CARDIOTOXICITY AND INTERSPECIES SENSITIVITY OF EARLY LIFE STAGE FISH TO POLYCYCLIC AROMATIC COMPOUNDS

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DEDICATION

I would like to dedicate this thesis to my parents, Lianne and David, for their unwavering support and for teaching me the value of hard work.

ABSTRACT

Polycyclic aromatic compounds (PACs) are a broad class of organic contaminants that are present in all types of hydrocarbon fuels and produced from the incomplete combustion of organic matter. During early development, PAC exposure induces sublethal and lethal toxicity in fish. Predictive tools such, as the target lipid model (TLM), have been developed to estimate these impacts. Although cardiotoxicity is a well-established outcome of early life stage crude oil and PAC exposure, few studies have compared cardiotoxicity endpoints across freshwater fish species, and applications of cardiotoxicity endpoints within a TLM framework remain limited. Therefore, the aim of this study was to compare lethal and cardiotoxicity endpoints of larval walleye (*Sander vitreus*), fathead minnow (*Pimephales promelas*), and rainbow trout (*Oncorhynchus mykiss*) following exposures to naphthalene, dibenzothiophene and benz(a)anthracene. Larvae from each species were exposed using a passive-dosing system to five serial concentrations of each PAC, with exposures initiated within 24 h post-hatch and terminated at seven days post-hatch. The TLM framework was able to effectively characterize lethality, bradycardia and ventricle to atrium length ratio (walleye only), enabling a critical target lipid body burden (CTLBB) for each endpoint and species. Although walleye exhibited significantly lower sensitivity to acute lethality relative to the other species, no significant differences between species were observed for bradycardia endpoints. Despite their high survival, walleye exhibited significant reductions in ventricle length and diminished cardiac function following exposure to PACs. This study is the first to successfully apply cardiotoxicity endpoints in fish to a TLM and the first to produce CTLBB data for walleye. Results from this study further support the integration of cardiac endpoints into TLM-based approaches for improving the prediction of sublethal impacts from oil spills.

CONTRIBUTION OF AUTHORS

The candidate is the main author of chapters 1-3. The candidate conducted the experiments, collected the data, and performed the data analysis stated in chapter 2. Dr. Steve Wiseman and Dr. Danielle Philibert contributed to experimental design, data interpretation, and scientific input of chapter 2, and edited chapters 1-3. Dr. Danielle Philibert advised on statistical analysis and completed chemical analysis for chapter 2.

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To my partner Caleb, thank you for supporting and encouraging me to pursue this chapter of my life. Thank you for your patience with the long hours, stress, and late nights. Thank you for loving and reassuring me, even on days when it wasn't easy.

Lastly, to my parents, Lianne and David, your unconditional love, support, and belief in me are the foundation of who I am and all that I have achieved. I am so lucky to have you as my parents. Every challenge I face is easier knowing you're behind me, cheering me on.

Table Of Contents

LIST OF TABLES

LIST OF FIGURES

LIST OF EQUATIONS

LIST OF ABBREVIATIONS

AhR	Aryl hydrocarbon receptor
ARNT	Aryl-hydrocarbon receptor nuclear translocator
AV	Atrioventricular conduction
BaA	Benz(a)anthracene
BaP	Benzo(a)pyrene
BE-SPME	Biomimetic extraction-solid phase microextraction
CAS	Chemical Abstract Services registry number
CCME	Canadian Council of Ministers of the Environment
CTLBB	Critical target lipid body burden
Cyp1a	Cytochrome P450 1A
DBT	Dibenzothiophene
dpf	Days post-fertilization
dph	Days post-hatch
EC	Effect concentration
HC5	Hazardous concentration 5%
HSP90	Heat shock protein 90
I_{Kr}	Delayed rectifier potassium channel current
LC	Lethal concentration
LOEC	Lowest observed effect concentration
Log K_{OW}	Log octanol-water partition coefficient
MeOH	Methanol
MS-222	Tricaine mesylate
MW	Molecular weight
NAP	Naphthalene
PAC	Polycyclic aromatic compound
PAH	Polycyclic aromatic hydrocarbon
PDMS	Polydimethylsiloxane
PHE	Phenanthrene
RPM	Rotations per minute
SSD	Species sensitivity distribution
TLM	Target lipid model
USEPA	United States Environmental Protection Agency
XAP2	Hepatitis B virus X-associated protein 2
XRE	Xenobiotic response elements

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1 Polycyclic aromatic compounds

Polycyclic aromatic compounds (PACs) are a class of organic compounds that naturally occur in hydrocarbon deposits (Patel et al., 2020). Characterized by having two or more fused benzene rings, PACs are primarily composed of carbon and hydrogen atoms but may also include heteroatoms like sulfur, nitrogen, or oxygen (e.g., dibenzothiophene (DBT), carbazole, and dibenzofuran) (Wallace et al., 2020). Polycyclic aromatic hydrocarbons (PAHs) are a class of PACs composed of aromatic rings and include only carbon and hydrogen atoms. Derived from natural and anthropogenic sources, PACs can be biogenic, petrogenic, or pyrogenic in origin (Patel et al., 2020). Biogenic PACs are produced when organisms such as fungi, plants, or bacteria synthesize organic material (Patel et al., 2020). Petrogenic PACs are naturally occurring constituents of hydrocarbon deposits in every utilized form of fossil fuel, such as coal, bitumen, oil, and gas (Patel et al., 2020). Pyrogenic PACs are produced through the incomplete combustion of organic matter or the burning of fossil fuels (Patel et al., 2020). Environmental PAC contamination is predominantly attributed to petrogenic and pyrogenic sources, which generate the greatest volume of emissions through hydrocarbon extraction, refining, and combustion (Hodson et al., 2020).

In 1976, the United States Environmental Protection Agency (USEPA) classified 16 PAHs as priority contaminants, based on their toxicities, relative abundances, and the availability of analytical standards (**Figure 1.1**; Keith, 2015; Roger et al., 2002). However, there have been questions regarding the suitability of the USEPA's list of priority PAHs when applied to environmental risk assessments, as the list may not be representative of the full range of toxic

potencies exhibited by all PAHs (Andersson & Achten, 2015). Several studies have found that relying entirely on this list may dramatically underestimate the amount of PAC contamination in the environment and, therefore, could lead to regulatory actions that are based on incomplete datasets (Barron & Holder, 2003; Larsson et al., 2013).

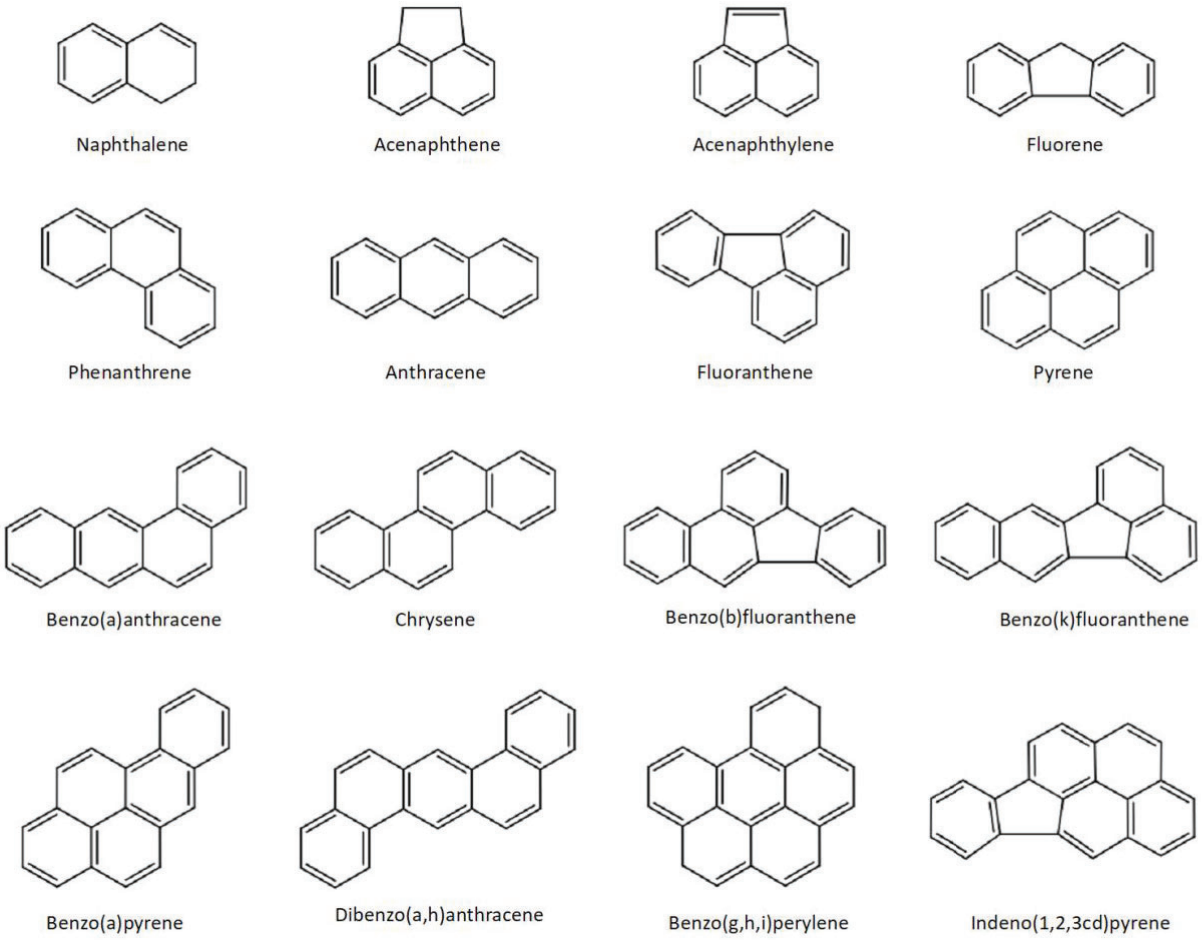

Figure 1.1 Molecular structure of the United States Environmental Protection Agency's (USEPA's) 16 priority pollutant PAHs, adapted from Rogers et al. (2002).

Due to their hydrophobic properties, PACs are persistent in aquatic environments and tend to accumulate in sediments where many fish species deposit their eggs (Hodson et al., 2020; Kurek et al., 2013). After hatching, yolk-sac larvae will spend much of their time lying on the lake or stream bed, resting on the potentially contaminated sediment layer before they reach the developmental stage where they have the buoyancy to remain in the water column. The lipophilic nature of PACs allows them to readily partition directly from water or sediments into the lipid-rich tissues of larval fish (Ramachandran et al., 2006). As hydrocarbon-related industries continue to expand, there is a need to understand the threat that PACs associated with these industries pose to our native fish species.

1.2 Modes of action

Polycyclic aromatic compounds cause toxicity through numerous mechanisms, resulting in a diverse range of adverse effects. Exposure of fish to PACs can cause developmental abnormalities, cardiotoxicity, and lethality, effects which are often not attributed to a singular mode of action (Hodson et al., 2020; Incardona, 2017; Wallace et al., 2020).

1.2.1 Nonspecific toxicity

Low molecular weight (MW) PACs, those with three or fewer rings, induce toxicity through nonspecific or baseline toxicity (i.e., narcosis) (Billiard et al., 2008). Characterized as a reversible state of arrested activity, narcosis toxicity occurs when chemicals act non-specifically to disrupt cell membrane function (Incardona, 2017; Hodson et al., 2020; van Wezel & Opperhuizen, 1995). Toxicity from this nonspecific mode of action occurs when membranes accumulate a quantity of a contaminant that is sufficient to cause an effect (Incardona, 2017; van Wezel & Opperhuizen, 1995). The impacts of narcotics are functionally compared to the effects

of anesthetizing agents, such as tricaine mesylate (MS-222), acting as a neuronal sodium (Na^+) channel blocker, effectively placing the organism in a state of arrested activity, and potentially leading to mortality (Incardona, 2017). Non-polar hydrophobic compounds, such as PACs, are lipophilic and tend to accumulate in the organism's lipid-rich tissues (Di Toro et al., 2000; Petersen & Kristensen, 1998; van Wezel & Opperhuizen, 1995). The toxicity of PACs, as it relates to narcosis, has a direct relationship with the lipophilicity of the compound (Di Toro et al., 2000). Lipophilicity can be expressed as the log octanol-water partition coefficient (log K_{OW}), which is a measure of a compound's ability to partition between a polar solvent (water) and a non-polar organic solvent (octanol) (Hodson et al., 2020). A critical assumption of narcosis-based toxicity is that PACs partition from water into lipid tissues at concentrations proportional to their log K_{OW} (Di Toro et al., 2000). The mechanisms involved in baseline toxicity are still poorly understood; however, there is a consistent correlation between PAC toxicity and its log K_{OW} (Di Toro et al., 2000; McGrath et al., 2005; Philibert et al., 2021).

1.2.2 AhR-dependent toxicity

Many PACs cause toxicity through modes of action other than nonspecific or baseline toxicity. One such mechanism is the dysregulation of the aryl hydrocarbon receptor (AhR). This protein is a ligand-activated transcription factor that, when inactive, is localized to the cytosol and is bound to chaperone proteins, including heat shock protein 90 (HSP90) (Shankar et al., 2020). While the endogenous role of the AhR is not fully known, activation of the AhR increases the expression of genes that encode a broad suite of enzymatic and non-enzymatic proteins from numerous physiological processes, including proteins that facilitate the biotransformation of xenobiotics (Harris et al., 2020). A classic example is cytochrome P450 1A (cyp1a), which

catalyzes phase I xenobiotic metabolism reactions, and whose expression is measured as a biomarker of AhR activation (**Figure 1.2**) (Harris et al., 2020).

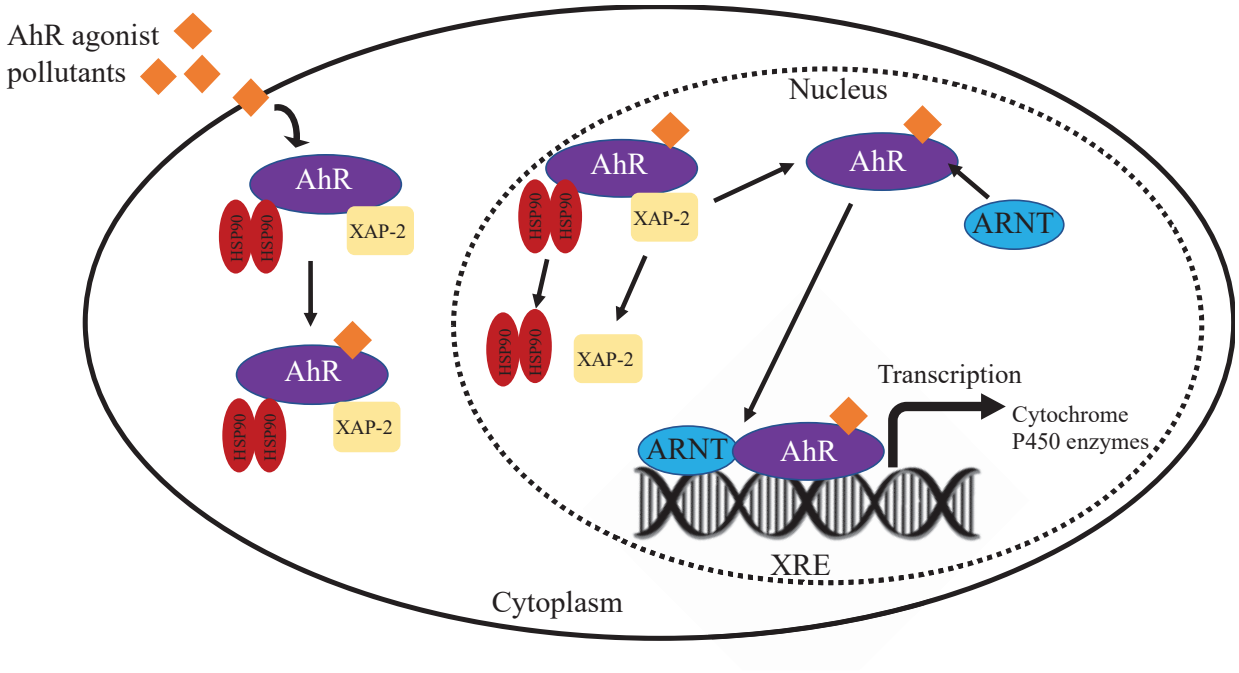

Figure 1.2 Schematic of the aryl hydrocarbon receptor (AhR) activation pathway in response to endogenous ligands, adapted from Vyavahare et al. (2024). An AhR agonist enters the cytosol through the cellular membrane and binds to AhR. Once ligand-bound, it undergoes a conformal change and translocates into the nucleus, dissociating from its chaperone protein (HSP90) and co-chaperone protein (XAP2), forming a heterodimer with the aryl-hydrocarbon receptor nuclear translocator (ARNT) protein. The AhR/ARNT complex binds to the xenobiotic response elements (XREs) on DNA, inducing cyp1a enzymes that work to metabolize the ligands. Biotransformation enzymes transform xenobiotics into more water-soluble metabolites that can then be excreted.

