ASSESSMENT OF ARYL HYDROCARBON RECEPTOR MEDIATED TOXICITY OF BENZOTRIAZOLE ULTRAVIOLET STABILIZERS (UV-P, UV-9, UV-090) TO FISHES

HUNTER MITCHEL JOHNSON
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HUNTER M. JOHNSON

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Dr. Steve B. Wiseman Thesis Supervisor	Associate Professor	Ph.D.
Dr. Tony Russell Thesis Examination Committee Member	Associate Professor	Ph.D.
Dr. Trushar R. Patel Thesis Examination Committee Member	Associate Professor	Ph.D.
Dr. Dmytro Yevtushenko Chair, Thesis Examination Committee	Associate Professor	Ph.D.

DEDICATION

I would like to dedicate this thesis to my parents Curtis and Tracy, along with all my other family and friends who continue to support me during my journey.

ABSTRACT

Benzotriazole ultraviolet stabilizers (BUVSs) are a class of chemical contaminants used to help counter UV-induced damage to manufactured goods, especially plastics. The broad applicability of BUVSs has resulted in their ubiquitous detection in aquatic ecosystems and biota. Although BUVSs are detected globally in aquatic ecosystems, a limited number of studies have investigated the potential toxic effects of BUVSs to fish. Of the limited toxicity data for BUVSs, studies suggest that certain BUVSs might dysregulate the aryl hydrocarbon receptor (AhR) causing early life-stage toxicity in fishes. Therefore, the objectives of this study were to use *in vivo* and *in vitro* approaches to characterize the toxicity of 2-(benzotriazol-2-yl)-4-methylphenol (UV-P), 2-(Benzotriazol-2-yl)-4-methyl-6-prop-2-enyl-phenol (UV-9), and 2-[3-(2H-benzotriazol-2-yl)-4-hydroxyphenyl]ethyl methacrylate (UV-090) as agonists of the AhR across a phylogenetically diverse number of fish species. *In vivo* toxicity was assessed by exposing zebrafish (*Danio rerio*) to BUVSs by microinjection and toxicities were assessed by recording embryo mortality and malformations including yolk sac and pericardial edema, and spinal curvature. Each of the tested BUVSs caused dose-dependent increases in embryo mortality following exposure. *In vitro* activation of the AhR by BUVSs was determined with a luciferase reporter gene (LRG) assay using COS-7 cells transfected with the AhR of zebrafish or eight other species. Results confirm that UV-P and UV-9, cause toxicity via AhR activation whereas, UV-090 lacked the ability to activate the AhR, indicating that its toxicity is independent of the AhR. Furthermore, interspecies differences in sensitivity to AhR activation by BUVSs was observed. Overall, this study fills knowledge gaps regarding the potential toxic effects of BUVSs to fishes and can help guide improved objective assessment of risks posed by BUVS that have AhR agonistic properties for the protection of Canada's diverse population of fish.

CONTRIBUTION OF AUTHORS

Hunter Johnson is the primary author of chapters 1-3. Dr. Zhe Lu completed the chemistry analysis for chapter 2 and wrote section 2.2.3. Justin Dubiel aided in experimental procedures and edited chapter 2. Andreas Eriksson contributed to the interpretation and statistics of data along with editing chapter 2. Cameron Collins helped with experimental procedures of chapter 2. Dr. Steve Wiseman and Dr. Jon Doering contributed to the experimental design, data interpretation, and scientific input of chapter 2 and edited chapters 1-3.

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I am also extremely grateful to our previous post doc Jon Doering for his dedication and patience towards teaching, provoking thoughtful discussion, and continuous feedback throughout the entirety of my undergrad and graduate studies. I would like to extend my gratefulness to each and every Wiseman lab member that has provided guidance and created a lab dynamic that can not be replaced.

I am deeply indebted to my family, particularly my parents Curtis and Tracy, who have continued to support me in everything that I do and push me to higher limits. I must say that my success thus far would not have been possible without your continuous support.

I would lastly like to thank my girlfriend Bailey who has cheered me on since the beginning and for all the love and support you have provided me during this chapter of my life.

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LIST OF ABBREVIATIONS

AOP	Adverse outcome pathway
AhR	Aryl hydrocarbon receptor
AhRR	Aryl hydrocarbon receptor repressor
AR	Androgen receptor
ARNT	Aryl hydrocarbon receptor nuclear translocator
BFR	Brominated flame retardant
CAS	Chemical Abstracts Service registry number
CAT	Catalase
cDNA	Complementary deoxyribonucleic acid
Cyp1a1	Cytochrome P450 1A1
DLC	Dioxin-like compounds
DMSO	Dimethylsulfoxide
EC	Effective concentration
ECThreshold	Effective concentration threshold
ELS	Early life stage
EROD	Ethoxyresorufin-O-deethylase
GC-MS	Gas chromatography-mass spectrometry
GCLC	Glutamate-cysteine ligase catalytic unit
GPX	Glutathione peroxidase
GST	Glutathione s-transferase
Hpf	Hours post Fertilization
HPLC	High performance liquid chromatography
HPT	Hypothalamic-pituitary-thyroid
Hsp90	Heat shock protein 90
LD	Lethal Dose
LogKow	Logarithmic octanol-water partitioning coefficient
LOD	Limit of detection
LRG	Luciferase reporter gene
MAE	Mean Absolute Error
MAPE	Mean Absolute Percentage Error
MIE	Molecular initiation event
MW	Molecular weight
OECD	Organization for economic co-operation and development
PAH	Polyaromatic hydrocarbons
PCB	Polychlorinated Biphenyls
PCDD	Polychlorinated dibenzo-p-dioxins
PCDF	Polychlorinated dibenzofurans
qAOP	Quantitative adverse outcome pathway

qPCR	Quantitative real-time polymerase chain reaction
ReP	Relative Potency
ReS	Relative Sensitivity
SD	Standard deviation
SE	Standard Error
SOD	Superoxide dismutase
SPE	Solid-phase extraction
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
UV	Ultraviolet
UV-X	Benzotriazole ultraviolet stabilizer where X identifies the specific compound
XAP-2	Hepatitis B virus X-associated protein
XRE	Xenobiotic response elements

NOTE: Throughout the thesis gene names are given in uppercase italics, mRNA is reported as lower-case italics, and protein are upper case non-italicized

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1 Environmental Pollution and Aquatic Ecosystems

As the global population continues to increase, society is constantly exploring innovative technologies to enhance our way of life and the products that shape it. As a result, a vast number of anthropogenically generated compounds are utilized to evolve and advance the products that we rely on. Many times, these compounds are utilized in large sectors such as military, agricultural, medical, and industrial departments.[1] An unintended consequence of the widespread use of anthropogenic compounds is contamination of our freshwater ecosystems, which can have negative impacts on their resident biota.[2]

One of the largest vectors of pollution both in marine and freshwater ecosystems is plastic materials which threaten all waterbodies and is cause for environmental concern.[3,4] In 2015 alone, a staggering 380 million tonnes of plastics were produced and due to inadequate disposal practices, a significant portion of this plastic entered water bodies.[5,6] Once these plastic products are sequestered into aquatic environments, they undergo weathering and fragmentation into microplastics (<5 mm) through mechanical and photochemical processes.[7] While microplastics pose a physical threat alone, only recently have scientists realized that chemicals used (e.g., Bisphenol A) to improve the durability, longevity, and quality of plastics can leach out, potentially affecting the organisms that reside in plastic contaminated water.[8,9] One specific contaminant that has gained considerable interest among scientists and regulatory bodies is benzotriazole ultraviolet stabilizers (BUVSs), which are added to plastics to prevent ultraviolet (UV)-induced discoloration and degradation.[10-13]

1.2 Characteristics of Benzotriazole Ultraviolet stabilizers

Benzotriazole ultraviolet stabilizers are added to a variety of industrial and consumer items to help mitigate damage and discoloration caused by UV light.[10,11,14-21] Structurally, BUVSs contain a benzotriazole moiety that is attached to a 2-hydroxyphenol group that can contain various alkyl substituents which form different BUVSs (**Figure 1-1**).[19,22,23] As a result of their diverse structure, BUVSs can absorb a broad range of UV light (280-400 nm) making them effective additives to a range of goods including cosmetics, coatings, adhesives, waxes, paints, motor oils, rubber, and plastics.[10,11,14-21] There are numerous BUVSs, all of which are structurally different and have unique properties such as molecular weight, functional groups, and logarithmic octanol-water partitioning coefficients ($LogK_{ow}$) between 3.00 and 7.67.[24] As indicated by their range of $LogK_{ow}$, BUVSs display relatively high hydrophobic tendencies.

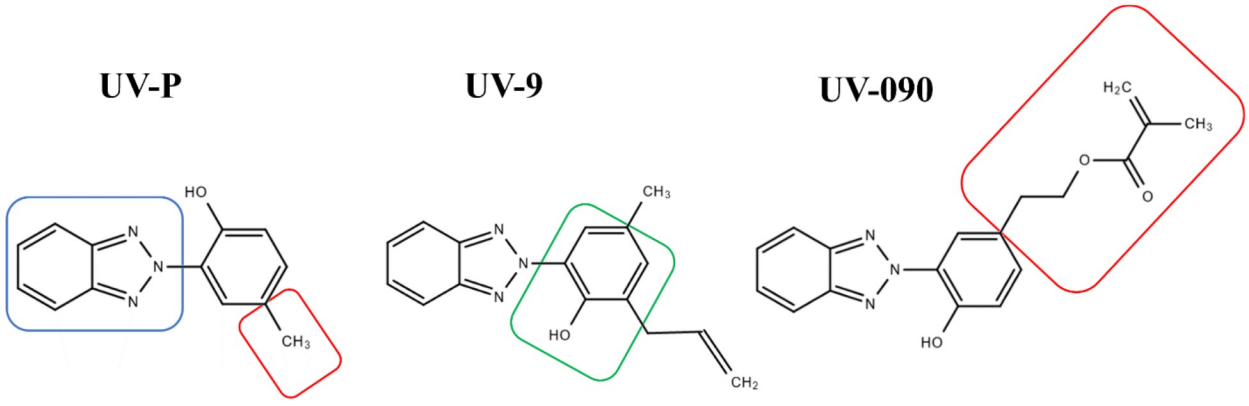
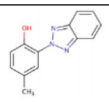
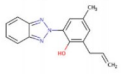
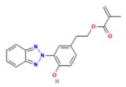
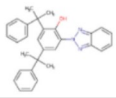
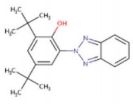
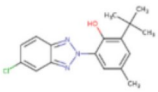
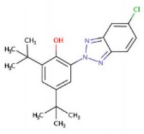
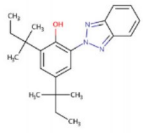
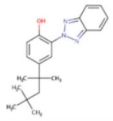
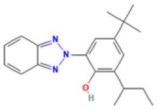
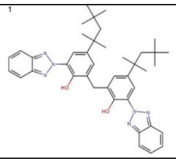


Figure 1-1: Chemical structure of benzotriazole ultraviolet stabilizers (BUVSs). Each BUVS contains a benzotriazole moiety (Blue), a 2-hydroxyphenol group (Green), and various alkyl substituents (Red) that are attached to the 2-hydroxyphenol group. Three BUVSs - UV-P, UV-9, and UV-090 - are the focus for this thesis however, there are several other benzotriazole ultraviolet stabilizers that are not depicted here.

1.3 BUVSs in the Environment

Due to the widespread usage of BUVSs, these contaminants are ubiquitously detected in aquatic ecosystems, globally. These contaminants have been identified and measured in various countries including India[25], China[26], Japan[13,27], Germany[28], Norway[29], and Canada[20,24]. Furthermore, BUVSs have been measured in different environmental matrices including water, sediment, wastewater treatment plant effluent, household dust, and biota (**Table 1-1**).[10,11,20,21,25,28,30] The detection of BUVSs in the environment and biota has raised alarm among scientists and regulatory bodies as their high volume of production, persistence, and ability to bioaccumulate suggests that BUVSs likely have the potential to reach high environmental concentrations. In May 2023, 2-(3,5-di-*tert*-amyl-2-hydroxyphenyl) benzotriazole (UV-328), a particular type of BUVS, was included in Annex A of the Stockholm Convention on persistent organic pollutants, a decision that will lead to a worldwide ban on production and usage of this chemical. This decision was driven by its extensive environmental transport and the potential for causing adverse effects on human health and the environment. BUVSs ability to bioaccumulate, and in some cases bio-magnify, stems primarily from their hydrophobicity ($\log K_{OW}$: 3.00 – 7.67).[12] As such, the stability, widespread usage, and global detection of BUVSs has caused concern for their potential toxicological effects on aquatic organisms, particularly fishes. However, there is a significant knowledge gap with respect to adverse effects of BUVSs on fishes, meaning it is essential that any potential toxicological effects be identified given that fish have economic, cultural, and ecological importance globally, including in Canada.

