al.* 1999). Wernersson (2003) also suggested a threshold value of 3% total PAHs as an initial screening technique for considering a sample to be phototoxic, as all products with < 3% total PAH were reported non-phototoxic. If we consider the 21 mg kg^{-1} threshold value, it is not surprising that larval amphibians did not exhibit photo-induced toxicity with exposure to the WAF of UNIVIS HVI 13. The eight phototoxic PAHs measured in the WAF were anthracene, benzo[a]anthracene, benzo[g,h,i]perylene, indeno[1,2,3,c,d]pyrene, benzo[a]pyrene, fluoranthene, pyrene, and dibenzo(a,h)anthracene, contributing to total phototoxic PAH concentrations of 2.67 μg L^{-1} and 0.037 μg L^{-1} in the 1:10 and 1:1 WAF loadings, respectively. Although the 1:10 WAF had high concentrations of total F2 and F3 hydrocarbons, these fractions were highly comprised of non-phototoxic compounds such as naphthalene and 2-methylnaphthalene.

Photo-induced toxicity of PAHs has been studied in a wide range of aquatic organisms, ranging from algae to fish (Arfsten *et al.,* 1996), and a number of models have been developed for predicting the photo-induced toxicity of PAHs to aquatic organisms (Marzooghi and Di Toro, 2017). As reported for other aquatic organisms including fish, we found that anthracene and benzo(a)pyrene exhibited clear photo-induced toxicity in early life stage *Xenopus* and wood

frogs. Although literature is limited, studies show that UV light can significantly enhance the toxicity of certain PAHs to amphibians. Exposure of *Lithobates pipiens* tadpoles to anthracene for 24 h in the dark resulted in no mortality; however, exposure to sunlight for 30 mins following anthracene exposure resulted in a significant increase in tadpole mortality (Kagan *et al.*, 1984). A significant increase in mortality was also observed in late embryonic stages of *L. pipiens* following 30 minutes co-exposure to sunlight and anthracene, fluoranthene or pyrene (Devillers and Exbrayat, 1992). Similarly, there was no mortality observed in newt embryos exposed to 500 $\mu g\ L^{-1}$ benzo(a)pyrene under fluorescent lighting but exposure to a much lower concentration (12.5 $\mu g\ L^{-1}$) and UV-A irradiation resulted in 100% mortality (Fernandez and l'haridon, 1994). In addition to mortality, developmental indices in amphibians are also impacted by PAH and UV exposure. Co-exposure of larval *X. laevis* and *L. pipiens* to 125 $\mu g\ L^{-1}$ fluoranthene and UV light increased the incidence of malformation (Hatch and Burton, 1998). We found increased mortality, decreased length, and increased deformities observed in *Xenopus* and wood frogs following exposure to UV light and anthracene and benzo(a)pyrene, demonstrating for the first time the photo-induced toxicity of PAHs to wood frogs.

Much of the limited amphibian toxicity data generated to date comes from studies using model, laboratory species such as *Xenopus* with the assumption that this data is protective of other amphibian species. In this study, *Xenopus* were more sensitive to the photo-induced toxicity of anthracene than wood frogs, with increased mortality following exposure to 2, 20 and 200 $\mu g\ L^{-1}$ anthracene and UV light. Comparatively, wood frog tadpoles exhibited a significant increase in mortality following exposure to 200 $\mu g\ L^{-1}$ anthracene and UV light. Conversely, wood frogs were more sensitive to the photo-induced toxicity of benzo(a)pyrene than *Xenopus* with significant increases in mortality occurring following exposure to 10 and 100 $\mu g\ L^{-1}$ benzo(a)pyrene and UV light, as compared to *Xenopus* that only exhibited a significant increase in mortality following exposure to 100 $\mu g\ L^{-1}$ benzo(a)pyrene and UV light. With regards to mortality, the increased sensitivity of *Xenopus* to the photo-induced toxicity of anthracene cannot be explained by species-specific differences in body burden as wood frogs accumulated higher concentrations of anthracene at 8 h compared to *Xenopus*. However, there was a significant positive correlation between tissue concentration of anthracene at 8 h and mortality at 96 h in *Xenopus*. These results suggest that lower tissue concentrations of anthracene at 8 h may be able to elicit photo-induced toxicity in larval *Xenopus* as compared to larval wood frog. The greater

sensitivity of wood frog to the photo-induced toxicity of benzo(a)pyrene could be explained by species-specific differences in body burden as wood frogs accumulated higher concentrations of benzo(a)pyrene at 8 h compared to *Xenopus*.