Biotransformation of toxicants usually occurs in two phases. During phase I, cyp1a enzymes work to oxidize, reduce, or hydrolyze compounds, after which phase II enzymes catalyze the conjugation of large hydrophilic moieties to phase I metabolites (Santos & Bueno dos Reis Martinez, 2020; Tierney et al., 2013). However, metabolism and biotransformation of PACs through cyp1a activity can form reactive metabolites that are more toxic than the parent compound (Harris et al., 2020; Tierney et al., 2013). Cyp1a metabolic activity is believed to be largely responsible for the oxidative stress and cellular damage caused by PACs (Incardona et al., 2005).

While not all PACs activate the AhR, the number of aromatic rings in the compound positively correlates to AhR activity (Amakura et al., 2016). Although a small number of low MW PACs can weakly activate the AhR, PACs with four or more rings are more likely to be strong AhR agonists (Amakura et al., 2016). In the early life stages of fish, AhR-dependent toxicity is associated with mortality and developmental abnormalities such as craniofacial deformities, pericardial and yolk-sac edema, cardiovascular deformities, and delayed development (Barron et al., 2004b; Dubiel et al., 2023).

1.2.3 Cardiotoxicity

Developmental cardiotoxicity can occur via mechanisms that are independent of narcosis or AhR activation, indicating that multiple mechanisms can occur in the same cells simultaneously (**Figure 1.3**) (Incardona, 2017).

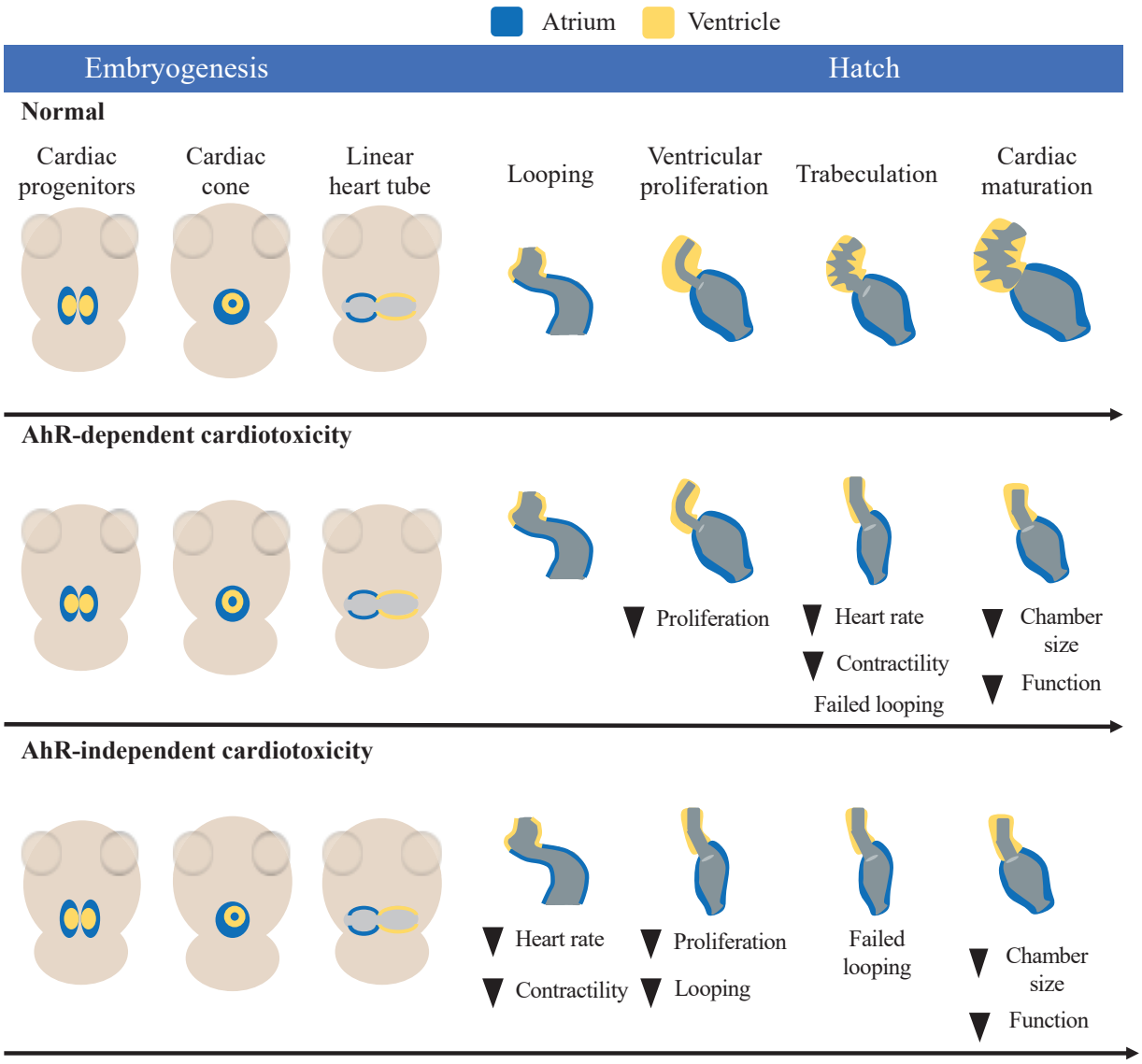

Figure 1.3 Visualization of normal, AhR-dependent, and AhR-independent cardiotoxicity in zebrafish (*Danio rerio*) adapted from Incardona (2017). Top: Typical progression of embryogenesis from early cardiogenic differesntiation to larval cardiac maturation. Middle: AhR-dependent toxicity reduces ventricular proliferation, resulting in failed looping and decreased cardiac contractility, chamber size, and function. Bottom: decreased heart rate and contractility during cardiac looping, resulting in decreased proliferation, chamber size, and function.

A link between crude oil exposure and cardiotoxicity in fish has been well established (Brette et al., 2017; Incardona, 2017; Sørhus et al., 2016). Because the developing fish heart has been recognized as a target of crude oil toxicity, individual PACs have been investigated for their roles in cardiotoxicity. Exposure of zebrafish (*Danio rerio*) embryos to two 3-ring PACs, DBT and phenanthrene (PHE), resulted in similar developmental cardiotoxicity and intracranial hemorrhaging with and without morpholino knockdown of AhR2 expression (Incardona et al., 2005). However, the AhR2 morphants experienced a higher degree of arrhythmia than the fish without AhR knockdown, indicating a degree of effective detoxification from cyp1a (Incardona et al., 2005).

Tricyclic PACs are strongly linked to cardiotoxic effects in early life stage fish and have been the focus of several studies investigating their disruption of cardiac function (Abramochkin et al., 2021; Ainerua et al., 2020; Brette et al., 2017; Incardona et al., 2004, 2015; Incardona & Scholz, 2016; Incardona, 2017; Sørhus et al., 2016). Rather than AhR activation, cardiotoxicity induced by tricyclic PACs could be the result of impacts to voltage-gated ion channels, effectively disrupting the regulation of potassium (K^+) and calcium (Ca^{2+}) ions, thereby altering cardiac excitability and rhythm (Billiard et al., 2008; Brette et al., 2017; Incardona et al., 2005; Langheinrich et al., 2003). Electrical activation of the fish heart begins with the opening of voltage-gated Na^+ channels, allowing Na^+ influx that depolarizes the cardiomyocyte. This is followed by a short K^+ efflux and the opening of L-type Ca^{2+} channels, where Ca^{2+} enters the cell to initiate excitation-contraction coupling and triggering of muscle contraction. Repolarization occurs via delayed rectifier K^+ channel current (I_{Kr}), followed by Na^+ / Ca^{2+} exchangers that restore resting ion gradients in preparation for the next cardiac cycle (Ainerua et al., 2020; Brette et al., 2017). One study found that brown trout (*Salmo trutta*) exposed to PHE experienced a

70% inhibition of the I_{Kr} and a 38% reduction in L-type Ca^{2+} currents, slowing repolarization and increasing the interval between ventricular repolarization and depolarization (Ainerua et al., 2020). Ventricular structure and function have also been shown to be particularly susceptible to the effects of crude oil (Sørhus et al., 2016). When Atlantic haddock (*Melanogrammus aeglefinus*) embryos were exposed to crude oil for seven days and transferred to clean water for hatch, the larvae experienced several cardiac defects, such as ventricular asystole (silent ventricle), reduced contractility, morphological underdevelopment, and arrhythmias (Sørhus et al., 2016). Additionally, 2:1 atrioventricular conduction (AV) blocks have been observed in larval fish following exposure to tricyclic PACs and crude oil, with this arrhythmia resulting from PAC-induced inhibition of the I_{Kr}, which delays ventricular repolarization (Ainerua et al., 2020; Incardona et al., 2004; Sørhus et al., 2016). A normal functioning cardiovascular system in all vertebrates, including fish, is essential for the fitness and survival of the organism. Negative impacts on the structure and or function of the cardiovascular system could threaten the survival of the fish, or at a minimum, impede metabolically demanding activities.

1.3 Target lipid models

The target lipid model (TLM) is widely utilized to assess the acute lethality of PACs based on a positive relationship between lethality and immobilization, and lipid solubility (Hodson et al., 2020). Adopted by the USEPA, the TLM has served as a foundation for developing regulatory criteria for PACs that act by a nonspecific mode of action (U.S. EPA, 2008). The primary application of TLMs is to predict the lethality and immobilization of organisms exposed to hydrocarbons and other non-ionic organic compounds, to derive hazardous concentration values for 5% of species (HC5), a concentration above which 95% of species should be protected from adverse effects (McGrath et al., 2018). As PACs are lipophilic, they

accumulate in the plasma membrane of the cells of lipid-rich tissue (Petersen & Kristensen, 1998; van Wezel & Opperhuizen, 1995). A linear relationship between lipophilicity and toxicity exists for compounds with a log K_{OW} of 2-5.5 (Incardona et al., 2004; Philibert et al., 2021). Compounds with a log K_{OW} of less than 2 are hydrophilic, making it unlikely that they will partition into lipids (Di Toro et al., 2000). For PACs with log K_{OW} > 5.5, the relationship with toxicity deviates from linearity as their aqueous solubility is too low to partition into lipids at a high enough concentration to exert toxicity (Di Toro et al., 2000; Incardona et al., 2004; Philibert et al., 2021). The narcotic effect of PACs can be quantified by the critical target lipid body burden (CTLBB) (Marzooghi & Di Toro, 2017). The CTLBB is an estimated concentration of a chemical in an organism's lipid tissue that is associated with a specified toxic effect and is an important variable in the TLM to adjust for interspecies differences in sensitivity. The conceptual foundation for this model is based on the relationship between a compound's predicted log K_{OW} and acute toxicity (**Equation 1.1**) (Di Toro et al., 2000).

$$\mathbf{\log(LC_{50i}) = m \log(K_{OWi}) + b} \qquad \mathbf{1.1}$$

m = slope

b = y-intercept

A universal narcosis slope of -0.94 is used in **Equation 1.1** as it is constant across species and compounds (Di Toro et al., 2000). While it is expected that all species will share the same lipid-octanol relationship (m = -0.94), the y-intercept can vary between species (**Equation 1.1**) (Di Toro et al., 2000). To accommodate for variation in species sensitivity, the y-intercept is adjusted to a species-specific CTLBB (**Equation 1.2**).

$$\log(CW_i) + \Delta c_i = -0.94 \log(K_{OWi}) + \log(CTLBB) \quad \textbf{1.2}$$

CW = Critical aqueous concentration for an endpoint (µmol/L)

Δc_i = Chemical class corrections if required ($\log_{10}$ µmol/L)

As the research described in this thesis addresses structurally different PACs, a polyaromatic hydrocarbon chemical class correction factor (Δc_i) of 0.364 will be applied to PACs without a heteroatom, and 0.47 to PACs containing a sulfur heteroatom. (**Equation 1.2**) (McGrath et al., 2021). To apply the TLM framework to predict PAC toxicity, the log K_{OW} of the compound and species-specific CTLBB must be known. For CTLBB data to be derived for a species-specific endpoint, L/EC data must first be collected through a toxicity assay. With the log K_{OW}, chemical class corrections, and the species-specific CTLBB, TLMs can be applied to predict species sensitivity to a wide variety of PACs (**Equation 1.2**).

Much of the acute toxicity data for animal taxa in the TLM database has been produced from measuring lethality or immobilization endpoints (McGrath et al., 2018; Tillmanns et al., 2023). However, results from Dubiel et al. (2024) suggest that TLMs may also be applicable to other sublethal endpoints. Dubiel et al. (2024) assessed cardiotoxicity in American lobster (*Homarus americanus*) larvae following a 48-hour exposure to ten PACs by measuring a decrease in heart rate (bradycardia). When the heart rate data was applied to a TLM, an American lobster CTLBB for bradycardia was produced, further expanding the CTLBB database to include an acute cardiotoxicity endpoint (Dubiel et al., 2024). As cardiotoxicity is a well-documented outcome of PAC and crude oil exposure, its integration into the TLM framework represents a promising yet underutilized application for predicting sublethal toxicity.

Target lipid models are particularly useful in determining toxicity to larvae as, during this life stage, there is a high surface area for chemical uptake and a high lipid content relative to body weight (Petersen & Kristensen, 1998). A study by McGrath et al. (2018) expanded the TLM framework by incorporating a larger and more phylogenetically diverse species dataset. It was found that including species with limited toxicity data did not produce higher uncertainty in the TLM coefficients (McGrath et al., 201). By including additional species that were underrepresented in earlier versions of the model, McGrath et al. (2018) addressed previous limitations of the TLM framework and produced a robust HC5 characterization for chronic exposure to hydrocarbons. In a follow-up study, it was confirmed that the TLM framework is also applicable for heterocyclic aromatic compounds in that their HC5s were protective of both sublethal and lethal chronic endpoints, thereby substantiating the broad application potential of TLMs and supporting its use in risk assessments of petroleum substances (McGrath et al., 2021). Despite the promising research that has been completed to date, several authors have criticized TLMs for the selection of narcosis as the primary mode of action, describing it as an ineffective characterization of PAC toxicity, arguing that there is a lack of evidence that demonstrates narcotic actions in organisms exposed to PACs (Incardona, 2017). While the specific mechanism(s) of narcosis are not well understood, there is a demonstrated correlation between toxicity and the lipophilicity of PACs, evidenced by the successful application of TLMs, and these models are continuously refined with additional species and compounds to improve the accuracy of their predictions (McGrath et al., 2021).

1.4 Interspecies sensitivity

While there are over 20,000 identified fish species (National Oceanic and Atmospheric Administration Fisheries, 2022), individual PAC toxicity data produced through robust

methodologies are unavailable for many fish species, emphasizing the need for further investigation (Blewett et al., 2024; Dubiel et al., 2023; Hodson et al., 2020). Existing studies indicate that sensitivity to PACs can vary widely among species. For example, a study examining activation of the AhR2 by benz(a)anthracene (BaA) across nine freshwater fish species reported a 141-fold difference in sensitivity among species (Dubiel et al., 2023). Additionally, a 10,000-fold difference in species sensitivity to acute lethality caused by exposure to benzo(a)pyrene (BaP), a strong AhR agonist PAH, has been established (**Figure 1.4**; Hodson et al., 2020).

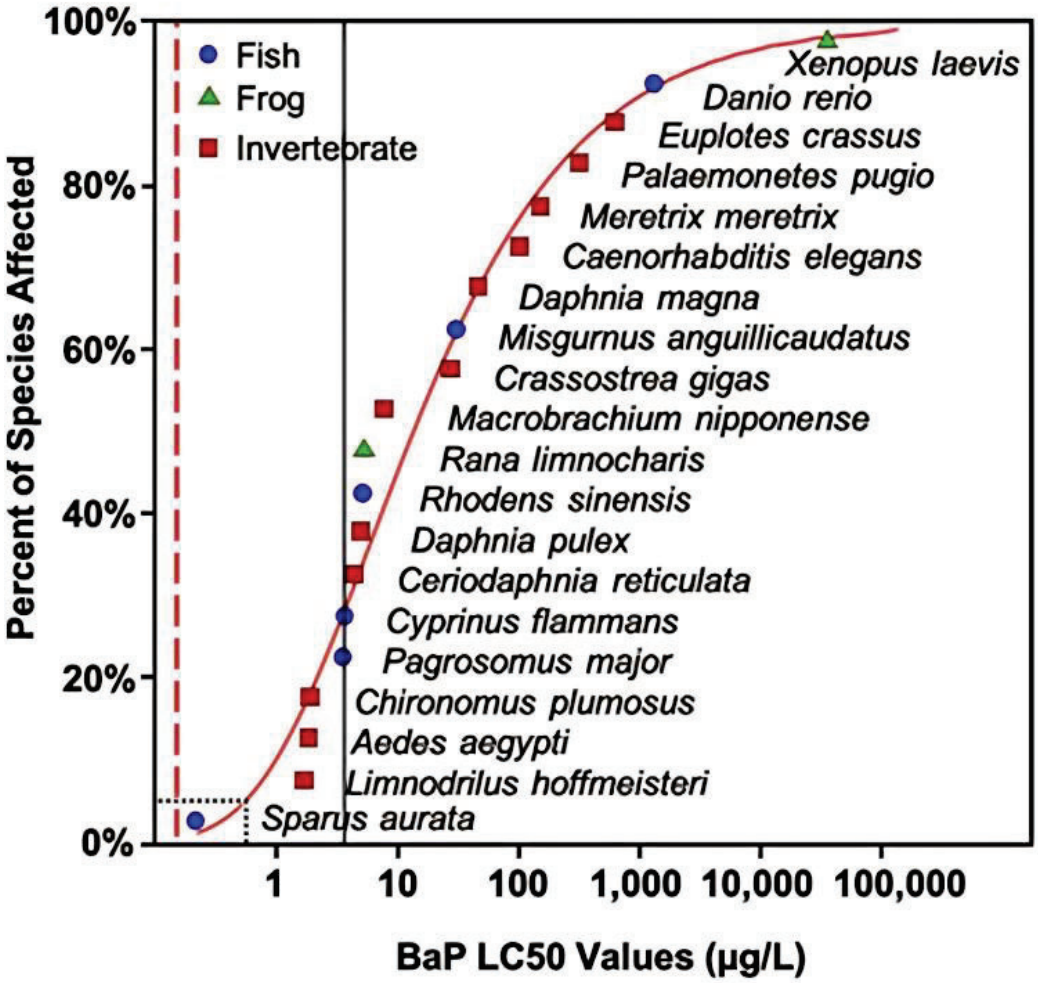

Figure 1.4 Species sensitivity distribution of 20 species LC_{50}s for benzo(a)pyrene (BaP); with six of the species represented in the distribution being fish. The black line represents the solubility limit of benzo(a)pyrene (4 µg/L), and the dashed red line represents the Canadian Council of Ministers of the Environment (CCME) guideline for the protection of freshwater life (0.015 µg/L). Reproduced from Hodson et al. (2020), "Polycyclic aromatic compounds (PACs) in the Canadian environment: The challenges of ecological risk assessments," © 2020 The Authors. Published by Elsevier Ltd. Licensed under CC BY-NC-ND 4.0 (https://creativecommons.org/licenses/by-nc-nd/4.0/). No modifications were made.