Table 1-1: Geographical location of benzotriazole ultraviolet stabilizers that have been detected in environmental matrices including sediment, surface water, wastewater & sewage, and aquatic organisms. This table is an updated version taken from Fujita, 2021.[31]

Name	Structure	Sediment	Surface water	Wastewater& sewage	Aquatic organisms
UV-P		USA[32] India[25,23] Japan[27] China[34] Canada[19] Germany[28]	India[25,33] Japan[27] China[35] Spain[36] Canada[24]	Portugal[37] Japan[27] China[35,38] Spain[36,37,39]	India[25] China[40] Philippines[41]
UV-9			India[25] Canada[24]		India[25] Philippines[41] Canada[24]
UV-090					Canada[24]
UV-234		Australia[42] Japan[27] China[34] Canada[19,24] Germany[28]	Australia[42] Canada[24]	Canada[43] China[38]	Philippines[41] China[40] Canada[24]
UV-320		USA[32] India[25,33] Japan[13] Canada[24]	India[25,33] Canada[24]	Portugal[37]	India[25] Japan[13] Philippines[41] Canada[24]
UV-326		USA[19,32] India[25,33] Japan[13,27] China[26,34] Canada[19] Germany[28] Spain[39]	India[25,33] Australia[42] Japan[27] Canada[24]	Japan[27] Portugal[37] Canada[43] China[26,38] Spain[36,37,39] Poland[44]	India[25] Japan[13] China[40] Philippines[41] Canada[21,24] Spain[39]
UV-327		USA[19,32] India[25,33] Australia[42] Japan[13,27] China[26,34] Canada[19] Germany[28]	India[25,33] Australia[42] Japan[27] Canada[24]	Portugal[37] Japan[27] Canada[43] China[26m38] Spain[36,37,39]	India[25] Japan[13] China[40] Philippines[41] Canada[24]
UV-328		USA[19,32] India[25,33] Australia[42] Japan[13,27] China[26,34] Canada[19,24] Germany[28] Spain[39]	India[25,33] Australia[42] Japan[27] Canada[24]	Portugal[37] Japan[27] Canada[43] China[26] Spain[36,39]	India[25] Japan[13] China[40] Philippines[41] Spain[39,45] Canada[24]
UV-329		Japan[27] China[34] Canada[19] Germany[28] India[33] Spain[39]	Australia[42] India[33] Canada[24]	Canada[43] Spain[36,37,39] China[38] Poland[44]	China[40] Philippines[41] Spain[39,45] Canada[21,24]
UV-350		Canada[24]	Canada[24]		Canada[21,24]
UV-360		Spain[39]		Spain[39]	Spain[39]

1.4 Adverse Effects of Benzotriazole Ultraviolet Stabilizers to Biota

The toxicological impacts of BUVSs on biota remains poorly understood. Research to date has demonstrated that BUVSs can disrupt a variety of physiological process in fishes, including dysregulation of processes related to the hypothalamic-pituitary-thyroid (HPT) axis, anti-androgenic activity, and disruption of sex steroid synthesis.[46-49] The BUVS, UV-328, induced alterations in the expression of genes associated with oxidative stress.[50] Some studies have shown that BUVSs can activate the aryl hydrocarbon receptor (AhR), which is the mechanism of toxicity that is the central focus of this thesis.[15,22,51,52]

Several BUVSs, including UV-P, UV-PS, UV-9, UV-090, UV-234, UV-320, UV-326, UV-327, UV-328, UV-329, UV-350, UV-571, and UV-360, have been assessed for their capacity to activate the AhR in mammals or fishes. Using yeast reporter gene and DR-EcoScreen cell assays, it was reported that four BUVSs, specifically UV-P, UV-9, UV-090, and UV-PS, function as agonists for both human and mouse AhRs.[15,22] A small number of studies have demonstrated that some BUVSs are able to activate the AhR of fish.[51,52] Exposure of zebrafish embryos to waterborne UV-P or UV-326 led to an increase in the transcript abundance of cytochrome P450 1A (*cyp1a*), a reliable indicator of AhR activation.[52] Additionally, another study demonstrated elevated transcript levels of *cyp1a* and *ahr2* in juvenile zebrafish exposed to UV-P and UV-329.[51] In either case, there were no increases in mortality or malformations at any of the tested concentrations.[51,52] In contrast to those findings, Japanese medaka exposed to foodborne UV-P exhibited no changes in transcript levels of *cyp1a*.[47] These findings collectively indicate that the ability of BUVSs to act as AhR agonists can vary among species.

1.5 Aryl Hydrocarbon Receptor

The AhR is a ligand-activated transcription factor that regulates the transcription of a suite of genes involved in physiological processes such as xenobiotic metabolism, cellular growth, and cell migration.[53,54] The inactive form of the AhR is localized to the cytoplasm of cells, in complex with multiple co-chaperone proteins, including two molecules of heat shock protein 90 (hsp90), hepatitis B virus X-associated protein (XAP-2), and p23, that maintain the AhR heterocomplex in a specific orientation that allows ligands to bind to it and prevents proteolytic degradation of the AhR (**Figure 1-2**).[55] Once a ligand binds to the AhR, a conformational change is initiated allowing for the AhR complex to be translocated into the nucleus where the co-chaperone complex dissociates, and the ligand bound AhR forms a heterodimer with the aryl hydrocarbon receptor nuclear translocator (ARNT) (**Figure 1-2**).[55] This AhR:ARNT complex can bind to xenobiotic response elements (XRE) located in the promoter regions of thousands of target genes which initiates recruitment of transcriptional machinery and permits transcription to commence.[53,55] One of the genes that is regulated by the AhR is *CYP1A*, which encodes for a CYP1A enzyme that is involved in the biotransformation and metabolism of various different lipophilic xenobiotic compounds, including AhR agonists like polycyclic aromatic hydrocarbons (PAHs) and Dioxins.[56,57] As such, *CYP1A* expression, measured as transcript abundance, protein abundance, or enzyme activity, has been widely accepted as a biomarker for activation of the AhR.[58] One of the methods to measure CYP1A activity is by performing an ethoxyresorufin-O-deethylase (EROD) assay, which quantifies the conversion of a fluorescent substrate, ethoxyresorufin, to a fluorescent product, resorufin, by CYP1A enzymes.[56,58] Another gene that is important in the regulation of AhR signalling is the AhR repressor (AhRR) which acts like the AhR but cannot bind ligands.[55] Rather than having an activation domain, the AhRR has a repression domain that regulates

transcription of AhR dependant genes through a negative feedback loop following its binding to XREs.[55] After successful activation of gene expression, the AhR is translocated back to the cytosol where it undergoes ubiquitination and degradation via the proteosome.[55,59]

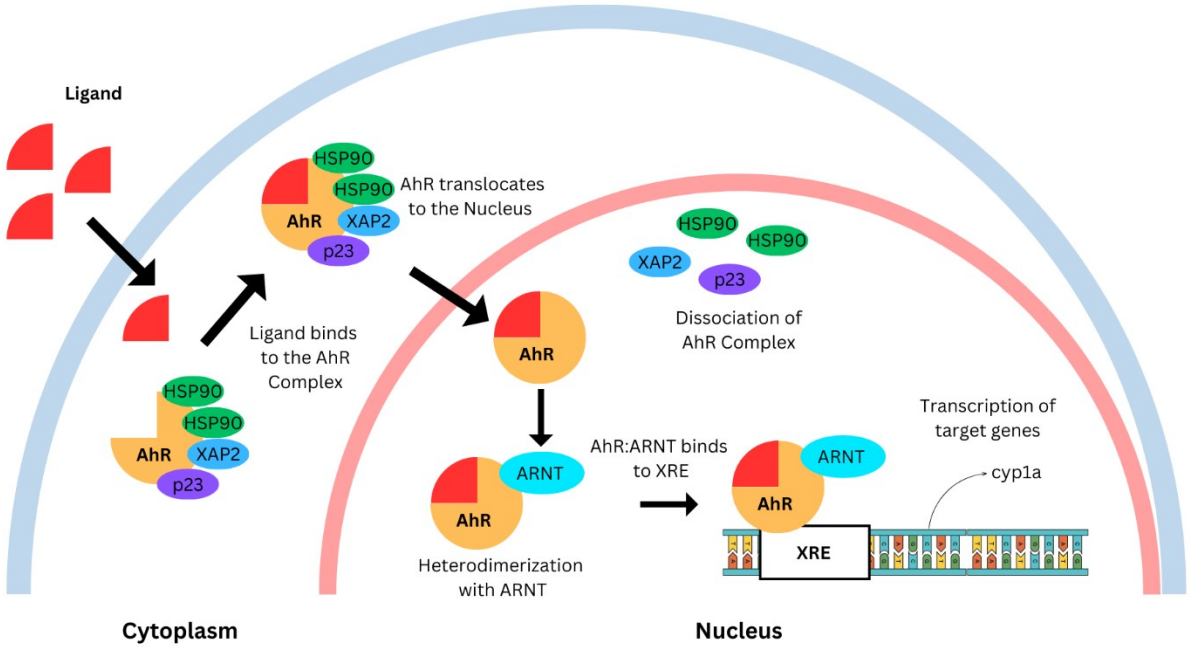


Figure 1-2: Basic illustration of the AhR pathway modified from Larigot et al., (2018).[55] Initially, a ligand enters the cell and binds with the AhR heterocomplex which includes co-chaperone proteins HSP90, XAP2, and p23. The ligand bound complex then is translocated into the nucleus where the complex dissociates. Following the dissociation of the AhR complex the ligand bound AhR heterodimerizes with the AhR nuclear translocator (ARNT) protein. The AhR:ARNT heterodimer then binds to xenobiotic response elements (XREs) initiating the transcription of targeted genes such as cytochrome P450 1a (*CYP1A*).

Various isoforms of the AhR exist in vertebrates (AhR1, AhR2, & AhR3); however, in fish, there is strong evidence that suggests that the activation of the AhR2 isoform is responsible for toxic effects during early life stages (ELS), although the AhR1 might also play a role in mediating

toxicities in certain species.[54,60-63] The various isoforms of the AhR exhibit distinct affinities for exogenous ligand binding and demonstrate diverse activities in response to ligand interactions.[64] In instances where species possess multiple AhR2 isoforms (AhR2α and AhR2β), the dominance of the AhR2β isoform in eliciting toxicity in ELS fish highlights the importance of selecting the appropriate AhR2 isoform for investigations on toxicity.[62] As a result of AhR2 dysregulation, fish embryos can experience dose-dependent mortality and adverse effects such as hepatotoxicity, immune suppression, reproductive and endocrine impairment, teratogenicity, carcinogenicity, and loss of weight.[53,65] Adverse effects that can be visually observed under a microscope include spinal and cranial malformations, yolk sac and pericardial edemas, cardiac dysfunction, and changes to swim bladder function (**Figure 1-3**).[53,65,66] However, the connection between AhR regulated gene expression and these adverse effects is not yet known due to the number of genes and pathways regulated by the AhR.[67]

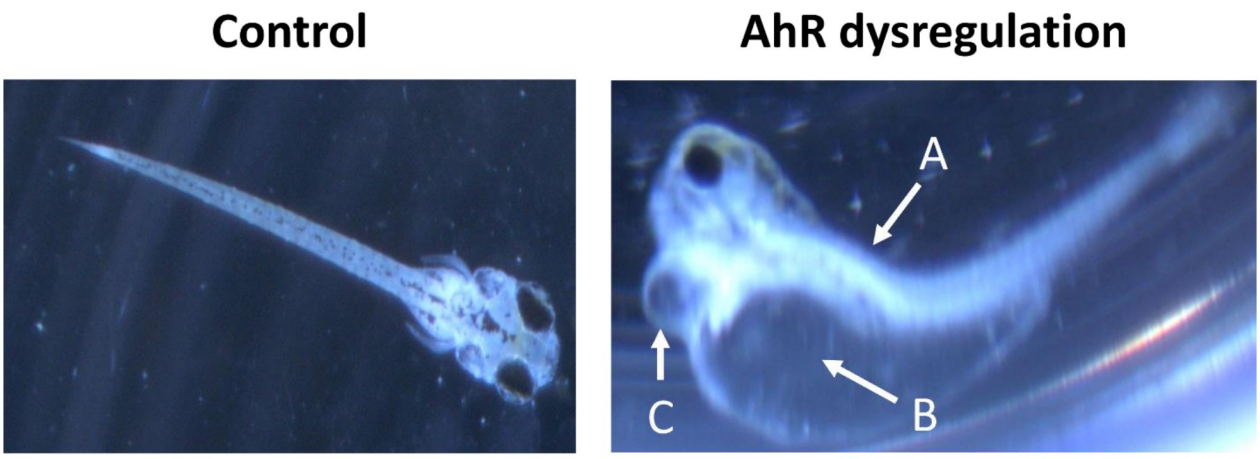


Figure 1-3: Representative images of AhR-mediated malformations in zebrafish (*Danio rerio*) exposed to Dimethylsulfoxide (DMSO) control or benzotriazole ultraviolet stabilizer (BUVS) UV-P. Malformations include spinal curvature (A), yolk sac edema (B), and pericardial edema (C).

1.6 Difference In Potencies Among AhR Agonists

Many different classes of chemical contaminants such as, PAHs and dioxin-like compounds (DLCs), can activate the AhR.[62,68] However, not all agonists of the AhR activate the receptor with equal potency.[69] For example, certain polychlorinated dibenzo-p-dioxins (PCDDs) such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), the prototypical AhR agonist, displays a great affinity for the AhR resulting in its high potency, while other PCDD congeners like 2,8-dichlorodibenzo-p-dioxin, have up to a 300-fold lower affinity for the AhR and are much less potent agonists.[70,71] The dramatic differences in potencies between chemicals from the same chemical class could stem from differences in AhR receptor binding kinetics and the chemical's physical properties.[72] For example, the potency of DLCs correlates with the molecular structure of the molecule, with planar configurations typically exhibiting the highest affinity for the AhR and consequently, the greatest potency.[58]

An *in vitro* study evaluating the agonistic potential of 13 BUVSs to activate the AhR in mice demonstrated that only four BUVSs (UV-P, UV-PS, UV-9, and UV-090) exhibited the ability to activate the mammalian AhR at varying magnitudes.[22] Although these findings indicate that not all BUVSs exhibit agonistic properties for the AhR, they do indicate that certain BUVSs can activate the AhR with varying potencies. However, the majority of studies assessing the potency of BUVSs as agonists of the AhR have been studied in mammals (i.e., mice and human AhRs).[15,22,73] In fish, differences in potencies for BUVSs to activate the AhR2 have not been well characterized. However, the ability for different BUVSs to alter the transcript abundance of *cyp1a* at varying magnitudes indicates that BUVSs could have differences in potencies for activating the AhR2 in fish.[51,52] Therefore, it is crucial to determine the relative potencies (RePs) of BUVSs compared to TCDD to gain a deeper understanding of the toxicities of these chemicals and the risks they might present to aquatic organisms.