Interestingly, early life stages of *Xenopus* were more sensitive to UV light treatment alone when compared to wood frog, as evidenced by the decreased length and increased incidence of deformities. Compared to native species such as the wood frog, *Xenopus* have significant differences in their genetics, life history, physical characteristics, and relative tolerance to environmental contaminants (McDiarmid and Mitchell, 2000). In a study by Hatch and Burton (1998), *Xenopus* exhibited increased mortality following UV exposure both with and without PAH as compared to *L. pipiens* (another Ranid frog). There have been several proposed explanations for increased sensitivity of *Xenopus* to UV light as compared to other amphibians. In the aquatic environment, eggs of native anurans such as *Ranids* are typically laid near vegetation or twigs in the shallow water of temporary ponds or wetlands and so these species are exposed to sunlight as embryos (Wright and Wright, 1949). Upon hatching, these tadpoles search for food near the bottom of shallow waters (Starret, 1973). In contrast, *Xenopus* tend to lay eggs in deep murky waters, which may suggest a lack of adaptation to development in sunlight (Blaustein *et al.*, 1994). Larval *Xenopus* develop with completely translucent skin whereas larval wood frogs develop with dark skin pigmentation. The dark skin of wood frogs may be an adaptation of increased exposure to UV light during development (Hatch and Burton, 1998) and could explain the differences in sensitivity to UV light between *Xenopus* and native species such as wood frog. *Xenopus* also have low photolyase levels, likely due to their adaptation to deep murky waters, which may also increase their sensitivity to UV-induced DNA damage (Blaustein *et al.*, 1994). Differences in skin pigmentation and photolyase expression during development may explain the differences in sensitivity to UV light observed in this study. Our data suggests that these morphological and physiological differences between *Xenopus* and wood frogs during early life stages may play a greater role than body burden with regards to differences in sensitivity to PAH photo-induced toxicity. However, further studies are required to directly measure and compare differences in skin pigmentation and photolyase activity/expression in the early life stages of both species.

To determine whether *Xenopus* is a good predictor for the photo-induced toxicity of PAHs to wood frog, we compared the LC_{50} values for both species exposed to the model

phototoxic compounds anthracene and benzo(a)pyrene. In the current study, the LC_{50} value for *Xenopus* exposed to anthracene and UV light was 5 μg L^{-1}, while the LC_{50} value for wood frogs exposed to anthracene and UV light was 124 μg L^{-1} leading us to conclude that for anthracene, toxicity thresholds based on *Xenopus* would be protective for the wood frog. The LC_{50} values for benzo(a)pyrene and UV light were more comparable between species with 45 μg L^{-1} for *Xenopus* and 17 μg L^{-1} for wood frogs. Based on the significant mortality to low concentrations of anthracene, and the increased sensitivity to UV treatment alone, *Xenopus* allows for conservative comparisons to the wood frog when assessing the photo-induced toxicity of PAHs. It is important to note however, that while *Xenopus* appears to be a suitable surrogate species for assessing toxicity of PAHs and UV light exposures with lethality as an endpoint, sub-lethal responses to exposure may not reflect those observed in other amphibian species. Changes in growth and incidence of deformities are likely more species-specific effects and may be attributed to unique physical characteristic of the species (e.g. pigmentation) or differences in toxicokinetics and biochemical/molecular response to the PAH and UV light. Therefore, while *Xenopus* may be useful for determining exposure thresholds for lethality and for generating data for risk assessments, studies aimed at elucidating modes of action and sub-lethal effects on amphibian development should utilize a wider range of toxicologically and ecologically relevant species.

There has been an alarming decline in global amphibian populations over the past 40 years with a number of environmental and chemical stressors being identified as potential factors for the decline including pathogens, climate change and contaminants (Collins, 2010). The decline is not solely due to a single stressor but rather multiple stressors. Aquatic organisms such as larval amphibians are rarely exposed to just one stressor in their aquatic environment, and this study highlights the importance of studying the effects of multiple stressors to aquatic organisms when conducting toxicity tests. Chemical stressors such as PAHs can interact with environmental stressors such as UV radiation to produce synergistic effects. Our results demonstrate that anthracene and benzo(a)pyrene have significantly greater acute toxicity to early life stages of *Xenopus* and wood frog in the presence of environmentally relevant intensities of UV light. Exposure to UV radiation is an important consideration when assessing the hazard of PAHs in aquatic environments, particularly shallow vernal pools, where developing embryo-larval amphibians are continuously exposed to the stressor of UV light.

