Assessing exposure of small-bodied fish to experimental crude oil spills in contained shoreline environments using biliary polycyclic aromatic compound metabolites

by

Jamie Dearnley

A Thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

In partial fulfillment of the requirements of the degree of

Master of Science

Department of Chemistry

University of Manitoba

Winnipeg

Abstract

Knowledge gaps pertaining to the remediation of freshwater lakes impacted by oil spills have persisted despite recent record highs for oil production and transportation across vulnerable regions in North America. The multiyear Freshwater Oil Spill Remediation Study (FOReSt), conducted at the IISD-Experimental Lakes Area in Canada, is focusing on the efficacy of minimally invasive methods for remediating oil spills in freshwater boreal lakes. In this thesis, the impacts and remediation of diluted bitumen (dilbit) and conventional heavy crude oil (CHV) spills were investigated (year 1), as were a variety of different remediation methods for spills of dilbit on different shoreline substrates (year 2). Two common small-bodied fish, fathead minnows (*Promephales promelas*) and finescale dace *(Chrosomus neogaeus*), were used to assess exposure to petrogenic polycyclic aromatic compounds (PACs) in model enclosed shoreline ecosystems impacted by spills and remediated using minimally invasive techniques. Short-term exposure to PACs, the most toxicologically relevant compounds in oil, was assessed in fish using biliary metabolite concentrations. In year one, finescale dace and fathead minnows residing in oil treated enclosures each had biliary pyrene metabolite concentrations that were positively correlated with pyrene concentrations in the water of the enclosures. Three months after the initial spills, fish in the enclosure receiving dilbit were significantly more exposed to PACs than fish in reference enclosures that did not receive oil. In year two, both finescale dace and fathead minnows residing in oil-treated exposures, regardless of shoreline substrate, showed increased exposure to PACs compared to fish in reference enclosures and the pristine lake environment two and a half months after the spills. No significant differences in exposure were observed among the remediation treatments. Biliary PAC metabolite concentrations were positively predicted by parent PAC concentrations in periphyton. PACs in periphyton two and a half months after oil introduction were positively correlated with PACs in the enclosures one week after spills, suggesting fish also had increased exposure to periphyton-bound alkyl-PACs. This thesis validates the use of small-bodied fish in assessing PAC exposure following freshwater oil spills and demonstrates the difficulties in estimating exposure using environmental concentrations in natural systems.

To Scatman John and the people of Scatland

Acknowledgements

This project could have been done by anyone; I was just the cog that happened to fit in the machine. It could not, however, have been done without the huge supporting cast that made it all possible and these are the people who deserve the most credit. The people on the title page (until the U of M example title page didn't have the committee listed, anyway) deserve an especially big thank you: Vince Palace and Gregg Tomy for all their support, time spent on revising and advising, plus all the usual clichés they've had lofted upon them in many theses past and Jörg Stetefeld for his invaluable contributions on the committee. I am also especially appreciative to Dr. Tom Ward who played a major role in getting the HPLC/F up and running; this thesis would look a lot different without him. Dr. Lisa Peters helped me out majorly with lab stuff and generated all the non-bile and non-periphyton data you'll soon be reading about. I am very appreciative of Ifeoluwa Idowu, Zhe Xia, and Thor Halldorson for showing me the ropes in the Tomy Lab and for being good company, along with everyone else who passed through our doors. The larger Freshwater Oil Spill Remediation Project and my part in it featured a tremendous number of contributors during the field season from set-up to sample collection to take-down and I've done my best to list them here in reverse alphabetical order: Sarah Warrack, Lauren Timlick, Adam Scott, Jose Luis Gil Rodriguez, Kenzie Perry, Brooke Palace, Sonya Michaleski, Cody Jackson, Lee Hrenchuk, Lauren Hayhurst, Marco Haust, Dr. Mark Hanson, Katarina Djordovic, Danielle Desrochers, Abby DeBofsky, Blake Cooney, Patrique Bulloch, Tyler Black, Alyssa Ball, Maria Armstrong, Phillip Ankley. You are the ones who made the magic happen. Lastly, thank you to the Vancouver Canucks who did a great job of not distracting me with winning hockey and exciting playoff runs over the course of this 3+ year thesis.

Preface

This main objective of this thesis was to assess the exposure of small-bodied fish, primarily fathead minnows (*Promelas primephales*) and finescale dace (*chrosomus neogaeus*), to polycyclic aromatic compounds (PACs) following model freshwater shoreline oil spills which had been remediated using minimally invasive techniques. This was accomplished using biliary PAC metabolites in the experimental fish. The first two chapters establish the foundation on which the latter chapters are built. Chapter 1 reviews contemporary literature and contextualizes the work of proceeding chapters. The toxicological effects of PACs to fish are examined, the rationale for using biliary PAC metabolites as a biomarker for exposure is explained, the methods of analysis for these metabolites are explored, and instances of the biomarker being used to assess PAC pollution globally are presented. Notably, this review is devoid of the use of biliary PAC metabolites in fathead minnows or finescale dace. This necessitated the work of Chapter 2, in which fathead minnows were exposed to a range of PAC concentrations to observe whether a dose-dependent response in biliary PAC metabolites was elicited. Fathead minnows, along with finescale dace, then feature prominently in the next two chapters, which focus on the work of the Freshwater Oil Spill Remediation Study (FOReSt) at IISD-Experimental Lakes Area in Northwest Ontario. Chapters 3 and 4 each focus on a different year of FOReSt in which oil spills in contained shoreline enclosures were studied. In Chapter 3, spills of diluted bitumen (dilbit) and conventional heavy crude oil were studied, while in Chapter 4 different minimally invasive remediation techniques were studied in response to dilbit introduction. In both chapters the exposure, as well as the routes of exposure, of PACs to small-bodied fish living in the enclosures was assessed several months after each spill and its subsequent remediation. Finally, the conclusions of the thesis are presented and future directions are explored.

Table of Contents

List of figures

List of tables

Chapter 1: Monitoring polycyclic aromatic compounds (PACs) exposure in fish using biliary metabolites

A version of this chapter has already been published as Jamie M. Dearnley, Charles Killeen, Rebecca L. Davis, Vince P. Palace & Gregg T. Tomy (2022) Monitoring polycyclic aromatic compounds exposure in fish using biliary metabolites, Critical Reviews in Environmental Science and Technology, 52:4, 475-519, DOI: 10.1080/10643389.2020.1825895. Reproduced with permission.

I wrote the entirety of the "Analytic methods for biliary metabolites" and "Environmental occurrence and monitoring" sections and contributed to each of the other sections. I was also responsible for editing to produce a cohesive manuscript with a consistent tone and message.

Abstract

Polycyclic aromatic compounds (PACs) are a class of organic contaminants whose toxicity and persistence pose a threat to fish health. In many locations, anthropogenic inputs represent the most prominent source of these compounds. Thus, quantifying fish exposure to PACs is important for assessing ecosystem health and anthropogenic inputs. Quantitation of biliary PAC metabolites is the most accurate method for determining short-term exposure of resident fish to these compounds and is often used as a biomarker for fish health. Such analyses have been conducted worldwide on many different species of fish and with a variety of instrumentation. Here, PAC metabolism and toxicity in fish are reviewed, as are the different methodologies used to analyze metabolites in bile. Recent environmental monitoring studies are presented, compared, and contrasted to highlight important findings and novel applications of the technique. These include instances of the biomarker being used to assess the extent of PAC pollution from oil spills and tracking the subsequent recovery of the affected region, establishing baseline PAC exposure in pristine areas, and assessing pollution in urban waterways. By presenting global perspectives of PAC exposure and metabolites in fish, comparisons of the most effective ways to implement environmental monitoring are explored.

1.1 Introduction

Very few chemicals have garnered more toxicological study, scrutiny, and screening than polycyclic aromatic compounds (PACs). Found in air, sediments, soils and water, PACs can impinge upon resident biota including fish. Driven by their lipophilicity, these compounds readily partition into the tissues of exposed organisms. Once assimilated by fish, many PACs are quickly metabolized and excreted into the bile and urine. Consequently, quantitation of biliary metabolites for PACs has become an important biomarker for exposure and potential deleterious effects on fish health.

Polycyclic aromatic compounds are ring-based aromatic compounds composed primarily of carbon and hydrogen (Figure 1.1). Those consisting solely of carbon and hydrogen comprise a subset of PACs known as polycyclic aromatic hydrocarbons (PAHs). Other PACs incorporate other elements, referred to as heteroatoms, into their structures while still maintaining

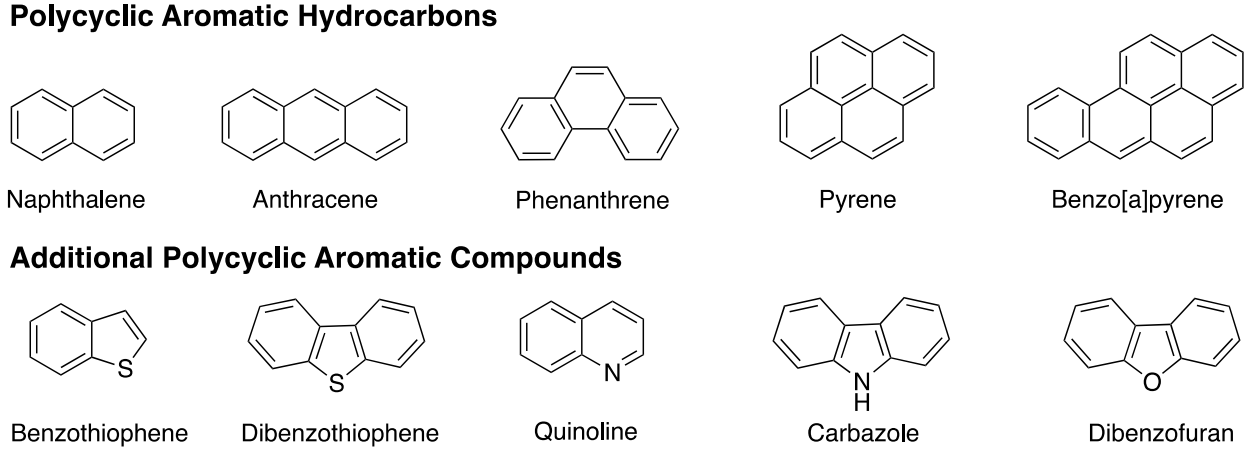


Figure 1.1: A selection of polycyclic aromatic compounds (PACs). These compounds may be comprised solely of hydrogen and carbon atoms, but can also contain heteroatoms (such as sulfur, nitrogen, and oxygen) while still maintaining aromaticity. PACs can also have their hydrogen atoms substituted with alkyl chains, rendering alkyl-PACs or serve as substrates for reactions which replace hydrogen atoms with moieties such as hydroxyl groups. Reproduced with permission from Dearnley et al. (2020).

aromaticity. Nitrogen, oxygen, and sulfur are the most common heteroatoms to be incorporated (Collier et al., 2013). With only two fused rings, naphthalene is the simplest PAC and PACs containing seven rings and more are commonly documented. PACs can also be substituted by one or more alkyl groups, rendering compounds termed alkyl-PACs. Most PACs are exceedingly non-polar, imparting them with high lipophilicity and low water solubility. This

leads to PACs generally being strongly bioaccumulative in fatty tissues, as evidenced by their high octanol-water partition coefficients (K_{OW}; Sette et al., 2013). This tendency to partition into lipids generally increases with the number of constituent rings; two-ringed naphthalene has log K_{OW} = 3.35, while most six-ringed PACs have log K_{OW} > 7 (Achten & Andersson, 2015). Aromatic compounds have some unique properties associated with their delocalized electronic structures including unusually high chemical stability and fluorescence (Bandeira & Meneses, 2013). In particular, the environmental persistence of PACs is due to the aromaticity (cyclic delocalized electrons) of their fused ring structures. These compounds are not readily degraded by common processes which destroy other common xenobiotics, such as atmospheric oxidation. Aromaticity is also responsible for the highly fluorescent nature of PACs, which makes fluorescence spectroscopy an ideal method for their analysis and quantitation.

In aquatic environments, PACs originate from either pyrogenic or petrogenic sources. Pyrogenic PACs arise from the incomplete combustion of carbonaceous material, whereas petrogenic PACs are naturally present in crude oil and enter ecosystems mainly through spills or from refined petroleum products and processing activities. These sources can be anthropogenic (oil spills, fuel combustion) or natural (oil seepages, forest fires). Petrogenic PAC signatures tend to include more 2-3 ring compounds that feature a high degree of alkylation. Conversely, pyrogenic PACs generally contain more 3+ ring compounds with a lower degree of alkylation (Abrajano et al., 2013). In fact, the ratio of alkyl-PACs to their non-alkylated counterparts in water and sediment can be used as a metric for determining the origin of site contamination. This extends to bile, where the ratio of smaller 2-3 ring PAC metabolites to 4+ ring ones can be used to differentiate between pyrogenic and petrogenic pollution (Beyer et al., 2010; Duarte et al., 2017). Fewer rings also tend to render petrogenic PACs more susceptible to degradation by microbes that have adapted to completely metabolize small PACs (Heitkamp & Cerniglia, 1987; Lima et al., 2005). Historically, most studies on PACs have focused on the 16 EPA Priority PAHs, though contemporary research has expanded its frontiers beyond them with many studies now including upwards of 50+ compounds of interest (Andersson & Achten, 2015). These include alkyl-PACs, heterocyclics, and/or hydroxylated PACs, the last of which is also used a biomarker for PAC exposure in fish.

1.2 Mechanisms of formation

Abiotic or biotic transformation of PACs can create derivatized products more toxic to fish than the parent compounds. While this review focuses mainly on metabolic transformation and products, abiotic transformation of PACs also occurs in the environment. The atmosphere is one source of abiotically functionalized PACs. Smaller, 2-3 ringed PACs are volatile enough to readily vaporize and enter the atmosphere in the gaseous phase, while larger PACs are primarily emitted into the atmosphere adsorbed to aerosolized smoke and soot particles from pyrogenic sources (Harrison et al., 2018; Lima et al., 2005). In the upper atmosphere, persistent reactive species such as ozone, nitrogen and sulfur oxides (NO_x, SO_x), and free radicals are present (Reisen & Arey, 2005). While ultraviolet (UV) light is capable of degrading PACs, it can also facilitate their functionalization by these reactive species, forming a suite of derivatives which are more polar, and potentially more toxic than the parent compounds. The most relevant atmospheric radical reactions of PACs primarily form nitro- and hydroxy-PACs (Harrison et al., 2018). These products, along with parent PACs, readily enter the aquatic environment by settling as smog or dust through agglomeration, or by adhering to constituent particles of atmospheric soot. Alternatively, they are simply carried down by rain and/or snow. The size of the compound affects its partitioning in the atmosphere and mode of deposition, with lighter (2-4 ring) PACs mainly found in the gas phase while heavier PACs tend to be adsorbed to particulate (Lima et al., 2005). Once in the aqueous environment, abiotic transformation of PACs and hydroxy-PACs can occur in surface waters given sufficient ultraviolet solar radiation and oxygen (Fasnacht & Blough, 2002; Ge et al., 2016). This photodegradation process leads to a wide variety of products including alcohols, aldehydes, ketones, and quinones. As a whole, these compounds have demonstrated comparable, if not higher, toxicity than their parent compounds in laboratory studies (Ge et al., 2016; Kang et al., 2019). Once in the ecosystem, exposure of fish to PACs and their transformation products occurs through three main routes of exposure: ingestion, transfer across the gills' epithelial surface, and dermal uptake (Logan, 2007). The dominant route of exposure can vary from compound to compound, and in general depends on factors such as species, habitat, and lifestyle. This is explored in greater detail in the "Environmental occurrence and monitoring" section.

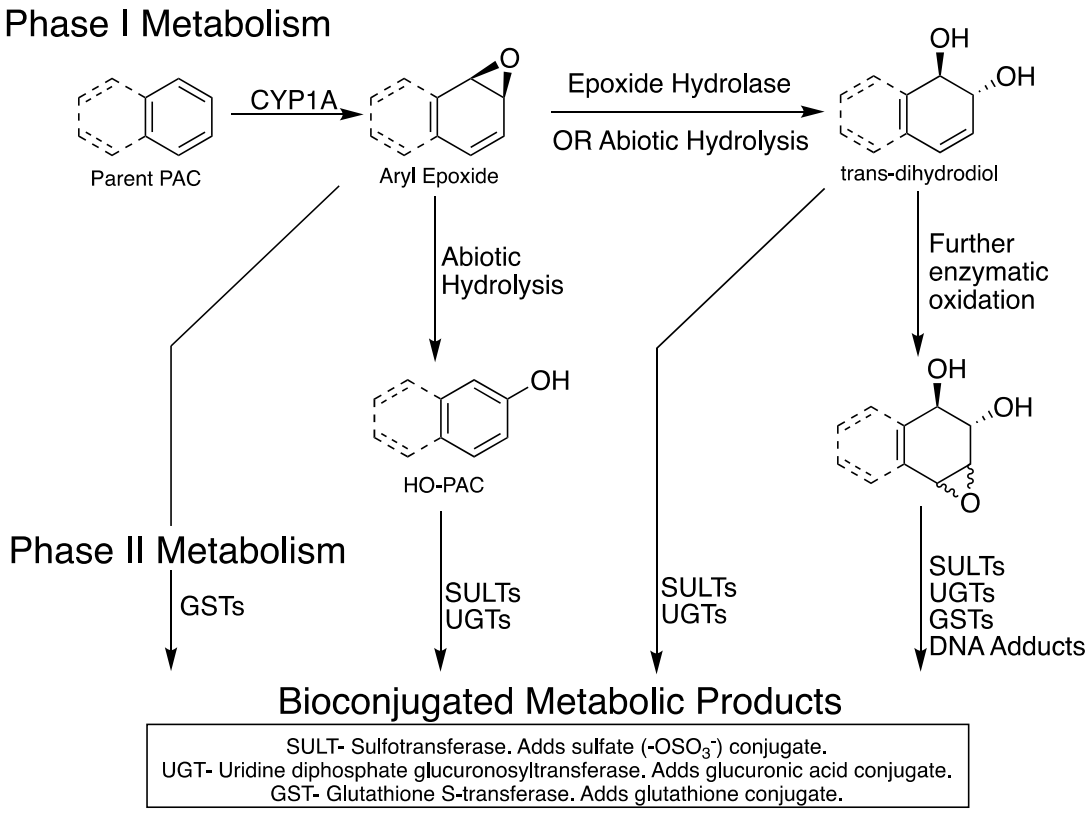


Figure 1.2: A diagram of the reactions, intermediates, and enzymes involved in polycyclic aromatic compound metabolism in fish (Pampanin & Sydnes, 2013; Tierney et al., 2013). Reproduced with permission from Dearnley et al. (2020).

Metabolic detoxification of PACs in fish generally proceeds through two steps (Figure 1.2). The first phase increases hydrophilicity by introducing oxygen into the aromatic ring portion of the compound and is referred to as Phase I metabolism. This creates an epoxide which is ultimately transformed into a hydroxylated PAC. Phase II metabolism uses the oxygen introduced in Phase I to attach large polar biomolecules to facilitate excretion of the entire complex in aqueous excretion media, including urine, feces and bile. This metabolism reduces toxic effects by reducing endogenous levels of PACs. However, many of the intermediate metabolites, in particular the epoxides, of Phase I transformation can be more reactive and toxic than the initial parent compounds. Phase I metabolism generally occurs via oxidation to aryl epoxides by enzymes of the cytochrome P4501A (CYP1A) family (Collier et al., 2013; Tierney et al., 2013). The highest concentrations of these enzymes are found in the liver, with lower activities appearing in the kidneys, intestines, gills, gonads, and vascular endothelium (Sarasquete & Segner, 2000). The reactive epoxides are readily hydrolyzed to trans-dihydrodiols by epoxide hydrolase enzymes, but can also be hydrolyzed abiotically. Phase II metabolism generally

consists of conjugation of the diol or phenol intermediates with an endogenous biomolecule to facilitate excretion (Tierney et al., 2013). Common functional groups consist of polar molecules which are abundant in biological systems, including sulfate (SO_4^{2-}) and acetate (CH_3COO^-). In fish, conjugation with glucuronic acid (to form glycoside compounds), sulfate, and glutathione are among the most common phase II conjugation reactions (Ikenaka et al., 2013). These metabolic steps facilitate excretion via bile or the urinary tract. In general, the process efficiently leads to elimination of many PACs in fish: naphthalene, acenaphthene, acenaphthylene, fluorene, pyrene, benz[a]anthracene, and benzo[a]pyrene have each been found to be metabolized quickly and therefore do not bioaccumulate in fish, though metabolic rates can vary significantly from species to species (Bleeker & Verbruggen, 2009; Pulster et al., 2017). The degree of PAC alkylation can play an important role in the rate of elimination, with increasing alkylation generally leading to slower excretion (Jonsson et al., 2004; Sørensen et al., 2017). With too much alkylation, however, a PAC can become too size limited in terms of passively entering an organism (Barron, 1990; Opperhulzen et al., 1985). The most commonly quantified biliary metabolite in environmental monitoring assessments is 1-pyrenol. An often cited study of estuarine European eel (*Anguilla anguilla*) found that, of the compounds targeted, 1-pyrenol accounted for the majority (up to 76%) of the total PAC metabolite concentrations (Ruddock et al., 2003), prompting researchers to make it a priority in subsequent analyses (Blahova et al., 2013; Floehr et al., 2015; Kammann et al., 2017). Other frequently targeted compounds include metabolites of benzo[a]pyrene, phenanthrene, and naphthalene. The expeditiousness of PAC metabolism in fish is seen in many exposure studies reliant on tissue burden quantitation. For instance, at the end of a 53 day study featuring continual exposure via diet, juvenile Chinook salmon (*Oncorhynchus tshawytscha*) metabolized 99% of the ingested PACs (Meador et al., 2006). Results like these underscore the utility of biliary metabolites as biomarkers for evaluating PAC exposure.

1.3 Toxicity

In principle, efficient metabolism of PACs in fish is beneficial but in practice the process can have deleterious effects with carcinogenesis the primary concern in adult fish. Reactive epoxide metabolic intermediates formed in Phase I metabolism can form hydrophobic, covalently bound adducts with DNA in fish. When these adducts form between an oncogene in the genomic DNA,

carcinogenesis can result. DNA adducts are associated with hepatic lesions, induction of hepatocarcinogenesis, and hepatic neoplasia in many species of fish (Collier et al., 2013; Pampanin & Sydnes, 2013; Reichert et al., 1998). Due to their persistence in fish liver, DNA adducts are often used as a biomarker to assess long-term exposure to PACs (Pampanin & Sydnes, 2013). The prospective metabolites of PACs differ in their capacity to form DNA adducts and promote carcinogenesis based on the parent PAC and site(s) of oxidation. One metabolite of benzo[a]pyrene, benzo[a]pyrene-7,8-diol-9,10-epoxide is particularly carcinogenic and its preferential formation in some species of fish compared to others may explain differences in susceptibility to PAC carcinogenesis (Willett et al., 2000).

When confronted with high ambient levels of PACs, fish can develop toxicity resistance. This was observed in mummichog (*Fundulus heteroclitus*) residing among sediments heavily contaminated with PACs from creosote spills in a nearby wood treatment facility (Atlantic Wood) in Virginia, USA. This extensively studied population has undergone several reviews and illustrates the impact high PAC pollution can have on resident fish (Di Giulio & Clark, 2015; Van Veld & Nacci, 2008). In mummichog from the most contaminated sites, an extremely high prevalence of hepatocellular carcinoma and other lesions was observed. In one study, 93% of fish displayed visible liver lesions (Vogelbein et al., 1990) and another found exceptionally high levels of DNA adducts in hematopoietic tissues (Rose et al., 2000). Yet, despite these afflictions the Atlantic Wood fish demonstrated resistance to acute toxicity. Whereas mummichog from reference sites typically developed visible symptoms (i.e., skin, gill and fin erosion; death) within days of being exposed to contaminated sediment, those from Atlantic Wood displayed none (Van Veld & Nacci, 2008). Similarly, embryos from reference site parents experienced 100% mortality when exposed to Atlantic Wood sediment, while embryos from Atlantic Wood mummichog parents experienced 76% survival and no cardiac malformations (Van Veld & Nacci, 2008; Williams, 1994). Additionally, Atlantic Wood mummichog were found to be recalcitrant to CYP1A induction following intraperitoneal injection of 3-methylcholanthrene (Di Giulio & Clark, 2015). Aspects of this toxicity resistance were demonstrated to be heritable, as F2 embryos from lab-reared F1 adults were resistant to toxic effects from polluted Atlantic Wood sediment (Meyer & Di Giulio, 2003; Ownby et al., 2002). At the molecular level, multiple sites across the Atlantic Wood mummichog genome have been identified as being associated with PAC resistance (Osterberg et al., 2018). Contrarily, some characteristics of the

Atlantic Wood mummichog, such as resistance to CYP1A induction, were found to be reduced in F2 embryos and largely lost by F3 (Clark et al., 2014; Meyer et al., 2002). Thus, PAC toxicity resistance in these fish does not appear to be fully genetically heritable and investigation continues (Di Giulio & Clark, 2015).