1.7 Interspecies Differences in Sensitivity to Activation of the AhR

Teleost's, which are comprised of about 27, 000 distinct species, represent the largest and most diverse group of vertebrates on earth.[74] With such diversity, it is common for certain species of fish to be more sensitive to exposure to certain chemical contaminants than others. For example, a previous study that assessed the effects of TCDD on ELS mortality and malformations of seven different species of fish identified differences in embryo mortality between species.[75] Furthermore, the timing and incident number of malformations such as cranial, pericardial, abnormal yolk sac edemas, and jaw deformities varied among species.[75] This demonstrates that the magnitude of apical toxicities (ie: embryo mortality or malformations) of fish exposed to the same chemical can differ between species. Species differences in sensitivities to AhR activation has also been demonstrated. Specifically, there is an approximate 200-fold difference in species sensitivity to the

activation of the AhR among 17 different species of fish exposed to TCDD.[62,75] For example, the least sensitive species to activation of the AhR by TCDD was shovelnose sturgeon (*Scaphirhynchus platorynchus*), while the most sensitive species was lake trout with species such as zebrafish, Japanese medaka, and fathead minnow having sensitivities near the middle of the range.[62] It is thought that species differences in sensitivities could be related to differences in conformation of the ligand binding domain of AhRs from different species.[76] Whether species differ in their sensitivity to AhR activation by BUVS is not currently known, but likely do exist, as it does for other AhR agonists. Understanding the diversity of species sensitivity to activation of the AhR by BUVSs will allow for more informed risk assessments for these chemicals and provide a starting point for informing which species are at the highest risk, given the limited knowledge regarding the adverse toxic effects of BUVSs.

1.8 In Vitro Methods for Testing AhR Activation

Several *in vitro* methods have been developed for the assessment of AhR-mediated activities of individual and complex chemical mixtures.[77] For example, yeast reporter gene assays and H4IIE-Luc assays have been developed to assess the *in vitro* activation of the mammalian AhR.[15,78] Additionally, several stably transfected cell lines have been used to assess AhR activation in fishes identified with a luciferases *cyp1a* reporter or coupled with 7-ethoxyresorufin-O-deethylase (EROD).[79-81] However, the luciferase reporter gene (LRG) assay, which is the assay used in this thesis, has been extensively used to assess *in vitro* AhR activation across a diverse range of different species of fish and chemical classes.[54,62,68,82-84] An LRG assay is a bioluminescence-based assay that uses a luciferase enzyme, commonly derived from firefly (*Lampyridae*) and a substrate, such as luciferin, to study gene expression and regulation at the level of transcription. The assay involves chemically transfecting mammalian COS-7 cells with a species

specific AhR2, white sturgeon ARNT, renilla luciferase vector, and rat cyp1a reporter construct.[82] The rat cyp1a reporter construct is a target gene used to assess AhR activation and is genetically engineered to control the expression of the luciferase gene and modified to contain a XRE which the AhR:ARNT complex can bind to. When the transfected cells are exposed to AhR agonists, the activity of the luciferase enzyme when coupled with the luciferin substrate generates luminesce that can be immediately quantified to generate *in vitro* dose-response curves to develop effective concentration (EC) values.

The LRG assay allows for testing of AhR activation in multiple difference species with ease when utilized in mammalian COS-7 cells, which lack an endogenous AhR pathway.[62] This is advantageous because the AhR agonist of interest will not be bio transformed or metabolized through phase I reactions allowing for a complete potency profile of the agonist. However, this generates issues regarding biotransformation and metabolism processes that occur in living organisms that may affect the potency of the chemical *in vivo* and draw inaccurate conclusions about potency based on *in vitro* screening alone. Lastly, and arguably the biggest advantage of the LRG assay is that any species that contains an AhR can be screened for AhR activation by a suite of chemicals that may be agonists of the AhR. This can be done by cloning the AhR from the desired species through a non-lethal tissue sample (ie: blood or fin clip) for testing in the LRG. Therefore, species that are particularly difficult to study in a laboratory setting, threatened, or endangered can be screened for potential toxicity associated risks without using a whole organism.

As a result of its extreme sensitivity, reliability, and reproducibility the LRG assay has been used to assess the potency of AhR activation by different classes of AhR agonist within and across species. The AhR2 of several freshwater fishes have been screen for their activation by AhR agonists, including brook trout (*Salvelinus fontinalis*), fathead minnow (*Pimephales promelas*),

Japanese maedaka (*Oryzias latipes*), lake sturgeon (*Acipenser fulvescens*), lake trout (*Salvelinus namaycush*), northern pike (*Esox Lucius*), rainbow trout (*Oncorhynchus mykiss*), red seabream (*Pagrus major*), white sturgeon (*Acipenser transmontanus*), white sucker (*Catostomus commersonii*), and zebrafish (*Danio rerio*).[62,64,69] Furthermore, AhR constructs have been developed for several avian species and amphibians.[85,86] In addition to the vast number of species screened in the LRG, several different agonists have been tested. These include PAHs, Dioxins and DLCs, polychlorinated biphenyls (PCBs), and polychlorinated dibenzofurans (PCDFs).[62,64,82,86] The *in vitro* EC values, obtained through the *in vitro* LRG assay, hold significance. When combined with the molar mass of the AhR agonist and input into a previously established model, these values can predict dose-response curves for ELS mortality of birds and fish species exposed to DLCs.[62] However, it's important to note that the existing model relies on the EC value causing 50% activation (EC50) *in vitro*, which may not be attainable in the case of weaker agonists of the AhR.[62] Consequently, a new model was devised, utilizing the first concentration in the LRG assay that significantly increases fluorescence compared to the control, known as the EC threshold ($EC_{Threshold}$).[69] While this model is slightly less accurate than the original, it still demonstrates the ability to predict *in vivo* ELS mortality within an order of magnitude on average from *in vitro* $EC_{Threshold}$ values obtained in the LRG assay.[69] This allows for identifying changes in gene expression and regulation that can be connected to adverse effects observed at the organismal level by a broad range of different AhR agonists.

1.9 Zebrafish as a Model Organism

Numerous fish species including fathead minnow, Japanese medaka, and zebrafish are used in ecotoxicology research to evaluate and characterize potential adverse effects, and associated mechanisms, of chemical contaminants. These species make excellent model organisms due to

their comprehensively studied development and physiology, fully sequenced genomes, short generation times, and ease of handling and culturing in aquatic facilities. Zebrafish, a small oviparous species that originates from parts of south Asia, have been extensively used as a model species in a wide range of scientific disciplines. Zebrafish, once sexually mature, are sexually dimorphic allowing for easy identification to set up breeding pairs. Additionally, they have the capacity to generate large numbers of embryos at each mating event, which is beneficial when assessing embryotoxicity. Furthermore, the ELS development of zebrafish embryos is external to the mother, rapid, and visually accessible allowing for easier identification of physical adverse effects that toxicants may exhibit at any stage of development. Lastly, their chorion is malleable which allows for easy manipulation during their ELS. Therefore, zebrafish have been commonly used in early life stage toxicity testing for many chemical contaminants in the Organization for Economic Co-operation and Development (OECD) Test No. 210: Fish, Early-Life Stage Toxicity Testing.[87]

Zebrafish have been used extensively to unravel the complexity of the AhR pathway. Early investigations on the AhR pathway discovered that unlike mammals, fish have several different isoforms of the AhR (ie: AhR1 & AhR2) resulting from whole genome duplication events.[88] As a result, fish AhR isoforms have distinct functions. For example, the AhR1 in zebrafish, is an ortholog of the mammalian AhR but lacks high-affinity binding of TCDD and lacks a functional transactivation domain.[88] As such, the AhR2 in fish drive most if not all toxicities relating to activation of the AhR by DLC.[62] As a result of the easy manipulation either molecularly, physiologically, or developmentally, zebrafish have become a desirable model species when investigating AhR-mediated pathways and toxicity testing.

1.10 Research Rational and Objectives

BUVSs have garnered considerable attention from both the scientific community and regulatory agencies due to their persistence, capacity for bioaccumulation and biomagnification, and their potential to cause toxicity.[10-13] BUVSs have been detected in various environmental matrices worldwide yet little is known about their toxicity to fish.[10,11,19,20,24] A small body of research suggests that some BUVS can potentially exert their toxicity by activating the AhR in fish.[49,51,52] However, the differences in potencies of BUVSs as agonists of the AhR is not currently well understood. It has also been demonstrated that large differences in sensitivity to AhR activation by PAHs and DLCs exists among fish species.[62] However, it is unknown if there are species differences in sensitivity to AhR activation by BUVSs. Based on the ubiquitous detection and activity towards activating the zebrafish and human AhR, UV-P, UV-9, and UV-090 were chosen to assess the toxicities towards activation of the AhR2 of phylogenetically diverse number of fish species.

Hypothesis

As a result of their proposed action as an AhR agonist, it is hypothesized that potencies as AhR agonists along with species differences in sensitivities will differ between UV-P, UV-9, and UV-090.

Objectives

The overarching objective of this thesis is to use *in vivo* and *in vitro* approaches to characterize the toxicity of UV-P, UV-9, and UV-090 as agonists of the AhR across phylogenetically diverse number of fish species. Specific objectives of this research are to:

1. Assess if BUVSs can exert toxicity in fishes through activation of the AhR2 and determine the potencies of UV-P, UV-9, and UV-090.
2. Assess whether, and to what extent, there are interspecies differences in the sensitivity to activation of the AhR2 by testing two model species that are not native to North America, and seven freshwater species native to North America.

CHAPTER 2: ASSESSING THE TOXICITY OF BENZOTRIAZOLE ULTRAVIOLET STABILIZERS TO FISHES: INSIGHTS INTO ARYL HYDROCARBON RECEPTOR-MEDIATED EFFECTS

Hunter M. Johnson[a], Justin Dubiel[a], Cameron H. Collins[c], Andreas N.M. Eriksson[a], Zhe Lu[b], Jon A. Doering[a,c]*, Steve Wiseman[a]*

[a] Department of Biological Science, University of Lethbridge, Lethbridge, AB, T1K 3M4, Canada

[b] Institut des Sciences de la Mer de Rimouski, Université du Québec à Rimouski, Rimouski, Québec G5L 3A1, Canada

[c] Department of Environmental Sciences, College of the Coast & Environmental, Louisiana State University, Baton Rouge, LA 70803, USA

Corresponding author

Hunter M. Johnson: hunter.johnson@uleth.ca

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2.0 ABSTRACT

Benzotriazole ultraviolet stabilizers (BUVSs) are chemicals used to mitigate UV-induced damage to manufactured goods. Their presence in aquatic environments and biota raises concerns as certain BUVSs activate the aryl hydrocarbon receptor (AhR), which is linked to adverse effects in fish. However, potencies of BUVSs as AhR agonists, and species sensitivities to AhR activation, are poorly understood. This study evaluated the toxicity of three BUVSs using embryotoxicity assays. Zebrafish (*Danio rerio*) embryos exposed to BUVSs by microinjection suffered dose-dependent increases in mortality, with LD_{50}s of 4,772, 11,608, and 56,292 ng/g-egg for UV-P, UV-9, and UV-090, respectively. The potencies and species sensitivities to AhR2 activation by BUVSs were assessed using a luciferase reporter gene assay with COS-7 cells transfected with the AhR2 of zebrafish and eight other fishes. AhR2s from all nine fishes had a rank order of potency of UV-P > UV-9 >UV-090. However, AhR2s among species differed in sensitivities to activation by up to 100-fold. An approximate reversed rank order of species sensitivity was observed compared to the rank order of sensitivity to 2,3,7,8-tetracholorodibenzo[p]dioxin, the prototypical AhR agonist. Despite this, a pre-existing quantitative adverse outcome pathway linking AhR activation to embryo lethality could predict embryotoxicities of BUVSs in zebrafish.

Keywords: Species sensitivity; Embryotoxicity; Quantitative adverse outcome pathway; AhR; CYP1A; Microinjection; Plastics associated chemicals

Synopsis: Little is known regarding the potency and species sensitivities to AhR activation by BUVSs in fish. This study shows that UV-P, UV-9, and UV-090 differ in potency and sensitivities to AhR activation are unique from dioxin-like compounds.

2.1 Introduction

Benzotriazole ultraviolet stabilizers (BUVSs) are a class of chemical contaminants that have recently raised concerns among regulatory bodies due to their persistence, bioaccumulation, biomagnification, and toxic characteristics.[10-13] BUVSs are characterized by the presence of a primary benzotriazole moiety attached to a 2-hydroxyphenol group, with significant structural diversity resulting from substituents attached at various positions.[19,22,23] Due to their photostability, BUVSs are additives in industrial and consumer goods including cosmetics, coatings, adhesives, waxes, paints, motor oils, rubber, and plastics to protect against degradation and discoloration caused by full-spectrum ultraviolet light (280-400 nm).[10,11,14-21] BUVSs are hydrophobic with logarithmic octanol-water partition coefficients ($LogK_{ow}$) greater than 3, providing a potential for environmental accumulation by sorbing to sediment or accumulating in fatty tissues of aquatic organisms where they can undergo bioaccumulation and biomagnification.[20,24,26,89] BUVSs enter the environment through manufacturing processes, wastewater effluent, sewage, and leaching from waste products.[26,32] As a consequence, BUVSs are ubiquitous in the environment, having been detected in surface waters, sediment, and tissues of biota including birds, invertebrates, mammals, and fishes.[10,11,13,17,20,21,26-28,32,38,90] Concentrations of BUVSs in freshwater rivers range from 0.7 ng/L to 701 ng/L.[25,27,35] Furthermore, sediment concentrations of BUVSs range from 0.16 µg/g – 35 µg/g dried weight.[13,19,27,91] Specifically, 2-(2-Hydroxy-5-methylphenyl)benzotriazole (UV-P), 2-(2H-benzotriazol-2-yl)-4-methyl-6-(2-propenyl)phenol (UV-9), and 2-[3-(2H-benzotriazol-2-yl)-4-hydroxyphenyl]ethyl methacrylate (UV-090) have been detected in sediment at concentrations ranging from 15 – 800 ng/g dried weight and in water at concentrations from 0.9 – 28.1 ng/L in several locations globally.[15,19,25] Additionally, concentrations of UV-P and UV-9 in muscle tissues of fish have been reported to range from 2.2 ng/g – 9.1 ng/g lipid weight and 1.3

ng/g – 14 ng/g wet weight, respectively.[25,92] Fish that are developing in BUVS-contaminated water or sediment, or that are potentially exposed to maternally transferred BUVSs, could experience adverse effects during their early life-stages.[11,15,52,92,93]