Fish species generally exhibit a baseline sensitivity to narcotic compounds that is well-predicted by their log K_{OW} (Li et al., 2018; McGrath et al., 2018; Tillmanns et al., 2023). However, several species, including five of the 10 most sensitive species in the chronic CTLBB database, are underrepresented with only one publication per species, indicating the potential for additional underrepresented species with high PAC sensitivity (Tillmanns et al., 2023). Acute CTLBB values varied by approximately 400-fold between the most and least sensitive species, ranging from 1.5 to 600.14 $\mu mol/g_{octanol}$ (Tillmanns et al., 2023). Additionally, conflicting results have been reported for the effects of PACs in the same species. While Incardona et al. (2004) found evidence of DBT toxicity in zebrafish, Peddinghaus et al. (2012) did not. For volatile compounds, including many PACs, differences in experimental protocols can produce inconsistent exposure concentrations, thus producing variable results (Butler et al., 2016). Vast differences in species sensitivity to PACs indicate that more data is needed for species of concern to improve toxicity predictions and refine the foundation of species sensitivity distributions (SSDs) used in water quality guideline development (Tillmanns et al., 2023).

1.6 Research rationale and objectives

Polycyclic aromatic compounds can exert toxicity to fish species through various mechanisms, however, crucial data on interspecies sensitivity to PACs is underdeveloped. This data is required to determine which aquatic species are most vulnerable to PAC contaminants by accounting for physiological differences across species. Extending toxicity data to include native species is essential for developing more informed and effective strategies to safeguard biodiversity and ecosystem health. Incorporating cardiotoxicity data into the TLM framework could extend the model's application beyond acute lethality, expanding its capacity to estimate sublethal impacts and provide more comprehensive assessments of risk. Therefore, the overall

objective of this research is to investigate the cardiotoxicity and interspecies sensitivity of three fish species to three structurally unique PACs - DBT, BaA, and naphthalene (NAP) - (**Figure 1.5**) and explore the efficacy of model species as a surrogate for walleye (*Sander vitreus*). This research further seeks to expand the TLM by integrating cardiotoxicity endpoints, extending its application beyond acute lethality to improve predictions of sublethal effects.

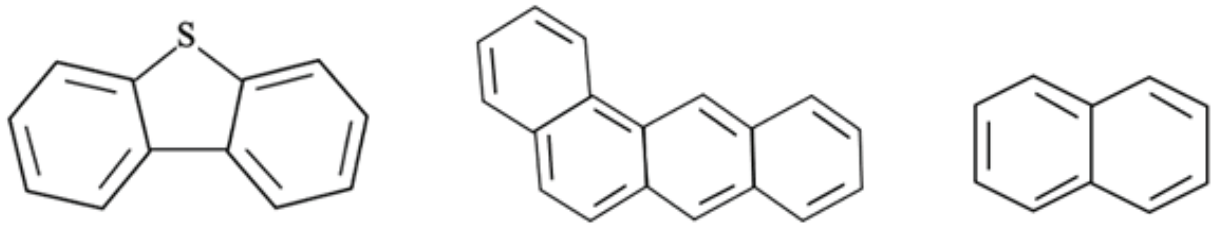

Figure 1.5 Molecular structure of dibenzothiophene (DBT, left), benz(a)anthracene (BaA, middle), and naphthalene (NAP, right).

Therefore, the specific objectives of this research are to:

1. Quantify and compare the lethal and sublethal effects of three model PACs - BaA, DBT, and NAP - on the early life stages of three species of freshwater fish - fathead minnows (*Pimephales promelas*), rainbow trout (*Oncorhynchus mykiss*), and walleye.
2. Calculate CTLBB values for lethal and cardiotoxicity endpoints for each species and assess whether cardiotoxicity endpoints agree with the relationship proposed by the TLM.

It is hypothesized that exposure to BaA, DBT, and NAP will induce lethal and sublethal effects on the early life stages of the three fish species, with each exhibiting unique, species-specific CTLBB values. These values will enable TLMs calibrated with cardiotoxic CTLBB data to predict sublethal cardiotoxic effects across model and non-model native fish species, enhancing the model's ecological relevance and application beyond acute mortality endpoints.

CHAPTER 2: ASSESSING CARDIOTOXICITY OF EARLY LIFE STAGE FISH TO POLYCYCLIC AROMATIC COMPOUNDS: INTERSPECIES VARIATION IN LETHAL AND SUBLETHAL RESPONSES

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2.1 Introduction

Polycyclic aromatic compounds (PACs) are a diverse group of hydrophobic organic contaminants that are ubiquitous in aquatic environments and are categorised by their composition of multiple fused aromatic rings, which may also contain heteroatoms such as sulfur, nitrogen, or oxygen. (Hodson et al., 2020; Kurek et al., 2013). Naturally occurring in hydrocarbon deposits and a byproduct of combustion reactions, PACs are introduced into the environment as complex mixtures through both natural and anthropogenic sources (Hodson et al., 2020; Kurek et al., 2013). Anthropogenic PACs can be categorized as originating from two sources: petrogenic, resulting from the extraction, production, transport, and spill of fossil fuels, and pyrogenic, which are predominantly formed during incomplete combustion of hydrocarbon fuels (Wallace et al., 2020).

Due to their low solubility, PACs bind to organic particles or sediments in the water column, where they can remain suspended for long periods of time (Marvin et al., 2021; Wallace et al., 2020). Exposure of larval fish to PACs can result from a variety of sources, including direct contact with contaminated sediment, aqueous exposure via respiratory membranes, and ingestion of contaminated sediment or prey (Lee, 2015; Wallace et al., 2020). Many freshwater fish species, including Salmonids, Percids, and Acipenserids, lay their eggs in lake or streambed sediments (Lane et al., 1996). After hatching, larval fish take days to weeks before they develop a functional swim bladder, used to maintain buoyancy in the water column, and thus frequently come into contact with potentially contaminated lake or stream bed sediments (Lindsey et al., 2010). Polycyclic aromatic compounds act via several modes of action to cause a variety of adverse effects to fish. Exposure to PACs has been shown to cause physical malformations, including spinal curvature and pericardial or yolk sac edema, as well as cardiac abnormalities,

immobility, and lethality (Hodson et al., 2020; Incardona, 2017; Wallace et al., 2020). One mechanism of PAC toxicity is referred to as baseline toxicity or narcosis (Dubiel et al., 2024; Incardona et al., 2021; McGrath & Di Toro, 2009; Philibert et al., 2021; Sørhus et al., 2016; van Wezel & Opperhuizen, 1995). Baseline toxicity, defined as the reversible non-specific disruption of lipid cell membrane function, is a term used to characterize the general acute toxic effects of chemicals like low molecular weight PACs (Incardona, 2017; van Wezel & Opperhuizen, 1995).

Cardiotoxicity is a well-established effect of crude oil on fish, where chemical contaminants, including PACs, impair cardiac structure or function, disrupt circulation, limit the heart's capacity to meet metabolic demands, and reduce survival (Brette et al., 2017; Hicken et al., 2011; Incardona et al., 2004, 2005; Sørhus et al., 2016, 2023a). During cardiogenesis, following formation of the cardiac tube, the heart bends, forming an S-shape (Incardona et al., 2004; 2005; Incardona, 2017; Stainier et al., 1993). This process, known as cardiac looping, is disrupted by both crude oil and individual PAC exposure and has been shown to cause permanent, long-term reductions in swimming performance, and ultimately, decreased fitness and survival (Hicken et al., 2011; Incardona et al., 2015, 2021; Mager et al., 2014; Sørhus et al., 2016, 2023a). One study, exposing Atlantic haddock (*Melanogrammus aeglefinus*) embryos to crude oil for three days during early embryogenesis, found considerable reductions in ventricle area, length, and circularity, producing functional impairments such as silent ventricles and 2:1 atrioventricular conduction (AV) blocks (Sørhus et al., 2016). Similarly, Incardona et al. (2021) exposed Pacific herring (*Clupea pallasi*) embryos to oiled gravel for ten days and found a concentration-dependent reduction in ventricular ballooning in larval stages, followed by abnormal trabeculation, and overgrowth of the spongy myocardium in juveniles reared to four months in clean seawater following the exposure. In both studies, cardiac abnormalities

originated during embryogenesis and were attributed to disrupted calcium (Ca^{2+}) cycling and excitation-contraction coupling (Incardona et al., 2021; Sørhus et al., 2016). Effects of PACs on larval fish vary widely between compounds and species. A 4-day exposure of zebrafish (*Danio rerio*) embryos to tricyclic PACs caused hatched larvae to develop a reduction or loss of circulation, bradycardia, and AV blocks (Incardona et al., 2004). However, a 4-day exposure of zebrafish embryos to the 2-ring PAC, naphthalene (NAP), did not result in any physical abnormalities, but did cause bradycardia (Incardona et al. 2004). Exposure of zebrafish embryos to benz(a)anthracene (BaA) resulted in incomplete cardiac looping, pericardial and yolk sac edema, intracranial hemorrhage, and reduced growth (Incardona et al., 2006). While these studies highlight the profound impacts of PACs on early cardiac development, the severity and manifestation of cardiotoxicity may differ substantially between species, highlighting the importance of interspecies comparisons.

Evaluating variations in species sensitivity is a crucial factor in assessing the risk of anthropogenic chemicals to aquatic species. One study measuring activation of the aryl hydrocarbon receptor 2 (AhR2) by BaA between nine species of freshwater fish found a 141-fold range in sensitivity between species (Dubiel et al., 2023). Incardona et al. (2014) demonstrated differences in species sensitivity to cardiotoxicity following embryonic exposure of three species of pelagic fish, Atlantic bluefin tuna (*Thunnus thynnus*), yellowfin tuna (*Thunnus albacares*), and amberjack (*Seriola dumerili*), to crude oil. Amberjack embryos exhibited an EC_{50} for pericardial edema that were 15.5-fold higher than that of Bluefin tuna (Incardona et al., 2014). While yellowfin and bluefin tuna had bradycardia EC_{50}s of 6.1 µg/L and 7.7 µg/L ΣPAH, respectively, amberjack had an EC_{50} of 18.2 µg/L ΣPAH (Incardona et al., 2014). Further, Incardona et al. (2015) found species-specific differences in sensitivity of Pacific herring and

pink salmon (*Oncorhynchus gorbuscha*) following embryonic exposure to oiled gravel effluent. After 7-8 months of growth in clean seawater, Pacific herring displayed significant reductions in swimming performance at concentrations as low as 0.23 µg/L ΣPAH, whereas pink salmon required substantially higher exposure concentrations, at 15.4 µg/L ΣPAH, to exhibit similar reductions in swimming performance. These studies highlight pronounced differences in interspecies sensitivity and thus necessitate further research into species sensitivity for both lethal and sublethal endpoints (Hodson et al., 2020; Incardona et al., 2014, 2015).

Evaluating the risks that PACs pose to aquatic species presents challenges as the toxicological effects of PACs vary greatly between compounds and species, and because PACs exist in the environment as complex mixtures (Hodson et al., 2020; Kurek et al., 2013). Various modelling tools have been developed to correlate chemical properties and toxicological effects (Hodson et al., 2020; Philibert et al., 2021). One such model, the target lipid model (TLM), predicts toxicity based on the linear relationship between the log half maximal lethal or effect concentration (L/EC_{50}) and the log octanol-water partition coefficient ($\log K_{OW}$) of a compound (Di Toro et al., 2000; McGrath et al., 2018). This relationship between L/EC_{50} and $\log K_{OW}$ is strongly defined within a $\log K_{OW}$ range of approximately 2-5.5 (Di Toro et al., 2000; Incardona et al., 2004; McGrath et al., 2018; Philibert et al., 2021). Within this range, toxicity increases with increasing lipophilicity, until a $\log K_{OW} > 5.5$, at which toxicity begins decreasing due to low water solubility and bioavailability (Incardona et al., 2004). This relationship is used to determine a species and effect endpoint-specific critical target lipid body burden (CTLBB) (Di Toro et al., 2000). The CTLBB quantifies the concentration of a chemical within the target lipid ($\mu mol/g_{octanol}$) required to cause the observed effect. This value is particularly useful for assessing species-specific sensitivities across numerous compounds, modelling the effects of complex

mixtures, and identifying exposure concentrations that ensure the protection of 95% of species within a given species distribution (HC5) (Di Toro et al., 2000; McGrath et al., 2018; Tillmanns et al., 2024).

The majority of the TLM database is based upon lethality, immobilization, reproduction, and growth endpoints (McGrath et al., 2018; Tillmanns et al., 2024). While cardiotoxicity is a known effect of exposure to crude oil, linked to some 3-ring PACs, only two studies have incorporated cardiotoxicity endpoints into a predictive or body burden framework, such as the TLM (Dubiel et al., 2024; Hedgpeth et al., 2019; McGrath et al., 2018; Tillmanns et al., 2024). One of the first studies to do so assessed the cardiotoxic effects of 10 PACs in American lobster (*Homarus americanus*) larvae, deriving bradycardia endpoints from heart rate measurements following a 48-hour exposure (Dubiel et al., 2024). When applied to a TLM, the American lobster bradycardia CTLBB was among the most sensitive points in the database (Dubiel et al., 2024). These findings suggest that bradycardia may represent a sensitive and ecologically relevant endpoint that can be incorporated into the TLM, though further research is needed to establish its application across taxa. Similarly, Hedgpeth et al. (2019) examined cardiac-related abnormalities in zebrafish embryos exposed to petroleum substances, using biomimetic extraction–solid phase microextraction (BE-SPME) to quantify bioavailable hydrocarbon concentrations as a surrogate for critical body burden. The BE-SPME methodology operates on a similar principle to the TLM, as hydrocarbons partition from the water onto the PDMS-coated fiber at a similar rate to their partitioning into organismal lipid (Hedgpeth et al., 2019). Although abnormal cardiac function was observed throughout the test, these effects were not as consistent or as sensitive as pericardial and yolk sac edema, tail curvature, and non-viability and were therefore excluded from further quantitative analysis.

Understanding endpoint and species-specific sensitivity to PACs is essential for accurately modelling oil spill impacts and identifying the aquatic species at greatest risk from petroleum-derived contamination. This data ensures that protective measures extend beyond laboratory model species to safeguard biodiversity and ecosystem health more effectively. Many studies relating to fish cardiotoxicity involve in vitro exposures, with far fewer investigating the structural and functional cardiac defects that result from larval-stage exposures (Hicken et al., 2011; Incardona et al., 2005, 2015, 2021; Incardona, 2017; Sørhus et al., 2016). Additionally, an increasing number of studies highlight the importance of differences in species sensitivity and the long-term impacts of non-lethal exposure (Dubiel et al., 2023, 2024; Hicken et al., 2011; Hodson et al., 2020; Incardona et al., 2015; McGrath et al., 2018). Most studies contributing to species sensitivity distributions compile single-species toxicity data from independent sources, while multi-species comparisons often focus on molecular-level responses. Many studies also rely on solvent carriers rather than more consistent methods like passive dosing to conduct PAC exposures. This can lead to variable exposure concentrations due to volatilization, ultimately reducing data quality (Dubiel et al., 2024; McGrath et al., 2018). Therefore, this study directly compares cardiotoxicity and lethality across three fish species, fathead minnows (*Pimephales promelas*), rainbow trout (*Oncorhynchus mykiss*), and walleye (*Sander vitreus*), exposed under identical conditions to three structurally distinct PACs: dibenzothiophene (DBT), BaA, and NAP. Cardiac endpoints may help inform predictions of long-term functional survival and improve the ecological relevance of oil spill risk assessments.

2.2 Materials and methods

2.2.1 Chemicals

Physicochemical properties of NAP (99%), DBT (≥99%), and BaA (99%) are provided in **Table 2.1**. Each PAC and HPLC-grade methanol (MeOH) was purchased from MilliporeSigma (Oakville, ON, Canada).