Beyond functioning as carcinogens, PACs can also act as endocrine disruptors and cause reproductive impairment in fish. In female fish, these effects are well documented. As noted by Collier et al. in their review (2013), PAC exposure in both natural and laboratory settings has been linked to decreased plasma estradiol concentrations, reduced ovarian estradiol production, suppression of vitellogenesis, reduced ovarian growth, ovarian atresia, and reduced fecundity (Bugel et al., 2010; Johnson et al., 1998, 2008; Khan, 2013; Thomas, 1990). Depression of plasma 17-β estradiol has also been observed in female fish exposed to individual PACs and mixtures (Monteiro et al., 2000; Pollino et al., 2009; Sol et al., 2000; Tintos et al., 2006, 2007). There is some evidence of reproductive effects from PAC exposure in male fish, but results are less consistent (Collier et al., 2013). In some studies exposure to PACs (from crude oil or processed water) led to reduced testicular development and/or reduction in spermatogenesis (Geraudie et al., 2014; Khan, 2013; Kiceniuk & Khan, 1987; Sundt & Bjöerkblom, 2011), while others found no effects on reproduction (Holth et al., 2008). In a recent study, Bender et al. (2016) exposed polar cod (*Boreogadus saida*) to dietary crude oil over a period of gonadal development. In both sexes gonadal development was not significantly impaired nor were plasma steroid levels, though sperm motility decreased in exposed males. A study exposing adult sheepshead minnow (*Cyprinodon variegatus*) to high energy water accommodated fractions (WAF) of oil found both egg production and fertilization rate to be negatively correlated with total tissue PACs, though the differences between exposed groups and a non-exposed control were not significant (Jasperse et al., 2019). PAC exposure has also been linked to other adverse outcomes in fish such as degradation of vision, immunosuppression, and decreased swim performance and shoal cohesion (Armstrong et al., 2019; Collier et al., 2013; Johansen & Esbaugh, 2017; Logan, 2007; Whyte et al., 2000).

1.3.1 Early life stage toxicity

Though fish health is impacted by PACs in the environment at all life stages, those in the embryonic state are particularly susceptible to deleterious effects. Embryonic fish lack the

metabolic capacity of adults, imparting them with a higher potential for bioaccumulation of PACs (Jung et al., 2015). Development is impacted from the earliest life stages as increased PAC concentrations negatively affect embryonic cleavage. This is caused by PACs disrupting the Wnt/β-catenin signalling pathway, which impairs establishment of the dorsal-ventral axis (Fairbairn et al., 2012). In severe cases, this disruption leads to mortality before hatching, though the effects of axial malformation impact the viability of the organism at all life stages. Research suggests all teleost embryos are susceptible to adverse developmental effects in response to petroleum exposure and toxic effects are consistently observed in habitats affected by oil spills (Incardona et al., 2009, 2014).

The most significant toxic effects PACs exert on embryonic fish target the developing heart, and this cardiotoxicity acts through multiple structure-dependent mechanisms. Larger (>3 rings) PACs are believed to primarily impact heart development through antagonism of the aryl hydrocarbon receptor (AhR) in myocardial tissue (Incardona, 2017). While AhR-mediated carcinogenesis is primarily related to the production of more toxic metabolites from parent PACs, persistent AhR activation in the developing heart leads to cardiotoxicity via a CYP1A-independent mechanism, typical of dioxins. Here, toxicity arises through downregulation of normal genetic function, which manifests as pericardial edema and cardiovascular dysfunction (Scott et al., 2011). In Japanese medaka (*Oryzias latipes*), pyrene and 1-methylpyrene demonstrate very similar toxicities, implying a mechanism of action that is not significantly altered by alkyl substitution and consistent with the AhR-mediated pathway (Barjhoux et al., 2014). Some three-ringed PACs such as retene (7-isopropyl-1-methylphenanthrene) also exhibit toxicity which is independent of CYP1A activation, and depends on an AhR-mediated mechanism (Scott et al., 2011). Thus, the degree of PAC alkylation also impacts the molecular mechanisms of embryonic toxicity. In contrast, most small (2-3 rings) PACs have been found to be poor AhR ligands, suggesting a mechanism of action which is independent of the AhR-mediated pathway (Incardona, 2017). Indeed, three-ringed alkyl-PACs are shown to be significantly more toxic than the parent non-alkylated compounds (Barron, Heintz, et al., 2004). The toxicity of three-ringed PAC congeners arises from their ability to inhibit potassium channels and disrupt calcium cycling in the myocardial tissue of the developing embryo (Incardona, 2017). Brette et al. (2014) investigated the effect of environmental oil samples

collected from the *Deepwater Horizon* cleanup effort on the action potential of isolated cardiomyocytes of bluefin tuna (*Thunnus orientalis*) and albacore (*Thunnus albacares*). This work demonstrated that action potential repolarization is prolonged by crude oil exposure in a concentration-dependent manner, and that the cardiotoxic potential of different oil samples correlates more closely to the concentration of three-ringed PAC congeners rather than total-PAC (tPAC) concentration in the oil sample. This disruption of the embryonic heartbeat can have permanent effects on heart shape and affect cardiac output at all life stages, increasing the potential for arrhythmias and limiting swim performance in adult fish. A basis for some of these effects has been studied at the cellular level. For instance, when exposed to saline solutions of high-energy WAF, mahi-mahi (*Coryphaena hippurus*) cardiomyocytes demonstrated reduced contractility, as well as prolonged action potentials and disrupted calcium cycling (Heuer et al., 2019). Moreover, cardiac muscle fibres from recently high-energy WAF exposed mahi-mahi showed reduced mitochondrial respiration and reduced mitochondrial ADP affinity (Kirby et al., 2019). Interestingly, a synergistic effect is observed when the toxicity of crude oil is compared to individual PACs (Hodson, 2017). This may arise from the ability of different PACs to be poor AhR ligands and good CYP substrates, or *vice versa*, with larger PACs that are good AhR ligands being capable of increasing the rate at which toxic metabolites of small PACs are produced (Incardona, 2017). This complicates the attribution of meaningful concentrations and biological effects of individual PACs in crude oil samples, which are by nature extremely complex. Meador & Nahrgang (2019) discuss this idea further and suggest that attributing the toxicity of crude oil to embryonic fish solely to PACs is premature based on existing literature. The authors assert there is insufficient evidence that individual PACs are capable of exerting their effects at the low levels reported (0.1-5 μg/L) without being part of a crude oil fraction, and conclude that additional water-soluble components of crude oil contribute to its environmental toxicity.

PACs have also been shown to have physiological impacts on the developing embryo unrelated to cardiotoxicity. Craniofacial deformity and hinderance of embryonic eye development are other aspects of PAC-based organocontamination with the potential for serious consequences at all life stages. Magnuson et al. (2018) demonstrated the impact of *Deepwater Horizon* crude oil WAFs on the eye-related behavioural and morphological development of embryonic red drum

(*Sciaenops ocellatus*) and sheepshead minnow. In this work, the negative effect of tPAC concentration on optomotor response was demonstrated, with an increase in tPAC correlated both to later post-fertilization development and a slowed optomotor response in test groups. In addition, histological analysis of PAC-exposed red drum larvae 10-12 days post-hatch showed significant reductions in retinal, epithelial, and lens diameters relative to a control group. In another study involving red drum embryos, Khursigara et al. (2017) found craniofacial deformity was as sensitive as pericardial edema to high energy WAF exposure. For zebrafish (*Danio rerio*), embryos exposed to phenanthrene showed a decrease in eye size, response to light, and reduced retinal cell proliferation relative to a control group. However, there were no significant differences observed in body shape or head size of the test group, suggesting the existence of compound and site-specific mechanisms of ocular toxicity analogous to those of cardiotoxicity (Huang et al., 2013). PAC exposure also affects embryonic skeletal development, leading to permanent spinal curvature, reduction in jaw size, and overall reduced growth (Barron, Carls, et al., 2004; Incardona et al., 2004). In some instances, effects of exposure are even seen in subsequent generations of fish. In a study on the heritable effects of PAC exposure, a population of adult (F0) zebrafish were exposed to benzo[a]pyrene and then bred to rear a second generation population (F1). This process was then repeated to rear second, third and fourth (F2, F3, F4) generations from the initial benzo[a]pyrene-exposed population. While increased mortality rates were observed in the F1 generation, no increase was found for subsequent generations. Body morphology differences were greatest in the F1 population and absent by the F3 generation, whereas craniofacial deformation was most significant in the F2 generation. Subsequent generations also showed higher incidences of abnormal body shape and reduced time to hatch, further demonstrating the heritability of PAC-induced toxic effects (Corrales et al., 2014). Bautista & Burggren (2019) provided further insight into transgenerational effects of PAC exposure, showing inherited phenotypes can be both adaptive and maladaptive. Following exposure of F0 adult zebrafish to high energy WAF dietarily, the F1 larvae of exposed adults were observed to have significantly decreased survival in clean water compared to F1 larvae of non-exposed adults. However, when reared in water spiked with high energy WAF, the F1 larvae of oil-exposed parents demonstrated significantly enhanced survival.

No discourse on PAC toxicity to embryonic fish is complete without addressing photo-enhanced toxicity, which was recently reviewed by Barron (2017). Arising from interactions of certain PACs with UV light *in vivo*, embryonic and larval fish are particularly susceptible as their small size and translucent bodies expose a relatively large amount of tissue to potential phototoxic effects. Phototoxicity is believed to act primarily through photosensitization in which incident (chiefly UVA) light first promotes a bioaccumulated PAC into an excited electronic state. Then, in the process of relaxing to its ground state, interactions of the PAC with oxygen or other biomolecules ultimately create reactive oxygen species and free radicals which are able to damage surrounding tissues by oxidizing cell structures. Again, this process occurs entirely *in vivo* with little or no enhanced toxicity occurring as a result of PAC transformations in the environment prior to assimilation by fish. With sufficient duration and intensity of UV exposure, death can result and the intensity of UV light required for photosensitization is found in natural waters (Barron, 2017; Willis & Oris, 2014). Photomodification is a second mechanism of phototoxicity in which parent PACs are photooxidized to toxic products, such as alcohols, ketones, and quinones. Products of PAC photooxidation can exhibit greater toxicity than the parent compound, though photooxidation can also lower phototoxicity through degradation of phototoxic PACs (Choi & Oris, 2003; Ge et al., 2016; Kang et al., 2019). While photomodification is an important aspect of phototoxicity, its importance is generally considered secondary to photosensitization (Barron, 2017). Overall, the toxicity of petroleum is increased two to 100+ fold by photoenhanced toxicity. Specific three to five ring PACs are believed to be responsible for phototoxicity; single PAC exposures have identified anthracene and pyrene as being phototoxic, with phenanthrene much less so (Willis & Oris, 2014). With toxicity arising from water column exposures well-established, recent work has provided the first demonstration of the photoenhanced toxicity of oiled sediment. In this study, zebrafish larvae exposed to weathered oil-contaminated sediment and 3.5 hours of unattenuated natural sunlight incurred a 100% mortality rate, compared to a 0% mortality rate among fish receiving no sunlight or 10% sunlight (Barron et al., 2018).

1.4 Analytic methods for biliary metabolites

The efficient biotransformation of many PACs by fish and their subsequent excretion into bile has allowed biliary PAC metabolite analysis to become an essential technique for determining

fish exposure. The necessity of the method is bolstered by the exceptionally fast rate at which parent PACs are metabolized, which can make tissue burden analysis unreliable for assessing PAC exposure (Budzinski et al., 2004). Moreover, as the body of literature demonstrating PAC toxicity has grown, so too has the urgency in assessing fish exposure. Consequently, biliary PAC metabolite analysis has flourished, based on an ability to determine immediate exposures and biological clearance rates. Biliary metabolite analysis represents the best method for assessing recent or ongoing exposure to PACs in fish and measured concentrations are important biomarkers in studies of fish health. Many quantification techniques for biliary metabolites are well established, with work in the field dating back more than forty years (Krahn et al., 1980). Fixed wavelength fluorescence (FF), synchronous fluorescence scanning (SFS), high-performance liquid chromatography with fluorescence detection (HPLC/F), gas chromatography coupled with mass spectrometry (GC/MS), and high-performance liquid chromatography with mass spectrometry (HPLC/MS) are most commonly employed. These techniques vary in time, equipment, and reagent demands; high specificity can be achieved, but at the cost of increased sample preparation requirements and analysis time. These methods are often used complimentarily, with a simpler screening technique used to determine which samples are fit for a more rigorous, time consuming, and costly analysis.

1.4.1 Semi-quantitative methods

The conjugated pi-bonds present in polycyclic aromatic rings of PACs give rise to fluorescence, with increased conjugation (i.e. more rings), leading to a higher wavelength of light required for excitation (Rivera-Figueroa et al., 2004). Fixed wavelength fluorescence, synchronous fluorescence scanning, and high-performance liquid chromatography with fluorescence detection each take advantage of this property. The excitation and emission wavelengths of a fluorometer can be optimized to detect compounds based on the number of aromatic rings they contain, which can then be used to estimate the number of 2-3 ring, 3-4 ring, and 4-5 ring compounds in a sample. Measurements based off fluorescence do not require any deconjugation of Phase II metabolites, as research indicates a minimal difference in the wavelength of maximum excitation for a Phase I metabolite compared to its corresponding Phase II metabolite, though Phase II metabolites usually fluoresce more intensely (Beyer et al., 2010; Pampanin et al., 2016; Singh & Wiebel, 1979). These analyses are quite straightforward with dilution the only sample

preparation required – typically with methanol or ethanol in a 50:50 mixture with water – to minimize matrix effects from other compounds in the bile such as pigments (Aas et al., 2000; Beyer et al., 2010). Synchronous fluorescence scanning was developed by Ariese et al (1993). In SFS, both the excitation and emission wavelengths are scanned through a range of wavelengths (in the ultraviolet and visible spectrum) with the difference in wavelength between emission and excitation held constant. This wavelength difference can be optimized for the compounds being studied, with 42 nm frequently used (Beyer et al., 2010). In this method, semi-quantitation occurs by comparing the signal from bile at a given wavelength with that of a pure standard. The concentration of the target analyte in the sample is then reported as equivalents of the given standard and a correction can be applied to compensate for differences in signal strength between conjugated metabolites and hydroxylated or pure standards. Conversion is accomplished by comparing the concentration found in the semi-quantitative analysis to that found in quantitative analysis of an identical sample (Tairova et al., 2012). Fixed wavelength fluorescence was introduced in 1996 by Lin et al. and is the quickest and simplest method for detection of PAC metabolites in bile. Following dilution of bile with 50% methanol or ethanol and water, the sample is scanned at fixed excitation/emission wavelength pairs on a fluorometer with different pairs having a maximal response for different sized PACs. The wavelength pairs employed are typically 290/335 nm for 2/3-ring PACs, 341/383 nm for 4-ring PACs, and 380/430 nm for 5/6-ring PACs (Beyer et al., 2010). As in SFS, signal strength from a sample is compared to that of pure or hydroxylated standards and concentrations are reported in equivalences. Research suggests FF cannot reliably be related to q-HPLC/F values with a universal conversion factor (Szlinder-Richert et al., 2014). Although these methods are undoubtedly useful as screening techniques, researchers should be cautious about drawing conclusions based solely on their use. For example, overestimation of PAC concentrations in Atlantic hagfish due to interfering fluorescent compounds in bile has been observed (Sundt et al., 2011). Conversely, FF/SFS have been found to drastically underestimate the concentrations of dihydrodiol metabolites in bile, which is problematic given these compounds are the most abundant metabolite for certain PACs in several species of fish (Pampanin et al., 2016).

Another more rigorous semi-quantitative method for measuring biliary PACs employs high performance liquid chromatography in conjunction with a fluorescence detector (HPLC/F). As in FF/SFS, no hydrolysis step is required and bile is analyzed directly (Krahn et al., 1980, 1984; Pulster, Gracia, Armenteros, et al., 2020). In the analysis, bile is directly injected onto a reverse-phase column with an initial 100% water mobile phase that progresses linearly to 100% methanol or acetonitrile (Krahn, Rhodes, et al., 1986; Leonard & Hellou, 2001; Pulster, Gracia, Armenteros, et al., 2020). Like SFS and FF, the detector (or a series of detectors) is programmed to measure different wavelength pairs depending on the number of rings in the analyte(s) being targeted (Krahn, Moore, et al., 1986; Snyder et al., 2019). Analysis time is roughly 30 minutes per sample with the increased time over SFS and FF affording additional information. The fingerprint of peaks in the chromatograms can help pinpoint the source of PAC exposure; fish exposed to petrogenic PAC sources (which contain larger amounts of alkylated compounds) will have chromatograms that are distinct from those exposed to urban PAC sources (Beyer et al., 2010; Krahn et al., 1993). Quantitation in the HPLC/F method occurs by summing the area of all peaks eluting after approximately seven minutes and comparing these responses to PAC or hydroxylated PAC reference standards (Beyer et al., 2010; Krahn et al., 1993; Snyder et al., 2015). As in SFS and FF, concentrations are reported as equivalents of the standard used.

There are many studies comparing semi-quantitative analytic techniques to more selective and quantitative methods. For instance, semi-quantitative HPLC/F concentrations correlate quite well ($r=0.93$, $p<0.0001$) with fully quantitative gas chromatography methods (Krahn et al., 1992). Similarly, there is research comparing SFS and FF to quantitative (q) HPLC/F (to be discussed below). Studies contrasting q-HPLC/F and FF concentrations found correlations ranging from $r^2=0.639$-0.942 for pyrene-like metabolites, $r^2=0.981$-0.990 for benzo[a]pyrene-type metabolites, and $r^2=0.894$-0.997 for naphthalene-type metabolite concentrations, with correlations dependent on the species of fish being studied (Lin et al., 1996; Ruczyńska et al., 2011; Vuontisjärvi et al., 2004). A comparison of SFS to q-HPLC/F concentrations found that q-HPLC/F concentrations of pyrene-type metabolites were 93±17% of concentrations found by SFS (Ariese et al., 1993). Moreover, a correlation of $r^2=0.89$, $p<0.001$ was found between 1-hydroxypyrene equivalents determined by SFS and 1-hydroxypyrene concentrations determined by quantitative HPLC/F, though the relationship was not one-to-one (Tairova et al., 2012). Strong correlations between PAC metabolite concentrations found by GC-MS and SFS are also

reported (Sturve et al., 2014). Semi-quantitative methods are often used in tandem with a quantitative method, as many studies involving biliary metabolite quantitation require large numbers of samples to be analyzed. The majority of the samples can be quickly analyzed and screened using the semi-quantitative techniques, with a subset undergoing verification using the more rigorous quantitative methods described below.

1.4.2 Quantitative methods

While quantitative methods are more demanding than those described above, they offer much better selectivity. In these methods, specific analytes are quantified, as opposed to the multitude of compounds that contribute to the measured signal in the semi-quantitative methods. Of the techniques available for quantitation of individual hydroxy metabolites, the two most common are gas chromatography-mass spectrometry (GC/MS) and HPLC/F.

Unlike the semiquantitative methods, quantitative analyses typically begin with a deconjugation step. Standards containing glucuronides and sulfates are often unavailable, rendering accurate quantitation of these different species impossible. Deconjugation of phase II metabolites is typically achieved through enzymatic preparations. Specifically, β-glucuronidase containing arylsulfatase is added to the bile and incubated for a short time at an optimized temperature (typically two hours at 37 °C; Beyer et al., 2010). In q-HPLC/F methods, following deconjugation, the now hydrolyzed samples are typically diluted with ethanol or methanol to denature added enzymes, then centrifuged or membrane filtered and injected onto the HPLC column. Acetonitrile/water gradients with a small amount of trifluoroacetic acid or ammonium acetate are most commonly used for the separation which typically employs a reverse phase column (Ariese et al., 2005; Beyer et al., 2010; Kammann et al., 2013). If only one analyte is being targeted, the fluorescence detector is set to an optimized wavelength pair; if several analytes are to be quantified the detector shifts to different wavelength pairs as the elution proceeds (Jonsson et al., 2003). Quantitation for q-HPLC/F is typically accomplished through external calibration (Ariese et al., 2005; Kammann et al., 2013). In contrast to the sq-HPLC/F method, in the q-HPLC/F method, only individual peaks corresponding to the analytes of interest are summed and the concentration of that specific metabolite is reported. Whereas samples are often analyzed immediately after hydrolysis in q-HPLC/F methods, in GC/MS samples typically undergo derivatization (into trimethylsilyl ethers commonly) prior to analysis as the polarity of

the hydroxy groups can be problematic for the GC/MS. Similarly, the derivatization also helps prevent the degradation of dihydrodiols in the analysis (Beyer et al., 2010; Jacob & Grimmer, 1987). Although, internal standards are sometimes used in q-HPLC/F analyses, their use is more necessary in GC/MS due to the volatile solvents and extensive sample preparation. These internal standards may be added before or after hydrolysis (Beyer et al., 2010; Kammann et al., 2013). Following derivatization, samples are analyzed by GC/MS. In both q-HPLC/F and GC/MS, peak identification may be aided by standard addition (Ariese et al., 2005; Jonsson et al., 2003).

Interlaboratory studies of identical bile samples prove useful in contrasting the strengths of q-HPLC/F and GC/MS. In the early 2000s a collaborative effort utilizing both analyses created fish bile reference materials replete with certified concentrations of 2-hydroxynaphthalene, 1-hydroxyphenanthrene, 1-hydroxypyrene, and 3-hydroxybenzo[a]pyrene. In these analyses, GC-MS excelled at quantifying the smaller compounds like 2-hydroxynaphthalene and 1-hydroxyphenanthrene, while struggling with the larger 3-hydroxbenzo[a]pyrene. Conversely, q-HPLC/F was unable to quantify 1-hydroxynaphthalene, largely due to peak overlap, but was superior in measuring larger compounds such as 3-hydroxybenzo[a]pyrene (Ariese et al., 2005; Jonsson et al., 2003). Another interlaboratory comparison performed in the early 2010s quantifying 1-hydroxypyrene in fish bile found q-HPLC/F, GC/MS, and SFS (with a conversion factor applied) yielded comparable results (Kammann et al., 2013). In general GC/MS is more expensive and labour-intensive than q-HPLC/F, but is preferred for analysis of smaller, more volatile PACs (Beyer et al., 2010). Unlike q-HPLC/F, GC/MS is also able to quantify metabolites of alkyl-PACs (Jonsson et al., 2004).

High performance liquid chromatography with mass spectrometry (HPLC/MS) is another technique for quantifying PAC metabolites in fish bile. The instrumentation was noted as having a great potential for analysis in a 2010 review by Beyer et al. but its use, while growing, remains limited. The instrumentation was first shown to be amenable to analysis of hydroxylated PACs in 1994 (Galceran & Moyano). Subsequently, HPLC/MS/MS, ultra high-performance LC/MS, and HPLC/MS have each been used on fish bile, probing a suite of phase I and II metabolites of individual PACs (Ikenaka et al., 2013; Sette et al., 2013; Willett et al., 2000; Zhu et al., 2008). HPLC/MS/MS has also begun to find use in environmental monitoring studies, quantifying a

suite of PAC metabolites in fish bile (Pheiffer et al., 2018). Instances of HPLC/MS use for biliary analysis can be expected to increase in the coming years.