The current empirical understanding of the adverse effects of exposure to BUVSs on aquatic wildlife, including fishes, is limited to a few studies. Transcriptomic and molecular studies of fish exposed to BUVS revealed potential dysregulation of molecular processes related to the hypothalamic-pituitary-thyroid axis, anti-androgenic activity, disruption of steroidogenesis, and activation of the aryl hydrocarbon receptor (AhR).[46-49,52] The AhR is a ligand-activated transcription factor that regulates the expression of genes involved in a multitude of physiological processes such as xenobiotic metabolism, cellular growth, and cell migration.[15,67,88,94,95] Several isoforms of the AhR exist in vertebrates, but in fishes, there is strong evidence that activation of the AhR2 isoform is causative of toxicities in early life-stages, including mortality, spinal and cranial malformations, yolk sac and pericardial edemas, and cardiac dysfunction.[60-63] As fishes transition out of their early life-stages, numerous toxicities have been associated with activation of the AhR2 including wasting syndrome, hepatotoxicity, and fin necrosis.[96-98] Regarding BUVSs, a prior study reported no significant increase in mortality or malformations in zebrafish (*Danio rerio*) embryos exposed to waterborne UV-P or 2-(3-tert-butyl-2-hydroxy-5-methylphenyl)-5-chlorobenzotriazole (UV-326); but there was increased transcript abundance of cytochrome P450 1A (*cyp*1a), a widely accepted biomarker for activation of the AhR.[52,58] In contrast, another study found no significant increase in the transcript abundance of *cyp1a* in adult Japanese medaka (*Oryzias latipes*) exposed to UV-P spiked food.[47] The differences in these effects of UV-P on transcript abundance of *cyp1a* could be due to the variability in AhR2 structure among fish species which can translate into a vast difference in relative sensitivities (ReS) among species to the same

agonist.[83,99] In addition to differences in ReSs, relative potencies (ReP) of AhR agonists can exceed several orders of magnitude within a species when compared to the prototypical AhR agonist 2,3,7,8-tetracholorodibenzo[p]dioxin (TCDD).[70] Lastly, screening of 13 structurally diverse BUVSs for their ability to activate the AhR of mice found that UV-P, UV-9, UV-090, and 2-(5-*tert*-Butyl-2-hydroxyphenyl)benzotriazole (UV-PS) were AhR agonists, indicating that some, but not all, BUVSs can act as agonists of the AhR.[22]

Potencies of BUVSs as agonists of the AhR2 in fishes are unknown. Since other classes of AhR agonists display a wide range of interspecies differences in potencies for activating the AhR, it is likely that a similar diversity exists for BUVSs. As such, investigating interspecies sensitivity to AhR activation by BUVSs will enable a more informed approach to environmental risk assessment of BUVSs that encompass a wide array of fish species. Therefore, the primary aim of this study was to determine if BUVSs can exert toxicity in fishes through activation of the AhR2. As an initial step towards accomplishing this, zebrafish embryos, a commonly used model test species, were exposed to three serial doses of UV-P, UV-9, or UV-090 through microinjection and assessed for AhR-mediated toxicities and response of *cyp1a*. The second goal of this study was to determine whether, and to what extent, there are interspecies differences in the sensitivity to activation of the AhR2 by testing zebrafish and Japanese medaka, two model species that are not native to North America, and seven freshwater species native to North America, namely, brook trout (*Salvelinus fontinalis*), fathead minnow (*Pimephales promelas*), lake sturgeon (*Acipenser fulvescens*), lake trout (*Salvelinus namaycush*), northern pike (*Esox lucius*), white sucker (*Catostomus commersonii*), and white sturgeon (*Acipenser transmontanus*) by quantifying activation of the AhR2 using a standardized *in vitro* luciferase reporter gene (LRG) assay of COS-

7 cells transfected with the AhR2 of each species. Results of this study will help inform whether activation of the AhR2 by BUVSs could represent a risk to native populations of fishes.

2.2 Material and methods

2.2.1 Embryotoxicity

2.2.1-1 Animal Care and Embryo Collection

Collection of embryos complied with University of Lethbridge Animal Welfare Protocol AWP#2114. Embryos were obtained from a breeding culture of zebrafish (Tupfel long fin strain) maintained in the aquatic research facility in the Alberta Water and Environmental Science Building at the University of Lethbridge (Lethbridge, AB, Canada). Zebrafish were housed in vertical flow-through racks (Tecniplast, Toronto, ON, Canada) supplied with 26°C ± 1°C City of Lethbridge municipal water that is dechlorinated, filtered, and UV sterilized. The breeding culture was fed artemia (Brine Shrimp Direct, Ogden, UT, United States) twice daily, until satiety, and kept on a 16:8 light:dark photoperiod. On the evening prior to microinjections, two sexually mature male and female zebrafish were randomly selected from the breeding culture and transferred into a 1.7 L sloped breeding tank (Tecniplast). Three to five breeding tanks were setup for each embryo collection. The next morning, embryos were collected from each tank approximately 1-hour post-fertilization (hpf) and viable eggs were pooled for use in microinjections.

*2.*2.1-2 Embryo Microinjection

Physiochemical properties of UV-P (purity >97%), UV-9 (purity >99%), and UV-090 (purity >99%) are provided (**Table 2-1**). Each BUVS was purchased from Sigma (Sigma, Mississauga, Ontario, Canada) and was prepared at a nominal concentration of 15 mg/mL (high) in dimethylsulfoxide (DMSO), which is near their maximal solubility. As a first step toward better

understanding the toxicities of BUVSs in fishes, the nominal concentrations were serially diluted 3-fold to generate additional dosing solutions at 5 mg/mL (medium) and 1.67 mg/mL (low).

Table 2-1: Benzotriazole ultraviolet stabilizer chemical properties. Chemical Abstracts Service registry number (CAS no.), molecular weight (MW) and formula, chemical purity, and Logarithmic octanol-water partitioning coefficient (log K_{ow}) values for each BUVS studied.

Abbreviation	CAS no.	Formula	MW (g/mol)	Purity (%)	log K_{ow}
UV-P	2440-22-4	$C_{13}H_{11}N_3O$	225.09	>97	3.0[24]
UV-9	2170-39-0	$C_{16}H_{15}N_3O$	265.31	>99	4.4[24]
UV-090	96478-09-0	$C_{18}H_{17}N_3O_3$	323.35	>99	3.9[24]

[24]Data obtained from Castilloux et al. (2022).

Microinjection of zebrafish embryos were performed based on previously described methods[100] with modifications[68]. This method of exposure was chosen over waterborne exposure as it bypasses the chorion, allows for the entirety of the dose to be delivered at once, allows for precise dosing, mitigates issues related to solubility in water, and allows for easy quantification of administered dosages. An IM-400 Electric Microinjector (Narishige Group, Tokyo, Japan) was calibrated to administer approximately 1.5 nL of DMSO prior to injection of embryos. A volume of 1.5 nL was chosen based on a method development experiment on the maximal volume that could be injected without causing embryo mortality (data not shown). Each dose of the BUVSs were injected directly into the yolk-sac prior to the completion of gastrulation (<6 hpf) to prevent developmental impacts caused by the microinjection process. All microinjections were performed at room temperature (~25°C). Three experimental replicates of approximately 100 zebrafish embryos per replicate were injected for every treatment group. Control embryos were injected with 1.5 nL of full-strength DMSO. A negative control was not employed since the assessment of effects were in excess of the control and results from previous studies indicated no effects caused by this volume of full strength DMSO on zebrafish embryos.[68] Each experimental replicate included a

DMSO control, and the three BUVS treatment groups all of which used embryos from independent breeding events. Lastly, 1 g of embryos (approximately 2500 embryos) were injected per concentration of each BUVS and DMSO control, then immediately frozen at -80 °C for quantification of dose (Sec 2.3).

2.2.1-3 Embryo Rearing and Assessment

Following injection, embryos were incubated for 24 h in plastic Petri dishes containing processed City of Lethbridge municipal water at 26°C ± 1°C. After 24 h, any embryos that were nonviable or dead were discarded and not included in the final mortality data. Mortalities were presumed to be due to the microinjection process as there were no dose-dependent effects of either BUVS on mortality within this period (data not shown). Twenty-four viable embryos were randomly selected and transferred into independent wells of a 24-well plate (Eppendorf Canada, Mississauga, ON, Canada) containing 2 mL of processed City of Lethbridge municipal water at 26°C ± 1°C and were reared until complete yolk sac utilization at 15 days post-fertilization (dpf).[68] The 15-day study duration was based on a previous study that assessed the same endpoints in zebrafish embryos microinjected with polycyclic aromatic hydrocarbons (PAHs).[68] Daily water renewals of 50% were performed. Remaining embryos from the high dose of each of the BUVSs were reared until 5 dpf, the completion of hatch, and then allocated into groups of 10 and immediately frozen at -80°C for quantitative real-time polymerase chain reaction (qPCR). For embryos transferred to the 24-well plates, daily assessments of pericardial and yolk sac edemas, spinal curvature, and mortality were performed for each embryo by use of a Zeiss Discovery.V12 stereo microscope (Carl Zeiss Canada, Toronto, ON, Canada). At the completion of yolk sac absorption (15 dpf), larvae that lacked response to mechanical stimulus and were orientated vertically were assessed as functionally dead. All assessments were in accordance with the

guidelines provided by the Organization for Economic Co-operation and Development Test No. 210: Fish, Early-Life Stage Toxicity Test.[87] Images were captured using a Zeiss Axiocam 105 Colour (Carl Zeiss Canada, Toronto, ON, Canada) and ZEN lite imaging software (Carl Zeiss Microscopy, Oberkochen, Germany).

2.2.1-4 Quantitative Real-Time PCR

Transcript abundance of *cyp1a* was quantified in 5 dpf embryos exposed to the highest dose of each BUVS to determine activation of the AhR. Isolation of total RNA from 10 randomly chosen larvae per treatment was performed using TRIzol™ reagent (Thermofisher Scientific, Ottawa, ON, Canada) according to the protocol provided by the manufacturer. The RNA concentration for each sample was quantified using a Nanodrop™ One© spectrophotometer (Thermo Scientific). Complementary DNA (cDNA) was synthesized from 2 µg of RNA by using A QuantiNova™ Reverse Transcription Kit, which uses a genomic DNA removal step (Qiagen Inc. Mississauga, ON, Canada). For qPCR, a 35 µL reaction mixture was created with SensiFAST™ SYBR® No-ROX Kit (Meridian Bioscience, Cincinnati, OH, United States), 1.75 µL primers (final concentration: 10 pM), RNAse free water, and 1.75 µL of cDNA. All reactions were performed in triplicate in 96-well plates using a CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Mississauga, ON, Canada). Prior to the initial qPCR cycle, reactions were denatured at 95°C for 2 min, then reactions were denatured at 95°C for 5 sec followed by annealing and elongation at 60°C for 10 sec for 40 complete cycles. To confirm the amplification of a single qPCR product, a melt curve was generated. No-template controls were included to ensure reaction mixtures were not contaminated. The abundance of *cyp1a* transcript was normalized to the abundance of *18s rRNA,* and changes in the abundance of *cyp1a* in larvae exposed to BUVSs were determined relative to

DMSO controls by use of the primer efficiency corrected Pfaffl method.[126] Primer sequences and reaction efficiencies for *cyp1a* and *18s* are provided (**Table 2-2**).

Table 2-2: Primer sequence, efficiency, target mRNA, and accession number for oligonucleotide primers used in quantitative real-time PCR (qPCR)

Target mRNA	Function	Primer Sequence	Efficiency (%)	Accession #	Reference
18s rRNA	House keeping	**F**: CCACTCCCGAGATCCAACTA **R**: CAAATTACCCATTCCCGACA	105	NR_145818.1	101
cyp1a	AhR activation	**F**: GCATTACGATACGTTCGATAAGGAC **R**: GCTCCGAATAGGTCATTGACGAT	102	NM_131879.2	102

2.2.2 Luciferase Reporter Gene Assay

The LRG assay followed a previously described protocol[84] with modifications[54]. In short, immortalized COS-7 cells that lack an endogenous AhR pathway were chemically transfected with 8 ng of species-specific AhR2[54,62,83,103], 1.55 ng of white sturgeon ARNT2[54], 20 ng of rat CYP1A reporter construct[104,105], and 0.75 ng of renilla luciferase vector to assess transfection efficiency (Promega, Madison, Wisconsin, USA) per well. In previous studies, the use of the ARNT of a single species did not affect species sensitivities to TCDD so it is unlikely to affect the results in the present study.[62] Additionally, several isoforms of the AhR (AhR1 & AhR2) exist in vertebrates because of gene duplication and diversification events.[88,106,107] The AhR2 was chosen because activation of this isoform has been linked with early life-stage mortality and malformations in fish.[60,62,63] After transfection, cells were dosed with 9 nominal concentrations of UV-P, UV-9, or UV-090, ranging from 0.33 nM – 30,000 nM, which were prepared in 100% DMSO from the 15 mg/mL stock solutions described in section 2.1.1. The concentration range of BUVSs were chosen with the goal of covering the entire concentration-response curve. However, due to the low potency

of BUVSs, the greatest tested concentration of 30,000 nM was selected based on concerns with cytotoxicity and solubility at greater concentrations as observed during initial range-finding studies (data not shown). Each chemical-specific assay was completed in triplicate with each replicate consisting of 4 independent wells per concentration. A SpectraMax i3x plate reader (Molecular Devices, San Jose, CA, USA) was used to measure the luminescence of the luciferase reporter. Maintenance, transfection, dosing of COS-7 cells, and plate reading were performed in the SynBridge core facility at the University of Lethbridge.

2.2.3 Analysis of BUVSs in Eggs

Chemical analysis was performed to determine doses of each BUVS in zebrafish eggs following microinjections, as previously described.[47] Anhydrous sodium sulfate, Supelclean ENVI-Florisil glass solid-phase extraction (SPE) tubes, high performance liquid chromatography (HPLC) grade acetonitrile, water, n-hexane, acetone and dichloromethane were purchased from Sigma-Aldrich (Oakville, Canada). The surrogate standard 2-(2H-benzotriazol-2-yl)-4,6- di-tert-pentylphenol-d4 (UV328-d_4) (purity: 99.8%) was purchased from ASCA GmbH (Berlin, Germany). Chemical analysis was performed to determine doses of each BUVS in zebrafish embryos following microinjections. Approximately 0.5 – 1g of zebrafish embryos were homogenized with 2g of anhydrous sodium sulfate (Na_2SO_4) in a mortar. Following homogenization, samples were transferred to a glass tube and spiked with a surrogate standard of UV328-d_4. Samples were vortexed with 5 mL of acetonitrile for 1 minute, followed by 10 minutes of sonification and 5 min of centrifugation at 1167 ×g. This process was performed in triplicate and samples were concentrated to 10 mL from which 1 mL was further concentrated to dryness and reconstituted in 0.5 mL of hexane for instrument analysis.