CHAPTER 4

GENERAL DISCUSSION

4.1. Major Conclusions of Research

In Chapter 2 of this thesis, the acute toxicity of the water-soluble constituents of UNIVIS HVI 13 was assessed due to the uncertainty of UNIVIS HVI 13 toxicity to aquatic organisms, and concern regarding the hydraulic fluid lubricating oil reaching aquatic ecosystems. In general, the acute toxicity of the WAF of fresh UNIVIS HVI 13 (UNIVIS-F) decreased from lower level trophic organisms to higher level trophic organisms with *D. magna* being the most sensitive organism with an estimated EC_{50} value of 114 g L^{-1} UNIVIS-F WAF. All estimated EC values for UNIVIS-F and UNIVIS-A were > 0.1 g L^{-1}, which is defined as practically non-toxic (United Nations, 2011). UNIVIS-A was more toxic than UNIVIS-F to embryo-larval *Xenopus* and this increased toxicity is hypothesized to be in part due to increased constituents (e.g., F1-F4 hydrocarbons and sulphur) in the aged oil. To our knowledge, this was the first study to assess the toxicity of UNIVIS HVI 13 oil to aquatic organisms. Overall, based on the derived EC values for UNIVIS-F and UNIVIS-A, we can conclude that the acute toxicity of the water-soluble constituents of UNIVIS HVI 13 pose minimal hazard to aquatic organisms.

Chemical analysis from Chapter 2 of this thesis confirmed the presence of PAHs in the WAF of UNIVIS HVI 13. Due to the presence of PAHs in the WAF, it was hypothesized that ecologically relevant intensities of UV light could increase the acute toxicity of the oil. Amphibians were chosen as the test organism for Chapter 3 because 1) amphibian populations are in decline due to many factors including climate change and anthropogenic pollution, 2) amphibians are found in aquatic habitats contaminated with PAHs and potentially UNIVIS HVI 13, and 3) early-life stages of amphibians are sensitive to photo-induced effects as they develop in shallow vernal pools and have a high potential to accumulate contaminants. There is potential for amphibians to be exposed to PAHs and UV radiation simultaneously in the environment, and

studying the effects of multiple stressors on amphibians is critical to further understanding the global decline. UV light can increase the toxicity of individual PAHs and petroleum-products containing complex mixtures of PAHs; therefore, including a UV light component for the assessment of UNIVIS HVI 13 was warranted. Currently, data gaps exist for assessing the photo-induced effects of PAHs in amphibians, and assessing whether the model amphibian *Xenopus* is a good predictor of PAH photo-induced toxicity in non-model native species such as the wood frog. Most risk assessments use toxicity data based on *Xenopus*; however, *Xenopus* has different genetics, life history, and physical characteristics as compared to the Ranidae family of frogs. For these reasons, Chapter 3 of this thesis investigated whether ecological relevant intensities of UV light increase the toxicity of three individual PAHs (anthracene, benzo(a)pyrene, naphthalene), or the WAF of an environmentally relevant PAH mixture, UNIVIS HVI 13 to two amphibian species, *Xenopus* and wood frog.

In contrast to recent studies reporting the photo-enhanced toxicity of oil products, UV light did not enhance the acute toxicity of UNIVIS HVI 13 WAF in larval *Xenopus* or wood frog. We concluded that the results were likely due to low concentrations of phototoxic PAHs in the WAF. Similar to studies with larval fish and invertebrates, UV light increased the acute toxicity of benzo(a)pyrene and anthracene in *Xenopus* and wood frog. Mortality occurred rapidly at higher concentrations of both compounds, with 80-100% mortality occurring within 2 h of UV light treatment. Without UV, mortality was low for both species when exposed to anthracene or benzo(a)pyrene across all concentrations tested (0-15%). *Xenopus* were more sensitive to the photo-induced toxicity of anthracene than wood frog, with increased mortality following UV exposure at 2, 20 and 200 $\mu g\ L^{-1}$ anthracene, while wood frogs were more sensitive to the photo-induced toxicity of benzo(a)pyrene than *Xenopus*, with increased mortality following exposure to 10 and 100 $\mu g\ L^{-1}$ benzo(a)pyrene and UV light. Body burden measurements of parent compounds of anthracene and benzo(a)pyrene at 8 h allowed us to compare uptake between species. Overall, wood frogs accumulated higher concentrations of both anthracene and benzo(a)pyrene at 8 h compared to *Xenopus.* Due to the increased body burden of anthracene in wood frog, the increased mortality of *Xenopus* in the presence of anthracene and UV light cannot be explained by species-specific differences in body burden. However, there was a significant positive correlation between tissue concentration of anthracene at 8 h and mortality at 96 h in *Xenopus*, suggesting that lower tissue concentrations of anthracene at 8 h may be able to elicit

photo-induced toxicity in *Xenopus* compared to wood frog. Conversely, the increased mortality of wood frog in the presence of benzo(a)pyrene and UV light could be explained by species-specific differences in body burden.