Quantitation of bile pigments has become a mandatory step in most modern PAC biliary analyses. When a fish feeds, its gall bladder is first emptied into the digestive tract and subsequently filled with dilute, watery bile. The gall bladder then begins accumulating PAC metabolites anew as they are processed by the liver, changing the density and volume of the bile until the cycle resets with the next feeding (Ariese et al., 1997; Collier & Varanasi, 1991). Consequently, feeding status can have a strong influence on the concentration of metabolites detected in a sample. To counteract this effect, quantitation of the bile pigment biliverdin has become commonplace (assays for protein content have also seen use; Couderc et al., 2015), against which PAC concentrations can be normalized. Biliverdin analysis is accomplished by diluting bile 100 times in a 50/50 mixture of ethanol/water and determining absorbance at 380 nm. Variations involving other wavelengths and dilution factors are also employed (Ariese et al., 1997; Nagel, Kammann, et al., 2012; Richardson et al., 2004). The method's goal is to reduce variation, but it has had mixed results. In some instances the technique has reduced variability in the reported data set (Kammann, 2007; Nagel, Wagner, et al., 2012; Ruddock et al., 2003), in others it has not (Pathiratne et al., 2010; Vethaak et al., 2016; Vuorinen et al., 2006). Thus, measurement of bile pigment concentration continues to be recommended as part of sampling regimes, but decisions on whether to use the normalized or non-normalized data must be made situationally. Such normalization is particularly important in situations where significant differences in feeding status between different fish are known to exist.

1.5 Environmental occurrence and monitoring

A growing awareness and appreciation for ecosystem health, combined with the pervasiveness of PACs in the environment, has led to increasing use of biliary PAC metabolite concentrations to assess exposure. This is aided by accessible semi-quantitative analyses, which are done without prohibitively expensive instrumentation and highly specialized analysts. As a result, quantification of biliary PAC metabolites has been embraced globally. Some studies focus solely on PAC metabolites, while in others they are among a suite of biomarkers measured to assess overall fish health. The most common motivation for biliary concentration research is to establish baseline concentrations and monitor exposure metrics. Many of these studies focus on

waterbodies subject to anthropogenic pollution from urban, industrial, and/or shipping traffic sources, though some focus on relatively unimpacted, pristine regions. Another important motivation for studies involving PAC biliary metabolite concentrations is assessment of fish exposure in response to known, acute point-source PAC inputs to their habitat – typically from oil or fuel spills. In these situations, biliary PAC metabolite concentration data provides vital information about the severity of PAC exposure to fish resulting from the spill, as well as its geographic scope. Subsequent recovery monitoring then tracks the return of biliary PAC metabolite concentrations to baseline levels, which ideally are already known from routine environmental monitoring. This allows researchers to determine when resident fish are no longer experiencing elevated exposure to PACs and the affected area has been remediated.

Most commonly, baseline studies are undertaken in regions already experiencing moderate-to-intermediate levels of anthropogenic pollution. Others probe relatively pristine which may be expected to have increasing PAC inputs in the near future, such as the Arctic (Jörundsdóttir et al., 2014; Tomy et al., 2014). In Europe, international conventions and agreements – in tandem with expert collaborations – have led to a comprehensive and well-developed network for environmental marine monitoring for xenobiotics and their biological effects in the Northeast Atlantic. Biliary PAC metabolite concentration was selected as one of the core indicators and is currently recommended for use in environmental monitoring (Hylland et al., 2017). Based on previous work in the region, background assessment concentrations (BAC) and environmental assessment criteria (EAC) have been established for numerous species of fish in Europe (ICES, 2013; Table 1.1). The BAC is defined as the 90th percentile of concentrations measured in fish in a remote reference area, while the EAC is a threshold concentration below which no chronic effects are expected to occur. The EAC is derived from toxicological experiment data (Davies & Vethaak, 2012; Kammann et al., 2017). These benchmarks provide an important context for interpreting and evaluating concentrations reported in monitoring studies. Many recent publications have focused on the North Atlantic, with data for species having established EAC and BAC shown in Table 1.2. On the whole, a concentration gradient was observed from the more pristine Iceland waters and off-shore North Sea locales to higher concentrations in coastal areas (Kammann et al., 2017). Overall, the data regarding PAC exposure of fish in European

Fish Species	*1-phenanthrol* BAC[a] (ng/mL)	*1-phenanthrol* EAC[b] (ng/g)	*1-pyrenol* BAC[a] (ng/mL)	*1-pyrenol* EAC[b] (ng/g)	*Pyrene-type* BAC[c] (ng/mL)	*Pyrene-type* EAC[c] (ng/mL)
Common dab (*Limanda limanda*)	3.7	-	16	-	150	22 000
Atlantic cod (*Gadhus morhua*)	2.7	528[a]	21	483	1 100	35 000
European flounder (*Platichthys flesus*)	3.7	-	16	-	1 300	29 000
Haddock (*Melanogrammus aeglefinus*)	0.8	-	13	-	1 900	35 000
Turbot (*Scophthalmus maximus*)	-	1 832	-	909	-	29 000
Atlantic halibut (*Hippoglossus hippoglossus*)	-	262	-	745	-	22 000
Viviparous eelpout (*Platichthys flesus*)	7.9	-	92	-	-	-
Atlantic herring (*Clupea harengus*)	2.6	-	143	-	-	-

Table 1.1: Background assessment concentrations (BAC) and environmental assessment criteria (EAC) from ICES (2013). Concentrations measured using [a]quantitative-HPLC/F (ng/mL), [b]GC/MS, [c]SFS at 341/383 nm. Quantitative-HPLC/F and GC/MS analyses have been reported to give comparable results in PAC biliary analysis for the compounds of the table, thus thresholds determined from one of these methods should be valid for the other (Kammann et al., 2017). Reproduced with permission from Dearnley et al. (2020).

marine waters is encouraging as they pertain to EAC levels, though many sites remain above background concentrations. Kammann et al. noted (2017) that only 7 fish in their study exceeded the EAC set for cod and none exceeded the EAC for halibut. Similarly all dab in Irish waters and cod in the North Atlantic fell below their respective EAC, while no flounder in the Baltic Sea exceeded any of the available EAC (Dabrowska et al., 2017; Giltrap et al., 2017; Karl et al., 2016). In a study of temporal trends for dab and flounder in Dutch marine waters, only 9.3% of the individual flounder (tested between 1998-2005) and 0.6% of dab (tested between 1996-2012) exceeded the EAC limit for halibut – a species cautiously noted to be apt for comparison (Vethaak et al., 2016). Encouragingly, 1-hydroxypyrene concentrations were generally found to be decreasing for the duration of that study. Eelpout in Scandinavia were found to have average concentrations well above their BAC indicating exposure to PAC pollution (Asker et al., 2016). The species has also been studied in Estonia and Denmark (Kreitsberg et al., 2012; Tairova et al., 2012). Overall, the European monitoring regime best realizes the utility and power of biliary PAC analysis. The EAC levels are invaluable in immediately connecting environmental concentrations to toxicological effects, thereby alerting researchers and policy makers to untenable, damaging levels of PAC pollution and exposure. Similarly, in the case of any petrochemical spills or discharges, little uncertainty is left as to the background PAC levels in affected fish. Moreover, the duration for which metabolite levels have been monitored in the Northeast Atlantic allows for easy, expeditious, reliable evaluation of the efficacy of any efforts to mitigate pollution. While PAC concentrations can be measured in water, sediment, and even lower trophic biota, then extrapolated to estimate exposure to fish, direct measurement of bile is the best and easiest way to determine exposure.

Year Collected	Location	Species	Method	1-phenanthrol (ng/ml)	1-pyrenol (ng/mL)	Pyrene-type (ng/mL)	Reference
2006/2012	Barents Sea	Atlantic cod (*Gadus morhua*)	q-HPLC/F		8.06 (<4.5-29.67)		(Karl et al., 2016)
2011	Greenland				<2.25 (<2.25-9.6)		
2006/2012	North Norway				9.93 (<4.5-27.29)		
2007	North Sea				37.39 (<4.5-123.69)		
2012	South Norway				6.61 (<4.5-93.36)		
2006	Western Baltic Sea				156.55 (31.3-1054.5)		
2008	Iceland southeast	Common dab (*Limanda limanda)*	q-HPLC/F	1.88 (<0.10-3.45)	7.64 (<4.50-13.72)		(Kammann et al., 2017)
2008	Iceland west			0.65(<0.10-3.66)	9.69 (<4.50-24.41)		
2008	North Sea off-shore southeast			1.45 (<0.10-2.86)	43.55 (20.75-92.58)		
2008	North Sea off-shore Denmark			3.45 (1.77-5.48)	39.53 (15.65-66.26)		
2008	North Sea off-shore west			6.11 (<0.10-39.87)	26.39 (10.81-59.92)		
2008	North Sea off-shore UK			<0.10 (<0.10-<0.33)	13.93 (<4.50-25.82)		
2008	German Bight south			2.38 (2.04-2.72)	88.48 (42.78-134.18)		
2008	German Bight north			5.83 (0.59-24.32)	83.21 (12.75-430.33)		
2008	Western Baltic			4.32 (2.71-7.45)	115.06 (81.61-175.56)		
2008	France Seine Estuary			2.31 (0.91-3.36)	345.38 (272.57-416.59)		
2005	Doggerbank, North Sea				38 (9-89)		(Vethaak et al., 2016)
2005	North Sea				134 (74-235)		
2005	North Sea				68 (23-214)		
2010	Dublin Bay, Ireland autumn		SFS			244	(Giltrap et al., 2017)
2010	Dublin Bay, Ireland summer					1181	
2010	Shannon, Ireland					181	
2010	Wexford, Ireland					330	
2010	Cork Harbour, Ireland					328	
2008	Iceland west		q-HPLC/F	0.38 (<0.10-0.68)	18.36 (15.21-21.51)		

2008	North Sea Dutch Coast	European flounder (*Platichthys flesus*)		1.96 (0.55-4.15)	76.99 (44.48-148.36)		(Kammann et al., 2017)
2008	Western Baltic			7.93 (2.71-33.77)	209.21 (77.08-653.72)		
2008	France Seine Estuary			7.49 (1.95-13.10)	316.49 (158.43-587.03)		
2008	UK Alde river			22.46 (8.21-56.02)	373.24 (207.65-653.87)		
2009	Eastern Scheldt, Netherlands				99 (35-315)		(Vethaak et al., 2016)
2012	Western Scheldt, Netherlands				157 (58-321)		
2012	North Sea coast, Netherlands				45 (13-63)		
2011	Waddenzee, Netherlands				85 (43-228)		
2012	Ems-Dollard, Netherlands				264 (104-568)		
2009	Gulf of Gdańsk east, Baltic Sea				47 (41-73)		(Dabrowska et al., 2017)
2009	Gulf of Gdańsk west, Baltic Sea				58 (41-104)		
2009	Baltic Sea				49 (31-71)		
2009	Baltic Sea				32 (25-43)		
2008	Iceland southeast	Haddock (*Melanogrammus aeglefinus*)	q-HPLC/F	<0.33 (<0.10-1.58)	<4.50 (<0.68-18.58)		(Kammann et al., 2017)
2008	Iceland west			<0.33(<0.10-1.99)	6.63 (<4.50-20.32)		
2008	North Sea off-shore			<0.33 (<0.10-0.65)	6.43 (<4.50-9.16)		
2009	Sweden west coast	Viviparous eelpout (*Platichthys flesus*)	q-HPLC/F		501		(Asker et al., 2016)
2009	Denmark				512		
2009	Germany				137		
2003	Aspholmamal, Sweden		GC/MS SFS	2400[a]	9600[a]		(Sturve et al., 2014)
2008	Vejle Fjord, Denmark					2120	(Tairova et al., 2012)
2008	Aarhus Bight, Denmark					1200	

Table 1.2: Mean concentrations of biliary PAC metabolite concentrations for fish species with determined BAC and/or EAC. Range shown in parentheses when reported. Additional information such as standard deviations, medians, additional sampling sites and/or time points, or sex-specific data, may be available from papers cited. Reproduced with permission from Dearnley et al. (2020). [a]Sum of all monohydroxylated forms of the given C0 PAC and in ng/g

Work in Europe, and the rest of the world, has not been limited to the previously discussed species. While no BAC nor EAC values exist for many organisms, their study can still lead to valuable insight. One species subject to recent scrutiny is the European eel (*Anguilla anguilla*). A collapse in the stocks of their "glass eel" early life-stage prompted work quantifying their biliary PAC metabolite concentrations, along with other persistent organic pollutants, which may reduce their reproductive success. The species is currently listed as critically endangered (Jacoby & Gollock, 2014). Biliary metabolite concentrations in the European eels from a wide variety of regions and different life stages are displayed in Table 1.3. In the yellow stage, the eel is resident in coastal, estuarine, and/or inland waters and is actively feeding to fuel growth, while in the silver stage, the fish is migratory and feeding ceases (Durif et al., 2005; Kammann et al., 2014). Thus, biliary PAC concentrations in the yellow stage are indicative of local contamination, while in silver eels are representative of a larger catchment area (Szlinder-Richert et al., 2014). The vast range in which the eel has been studied allows for broad geographic comparisons, and the concentrations can indicate potentially dangerous levels of exposure. While PAC levels had never been tested in Moroccan fish prior to the eel studies (Wariaghli et al., 2015), comparison of their concentrations with those in Europe suggest the Moroccan waters are relatively pristine. The utility of PAC biliary metabolite measurement in evaluating PAC exposure is demonstrated by Nagel, Kammann, et al. (2012), who noted eels from the Elbe and Oder were only moderately contaminated, despite being caught in major shipping routes and/or in the immediate vicinity of urban centers. Lastly, the unique life stages of the eel have made it an ideal candidate for normalization of reported PAC concentrations to bile pigments. While this normalization did not reduce variation among eels of the same sex and life stage (Nagel, Kammann, et al., 2012), it did greatly reduce influence of life stage on measured concentrations (Nagel, Wagner, et al., 2012).

Outside of Northwest Europe, instances of biliary metabolite analysis continue to grow as an important metric of fish health and a convenient indicator of PAC pollutions. Many of these assessments are the first of their kind in their respective regions or represent the first baseline measurements of a resident species. Incidentally, this geographic expansion has led to an increased number of species in which biliary metabolites have been quantified. While this complicates comparison of PAC exposure levels to other regions of the world – due to

Year Collected	Location	Life Stage	1-phenanthrol (ng/ml)	1-pyrenol (ng/mL)	Reference
2011	Vistula Lagoon, Poland	Yellow	64.0 (28-320)	57.8 (3-378)	(Szlinder-Richert et al., 2014)[a]
2011	Szczecin Lagoon, Poland	Yellow	63 (13-176)	11 (3-152)	
2011	Puck Bay, Poland	Silver	929 (219-2561)	1642 (10-5364)	
2011	Lake Sniardwy, Poland	Yellow	83 (30-382)	99 (13-337)	
2009	Ems River, Germany	Yellow	52.7 ± 23.5	394.5 ± 225.0	(Nagel et al., 2012)
2009	Havel River, Germany	Yellow	82.7 ± 40.3	305.6 ± 147.9	
2009	Trave River, Germany	Yellow	632.4 ± 178.9	2420.8 ± 638.2	
2009	Warnow River, Germany	Yellow	438.0 ± 391.2	655.5 ± 514.8	
2009	Peene River, Germany	Yellow	278.0 ± 96.8	415.5 ± 167.5	
2009	Uecker River, Germany	Yellow	155.8 ± 74.9	110.9 ± 52.4	
2009	Oder River, Germany	Yellow	299.4 ± 85.7	651.6 ± 258.7	
2011	Eider River, Germany	Yellow	222	929	(Kammann et al., 2014)[b]
2011	Eider River, Germany	Silver	367	1118	
2011	Elbe River, Germany	Yellow	156	619	
2011	Elbe River, Germany	Silver	318	1602	
2011	Rhine River, Germany	Silver	587	3124	
2011	Schlei/Trave Catchment, Germany	Silver	445	2317	
2011	Weser River, Germany	Silver	210	323	
2012	Varades, France	Yellow	4.1 (1.1-7.7)	60.7 (30.7-148.3)	(Couderc et al., 2015)
2012	Bellevue, France	Yellow	4.3 (0.3-13.2)	27.8 (23.1-34.3)	
2012	Hautre Indre, France	Yellow	3.6 (0.4-6.7)	25.5 (15.5-41.0)	
2011	Loire Estuary, France	Silver	38.0 (21.8-53.3)	100.5 (62.2-156.5)	
2014/2015	Moulay Bousselham Lagoon, Morocco	N/A	6.97(5.23-9.62)	8.43 (7.34-15.56)	(Baali et al., 2016)
2009	Sebou upriver, Morocco	Yellow	61 (18-152)	52 (12-221)	
2009	Sebou downriver, Morocco	Yellow	65 (29-183)	154 (59-545)	(Wariaghli et al., 2015)
2009	Loukkos, Morocco	Yellow	73 (10-305)	210 (56-453)	

Table 1.3: Mean concentrations of biliary PAC metabolite concentrations in European eel (*Anguilla Anguilla*) as measured by quantitative-HPLC/F. Range (in parentheses) or standard deviation (±) is given when available, with range preferred if both are reported. Additional information such as standard deviations, medians, additional sampling sites and/or time points, or sex-specific data may be available from papers cited. Reproduced with permission from Dearnley et al. (2020). [a]Median reported. [b]Yellow life stage data calculated from eels in silvering stage I and II, silver life stage data calculated from eels in silvering stage

species-species differences – local reference sites serve important roles in interpreting results. A selection of measured concentrations are displayed in Table 1.4, with the studies of Morocco, Zanzibar, and South Africa being the first analyses of fish bile in their regions, respectively (Juma et al., 2018; Pheiffer et al., 2018; Wariaghli et al., 2015). In general, the concentrations found in Table 1.4 are well below any of the European EAC and many are below the BAC. Though no definitive conclusions on fish health or contamination can be drawn from this comparison – again due to species-species differences – it suggests PAC exposure to fish in these regions is not currently of major concern. Notable exceptions to this were seen in the Three

Year Collected	Location	Fish	Method	1-phenanthrol (ng/ml)[a]	1-pyrenol (ng/mL)[a]	Pyrene-type (ng/mL)	Reference
2014-15	Moulay Bousselham Lagoon, Morocco	Moray (*Muraenidae*)	q-HPLC/F	0.37 (<LOD-1.14)	2.97 (0.65-13.28)		(Baali et al., 2016)
2014-15	Boujdor, Morocco	Conger eel (*Conger conger*)	q-HPLC/F	0.03 (<LOD-0.35)	0.5 (<LOD-1.63)		(Baali et al., 2016)
2013	Fleurhof Dam, Klip River, RSA	African sharptooth catfish (*Clarias gariepinus*)	HPLC/MS		55 (0-116)		(Pheiffer et al., 2018)
2013	Orlando Dam, Klip River, RSA				<LOD		
2013	Lenasia Dam, Klip River, RSA				86 (26-146)		
2012	Daning River, Wuhan, China	Darkbarbel catfish (*Pelteobagrus vachellii*)	q-HPLC/F		2428 ± 871		(Floehr et al., 2015)
2012	Baijiaxi River, China (Reference)				328 ± 201		
2012	Pengxi River/Xiao River, Yunyang, China				211 ± 88		
2012	Jiang River, Chongqing, China				1120 ± 296		
2012	Long River, Fengdu, China				1211 ± 460		
2012	Hanfeng Lake, Kaxian, China				1562 ± 212		
2009	Catalan Slope, Spain	Risso's smooth-head (*Alepocephalus rostratus*)	GC/MS		0.6 (<LOD-4.6)		(Koenig et al., 2013)
2009	Western Mediterranean, Spain				<LOD		
2012	Ripoll River, Spain	Ebro chub (*Squalius laietanus*)	GC/MS		34 ± 5		(Blanco et al., 2019)
2012	Ripoll River, Spain	Mediterranean barbel (*Barbus meridionalis*)	GC/MS		56 ± 10		(Blanco et al., 2019)
2013	Ebro River, Spain	Common roach (*Rutilus rutilus*)	GC/MS		19		(Blanco et al., 2018)
2008	Urdaibai estuary, Spain	Thicklip grey mullet (*Chelon labrosus*)	GC/MS		135		

2008	Arriluze, Spain				1850		(Puy-Azurmendi et al., 2010)
2001-02	Hinlopen, Arctic Ocean	Polar cod (*Boreogadus saida*)	GC/MS	27 ± 9	48 ± 16		(Jonsson et al., 2010)
2001-02	Isfjorden, Arctic Ocean			19 ± 10	56 ± 18		
2011	Athabasca and Slave River, Canada	Burbot (*Lota lota*)	SFS			1400 ± 700[b]	(Ohiozebau et al., 2016)
2011	Athabasca and Slave River, Canada	Goldeye (*Hiodon alosoides*)	SFS			2300 ± 700[b]	(Ohiozebau et al., 2016)
2011	Athabasca and Slave River, Canada	Jackfish/Northern pike (*Esox luscius*)	SFS			900 ± 400[b]	(Ohiozebau et al., 2016)
2011	Athabasca and Slave River, Canada	Walleye (*Sander vitreus*)	SFS			900 ± 400[b]	(Ohiozebau et al., 2016)
2011	Athabasca and Slave River, Canada	Whitefish (*Coregonus clupeaformis*)	SFS			1800 ± 1200[b]	(Ohiozebau et al., 2016)
2007	Newark Bay, USA	Mummichog (*Fundulus heteroclitus*)	SFS			4570	(Bugel et al., 2010)
2015	Kinazini, Zanzibar	Goby fish (*Periophthalmus sobrinus*)	SFS			690	(Juma et al., 2018)
2015	Mbweni, Zanzibar					99	
2015	Bumbwini Makoba, Zanzibar					239	
2015	Donge Muwanda, Zanzibar					137	
2015	Zanzibar	Rabbit fish (*Siganus sutor*)	SFS			ND	(Juma et al., 2018)
2014	Site 1, Gulf, Kuwait	Fourlined terapon (*Pelates quadrilineatus*)	SFS			312.8 ± 108.1	(Al-Zaidan et al., 2015)
2014	Site 3, Gulf, Kuwait					417.2 ± 167.8	
2014	Site 1, Gulf, Kuwait	Giant sea catfish (*Arius thalassinus*)	SFS			185.3 ± 51.8	(Al-Zaidan et al., 2015)
2014	Site 2, Gulf, Kuwait					477.5 ± 290.7	

2014	Site 3, Gulf, Kuwait	129.0 ± 50.7
2014	Site 5, Gulf, Kuwait	163.2 ± 77.4

Table 1.4: Mean concentrations of biliary PAC metabolite concentrations in fish without existing EAC or BAC as analyzed by quantitative-HPLC/F, HPLC/MS, GC/MS, or synchronous fluorescence scanning. Range (in parentheses) or standard deviation (±) is given when available, with range preferred if both are reported. Additional information such as standard deviations, medians, additional sampling sites and/or time points, or sex-specific data may be available in papers cited. Reproduced with permission from Dearnley et al. (2020). [a]GC/MS results are ng/g bile (density is similar to water). [b]Chrysene-type

Gorges Reservoir watershed of China where elevated PAC levels led authors to conclude there may be concern for fish health (Floehr et al., 2015) and in Arriluze, Spain which was selected as a high-pollution reference site (Puy-Azurmendi et al., 2010). The potential pitfalls of cross-species comparison are illustrated by Juma et al. (2018) who quantified biliary PAC metabolites in Goby fish (*Periophthalmus sobrinus),* but found no measurable metabolites in rabbit fish (*Siganus sutor*) from the same region. Though both species feed on bottom dwelling organisms, goby fish spend more time buried in or in proximity to muddy sediments inside mangrove forests compared to rabbit fish who are commonly found in sea grass beds (Almeida et al., 1999; Murdy, 1989) and these differences in habitation may help explain the study's findings. Differences in metabolite concentrations between species is corroborated by de Albergaria-Barbosa et al. (2018) who, in studying Rake stardrum (*Stellifer rastrifer*), Whitemouth croaker (*Micropogonias furnieri*), Smalleye croaker (*Nebris microps*), and Checkered puffer (*Sphoeroides testudineus*) in Brazil, found significant differences in phenanthrene-type and benzo[a]pyrene-type metabolite concentrations between the species. A contrary incidence is found by Ohiozebau et al. (2016), who, in studying lake whitefish (*Coregonus clupeaformis*), northern pike (*Esox luscius*), walleye (*Sander vitreus*), goldeye (*Hiodon alosoides*), and burbot (*Lota lota*) in Canadian rivers, found no significant difference in biliary metabolite concentrations between the species. Each of these species are mostly piscivorous top predators which may explain the similarity in metabolite concentrations between them. Taken together, these findings further underline how the validity of cross-species comparisons can be difficult to determine *a priori*. This does not, however, mean homogeny of studied species should be universally striven for, as specific species can be used to investigate different parts of the ecosystem. For example, in one study the deep-sea Risso's smooth-head (*Alepocephalus rostratus*) was selected to assess exposure at oceanic depths up to 2000 m (Koenig et al., 2013). Lastly, unlike many locations in Table 1.4, the oil producing Arabian/Persian Gulf features both newly measured species in Giant sea catfish (*Arius thalassinus*) and Fourlined terapon (*Pelates quadrilineatus*; Al-Zaidan et al., 2015) and continued monitoring of yellowfin seabream (*Acanthopagrus latus*) which allowed researchers to demonstrate no significant increase in PAC exposure between 2005 and 2015 (Beg et al., 2018).