Gas chromatography-mass spectrometry (GC-MS) was used for sample analysis. The GC-MS consisted of a Thermo Trace GC and an Ultra-PolarisQ MS and details are shown in a previous study. The quantification ions were *m/z* 225 for UV-P, *m/z* 250 for UV-9, and *m/z* 237 for UV-090. The qualification ions were *m/z* 168, 265, and 180 for UV-P, UV-9 and UV-090, respectively. The quantification ion for the surrogate standard UV-328-d_4 was *m/z* 326. Quantitation was done by relative response to the surrogate standard using a solvent standard curve. Procedural blanks (*n*=3) were included in the experiment, but none of the target compounds were detected. The recovery of surrogate standard was 83±6% (mean± standard error; 32 samples). Limit of detection (LOD), which was estimated as the concentration that produced 3 times of the signal to noise ratio in the matrices, were 0.2, 0.3, and 1.9 ng/g for UV-P, UV-9, and UV-090, respectively.

2.2.4 Statistics and Data Analysis

Measured doses were used for all analyses of *in vivo* data. Differences in *cyp1a* transcript abundance between treatments and controls were determined using IBM SPSS Statistics 20 software. Data was assessed for normality using Shapiro-Wilk test and homogeneity of variance usings a Levene's test. A Kruskal-Wallis test was used based on outputs from normality and homogeneity of variance tests. GraphPad Prism 9 software v.9.4.1 for windows (GraphPad Software, San Diego, CA, USA) was used to generate *in vivo* dose-response curves. Curves were fit to a four-parametric logistic model and doses that caused 0%, 10%, and 50% lethality (LD_0, LD_{10}, and LD_{50}) were calculated for zebrafish embryos exposed to each BUVS. Control background mortality was normalized to 0 using Min-Max Scaling under the "Normalize" function in GraphPad Prism. A Fisher's exact test, conducted using GraphPad Prism, was used to identify statistical significance ($p<0.05$) between control mortality and treatment mortality for each BUVS. Figures were generated using GraphPad Prism.

Statistical analysis for *in vitro* AhR activation was performed in R v.4.2.2 (The R Foundation for Statistical Computing, 2022) coupled to RStudio v.2022.12.0.353 (Rstudio Team 2022) as described in Dubiel et al.[82] Outliers were determined by preforming a Chi-squared test (statistical cut-off: $p \leq 0.05$) using the Tests for Outliers package and then removed from the finalized data set.[108] Dose-response curves were generated in Rstudio and fit using a four-parameter log-logistic function using the Analysis of Dose-Response Curves package[109] and effective concentration threshold ($EC_{Threshold}$) of activation of the AhR2 in COS-7 cells were established. The $EC_{Threshold}$ was identified as the first chemical concentration that caused a significant increase in response ($p \leq 0.05$) from the DMSO control with all subsequent doses also having significant increases in response. $EC_{Threshold}$ was selected because it is independent of the concentration-response curve reaching maximal response. Half maximal effect concentration (EC_{50}) was not calculated because BUVSs did not reach a clear maximal response in most, if not all, species. Due to few replicates (n=3), normality was not assessed and significant differences between treatments and controls were determined using Kruskal-Wallis H test combined with Dunn's post hoc test not adjusted for multiple comparisons when assessing $EC_{threshold}$. Relative potencies and ReSs were calculated by dividing the $EC_{threshold}$ of the reference chemical or species by the $EC_{threshold}$ of the chemical or species of interest. Providing the $EC_{Threshold}$ generated by the LRG assay along with the molar mass of each chemical as input variables into the qAOP model provided in Doering et al.[69] gives the predicted LD_{10}, LD_{50}, and LD_{100} as output variables. The accuracy of the qAOP for predicting dose response curves for BUVSs from $EC_{threshold}$ was evaluated using mean absolute error (MAE), mean absolute percentage error (MAPE), and fold-difference. Concentrations used for the LRG assay were carefully designed as 3-fold dilutions, a choice made to minimize the intervals between each concentration. This enhances the precision of the $EC_{Threshold}$ measurement,

ensuring it remains within a range less than 3-fold, all while guaranteeing capture of the entire concentration-response curve.

2.3 Results

2.3.1 Embryotoxicity

Exposure of zebrafish embryos to UV-P, UV-9, or UV-090 caused a dose-dependent increase in mortality, with a rank order of potency of UV-P > UV-9 > UV-090 (**Table 2-3**). The LD_{50}s were 4,772, 11,608, and 56,292 ng/g-egg for UV-P, UV-9, and UV-090, respectively (**Table 2-3**).

Table 2-3: Calculated and predicted lethal doses (LDs) (ng/g-egg) that caused 10% and 50% mortality of zebrafish embryos exposed to doses of UV-P, UV-9, or UV-090. The predicted LD_0, LD_{10}, and LD_{50} were established using a quantitative adverse outcome pathway for low potency agonists of the AhR[61] The range of values presented in brackets represent the 95% confidence interval.

Chemical	LD_0	LD_{10}	LD_{50}	Predicted LD_0	Predicted LD_{10}	Predicted LD_{50}
UV-P	112 (0.28-822)	854 (10-2630)	4772 (3064-8313)	71	148	426
UV-9	1323 (270-6493)	1724 (489-6073)	11608 (8687-16747)	187	401	1212
UV-090	634 (55-7367)	7190 (1327-38956)	56292 (38656-123551)	NA	NA	NA

LD values are calculated based on measured doses of each BUVS
Predicted values are calculated on metrics outlined in Table 2-8
NA=Not Applicable

In addition to mortality, the proportion of embryos exhibiting malformations (yolk sac edema, pericardial edema, and spinal curvature) was increased following exposure to each chemical (**Table 2-4**). However, the occurrence of malformations did not increase significantly with increasing

doses of either BUVS. Representative images of observed malformations are provided (**Figure 2-1**).

Table 2-4: Measurement parameters of zebrafish embryos used in early life-stage toxicity testing that were exposed to a DMSO control and each of the BUVSs (UV-P, UV-9, and UV-090). The values presented in brackets are mean ± standard deviation (±SD).

Treatment	Nominal Dosing Solution Concentration (ng/nL)	Nominal Embryo Concentration (ng/g-egg)	Measured Embryo Concentration (ng/g-egg)	Malformations (%)	Mortality (%)	Mortality Normalized to Control
DMSO Control	ND	ND	ND	10 (5)	32 (8)	0 (0)
UV-P	1.67	4167	3090	13 (9)	51 (19)*	31 (20)
	5.00	12500	2700	14 (8)	56 (17)*	36 (19)
	15.00	37500	5210	18 (8)	65 (14)*	51 (13)
UV-9	1.67	4167	5910	22 (7)	56 (2)*	24 (14)
	5.00	12500	10500	22 (12)	74 (5)*	56 (8)
	15.00	37500	37500	22 (14)	84 (5)*	73 (9)
UV-090	1.67	4167	10400	14 (5)	46 (4)	12 (11)
	5.00	12500	18800	6 (5)	46 (12)*	23 (13)
	15.00	37500	63400	19 (2)	58 (19)*	48 (18)

Malformations: spinal curvature, yolk sac edema, and pericardial edema
* Significant difference between control mortality and treatment mortality ($p<0.05$, Fisher's exact test)
ND = Not Detected

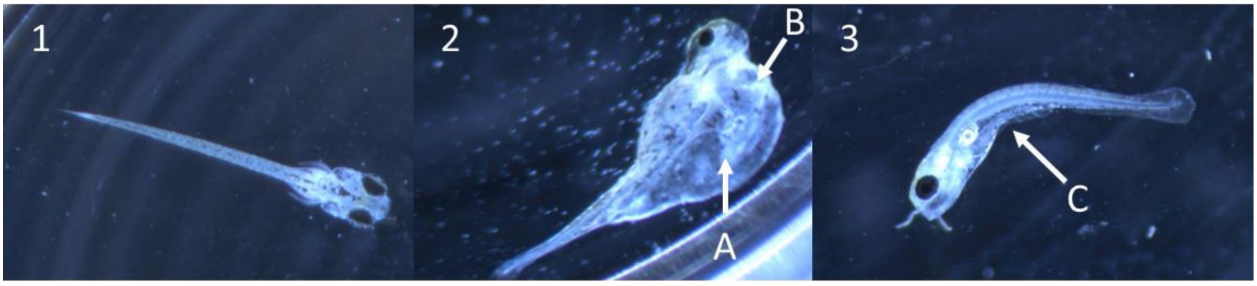

Figure 2-1. Representative images of control larvae (1) and larvae exposed to UV-P (2), UV-9 (3), and UV-090. Zebrafish larvae that were exposed to BUVSs developed malformations including yolk sac edema (A), pericardial edema (B), and spinal curvature (C).

Transcript abundance of *cyp*1a was assessed to confirm *in vivo* activation of the zebrafish AhR2. Zebrafish embryos exposed to the maximal dose of UV-P and UV-9, which caused 51% and 73% embryo mortality, respectively, showed a 5.9-fold and 42.2-fold significant increase in cyp1a transcript abundance, respectively. (**Figure 2-2**). Furthermore, there was no significant increase in the transcript abundance *cyp1a* in embryos exposed to the maximal dose of UV-090, which caused 48% embryo mortality, compared to the control group (**Figure 2-2**).

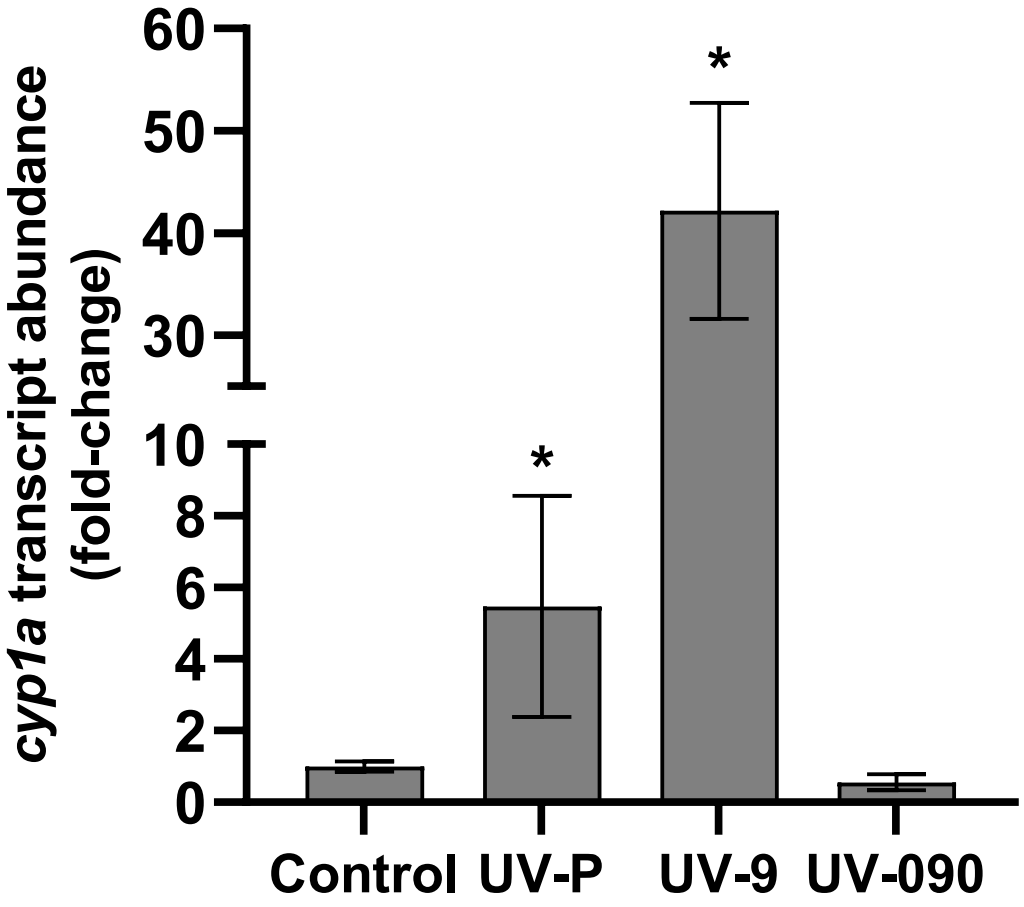

Figure 2-2: Effect of exposure to maximal measured concentrations of UV-P, UV-9, and UV-090 on transcript abundance of *cyp1a* in zebrafish embryos collected at 120 hours post-fertilization. Data is expressed as mean ± standard error (± SE) relative to the processed control. Statistical significance ($p<0.05$, Levene's statistic with Kruskal-Wallis test combined with Dunn's post hoc test) is indicated by asterisk.

2.3.2 AhR2 Transactivation

The AhR2 of zebrafish was activated in a concentration-dependent manner by UV-P and UV-9 but not by UV-090 (**Figure 2-3 and Table 2-5**). The rank order of potency for activation of the AhR2 in zebrafish was UV-P > UV-9 > UV-090 (**Table 2-5**) and the $EC_{threshold}$ to activate the zebrafish AhR2 was 1,000 nM for UV-P and 3,000 nM for UV-9. UV-P activated the AhR2s of each of the other eight species in a concentration-dependent manner (**Figure 2-3**); the $EC_{threshold}$ ranged from 300 nM in white sucker to 30,000 nM in Japanese medaka (**Table 2-5; Figure 2-3**). UV-9 only activated the AhR2s of zebrafish, lake sturgeon, and northern pike in a concentration-dependent manner which all had an $EC_{threshold}$ of 3,000 nM (**Figure 2-3; Table 2-5**). Lastly, UV-090 did not activate the AhR2 of any of the tested species (**Figure 2-3; Table 2-5**). The rank order of potencies for AhR2 activation was UV-P > UV-9 > UV-090 for each of the species tested, except for lake sturgeon and northern pike, where UV-P and UV-9 were equipotent (**Table 2-5**).