Exposure to PAHs and UV light resulted in sub-lethal effects on morphometric parameters, specifically whole-body length and incidence of deformities. Wood frogs exposed to 20 and 200 μg L^{-1} anthracene followed by exposure to UV light had decreased total body length compared to PAH exposure alone. Although there was no interaction between PAHs and UV light on *Xenopus* morphometrics or deformities, UV light treatment alone resulted in decreased *Xenopus* whole-body length and significantly increased the incidence of deformities in *Xenopus*. We hypothesize that the increased sensitivity of *Xenopus* to UV light treatment alone compared to wood frog may be due to differences in morphology (i.e., skin pigmentation) and physiology (i.e., photolyase expression) between *Xenopus* and wood frog at the tadpole stage.

Based on the significant mortality to low concentrations of anthracene, and the increased sensitivity to UV light alone in Chapter 3, *Xenopus* allows for conservative comparisons to the wood frog when assessing the photo-induced toxicity of PAHs. The results of Chapter 3 suggest that *Xenopus* is useful for assessing toxicity of PAHs and UV light exposures with lethality as an endpoint but may not be applicable for assessing and comparing sub-lethal responses for other amphibian species. Overall, with regards to studying photo-induced toxicity of PAHs in amphibians, we conclude that *Xenopus* is better suited for determining exposure thresholds for lethality and for generating data for risk assessments, but less suited for studies elucidating modes of action and sub-lethal effects on development.

4.2 Limitations of research and future directions

This research evaluated the photo-induced toxicity of PAHs in early life stages of larval amphibians using 96 h toxicity tests. The early larval stages (early premetamorphosis) were chosen in order to follow standardized guidelines (ASTM, 2012) for acute exposures in amphibians and to compare our results to other studies in this field of research (Kagan *et al.*, 1984; Fernandez and l'Haridon, 1994; Hatch and Burton, 1998; Monsoon *et al.*, 1999). We did not evaluate photo-induced toxicity of our test compounds on later metamorphic stages of amphibians, when there are dramatic changes in morphology and physiological processes. Early exposure to UVB radiation decreased immune function in later developmental stages of the

striped marsh frog (*Limnodynastes peronei*), demonstrating that early UV exposure can have carry-over effects on later stages even if the stressor has no immediate effect (Ceccato *et al.*, 2016). Similarly, exposure of embryonic/larval stage mahi-mahi (*Coryphaena hippurus*) to a ΣPAH concentration of 1.2 μg L^{-1} resulted in decreased swimming performance, but this adverse effect did not manifest until ~25 days following exposure. In our study, early life stage tadpoles were exposed to PAHs and UV in acute toxicity tests (96 h) and individuals were not monitored long-term to evaluate any potential latent adverse effects. It would be warranted to conduct follow-up studies based on sub-lethal combinations of PAHs and UV and evaluating additional endpoints such as swimming behavior, predator avoidance, and immunocompetence at additional time points following exposure to determine whether these processes are delayed or permanently affected.

There are also recent studies examining the effects of UV exposure on the immune function of aquatic organisms. Enhanced levels of UVB caused adverse effects on growth, body condition and immune responses in three-spined sticklebacks (*Gasterosteus aculeatus*) (Vitt *et al.*, 2017), and UVB exposure negatively affected the immune system and promoted nuclear abnormalities in bullfrog tadpoles (Franco-Belussi *et al.*, 2018). Moreover, the adverse effects of UV on immune function are not usually observed immediately following exposures but instead carried-over to the adult life-stages of fish or metamorphs in amphibians (Ceccato *et al.*, 2016; Jokinen et al, 2008). Strong evidence also exists that PAH exposure and PAH metabolites, particularly B(a)P can induce immunotoxicity in fish and mammals (White *et al.*, 1994; Carlson and Zelikoff, 2002; Gao *et al.*, 2005; Reynaud and Deschaux, 2006; Moller *et al.*, 2014). There is high potential that exposure of tadpoles to both PAHs and UV could compromise the immune capacity of tadpoles and resulting juvenile frogs, which has the potential to increase their susceptibility to pathogens and risk of disease. Our study examined the acute effects of PAH and UV exposure on growth and confirmed PAH uptake in early life stage tadpoles but did not consider immediate or latent effects on immune function. Given the evidence in fish for immunosuppression by both UV and PAH, further studies should determine impacts of these stressors on immune function in amphibians and elucidate the mechanisms through which immune system dysfunction may influence disease susceptibility.