In many ways, the different techniques for measuring biliary metabolite concentrations can be thought of as different languages evolved from a common ancestor. Results from different

methods share many correlations but cannot truly "speak" to one another in the absence of conversion factors, as results are not one-to-one. Thus, measurements made with sq-HPLC/F – which is particularly popular in the Americas – cannot be compared meaningfully to threshold values developed for other methods. However, non-species specific threshold concentrations linking measured biliary concentrations to pollution severity have been cited for this method (de Albergaria-Barbosa et al., 2018; Fuchsman et al., 2001; Pinkney et al., 2001). These, along with results from recent published studies are shown in Table 1.5 and reveal medium-to-high PAC pollution in many locations assessed. Toxicity threshold concentrations identified for adverse effects (akin to EAC) using sq-HPLC/F are 2 μg phenanthrene-equivalents/mg biliary protein for juvenile salmonids and 5 μg phenanthrene-equivalents/mg biliary protein for Dolly varden (*Salvelinus malma;* Meador et al., 2008; Sol et al., 2000). The former was used to assess juvenile Chinook salmon (*Oncorhynchus tshawytscha*) in the Lower Columbia River estuary, USA and was exceeded by mean concentrations in four of the seven sites tested (Yanagida et al., 2012).

Fixed wave fluorescence (FF) is another method by which biliary PAC metabolite concentrations are assessed. For this method, no recently cited threshold values combined with a dearth of comparable contemporary data preclude its quantitative inclusion in this review. In some studies, no analytic standards are used and "concentrations" are reported in arbitrary units of fluorescence. Meaningful comparisons can still be made from this data between different sites tested in the given study, but the values remain insular with respect to other research as the arbitrary units are idiosyncratic to the instrument used. Others only provide data normalized to protein content. Nevertheless, a study of bluehead chub (*Nocomis leptocephalus*), creek chub (*Semotilus atromaculatus*), bluegill (*Lepomis macrochirus*), and redbreast sunfish (*Lepomis auritus*) in a southeastern USA river used FF to implicate PACs in biochemical effects on resident fish (van den Hurk & Haney, 2017), as did a study of Madamango sea catfish (*Cathorops spixii*) in Brazil (Gusso-Choueri et al., 2016). Fixed wave fluorescence has also been used in assessments of pollution and fish health in Yellowtail scad (*Trachurus novaezelandiae*), Skipjack trevally (*Pseudocaranx wrighti*), and Black bream (*Acanthopagrus butcheri*) in Australia (Duarte et al., 2017; Gagnon & Rawson, 2016); Lionfish (*Pterois volitans*)

Year Collected	Location	Fish	2-3 ring equivalents (µg/g)	3-4 ring equivalents (ng/g)	5-6 ring equivalents (ng/g)	Reference
	HIGH POLLUTION		500	50 000	1 000	(Pinkney et al., 2001)
	MODERATE POLLUTION		300	10 000	200	
2011	Northern Gulf of Mexico, USA	Red Snapper (*Lutjanus campechanus*)	120 (41-470)		280 (94-590)	(Snyder et al., 2015)
2012			61 (20-130)		220 (68-540)	
2013			51 (13-140)		380 (310-1 500)	
2012	Northern Gulf of Mexico, USA	Golden tilefish (*Lopholatilus chamaeleonticeps*)	240 (110-340)		170 (51-470)	(Snyder et al., 2015)
2013			220 (22-480)		370 (71-3 030)	
2012	Northern Gulf of Mexico, USA	King snake eel (*Ophichthus rex*)	38 (11-88)		260 (46-850)	(Snyder et al., 2015)
2013			24 (3.6-210)		160 (34-880)	
2009	Santos Estuary, Sao Paolo, Brazil	White mullet (*Mugil curema*)	82.4 (3.91-468)	8 740 (<120-60 800)	626 (150-5 120)	(de Albergaria-Barbosa et al., 2017)
2008	Cananéia Estuary, Brazil		9.52 (1.17-30.3)	1 090 (<120-3 700)	138 (<90-450)	
2005	Santos Bay, Brazil	Rake Stardrum (*Stellifer rastrifer*)	210	64 100	1 200	(de Albergaria-Barbosa et al., 2018)
2005	Santos Bay, Brazil	Whitemouth croaker (*Micropogonias furnieri*)	330	19 100	580	(de Albergaria-Barbosa et al., 2018)
2005	Santos Bay, Brazil	Smalleye croaker (*Nebris microps*)	230	15 700	290	(de Albergaria-Barbosa et al., 2018)
2005	Santos Bay, Brazil	Checkered puffer (*Sphoeroides testudineus*)	222	31 100	1 120	(de Albergaria-Barbosa et al., 2018)
2000-04	Hospital Beach, Canada	English sole (*Parophrys vetulus*)		34 000 ± 4 900	1 200 ± 230	(Johnson et al., 2015)

2000-04	Kitimat Arm, Canada	24 500	718
2000-04	Kitlope (Ref), Canada	24 000 ± 2 600	1 400 ± 280
2000-04	Kildala Arm (Ref), Canada	23 300 ± 2 600	600 ± 120

Table 1.5: Mean concentrations of biliary PAC metabolite concentrations in fish as measured by semi quantitative-HPLC/F. Range (in parentheses) or standard deviation (±) is given when available, with range preferred if both are reported. Additional information such as standard deviations, medians, additional sampling sites and/or time points, or sex-specific data may be available in papers cited. Reproduced with permission from Dearnley et al. (2020).

in the Florida Keys, USA (van den Hurk et al., 2020); and sole (*Solea solea*, *Solea senegalensis*) in the Mediterranean Sea (Solé et al., 2013). PAC metabolites have also been quantified in European chub (*Leuciscus cephalus L.*) in Czechia rivers (Blahova et al., 2013).

Polycyclic aromatic compound metabolite biliary analysis is perhaps most useful in the aftermath of oil and fuel spills. In these situations, metabolite concentrations can tell us how far toxic PACs have spread, which species are most affected, and provide an objective evaluation of the efficacy of remediation efforts and progress of natural dissipation. The largest of these events is the *Deepwater Horizon* blowout of 2010 in the Gulf of Mexico. Hampered by a lack of pre-spill background data, this prompted a multinational health survey of fish in the Gulf of Mexico, including PAC biliary metabolite quantitation of 1800 fish from 75 offshore species (Pulster, Gracia, Armenteros, et al., 2020; Pulster, Gracia, Snyder, et al., 2020; Struch et al., 2019). This data is publicly available through the Gulf of Mexico Research Initiative Information & Data Cooperative (Pulster et al., 2019). Between 2011 and 2015, twelve species tested demonstrated declines in total biliary PAC concentrations and the region of the Gulf nearest to the blowout maintained significantly higher concentrations than those further away (Pulster, Gracia, Snyder, et al., 2020). These trends are consistent with an episodic exposure from a point source (Snyder et al., 2015). The most extreme drop was seen in yellow conger eels (*Rhynchoconger flavus*), whose levels of total PAC metabolites dropped 90%. Conversely, for some species, biliary concentrations of metabolites increased in the years following the blowout. One species that followed this trend is Golden tilefish (*Lopholatilus chamaeleonticeps*), which was scrutinized particularly closely. These fish continued to experience significant increases in total biliary PACs from 230 μg/g in 2012 to 640 μg/g in 2017 indicating continued PAC exposure, even seven years after the incident. This has been attributed to its burrowing lifestyle, sediment ingestion, and benthic diet in tandem with resuspension of oil deposited on the seafloor (Snyder et al., 2015, 2019). The data set amassed in the wake of the *Deepwater Horizon* blowout illustrates the benefit and necessity of monitoring different species to assess PAC exposure in the aquatic environment. Selection of a diversity of fish species to study is a tool for effectively determining exposure from different parts of the environment and trophic levels and its absence can have disastrous effects. Had researchers chosen only to study yellow conger eels, for

example, they would likely have erroneously concluded residual PAC pollution from the released oil was no longer affecting the ecosystem.

While the *Deepwater Horizon* blowout is the most notable release of petroleum in recent memory, there has been no shortage of other smaller spills to attract researchers' attention and prompt concern for resident fish health. Three years after the *Deepwater Horizon* blowout, the Gulf of Mexico was afflicted by the burning of a marine gas rig. No correlation was observed between PAC metabolite concentrations in red snapper (*Lutjanus campechanus*) and distance to the rig, suggesting the incident had minimal impact on fish health (Romero et al., 2016). In South Korea, a 2007 tanker collision-caused crude oil spill led to studies of rockfish (*Sebastes schlegeli*) and marbled flounder (*Pseuodopleuronectes yokohamae*; Jung et al., 2011). Metabolite concentrations peaked after 5 days in both species, were halved by day 35, and steadily declined in the 11 months following the spill. A 2003 bunker oil spill in Sweden led to assessment of common eelpout in the region and underscores the utility of baseline studies (Sturve et al., 2014). Biomonitoring had occurred in the region just one month prior to incident and allowed researchers to definitively conclude baseline levels had been reached five months after the incident. Conversely, two weeks after the oil release, concentrations (Table 1.5) of hydroxylated phenanthrene and pyrene metabolites, respectively, exceeded all available EAC. Following a 2006 spill of approximately 40 tons of heavy fuel oil off the Estonian coast, PAC metabolite concentrations in European Flounder steadily declined over the following two years, ultimately ending up 82% lower than their post-spill peak (Kreitsberg et al., 2010).

Biliary concentrations have also been used to investigate specific PAC sources outside of oil spills. Offshore oil platforms represent potential PAC point-sources which is problematic given the large number of resident fish they harbour. Fortunately, a study of Pacific sanddab (*Citharichthys sordidus*), kelp rockfish (*Sebastes atrovirens*), and kelp bass (*Paralabrax clathratus*) living near such structures in California were found to have no measurable 1-hydroxypyrene (<5 ng/mL) and no elevated concentrations of other PACs compared to a reference site (Gale et al., 2012). In Norway, common minnow (*Phoxinus phoxinus*) living in sedimentation ponds were studied to assess PAC exposure from road runoff and had median biliary concentrations exceeding all EAC for 1-hydroxypyrene and 1-hydroxyphenanthrene

(Grung et al., 2016). Lastly, in British Columbia, Canada a remote smelter was identified as a potential source of PAC pollution, prompting assessment of English sole (*Parophrys vetulus*) for exposure. These fish had biliary PAC metabolite concentrations consistent with moderate-to-high pollution, with fish captured from the site nearest to the smelter (Hospital Beach) having higher concentrations than those from other sites within the inlet (Kitimat Arm). However, these fish were generally no more exposed to PACs than those from reference locations who harboured surprisingly high metabolite concentrations (Johnson et al., 2015).

The plethora of studies and environmental monitoring programs involving biliary PAC metabolite analysis provide important corollaries on how the technique can be most effectively used. In many sampling regimes, these practices are already in place. To best make reliable comparisons between different measured concentrations, especially for long-term environmental monitoring, consistency is critical. As it pertains to fish sampling this can take on many different facets, but reducing temporal variation is perhaps most important. Studies encompassing a variety of species of fish frequently find significant differences in biliary PAC metabolite concentrations across different climatic seasons (Asker et al., 2016; de Albergaria-Barbosa et al., 2017; Giltrap et al., 2017; Nahrgang et al., 2013; Ohiozebau et al., 2016; Tairova et al., 2012). Significant differences in concentrations between different sexes have also been observed (Snyder et al., 2015; Tairova et al., 2012), though such findings are not universal (de Albergaria-Barbosa et al., 2017; Giltrap et al., 2017; Kammann, 2007; Ohiozebau et al., 2016; Snyder et al., 2019; Solé et al., 2013). As previously discussed other factors like species, feeding status, and life stage each have the potential to influence metabolite concentrations. These variables must be considered when designing studies, collecting samples, and interpreting data.

While biliary metabolite concentrations represent the best approach for determining short-term exposure of fish to PACs, the method suffers some limitations and drawbacks which require additional consideration when planning studies and interpreting results. Few of these hindrances are insurmountable, though the requirement of fish to metabolize PACs and excrete the metabolites into the bile is not. Though this may seem trivial, an important implication is that it precludes early life stage fish from being subject to such analysis. For these organisms, PAC exposure is best quantified through body burdens of the PACs themselves. Other challenges can

arise from the interpretation of biliary metabolite concentrations, especially data originating from wild fish. Whenever PAC metabolite concentrations are measured in a sample, only the capture location of the fish is definitively known; neither the duration of exposure nor the precise location of exposure can be conclusively determined. This issue is of less concern for environmental monitoring of relatively large regions, but can be especially problematic when studying localized contamination (such as small oil spills or industrial point-sources) as the site fidelity of captured fish may not be assured. Such concerns can be mitigated by sampling species whose habitat preferences and ranges are well known. Ease of analysis is one of the major benefits of biliary PAC monitoring, but as these additional considerations demonstrate the concentrations found are only useful if the sampling is thoughtfully planned.

The European system of EAC thresholds for toxic effects, despite its many benefits, also presents its own limitations and cautions. One requirement of EAC, which have been developed for a variety of contaminants and their biomarkers, is that contaminant-related biological effects must be separable from influences caused by other factors (such as natural variation or feeding status; Davies & Vethaak, 2012; Vethaak et al., 2017). While this is largely true for biliary PAC metabolites in relation to PAC exposure, previously discussed extraneous factors have the potential to exert influence on biliary PAC concentrations. Thus, these variables can also affect the proportion of fish exceeding their EAC. This again reinforces the importance of consistent sampling and the role study design can play in minimizing the influence of some of these variables. For instance, the effect of feeding status can be mitigated by holding fish in tanks for several days upon capture (Davies & Vethaak, 2012). Moreover, research to ensure seasonal fluctuations are not too extreme is advisable. Researchers must also be aware that PAC pollution causing sub-EAC metabolite concentrations still has the potential to cause adverse health effects in fish. The EAC are developed based on laboratory exposure studies to PACs only, but PACs may act synergistically with other contaminants in natural environments and cause detrimental health effects below the EAC thresholds. As has been previously discussed, researchers must also be cautious in applying EAC concentrations to species outside of the one for which they were developed. While the EAC concentrations are not infallible, the establishment of these toxic thresholds still represent the field's most comprehensive attempt at linking PAC pollution

and exposure with biological effects in fish and are invaluable in allowing for quick interpretation of environmental monitoring data.

From their first use four decades ago to present day, biliary metabolite concentrations continue to be an essential tool in assessing short-term exposure of fish to PACs. They serve an important role in assessing urban pollution and are unparalleled in determining the severity, longevity, and geographic extent of PAC contamination in fish following oil and petroleum product spills and in evaluating subsequent recovery of the ecosystem. To this end, it is imperative that baseline testing be conducted in regions at high risk for such releases. The ease of analysis for biliary PAC metabolites render such endeavours elementary and in the event of PAC discharges this information proves invaluable. Beyond expanding monitoring efforts, future studies directed at establishing toxicity threshold concentrations for a wider variety of fish species would be a boon for environmental monitoring efforts. The EAC of Europe have proven their utility in assessing urban pollution, but their use is limited geographically due to habitat ranges. Expansion of threshold concentrations to a greater number of species would ease data interpretation and risk assessment efforts globally.

Lastly, while this review has focused on metabolite quantitation in bile, the future of monitoring short-term PAC exposure may lie in other matrices. As biological sciences continue to strive for non-lethal sampling methods, urine and/or feces (where bile is ultimately excreted) may become the default matrices for PAC metabolite quantitation. This shift would involve new challenges pertaining to sample acquisition, establishment of dose-dependent response, and potential normalization for metabolite dilution, though analytic methods have already been developed for PAC metabolites in fish urine (Kreitsberg et al., 2010; Tairova et al., 2012). While it is acknowledged that many studies featuring biliary quantitation involve other analyses that necessitate lethal sampling, any efforts to reduce research related mortality will be greatly appreciated by our piscine friends.

1.6 References

Aas, E., Beyer, J., & Goksoyr, A. (2000). Fixed wavelength fluorescence (FF) of bile as a monitoring tool for polyaromatic hydrocarbon exposure in fish : an evaluation of compound

specificity , inner filter effect and signal interpretation. *Biomarkers*, *5*(1), 9–23. https://doi.org/10.1080/135475000230505

Abrajano, T. A., Yan, B., & O'Malley, V. (2013). High Molecular Weight Petrogenic and Pyrogenic Hydrocarbons in Aquatic Environments. In *Treatise on Geochemistry: Second Edition* (2nd ed., Vol. 11). Elsevier Ltd. https://doi.org/10.1016/B978-0-08-095975-7.00913-X

Achten, C., & Andersson, J. T. (2015). Overview of Polycyclic Aromatic Compounds (PAC). *Polycyclic Aromatic Compounds*, *35*(2–4), 177–186. https://doi.org/10.1080/10406638.2014.994071

Al-Zaidan, A. S., Al-Sarawi, H. A., Massoud, M. S., Al-Enezi, M., Smith, A. J., Bignell, J. P., Green, M. J., Askem, C., Bolam, T. P. C., Barber, J. L., Bersuder, P., & Lyons, B. P. (2015). Histopathology and contaminant concentrations in fish from Kuwait's marine environment. *Marine Pollution Bulletin*, *100*(2), 637–645. https://doi.org/10.1016/j.marpolbul.2015.07.030

Almeida, A. J., Marques, A., & Saldanha, L. (1999). Some aspects of the biology of three fish species from the seagrass beds at Inhaca Island, Mozambique. Cybium, 23(4).

Andersson, J. T., & Achten, C. (2015). Time to Say Goodbye to the 16 EPA PAHs? Toward an Up-to-Date Use of PACs for Environmental Purposes. *Polycyclic Aromatic Compounds*, *35*(2–4), 330–354. https://doi.org/10.1080/10406638.2014.991042

Ariese, F., Beyer, J., & Wells, D. (2005). Two fish bile reference materials certified for PAH metabolites. *Journal of Environmental Monitoring*, *7*(9), 869–876. https://doi.org/10.1039/b504991a

Ariese, F., Burgers, I., Oudhoff, K., Rutten, T., Stroomberg, G. J., & Vethaak, D. (1997). *Comparison of analytical approaches for PAH metabolites in fish bile samples for marine and estuarine monitoring*. *R-97/9*, 1–29.

Ariese, F., Kok, S. J., Verkaik, M., & Gooijer, C. (1993). Synchronous fluorescence spectrometry of fish bile : A rapid screening method for the biomonitoring of PAH exposure. *Aquatic Toxicology*, *26*(3), 273–286.

Armstrong, T., Khursigara, A. J., Killen, S. S., Fearnley, H., Parsons, K. J., & Esbaugh, A. J. (2019). Oil exposure alters social group cohesion in fish. *Scientific Reports*, *9*(1), 1–9. https://doi.org/10.1038/s41598-019-49994-1

Asker, N., Albertsson, E., Wijkmark, E., Bergek, S., Parkkonen, J., Kammann, U., Holmqvist, I., Kristiansson, E., Strand, J., Gercken, J., & Förlin, L. (2016). Biomarker responses in eelpouts from four coastal areas in Sweden, Denmark and Germany. *Marine Environmental Research*, *120*, 32–43. https://doi.org/10.1016/j.marenvres.2016.07.002

Baali, A., Kammann, U., Hanel, R., El Qoraychy, I., & Yahyaoui, A. (2016). Bile metabolites of polycyclic aromatic hydrocarbons (PAHs) in three species of fish from Morocco. *Environmental Sciences Europe*, *28*(1). https://doi.org/10.1186/s12302-016-0093-6

Bandeira, G. C., & Meneses, H. E. (2013). Handbook of polycyclic aromatic hydrocarbons: Chemistry, occurrence and health issues. In *Handbook of Polycyclic Aromatic Hydrocarbons: Chemistry, Occurrence and Health Issues*.

Barjhoux, I., Cachot, J., Gonzalez, P., Budzinski, H., Le Menach, K., Landi, L., Morin, B., & Baudrimont, M. (2014). Transcriptional responses and embryotoxic effects induced by pyrene and methylpyrene in Japanese medaka (Oryzias latipes) early life stages exposed to spiked sediments. *Environmental Science and Pollution Research*, *21*(24), 13850–13866. https://doi.org/10.1007/s11356-014-2895-7

Barron, M. G. (1990). Bioconcentration: Will water-borne organic chemicals accumulate in aquatic animals? *Environmental Science and Technology*, *24*(11), 1612–1618. https://doi.org/10.1021/es00081a001

Barron, M. G. (2017). Photoenhanced Toxicity of Petroleum to Aquatic Invertebrates and Fish. *Archives of Environmental Contamination and Toxicology*, *73*(1), 40–46. https://doi.org/10.1007/s00244-016-0360-y

Barron, M. G., Carls, M. G., Heintz, R., & Rice, S. D. (2004). Evaluation of fish early life-stage toxicity models of chronic embryonic exposures to complex polycyclic aromatic hydrocarbon mixtures. *Toxicological Sciences*, *78*(1), 60–67. https://doi.org/10.1093/toxsci/kfh051

Barron, M. G., Heintz, R., & Rice, S. D. (2004). Relative potency of PAHs and heterocycles as aryl hydrocarbon receptor agonists in fish. *Marine Environmental Research*, *58*(2–5), 95–100. https://doi.org/10.1016/j.marenvres.2004.03.001

Barron, M. G., Krzykwa, J., Lilavois, C. R., & Raimondo, S. (2018). Photoenhanced Toxicity of Weathered Crude Oil in Sediment and Water to Larval Zebrafish. *Bulletin of Environmental Contamination and Toxicology*, *100*(1), 49–53. https://doi.org/10.1007/s00128-017-2228-x

Bautista, N. M., & Burggren, W. W. (2019). Parental stressor exposure simultaneously conveys both adaptive and maladaptive larval phenotypes through epigenetic inheritance in the zebrafish (Danio rerio). *Journal of Experimental Biology*, *222*(17). https://doi.org/10.1242/jeb.208918

Beg, M. U., Butt, S. A., Al-Dufaileej, S., Karam, Q., Al-Sharrah, T. K., & Saeed, T. (2018). Biomarkers in fish as a measure of the state of marine environment of Kuwait. *Environmental Monitoring and Assessment*, *190*(6). https://doi.org/10.1007/s10661-018-6704-5

Bender, M. L., Frantzen, M., Vieweg, I., Falk-Petersen, I. B., Johnsen, H. K., Rudolfsen, G., Tollefsen, K. E., Dubourg, P., & Nahrgang, J. (2016). Effects of chronic dietary petroleum exposure on reproductive development in polar cod (Boreogadus saida). *Aquatic Toxicology*, *180*, 196–208. https://doi.org/10.1016/j.aquatox.2016.10.005

Beyer, J., Jonsson, G., Porte, C., Krahn, M. M., & Ariese, F. (2010). Analytical methods for determining metabolites of polycyclic aromatic hydrocarbon (PAH) pollutants in fish bile: A review. *Environmental Toxicology and Pharmacology*, *30*(3), 224–244. https://doi.org/10.1016/j.etap.2010.08.004

Blahova, J., Leontovycova, D., Kodes, V., & Svobodova, Z. (2013). Study of polycyclic aromatic hydrocarbon contamination of major rivers in the Czech Republic using biliary metabolite in chub, Leuciscus cephalus L. *Bulletin of Environmental Contamination and Toxicology*, *90*(5), 521–524. https://doi.org/10.1007/s00128-013-0972-0