Table 2.1 Polycyclic aromatic compound chemical properties. Chemical Abstracts Service registry number (CAS no.), chemical formula, molecular weight (MW), chemical purity, and log octanol-water partition coefficient (log K_{OW}) values for each chemical studied.

Abbreviation	Formula	CAS no.	MW (g/mol)	Purity (%)	log K_{OW}
NAP	C10H8	91-20-3	128.17	99	3.17[1]
DBT	C12H8S	132-65-0	184.26	≥99	4.29[1]
BaA	C18H12	56-55-3	228.29	99	5.52[1]

[1]Value obtained from EPISuite v4.11 (2025).

2.2.2 Animal care

Each species was hatched at species-specific temperatures, in continuously aerated dechlorinated City of Calgary tap water, and kept on a 16:8 light:dark photoperiod. Fathead minnow larvae were obtained from a breeding culture maintained at Nautilus Environmental (Calgary, AB, Canada). Fertilized fathead minnow embryos were cultured at 25 ± 2°C, in 10L plastic pails with complete daily manual water exchanges. Rainbow trout embryos were obtained from Trout Lodge (Bonney Lake, Washington, United States) and cultured in a flow-through vertical egg tray incubator at 12 ± 2 °C (Fish Farm Supply Co, Elmira, Ontario, Canada) at Nautilus Environmental. Walleye embryos were sourced from the Government of Alberta spawn camp (Lac Ste. Anne, AB, Canada). Walleye embryos were transported to Nautilus Environmental and cultured in a McDonald-type hatching jar (Fish Farm Supply Co). Upon arrival, walleye embryos were initially reared at 9 °C for one week, after which the temperature was increased by 1 °C every two days until hatch, reaching a final temperature of 15 °C. At the end of each test protocol, all surviving test organisms were humanely euthanized with an overdose of buffered MS-222 according to University of Lethbridge animal welfare protocols.

2.2.3 Passive dosing system

Polycyclic aromatic compounds were loaded into polydimethylsiloxane (PDMS) O-rings (Silicone O-rings West; 2.5 cm diameter, approximately 1.06 g, 2.33 mm thick, product no. AS568B-210) following procedures outlined in Philibert et al. (2021) and Butler et al. (2016). Concentrations followed an approximate 1:3 dilution series, with the highest concentration set at each compound's limit of solubility in MeOH. Stock solutions were prepared by independently dissolving five target concentrations of each PAC directly into 350 mL of HPLC-grade MeOH. Clean O-rings were added to each PAC stock solution in capped 1L amber bottles. The O-rings

were vigorously agitated at room temperature for 72 h on an orbital shaker, at 250 rotations per minute (RPM), to allow for partitioning of the PAC to the O-rings. Once complete, loaded O-rings were rinsed with deionized water, added to vessels containing dechlorinated freshwater, with water hardness ranging between 175-240 mg/L CaCO3, and equilibrated at test temperature on a shaker (150 RPM) for 24 h prior to test initiation (**Table 2.2**). O-ring equilibration occurs quickly, with 24 h providing adequate time to achieve equilibrium (Stibany et al., 2017).

2.2.4 Toxicity assay

Healthy yolk-sac larvae, less than 24 hours post-hatch, were exposed to five serial concentrations of BaA, DBT, and NAP for seven days. Each concentration was prepared as a single batch and then aliquoted into three replicate exposure vessels, with species-specific test conditions outlined in **Table 2.2**.

Table 2.2 Test conditions that differed between species. Temperature, water exchanges, and organisms per replicate for each species studied.

Test Conditions	Fathead minnow	Rainbow trout	Walleye
Temperature (°C)	25 ± 2	12 ± 2	15 ± 2
Water Exchanges	3	3	2
Organisms per Replicate	6	5	10

Tests were conducted in 500mL mason jars, sealed to minimize PAC loss during exposure, with each vessel containing approximately 400mL of dechlorinated City of Calgary tap water. The frequency of water exchanges and organisms per replicate were adjusted with considerations given to temperature and loading density to minimize ammonia concentrations. Testing procedures for fathead minnows were based on environment Canada (2011) methods where applicable. Tests were visually assessed daily for mortality, and dead organisms were removed once full body tissue necrosis was observed, ensuring no immobilized living organisms were incorrectly removed and classified as mortality. Temperature was maintained through ambient air temperatures in a climate-controlled chamber with a 16:8 light:dark photoperiod. Because of their smaller size, walleye and fathead minnows were kept in a secondary containment vessel, a glass cylinder with a Nitex screen bottom, within the test container to allow for daily visual assessments. Additionally, secondary containment vessels prevented the larvae from being poured out during water exchanges, minimizing handling stress during the trial. Trials were considered valid if at test termination the mean survival of controls was ≥ 80%, dissolved oxygen remained ≥ 40%, and unionized ammonia remained ≤ 0.019 mg/L (CCME, 2010; Environment Canada, 1990, 2011). Exposure concentrations were measured at test initiation, before and after water exchanges, and at test termination using 2D emission fluorometry using an Aqualog Benchtop Fluorometer (Horiba Instruments) (Dubiel et al., 2024; Philibert et al., 2021). Measured PAC concentrations collected throughout the test duration were averaged to determine the test concentrations used for data analysis. Water quality parameters, including pH, dissolved oxygen, and un-ionized ammonia, were measured before water exchanges and at test termination.

2.2.5 Cardiac assessment

Heart rate was assessed at test termination based on previously described methods from Dubiel et al. (2024), with decreases in heart rate (bradycardia) being the only effect on heart rate that was observed. Due to their large size relative to the other species, rainbow trout larvae were lightly sedated by a brief submersion in 20mg/L buffered MS-222 prior to recordings (Eriksson et al., 2024). For each species, a transfer pipette was used to move surviving larvae from test vessels onto a microscope slide with a small volume of water. Larvae were positioned on their side and heartbeats were recorded in a 15 to 20-second video, using a Leica DMLB fluorescence microscope attached to a camera. In groups with high survival, a minimum of 10 organism recordings were collected per treatment, but all organisms were used in groups with fewer than 10 surviving organisms. A subset of 10 random recordings was counted blind to ensure unbiased data collection. Heartbeats were visually counted and multiplied to determine beats per minute. A total of four videos out of 425 (0.94 %) were removed from analysis because of poor video quality or poor organism position during the video.

Cardiac morphology in walleye was assessed based on the relative length of the ventricle compared to the atrium. The fathead minnows and rainbow trout had pigmentation and/or overlying anatomical structures partially obscuring cardiac structures, preventing full structural assessment. Still images captured at peak ventricular diastole, identified by manually forwarding through video frames, were taken from a minimum of five individuals per concentration. Atrium length was measured as the distance between the atrium-ventricular canal and the sinus venosus. Ventricle length was measured from the entrance of the bulbus arteriosus to the opposing wall of the ventricle. Atrium and ventricle lengths were measured using the freehand trace tool in ImageJ (ImageJ 1.53m, National Institutes of Health, Bethesda, Maryland, USA,

https://imagej.net/ij/). A total of 91 images (44%) were excluded from analysis because one or both cardiac chambers were obscured, preventing accurate measurement. Pericardial edema was visually assessed on the absence or presence of excess fluid in the pericardial space surrounding the heart (Incardona et al., 2013; Philibert et al., 2016). A total of 20 videos out of 425 (4.7%) were removed from analysis because the visibility of the heart in relation to the pericardial wall was not clear. Yolk sac edema was also scored based on the presence or absence of fluid accumulation around the yolk sac in each individual. Swim bladder inflation was assessed only in fathead minnows, as they were the only species expected to have an inflated swim bladder by 7 days post-hatch (dph). Atrioventricular conduction blocks were scored based on presence or absence in each individual and defined as one or more occurrences of the atrium contracting more than once per ventricular contraction (Sørhus et al., 2016). Silent ventricles were scored when there was no ventricular contraction and no visible blood movement into or out of the chamber for the entire duration of the video (Sørhus et al., 2016, 2023b). Fish exhibiting silent ventricles were excluded from AV block assessments to analyze only those with functional ventricles. Therefore, 20 videos out of 425 (4.7%) videos were removed from silent ventricle assessment due to lack of visibility, and 36 out of 425 (8.47%) videos were excluded from AV block assessment based on lack of visibility and or the presence of a silent ventricle.

2.2.6 Statistical analysis

Statistical analysis was performed using the software R (R-core v4.2.2). Prior to statistical comparisons, data was tested for homogeneity of variance using a Levene's test and for normality using the Shapiro-Wilks test. No significant differences were detected between O-ring and freshwater controls for walleye, rainbow trout, or fathead minnows (two-sample t-tests, $p > 0.05$), supporting the decision to pool control groups within species. All treatment effects on

heart rate were calculated as a percent effect relative to control values. The lowest observed effect concentration (LOEC), defined as the lowest concentration where it and all subsequent concentrations differed significantly ($p < 0.05$) from the control, was identified using the Kruskal-Wallis test followed by an unadjusted Dunn's post hoc test to account for variation in sample sizes. Half maximal effect concentration values (EC_{50}) for bradycardia were calculated using a four-parameter log-logistic function in the Analysis of Dose Response Curves package for R (Ritz et al., 2015). Ventricle size EC_{25}s were calculated using a three-parameter log-logistic function in CETIS™ (Tidepool Scientific Software, 2022). Mortality data was modeled using a two-parameter log-logistic function to estimate half-maximal lethal concentration values (LC_{50}) (Ritz et al., 2015). As none of the data was normally distributed, when needed, Spearman's rank correlation was used to assess significant trends between data sets. Statistically significant differences between LC_{50} and EC_{50} values were calculated using the ratio test described in Wheeler et al. (2006).

2.2.7 Target lipid model

The TLM was used to calculate a species and endpoint specific CTLBB following the methods outlined in Dubiel et al. (2024) and McGrath et al. (2021). Linear regressions for the cardiotoxicity EC_{25}s, EC_{50}s, and LC_{50}s were performed using the previously described effect concentrations, and each species and endpoint specific CTLBB was estimated from the fitted intercept in **Equation 2.1** (McConville et al., 2018).

$$\mathbf{\log(L/EC_{50i}) + \Delta c_i = -0.94 \log(K_{OWi}) + \log(CTLBB)} \qquad \mathbf{2.1}$$

Lethal concentration values and effect concentrations for bradycardia and ventricle-to-atrium size ratio were used to estimate lethal and sublethal CTLBBs, following methods described by McGrath et al. (2021), where Δci represents a PAC class-specific correction. Individualized class corrections were applied to the PACs (DBT Δc = 0.470) and PAHs (NAP and BaA; Δc = 0.364) (McGrath et al., 2021). The L/EC_{50} values were fit to **Equation 2.1** by minimizing squared residual errors using the goal-seek algorithm in Microsoft Excel. Any PACs that did not reach the threshold for a cardiotoxicity EC_{50} or an LC_{50} were not included in the analysis.

2.3 Results

2.3.1 Lethality

Exposure to NAP and DBT caused concentration-dependent increases in mortality for fathead minnows and rainbow trout. The most sensitive species to NAP was rainbow trout, with an LC_{50} of 14.23 ± 7.13 µmol/L, whereas the most sensitive species to DBT was fathead minnows, with an LC_{50} of 1.88 ± 0.19 µmol/L (**Table 2.3**). LC_{50} values could not be calculated for walleye exposed to either PAC due to insufficient mortality; however, the LC_{25} for walleye exposed to NAP was estimated at 55.4 ± 11.88 µmol/L. For NAP, ratio tests showed that walleye were significantly less sensitive than both rainbow trout ($p = 0.0048$) and fathead minnows ($p < 0.0001$) at the LC_{25} level, while no significant difference was detected between trout and fathead minnows (**Table 2.3**). For DBT, the fathead minnows were significantly more sensitive than rainbow trout ($p = 0.010$), based on LC_{50}. Rainbow trout and fathead minnows exhibited a steep concentration response for NAP, with an LC_{50}/LC_{10} ratio of 1.22 and 1.44 (**Table 2.3**). Following the same pattern, LOEC values ranged from 44.38 µmol/L for walleye to 18.13

μmol/L for rainbow trout exposed to NAP. For DBT and NAP, the most-to-least sensitive species differed 1.9 and 3.3-fold, respectively.

Table 2.3 Estimated LC_{50}, LC_{25}, and LC_{50}/LC_{10} ratios for walleye, rainbow trout, and fathead minnows following exposure to naphthalene (NAP), dibenzothiophene (DBT), and benz(a)anthracene (BaA) with 95% confidence interval for each value in parentheses.

Compound	Species	LC_{50} (µmol/L)	LC_{25} (µmol/L)	$LC_{50/10}$ (µmol/L)
NAP	Walleye	>44.38	55.41 (29.73-81.08)	N/A
	Fathead minnow	17.22 (13.70 -20.73)	12.88 (7.25-18.51)	1.44
	Rainbow trout	14.23 (-0.88-29.33)	12.87 (-6.43-32.16)	1.22
DBT	Walleye	>2.73	>2.73	N/A
	Fathead minnow	1.88 (1.46 - 2.26)	1.38 (0.99-1.77)	1.86
	Rainbow trout	3.64 (1.71-5.56)	2.44 (1.37-3.52)	2.22
BaA	Walleye	>0.031	>0.031	N/A
	Fathead minnow	>0.036	>0.036	N/A
	Rainbow trout	>0.028	>0.028	N/A

2.3.2 Effect on heart rate

Exposure of walleye, fathead minnows, and rainbow trout to DBT and NAP resulted in concentration-dependent bradycardia in all three species (**Figure 2.1**). Walleye experienced the greatest response for bradycardia, relative to the control, after exposure to 44.38 µmol/L NAP with an average decrease in heart rate of 52% (**Figure 2.1**). For fathead minnows and rainbow trout, the greatest response for bradycardia, relative to the control, was observed following exposure to 3.3 and 2.88 µmol/L DBT, respectively, with an average reduction in heart rate of 61% and 49%, respectively (**Figure 2.1**).

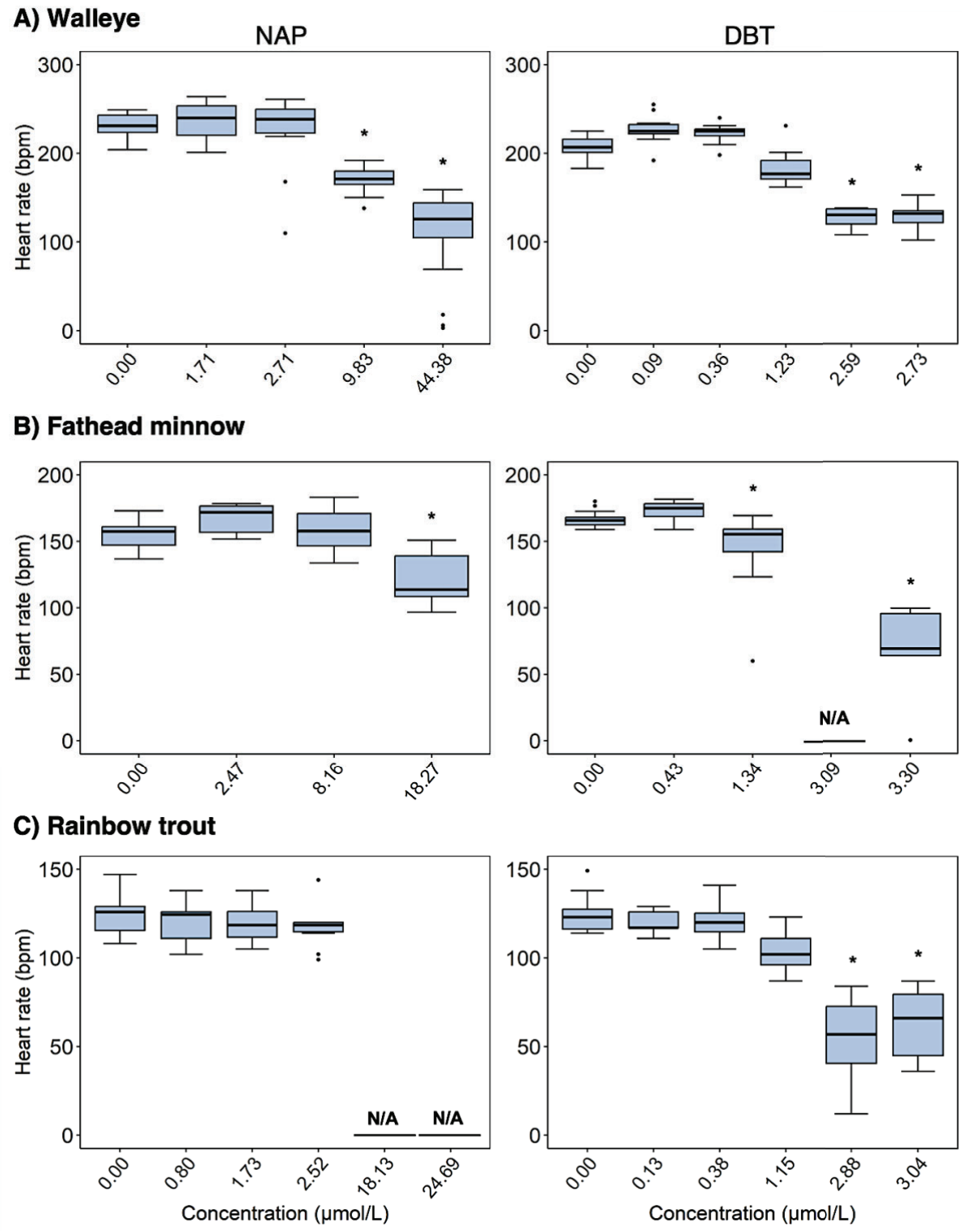

Figure 2.1 Differences in the heart rates for (A) walleye, (B) fathead minnow, (C) rainbow trout after exposure to naphthalene (NAP) and dibenzothiophene (DBT). No effects were observed in the benz(a)anthracene exposures. Significant reductions in heart rate compared to the control ($p < 0.05$) are marked with an asterisk.