Another important consideration in interpretation of our data is that we only assessed potential photosensitization rather than photomodification of anthracene and benzo(a)pyrene. As

stated in Chapter 1, it generally considered that photosensitization is the most important mechanism for the photo-induced toxicity of PAHs to aquatic organisms; however, photo-modification has also been recognized as an important mechanism of toxicity in invertebrates, plants and bacteria (McKonkey *et al.,* 1997; Choi and Oris, 2003; Brack *et al.,* 2003; Lampi *et al.,* 2006). Two photoproducts of benzo(a)pyrene (1,6- and 3,6-benzo(a)pyrenequinone), and a photoproduct of anthracene (benz(a)anthraquinone) were highly toxic to *Daphnia magna* (Lampi *et al.,* 2006). For this reason, a follow-up study could evaluate the effects of anthracene and benzo(a)pyrene photomodification to tadpoles and compared to photosensitization results obtained in this thesis research. Additionally, this study only measured parent concentrations of anthracene and benzo(a)pyrene for body burden analysis rather than metabolites or photo-products formed during metabolism or photosensitization. Previous work has shown that photo-induced toxicity is based off concentrations of parent compound, and that phase I and II metabolites of PAHs play minimal role in the photosensitization mechanism of action (Roberts *et al.,* 2017). That being said, quantification of quinone photoproducts of anthracene and benzo(a)pyrene would provide valuable information on the photosensitization mechanism of toxicity observed in this study. Without quantification of quinones and other photoproducts, we cannot directly link observed effects with specific compounds. However, based on the extensive literature on photosensitization of PAHs and design of the study in Chapter 3, we can speculate that observed photo-induced toxicity of anthracene and benzo(a)pyrene in tadpoles is due to photosensitization of parent PAHs.

Although the acute toxicity of UNIVIS HVI 13 WAF to aquatic organisms was minimal, the estimated EC values for specific hydrocarbon fractions (F1-F3) generated in thesis Chapter 2 indicated oil hydrocarbons may pose a hazard to aquatic organisms if they reached aquatic ecosystems via groundwater migration. However, based on the low water solubility and short half-life of the oil, the probability of oil hydrocarbons reaching off-site aquatic ecosystems is expected to be low. The risk assessment of UNIVIS HVI 13 is a collaborative project and other research projects are currently assessing the environmental fate and migration of UNIVIS HVI 13 in soil. Future research into the environmental fate of UNIVIS HVI 13 in soil would provide a better understanding of the risk of oil hydrocarbons reaching aquatic organisms in nearby surface water. In addition, potential chronic toxicity of the oil to aquatic organisms remains unknown. The oil leaking from actuator valves at compressor stations along pipelines is occurring in

continuous pulses 365/year; therefore, there is potential for soil saturation, continuous migration to nearby aquatic ecosystems, and bioaccumulation in aquatic organisms. For these reasons, further research should assess and compare the chronic toxicity of fresh and aged UNIVIS HVI 13 WAF.

Our findings strengthen once more, that UV can enhance the toxicity PAHs to increase mortality and affect the growth of developing amphibians. Future research should focus on understanding the underlying physiological mechanisms of these detrimental effects. Based on results in thesis Chapter 3, it is hypothesized that the sensitivity of larval *Xenopus* to UV light is due to their translucent skin pigmentation at this life stage and this hypothesis could be addressed by quantifying and comparing melanin and/or pigment abundance in the two amphibian species. A previous study performed non-invasive precise melanin quantification in human subjects using the spectrum resolution method and a spectrophotometer (Masuda *et al.,* 2009). In animals, pigment quantification was performed in the brown shrimp *Crangon crangon* using the pigment cover (PiC) method in Image J (Siegenthaler *et al.,* 2017). It was also suggested that the sensitivity of *Xenopus* to UV light is due to low expression of photolyase during development (Hatch and Burton, 1998). Comparing basal photolyase activity or gene expression across species as well as how photolyase responds following sub-lethal exposure to UV light would provide some insight into why there are species differences in sensitivity to UV light. Finally, one of the major mechanisms of PAH photo-induced toxicity is through oxidative damage (Choi and Oris, 2000; Fu *et al.,* 2012). Whole-body ROS generation, antioxidant enzyme activity, lipid peroxidation and related gene expression could be measured and compared between *Xenopus* and wood frog to determine if oxidative stress responses to PAH and UV exposure correlate with the degree of photo-induced toxicity observed for each species.

4.3 Applicability of research findings

For this purpose of this thesis research, we successfully designed and implemented an amphibian embryo-larval bioassay as a tool to evaluate and compare effects of PAHs and UV on amphibian development between two amphibian species. This approach could be used to assess the potential photo-induced toxicity of other contaminants in aquatic systems that pose a risk to amphibian populations (i.e., pesticides, pharmaceuticals, personal care products). This research also provided the first toxicity data for wood frogs exposed to PAHs and UV light, PAH body

burden data for two amphibian species, and the first toxicity data for an environmentally relevant oil used in Canada (UNIVIS HVI 13).