Blanco, M., Fernández, P., Fernandes, D., Caiola, N., Huertas, D., Porte, C., & Rizzi, J. (2018). The combined use of chemical and biochemical markers in Rutilus rutilus to assess the

effect of dredging in the lower course of the Ebro River. *Ecotoxicology and Environmental Safety*, *155*, 9–16. https://doi.org/10.1016/j.ecoenv.2018.02.060

Blanco, M., Rizzi, J., Fernandes, D., Colin, N., Maceda-Veiga, A., & Porte, C. (2019). Assessing the impact of waste water effluents on native fish species from a semi-arid region, NE Spain. *Science of the Total Environment*, *654*, 218–225. https://doi.org/10.1016/j.scitotenv.2018.11.115

Bleeker, E. A. J., & Verbruggen, E. (2009). Bioaccumulation and biomagnification of polycyclic aromatic hydrocarbons in aquatic organisms. In *RIVM Report 601779002*. https://doi.org/10.1016/S0304-4203(96)00042-4

Brette, F., Machado, B., Cros, C., Incardona, J. P., Scholz, N. L., & Block, B. A. (2014). Crude oil impairs cardiac excitation-contraction coupling in fish. *Science*, *343*(6172), 772–776. https://doi.org/10.1126/science.1242747

Budzinski, H., Mazéas, O., Tronczynski, J., Désaunay, Y., Bocquené, G., & Claireaux, G. (2004). Link between exposure of fish (Solea solea) to PAHs and metabolites: Application to the "Erika" oil spill. *Aquatic Living Resources*, *17*(3), 329–334. https://doi.org/10.1051/alr:2004040

Bugel, S. M., White, L. A., & Cooper, K. R. (2010). Impaired reproductive health of killifish (Fundulus heteroclitus) inhabiting Newark Bay, NJ, a chronically contaminated estuary. *Aquatic Toxicology*, *96*(3), 182–193. https://doi.org/10.1016/j.aquatox.2009.10.016

Choi, J., & Oris, J. T. (2003). Assessment of the toxicity of anthracene photo-modification products using the topminnow (Poeciliopsis lucida) hepatoma cell line (PLHC-1). *Aquatic Toxicology*, *65*(3), 243–251. https://doi.org/10.1016/S0166-445X(03)00139-5

Clark, B. W., Bone, A. J., & Di Giulio, R. T. (2014). Resistance to teratogenesis by F1 and F2 embryos of PAH-adapted Fundulus heteroclitus is strongly inherited despite reduced recalcitrance of the AHR pathway. *Environmental Science and Pollution Research*, *21*(24), 13898–13908. https://doi.org/10.1007/s11356-013-2446-7

Collier, T. K., Anulacion, B. F., Arkoosh, M. R., Dietrich, J. P., Incardona, J. P., Johnson, L. L., Ylitalo, G. M., & Myers, M. S. (2013). Effects on Fish of Polycyclic Aromatic

Hydrocarbons (PAHS) and Naphthenic Acid Exposures. In K. B. Tierney, A. P. Farrell, & C. J. Brauner (Eds.), *Organic Chemical Toxicology of Fishes* (First Edit, Vol. 33, pp. 195–255). Elsevier Inc. https://doi.org/10.1016/B978-0-12-398254-4.00004-2

Collier, T. K., & Varanasi, U. (1991). Hepatic activities of xenobiotic metabolizing enzymes and biliary levels of xenobiotics in english sole (Parophrys vetulus) exposed to environmental contaminants. *Archives of Environmental Contamination and Toxicology*, *20*, 462–473. http://download.springer.com/static/pdf/171/art%253A10.1007%252FBF01065834.pdf?originUrl=http%253A%252F%252Flink.springer.com%252Farticle%252F10.1007%252FBF01065834&token2=exp=1453740322~acl=%252Fstatic%252Fpdf%252F171%252Fart%2525253A10.1007%2525252FBF01

Corrales, J., Thornton, C., White, M., & Willett, K. L. (2014). Multigenerational effects of benzo[a]pyrene exposure on survival and developmental deformities in zebrafish larvae. *Aquatic Toxicology*, *148*, 16–26. https://doi.org/10.1016/j.aquatox.2013.12.028

Couderc, M., Poirier, L., Zalouk-Vergnoux, A., Kamari, A., Blanchet-Letrouvé, I., Marchand, P., Vénisseau, A., Veyrand, B., Mouneyrac, C., & Le Bizec, B. (2015). Occurrence of POPs and other persistent organic contaminants in the European eel (Anguilla anguilla) from the Loire estuary, France. *Science of the Total Environment*, *505*, 199–215. https://doi.org/10.1016/j.scitotenv.2014.09.053

Dabrowska, H., Kopko, O., Lehtonen, K. K., Lang, T., Waszak, I., Balode, M., & Strode, E. (2017). An integrated assessment of pollution and biological effects in flounder, mussels and sediment in the southern Baltic Sea coastal area. *Environmental Science and Pollution Research*, *24*(4), 3626–3639. https://doi.org/10.1007/s11356-016-8117-8

Davies, I. M., & Vethaak, D. (2012). *Integrated marine environmental monitoring of chemicals and their effects Editors* (Issue October 2018).

de Albergaria-Barbosa, A. C. R., da Silva, D. A. M., da Silva Rocha, A. J., Taniguchi, S., Patire, V. F., Dias, J. F., Fernandez, W. S., & Bícego, M. C. (2018). Evaluation of polycyclic aromatic hydrocarbons bioavailability on Santos Bay (Brazil) through levels of biliary metabolites. *Marine Pollution Bulletin*, *129*(2), 822–828. https://doi.org/10.1016/j.marpolbul.2017.10.006

de Albergaria-Barbosa, A. C. R., Patire, V. F., Taniguchi, S., Fernandez, W. S., Dias, J. F., & Bícego, M. C. (2017). Mugil curema as a PAH bioavailability monitor for Atlantic west sub-tropical estuaries. *Marine Pollution Bulletin*, *114*(1), 609–614. https://doi.org/10.1016/j.marpolbul.2016.09.039

Di Giulio, R. T., & Clark, B. W. (2015). The Elizabeth River Story: A Case Study in Evolutionary Toxicology. *Journal of Toxicology and Environmental Health - Part B: Critical Reviews*, *18*(6), 259–298. https://doi.org/10.1080/15320383.2015.1074841

Duarte, R. M., Sadauskas-Henrique, H., de Almeida-Val, V. M. F., Val, A. L., Nice, H. E., & Gagnon, M. M. (2017). Biomarker responses and PAH ratios in fish inhabiting an estuarine urban waterway. *Environmental Toxicology*, *32*(10), 2305–2315. https://doi.org/10.1002/tox.22447

Durif, C., Dufour, S., & Elie, P. (2005). The silvering process of Anguilla anguilla: a new classification from the yellow resident to the silver migrating stage. *Journal of Fish Biology*, *66*(4), 1025–1043. https://doi.org/10.1111/j.1095-8649.2005.00662.x

Fairbairn, E. A., Bonthius, J., & Cherr, G. N. (2012). Polycyclic aromatic hydrocarbons and dibutyl phthalate disrupt dorsal-ventral axis determination via the Wnt/β-catenin signaling pathway in zebrafish embryos. *Aquatic Toxicology*, *124–125*, 188–196. https://doi.org/10.1016/j.aquatox.2012.08.017

Fasnacht, M. P., & Blough, N. V. (2002). Aqueous photodegradation of polycyclic aromatic hydrocarbons. *Environmental Science and Technology*, *36*(20), 4364–4369. https://doi.org/10.1021/es025603k

Floehr, T., Scholz-Starke, B., Xiao, H., Hercht, H., Wu, L., Hou, J., Schmidt-Posthaus, H., Segner, H., Kammann, U., Yuan, X., Roß-Nickoll, M., Schäffer, A., & Hollert, H. (2015). Linking Ah receptor mediated effects of sediments and impacts on fish to key pollutants in the Yangtze Three Gorges Reservoir, China - A comprehensive perspective. *Science of the Total Environment*, *538*, 191–211. https://doi.org/10.1016/j.scitotenv.2015.07.044

Fuchsman, P. C., Leigh, K. B., & Barber, T. R. (2001). Ecological Assessment of PAHs in Fish. In *Sediments Guidance Compendium*.

Gagnon, M. M., & Rawson, C. A. (2016). Integrating Multiple Biomarkers of Fish Health: A Case Study of Fish Health in Ports. *Archives of Environmental Contamination and Toxicology*, *70*(2), 192–203. https://doi.org/10.1007/s00244-015-0258-0

Galceran, M. T., & Moyano, E. (1994). High-performance liquid chromatography-mass spectrometry (pneumatically assisted electrospray) of hydroxy polycyclic aromatic hydrocarbons. *Journal of Chromatography A*, *683*(1), 9–19. https://doi.org/10.1016/S0021-9673(94)89097-8

Gale, R. W., Tanner, M. J., Love, M. S., Nishimoto, M. M., & Schroeder, D. M. (2012). Comparison of Concentrations and Profiles of Polycyclic Aromatic Hydrocarbon Metabolites in Bile of Fishes from Offshore Oil Platforms and Natural Reefs Along the California Coast. In *U.S. Geological Survey Open-File Report 2012–1248*.

Ge, L., Na, G., Chen, C. E., Li, J., Ju, M., Wang, Y., Li, K., Zhang, P., & Yao, Z. (2016). Aqueous photochemical degradation of hydroxylated PAHs: Kinetics, pathways, and multivariate effects of main water constituents. *Science of the Total Environment*, *547*, 166–172. https://doi.org/10.1016/j.scitotenv.2015.12.143

Geraudie, P., Nahrgang, J., Forget-Leray, J., Minier, C., & Camus, L. (2014). In vivo effects of environmental concentrations of produced water on the reproductive function of polar cod (Boreogadus saida). *Journal of Toxicology and Environmental Health - Part A: Current Issues*, *77*(9–11), 557–573. https://doi.org/10.1080/15287394.2014.887420

Giltrap, M., Ronan, J., Bignell, J. P., Lyons, B. P., Collins, E., Rochford, H., McHugh, B., McGovern, E., Bull, L., & Wilson, J. (2017). Integration of biological effects, fish histopathology and contaminant measurements for the assessment of fish health: A pilot application in Irish marine waters. *Marine Environmental Research*, *129*, 113–132. https://doi.org/10.1016/j.marenvres.2017.04.004

Grung, M., Petersen, K., Fjeld, E., Allan, I., Christensen, J. H., Malmqvist, L. M. V., Meland, S., & Ranneklev, S. (2016). PAH related effects on fish in sedimentation ponds for road runoff and potential transfer of PAHs from sediment to biota. *Science of the Total Environment*, *566–567*(0349), 1309–1317. https://doi.org/10.1016/j.scitotenv.2016.05.191

Gusso-Choueri, P. K., Choueri, R. B., Santos, G. S., de Araújo, G. S., Cruz, A. C. F., Stremel, T., de Campos, S. X., Cestari, M. M., Ribeiro, C. A. O., & de Sousa Abessa, D. M. (2016). Assessing genotoxic effects in fish from a marine protected area influenced by former mining activities and other stressors. *Marine Pollution Bulletin*, *104*(1–2), 229–239. https://doi.org/10.1016/j.marpolbul.2016.01.025

Harrison, R. M., Jang, E., Alam, M. S., & Dang, J. (2018). Mechanisms of reactivity of benzo(a)pyrene and other PAH inferred from field measurements. *Atmospheric Pollution Research*, *9*(6), 1214–1220. https://doi.org/10.1016/j.apr.2018.05.009

Heitkamp, M. A., & Cerniglia, C. E. (1987). Effects of chemical structure and exposure on the microbial degradation of polycyclic aromatic hydrocarbons in freshwater and estuarine ecosystems. *Environmental Toxicology and Chemistry*, *6*(7), 535–546. https://doi.org/10.1002/etc.5620060706

Heuer, R. M., Galli, G. L. J., Shiels, H. A., Fieber, L. A., Cox, G. K., Mager, E. M., Stieglitz, J. D., Benetti, D. D., Grosell, M., & Crossley, D. A. (2019). Impacts of Deepwater Horizon Crude Oil on Mahi-Mahi (Coryphaena hippurus) Heart Cell Function. *Environmental Science and Technology*, *53*(16), 9895–9904. https://doi.org/10.1021/acs.est.9b03798

Hodson, P. V. (2017). The Toxicity to Fish Embryos of PAH in Crude and Refined Oils. *Archives of Environmental Contamination and Toxicology*, *73*(1), 12–18. https://doi.org/10.1007/s00244-016-0357-6

Holth, T. F., Nourizadeh-Lillabadi, R., Blaesbjerg, M., Grung, M., Holbech, H., Petersen, G. I., Aleström, P., & Hylland, K. (2008). Differential gene expression and biomarkers in zebrafish (Danio rerio) following exposure to produced water components. *Aquatic Toxicology*, *90*(4), 277–291. https://doi.org/10.1016/j.aquatox.2008.08.020

Huang, L., Wang, C., Zhang, Y., Wu, M., & Zuo, Z. (2013). Phenanthrene causes ocular developmental toxicity in zebrafish embryos and the possible mechanisms involved. *Journal of Hazardous Materials*, *261*, 172–180. https://doi.org/10.1016/j.jhazmat.2013.07.030

Hylland, K., Burgeot, T., Martínez-Gómez, C., Lang, T., Robinson, C. D., Svavarsson, J., Thain,

J. E., Vethaak, A. D., & Gubbins, M. J. (2017). How can we quantify impacts of contaminants in marine ecosystems? The ICON project. *Marine Environmental Research*, *124*, 2–10. https://doi.org/10.1016/j.marenvres.2015.11.006

ICES. (2013). *Report of the Working Group on the Biologial Effects of Contaminants (WGBEC)* (Issues 10-15 March 2013, San Pedro del Pinatar, Spain).

Ikenaka, Y., Oguri, M., Saengtienchai, A., Nakayama, S. M. M., Ijiri, S., & Ishizuka, M. (2013). Characterization of phase-II conjugation reaction of polycyclic aromatic hydrocarbons in fish species: Unique pyrene metabolism and species specificity observed in fish species. *Environmental Toxicology and Pharmacology*, *36*(2), 567–578. https://doi.org/10.1016/j.etap.2013.05.018

Incardona, J. P. (2017). Molecular Mechanisms of Crude Oil Developmental Toxicity in Fish. *Archives of Environmental Contamination and Toxicology*, *73*(1), 19–32. https://doi.org/10.1007/s00244-017-0381-1

Incardona, J. P., Carls, M. G., Day, H. L., Sloan, C. A., Bolton, J. L., Collier, T. K., & Schoiz, N. L. (2009). Cardiac arrhythmia is the primary response of embryonic pacific herring (Clupea pallasi) exposed to crude oil during weathering. *Environmental Science and Technology*, *43*(1), 201–207. https://doi.org/10.1021/es802270t

Incardona, J. P., Collier, T. K., & Scholz, N. L. (2004). Defects in cardiac function precede morphological abnormalities in fish embryos exposed to polycyclic aromatic hydrocarbons. *Toxicology and Applied Pharmacology*, *196*(2), 191–205. https://doi.org/10.1016/j.taap.2003.11.026

Incardona, J. P., Gardner, L. D., Linbo, T. L., Brown, T. L., Esbaugh, A. J., Mager, E. M., Stieglitz, J. D., French, B. L., Labenia, J. S., Laetz, C. A., Tagal, M., Sloan, C. A., Elizur, A., Benetti, D. D., Grosell, M., Block, B. A., & Scholz, N. L. (2014). Deepwater horizon crude oil impacts the developing hearts of large predatory pelagic fish. *Proceedings of the National Academy of Sciences of the United States of America*, *111*(15). https://doi.org/10.1073/pnas.1320950111

Jacob, J., & Grimmer, G. (1987). Capillary gas chromatographical analysis and mass

spectrometric identification of polycyclic aromatic hydrocarbon metabolites from biological materials. *Reviews in Analytical Chemistry*, *9*(1). https://doi.org/10.1515/REVAC.1987.9.1.49

Jacoby, D., & Gollock, M. (2014). Anguilla anguilla. *The IUCN Red List of Threatened Species*, 1–29. https://doi.org/http://dx.doi.org/10.2305/IUCN.UK.2014-1.RLTS.T60344A45833138.en

Jasperse, L., Levin, M., Rogers, K., Perkins, C., Bosker, T., Griffitt, R. J., Sepúlveda, M. S., & De Guise, S. (2019). Transgenerational effects of polycyclic aromatic hydrocarbon exposure on sheepshead minnows (Cyprinodon variegatus). *Environmental Toxicology and Chemistry*, *38*(3), 638–649. https://doi.org/10.1002/etc.4340

Johansen, J. L., & Esbaugh, A. J. (2017). Sustained impairment of respiratory function and swim performance following acute oil exposure in a coastal marine fish. *Aquatic Toxicology*, *187*(April), 82–89. https://doi.org/10.1016/j.aquatox.2017.04.002

Johnson, L. L., Arkoosh, M. R., Bravo, C. F., Collier, T. K., Krahn, M. M., Meador, J. P., Myers, M. S., Reichert, W. L., & Stein, J. E. (2008). The effects of polycyclic aromatic hydrocarbons in fish from Puget Sound, Washington. In R. T. Di Giulio & D. E. Hinton (Eds.), *The Toxicology of Fishes* (1st ed., pp. 877–924). Taylor & Francis Group. https://doi.org/10.1201/9780203647295

Johnson, L. L., Landahl, J. T., Kubin, L. A., Horness, B. H., Myers, M. S., Collier, T. K., & Stein, J. E. (1998). Assessing the effects of anthropogenic stressors on Puget Sound flatfish populations. *Journal of Sea Research*, *39*(1–2), 125–137. https://doi.org/10.1016/s1385-1101(97)00057-9

Johnson, L. L., Ylitalo, G. M., Myers, M. S., Anulacion, B. F., Buzitis, J., & Collier, T. K. (2015). Aluminum smelter-derived polycyclic aromatic hydrocarbons and flatfish health in the Kitimat marine ecosystem, British Columbia, Canada. *Science of the Total Environment*, *512–513*(C), 227–239. https://doi.org/10.1016/j.scitotenv.2015.01.017

Jonsson, G., Bechmann, R. K., Bamber, S. D., & Baussant, T. (2004). Bioconcentration, biotransformation, and elimination of polycyclic aromatic hydrocarbons in sheepshead

minnows (Cyprinodon variegatus) exposed to contaminated seawater. *Environmental Toxicology and Chemistry*, *23*(6), 1538–1548. https://doi.org/10.1897/03-173

Jonsson, G., Beyer, J., Wells, D., & Ariese, F. (2003). The application of HPLC-F and GC-MS to the analysis of selected hydroxy polycyclic hydrocarbons in two certified fish bile reference materials. *Journal of Environmental Monitoring*, *5*(3), 513–520. https://doi.org/10.3233/978-1-60750-938-7-19

Jörundsdóttir, H. Ó., Jensen, S., Hylland, K., Holth, T. F., Gunnlaugsdóttir, H., Svavarsson, J., Ólafsdóttir, Á., El-Taliawy, H., Rigét, F., Strand, J., Nyberg, E., Bignert, A., Hoydal, K. S., & Halldórsson, H. P. (2014). Pristine Arctic: Background mapping of PAHs, PAH metabolites and inorganic trace elements in the North-Atlantic Arctic and sub-Arctic coastal environment. *Science of the Total Environment*, *493*, 719–728. https://doi.org/10.1016/j.scitotenv.2014.06.030

Juma, R. R., Salum, N. S., Tairova, Z., Strand, J., Bakari, S. S., & Sheikh, M. A. (2018). Potential of Periophthalmus sobrinus and Siganus sutor as bioindicator fish species for PAH pollution in tropical waters. *Regional Studies in Marine Science*, *18*, 170–176. https://doi.org/10.1016/j.rsma.2017.09.016

Jung, J. H., Kim, M., Yim, U. H., Ha, S. Y., An, J. G., Won, J. H., Han, G. M., Kim, N. S., Addison, R. F., & Shim, W. J. (2011). Biomarker responses in pelagic and benthic fish over 1 year following the Hebei Spirit oil spill (Taean, Korea). *Marine Pollution Bulletin*, *62*(8), 1859–1866. https://doi.org/10.1016/j.marpolbul.2011.04.045

Jung, J. H., Kim, M., Yim, U. H., Ha, S. Y., Shim, W. J., Chae, Y. S., Kim, H., Incardona, J. P., Linbo, T. L., & Kwon, J. H. (2015). Differential Toxicokinetics Determines the Sensitivity of Two Marine Embryonic Fish Exposed to Iranian Heavy Crude Oil. *Environmental Science and Technology*, *49*(22), 13639–13648. https://doi.org/10.1021/acs.est.5b03729

Kammann, U. (2007). PAH metabolites in bile fluids of dab (Limanda limanda) and flounder (Platichthys flesus): Spatial distribution and seasonal changes. *Environmental Science and Pollution Research*, *14*(2), 102–108. https://doi.org/10.1065/espr2006.05.308

Kammann, U., Akcha, F., Budzinski, H., Burgeot, T., Gubbins, M. J., Lang, T., Le Menach, K.,

Vethaak, A. D., & Hylland, K. (2017). PAH metabolites in fish bile: From the Seine estuary to Iceland. *Marine Environmental Research*, *124*, 41–45. https://doi.org/10.1016/j.marenvres.2016.02.014

Kammann, U., Askem, C., Dabrowska, H., Grung, M., Kirby, M. F., Koivisto, P., Lucas, C., McKenzie, M., Meier, S., Robinson, C., Tairova, Z. M., Tuvikene, A., Vuorinen, P. J., & Strand, J. (2013). Interlaboratory proficiency testing for measurement of the polycyclic aromatic hydrocarbon metabolite 1-hydroxypyrene in fish bile for marine environmental monitoring. *Journal of AOAC International*, *96*(3), 635–641. https://doi.org/10.5740/jaoacint.12-080

Kammann, U., Brinkmann, M., Freese, M., Pohlmann, J. D., Stoffels, S., Hollert, H., & Hanel, R. (2014). PAH metabolites, GST and EROD in European eel (Anguilla anguilla) as possible indicators for eel habitat quality in German rivers. *Environmental Science and Pollution Research*, *21*(4), 2519–2530. https://doi.org/10.1007/s11356-013-2121-z

Kang, H. J., Jung, Y., & Kwon, J. H. (2019). Changes in ecotoxicity of naphthalene and alkylated naphthalenes during photodegradation in water. *Chemosphere*, *222*, 656–664. https://doi.org/10.1016/j.chemosphere.2019.01.153

Karl, H., Kammann, U., Aust, M. O., Manthey-Karl, M., Lüth, A., & Kanisch, G. (2016). Large scale distribution of dioxins, PCBs, heavy metals, PAH-metabolites and radionuclides in cod (Gadus morhua) from the North Atlantic and its adjacent seas. *Chemosphere*, *149*, 294–303. https://doi.org/10.1016/j.chemosphere.2016.01.052

Khan, R. A. (2013). Effects of Polycyclic Aromatic Hydrocarbons on Sexual Maturity of Atlantic Cod, Gadus morhua, Following Chronic Exposure. *Environment and Pollution*, *2*(1), 1–10. https://doi.org/10.5539/ep.v2n1p1

Khursigara, A. J., Perrichon, P., Martinez Bautista, N., Burggren, W. W., & Esbaugh, A. J. (2017). Cardiac function and survival are affected by crude oil in larval red drum, Sciaenops ocellatus. *Science of the Total Environment*, *579*, 797–804. https://doi.org/10.1016/j.scitotenv.2016.11.026

Kiceniuk, J. W., & Khan, R. A. (1987). Effect of petroleum hydrocarbons on Atlantic cod,

Gadus morhua , following chronic exposure . *Canadian Journal of Zoology*, *65*(3), 490–494. https://doi.org/10.1139/z87-076

Kirby, A. R., Cox, G. K., Nelson, D., Heuer, R. M., Stieglitz, J. D., Benetti, D. D., Grosell, M., & Crossley, D. A. (2019). Acute crude oil exposure alters mitochondrial function and ADP affinity in cardiac muscle fibers of young adult Mahi-mahi (Coryphaena hippurus). *Comparative Biochemistry and Physiology Part - C: Toxicology and Pharmacology*, *218*(January), 88–95. https://doi.org/10.1016/j.cbpc.2019.01.004

Koenig, S., Porte, C., Solé, M., & Sturve, J. (2013). Biliary PAH and alkylphenol metabolites, biomarker enzyme activities, and gene expression levels in the deep-sea fish alepocephalus rostratus. *Environmental Science and Technology*, *47*(6), 2854–2861. https://doi.org/10.1021/es304345s

Krahn, M. M., Brown, D. W., Collier, T. K., Friedman, A. J., Jenkins, R. G., & Malins, D. C. (1980). Rapid analysis of naphthalene and its metabolites in biological systems: Determination by high-performance liquid chromatography/fluorescence and by plasma desorption/chemical ionization mass spectrometry. *Journal of Biochemical and Biophysical Methods*, *2*(4), 233–246. https://doi.org/10.1016/0165-022X(80)90038-X

Krahn, M. M., Burrows, D. G., Ylltalo, G. M., Brown, D. W., Wlgren, C. A., Collier, T. K., Sin-Lam, C., & Usha, V. (1992). Mass Spectrometric Analysis for Aromatic Compounds in Bile of Fish Sampled after the Exxon Valdez Oil Spill. *Environmental Science and Technology*, *26*(1), 116–126. https://doi.org/10.1021/es00025a012

Krahn, M. M., Moore, L. K., & Macleod, W. D. J. (1986). *NOAA Technical Memorandum NMFS F / NWC-92 Standard Analytical Procedures of the NOAA National Analytical Facility*. *October 1985*, 120.