Fathead minnows were the most sensitive species to heart rate reduction following NAP exposure, with an EC_{50} of 23.23 ± 6.1 µmol/L, followed by walleye and rainbow trout, which had EC_{50} values of 36.25 ± 5.0 and 42.14 ± 26.9 µmol/L, respectively (**Table 2.4**). The high mortality observed in rainbow trout exposed to NAP resulted in an elevated EC_{50} value (**Figure 2.1; Table 2.4**). Exposure of walleye, fathead minnows, and rainbow trout to DBT resulted in EC_{50}s of 3.20 ± 0.13, 2.72 ± 0.24, and 2.83 ± 0.19 µmol/L, respectively (**Table 2.4**). Ratio tests indicated that bradycardia effect concentrations for NAP and DBT did not differ significantly among species ($p > 0.05$). When possible, LOECs were calculated for each species and PAC, ranging from 18.27 µmol/L in fathead minnows to 9.83 µmol/L in walleye for NAP, and from 2.88 µmol/L in rainbow trout to 1.34 µmol/L in fathead minnows for DBT (**Table 2.4**). Exposure to BaA had no effect on the heart rate of either species at any concentration tested. Fathead minnows exhibited the steepest concentration response for NAP, with an EC_{50}/EC_{10} ratio of 1.58, compared to walleye with the highest ratio of 11.58. However, for DBT, the EC_{50}/EC_{10} ratio had a more moderate spread at 2.43 for fathead minnows and 3.72 for rainbow trout (**Table 2.4**).

Table 2.4 Effect concentrations (EC_{50} and EC_{10}) for decreased heart rate (bradycardia) and corresponding $EC_{50/10}$ ratios in walleye, fathead minnows, and rainbow trout following exposure to naphthalene (NAP), dibenzothiophene (DBT), and benz(a)anthracene (BaA). Ninety-five percent confidence intervals are shown in parentheses.

Chemical	Species	EC_{50} (µmol/L)	EC_{10} (µmol/L)	$EC_{50/10}$ (µmol/L)
NAP	Walleye	36.25 (26.36-46.15)	3.13 (0.88-5.38)	11.58
	Fathead minnow	23.23 (10.94-35.53)	14.72 (7.30-22.14)	1.58
	Rainbow trout	42.14 (-12.08- 96.35)	5.20 (0.026-10.38)	8.10
DBT	Walleye	3.20 (2.95-3.46)	0.95 (0.74-1.16)	3.37
	Fathead minnow	2.72 (2.25-3.20)	1.12 (0.72 - 1.51)	2.43
	Rainbow trout	2.83 (2.44-3.21)	0.76 (0.40- 1.13)	3.72
BaA	Walleye	>0.031	>0.031	N/A
	Fathead minnow	>0.036	>0.036	N/A
	Rainbow trout	>0.028	>0.028	N/A

2.3.3 Effect on walleye ventricle morphology

Following the 7-day exposure of walleye to each PAC, significant ($p < 0.05$) reductions in ventricle length were observed (**Figure 2.2**). Decrease in ventricle length was concentration-dependent for NAP, DBT, and BaA (**Figure 2.2**). There was a significant correlation between reduction in ventricle length and bradycardia across concentrations for NAP (Spearman's $\rho = 0.90$, $p = 0.037$).

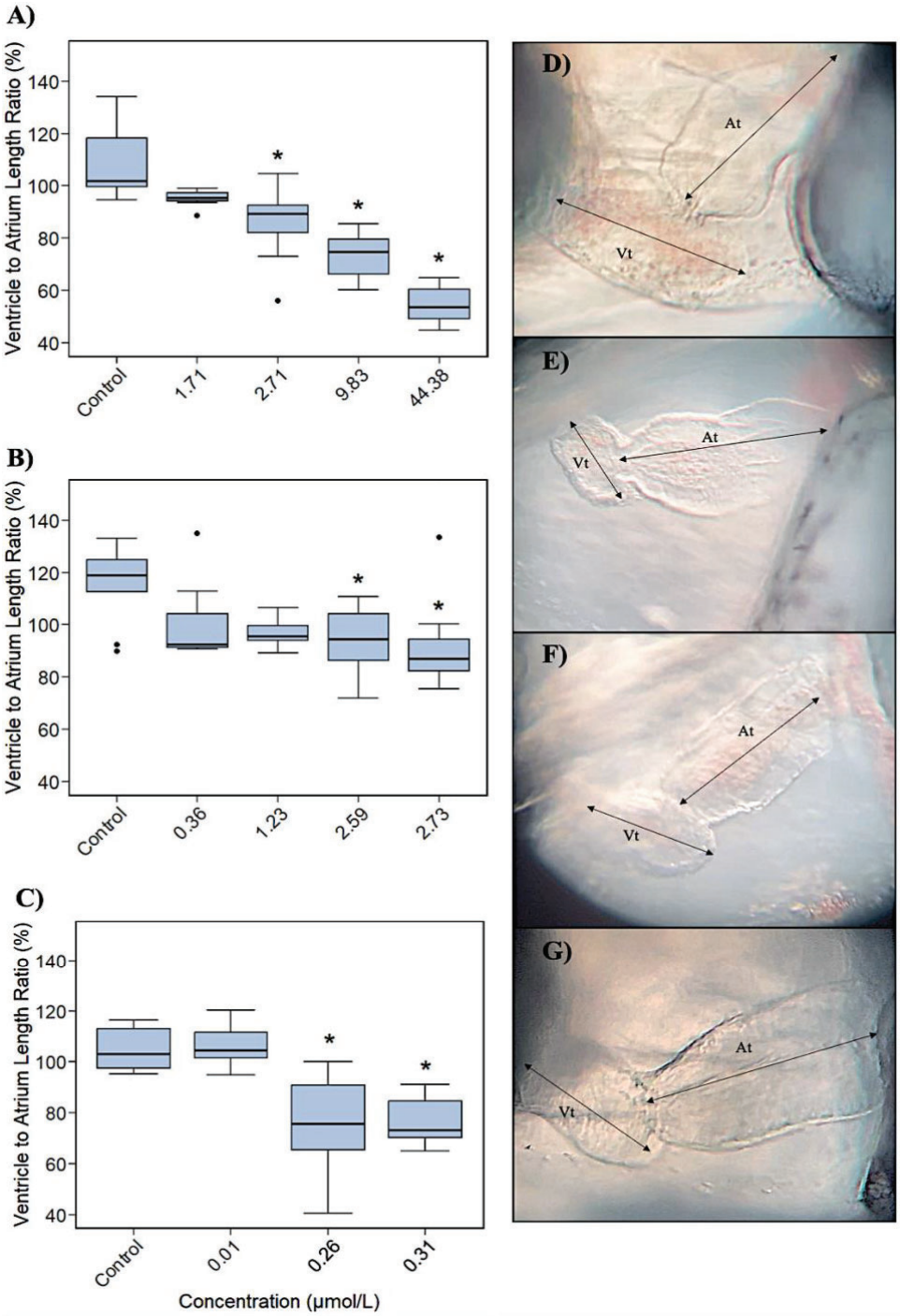

Figure 2.2 Ventricle to atrium length ratio for 7 dph walleye following exposure to (A) naphthalene (NAP), (B) dibenzothiophene (DBT), and (C) benz(a)anthracene (BaA). Significant reductions in ventricle size compared to the control ($p < 0.05$) are marked with an asterisk, data outliers are marked with a black dot, the median heart rate is shown as a solid line, box boundaries represent the middle 50% of the data, and whiskers indicate the full range. Representative still images of 7 dph walleye hearts after exposure to (D) control water, (E) 44.38 (µmol/L) NAP, (F) 2.73 (µmol/L) DBT, and (G) 0.031 (µmol/L) BaA. Vt= ventricle and At= atrium.

To enable consistent comparisons, EC_{25} values for reduction in ventricle length were calculated for all compounds, as only NAP elicited ≥50% effect. Exposure of larval walleye to NAP, DBT, and BaA yielded ventricle length reduction EC_{25}s of 4.88, 4.16, and 0.026 μmol/L, respectively (**Table 2.5**). The ventricle to atrium length ratio was the only endpoint that gave a measurable effect for BaA (**Table 2.5**).

Table 2.5 Ventricle to atrium length ratio estimated EC_{25} values with 95% confidence intervals in parentheses, LOECs, and incidence of cardiac abnormalities in walleye larvae exposed to naphthalene (NAP), dibenzothiophene (DBT), and benz(a)anthracene (BaA). Ventricle to atrium length ratios expressed as percent reduction relative to the control.

Chemical	Concentration (µmol/L)	AV length ratio as a percent of control	EC_{25} (µmol/L)	EC_{10} (µmol/L)	LOEC (µmol/L)
NAP	Control	-	4.53 (2.83-6.91)	0.47 (0.13-1.13)	2.71
	1.71	13.85			
	2.71	21.99			
	9.83	33.70			
	44.38	50.49			
DBT	Control	-	2.52 (1.50-3.96)	0.33 (0.02-1.15)	2.59
	0.36	8.26			
	1.23	12.19			
	2.59	14.75			
	2.73	21.26			
BaA	Control	-	0.021 (0.018-0.027)	0.015 (0.0063-0.024)	0.026
	0.0095	3.48			
	0.026	30.32			
	0.031	30.55			

2.3.4 Other sublethal developmental phenotypes

Walleye exhibited the greatest diversity of cardiotoxic phenotypes. Frequent incidences of AV conduction blocks were observed in walleye exposed to the two highest concentrations of DBT (**Figure 2.3**). In addition, silent ventricles, an absence of ventricular contractions while atrial beating continues, were observed in walleye following exposure to all three PACs. The highest incidence occurred in 44.38 μmol/L NAP, where 82.4% of larvae displayed silent ventricles. Pericardial edema was the most common phenotype overall in walleye and was detected across exposures to all three PACs. Yolk sac edema was also present in BaA treatments, although it was not quantified (**Figure 2.4**). Representative images of these malformations are provided in **Figure 2.4**. Fathead minnows and rainbow trout displayed fewer phenotypes than walleye. Across PACs, fathead minnows primarily showed impaired swim bladder inflation and, to a lesser extent, silent ventricles (**Figure 2.3**). Both NAP and DBT inhibited swim bladder inflation at their highest concentrations, with only 37.5% and 40% of larvae, respectively, exhibiting inflated swim bladders at 7 dph. High incidences of yolk sac edema were observed following exposure to 2.88 and 3.04 μmol/L DBT, making DBT the only PAC to elicit observable malformations in this species. Additionally, AV blocks were detected in rainbow trout exposed to the two highest DBT concentrations (**Figure 2.3**).

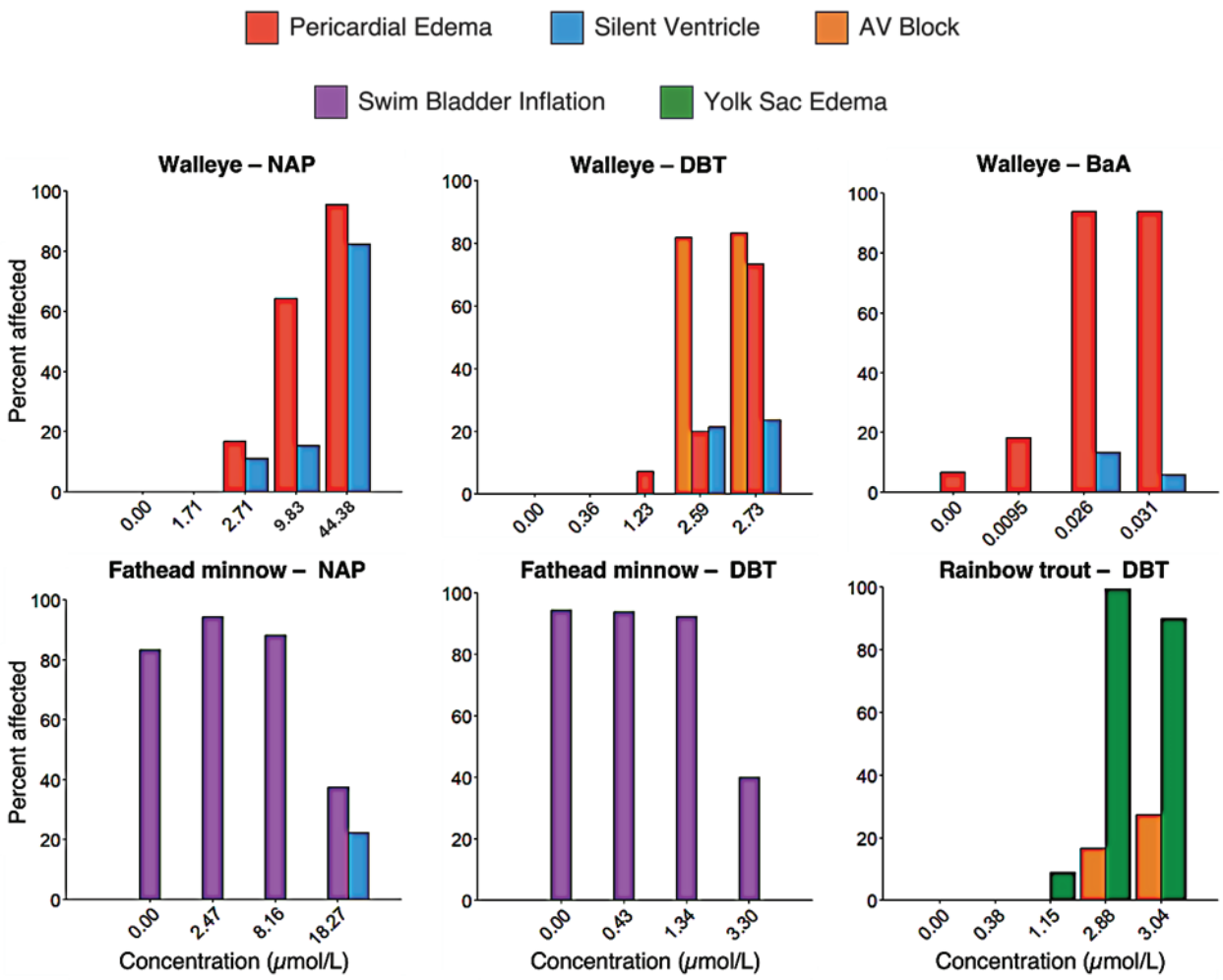

Figure 2.3 Percent of larvae affected by cardiac and developmental abnormalities in walleye, fathead minnows, and rainbow trout following exposure to naphthalene (NAP), dibenzothiophene (DBT), and benz(a)anthracene (BaA). Bar graphs represent the incidence of five phenotypes: pericardial edema, silent ventricle, 2:1 atrioventricular conduction (AV) block, inhibited swim bladder inflation, and yolk sac edema. Each colour corresponds to a specific sublethal effect and is consistent across all graphs. If a species-chemical combination is not shown, no sublethal effects were observed in that treatment and species. Absence of a given effect within a panel indicates that the phenotype was not observed in that treatment group.

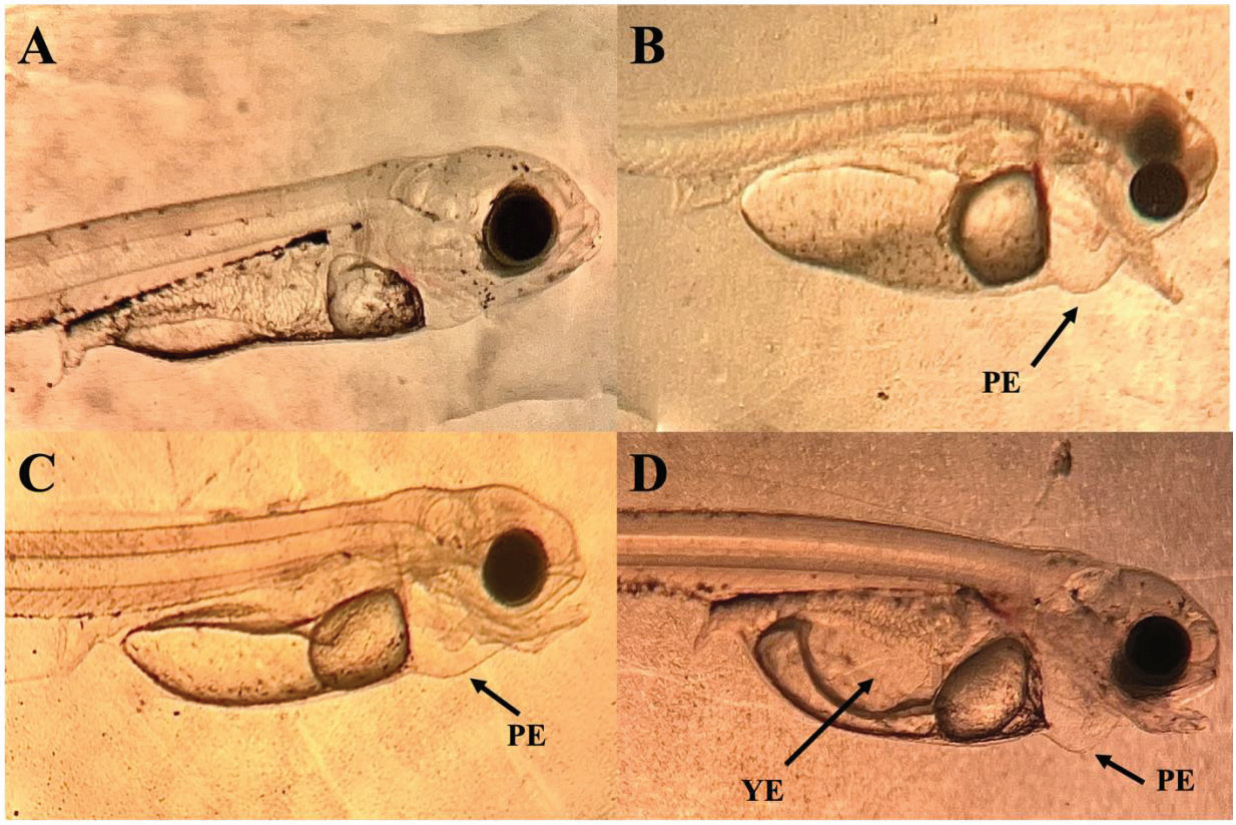

Figure 2.4 Representative images of control 7 dph walleye (A), and larvae exposed to 44.38 µmol/L naphthalene (NAP) (B), 2.73 µmol/L dibenzothiophene (DBT) (C), and 0.031 µmol/L benz(a)anthracene (BaA) (D). PE = pericardial edema, YE = yolk sac edema.