The acute toxicity of UNIVIS HVI 13 to aquatic organisms was assessed in this thesis as part of a collaborative industry project. Due to concern of oil reaching aquatic receptors, industry partners requested the hazard of UNIVIS HVI 13 to aquatic organisms be evaluated. This research provided industry partners with 1) toxicity values for six aquatic organisms (Microtox, *Daphnia*, duckweed, *Xenopus*, wood frog and fathead minnow) exposed to fresh UNIVIS HVI 13 and 2) toxicity values for aged UNIVIS HVI 13 in the model organisms *Xenopus* and *Daphnia*. These toxicity values may be used for further evaluation and risk assessment of UNIVIS HVI 13.

Although UV light did not enhance the toxicity of our environmentally relevant PAH mixture (UNIVIS HVI 13), photo-induced toxicity is still an important consideration when assessing the hazard of PAH mixtures in the environment. Oil spills are a major anthropogenic source of PAH exposure in the environment. For example, Accidental oil spills like the Deepwater Horizon oil spill in the Gulf of Mexico can have drastic effects on an ecosystem, and UV radiation has been shown to play a significant role on oil toxicity (Alloy *et al.*, 2016). In Canada, the Alberta oil sands is the third largest oil reservoir in the world, and the deposits underlie 142,200 km^2 of boreal forest in northern Alberta (CAPP, 2016; AEMERA and ECCC 2016). Crude oil production in Alberta oil sands is predicted to rise over the next 15 years, and consequently, the environmental impacts associated with oil sands extraction will continue to impact the surrounding ecosystems. The major pipelines that cross ecoregions in Canada also inevitably release contaminants (i.e., PAHs) to surrounding ecosystems. The recent Husky oil spill in Saskatchewan is an example of a major exposure pathway for complex PAH mixtures releasing into the environment. Oil spills expose aquatic organisms to PAHs and occur where sunlight can penetrate surface water, simultaneously exposing these organisms to PAHs and UV radiation. Although acute toxicity tests employing mortality as an endpoint do have limitations in fully understanding influence of UV on PAH-toxicity interactions, the results of this research provide a foundation for longer-term laboratory studies or mesocosm studies to further examine such interactions and aid in understanding implication of UV radiation in risk management of PAH and PAH mixtures. The research presented in this thesis, together with several other studies, supports the conclusion that a UV component is important to a complete understanding of the

toxicity of PAHs and complex mixtures containing PAHs to aquatic organisms, or hazard may be greatly underestimated.

4.4 Concluding statement

Aquatic organisms are rarely exposed to just one stressor in their aquatic environment - rather multiple stressors that can interact to result in greater toxicological effects then expected from either of the stress types alone. Specifically, assessing multiple stressors in amphibians is warranted due to the alarming global decline in populations, which is not necessarily due to a single stressor, but rather multiple stressors (including increased pollution and UV radiation) interacting together. UV light can substantially increase the toxicity of contaminants such as PAHs to aquatic organisms and the challenge for future studies will therefore be to include aspects of combined stressors in effect and risk assessment of chemicals in the environment. Studying multiple stressors in amphibians is critical to gaining a better understanding of the population decline, and producing valuable data that can be used for developing strategies to prevent further loss.

APPENDICES

Appendix A: Average chemical analysis (n=3) of polycyclic aromatic hydrocarbons (PAHs) in 1:1 loadings of fresh and aged UNIVIS HVI 13 WAF. The "<" indicates concentrations reported below limit of detection for respective compound.

Polycyclic Aromatic Hydrocarbon (PAH)	Oil type (concentration)	
	1:1 Fresh ($\mu g\ L^{-1}$)	1:1 Aged ($\mu g\ L^{-1}$)
Benzo[a]pyrene equivalency	<0.010	0.093
Acenaphthene	<0.10	<1.0
Acenaphthylene	<0.10	7.0
Acridine	<0.20	3.7
Anthracene	0.037	0.48
Benzo(a)anthracene	<0.0085	<0.085
Benzo(b&j)fluoranthene	<0.0085	<0.085
Benzo(k)fluoranthene	<0.0085	<0.085
Benzo(g,h,i)perylene	<0.0085	<0.085
Benzo(c)phenanthrene	<0.050	<0.50
Benzo(a)pyrene	<0.0075	<0.075
Benzo(e)pyrene	<0.050	<0.50
Chrysene	<0.0085	<0.0.085
Dibenz(a,h)anthracene	<0.0075	<0.075
Fluoranthene	<0.010	<0.10
Fluorene	0.13	1.6
Indeno(1,2,3-cd)pyrene	<0.0085	<0.085
2-Methylnaphthalene	0.65	1.5
Naphthalene	0.18	<1.0
Phenanthrene	0.1	2.7
Perylene	<0.050	<0.50
Pyrene	<0.020	<0.20

Appendix B: Average chemical analysis (n=3) of CCME regulated metals in 1:1 loadings of fresh and aged UNIVIS HVI 13 WAF. The "<" indicates concentrations reported below limit of detection for respective compound.