Krahn, M. M., Myers, M. S., Burrows, D. G., Malins, D. C., Krahn, M. M., Myers, M. S., Burrows, D. G., & Malins, D. C. (1984). Determination of metabolites of xenobiotics in the bile of fish from polluted waterways. *Xenobiotica*, *14*(8), 633–646. https://doi.org/10.3109/00498258409151461

Krahn, M. M., Rhodes, L. D., Myers, M. S., Moore, L. K., MacLeod, W. D., & Malins, D. C.

(1986). Associations between metabolites of aromatic compounds in bile and the occurrence of hepatic lesions in English sole (Parophrys vetulus) from Puget Sound, Washington. *Archives of Environmental Contamination and Toxicology*, *15*(1), 61–67. https://doi.org/10.1007/BF01055249

Krahn, M. M., Ylitalo, G. M., Buzitis, J., Chan, S. L., & Varanasi, U. (1993). Rapid high-performance liquid chromatographic methods that screen for aromatic compounds in environmental samples. *Journal of Chromatography A*, *642*(1–2), 15–32. https://doi.org/10.1016/0021-9673(93)80073-H

Kreitsberg, R., Tuvikene, A., Baršiene, J., Fricke, N. F., Rybakovas, A., Andreikenaite, L., Rumvolt, K., & Vilbaste, S. (2012). Biomarkers of environmental contaminants in the coastal waters of Estonia (Baltic Sea): Effects on eelpouts (Zoarces viviparus). *Journal of Environmental Monitoring*, *14*(9), 2298–2308. https://doi.org/10.1039/c2em30285c

Kreitsberg, R., Zemit, I., Freiberg, R., Tambets, M., & Tuvikene, A. (2010). Responses of metabolic pathways to polycyclic aromatic compounds in flounder following oil spill in the Baltic Sea near the Estonian coast. *Aquatic Toxicology*, *99*(4), 473–478. https://doi.org/10.1016/j.aquatox.2010.06.005

Leonard, J. D., & Hellou, J. (2001). Separation and characterization of gall bladder bile metabolites from speckled trout, Salvelinus fontinalis, exposed to individual polycyclic aromatic compounds. *Environmental Toxicology and Chemistry*, *20*(3), 618–623. https://doi.org/10.1002/etc.5620200322

Lima, A. L. C., Farrington, J. W., & Reddy, C. M. (2005). Combustion-derived polycyclic aromatic hydrocarbons in the environment - A review. *Environmental Forensics*, *6*(2), 109–131. https://doi.org/10.1080/15275920590952739

Lin, E. L. C., Cormier, S. M., & Torsella, J. A. (1996). Fish biliary polycyclic aromatic hydrocarbon metabolites estimated by fixed-wavelength fluorescence: Comparison with HPLC-fluorescent detection. *Ecotoxicology and Environmental Safety*, *35*(1), 16–23. https://doi.org/10.1006/eesa.1996.0077

Logan, D. T. (2007). Perspective on ecotoxicology of PAHs to fish. *Human and Ecological Risk*

Assessment, *13*(2), 302–316. https://doi.org/10.1080/10807030701226749

Magnuson, J. T., Khursigara, A. J., Allmon, E. B., Esbaugh, A. J., & Roberts, A. P. (2018). Effects of Deepwater Horizon crude oil on ocular development in two estuarine fish species, red drum (Sciaenops ocellatus) and sheepshead minnow (Cyprinodon variegatus). *Ecotoxicology and Environmental Safety*, *166*, 186–191. https://doi.org/10.1016/j.ecoenv.2018.09.087

Meador, J. P., Buzitis, J., & Bravo, C. F. (2008). Using fluorescent aromatic compounds in bile from juvenile salmonids to predict exposure to polycyclic aromatic hydrocarbons. *Environmental Toxicology and Chemistry*, *27*(4), 845–853. https://doi.org/10.1897/07-434.1

Meador, J. P., & Nahrgang, J. (2019). Characterizing Crude Oil Toxicity to Early-Life Stage Fish Based on a Complex Mixture: Are We Making Unsupported Assumptions? *Environmental Science and Technology*, *53*(19), 11080–11092. https://doi.org/10.1021/acs.est.9b02889

Meador, J. P., Sommers, F. C., Ylitalo, G. M., & Sloan, C. A. (2006). Altered growth and related physiological responses in juvenile Chinook salmon (Oncorhynchus tshawytscha) from dietary exposure to polycyclic aromatic hydrocarbons (PAHs). *Canadian Journal of Fisheries and Aquatic Sciences*, *63*(10), 2364–2376.

Meyer, J. N., & Di Giulio, R. T. (2003). Heritable adaptation and fitness costs in killifish (Fundulus heteroclitus) inhabiting a polluted estuary. *Ecological Applications*, *13*(2), 490–503. https://doi.org/10.1890/1051-0761(2003)013[0490:HAAFCI]2.0.CO;2

Meyer, J. N., Nacci, D. E., & Di Giulio, R. T. (2002). Cytochrome P4501A (CYP1A) in killifish (Fundulus heteroclitus): Heritability of altered expression and relationship to survival in contaminated sediments. *Toxicological Sciences*, *68*(1), 69–81. https://doi.org/10.1093/toxsci/68.1.69

Monteiro, P. R. R., Reis-Henriques, M. A., & Coimbra, J. (2000). Plasma steroid levels in female flounder (Platichthys flesus) after chronic dietary exposure to single polycyclic aromatic hydrocarbons. *Marine Environmental Research*, *49*(5), 453–467. https://doi.org/10.1016/S0141-1136(99)00085-9

Murdy, E. O. (1989). A taxonomic revision and cladistic analysis of the oxudercine gobies

(Gobiidae: Oxudercinae). Records of the Australian Museum, Supplement, 11. https://doi.org/10.3853/j.0812-7387.11.1989.93

Nagel, F., Kammann, U., Wagner, C., & Hanel, R. (2012). Metabolites of polycyclic aromatic hydrocarbons (PAHs) in bile as biomarkers of pollution in European eel (Anguilla anguilla) from German rivers. *Archives of Environmental Contamination and Toxicology*, *62*(2), 254–263. https://doi.org/10.1007/s00244-011-9693-8

Nagel, F., Wagner, C., Hanel, R., & Kammann, U. (2012). The silvering process of European eel (Anguilla anguilla) influences PAH metabolite concentrations in bile fluid: Consequences for monitoring. *Chemosphere*, *87*(1), 91–96. https://doi.org/10.1016/j.chemosphere.2011.11.071

Nahrgang, J., Brooks, S. J., Evenset, A., Camus, L., Jonsson, M., Smith, T. J., Lukina, J., Frantzen, M., Giarratano, E., & Renaud, P. E. (2013). Seasonal variation in biomarkers in blue mussel (Mytilus edulis), Icelandic scallop (Chlamys islandica) and Atlantic cod (Gadus morhua)-Implications for environmental monitoring in the Barents Sea. *Aquatic Toxicology*, *127*(9037), 21–35. https://doi.org/10.1016/j.aquatox.2012.01.009

Ohiozebau, E., Tendler, B., Hill, A., Codling, G., Kelly, E., Giesy, J. P., & Jones, P. D. (2016). Products of biotransformation of polycyclic aromatic hydrocarbons in fishes of the Athabasca/Slave river system, Canada. *Environmental Geochemistry and Health*, *38*(2), 577–591. https://doi.org/10.1007/s10653-015-9744-6

Opperhulzen, A., Volde, E. W. v. d., Gobas, F. A. P. C., Liem, D. A. K., Steen, J. M. D. v. d., & Hutzinger, O. (1985). Relationship between bioconcentration in fish and steric factors of hydrophobic chemicals. *Chemosphere*, *14*(11–12), 1871–1896. https://doi.org/10.1016/0045-6535(85)90129-8

Osterberg, J. S., Cammen, K. M., Schultz, T. F., Clark, B. W., & Di Giulio, R. T. (2018). Genome-wide scan reveals signatures of selection related to pollution adaptation in non-model estuarine Atlantic killifish (Fundulus heteroclitus). *Aquatic Toxicology*, *200*(February), 73–82. https://doi.org/10.1016/j.aquatox.2018.04.017

Ownby, D. R., Newman, M. C., Mulvey, M., Vogelbein, W. K., Unger, M. A., & Arzayus, L. F.

(2002). Fish (Fundulus Heteroclitus) Populations With Different Exposure Histories Differ in Tolerance of Creosote-Contaminated Sediments. *Environmental Toxicology and Chemistry*, *21*(9), 1897. https://doi.org/10.1897/1551-5028(2002)021<1897:ffhpwd>2.0.co;2

Pampanin, D. M., Kemppainen, E. K., Skogland, K., Jørgensen, K. B., & Sydnes, M. O. (2016). Investigation of fixed wavelength fluorescence results for biliary metabolites of polycyclic aromatic hydrocarbons formed in Atlantic cod (Gadus morhua). *Chemosphere*, *144*, 1372–1376. https://doi.org/10.1016/j.chemosphere.2015.10.013

Pampanin, D. M., & Sydnes, M. O. (2013). Polycyclic Aromatic Hydrocarbons a Constituent of Petroleum: Presence and Influence in the Aquatic Environment. In V. G. Kutcherov & A. Kolesnikov (Eds.), *Hydrocarbon* (pp. 83–118). IntechOpen. https://doi.org/10.5772/48176

Pathiratne, A., Hemachandra, C. K., & Pathiratne, K. A. S. (2010). Assessment of bile fluorescence patterns in a tropical fish, Nile tilapia (Oreochromis niloticus) exposed to naphthalene, phenanthrene, pyrene and chrysene using fixed wavelength fluorescence and synchronous fluorescence spectrometry. *Bulletin of Environmental Contamination and Toxicology*, *84*(5), 554–558. https://doi.org/10.1007/s00128-010-9987-y

Pheiffer, W., Bortey-Sam, N., Ikenaka, Y., Nakayama, S. M. M., Mizukawa, H., Ishizuka, M., Smit, N. J., & Pieters, R. (2018). First report on OH-PAHs in South African Clarias gariepinus bile from an urban impacted system. *African Journal of Aquatic Science*, *43*(3), 305–312. https://doi.org/10.2989/16085914.2018.1511408

Pinkney, A. E., Harshbarger, J. C., May, E. B., & Melancon, M. J. (2001). Tumor Prevalence and Biomarkers of Exposure in Brown Bullheads (Ameiurus Nebulosus) From the Tidal Potomac River, Usa, Watershed. *Environmental Toxicology and Chemistry*, *20*(6), 1196. https://doi.org/10.1897/1551-5028(2001)020<1196:tpaboe>2.0.co;2

Pollino, C. A., Georgiades, E., & Holdway, D. A. (2009). Physiological changes in reproductively active rainbowfish (Melanotaenia fluviatilis) following exposure to naphthalene. *Ecotoxicology and Environmental Safety*, *72*(4), 1265–1270. https://doi.org/10.1016/j.ecoenv.2009.01.012

Pulster, E. L., Gracia, A., Armenteros, M., Carr, B. E., Mrowicki, J., & Murawski, S. A. (2020). Chronic PAH exposures and associated declines in fish health indices observed for ten grouper species in the Gulf of Mexico. *Science of the Total Environment*, *703*, 135551. https://doi.org/10.1016/j.scitotenv.2019.135551

Pulster, E. L., Gracia, A., Snyder, S. M., Romero, I. C., Carr, B., Toro-Farmer, G., & Murawski, S. A. (2020). Polycyclic Aromatic Hydrocarbon Baselines in Gulf of Mexico Fishes. In S. A. Murawski, C. H. Ainsworth, S. Gilbert, D. J. Hollander, C. B. Paris, M. Schlüter, & D. L. Wetzel (Eds.), *Scenarios and Responses to Future Deep Oil Spills* (pp. 253–271). https://doi.org/10.1007/978-3-030-12963-7

Pulster, E. L., Main, K., Wetzel, D., & Murawski, S. (2017). Species-specific metabolism of naphthalene and phenanthrene in 3 species of marine teleosts exposed to Deepwater Horizon crude oil. *Environmental Toxicology and Chemistry*, *36*(11), 3168–3176. https://doi.org/10.1002/etc.3898

Pulster, E. L., Snyder, S. M., Murawski, S. A., Carr, B., Mrowicki, J., Schwaab, M., Struch, R. E., & Toro-Farmer, G. (2019). *Biliary polycyclic aromatic hydrocarbon (PAH) equivalents for 92 species of fish collected aboard multiple research and fishing vessels in the Gulf of Mexico from 2011-08-16 to 2018-08-22*. Gulf of Mexico Research Initiative Information and Data Cooperative (GRIIDC), Harte Research Institute, Texas A&M University–Corpus Christi. https://doi.org/doi:10.7266/N7X34W1J

Puy-Azurmendi, E., Navarro, A., Olivares, A., Fernandes, D., Martínez, E., López de Alda, M., Porte, C., Cajaraville, M. P., Barceló, D., & Piña, B. (2010). Origin and distribution of polycyclic aromatic hydrocarbon pollution in sediment and fish from the biosphere reserve of Urdaibai (Bay of Biscay, Basque country, Spain). *Marine Environmental Research*, *70*(2), 142–149. https://doi.org/10.1016/j.marenvres.2010.04.004

Reichert, W. L., Myers, M. S., Peck-Miller, K., French, B., Anulacion, B. F., Collier, T. K., Stein, J. E., & Varanasi, U. (1998). Molecular epizootiology of genotoxic events in marine fish: Linking contaminant exposure, DNA damage, and tissue-level alterations. *Mutation Research - Reviews in Mutation Research*, *411*(3), 215–225. https://doi.org/10.1016/S1383-5742(98)00014-3

Reisen, F., & Arey, J. (2005). Atmospheric reactions influence seasonal PAH and nitro-PAH concentrations in the Los Angeles basin. *Environmental Science and Technology*, *39*(1), 64–73. https://doi.org/10.1021/es035454l

Richardson, D. M., Gubbins, M. J., Davies, I. M., Moffat, C. F., & Pollard, P. M. (2004). Effects of feeding status on biliary PAH metabolite and biliverdin concentrations in plaice (Pleuronectes platessa). *Environmental Toxicology and Pharmacology*, *17*(2), 79–85. https://doi.org/10.1016/j.etap.2004.03.003

Rivera-Figueroa, A. M., Ramazan, K. A., & Finlayson-Pitts, B. J. (2004). Fluorescence, Absorption, and Excitation Spectra of Polycyclic Aromatic Hydrocarbons as a Tool for Quantitative Analysis. *Journal of Chemical Education*, *81*(2), 242–245. https://doi.org/10.1021/ed081p242

Romero, I. C., Özgökmen, T., Snyder, S., Schwing, P., O'Malley, B. J., Beron-Vera, F. J., Olascoaga, M. J., Zhu, P., Ryan, E., Chen, S. S., Wetzel, D. L., Hollander, D., & Murawski, S. A. (2016). Tracking the Hercules 265 marine gas well blowout in the Gulf of Mexico. *Journal of Geophysical Research: Oceans*, *121*(1), 706–724. https://doi.org/10.1002/2015JC011037

Rose, W. L., French, B. L., Reichert, W. L., & Faisal, M. (2000). DNA adducts in hematopoietic tissues and blood of the mummichog (Fundulus heteroclitus) from a creosote-contaminated site in the Elizabeth River, Virginia. *Marine Environmental Research*, *50*(1–5), 581–589. https://doi.org/10.1016/S0141-1136(00)00252-X

Ruczyńska, W. M., Szlinder-Richert, J., Malesa-Ciećwierz, M., & Warzocha, J. (2011). Assessment of PAH pollution in the southern Baltic Sea through the analysis of sediment, mussels and fish bile. *Journal of Environmental Monitoring*, *13*(9), 2535–2542. https://doi.org/10.1039/c1em10423c

Ruddock, P. J., Bird, D. J., McEvoy, J., & Peters, L. D. (2003). Bile metabolites of polycyclic aromatic hydrocarbons (PAHs) in European eels Anguilla anguilla from United Kingdom estuaries. *Science of the Total Environment*, *301*(1–3), 105–117. https://doi.org/10.1016/S0048-9697(02)00292-9

Sarasquete, C., & Segner, H. (2000). Cytochrome P4501A CYP1A in teleostean fishes. *The Science of the Total Environment*, *247*, 313–332.

Scott, J. A., Incardona, J. P., Pelkki, K., Shepardson, S., & Hodson, P. V. (2011). AhR2-mediated, CYP1A-independent cardiovascular toxicity in zebrafish (Danio rerio) embryos exposed to retene. *Aquatic Toxicology*, *101*(1), 165–174. https://doi.org/10.1016/j.aquatox.2010.09.016

Sette, C. B., Pedrete, T. D. A., Felizzola, J., Nudi, A. H., Scofield, A. de L., & Wagener, A. de L. R. (2013). Formation and identification of PAHs metabolites in marine organisms. *Marine Environmental Research*, *91*, 2–13. https://doi.org/10.1016/j.marenvres.2013.02.004

Singh, J., & Wiebel, F. J. (1979). A Highly Sensitive and Rapid Fluorometric Assay for Using 3-Hydroxybenzo(a)pyrene as Substrate. *Analytical Biochemistry*, *98*(2), 394–401. https://doi.org/10.1016/0003-2697(79)90158-1

Snyder, S. M., Pulster, E. L., & Murawski, S. A. (2019). Associations Between Chronic Exposure to Polycyclic Aromatic Hydrocarbons and Health Indices in Gulf of Mexico Tilefish (Lopholatilus chamaeleonticeps) Post Deepwater Horizon. *Environmental Toxicology and Chemistry*, *38*(12), 2659–2671. https://doi.org/10.1002/etc.4583

Snyder, S. M., Pulster, E. L., Wetzel, D. L., & Murawski, S. A. (2015). PAH Exposure in Gulf of Mexico Demersal Fishes, Post- Deepwater Horizon. *Environmental Science and Technology*, *49*(14), 8786–8795. https://doi.org/10.1021/acs.est.5b01870

Sol, S. Y., Johnson, L. L., Horness, B. H., & Collier, T. K. (2000). Relationship between oil exposure and reproductive parameters in fish collected following the Exxon Valdez oil spill. *Marine Pollution Bulletin*, *40*(12), 1139–1147. https://doi.org/10.1016/S0025-326X(00)00074-6

Solé, M., Manzanera, M., Bartolomé, A., Tort, L., & Caixach, J. (2013). Persistent organic pollutants (POPs) in sediments from fishing grounds in the NW Mediterranean: Ecotoxicological implications for the benthic fish Solea sp. *Marine Pollution Bulletin*, *67*(1–2), 158–165. https://doi.org/10.1016/j.marpolbul.2012.11.018

Sørensen, L., Sørhus, E., Nordtug, T., Incardona, J. P., Linbo, T. L., Giovanetti, L., Karlsen, Ø.,

& Meier, S. (2017). Oil droplet fouling and differential toxicokinetics of polycyclic aromatic hydrocarbons in embryos of Atlantic haddock and cod. *PLoS ONE*, *12*(7), 1–26. https://doi.org/10.1371/journal.pone.0180048

Struch, R. E., Pulster, E. L., Schreier, A. D., & Murawski, S. A. (2019). Hepatobiliary Analyses Suggest Chronic PAH Exposure in Hakes (Urophycis spp.) Following the Deepwater Horizon Oil Spill. *Environmental Toxicology and Chemistry*, *38*(12), 2740–2749. https://doi.org/10.1002/etc.4596

Sturve, J., Balk, L., Liewenborg, B., Adolfsson-Erici, M., Förlin, L., & Carney Almroth, B. (2014). Effects of an oil spill in a harbor assessed using biomarkers of exposure in eelpout. *Environmental Science and Pollution Research*, *21*(24), 13758–13768. https://doi.org/10.1007/s11356-014-2890-z

Sundt, R. C., Beyer, J., Vingen, S., & Sydnes, M. O. (2011). High matrix interference affecting detection of PAH metabolites in bile of Atlantic hagfish (Myxine glutinosa) used for biomonitoring of deep-water oil production. *Marine Environmental Research*, *71*(5), 369–374. https://doi.org/10.1016/j.marenvres.2011.04.006

Sundt, R. C., & Bjöerkblom, C. (2011). Effects of produced water on reproductive parameters in prespawning atlantic cod (Gadus morhua). *Journal of Toxicology and Environmental Health - Part A: Current Issues*, *74*(7–9), 543–554. https://doi.org/10.1080/15287394.2011.550563

Szlinder-Richert, J., Nermer, T., & Szatkowska, U. (2014). PAH metabolites in European eels (Anguilla anguilla) as indicators of PAH exposure : Different methodological approaches. *Science of the Total Environment, The*, *496*, 84–91. https://doi.org/10.1016/j.scitotenv.2014.07.037

Tairova, Z. M., Strand, J., Chevalier, J., & Andersen, O. (2012). PAH biomarkers in common eelpout (Zoarces viviparus) from Danish waters. *Marine Environmental Research*, *75*, 45–53. https://doi.org/10.1016/j.marenvres.2011.09.005

Thomas, P. (1990). Teleost model for studying the effects of chemicals on female reproductive endocrine function. *Journal of Experimental Zoology*, *256*(S4), 126–128.

https://doi.org/10.1002/jez.1402560421

Tierney, K. B., Kennedy, C. J., Gobas, F., Gledhill, M., & Sekela, M. (2013). Organic Contaminants and Fish. In K. B. Tierney, A. P. Farrell, & C. J. Brauner (Eds.), *Organic Chemical Toxicology of Fishes* (First Edit, Vol. 33, pp. 1–52). Elsevier Inc. https://doi.org/10.1016/B978-0-12-398254-4.00001-7

Tintos, A., Gesto, M., Alvarez, R., Míguez, J. M., & Soengas, J. L. (2006). Interactive effects of naphthalene treatment and the onset of vitellogenesis on energy metabolism in liver and gonad, and plasma steroid hormones of rainbow trout Oncorhynchus mykiss. *Comparative Biochemistry and Physiology - C Toxicology and Pharmacology*, *144*(2), 155–165. https://doi.org/10.1016/j.cbpc.2006.07.009

Tintos, A., Gesto, M., Míguez, J. M., & Soengas, J. L. (2007). Naphthalene treatment alters liver intermediary metabolism and levels of steroid hormones in plasma of rainbow trout (Oncorhynchus mykiss). *Ecotoxicology and Environmental Safety*, *66*(2), 139–147. https://doi.org/10.1016/j.ecoenv.2005.11.008

Tomy, G. T., Halldorson, T., Chernomas, G., Bestvater, L., Danegerfield, K., Ward, T., Pleskach, K., Stern, G., Atchison, S., Majewski, A., Reist, J. D., & Palace, V. P. (2014). Polycyclic aromatic hydrocarbon metabolites in Arctic cod (Boreogadus saida) from the Beaufort Sea and associative fish health effects. *Environmental Science and Technology*, *48*(19), 11629–11636. https://doi.org/10.1021/es502675p

van den Hurk, P., Edhlund, I., Davis, R., Hahn, J. J., McComb, M. J., Rogers, E. L., Pisarski, E., Chung, K., & DeLorenzo, M. (2020). Lionfish (Pterois volitans) as biomonitoring species for oil pollution effects in coral reef ecosystems. *Marine Environmental Research*, *156*(February), 104915. https://doi.org/10.1016/j.marenvres.2020.104915

van den Hurk, P., & Haney, D. C. (2017). Biochemical effects of pollutant exposure in fish from urban creeks in Greenville, SC (USA). *Environmental Monitoring and Assessment*, *189*(5). https://doi.org/10.1007/s10661-017-5918-2

Van Veld, P. A., & Nacci, D. E. (2008). Toxicity resistance. In R. T. Di Giulio & D. E. Hinton (Eds.), *The Toxicology of Fishes* (1st ed., pp. 597–642). Taylor & Francis Group.

https://doi.org/10.1201/9780203647295

Vethaak, A. D., Baggelaar, P. K., van Lieverloo, J. H. M., & Ariese, F. (2016). Decadal Trends in Polycyclic Aromatic Hydrocarbon (PAH) Contamination Assessed by 1-Hydroxypyrene in Fish Bile Fluid in the Netherlands: Declining in Marine Waters but Still a Concern in Estuaries. *Frontiers in Marine Science*, *3*(November), 1–13. https://doi.org/10.3389/fmars.2016.00215

Vethaak, A. D., Davies, I. M., Thain, J. E., Gubbins, M. J., Martínez-Gómez, C., Robinson, C. D., Moffat, C. F., Burgeot, T., Maes, T., Wosniok, W., Giltrap, M., Lang, T., & Hylland, K. (2017). Integrated indicator framework and methodology for monitoring and assessment of hazardous substances and their effects in the marine environment. *Marine Environmental Research*, *124*, 11–20. https://doi.org/10.1016/j.marenvres.2015.09.010

Vogelbein, W. K., Van Veld, P. A., Huggett, R. J., & Fournie, J. W. (1990). Hepatic Neoplasms in the Mummichog Fundulus heteroclitus from a Creosote-contaminated Site. *Cancer Research*, *50*(18), 5978–5986.