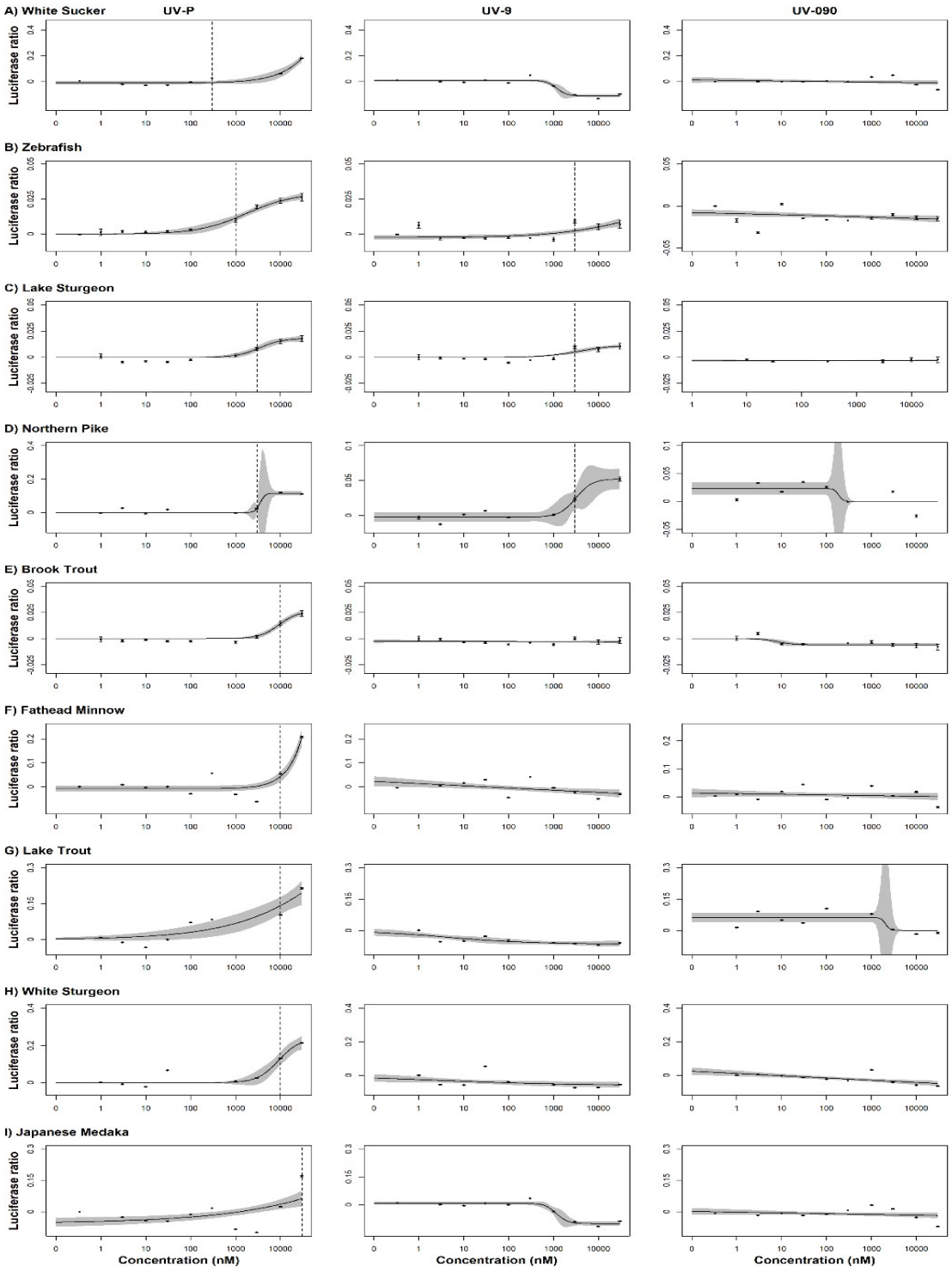

Figure 2-3: Dose-response curves of COS-7 cells transfected with the AhR2 of A) white sucker B) zebrafish C) lake sturgeon D) northern pike E) brook trout F) fathead minnow G) lake trout H) white sturgeon and I) Japanese Medaka following the exposure to UV-P, UV-9, or UV-090. Data is presented as mean ± standard error (±SE) based on three independent assays, each performed with four replicates per chemical concentration. 95% confidence intervals are represented by grey shaded area and vertical dotted lines represent $EC_{threshold}$.

Table 2-5: Calculated effective concentrations that pass the minimum threshold activation ($EC_{Threshold}$) of each investigated species specific AhR2 by UV-P, UV-9, or UV-090.

Species	Chemical	$EC_{Threshold}$ (nM)
White Sucker	UV-P	300
	UV-9	NA
	UV-090	NA
	TCDD	1.0[a]
Zebrafish	UV-P	1000
	UV-9	3000
	UV-090	NA
	TCDD	1.0
Lake Sturgeon	UV-P	3000
	UV-9	3000
	UV-090	NA
	TCDD	0.1[b]
Northern Pike	UV-P	3000
	UV-9	3000
	UV-090	NA
	TCDD	1.00[a]
Brook Trout	UV-P	10000
	UV-9	NA
	UV-090	NA
	TCDD	0.10[a]
Fathead Minnow	UV-P	10000
	UV-9	NA
	UV-090	NA
	TCDD	0.3[a]
Lake Trout	UV-P	10000
	UV-9	NA
	UV-090	NA
	TCDD	0.03[a]
White Sturgeon	UV-P	10000
	UV-9	NA
	UV-090	NA
	TCDD	0.03[c]
Japanese Medaka	UV-P	30000
	UV-9	NA
	UV-090	NA
	TCDD	0.3[a]

[a]Data obtained from Doering et al.[62]
[b]Data obtained from Doering et al.[83]
[c]Data obtained from Doering et al.[54]
NA = Not Applicable

Species sensitivity to AhR2 activation by UV-P ranged 100-fold based on the $EC_{threshold}$, with a rank order of sensitivity of white sucker > zebrafish > lake sturgeon = northern pike > brook trout = fathead minnow = lake trout = white sturgeon > Japanese medaka (**Table 2-5**). No such difference in species sensitivity for AhR2 activation by UV-9 or UV-090 was observed. The predicted LD_{50}s, derived from the qAOP[69] for low-potency agonists of the AhR, were 162, 426, 1028, 1028, 2701, 2701, 2701, 2701, and 6522 ng/g-egg for white sucker, zebrafish, lake sturgeon, northern pike, brook trout, fathead minnow, lake trout, white sturgeon, and Japanese medaka for UV-P respectively (**Table 2-6**). Furthermore, zebrafish, lake sturgeon, and northern pike had predicted LD_{50}s of 1212 ng/g-egg for UV-9 (**Table 2-6**).

Table 2-6: Predicted 20%, 50%, and 100% lethal doses (ng/g-egg) for UV-P and UV-9 based on $EC_{Threshold}$ value determined from the luciferase reporter gene assay.

Species	Chemical	$EC_{threshold}$ (nM)	Predicted LD_{10}	Predicted LD_{50}	Predicted LD_{100}
White sucker	UV-P	300	60	162	1097
zebrafish	UV-P	1000	148	426	3465
	UV-9	3000	401	1212	11661
Lake sturgeon	UV-P	3000	340	1028	9894
	UV-9	3000	401	1212	11661
Northern pike	UV-P	3000	340	1028	9894
	UV-9	3000	401	1212	11661
Brook trout	UV-P	10000	845	2701	31243
Fathead minnow	UV-P	10000	845	2701	31243
Lake trout	UV-P	10000	845	2701	31243
White sturgeon	UV-P	10000	845	2701	31243
Japanese medaka	UV-P	30000	1938	6522	89218

2.4. Discussion

BUVSs are an environmentally relevant class of persistent contaminants, but current understanding of their toxicity to fishes is limited. There is a growing body of evidence that some BUVS, including UV-P, can act as agonists of the AhR based on studies of the human AhR in yeast reporter assays and increase of *cyp1a* expression in zebrafish.[15,51,52] In contrast, studies of Japanese medaka show no evidence of AhR activation by UV-P.[47] This suggests that BUVSs have the potential to cause adverse effects via activation of the AhR, but that potency might differ among species. These differences in potency could result from species-specific differences in sensitivities to activation of the AhR by BUVSs that are driven by differences in structure of the AhR protein. However, whether potencies of BUVSs for activation of the AhR differ among fish species was unknown. Therefore, the present study explored the toxicities of three commonly detected BUVSs to zebrafish embryos and their ability to activate the AhR of zebrafish and eight other species as a mode of toxicity. Results suggest that UV-P and UV-9, but not UV-090, are low potency agonists of the AhR2 of zebrafish and cause AhR-mediated toxicity in embryos, but there likely are substantial differences in sensitivities to BUVSs across distinct species of fish.

Exposure to UV-P, UV-9, and UV-090 caused dose-dependent increases in mortality among zebrafish embryos, with potencies relative to TCDD that are comparable to low potency agonists of the AhR, such as coplanar polychlorinated biphenyls (PCBs; **Figure 2-4; Table 2-7**).[110] The most potent BUVS, UV-P, had an LD_{50} of 4,772 ng/g-egg, followed by UV-9 with an LD_{50} of 11,608 ng/g-egg, and the least potent compound, UV-090, with an LD_{50} of 56,292 ng/g-egg **(Figure 2-4; Table 2-3**). While derived from just three serial concentrations, these LD_{50} values serve as an initial foundation for understanding the potencies of BUVSs. Furthermore, this is the first study to demonstrate mortality following exposure to BUVSs, but prior studies used

waterborne or dietary exposure which likely resulted in a lesser effective dose relative to microinjection.[47,51,52,111] In addition to mortality, zebrafish embryos exposed to UV-P, UV-9, and UV-090 in the present study showed increased incidences of AhR-mediated toxicity related to Blue Sac Disease, which encompasses yolk sac and pericardial edemas, and spinal curvature.[60,63] Further, transcript abundance of *cyp1a* increased in zebrafish embryos exposed to UV-P and UV-9, which aligns with previous findings and further suggests activation of the AhR (**Figure 2-2**).[15,52] In contrast, zebrafish embryos exposed to UV-090 did not show increased transcript abundance of *cyp1a* (**Figure 2-2**). Results from the *in vitro* AhR transactivation assay show activation of the zebrafish AhR2 by UV-P and UV-9 with $EC_{thresholds}$ of 1000 nM and 3000 nM, respectively, but zebrafish AhR2 was not activated by UV-090 up to the greatest tested concentration of 30,000 nM (**Table 2-5, Figure 2-3**). Activation of the zebrafish AhR2 by UV-P and UV-9, but absence of any activation by UV-090 is consistent with the *cyp1a* response in embryos and suggests that the embryo mortality following exposure to UV-P and UV-9 is, at least partially, mediated by the AhR. Considering the absence of increased *cyp1a* transcript abundance *in vivo* and the lack of AhR activation in the LRG assay, these findings strongly suggest that toxicity induced by UV-090 is not mediated by the AhR and instead occurs through a different mechanism. Further research is needed to identify the mechanism. It is more plausible that the observed *in vivo* mortality of zebrafish embryos exposed to UV-090 is due to a mode of toxicity that is independent of AhR activation. The *in vitro* AhR transactivation assay utilized COS-7 cells which lack an endogenous AhR pathway and have evidence of essentially no intrinsic expression of *cyp1a* that is responsible for phase I biotransformation of xenobiotics.[112] Therefore, it is unlikely that UV-090 is being hydroxylated through phase I metabolism *in vitro.* Although the mechanism by which UV-090 caused embryotoxicity is not currently known and is beyond the scope of the present investigation,

toxicities related to Blue Sac Disease can occur in fish through AhR-independent mechanisms and should be further investigated.[113-115]

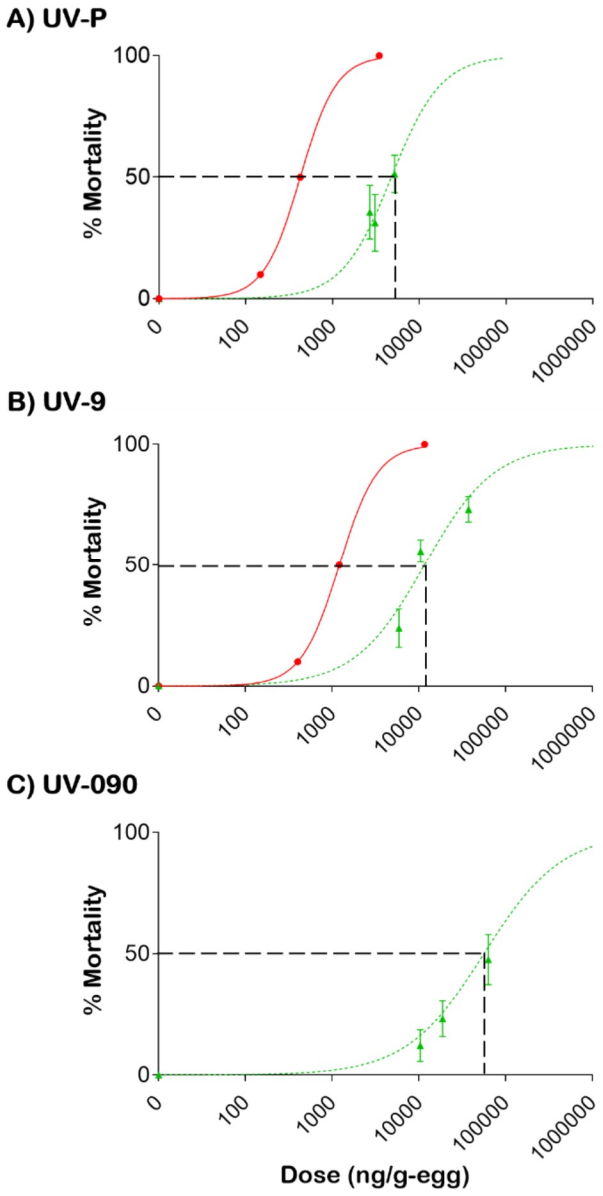

Figure 2-4: Dose response curves generated from early life-stage mortality of zebrafish embryos (<15 dpf) that were exposed to UV-P (A), UV-9 (B), or UV-090 (C) (green triangles and dotted line). Data is presented as mean ± standard error (±SE) based on three replicate studies, each with n = 24 ± 4 embryos per dose of chemical. Data are normalized for the control background mortality to equal 0. Intersect of long dashed lines indicates calculated lethal doses causing 50% mortality (LD_{50}) for each BUVS. Red circle and solid line dose-response curve is a prediction of the LD_{50} generated by using the quantitative adverse outcome pathway of low potency AhR agonists based on $EC_{threshold}$[69].