Metal	Oil type (concentration)	
	1:1 Fresh ($mg\ L^{-1}$)	**1:1 Aged ($mg\ L^{-1}$)**
Total Aluminum (Al)	0.045	0.046
Total Antimony (Sb)	<0.00060	<0.00060
Total Arsenic (As)	0.00031	0.00035
Total Barium (Ba)	0.044	0.041
Total Beryllium (Be)	<0.0010	<0.0010
Total Boron (B)	0.086	0.034
Total Cadmium (Cd)	<0.020	<0.020
Total Calcium (Ca)	41	39
Total Chromium (Cr)	<0.0010	0.0033
Total Cobalt (Co)	<0.00030	<0.00030
Total Copper (Cu)	0.0025	0.0074
Total Iron (Fe)	<0.060	0.064
Total Lead (Pb)	<0.00020	0.00070
Total Lithium (Li)	<0.020	<0.020
Total Magnesium (Mg)	20	19
Total Manganese (Mn)	<0.0040	<0.0040
Total Molybdenum (Mo)	0.0034	0.0018
Total Nickel (Ni)	0.0012	0.0022
Total Phosphorus (P)	0.12	0.35
Total Potassium (K)	3.9	3.7
Total Selenium (Se)	0.00042	0.00059
Total Silicon (Si)	0.69	0.94
Total Silver (Ag)	<0.00010	<0.00010
Total Sodium (Na)	33	930
Total Strontium (Sr)	0.26	0.23
Total Sulphur (S)	36	1300
Total Thallium (Tl)	<0.00020	<0.00020
Total Tin (Sn)	<0.0010	<0.0010
Total Titanium (Ti)	<0.0010	0.0015
Total Uranium (U)	0.00075	0.0011
Total Vanadium (V)	<0.0010	<0.0010
Total Zinc (Zn)	<0.0030	0.54

Appendix C: Model-fitting parameters and associated EC standard errors for three-parameter log-logistic (LL-3) model used for UNIVIS HVI 13 toxicity tests. "b" indicates relative slope around "e", the EC_{50}. Upper and lower limits were set at 100 and 0 for all toxicity value estimates.

A) Daphnia mortality (UNIVIS-F)

	Estimate	**Std. error**	**t-value**	**p-value**
b: (intercept)	-1.27	0.25	-5.09	0.00
e: (intercept)	114.04	16.91	6.74	0.00

Residual standard error (degrees of freedom):
13.87 (19)

Effective concentration	**Std. error**
EC_{10}	7.99
EC_{25}	11.76
EC_{50}	16.91
EC_{90}	221.21

B) Daphnia mortality (UNIVIS-A)

	Estimate	**Std. error**	**t-value**	**p-value**
b: (intercept)	-1.52	0.24	-6.35	0.00
e: (intercept)	361.81	41.94	8.63	0.00

Residual standard error (degrees of freedom):
11.76 (19)

Effective concentration	**Std. error**
EC_{10}	21.39
EC_{25}	27.92
EC_{50}	41.94
EC_{90}	400.02

C) Microtox inhibition (UNIVIS-F)

	Estimate	**Std. error**	**t-value**	**p-value**
b: (intercept)	-1.15	0.08	-14.51	0.00
e: (intercept)	246.03	14.80	16.62	0.00

Residual standard error (degrees of freedom):
6.83 (28)

Effective concentration	**Std. error**
EC_{10}	5.05
EC_{25}	8.01
EC_{50}	14.80
EC_{90}	251.86

D) Duckweed fresh weight inhibition (UNIVIS-F)

	Estimate	Std. error	t-value	p-value
b: (intercept)	-1.93	0.85	-2.26	0.04
e: (intercept)	775.38	212.92	3.64	0.00

Residual standard error (degrees of freedom):
27.13 (16)

Effective concentration	Std. error
EC_{10}	119.91
EC_{25}	132.25
EC_{50}	212.92
EC_{90}	1576.44

E) Duckweed growth inhibition (UNIVIS-F)

	Estimate	Std. error	t-value	p-value
b: (intercept)	-0.63	0.06	-10.95	0.00
e: (intercept)	1171.11	166.75	7.02	0.00

Residual standard error (degrees of freedom):
4.00 (16)

Effective concentration	Std. error
EC_{10}	7.83
EC_{25}	19.36
EC_{50}	166.75
EC_{90}	17108.83

F) Xenopus mortality (UNIVIS-A)