Vuontisjärvi, H., Keinänen, M., Vuorinen, P. J., & Peltonen, K. (2004). A comparison of HPLC with fluorescence detection and fixed wavelength fluorescence methods for the determination of polycyclic aromatic hydrocarbon metabolites in fish bile. *Polycyclic Aromatic Compounds*, *24*(4–5), 333–342. https://doi.org/10.1080/10406630490468478

Vuorinen, P. J., Keinänen, M., Vuontisjärvi, H., Baršiene, J., Broeg, K., Förlin, L., Gercken, J., Kopecka, J., Köhler, A., Parkkonen, J., Pempkowiak, J., & Schiedek, D. (2006). Use of biliary PAH metabolites as a biomarker of pollution in fish from the Baltic Sea. *Marine Pollution Bulletin*, *53*(8–9), 479–487. https://doi.org/10.1016/j.marpolbul.2005.11.020

Wariaghli, F., Kammann, U., Hanel, R., & Yahyaoui, A. (2015). PAH metabolites in bile of european eel (Anguilla anguilla) from Morocco. *Bulletin of Environmental Contamination and Toxicology*, *95*(6), 740–744. https://doi.org/10.1007/s00128-015-1586-5

Whyte, J. J., Herbert, K. L., Karrow, N. A., Dixon, D. G., Sivak, J. G., & Bols, N. C. (2000). Effect of maintaining rainbow trout in creosote microcosms on lens optical properties and liver 7-ethoxyresorufin-O-deethylase (EROD) activity. *Archives of Environmental*

Contamination and Toxicology, *38*(3), 350–356. https://doi.org/10.1007/s002449910046

Willett, K. L., Gardinali, P. R., Lienesch, L. A., & Di Giulio, R. T. (2000). Comparative Metabolism and Excretion of Benzo(a)pyrene in 2 Species of Ictalurid Catfish. *Toxicological Sciences*, *58*(1), 68–76. https://doi.org/10.1093/toxsci/58.1.68

Williams, C. A. H. (1994). *Toxicity Resistance in Mummichog (Fundulus heteroclitus) from a Chemically Contaminated Environment*.

Willis, A. M., & Oris, J. T. (2014). Acute photo-induced toxicity and toxicokinetics of single compounds and mixtures of polycyclic aromatic hydrocarbons in zebrafish. *Environmental Toxicology and Chemistry*, *33*(9), 2028–2037. https://doi.org/10.1002/etc.2648

Yanagida, G. K., Anulacion, B. F., Bolton, J. L., Boyd, D., Lomax, D. P., Paul Olson, O., Sol, S. Y., Willis, M., Ylitalo, G. M., & Johnson, L. L. (2012). Polycyclic aromatic hydrocarbons and risk to threatened and endangered Chinook Salmon in the lower Columbia River Estuary. *Archives of Environmental Contamination and Toxicology*, *62*(2), 282–295. https://doi.org/10.1007/s00244-011-9704-9

Zhu, S., Li, L., Thornton, C., Carvalho, P., Avery, B. A., & Willett, K. L. (2008). Simultaneous determination of benzo[a]pyrene and eight of its metabolites in Fundulus heteroclitus bile using ultra-performance liquid chromatography with mass spectrometry. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*, *863*(1), 141–149. https://doi.org/10.1016/j.jchromb.2008.01.018

Chapter 2: Investigation of dose-dependent responses of biliary polycyclic aromatic compounds (PACs) in fathead minnows (*Pimephales promelas*) following exposure to water accommodated fractions of diluted bitumen

Abstract

Monitoring of biliary polycyclic aromatic compound (PAC) concentrations is used globally to assess short-term PAC exposure in fish affected by anthropogenic pollution and oil spills. Dose-dependent responses have been identified in many species of fish but have yet to be demonstrated in fathead minnows (*Pimephales promelas*) from exposure to a mixture of PACs. Fathead minnows are an ideal candidate for biliary monitoring given their wide geographic range in North America and frequent use in laboratory studies. This study attempted to identify dose-dependent responses of phenanthrene, pyrene, chrysene, and benzo[a]pyrene metabolites following exposure to water accommodated fractions of diluted bitumen with concentrations of 0%, 3%, 10%, and 30%. Following a seven day exposure, metabolites of phenanthrene, pyrene, chrysene were identified by HPLC/F in the gall bladders of experimental fish. A significant and positive relationship was observed between phenanthrol metabolites and aqueous phenanthrene concentrations in WAFs. Metabolites 1-pyrenol and 2-chrysenol were detected in most fish, but these compounds did not demonstrate a dose-dependent response to aqueous parent PAC concentrations. The lack of response of 1-pyrenol and 2-chrysenol was due to either biological or experimental factors.

2.1 Introduction

Polycyclic aromatic compounds (PAC) are ubiquitous contaminants in aquatic environments that can pose a threat to fish health. In fish, efficient metabolism of PACs can make tissue burden analysis unreliable for assessing exposure, making biliary PAC metabolites analysis an essential technique for assessing short-term exposure (Budzinski et al., 2004; Dearnley et al., 2020). The motivations for quantifying these compounds are diverse and include commercial pollution assessment, toxicological lab studies, and oil spill recovery monitoring. A dose-dependent response between PAC exposure and biliary metabolite concentrations has been demonstrated for many species of fish in numerous studies (Beyer et al., 2010). Such a relationship, however, has not yet been reported in fathead minnows (*Pimephales promelas*). There are several important motivations for using fathead minnows in this exposure. First, the species has been used extensively in regulatory testing and research (Ankley & Villeneuve, 2006) providing a wealth of applicable data. Furthermore, the species' extensive habitat range imparts them with a high potential for monitoring PAC exposure in the event of a freshwater oil spill in North America. Additionally, they are easily sexed, have well characterized reproductive biology, and are amenable to being cultured in a lab (Ankley & Villeneuve, 2006).

In fish, PACs are generally metabolized by enzymes of the cytochrome P4501A (CYP1A) family. This metabolism occurs in two phases: in the first phase, an oxygen atom is added into the aromatic portion of the PAC to create an epoxide and in the second phase a large polar biomolecule is attached to the oxygen atom to facilitate excretion (Collier et al., 2013; Tierney et al., 2013). These biomolecules include glucuronic acid, sulfate and glutathione (Ikenaka et al., 2013). While CYP1A enzymes are found in various locations within fish, they are found at their highest concentrations in the liver thereby leading to excretion into the gall bladder, though metabolites can also be found in urine (Dearnley et al., 2020; Sarasquete & Segner, 2000).

Many studies have demonstrated correlations between PAC exposure and biliary PAC concentrations in fish and researched species include Atlantic cod (*Gadus Morhua*), lionfish (*Pterois volitans*), eelpout (*Zoarces viviparus*), and English sole (*Parophrys vetulus*) among others (Aas et al., 2000; Beyer et al., 2010; Collier & Varanasi, 1991; Sturve et al., 2014; van den Hurk et al., 2020). In fathead minnows a link between biliary metabolite concentration and aqueous benzo[a]pyrene concentration was recently identified (Grimard et al., 2020), but dose-

dependent responses have not yet been identified for the metabolites of this present study, nor has a response to a mixture of PACs been observed. Research involving fathead minnow exposure to benzo[a]anthracene in water over the span of 5-336 h attributed 87% of total elimination of the compound in the fish to biotransformation (De Maagd et al., 1998) – a process that results in biliary metabolites. A sigmoidal increase in both CYP1A and glutathione S-transferase mRNA – enzymes involved in the metabolism of PACs – in response to total petroleum hydrocarbon concentration was observed in fathead minnows exposed to diluted bitumen (dilbit) water accommodated fractions (WAFs; Alsaadi et al., 2018).

Quantitation of biliary PAC metabolites is motivated by the toxic effects PACs exert on fish. There is a vast body of literature on the subject, with no shortage of research on fathead minnows. Lab studies on the species have found PACs, sometimes individually and sometimes as a mixture, cause effects such as decreased reproductive output and reduced survival, teratogenic effects, mucosal cell damage, and reduced egg viability (Diamond et al., 1995; Parrott et al., 2019; Tilghman Hall & Oris, 1991; Weinstein et al., 1997). Contemporary research has focused largely on oil products and byproducts, with LC50 and IC25 having been determined on fathead minnows and their larvae for a variety of fresh and unweathered dilbits (Barron et al., 2018; Robidoux et al., 2018). This abundance of toxic effects in fathead minnows frame the importance of establishing a dose-dependent response of biliary PAC metabolites to PAC exposure.

In this study, juvenile fathead minnows were exposed to three different concentrations of dilbit WAFs. After a seven day exposure, gall bladders were collected and the bile was analyzed by HPLC/F for four different decoupled phase II metabolites – phenanthrols, 1-pyrenol, 2-chrysenol, and 3-benzo[a]pyrenol. This tested the hypothesis that the concentration of biliary PAC metabolites in fathead minnows correlate with the concentration of their respective parent PACs in the WAFs.

2.2 Methods and Materials

2.2.1 Chemicals

1-pyrenol (>98.0%, 1-PYR) and 2-naphthol (>99.0%, 2-NAP) were purchased from TCI America (Portland, OR). 1-phenanthrol (98.0%, 1-PHE), 2-phenanthrol (99.4%), 3-phenanthrol (98.0%), 4-phenanthrol (99.2%), 9-phenanthrol (97.0%), 2-chrysenol (98.0%, 2-CHR), and 3-benzo[a]pyrenol (95.1%, 3-BaP) were acquired from Toronto Research Chemicals (Toronto, ON). B-glucuronidase (from *Helix pomatia*, aqueous solution ≥100,000 units/mL with ≤7,500 units/mL sulfatase) was purchased from Sigma-Aldrich Canada (Oakville, ON). Methanol (HPLC grade) and ascorbic acid were each acquired from Fisher Scientific (Ottawa, ON). Calibration PAC stocks were prepared in methanol and stored at -80°C, while calibration standards were prepared in 50/50 methanol/water (v/v) containing 0.5% ascorbic acid and stored at -20°C. Standards and stocks were stored in amber vials and exposure to light was minimized. The 0.5% ascorbic acid proved necessary to prevent degradation during analysis. Ascorbic acid was also used create a buffer (0.5% ascorbic acid w/w, pH = 5.0) which was stored in the dark at 4°C and remade monthly. Diluted bitumen was provided by the Canadian Association of Petroleum Producers (CAPP) from pipeline certified stocks.

2.2.2 Test organisms

Details regarding fish exposure and husbandry have previously been described by Bulloch (2022). Briefly, juvenile fathead minnows aged <3 months were obtained from Aquatic Research Organisms (Hampton, NH, USA) and housed at the Aquatic Toxicology Research Facility at the University of Saskatchewan (Saskatoon, Canada). Fish acclimation occurred in a circular holding tank equipped with a flow-through system and filled with dechlorinated municipal tap water maintained at 25±1°C. Minnows were fed 2% total body weight of 70% thawed Hikari frozen bloodworms and 30% Nutrifin Max Goldfish flakes twice daily and subject to a photoperiod of 12h:12h light/dark cycling. Following the acclimation period, sexually immature (sub-adult) fish were randomly selected for exposure groups; fish with visible tubercles, mating coloration, or ovipositors were excluded.

2.2.3 Preparation of water accommodated fractions and exposure water

Preparation of the water accommodated fraction (WAF) stock was adapted from the procedures described by (Singer et al., 2001). Briefly, one part diluted bitumen (dilbit) was added to 9 parts dechlorinated municipal tap water in a 1 L aspiration flask and mixed with a magnetic stir bar at 60 revolutions per minute. After 18 h of stirring, the solution was allowed to settle for 1 h before

being decanted into an amber glass bottle. WAF stocks were then diluted to achieve target concentrations for dilbit exposures. Nominal experimental PAC concentrations were determined from GC-MS/MS measurements of matrix matched standards with nominal concentrations of 0, 3, 6, 12.5, 25, and 50% WAF. Details can be found in Bulloch (2022).

2.2.4 Dilbit exposure

WAF concentrations of 0% (control), 3.0% (low), 10.0% (medium), and 30.0% (high) were each prepared and added to 1 L borosilicate glass beakers. Juvenile fathead minnows were randomly assigned to the four experimental groups, with 2 fish per beaker. Aeration was provided continuously with aeration stones and the beakers were placed in a water bath to maintain temperatures at 25±1°C. For the first 6 days minnows were provided 4% body weight of food once daily and were fasted for the final 48 h to allow for bile accumulation. Static water changes were performed every 48h by siphoning 50% of the holding water from each beaker and replacing it with freshly prepared 25±1°C WAF solution.

2.2.5 Gall bladder collection and preceding measurements

Upon completion of the 7-day exposure, beakers were transferred to an insulated cooler for ultransonography and the fish were anesthetized using 10 mg/L Aquacalm (metomidate hydrochloride; Syndal Canada, Nanaimo, BC). Once stage 3 light anesthesia (Zahl et al., 2011) was reached, additional biometrics (see Bulloch, 2022 for details) were recorded and fish were then euthanized with an overdose of MS-222 (~0.4 g/L). Tissues were immediately harvested, and gall bladders were placed in sterile 0.5 mL microcentrifuge tubes and snap frozen in liquid nitrogen. They were then transported back to Winnipeg, MB on dry ice and stored at -80°C with exposure to light minimized at all times.

2.2.6 Preparation of bile samples

Preparation and HPLC-F analysis of bile followed methods of Jonsson et al (2003) and Grung et al (2016), each acknowledging Krahn et al (1987, 1992) as providing an initial analytic framework. Due to the small size of each gallbladder, bile was determined by weight. For analysis, gallbladders were allowed to thaw and were weighed on an analytical balance (0.0001g) while still in the microcentrifuge tube used for storage. Once weighed, each microcentrifuge tube had 50 μL of 0.5% ascorbic acid buffer (pH: 5.0) added to it, was briefly

vortexed, and was placed in a centrifuge for 5 min at 9,600 g. The gallbladders were then minced with a pipette tip to ensure full extraction of bile and the resulting bile/buffer mixture was transferred to a 1.5 mL microcentrifuge tube containing 5 μL of β-glucuronidase solution, vortexed and placed in a 37°C incubation bath in the dark for 2-5 hours. Following incubation, 50 μL of methanol containing 0.5% ascorbic acid was added, the tubes were briefly vortexed and then centrifuged for 5 min at 9,600 g with the supernatant analyzed. During incubation, tubes used for gall bladder storage were dried and re-weighed to determine the mass of the empty gallbladders and to allow calculation of bile mass. Experimental work was permitted by University of Saskatchewan animal use protocol # 20020084.

2.2.7 HPLC-F analysis

The HPLC system consisted of a Varian ProStar 410 Autosampler, Varian ProStar 210 pumps, and a Varian ProStar 363 fluorescence detector. Optimum excitation/emission (ex/em) wavelength pairs for each analyte were determined by direct injection into the fluorescence detector with a syringe pump while scanning through a range of emission or excitation wavelengths. The optimal ex/em pairs used were 275/361 nm for 2-naphthol, 300/383 nm for 1-phenanthrol, 340/384 nm for 1-pyrenol, 265/380 nm for 2-chrysenol, and 381/435 nm for 3-benzo[a]pyrenol. Ultrapure water (A) and methanol (B) comprised the mobile phase for the elution. The elution profile was as follows: 0-1 minute, isocratic 38% B; 1-35 min, 38-95% B linear gradient; 35-37 min, isocratic 95% B; 37-45 min, 95-38% B linear gradient; 45-50 min, isocratic 38% B. The flow rate was 0.30 mL/min. The chromatography column was held at 40°C during analysis and the autosampler delivered 10 μL for each injection. The samples were run on ZORBAX RR Eclipse XDB-C18, 80Å, 2.1 x 50 mm, 3.5 μm columns.

2.2.8 Calculations

The concentrations of 1-pyrenol were calculated from an average response factor obtained from a 10 ng/mL mixed standard containing all analytes run at the beginning, middle, and end of each sample run. The concentration of 2-chrysenol was calculated from the recovery of the metabolite in a reference sample spiked to 10 ng/mL with each analyte. The concentration of phenanthrols was estimated based on the average response factor of 1-phenanthrol in the 10 ng/mL mixed standard. When other isomers were detected, the response factor of 1-phenanthrol was corrected with the relative response factor of the isomer in question. When isomers

coeluted, their concentrations were estimated based on the relative amount of each co-eluting isomer in fully resolved samples. For calculations, the density of bile was assumed to be 1.00 g/mL, which slightly higher than the 0.975 g/mL reported for sheepshead minnow (*Cyprinodon variegatus*; G. Jonsson et al., 2003).

2.2.9 Statistical Analysis

Statistical tests were performed using RStudio software with statistical significance accepted at $p < 0.05$. A least squares linear regression was run for the concentration of each metabolite as the response variable and its parent compound as the predictor. Differences among treatments for each metabolite were evaluated using ANOVA followed by a post hoc Tukey's test, when applicable. The assumptions of normality and homogeneity of variance were confirmed graphically using histograms and quantile-quantile plots of the residuals and a heteroscedasticity plot of the residuals, respectively. Metabolite data was natural log-transformed as necessary to meet the assumptions of the models and achieve the best possible fit.

2.3 Results

Fish exposure to the various WAF concentrations proceeded as designed, with metabolites generally found above the limits of detection in each treatment group (Figure 2.1). 3-benzo[a]pyrenol was the exception and was not detected above the LOQ and, due to interference, was not reliably detected above LOD in any of the samples. Discrepancies in the number of bile samples between the different experimental groups was generally a function of successful gall bladder retrievability from the test organisms not fish mortalities. Additional information on percent detections and bile quantities are available in Supplemental Information I.

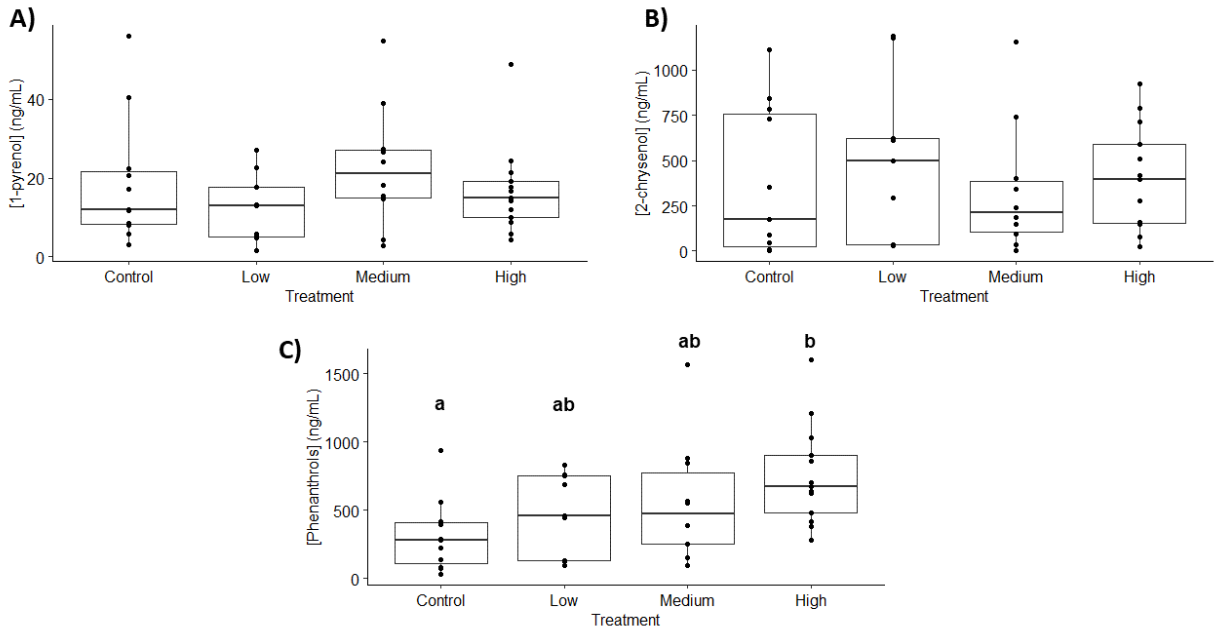


Figure 2.1: Concentrations of targeted bile metabolites (1-pyrenol (A), 2-chrysenol (B), and phenanthrols (C)) in control and dilbit exposed fathead minnows. Box plots show the 25th percentile (bottom hinge), median (centre line), 75th percentile (top hinge), and whiskers extending to 1.5 interquartile range. Significant differences ($p<0.05$) between treatments, when detected, are denoted by lowercase letters.

2.3.1 Method Validation

The analytic method was validated according to Eurachem guidelines (Magnusson, B.; Örnemark, 2014). The determined limits of detection (LOD) and limits of quantitation (LOQ) are shown in Table 2.1. LODs and LOQs were obtained from replicate measurements of each analyte in reference material (archived walleye (*Sander vitreus*) bile from Lake Winnipeg) after spiked reagent blanks yielded poor, unrepresentative recoveries in comparison. A high concentration of 1-pyrenol and non-detect of 2-chrysenol in the reference material prevented accurate determination of their LOD and LOQ. For each analyte, linearity was demonstrated over the range 1-250 ng/mL (see Supplemental Information I). The method was shown to be robust to changes in incubation times (1-8 hours), bile volume (3-10 µL; 5 µL was the default volume for method development),

Analyte	Limit of detection (ng/mL)	Limit of detection (pg)	Limit of quantitation (ng/mL)	Limit of quantitation (pg)
1-Phenanthrol	0.14	1.45	0.48	4.82
3-BaPol	0.05	0.54	0.18	1.81

Table 2.1: Limits of detection and quantitation (LOD/LOQ) for the analytes of the exposure study. These quantities reflect concentrations found in the bile/buffer/methanol solution run on the column. LOD/LOQ in bile will vary from sample to sample based on the volume of bile extracted. Under the method, the reported LOQs correspond to concentrations of 9.54 ng/mL (1-phenanthrol) and 3.58 ng/mL (3-benzo[a]pyrenol) in a 5 µL sample of bile.

and chromatography column changes (Waters XSelect HSS T3 XP Column, 100Å, 2.5 µm, 2.1 mm X 50 mm, specifically). While 2-naphthol standards demonstrated excellent potential for analysis, interference in bile samples precluded their quantitation in this study. Triphenylamine, often used as an internal standard in HPLC-F biliary metabolite analysis (Holth et al., 2008; Nahrgang et al., 2013), was tested for use in this method, but demonstrated unsuitably low recoveries. In its absence, each sample run contained a sample of reference bile and a sample of reference bile spiked to achieve an additional 10 ng/mL of each metabolite, which assessed metabolite recoveries and the efficiency of the enzymatic decoupling of parent metabolites.