2.3.4 Target lipid model

Class-corrected log LC_{50} and EC_{50} values were in strong agreement with the relationship proposed by the TLM (**Figure 2.5**). This allowed calculation of bradycardia and lethality CTLBBs for each species. As LC_{50} values could not be calculated for walleye, no acute lethal CTLBB could be produced. From the adjusted EC_{50} values, the bradycardia CTLBB was determined to be 87.2 ± 0.7, 64.4 ± 2.4, and 88.4 ± 0.0 $\mu mol/g_{octanol}$ for walleye, fathead minnow, and rainbow trout, respectively (**Table 2.6**). Acute lethality CTLBBs, derived from the adjusted LC_{50} values, were 46.1 ± 1.3 $\mu mol/g_{octanol}$ for fathead minnows and 58.2 ± 14.6 $\mu mol/g_{octanol}$ for rainbow trout (**Table 2.6**). For walleye, a CTLBB of 17.6 ± 33.2 $\mu mol/g_{octanol}$ was derived from the adjusted ventricle length to atrium ratio EC_{25} values (**Table 2.6**).

Table 2.6 Calculated critical target lipid body burdens (CTLBB) (μmol/$g_{octanol}$) for larval walleye, rainbow trout, and fathead minnows for lethality (LC_{50}), bradycardia (EC_{50}), and ventricle length (EC_{25}) endpoints. Standard error (SE) is shown in parentheses.

Species	Lethality (LC_{50})	Bradycardia (EC_{50})	Ventricle length (EC_{25})
	CTLBB (SE)	CTLBB (SE)	CTLBB (SE)
Walleye	N/A[a]	87.2 (0.7)	17.6 (33.2)
Fathead minnow	46.1 (1.3)	64.4 (2.4)	N/A[b]
Rainbow trout	58.2 (14.6)	88.4 (0.0)	N/A[b]

[a] Value not available as no effect was observed.
[b] Value not available as no effect was measured.

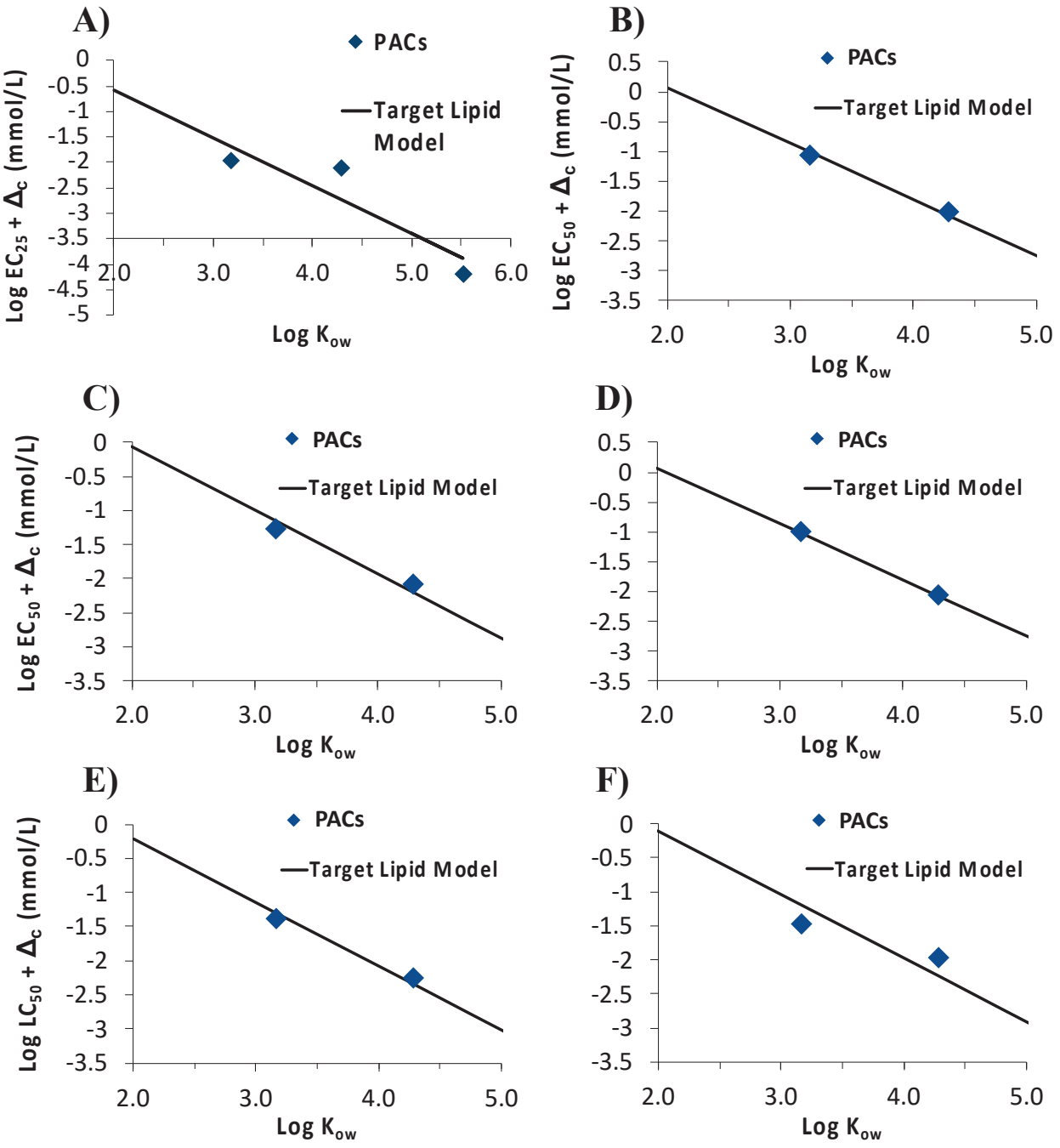

Figure 2.5 Class-corrected walleye ventricle length EC_{25} values for walleye (A), class-corrected bradycardia EC_{50} values for walleye (B), fathead minnow (C), and rainbow trout (D), class-corrected LC_{50} values for fathead minnows (E) and rainbow trout (F) plotted against the log octanol-water partition coefficient (log K_{OW}). Target lipid model predictions are represented by the black line.

A species sensitivity distribution (SSD) of acute CTLBBs was compiled from the McGrath et al. (2018) database, with the bradycardia and acute lethality CTLBBs for each species in this study highlighted to show their relative sensitivity within the distribution (**Figure 2.6)**. The most sensitive species and acute endpoint CTLBB in this study was fathead minnow lethality at a percent rank of 29.90%, and the least sensitive was rainbow trout bradycardia, ranked at 52.45% (**Figure 2.6**).

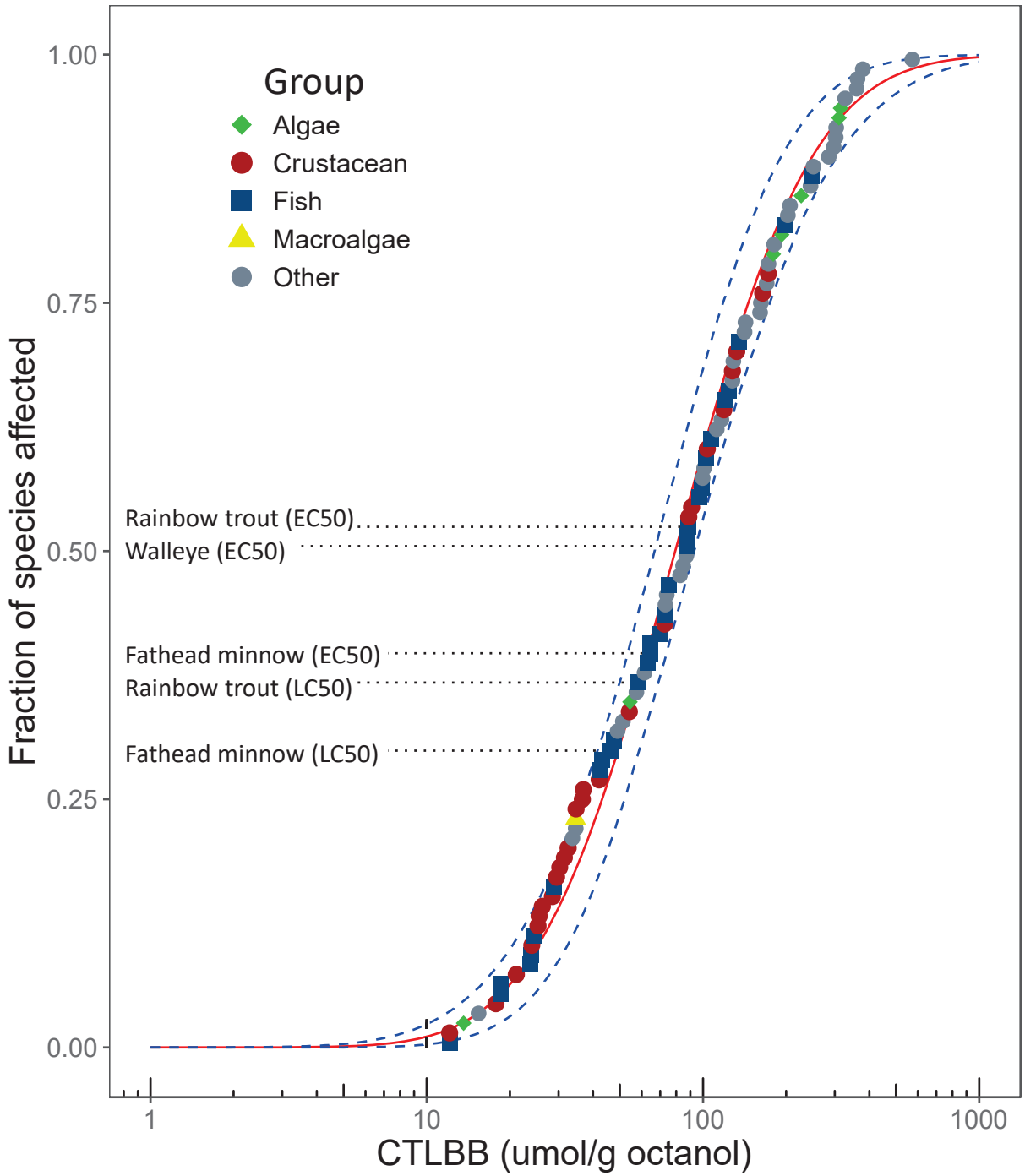

Figure 2.6 Species sensitivity distribution of acute critical target lipid body burdens (CTLBBs) compiled from McGrath et al. (2018), with the bradycardia and acute lethality CTLBBs for walleye, fathead minnows, and rainbow trout indicated. The legend represents taxonomic groups to illustrate the distribution of CTLBBs across different organism types.

2.4 Discussion

Polycyclic aromatic compounds are globally pervasive environmental contaminants that pose a threat to aquatic organisms, including fish. Toxicity from PACs occurs through both AhR-dependent and AhR-independent mechanisms that can produce developmental abnormalities in early life stage fish, including cardiotoxicity, reduced growth, and mortality. While species-specific differences in sensitivity to PACs are widely recognized, most species sensitivity data are derived from multiple independent single-species studies conducted using varying methodologies (McGrath et al., 2018, 2021; Tillmanns et al., 2024). This research addresses these gaps by evaluating the effects of PACs on three fish species under similar exposure conditions, focusing on cardiotoxicity and acute lethality, with cardiotoxicity as a potentially underutilized indicator of long-term survival and species sensitivity in oil spill models. The results of this study produced the first cardiac CTLBBs for freshwater fish, the first quantitative characterization of cardiotoxicity in walleye exposed to individual PACs, and the first CTLBB values for a species within the order Percidae (Mcrath et al., 2018).

Mortality was observed in the NAP and DBT exposures with rainbow trout and fathead minnow larvae, and minimal walleye mortality was observed in NAP only. Rainbow trout and fathead minnows are widely used model species, with rainbow trout known for their sensitivity to a wide range of toxicants (Adams et al., 2017; Environment Canada, 1990, 2011; Teather & Parrott, 2006). One study by Teather and Parrott (2006) comparing the 96-h LC_{50}s of nine species to 190 diverse chemicals (including metals, PACs, organophosphates, and others) reported that rainbow trout were generally more sensitive than fathead minnows, although overall differences in sensitivity differed less than 10-fold and were dependent on chemical class. Similarly, a study by DeGraeve et al. (1982) found that the 96-h LC_{50} of NAP was 4.9-fold lower

for juvenile rainbow trout than for fathead minnows, aligning with the results of the present study that rainbow trout are more sensitive than fathead minnows to NAP. However, when Parrott et al. (2019) compared the sensitivity of fathead minnows and walleye to oil sands tailings pond sediments, they reported embryo larval LC_{50}s of 0.097 g/L and 0.019 g/L, respectively, indicating greater sensitivity of walleye. The authors suggested that this difference may have been due to the longer embryonic period of walleye, 18 days compared with 5 days in fathead minnows, and their longer total exposure period of 31 days compared with 20 days in fathead minnows, which likely increased uptake of PAHs and contributed to the lower LC_{50} values observed in walleye (Parrott et al., 2019; Raine et al., 2017). These findings highlight how species-specific differences in developmental timing can influence apparent sensitivity, as evidenced by the greater lethality sensitivity of walleye in Parrott et al. (2019), whereas in the present study, walleye exhibited the lowest lethality sensitivity. There are questions as to whether model species used in regulatory testing are protective of native species (Hodson et al., 2020; Sappington et al., 2001; Wallace et al., 2020). With respect to acute lethality, the LC_{50} values of both fathead minnows and rainbow trout are protective of walleye acute lethality, demonstrating the suitability of these two model species as surrogates for walleye when direct acute lethality data is unavailable.

The LC_{50} values for fathead minnows and rainbow trout in this study differed from those reported in the CTLBB database (McGrath et al., 2018). These differences are expected because of variation in experimental design. The fathead minnow acute CTLBB value reported by McGrath et al. (2018) was extrapolated from four 96-hour exposures, resulting in an acute CTLBB of 99 $\mu mol/g_{octanol}$. It is not unexpected that the CTLBBs of 46.1 $\mu mol/g_{octanol}$ (**Table 2.6**) that were determined in the present study were lower than those derived by the 96-hour

CTLBB reported by McGrath et al. (2018), as a 7-day exposure allows for greater cumulative toxicity and thus a lower LC_{50}. McGrath et al (2018) reported that fathead minnows used in their acute toxicity assays were either juvenile or unspecified, which is important to consider as toxicity responses can vary significantly across life stages (Wang et al., 2021). While the rainbow trout CTLBB derived in this study is higher than the acute lethality CTLBB reported by McGrath et al. (2018) for rainbow trout, the life stage used in their study was unspecified, making direct comparisons challenging. Although McGrath et al. (2018) reported greater acute lethality sensitivity in rainbow trout than fathead minnows based on CTLBB values, the present study found fathead minnows to be more sensitive. This difference might be reflective of test duration. In this study, the majority of rainbow trout mortality occurred on or before day five, but all fathead minnow mortality occurred near the end of the exposure period, on days five to seven, indicating that extending the test period captured additional late mortality in fathead minnows. Although the McGrath et al. (2018) database derived their CTLBBs from studies using measured concentrations, aqueous PAC levels are prone to rapid loss through volatilization, sorption, and degradation (Butler et al., 2016). Notably, the exposure methods varied across those studies (static, static renewal, flow-through), whereas in the present study, all three species were tested using the same exposure method. Passive dosing remains a more reliable approach for maintaining tighter control of exposure concentrations compared to static, flow-through, or static renewal methods, which are likely more susceptible to fluctuations in PAC concentrations (Butler et al., 2016; de Jourdan et al., 2025).