	Estimate	Std. error	t-value	p-value
b: (intercept)	-0.32	0.13	-2.45	0.02
e: (intercept)	372.17	207.02	1.80	0.09

Residual standard error (degrees of freedom):
15.21 (19)

Effective concentration	Std. error
EC_{10}	1.04
EC_{25}	14.66
EC_{50}	207.02
EC_{90}	1.05e+06

G) Fathead minnow mortality (UNIVIS-F)

	Estimate	Std. error	t-value	p-value
b: (intercept)	-2.89	676.26	-4.27	0.00
e: (intercept)	3.11e+04	3.32e+04	0.94	0.36

Residual standard error (degrees of freedom):
10.58 (26)

Effective concentration	Std. error
EC_{10}	45.42
EC_{25}	1.37e+03
EC_{50}	9.92e+04
EC_{90}	4.20e+08

Appendix D: Average chemical analysis (*n*=3) of petroleum hydrocarbon fractions (F1-F4) in 1:10 and 1:1 loadings of UNIVIS HVI 13 WAF.

Petroleum hydrocarbon fraction (concentration)	WAF loading	
	1:10	**1:1**
F1 (mg L^{-1})	0.35	0.51
F2 (mg L^{-1})	1043	2.18
F3 (mg L^{-1})	1033	3.0
F4 (mg L^{-1})	5.60	< 0.20

Appendix E: Average chemical analysis (*n* =3) of PAHs in 1:10 and 1:1 loadings of UNIVIS HVI 13 WAF.

Polycyclic Aromatic Hydrocarbon (PAH)	WAF loading (concentration)	
	1:10 (μg L^{-1})	1:1 (μg L^{-1})
Benzo[a]pyrene equivalency	0.65	<0.010
Acenaphthene	3.1	<0.10
Acenaphthylene	<1.0	<0.10
Acridine	9.5	<0.20
Anthracene	0.54	0.037
Benzo(a)anthracene	<0.085	<0.0085
Benzo(b&j)fluoranthene	<0.085	<0.0085
Benzo(k)fluoranthene	<0.085	<0.0085
Benzo(g,h,i)perylene	<0.085	<0.0085
Benzo(c)phenanthrene	101	<0.050
Benzo(a)pyrene	<0.075	<0.0075
Benzo(e)pyrene	<0.50	<0.050
Chrysene	<0.085	<0.0085
Dibenz(a,h)anthracene	<0.075	<0.0075
Fluoranthene	0.7	<0.010
Fluorene	27	0.13
Indeno(1,2,3-cd)pyrene	<0.085	<0.0085
2-Methylnaphthalene	527	0.65
Naphthalene	125	0.18
Phenanthrene	<0.50	0.1
Perylene	<0.50	<0.050
Pyrene	0.78	<0.020

Appendix F: Average chemical analysis (*n* =3) of CCME regulated metals in 1:10 and 1:1 loadings of UNIVIS HVI 13 WAF.

Metal	WAF loading (concentration)	
	1:10 (mg L^{-1})	**1:1 (mg L^{-1})**
Total Aluminum (Al)	0.016	0.045
Total Antimony (Sb)	<0.00060	<0.00060
Total Arsenic (As)	0.00096	0.00031
Total Barium (Ba)	0.042	0.044
Total Beryllium (Be)	<0.0010	<0.0010
Total Boron (B)	0.047	0.086
Total Cadmium (Cd)	0.000046	<0.020
Total Calcium (Ca)	40	41
Total Chromium (Cr)	<0.0010	<0.0010
Total Cobalt (Co)	<0.00030	<0.00030
Total Copper (Cu)	0.0022	0.0025
Total Iron (Fe)	0.065	<0.060
Total Lead (Pb)	<0.00020	<0.00020
Total Lithium (Li)	<0.020	<0.020
Total Magnesium (Mg)	19	20
Total Manganese (Mn)	<0.0040	<0.0040
Total Molybdenum (Mo)	0.0017	0.0034
Total Nickel (Ni)	0.0011	0.0012
Total Phosphorus (P)	0.10	0.12
Total Potassium (K)	4.0	3.9
Total Selenium (Se)	0.00052	0.00042
Total Silicon (Si)	1.4	0.69
Total Silver (Ag)	<0.00010	<0.00010
Total Sodium (Na)	27	33
Total Strontium (Sr)	0.23	0.26
Total Sulphur (S)	33	36
Total Thallium (Tl)	<0.00020	<0.00020
Total Tin (Sn)	<0.0010	<0.0010
Total Titanium (Ti)	0.0019	<0.0010
Total Uranium (U)	0.0013	0.00075
Total Vanadium (V)	0.0011	<0.0010
Total Zinc (Zn)	0.0055	<0.0030

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