Table: 2-7: Potencies of BUVSs (UV-P, UV-9, UV-090) relative to TCDD for each species investigated in this study based on the calculated $EC_{threshold}$ and EC_{50} values.

Species	Chemical	ReP to TCDD	Order of Potency
White Sucker	UV-P	0.003	UV-P > UV-9 & UV-090
	UV-9	NA	
	UV-090	NA	
Zebrafish	UV-P	0.001	UV-P > UV-9 > UV-090
	UV-9	0.0003	
	UV-090	NA	
Lake sturgeon	UV-P	0.00003	UV-P & UV-9 > UV-090
	UV-9	0.00003	
	UV-090	NA	
Northern pike	UV-P	0.0003	UV-P & UV-9 > UV-090
	UV-9	0.0003	
	UV-090	NA	
Brook trout	UV-P	0.00001	UV-P > UV-9 & UV-090
	UV-9	NA	
	UV-090	NA	
Fathead Minnow	UV-P	0.00003	UV-P > UV-9 & UV-090
	UV-9	NA	
	UV-090	NA	
Lake trout	UV-P	0.000003	UV-P > UV-9 & UV-090
	UV-9	NA	
	UV-090	NA	
White sturgeon	UV-P	0.000003	UV-P > UV-9 & UV-090
	UV-9	NA	
	UV-090	NA	
Japanese Medaka	UV-P	0.00001	UV-P > UV-9 & UV-090
	UV-9	NA	
	UV-090	NA	

NA = Not Applicable

In fishes, interspecies differences in sensitivities to activation of the AhR2 can span several orders of magnitude between the most sensitive and least sensitive species.[62,82] Specifically, TCDD and other chlorinated dioxin-like compounds show clear differences in species sensitivities to AhR2 activation and associated early-life toxicities, with the zebrafish AhR2 being among the least sensitive to activation by TCDD.[62,76] As such, it is likely that zebrafish do not provide the optimal representation of sensitivities for native species of environmental and regulatory concern. Therefore, activation of the AhR2 of Japanese medaka, another model species, and seven native

fish species – brook trout, fathead minnow, lake sturgeon, lake trout, northern pike, white sucker, and white sturgeon – by UV-P, UV-9, and UV-090 was determined in the *in vitro* AhR transactivation assay. Across all species there was a 100-fold range of sensitivity to activation of the AhR2 by UV-P, with $EC_{thresholds}$ ranging from 300 nM to 30,000 nM (**Table 2-5**). The rank order of species sensitivities was white sucker > zebrafish > lake sturgeon = northern pike > brook trout = fathead minnow = lake trout = white sturgeon > Japanese medaka (**Table 2-5**). In a previous study, exposure of Japanese medaka, the species with the least sensitive AhR2 to activation by BUVSs, to UV-P showed no evidence of AhR activation *in vivo* based on abundance of *cyp1a* transcript and AhR-mediated embryotoxicities.[47] In studies with zebrafish, which possess one of the most sensitive AhR2's to activation by BUVSs in the transactivation assay, there was evidence of activation of the AhR2 based on increased transcript abundance of *cyp1a.*[15,52] These results suggest that the differences in species sensitivities observed in the *in vitro* transactivation assay translate to differences in sensitivity to AhR activation observed *in vivo*. TCDD has a similar range of sensitivity to activation of the AhR2 among species, being 33-fold based on $EC_{Threshold}$ (**Table 2-5**). However, the rank order of sensitivity to TCDD - lake trout > white sturgeon > brook trout > fathead minnow > lake sturgeon > Japanese medaka > white sucker > zebrafish > northern pike – is almost a reversed rank order of sensitivity to UV-P.[54,62] Similarly, UV-9 activated the AhR2 of the fishes that were among the least sensitive to TCDD (zebrafish, lake sturgeon, northern pike), but not the fishes that were among the most sensitive to TCDD (white sturgeon, brook trout, lake trout) (**Table 2-5**). However, there was no difference in the sensitivities to activation of the AhR2 among zebrafish, lake sturgeon, and northern pike which all had an $EC_{threshold}$ of 3,000 nM (**Table 2-5**). Due to the constraints of the LRG assay, it is possible for the $EC_{Thresholds}$ to exhibit an error of up to 3-fold. In terms of risk assessment, when considering that the maximal disparity in sensitivity to AhR activation is also 3-fold, it can be inferred that the species sensitivity to activation of the

AhR by UV-9 remains essentially consistent between these three species. In contrast, UV-090 did not activate the AhR2 of any of the species at any concentration tested (**Figure 2-3**). Taken together and based on the fishes tested here, results for UV-P and UV-9 are unique in that the rank order of species sensitivities to activation of the AhR2 appears to be approximately reversed when compared to TCDD. Therefore, species considered at least risk to chlorinated dioxin-like compounds could be at greatest risk to BUVSs that are agonists of the AhR. Several species are known to have AhRs that are substantially less sensitive to activation by TCDD relative to the fishes studied here and could be at elevated risk, including Atlantic cod (*Gadus morhua*), seabirds, and amphibians.[116-119]

Challenges related to assessing the risk of chemical pollutants based on measurement of early molecular changes, such as *in vitro* AhR transactivation, led to the development of adverse outcome pathways (AOPs) which provide a systematic way to understand and describe a sequence of events that lead from molecular-level occurrences, termed the molecular initiating event (MIE), to an adverse outcome (AO) of regulatory concern.[40,62] A mechanism-based biological model, referred to as a quantitative adverse outcome pathway (qAOP), quantifies the magnitude of the MIE to predict the likelihood or severity of subsequent AOs. As a result of variations in sensitivities among species to AhR activation, a qAOP has been developed that utilizes $EC_{threshold}$ from the standardized *in vitro* AhR transactivation assay utilized in the present study, which represents the MIE in this qAOP, to predict full dose-response curves of early-life stage mortality among fishes and other taxa, which denotes the AO of this qAOP, to within an order of magnitude, on average.[69] This qAOP has been validated for a suite of chlorinated dioxin-like compounds that range in potency by more than 30,000-fold.[69] Recently, it was demonstrated that this qAOP cannot predict early life toxicities for PAHs, likely resulting from complexity related to biotransformation.[68]

However, whether this qAOP could accurately predict dose-response curves of BUVSs was unknown. By use of $EC_{threshold}$ values from the present study as input into the qAOP model, the predicted LD_{50}s for UV-P and UV-9 in zebrafish were 426 ng/g-egg and 1,212 ng/g-egg (**Table 2-6**) while the calculated LD_{50}s were 4,772 ng/g-egg and 11,608 ng/g-egg, respectively (**Figure 2-4 & Table 2-3**). The fold-difference between the predicted and the experimental LD_{50}s was 6.2 lower for UV-P and 7.0 lower for UV-9 (**Table 2-8**). The MAEs for BUVSs are slightly higher than what had been previously reported for low-potency agonists of the AhR, however, the MAPE and fold-difference are within the range of accuracy determined for other low potency agonists using this model and represents an order of magnitude estimate commonly employed in risk assessments.[69] Therefore, these findings provide the first support for the qAOP having a chemical applicability domain beyond chlorinated dioxin-like chemicals and suggest that this model could be used to generate order of magnitude estimates of the toxicity of BUVSs to other species using $EC_{threshold}$ from the *in vitro* AhR transactivation assay.

Table 2-8: Metrics used to calculate the predicted lethal doses causing 0%, 10% and 50% mortality (LD_0, LD_{10} and LD_{50}) for both UV-P and UV-9 which include mean absolute error (MAE), mean absolute percent error (MAPE), and fold-difference.

Low-potency qAOP	MAE (ng/g egg)	MAPE (%)	Fold difference
UV-P	1701	71.8	6.2
UV-9	4285	84.1	7.0
Average	2993	78.0	6.6

Values used in calculating these metrics are presented in Table 1:
qAOP = quantitative adverse outcome pathway

Concentrations of BUVSs in fresh water can range from 0.7 ng/L – 701 ng/L and dried weight sediment concentrations can range from 0.16 µg/g – 35 µg/g.[13,15,19,25,27,35,91] Concentrations of certain BUVSs (UV-P, UV-329, UV-326, UV-234, UV-328, and UV-327), as high as 377 ng/g lipid-weight have been detected in the belly fat of fishes and approximately 0.9 ng/g, 0.64 ng/g,

and 0.64 ng/g median wet weight in the blood plasma of bottle nose dolphins (*Tursiops truncates)* *snapping turtles* (*Chelydra serpentina)*, and double-crested cormorant (*Phalacrocorax auritu),* respectively.[20,40,120] Results from the present study suggest that *in vivo* toxicity of BUVSs begins to occur at nearly 1500 ng/g for zebrafish embryos, which were one of the most sensitive species to AhR2 activation by BUVSs (**Table 2-3**). Therefore, based on the current reported environmental concentrations of BUVSs and the results of the present study, it is unlikely that BUVSs pose a significant threat to fishes through AhR-mediated pathways. A previous study found that several BUVS, including UV-P, accumulated in tissues, including ovaries, of zebrafish with a bioconcentration factor exceeding 10.[111] Given the lipophilicity of BUVSs, if the widespread use of BUVSs continues to increase globally, bioaccumulation and biomagnification of BUVSs could result in elevated exposure to BUVSs which in turn could lead to AhR-mediated effects. Japanese medaka, the least sensitive species tested in the AhR transactivation assay had no evidence of AhR activation in *in vivo* studies, suggesting that other species found to have AhRs that are insensitive, such as brook trout, fathead minnow, lake trout, and white sturgeon, are similarly unlikely to suffer from AhR-mediate toxicities from BUVSs. However, the evidence of zebrafish having sensitivities similar to PCB's suggests that other sensitive species, such as white sucker, northern pike, and lake sturgeon, might suffer AhR-mediated toxicities to BUVSs at toxicity thresholds similar to PCB's. If the reverse rank order of species sensitivity to AhR2 activation by BUVSs holds true *in vivo*, this becomes more problematic for other species that possess AhRs that are substantially less sensitive to TCDD than the species tested in the present study, such as Atlantic cod, seabirds, and amphibians.[116-119] Therefore, investigations on the sensitivity of AhR activation in these species should be explored as they could be at a greater risk for BUVS-induced AhR-mediated toxicities. To the best of our knowledge, this study presents the first evidence that AhR agonists beyond chlorinated dioxin-like chemicals are applicable to the previously developed qAOP.[69] Furthermore,

this study shows promise that the qAOP can be used to pragmatically assess sensitivities to BUVSs to a diversity of species from data generated from a standardized *in vitro* AhR transactivation assay. Utilizing this model could be essential for efficiently assessing potential risks to other species posed by BUVSs, including those species that could be at greatest risk.

CHAPTER 3: GENERAL DISCUSSION AND CONCLUSIONS

3.1 Introduction

Benzotriazole ultraviolet stabilizers (BUVSs), have garnered significant attention due to their widespread presence in the environment. These chemicals have been detected globally in various environmental compartments including, water, soil, wastewater, and biota.[10,11,13,20,26-28,32,38,90] There is concern that the environmental concentration of BUVSs will increase due to their widespread usage in consumer and industrial products, especially plastics. Their persistence and ubiquity raise questions about the potential consequences of acute and prolonged exposure to these compounds. Some studies have highlighted the potential for BUVSs to cause toxicity by activating the aryl hydrocarbon receptor (AhR), a crucial cellular transcription factor that plays a pivotal role in mediating responses to some environmental contaminants.[15,52] Therefore, the overall objective this research was to use a combination of *in vivo* and *in vitro* methods to assess if three BUVSs; UV-P, UV-9, and UV-090, that have been suggested to be AhR agonists, can cause AhR-mediated toxicities in fish. Agonists of the AhR, such as dioxin-like chemicals (DLCs), can have a wide range of potencies for receptor activation.[62] However, it is unknown if BUVSs can vary in their potency for AhR activation. Therefore, the first goal of this study was to investigate differences in potencies between BUVSs. Furthermore, species of fish can differ dramatically in their sensitivity to AhR-mediated toxicities.[76] As such, the second goal of this study was to characterize and assess the potential for species differences in sensitivity to AhR activation by BUVSs. The findings from this research will contribute to determining whether the activation of the AhR2 by BUVSs poses a potential risk to native fish populations.

3.2 Summary of Research Findings

3.2.1 in vivo toxicity testing of BUVSs

To assess AhR-mediated toxicities *in vivo*, freshly fertilized zebrafish embryos (less than 6 hours post-fertilization) were exposed through microinjection with a full-strength DMSO control or varying concentrations (1.67 ng/g-egg, 5.0 ng/g-egg, and 15.0 ng/g-egg) of UV-P, UV-9, or UV-090. Following microinjection, embryos were reared until they reached complete yolk sac absorption at 15 days post-fertilization, ensuring the full utilization of the administered dose by the developing embryos. Throughout the rearing period, the embryos were regularly examined at 24-hour intervals for any signs of malformations associated with Blue Sac Disease, including yolk sac edema, pericardial edema, spinal curvature, and mortality. A subset of embryos was selected shortly after hatching (at 5 days post-fertilization) and used for qPCR analysis to quantify mRNA abundance of the *cyp1a* gene, a biomarker for AhR activation. In addition, approximately 2,500 embryos were injected at each concentration of the respective BUVS and DMSO control to accurately measure the dosing concentrations.