Exposure to DBT caused concentration-dependent decreases in heart rate in all three species, whereas NAP only caused a significant bradycardia response in fathead minnows and walleye (**Figure 2.1; Table 2.4**). In rainbow trout exposed to NAP, the EC_{50}/EC_{10} ratio for

bradycardia was 8.1, whereas the ratio for acute lethality was 1.2. This pattern is indicative of NAP causing significant mortality at high concentrations, but that this effect dropped off sharply, leaving surviving fish in concentrations that didn't induce a strong bradycardic response (**Table 2.4)**. This pattern is supported by little to no bradycardia in walleye or fathead minnows at similar low concentrations of NAP (**Figure 2.1**). Estimated effect concentrations are typically lower than lethal concentrations (Dubiel et al., 2024; McGrath et al., 2018; Tillmanns et al., 2024), but this pattern was also not consistent in fathead minnows. While NAP EC_{50} did not differ significantly from the LC_{50} ($p = 0.285$), the DBT EC_{50} was significantly higher than the LC_{50} ($p = 0.003$; **Table 2.3; Table 2.4**). In the fathead minnow DBT assay, mortality still aligned with nominal concentrations, with 100% mortality observed in the highest nominal group. However, measured concentrations did not align perfectly with nominal concentrations, as the highest nominal group corresponded to only the second-highest measured concentration. As both LC_{50} and EC_{50} values were calculated from measured concentrations, without the highest nominal group in the heart rate analysis, it resulted in an EC_{50} that was higher than the LC_{50}. This unexpected pattern occurred only in the DBT fathead minnow test and although this result most likely occurred as a result of human and or analytical error, further testing should be conducted to validate this result. Though BaA caused no effect on heart rate or lethality in any species, this outcome is not unexpected as BaA's log K_{OW} of 5.52 (**Table 2.1**) marginally exceeds the threshold of what is typically thought to be readily bioavailable in waterborne exposures (Dubiel et al., 2024; McGrath et al., 2018; Philibert et al., 2021).

Fathead minnows showed the strongest bradycardic response to NAP at the end of the test (7 dph), though this likely reflects that walleye were comparatively less sensitive, and rainbow trout experienced almost total mortality at test concentrations that only caused

bradycardia in fathead minnows. In fathead minnows, apart from bradycardia and a 22.22% incidence of silent ventricles at 18.27 µmol/L NAP, no additional phenotypes were observed at test termination. This pattern for NAP is similar to observations by Incardona et al. (2004), where zebrafish larvae at 4 days post-fertilization (dpf) had normal development following embryonic exposure to NAP, but experienced delayed or failed swim bladder inflation. Because fathead minnows develop more rapidly than walleye and rainbow trout (Ankley & Villeneuve, 2006; Food and Agriculture Organization, 2009; Summerfelt & Johnson, 2015), the 7-day exposure represents a greater proportion of their early development. Their accelerated development may reduce their ability to compensate for structural malformations through the entire exposure duration, with individuals developing significant defects succumbing during the exposure, leaving few observable phenotypes among survivors (**Figure 2.3**). This difference in developmental timing may also be supported by the observation that DBT caused 2:1 AV blocks in larval walleye at ≥2.59 µmol/L, and in rainbow trout in ≥2.88 µmol/L but did not result in 2:1 AV blocks in fathead minnows at any concentration (**Figure 2.3**). Incardona et al. (2004) did observe morphological abnormalities and AV blocks in zebrafish 4 at dpf following embryonic exposure to DBT, though no mortality occurred in the larvae until 6 or 7 dpf. The delayed mortality of zebrafish exposed to DBT reported by Incardona et al. (2004) aligns with the timing of mortality of fathead minnows in the present study, with much of the mortality occurring in the latter half of the exposure period. Comparatively, walleye had silent ventricles and high occurrences of pericardial edema from all three compounds. Rainbow trout had high occurrences of yolk sac edema from DBT. These observations indicate that walleye demonstrated a greater capacity for acute survival while exhibiting pronounced cardiac defects and physical abnormalities than rainbow trout or fathead minnows. Although walleye exhibited very little

mortality compared to fathead minnows and rainbow trout, the bradycardia endpoints were notably similar across all three species, varying less than 2-fold in their EC_{50}s. If assessed by mortality alone, walleye would appear relatively insensitive to PAC exposure, however, cardiac and morphological effects were evident at sublethal concentrations. This underscores the importance of incorporating sublethal endpoints to fully capture physiological impacts that occur well below lethal concentrations.

Previous studies with crude oil and individual PAC exposures across multiple fish species have consistently reported impacts to heart morphology in early life stage fish (Incardona et al., 2004, 2013, 2015, 2021; Sørhus et al., 2016, 2023a, 2023b). For example, exposure to PACs can disrupt early cardiac looping, resulting in poorly formed ventricles in larvae (Incardona et al., 2006, 2015, 2021; Sørhus et al., 2016, 2023a, 2023b). In the present study, ventricular size deficits occurred despite exposures beginning after the looping stage, suggesting that this ventricular underdevelopment reflects interruptions in normal post-hatch cardiac growth, and doesn't only interrupt embryonic cardiogenesis. This finding aligns with a study by Sørhus et al. (2023a), where exposure of Atlantic cod (*Gadus morhua*) embryos to phenanthrene (PHE) prior to and post cardiac looping both resulted in undersized ventricles. Normal cardiac function during early life stage development is crucial for the functional maturation of the heart (Ainerua et al., 2020; Brette et al., 2017; Sørhus et al., 2016). Interruption to early cardiac function has been shown to profoundly impact subsequent morphological cardiac development (Ainerua et al., 2020; Hicken et al., 2011; Incardona et al., 2015, 2021; Sørhus et al., 2023a, 2023b). To understand these effects more fully, it is necessary to explore the mechanistic pathways underlying cardiotoxicity.

Cardiotoxicity from exposure to PACs can arise through multiple mechanisms, including AhR-dependent or independent mechanisms. Brette et al. (2017) described an AhR-dependent cardiotoxicity mechanism, whereby activation of the AhR by four and five-ring PACs resulted in primary defects in cardiac morphogenesis, including impaired chamber looping and reduced cardiomyocyte proliferation, followed by secondary functional abnormalities. As BaA is a known agonist of the AhR (Dubiel et al., 2023), the reduction in ventricle size observed in walleye likely resulted from AhR-mediated disruption of cardiac development (Goodale et al., 2013). However, future studies should verify this mechanism in walleye. For fish exposed to NAP, a compound that does not activate the AhR, there was a significant correlation between reduction in ventricle length and bradycardia across concentrations, indicating that increased bradycardia was correlated with reductions in ventricle size. The bradycardic effects of NAP observed in the present study (**Table 2.3**) align with findings from other studies (Incardona et al., 2004; Dubiel et al., 2024), with this significant depression of cardiac function likely contributing to ventricular underdevelopment by reducing the pumping activity required for normal cardiac growth (Sørhus et al., 2016). Ventricular deformities from DBT, not an AhR agonist, align with the well-established mechanism whereby tricyclic PACs inhibit delayed rectifier potassium channel current (I_{Kr}) and L-type Ca^{2+} currents and depress contractility (Abramochkin et al., 2021; Ainerua et al., 2020; Brette et al., 2017; Incardona et al., 2004, 2014; Incardona & Scholz, 2016; Incardona, 2017; Incardona et al., 2021; Sørhus et al., 2016, 2023a). Unlike NAP, there was no significant correlation between reductions in ventricle size and bradycardia in fish exposed to DBT. This likely indicates that the reduction in ventricle size in fish exposed to DBT can be attributed to disruptions in intracellular Ca^{2+} cycling and excitation-contraction coupling (Abramochkin et al., 2021; Ainerua et al., 2020; Brette et al., 2017; Sørhus et al., 2023a).

Although tricyclic PACs are known to induce cardiotoxicity in fish, a study by Hedgpeth et al. (2019) found no clear link between specific 3-ring PACs and the observed sublethal heart effects in zebrafish embryos. Instead, the results from that study support that the overall bioavailable fraction of petroleum substances, through non-specific modes of action, is the primary driver of cardiac impacts (Hedgpeth et al., 2019). This pattern is consistent with the results of the present study, which showed cardiac effects following exposure to the 3-ring PAC (DBT), as well as cardiac malformations following exposure to 2-ring NAP and 4-ring BaA (**Figure 2.2**).

Disruptions to cardiac function can be transient, and some impairments may recover in a matter of hours to days post-exposure by transferring larvae to clean water (Incardona et al., 2004). However, while reductions in heart rate and 2:1 AV blocks are reversible after PAC exposure has ceased, impacts to heart morphology caused by physiological dysfunction result in permanent damage and often delayed mortality (Hicken et al., 2011; Incardona et al., 2004, 2015; Sørhus et al., 2023a, 2023b). A 96-hour embryonic exposure of zebrafish to DBT and PHE, followed by rearing in clean water, still resulted in 100% larval mortality by 6-7 dpf, despite no mortality occurring during the exposure period (Incardona et al., 2004). A study by Sørhus et al. (2023b) observed that the concentration of total polycyclic aromatic hydrocarbons (TPAH) required to induce mortality decreased over time following an 8-day exposure of Atlantic haddock embryos to crude oil, with LC_{50}s of 2.22, 0.39, and 0.27 µg TPAH/L observed at the embryonic, larval, and juvenile stages, respectively. The study by Sørhus et al. (2023b) demonstrated the delayed toxicity potential of early life stage PAC exposure, as lower exposure levels were sufficient to cause mortality at later life stages, likely resulting from persistent structural damage to the heart sustained during early life stage cardiac development.

In fish, cardiac output is primarily regulated through increases in stroke volume rather than heart rate (Farrell, 1991). Defined as the difference between diastolic and systolic ventricular volumes, stroke volume represents the volume of blood ejected by the heart with each contraction (Farrell, 1991; Shiels et al., 2006). Though stroke volume varies between species, a large ventricle in active fish species is correlated with maximum stroke volumes that are 50-100% higher than those in less active species (Farrell, 1984, 1991, 1996; Shiels et al., 2006). For example, in rainbow trout, stroke volume has been shown to increase by approximately 200% during sustained swimming, while heart rate increases by only 50% (Jones & Randall, 1978; Farrell, 1991). This reliance on volume-driven cardiac output highlights the functional importance of a well-developed and contractile ventricle. Though no mortality occurred, walleye exposed to PACs exhibited markedly small and silent ventricles, a phenotype that likely reflects reduced ventricular filling and contractility. Given the importance of stroke volume in supporting oxygen delivery during aerobic activity, these structural impairments would be expected to severely limit cardiac output and, consequently, the fish's capacity for sustained swimming (Hicken et al., 2011; Incardona & Scholz, 2016; Shiels et al., 2006). Thus, the absence of mortality during PAC exposure does not exclude the potential for lethal effects during later life stages (Incardona et al., 2004; Mager et al., 2014; Sørhus et al., 2023a, 2023b). Given that similar cardiac impairments have resulted in delayed mortality in other species (Incardona et al., 2004, 2015; Sørhus et al., 2023b), the presence of structural heart damage in walleye raises concern for potential latent effects that may not be captured in acute toxicity assays.

There was strong agreement between the bradycardia EC_{50}s, ventricle-to-atrium length reduction EC_{25}s, and the relationship predicted by the TLM, supporting the integration of cardiac-specific endpoints into the TLM framework. Consistent with Dubiel et al. (2024), this

study demonstrates that the relationship between log K_{OW} and bradycardia is applicable across taxa, extending from marine invertebrates to freshwater fish. Although the CTLBB derived from reductions in ventricle-to-atrium length ratio exhibited greater variability than those derived from bradycardia, the observed correlation suggests that the TLM may also be applicable to cardiac structural effects (**Table 2.6**). This represents a novel extension of the TLM to ventricular size reduction, however, further research is required to confirm the validity and reproducibility of this relationship.

Based on the lethal and effect concentrations observed in this study, the HC5 of the species calculated by McGrath et al. (2018) would be protective of cardiotoxic and lethal effects in larval walleye, fathead minnows, and rainbow trout, which is important in the context of PAC risk assessment in freshwater systems (**Figure 2.6**). As environmental concentrations of PACs rise with continued industrial activity, regulatory frameworks must evolve to account for their complex sublethal and lethal toxicological impacts (Cooke & Drevnick, 2022; Kurek et al., 2013; Marvin et al., 2021; Wallace et al., 2020). Currently, there are no established water quality guidelines for DBT, and only two for BaA across developed nations (Tillmanns et al., 2024). This regulatory gap highlights the relevance of predictive modelling approaches, such as the TLM, which allow for toxicity estimation based on a compound's log K_{OW}. The existing CTLBB database and the TLM provide, at a minimum, a practical, predictive framework to establish interim guideline values, particularly when no empirical standards exist. Given that the TLM is a strong predictor of PAC toxicity across species and endpoints, it remains underutilized in regulatory contexts where even conservative estimates could strengthen environmental protections. Expanding the TLM to include diverse sublethal effects, such as cardiotoxicity,

enhances its reliability and utility as a predictive tool for environmental risk assessment across varied species and exposure scenarios.

CHAPTER 3: GENERAL DISCUSSION AND CONCLUSIONS

3.1 Introduction

Polycyclic aromatic compounds (PACs) are a group of hydrophobic organic contaminants that are present globally in the environment (Wallace et al., 2020). Fish are exposed to PACs through multiple routes, including water (aqueous), sediment (contact), and diet (Wallace et al., 2020). Aquatic exposures are highest near urban centers, in areas with high industrial activity such as hydrocarbon extraction, or close to natural sources like forest fires (Cooke et al., 2022; Hodson et al., 2020; Kurek et al., 2013; Wallace et al., 2020). Many PACs are toxic to fish, with effects ranging from sublethal effects such as cardiotoxicity, developmental abnormalities, and impaired growth, to lethality (Incardona et al., 2004, 2014; Incardona & Scholz, 2016; Incardona, 2017; Sørhus et al., 2023a, 2023b; Wallace et al., 2020). The mechanism of action varies between compounds but can include nonspecific toxicity (i.e., narcosis), disruption of cardiac ion channel function, and activation of molecular pathways such as the aryl hydrocarbon receptor (AhR) signaling pathway (Dubiel et al., 2024; Incardona et al., 2004, 2014; Incardona & Scholz, 2016; Incardona, 2017; Philibert et al., 2021; Sørhus et al., 2023a, 2023b; Wallace et al., 2020). The target lipid model (TLM) is a tool used to predict the toxicity of non-ionic organic compounds to aquatic organisms by relating the chemical's log octanol-water partition coefficient (log K_{OW}), a measure of hydrophobicity, to a critical membrane lipid burden associated with adverse effects (Di Toro et al., 2000; McGrath et al., 2018; Philibert et al., 2021; Tillmanns et al., 2024). To apply the TLM, individual toxicity data for a given PAC must be available to calculate its species-specific critical target lipid body burden (CTLBB), as sensitivity to PACs can vary significantly among species (McGrath et al., 2018; Tillmanns et al., 2024). Therefore, the first goal of the research conducted in this thesis

was to compare the lethality and cardiotoxicity of three structurally diverse PACs between two model and one non-model Canadian native fish species. The second goal was to evaluate whether bradycardia and ventricular length endpoints could be applied within the TLM framework to predict PAC cardiotoxicity. The findings from this research will contribute to assessing the utility of sublethal endpoints in predictive modelling approaches to better characterize PAC toxicity in early life stage fish.

3.2 Summary of research findings

3.2.1 Larval toxicity assays

To assess differences in species sensitivity to PACs, less than 24 hours post-hatch walleye (*Sander vitreus*), fathead minnows (*Pimephales promelas*), and rainbow trout (*Oncorhynchus mykiss*) larvae were exposed to five concentrations of naphthalene (NAP), dibenzothiophene (DBT), and benz(a)anthracene (BaA) under species-specific temperature conditions. Exposures were conducted utilizing a passive dosing methodology, with polydimethylsiloxane (PDMS) O-rings to maintain stable aqueous concentrations of PACs, and two control treatments, one with no O-rings and one with unloaded O-rings (Philibert et al., 2021; Dubiel et al., 2024). Aqueous PAC concentrations were measured at test initiation, before and after water exchanges, and at test termination using 2D emission fluorometry. Trials were run for seven days, during which mortality was assessed daily. Cardiotoxicity was assessed at the end of the exposure period by capturing 15 to 20-second videos of surviving larvae using high-magnification video microscopy and manually counting heartbeats. Bradycardia was identified as a reduction in heart rate relative to the controls. Additional sublethal endpoints were also evaluated, including the ratio of ventricle-to-atrium length (walleye only), pericardial and yolk

sac edema, swim bladder inflation (fathead minnows only), 2:1 atrioventricular conduction (AV) blocks, and silent ventricles. Concentration response relationships for the half maximal lethal concentration (LC_{50}), bradycardia half maximal effect concentration (EC_{50}), and ventricle-to-atrium length ratio EC_{25} were generated for each species and PAC combination. These values were then applied within the TLM framework to derive a species-specific CTLBB value for each species and applicable endpoint.

Lethality varied by species and PAC. No mortality was observed in response to BaA in any species. For DBT, fathead minnows exhibited a significantly lower LC_{50}, 1.88 ± 0.19 µmol/L, compared with rainbow trout, 3.64 ± 0.91 µmol/L. Lethal concentration endpoints for DBT could not be calculated for walleye due to low mortality across all test concentrations. For NAP, rainbow trout and fathead minnows exhibited LC_{50}s of 14.23 ± 7.13 and 17.22 ± 1.66 µmol/L, respectively, while walleye again showed minimal mortality, preventing LC_{50} determination. However, a NAP LC_{25} was calculated for all three species, allowing for cross-species comparisons. For exposure to NAP, rainbow trout and fathead minnows exhibited nearly identical LC_{25}s, at 12.87 ± 9.10 and 12.88 ± 2.66 µmol/L, respectively, whereas walleye were significantly less sensitive, with an LC_{25} of 55.40 ± 11.88 µmol/L. The walleye LC_{25} was approximately 3.3-fold higher than that of rainbow trout and fathead minnow. For both NAP and DBT, walleye was the least sensitive to acute lethality. For DBT, the fathead minnow LC_{50} was significantly more sensitive than that of rainbow trout, and walleye had no mortality.

Each species exhibited cardiotoxicity following PAC exposure, but the sensitivity and severity of effects varied between species and PAC. No change in heart rate was observed in any species following exposure to BaA. However, concentration-dependent effects on heart rate were

observed in all three species following exposure to NAP and DBT, with the exception of rainbow trout exposed to NAP, likely due to high mortality. For NAP and DBT, fathead minnows exhibited the greatest sensitivity to a decrease in heart rate (bradycardia), with EC_{50}s of 23.23 ± 6.1 and 2.72 ± 0.24 µmol/L, respectively. The most pronounced bradycardia from DBT exposure occurred in fathead minnows, which exhibited a 61% reduction at 3.30 µmol/L. At the highest tested NAP concentration, 44.38 µmol/L, walleye showed the greatest average heart rate reduction among species, with a 52% decrease relative to controls and an EC_{50} of 36.25 ± 5.0. Reductions in ventricle-to-atrium length ratio were observed following exposure of walleye to all three PACs. The greatest reduction, relative to the control, occurred after exposure to 44.38 µmol/L NAP, followed by 0.031 µmol/L BaA, and 2.73 µmol/L DBT, at 51%, 31% and 22%, respectively. These significant decreases in ventricle size indicate that PACs impact the cardiac development of walleye, without causing significant acute mortality. Pericardial edema was observed in walleye following exposure to all three PACs, and yolk sac edema was observed in rainbow trout exposed to DBT. Silent ventricles were present in walleye exposed to each PAC, and to a lesser extent in fathead minnows exposed to NAP. Following exposure to DBT, 2:1 AV conduction blocks were observed in walleye and rainbow trout. Lethality, bradycardia, and ventricle-to-atrium length ratio endpoints were used to calculate and plot class-corrected LC_{50}, EC_{50}, and EC_{25}s, in which there was good agreement with all endpoints and the relationship proposed by the TLM. Overall, findings from this study suggest that larval exposure to PACs induces both lethal and sublethal cardiotoxic effects in early life stage fish with species and chemical-specific ranges of sensitivity across endpoints.

3.2.2 Mechanisms of early life stage cardiotoxicity

The three PACs evaluated in this study cause distinct but partially overlapping presentations of cardiotoxic action in the early life stage of fish. Numerous studies using zebrafish (*Danio rerio*) embryos have established cardiotoxicity of many individual PACs, the result of which has led to a binary division of mechanisms of toxicity, either AhR-dependent and AhR-independent (Brette et al., 2017; Incardona, 2017). As an agonist of the AhR, BaA has been shown to induce cytochrome P450 1A (cyp1a) in myocardial cells and down-regulate genes encoding for calcium (Ca^{2+}) handling proteins, required for regulating cardiac contractility in the heart (Dubiel et al., 2023; Goodale et al., 2013; Incardona et al., 2006, 2011; Incardona, 2017). This AhR-dependent cardiotoxicity caused by PACs that are ligands of the AhR has been shown to cause defects in cardiac development, manifesting as reductions in ventricular cardiomyocyte proliferation without impacts to cardiac function (Clark et al., 2010; Goodale et al., 2013; Incardona, 2017). The observation of reduced ventricle length in walleye, coupled with no bradycardia or arrhythmias observed in any species in this study, aligns well with this proposed mechanism for BaA cardiotoxicity.

Mechanistically, tricyclic PACs are not strong agonists of the AhR and instead cause toxicity by disrupting cardiac electrical activity by targeting ion channels essential for action potential repolarization and excitation-contraction coupling (Ainerua et al., 2020; Brette et al., 2017; Incardona & Scholz, 2016; Incardona, 2017). Specifically, tricyclic PACs inhibit the delayed rectifier potassium current (I_{Kr}), which plays a critical role in repolarizing the cardiomyocyte membrane (Ainerua et al., 2020; Brette et al., 2017; Incardona & Scholz, 2016). Blockage of the I_{Kr} has been attributed to the occurrence of 2:1 AV conduction blocks, a distinct rhythm observed following exposure to crude oil and individual tricyclic PACs, as observed with

DBT in this study (Incardona et al., 2004, 2005; Sørhus et al., 2016). Tricyclic PACs, namely phenanthrene (PHE), reduce L-type Ca^{2+} current by inhibiting the corresponding voltage-gated Ca^{2+} channels (Brette et al., 2017). This action limits both extracellular Ca^{2+} influx and impairs Ca^{2+} release from the sarcoplasmic reticulum, ultimately reducing contractile force (Ainerua et al., 2020; Brette et al., 2017; Incardona et al., 2004; Incardona & Scholz, 2016). However, Brette et al. (2017) did not observe a significant difference in Ca^{2+} flux in ventricular Pacific mackerel (*Scomber japonicas*) cardiomyocytes compared to controls following exposure to DBT, and thus, the impacts of DBT, and Ca^{2+} flux are not confirmed. The impacts on ion channels result in bradycardia, abnormal rhythms, and weakened cardiac contractions, reflecting the collection of phenotypes observed in this study following DBT exposure (Brette et al., 2017; Incardona et al., 2004; Incardona & Scholz, 2016).

Exposure of early life-stage fish to NAP caused several effects characteristic of nonspecific toxicity. Baseline toxicity is a non-specific mode of action caused by the accumulation of hydrophobic compounds in biological membranes, leading to generalized disruption of cellular function (Su et al., 2014). Surviving walleye and fathead minnows, in the highest NAP concentrations, exhibited no response to external stimuli, had significant reductions in heart rate, and, in a select few individuals, survived with no observed heartbeat at test termination. Further, the swim bladder was inflated in only 40% of fathead minnows that survived exposure to the highest concentration of NAP, compared to the control group at 94%. These findings are characteristic of narcotic effects, which in some cases lack severe structural malformations but manifest as functional impairments. With similar findings, Incardona et al. (2004) observed no physical abnormalities in zebrafish following embryonic exposure to NAP, except delayed or failed swim bladder inflation. The absence of arrhythmias or AV conduction

blocks further supports a non-specific depressive mechanism, as opposed to targeted ion channel interference observed with tricyclic PACs. While narcotic effects are generally considered reversible, the prolonged exposure window likely increases the probability of persistent impacts in surviving fish. The most significant reductions in ventricle length were observed in larval walleye following exposure to 44. 38 µmol/L NAP. Additionally, NAP was the only compound that showed a statistically significant relationship between ventricle length reductions and bradycardia, suggesting that the observed ventricular deformities occurred as a result of the depressed heart rate.

Comprehensive evaluations of the sensitivity of aquatic species to chemical stressors require consideration of both lethal and sublethal toxicity. Sublethal effects provide deeper insight into the physiological impacts of PAC exposure, beyond what acute lethality could indicate. While this study found that walleye exhibited little to no mortality within the 7-day exposure period and exhibited bradycardia at concentrations comparable to those of fathead minnows and rainbow trout, these results do not necessarily account for downstream impacts from the prevalent ventricular abnormalities observed in walleye following NAP and DBT exposure. Previous studies of fish exposed to crude oil observed that impacts to cardiac development in early life stages, such as reduced chamber size, resulted in impaired cardiac function in later life stages (Hicken et al., 2011; Incardona et al., 2015, 2021; Sørhus et al., 2023b). The shape and function of the ventricle in fish are directly linked to cardiac output capacity, and therefore, swimming performance (Hicken et al., 2011). Damage sustained to the ventricle from early life stage crude oil exposure has resulted in reduced adult swimming performance, as shown by Hicken et al. (2011), thereby limiting their ability to capture prey and increasing their susceptibility to predation in the wild. These functional effects did not manifest

as mortality in walleye during the timeframe of the acute laboratory exposure, yet they represent ecologically relevant effects that can impact long-term survival, thereby influencing population dynamics and ecosystem stability.

3.2.4 Target lipid model

Walleye are one of many species of fish that are not readily accessible for inclusion in regulatory testing and have not been comprehensively characterized in toxicological studies. Traditional toxicity testing frameworks, including those used for environmental effect monitoring, typically rely on a limited number of model species that are widely available, well characterized, ecologically relevant to the receiving environment, and easily maintained in a laboratory culture. While the use of model species is robust for standardized and consistent toxicity benchmarks, they may not fully capture the sensitivities of understudied native species for which little toxicity data exists. To address these limitations, models such as the TLM are an effective tool for predicting the toxicity of PACs (Di Toro et al., 2000; McGrath et al., 2018; Tillmanns et al., 2023). The TLM is a quantitative structure-activity framework that operates on the relationship between the log K_{OW} of a compound and its observed toxicity. The model assumes that toxicity occurs when a critical concentration of a contaminant accumulates in the lipid tissues of an organism, resulting in lethality or an adverse effect (Di Toro et al., 2000; McGrath et al., 2018; McGrath and Di Toro, 2009). The TLM can be applied to establish and compare a species- and endpoint-specific CTLBB (Di Toro et al., 2000). After determining a CTLBB for a given species and endpoint, the CTLBB values can be further applied to large-scale species sensitivity distributions (SSDs), enabling the extrapolation of toxicity thresholds across numerous diverse taxa to determine the exposure concentration that would cause effects in only 5 % of species tested (HC5). This hazard concentration, above which 95% of species should be

protected, is used as the basis for water quality guidelines in numerous jurisdictions such as Canada, Australia, New Zealand, and Europe (Australian and New Zealand Environment and Conservation Council [ANZECC], 2000; Canadian Council of Ministers of the Environment [CCME], 1999; European Union, 2011). Research using single PACs to derive toxicity endpoints for understudied native species expands the applicability of the TLM and supports assessments using species-specific data directly relevant to the area of concern.

Across North America, the European Union, Australia, and New Zealand, regulatory frameworks for PACs are limited, with only a select few PACs being regulated for the protection of freshwater life (**Table 3.1**) (ANZECC, 2000; British Columbia Ministry of Environment, Lands and Parks, 1993; CCME, 1999; European Union, 2011; Michigan Department of Environment, Great Lakes and Energy, Water Resources Division, 2016). Despite this, the application of TLMs in regulatory bodies worldwide remains limited (**Table 3.1**).

Table 3.1 Acute and chronic guidelines of the PACs in the present study regulated for the protection of freshwater aquatic life.

Jurisdiction	Type	NAP (ug/L)	DBT (ug/L)	BaA (ug/L)
[1]AUS & NZ	Acute	-	-	-
	Chronic	16	-	-
[2]BC	Acute	1	-	-
	Chronic	1	-	0.1
[3]CAN	Acute	-	-	-
	Chronic	1.1	-	0.018
[4]EU	Acute	130	-	-
	Chronic	2	-	-
[5]MI	Acute	200	-	-
	Chronic	11	-	-

[1]Australian and New Zealand Environment and Conservation Council
[2] British Columbia Ministry of Environment, Lands and Parks
[3]Canadian Council of Ministers of the Environment.
[4]European Union.
[5]Michigan Department of Environment, Great Lakes and Energy.

Class-corrected LC_{50}, EC_{50}, and EC_{25} values in this study aligned well with TLM predictions, supporting the model's relevance for application to cardiotoxic endpoints (Dubiel et al., 2024; McGrath et al., 2018). The HC5 values calculated by McGrath et al. (2018) would be protective of both lethal and bradycardic effects in all tested species. In addition to their utility for use in creating interim individual PAC guideline values, calculated CTLBB values derived from the TLM can also be integrated into more complex exposure models such as the PetroTox model. This model applies species-specific CTLBB values with environmental concentrations of complex petroleum mixtures to estimate acute toxicity in aquatic organisms following oil spills or other petroleum discharges (Redman et al., 2012). The PetroTox model can be used for spill response and site-specific evaluations to estimate ecological hazards to aquatic species based on petroleum mixture composition. Predictive toxicity modelling tools provide quick toxicity estimates, offering a faster alternative or interim guidance than conducting laboratory tests or compiling large experimental datasets in response to a disaster. These applications highlight the utility of the TLM and CTLBB database as cost-effective tools to estimate toxicity and guide interim standards when baseline estimates could strengthen environmental protections and responses.

3.3 Future research

Environmental disasters such as the 1989 Exxon Valdez and 2010 Deepwater Horizon spills released millions of litres of crude oil into surrounding marine ecosystems, exposing them to PACs. In response, several studies have examined long-term post-exposure outcomes in marine pelagic species (Esbaugh et al., 2016; Incardona et al., 2014, 2015, 2021, 2023; Sørhus et al., 2023b), as well as in the well-characterized model species, zebrafish (Hicken et al., 2011; Incardona et al., 2005).

However, long-term post-exposure data for most freshwater species remain scarce. As the extensive ventricular damage observed in walleye raises concerns about lasting impacts, future research should investigate the long-term survival and fitness of freshwater species following early life-stage exposure to PACs. The need for long-term toxicity studies on freshwater species is highlighted by the scale of industrial development and resource extraction occurring in and around Canadian freshwater ecosystems (Hodson et al., 2020; Kurek et al., 2013; Wallace et al., 2020). Additionally, the widespread transport of hydrocarbon-based fuels presents an ongoing risk of freshwater contamination (Hodson et al., 2020; Wallace et al., 2020). Indigenous communities in Canada have voiced growing concern over the impacts of hydrocarbon resource extraction on native species, particularly those of cultural, resource, and ecological importance, many of which lack toxicity data needed to comprehensively assess their vulnerability to PACs (Brunet et al., 2020). Major waterbodies near large-scale oil sands development in Alberta, including the Athabasca River and its tributaries, support numerous fish populations that hold ecological, cultural, and resource value for First Nations communities in the region (Ohiozebau et al., 2017). Fish species, including walleye, from the Athabasca and Slave rivers show higher tissue concentrations of PACs near extraction and upgrading sites relative to downstream locations (Ohiozebau et al., 2016, 2017). These observations indicate that fish in the region are exposed to PACs, although the ecological consequences of that exposure remain uncertain. Additional research is needed to bridge the gap between acute laboratory assays and the ecological impacts of PAC exposure. As observed in this study, walleye, a species that exhibited little to no mortality during the 7-day toxicity assay, sustained extensive impacts to ventricular development and function. Previous studies observed long-term impacts on juvenile and adult cardiac morphology and aerobic capacity following early life-stage crude oil exposure (Hicken et

al., 2011; Incardona et al., 2014, 2015, 2021; Mager et al., 2014; Sørhus et al., 2023b), suggesting that the surviving walleye in this study would likely face substantial reductions in survival and fitness due to compromised cardiac function, despite their high acute survival. Given the extensive cardiac damage observed in walleye during the 7-day exposure, future studies should investigate long-term impacts of acute early life stage exposure on swimming performance and survival of walleye and other freshwater species by transferring exposed fish to clean water and monitoring outcomes into juvenile or adult life stages. This would enable comparisons of delayed responses with acute test endpoints, exploring how well acute toxicity captures long-term survival and fitness outcomes. Deepening our understanding of the long-term implications of sublethal cardiotoxicity will further enhance its integration into risk assessment frameworks and ensure that evaluations reflect outcomes relevant to environmental survival scenarios.

3.4 Conclusions

This study provides insight into species-specific sensitivity to PAC-induced lethality and cardiotoxicity during the early life stages of freshwater fish. Many Canadian aquatic environments are contaminated by PACs, largely due to expanding industrial development and urbanization (Hodson et al., 2020; Kurek et al., 2013; Ohiozebau et al., 2016, 2017; Wallace et al., 2020). As a result, PACs are now regularly detected in the tissues of native fish species residing in affected regions, raising concerns about the implications on ecological health (Ohiozebau et al., 2016, 2017). Evident by the large variation in lethality between walleye, fathead minnows, and rainbow trout, walleye appear to be more capable of withstanding cardiotoxic effects over the 7-day exposure period. Rainbow trout experienced rapid mortality early in the test, while fathead minnows mostly succumbed in the latter half of the exposure

period, suggesting species-specific differences in the tolerance of cardiotoxic impacts. Although walleye exhibited high survival during acute exposures, they still exhibited strong morphological and functional cardiac defects from PACs, indicating that tolerance to acute lethality does not translate to long-term organism viability. The findings from this study demonstrated that interspecies differences in sensitivity to PACs narrow considerably when sublethal cardiotoxicity endpoints were compared, a pattern that may carry important implications for the construction of species sensitivity distributions and the derivation of protective thresholds. Furthermore, this study successfully integrated bradycardia endpoints and introduced the novel application of cardiac chamber size into the TLM, demonstrating its potential utility as a predictive tool for cardiotoxicity. Therefore, gaining a broader understanding of species-specific sensitivity to cardiotoxicity will improve the evaluation of the long-term ecological impacts of PAC exposure and aid in the development of protective thresholds for fish species.

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