Zebrafish embryos that were exposed to BUVSs by microinjection suffered dose-dependent increases in mortality with LD_{50}s of 4,772, 11,608, and 56,292 ng/g-egg for UV-P, UV-9, and UV-090, respectively. These findings suggest that the tested BUVSs are low-potency agonists of the AhR which are comparable to other low potency agonists of the AhR such as coplanar PCBs.[110] Additionally, embryos exposed to each BUVSs suffered greater instances of malformations, including yolk sac edema, pericardial edema, and spinal curvature, all of which can be caused by activation of the zebrafish AhR2. Transcript abundance of *cyp1a* increased by 5.9-fold and 42.2-fold in larvae exposed to the maximum concentrations of UV-P and UV-9, respectively, providing

strong indication that UV-P and UV-9 are AhR agonists, and the mortality and malformations observed *in vivo* are at least partially AhR-mediated. However, UV-090 did not significantly increase transcript abundance of *cyp1a* at the maximum injected concentration. While Blue Sac Disease-related malformations and a dose-dependent increase in mortality of zebrafish embryos exposed to UV-090 were observed, the absence of increased transcript abundance of *cyp1a* strongly suggests that the observed toxicities *in vivo* were not driven by activation of the AhR, suggesting that UV-090 is not an agonist of the zebrafish AhR2, and the observed toxicities *in vivo* are likely attributed to AhR-independent mechanisms, which is discussed in section 3.3.3.

3.2.2 in vitro AhR activation by BUVSs

To assess differences in species sensitivity to AhR activation by each BUVSs, a standardized *in vitro* LRG assay was utilized to quantify the activation of the AhR2's from two model species - zebrafish and Japanese medaka – that are not native to North America, and seven fish species that are native to North America, namely brook trout, fathead minnow, lake sturgeon, lake trout, northern pike, white sucker, and white sturgeon. COS-7 cells, which lack an endogenous AhR pathway, were chemically transfected with all the necessary components to assess AhR activation, including the AhR2 of each species[54,62,83,103], white sturgeon ARNT2[54], rat CYP1A reporter construct[104,105], and renilla luciferase vector to assess transfection efficiency. Cells were dosed with 9 serial concentrations (0.3 nM – 30,000 nM) of the chosen BUVS and luminescence was used to quantify activation of the AhR2 of each species.

The AhR2 of zebrafish was activated in a concentration-dependent manner by UV-P ($EC_{Threshold}$ of 1,000 nM) and UV-9 ($EC_{Threshold}$ of 3,000 nM), but not by UV-090. These findings support the evidence from the *in vivo* component of this work that UV-P and UV-9 act as agonists of the AhR2 of zebrafish, and that the mortality and malformations observed *in vivo* were caused

at least partly by AhR-mediated mechanisms. The lack of activation of the AhR by the maximal dose of UV-090 provides additional evidence that UV-090 is not an agonist of the AhR, further supporting results from the embryo toxicity study. Based on the results from the LRG, the rank order of potency for activating the AhR2 of zebrafish was UV-P > UV-9 > UV-090. The *in vitro* rank order of potency for the three BUVSs follows the same rank order of potency observed in the *in vivo* zebrafish embryotoxicity testing. UV-P activated the AhR2s of each of the nine species in a concentration-dependent manner with $EC_{Thresholds}$ ranging from 300 nM for white sucker and 30,000 nM for Japanese medaka. These results highlight that species differences in sensitivity to activation of the AhR by BUVSs exists and ranges up to 100-fold based on the nine tested species of fish. UV-9 also displayed concentration-dependent activation in zebrafish, lake sturgeon, and northern pike. However, there was no difference in the sensitivities to activation of the AhR2 among zebrafish, lake sturgeon, and northern pike, which all had an $EC_{threshold}$ of 3,000 nM. Due to the constraints of the LRG assay, it is possible for the $EC_{Thresholds}$ to exhibit an error of up to 3-fold. This means that there may be slight species differences in sensitivity between zebrafish, lake sturgeon, and northern pike exposed to UV-9 that were not detected in the LRG assay. UV-090 did not activate the AhRs of any of the tested species even at the maximal concentration. This further supports the notion that UV-090 is likely not an agonist of the AhR in fish. The rank order of species sensitivity to UV-P was white sucker > zebrafish > lake sturgeon = northern pike > brook trout = fathead minnow = lake trout = white sturgeon > Japanese medaka. However, the rank order of sensitivity to TCDD is lake trout > white sturgeon > brook trout > fathead minnow > lake sturgeon > Japanese medaka > white sucker > zebrafish > northern pike – which is almost a reversed rank order of sensitivity to UV-P. This means that current ecological risk assessments that are primarily based on TCDD may not be entirely applicable to BUVSs as species considered at least risk to chlorinated dioxin-like compounds could be at greatest risk to BUVSs that are agonists

of the AhR. Several species are known to have AhRs that are substantially less sensitive to activation by TCDD relative to the fishes studied here and could be at elevated risk, including Atlantic cod (*Gadus morhua*), seabirds, and amphibians.[116-119]

3.3 Future Research

3.3.1 In vivo Confirmation of in vitro Results

In this study, an initial effort was made to characterize the potential for three BUVSs –UV-P, UV-9, and UV-090 – to cause AhR mediated toxicities to fish. For *in vivo* assessment, zebrafish were used as they are a commonly used model species in chemical toxicity assessments, have well described embryo development, and obtaining large numbers of embryos is routine. Zebrafish embryos microinjected with UV-P and UV-9 displayed phenotypes that are characteristic of AhR mediated toxicity, and also had greater mRNA abundance of *cyp1a*. Using an *in vitro* LRG assay, the AhR2 of zebrafish was activated by UV-P and UV-9, supporting that *in vivo* toxicity is likely caused by AhR activation. However, for the other eight species used in this study - brook trout, fathead minnow, Japanese medaka, lake sturgeon, lake trout, northern pike, white sucker, and white sturgeon - the potential for AhR mediated toxicity is based only on activation of their respective AhR2's in the LRG assay. While the *in vitro* results suggested that these species might differ by as much as 100-fold in their sensitivity to AhR-mediated toxicity, it is advisable to investigate the toxicities of BUVSs in other species with accessible embryos to confirm the observed activation in the *in vitro* LRG assay thus, allowing for better informed ecological risk assessment of BUVSs.

3.3.2 Metabolism of BUVSs

Aquatic organisms are constantly exposed to a variety of xenobiotics and need ways to process these compounds to mitigate their toxicities. Organisms can metabolize a variety of

xenobiotic compounds through phase I and phase II biotransformation enzymes to increase their water solubility and facilitate excretion, and removal of xenobiotic compounds through efflux pumps during phase III biotransformation.[121,122] Cytochrome p450 (CYP450) enzymes, such as CYP1A, CYP1B, and CYP3A4, catalyse the biotransformation of substrates during phase I detoxification.[121] In phase II detoxification, the products of phase I reactions are attached to other molecules, such as glutathione, sulfate, glycine, or glucuronic acid, by different enzymes, making them more soluble and easier to eliminate.[121,122] To date, understanding of the metabolism of BUVSs is limited to a small number of studies, however, several BUVSs have been investigated with respect to metabolism and biotransformation. Reports have indicated that certain BUVSs, specifically UV-P and UV-328, undergo biotransformation mediated by CYP3A4, leading to alterations in their activity as agonists of the human androgen receptor (AR).[46] Interestingly, effects of CYP3A4 mediated metabolism were chemical specific as the potency of UV-P as an AR agonist was decreased but potency of UV-328 increased.[46] Furthermore, it has been demonstrated that UV-P, UV-326, UV-327, UV-328, and UV-329 are bio-transformed to add hydroxyl or carbonyl moieties in the livers of zebrafish.[111] The hydroxylation process has the potential to decrease the hydrophobic nature of BUVSs, thereby influencing the interactions between the substances and biomacromolecules.[111] Additionally, several hydroxylated and oxidative phase I metabolites have been detected for UV-327 and UV-328.[123,124] These results suggest that biotransformation of BUVS may play a pivotal role in mediating their toxicity.[46,111,123,124] Therefore, this is an area of research that needs more attention and investigation to understand the potential toxic effects of BUVSs to fishes. As such, investigating the impact and role of phase I biotransformation on modifying the potency of BUVSs as agonists of the AhR is warranted.

3.3.3 Embryotoxicity of UV-090

In fishes, oxidative stress is a common mode of toxicity caused by various contaminants present in aquatic environments. For example, fish exposed to substances such as metformin, brominated flame retardants (BFR's), and selenomethionine can generate reactive oxygen species and induce oxidative stress.[113-115] Additionally, following the exposure to these contaminants, fish have been observed to have elevated instances of malformations, including tail scoliosis, pericardial edema, yolk deformation, and cranial facial deformities.[113-115] In this thesis, UV-090 caused developmental phenotypes consistent with AhR activation, but also consistent with induction of oxidative stress. The lack of greater mRNA abundance of *cyp1a* in embryos injected with UV-090, and the lack of AhR activation *in vitro*, is strong evidence that the observed developmental phenotypes are caused by mechanisms that are independent of the AhR, potentially driven by oxidative stress. Certain biomarkers are used to identify oxidative stress in fish. These include lipid peroxidation, protein carbonyl content, and hydroperoxide content, as well as greater expression and activity of the enzyme's glutamate-cysteine ligase catalytic subunit (GCLC), catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPX), and glutathione s-transferase (GST).[101,113] Future studies should assess whether UV-090 causes embryotoxicity via oxidative stress.

3.3.4 Screening of Additional BUVSs and BUVS Mixtures

BUVSs are a broad class of chemical contaminants that encompass numerous individual compounds that have the potential to be toxic to aquatic organisms. Results from previous studies have indicated that certain BUVSs can activate the AhR.[52] For example, a prior study that screened seven BUVSs including, benzotriazole (1HBT), UV-P, UV-320, UV-326, UV-327, UV-328, and

UV-329, reported increased *cyp1a* transcript abundance in zebrafish embryos exposed to waterborne UV-P or UV-326.[52] In another study, screening of 13 structurally diverse BUVSs for their ability to activate the AhR of mice found that UV-P, UV-9, UV-090, and UV-PS were agonists of the mammalian AhR.[22] As such, it is likely that other BUVSs, that were not assessed in this study, have the potential to interact and activate the AhR causing adverse effects. Based on the results from this study, the three tested BUVSs had substantial differences in potency for activating the AhR in fish with a rank order of potencies being UV-P > UV-9 > UV-090. Therefore, it is possible that other BUVSs are agonists of fish AhR2 and that could be more or less potent than the BUVSs screened in this study. Screening of other BUVSs *in vitro* and *in vivo* is needed to more fully characterise the risk that BUVSs pose to fish.

BUVSs have been detected in the environment in several different matrices and at different concentration levels.[24,25] In many cases, multiple BUVSs are detected in a single environmental sample.[19,25] Therefore, it is likely that aquatic organisms that are developing in BUVSs contaminated water or sediment are exposed to multiple BUVSs at one given time. However, knowledge regarding synergistic or additive effects of BUVSs is currently unknown. Other classes of chemical contaminants such as some PAHs, can cause a synergistic activation of the AhR.[125] As such, it is possible that co-exposure to BUVSs could result in synergistic or additive toxic effects. Therefore, research pertaining to the toxicity of BUVS mixtures to aquatic organisms is warranted.

3.3.5 Potency of BUVS in other organisms

Previous studies have identified that species of fish can vary in their sensitivity to AhR activation by other agonists like DLCs and PAHs by several orders of magnitude between the most and least sensitive species.[62,82] In this study an initial effort was made to assess if differences in species sensitivity to AhR activation by BUVSs exists. Across all species, there was a 100-fold

range in species sensitivity to AhR activation by UV-P between Japanese medaka and white sucker the least and most sensitive species, respectively, based on $EC_{Threshold}$. The rank order of species sensitivity to AhR activation by UV-P was white sucker > zebrafish > lake sturgeon = northern pike > brook trout = fathead minnow = lake trout = white sturgeon > Japanese medaka. However, the rank order of species sensitivity to TCDD is lake trout > white sturgeon > brook trout > fathead minnow > lake sturgeon > Japanese medaka > white sucker > zebrafish > northern pike.[54,62] When comparing the rank order of species sensitivities between TCDD and BUVSs, it appears that BUVSs have an approximate reverse rank order of species sensitivity to AhR activation between the two chemicals. This means that current ecological risk assessments that are primarily based on TCDD may not be entirely applicable to BUVSs as species considered at least risk to chlorinated DLCs could be at greatest risk to BUVSs that are agonists of the AhR. Several species are known to have AhRs that are substantially less sensitive to activation by TCDD relative to the fishes studied here and could be at elevated risk, including Atlantic cod, seabirds, and amphibians.[116-119] Therefore, assessing AhR mediated toxicities in species that are less sensitive to TCDD is advisable as if the approximate reverse species sensitivity to BUVSs remains true, these species may be at more risk to the toxicities of BUVSs. This will broaden the scope of knowledge beyond fish alone to better informed ecological risk assessment of aquatic organisms as a whole.

3.4 Conclusions

This study offers fresh insights into the potential harmful effects of BUVSs on fish, contributing to more informed ecological risk assessments for the diverse fish species found in Canada. Concentrations of BUVSs in the environment are presently low. However, due to their widespread usage and high lipophilic nature, combined with suboptimal disposal waste practices, BUVS concentrations are likely to increase in the future. Therefore, developing, and mature fish

are likely to be exposed to elevated concentration in the future. This study demonstrates that BUVSs have the capability to exert toxicity through AhR-mediated pathways and provides an initial understanding of the potential toxicity of these chemicals to fishes. Furthermore, this study provides evidence that a previously developed qAOP used for low-potency agonists of the AhR is applicable and can serve as a valuable tool for screening species that are difficult to study in a laboratory setting, threatened, or endangered. Lastly, the results from this study suggest that current ecological risk assessments based on AhR activation by DLCs may not be applicable to BUVSs as an approximate reverse species sensitivity to AhR activation relationship was observed. This means that organisms with more tolerant AhRs to TCDD such as, Atlantic cod, amphibians, and sea birds may be at elevated risks pertaining to BUVSs. Therefore, understanding the potential adverse impacts of BUVSs and their mode of toxicity is imperative for determining whether BUVSs pose a significant threat to fish populations.

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