2.3.2 Phenanthrols

		Phenanthrene	**Phenanthrols (ng/mL)**				
Dose	**Fish**	**(ng/L)**	**Mean**	**Median**	**Std dev**	**Min**	**Max**
Control	11	1.72	278.4	312.0	264.9	29.2	939.1
Low	9	16.4	477.4	459.4	299.2	97.2	830.2
Medium	10	52.7	556.0	473.5	446.2	99.3	1566
High	13	157.4	754.0	676.4	367.8	279.0	1599

Table 2.2: Summary data for phenanthrol metabolites in exposed fathead minnows and experimental aqueous phenanthrene concentrations

There was a significant and positive relationship between treatment and the concentration of phenanthrol metabolites ($F(3,30) = 4.08$, $p = 0.013$). Log-transformed metabolite data was used for this analysis. The only significant difference in metabolite concentrations was found between

the high and control doses (p = 0.007). A regression of log-transformed metabolite and water concentrations resulted in the best fitting model for the data (Figure 2.2). It yielded a significant positive correlation between aqueous phenanthrene concentration and phenanthrol metabolite concentration. The fitted regression model was: ln[Phenanthrols] = 0.245*ln[water phenanthrene] + 5.20. The overall regression was statistically significant (R^2 = 0.211, F(1,41) = 12.22, p = 0.002), with ln[water phenanthrene] predicting ln[phenanthrol] (β = 0.245±0.142 (95% CI), p = 0.002).

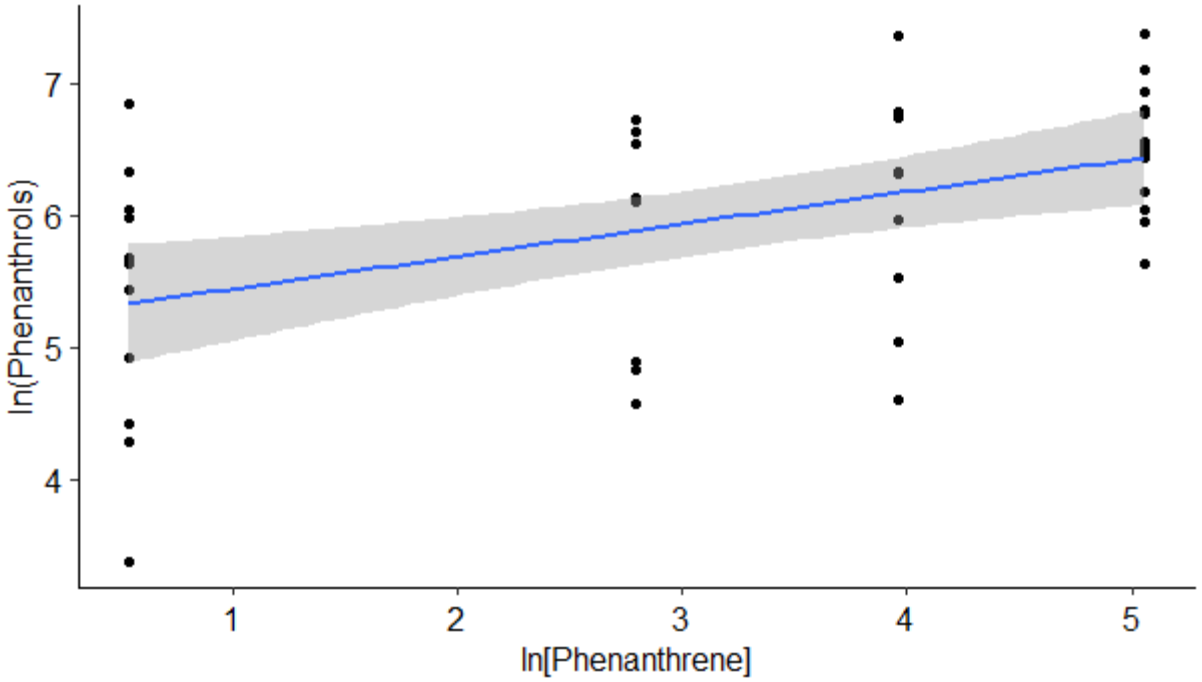


Figure 2.2: Least squares regression of aqueous phenanthrene predicting biliary phenanthrol concentrations in fathead minnows across treatments. The 95% confidence interval of the regression is shaded.

2.3.3 2-chrysenol

There was no significant effect of treatment on 2-chrysenol concentration (p = 0.810). Similarly, no significant relationship was observed between aqueous chrysene concentration and biliary metabolites (p = 0.254). The ANOVA was run on the original data, while log-transformed data was used for the regression (Table 2.3).

Dose	Fish	Chrysene (ng/L)	2-chrysenol (ng/mL) Mean	Median	Std Dev	Low	High
Control	11	0.45	376.2	176.6	411.8	ND	1112
Low	9	0.80	498.4	500.0	455.2	27.9	1186
Medium	10	1.69	333.9	212.6	360.4	4.7	1157
High	13	5.37	397.6	396.1	291.1	23.0	924.4

Table 2.3: Summary data for 2-chrysenol metabolites in exposed fathead minnows and experimental aqueous chrysene concentrations. ND ≡ no detect.

2.3.4 1-pyrenol

Dose	Fish	Pyrene (ng/L)	1-pyrenol (ng/mL) Mean	Median	Std dev	Low	High
Control	11	0.47	18.7	12.1	16.2	3.0	56.0
Low	9	0.87	12.3	13.1	8.8	1.5	27.2
Medium	10	2.27	22.7	21.2	15.6	2.8	54.7
High	13	3.57	16.8	15.0	11.3	4.4	48.8

Table 2.4: Summary data for 1-pyrenol metabolites in exposed fathead minnows and experimental aqueous pyrene concentrations.

Much like 2-chrysenol, ANOVA showed no significant effect of treatment on 1-pyrenol concentration ($p = 0.404$). No significant relation between water concentration of pyrene and metabolite concentration was observed in the experimental fish ($p = 0.447$). The ANOVA was run on the original data, while log-transformed data was used for the regression (Table 2.3).

2.4 Discussion

Groups of fathead minnows were exposed to a concentration gradient of aqueous PACs to see if a dose-dependent of response of biliary PAC concentration was elicited. A dose-dependent response was observed for phenanthrene and its metabolites, but not for pyrene nor chrysene. The dose-dependent response observed for aqueous phenanthrene exposures adds to a large body of literature supporting the use of PAC metabolites as suitable biomarkers for short-term PAC exposure and validates its use in small bile samples obtained from fathead minnows. The lack of response observed for pyrene and chrysene may be due to biological or experimental factors which are explored below.

For phenanthrene and its suite of monohydroxylated metabolites, although a statistically significant dose-dependent response was observed, many other factors influenced biliary metabolite concentration. This was demonstrated by both the relatively low R^2 value of the regression ($R^2 = 0.211$) and the large coefficients of variation (CV). Each parent PAC/metabolite pair studied had a CV > 85% in at least one of the treatment levels and the large variation is evident in Figure 2.1. Many factors influence biliary metabolite concentrations; some are exposure related, others biological. The feeding status of fish has been identified as one of these, with a recently emptied gallbladder likely to be filled with more dilute bile. As the fish continues to process PACs, bile metabolites accumulate until feeding when the bile is expelled to the lumen of the gut and the process starts anew (Dearnley et al., 2020). While starving the fish for the final 48 hours of the exposure was intended to control for this, the process may still have contributed to the variation in data, especially if food was not immediately consumed which would introduce variability into the starvation times of the fish. Several methods (bile pigment quantitation, most commonly) have been used to help account for the post-feeding bile dilution, though in practice they have been applied with mixed success for reducing variation in PAC metabolite concentrations (Dearnley et al., 2020). Nevertheless, the small quantities of bile retrievable from the fathead minnows and the method in which they were collected precluded the use of bile pigments for normalizing bile concentrations in this study. Bile masses of < 2 mg from experimental gall bladders were not uncommon. The food itself may represent an additional PAC input and may help to explain non-zero concentrations in the controls, but should not contribute to variation between experimental groups. The use of juvenile fish should have prevented variation due to differences in the life stage or sex of the fish in the study.

Work on examining and establishing dose-dependent biliary metabolite responses in fish, both in the wild and in laboratories, dates back to at least 1984, and new species continue to be validated for use in monitoring programs (H. Jonsson et al., 2010; Krahn et al., 1984; van den Hurk et al., 2020). Thus, any contrary findings such as the ones found in this study for pyrene and chrysene merit additional scrutiny. Whereas the nominal phenanthrene concentrations in the different exposures ranged from 1.72-157.4 ng/L, the ranges for chrysene and pyrene were 0.45-5.37 ng/L and 0.47-3.57 ng/L, respectively; the range of exposure of fish to phenanthrene was more than an order of magnitude greater than the other two parent PACs. This compressed range for the four

ringed compounds may help explain why no statistical difference between treatment groups was observed for them. Literature gives conflicting results as to whether this concentration difference is sufficient to observe a dose-dependent response in fish. For instance, a study exposing Atlantic cod (*Gadus morhua*) to a mixture of aqueous PACs and alkyl phenols found a significant difference in biliary 1-pyrenol concentrations in fish subject to 0.6 ng/L pyrene compared to those whose water contained 6.0 ng/L pyrene after 2 months of exposure (Grung et al., 2009). A study of fathead minnows exposed to aqueous benzo[a]pyrene concentrations of 30 (low), 80 (medium), and 1340 (high) ng/L, and a control also provides insight (Grimard et al., 2020). The Grimard et al. study did not find a significant difference in 3-benzo[a]pyrenol biliary metabolite concentrations between the control and the lowest treatment after 4, 7, or 14 days. However, after 4 days a significant difference was found between the medium dose and the treatments with lower aqueous concentrations of benzo[a]pyrene. This trend appeared to hold after 7 and 14 days but was not statistically significant, likely due to a small sample size (n=2). Similar results in that study were observed for the glucuronide metabolites (metabolites were not decoupled prior to analysis in that study), though the low treatment was not significantly different than the medium treatment after 4 days. Grimard et. al (2020) also noted the *in vitro* clearance rates of benzo[a]pyrene in their experimental fish were comparable to other species such as rainbow trout (*Oncorhynchus mykiss*) and bluegill sunfish (*Lepomis macrochirus*) and dispel the notion that CYP1A activity and inducibility in fathead minnows is lower than in other species, which had been previously suggested in literature. If the concentration gradients Grimard et. al (2020) found were necessary to elicit a dose-dependent response of benzo[a]pyrene extend to the PACs of this study, the lack of dose-dependent response in three of the four metabolites in this study are substantiated. However, if the findings of the Atlantic cod study are applicable, other explanations for the lack of dose-dependent response observed in this present study must be explored.

While Grimard et al.'s results (2020) suggest the concentration differences between doses of chrysene and pyrene may have been insufficient to invoke statistical differences in metabolites in this study, experimental factors may also have contributed to these results. Once again, their work may offer insight into the absence of dose-dependent responses of this study. In Grimard et al. (2020) nominal aqueous benzo[a]pyrene concentrations of 1,300, 4,000, and 12,000 ng/L, were measured at 30, 80, and 1340 ng/L, respectively, in the exposures. The loss of compound

was attributed to sorption to aquaria and food, degradation, and incomplete solubility, with the first two factors being applicable to our study. In this context, the lack of 3-benzo[a]pyrenol observed in our fish is well explained, as the highest nominal concentration (see Supplemental Info I) of benzo[a]pyrene among experimental doses was only 10 ng/L. With metabolite concentrations in this study only available for the WAF and not the experimental tanks, it is unclear whether similar losses were suffered. Sorption losses for benzo[a]pyrene should be higher than for the other parent PACs of this study, which all have lower K_{OW} values (Achten & Andersson, 2015). Nevertheless, even small losses of PACs in this study would result in a incredibly narrow concentration gradients for chrysene and pyrene, neither of which induced a dose-dependent response.

The duration of exposure is unlikely to have impacted results for this study. The significant differences in concentrations observed for phenanthrene metabolites suggest the exposure period was of sufficient duration. This is corroborated by results of several other studies, such as the Grimard et al. (2020) study, in which concentrations of BaP metabolites are similar, and in some cases lower on day 4 of exposure than day 7. Similarly, a study of intraperitoneal injection of benzo[a]pyrene on English sole (*Parophrys vetulus*) found biliary metabolite concentrations peaked after 4 days (Collier & Varanasi, 1991) and work on aqueous exposure of sheepshead minnow (*Cyprinodon variegatus*) showed peak PAC metabolite concentrations are reached within 2-6 days of exposure (G. Jonsson et al., 2004). In sheepshead minnows, this was found for alkylated and unalkylated naphthalenes, alkylated and unalkylated phenanthrenes, and pyrene. Those findings, combined with the results for benzo[a]pyrene in Grimard et al. (2020), mean each compound in this WAF exposure study should have reached the steady state biliary concentration commensurate with the aqueous PAC concentrations. The steady state concentrations will differ based on uptake and excretion rates of the different compounds. Of course, these assumptions are predicated on sufficient activities of CYP1A in the fish to metabolize each parent PAC, but given the extent of metabolism of phenanthrene it's likely pyrene, chrysene, and benzo[a]pyrene were also being metabolized. In fact, in fathead minnows, pyrene is less bioaccumulative than phenanthrene and therefore should be metabolized even more efficiently (Bleeker & Verbruggen, 2009; Carlson et al., 1979). Moreover, noted instances of PAC metabolite accumulation in fish in the absence of CYP1A induction further allay any concerns of sufficient metabolic activity to observe a dose-dependent response in pyrene and

chrysene (Aas et al., 2000; Boleas et al., 1998). Lastly, it is also assumed that a sufficient proportion of each metabolite was excreted into the bile to detect differences in exposure. This was the case for phenanthrols but does not necessarily mean it was the case for the other metabolites.

2.5 Conclusion

After exposure to different concentrations of water accommodated fractions of diluted bitumen, a dose-dependent response was observed for phenanthrene and phenanthrol in fathead minnows. Combined with previous research observing a dose-dependent response for benzo[a]pyrene exposure, this finding validates the use of using biliary metabolites for assessing exposure to selected PACs in fathead minnows. For causes unknown, a dose-dependent response was not identified for chrysene and pyrene and their respective metabolites. Insufficient experimental exposure range, overestimation of exposure range, and lack of enzymatic metabolic induction are among potential explanations for this observation.

2.6 Connection to later chapters

Future chapters of this thesis focus on using biliary metabolites to monitor exposure of fathead minnows to PACs in model oil spills in a natural shoreline environment. Without the dose-dependent response demonstrated in this study, any findings from the subsequent studies would at best come with a qualifier and at worse be inadmissible. Though this study was concerned solely with waterborne exposure, the fish in the following sections were subject to additional routes of exposure, such as ingestion via food and dermal uptake from the sediment. Though these PACs arrived from different sources, their metabolites still ended up in the gall bladder, were analyzed in the bile the same way, and were subject to the same dose-dependent response demonstrated in this chapter. Like this chapter, PAC concentrations in the fish's environment were compared to biliary metabolite concentrations to see if the relationships found in the lab persist in the wild. As importantly, the metabolites concentrations themselves were used to compare PAC exposure between different experimental groups of fish. This would not be possible without the dose-dependent response observed in this chapter.

2.7 References

Aas, E., Beyer, J., & Goksoyr, A. (2000). Fixed wavelength fluorescence (FF) of bile as a monitoring tool for polyaromatic hydrocarbon exposure in fish : an evaluation of compound specificity , inner filter effect and signal interpretation. *Biomarkers*, *5*(1), 9–23. https://doi.org/10.1080/135475000230505

Achten, C., & Andersson, J. T. (2015). Overview of Polycyclic Aromatic Compounds (PAC). *Polycyclic Aromatic Compounds*, *35*(2–4), 177–186. https://doi.org/10.1080/10406638.2014.994071

Alsaadi, F. M., Madison, B. N., Brown, R. S., Hodson, P. V., & Langlois, V. S. (2018). Morphological and molecular effects of two diluted bitumens on developing fathead minnow (Pimephales promelas). *Aquatic Toxicology*, *204*(June), 107–116. https://doi.org/10.1016/j.aquatox.2018.09.003

Ankley, G. T., & Villeneuve, D. L. (2006). The fathead minnow in aquatic toxicology: Past, present and future. *Aquatic Toxicology*, *78*(1), 91–102. https://doi.org/10.1016/j.aquatox.2006.01.018

Barron, M. G., Conmy, R. N., Holder, E. L., Meyer, P., Wilson, G. J., Principe, V. E., & Willming, M. M. (2018). Toxicity of Cold Lake Blend and Western Canadian Select dilbits to standard aquatic test species. *Chemosphere*, *191*, 1–6. https://doi.org/10.1016/j.chemosphere.2017.10.014

Beyer, J., Jonsson, G., Porte, C., Krahn, M. M., & Ariese, F. (2010). Analytical methods for determining metabolites of polycyclic aromatic hydrocarbon (PAH) pollutants in fish bile: A review. *Environmental Toxicology and Pharmacology*, *30*(3), 224–244. https://doi.org/10.1016/j.etap.2010.08.004

Bleeker, E. A. J., & Verbruggen, E. (2009). Bioaccumulation and biomagnification of polycyclic aromatic hydrocarbons in aquatic organisms. In *RIVM Report 601779002*. https://doi.org/10.1016/S0304-4203(96)00042-4

Boleas, S., Fernandez, C., Beyer, J., Tarazona, J. V., & Goksøyr, A. (1998). Accumulation and effects of benzo(a)pyrene on cytochrome P450 1A in waterborne exposed and intraperitoneal injected juvenile turbot (Scophthalmus maximus). *Marine Environmental*

Research, *46*(1–5), 17–20. https://doi.org/10.1016/S0141-1136(98)00022-1

Budzinski, H., Mazéas, O., Tronczynski, J., Désaunay, Y., Bocquené, G., & Claireaux, G. (2004). Link between exposure of fish (Solea solea) to PAHs and metabolites: Application to the "Erika" oil spill. *Aquatic Living Resources*, *17*(3), 329–334. https://doi.org/10.1051/alr:2004040

Bulloch, P. (2022). *Novel, non-lethal analysis of F2-Isoprostanes in the epidermal mucus of wild fathead minnows exposed to diluted bitumen at the IISD - Experimental Lakes Area*. University of Manitoba.

Carlson, R. M., Oyler, A. R., Gerhart, E. H., Caple, R., Welch, K. J., Kopperman, H. L., Bodenner, D., & Swanson, D. (1979). *Implications to the aquatic environment of polynuclear aromatic hydrocarbons liberated from Great Plains coal* (p. 170). United States Environmental Protection Agency.

Collier, T. K., Anulacion, B. F., Arkoosh, M. R., Dietrich, J. P., Incardona, J. P., Johnson, L. L., Ylitalo, G. M., & Myers, M. S. (2013). Effects on Fish of Polycyclic Aromatic Hydrocarbons (PAHS) and Naphthenic Acid Exposures. In K. B. Tierney, A. P. Farrell, & C. J. Brauner (Eds.), *Organic Chemical Toxicology of Fishes* (First Edit, Vol. 33, pp. 195–255). Elsevier Inc. https://doi.org/10.1016/B978-0-12-398254-4.00004-2

Collier, T. K., & Varanasi, U. (1991). Hepatic activities of xenobiotic metabolizing enzymes and biliary levels of xenobiotics in english sole (Parophrys vetulus) exposed to environmental contaminants. *Archives of Environmental Contamination and Toxicology*, *20*, 462–473. http://download.springer.com/static/pdf/171/art%253A10.1007%252FBF01065834.pdf?originUrl=http%253A%252F%252Flink.springer.com%252Farticle%252F10.1007%252FBF01065834&token2=exp=1453740322~acl=%252Fstatic%252Fpdf%252F171%252Fart%2525253A10.1007%2525252FBF01

De Maagd, P. G. J., De Poorte, J., Opperhuizen, A., & Sijm, D. T. H. M. (1998). No influence after various exposure times on the biotransformation rate constants of benzo(a)anthracene in fathead minnow (Pimephales promelas). *Aquatic Toxicology*, *40*(2–3), 157–169. https://doi.org/10.1016/S0166-445X(97)00053-2

Dearnley, J. M., Killeen, C., Davis, R. L., Palace, V. P., & Tomy, G. T. (2020). Monitoring polycyclic aromatic compounds exposure in fish using biliary metabolites. *Critical Reviews in Environmental Science and Technology*, *0*(0), 1–45. https://doi.org/10.1080/10643389.2020.1825895

Diamond, S. A., Oris, J. T., & Guttman, S. I. (1995). Adaptation to fluoranthene exposure in a laboratory population of fathead minnows. *Environmental Toxicology and Chemistry*, *14*(8), 1393–1400. https://doi.org/10.1002/etc.5620140816

Grimard, C., Mangold-Döring, A., Schmitz, M., Alharbi, H., Jones, P. D., Giesy, J. P., Hecker, M., & Brinkmann, M. (2020). In vitro-in vivo and cross-life stage extrapolation of uptake and biotransformation of benzo[a]pyrene in the fathead minnow (Pimephales promelas). *Aquatic Toxicology*, *228*(May). https://doi.org/10.1016/j.aquatox.2020.105616

Grung, M., Holth, T. F., Jacobsen, M. R., & Hylland, K. (2009). Polycyclic aromatic hydrocarbon (PAH) metabolites in Atlantic cod exposed via water or diet to a synthetic produced water. *Journal of Toxicology and Environmental Health - Part A: Current Issues*, *72*(3–4), 254–265. https://doi.org/10.1080/15287390802539210

Grung, M., Petersen, K., Fjeld, E., Allan, I., Christensen, J. H., Malmqvist, L. M. V., Meland, S., & Ranneklev, S. (2016). PAH related effects on fish in sedimentation ponds for road runoff and potential transfer of PAHs from sediment to biota. *Science of the Total Environment*, *566–567*(0349), 1309–1317. https://doi.org/10.1016/j.scitotenv.2016.05.191

Holth, T. F., Nourizadeh-Lillabadi, R., Blaesbjerg, M., Grung, M., Holbech, H., Petersen, G. I., Aleström, P., & Hylland, K. (2008). Differential gene expression and biomarkers in zebrafish (Danio rerio) following exposure to produced water components. *Aquatic Toxicology*, *90*(4), 277–291. https://doi.org/10.1016/j.aquatox.2008.08.020

Ikenaka, Y., Oguri, M., Saengtienchai, A., Nakayama, S. M. M., Ijiri, S., & Ishizuka, M. (2013). Characterization of phase-II conjugation reaction of polycyclic aromatic hydrocarbons in fish species: Unique pyrene metabolism and species specificity observed in fish species. *Environmental Toxicology and Pharmacology*, *36*(2), 567–578. https://doi.org/10.1016/j.etap.2013.05.018

Jonsson, G., Bechmann, R. K., Bamber, S. D., & Baussant, T. (2004). Bioconcentration, biotransformation, and elimination of polycyclic aromatic hydrocarbons in sheepshead minnows (Cyprinodon variegatus) exposed to contaminated seawater. *Environmental Toxicology and Chemistry*, *23*(6), 1538–1548. https://doi.org/10.1897/03-173

Jonsson, G., Beyer, J., Wells, D., & Ariese, F. (2003). The application of HPLC-F and GC-MS to the analysis of selected hydroxy polycyclic hydrocarbons in two certified fish bile reference materials. *Journal of Environmental Monitoring*, *5*(3), 513–520. https://doi.org/10.3233/978-1-60750-938-7-19

Jonsson, H., Sundt, R. C., Aas, E., & Sanni, S. (2010). The Arctic is no longer put on ice: Evaluation of Polar cod (Boreogadus saida) as a monitoring species of oil pollution in cold waters. *Marine Pollution Bulletin*, *60*(3), 390–395. https://doi.org/10.1016/j.marpolbul.2009.10.014

Krahn, M. M., Burrows, D. G., Ylltalo, G. M., Brown, D. W., Wlgren, C. A., Collier, T. K., Sin-Lam, C., & Usha, V. (1992). Mass Spectrometric Analysis for Aromatic Compounds in Bile of Fish Sampled after the Exxon Valdez Oil Spill. *Environmental Science and Technology*, *26*(1), 116–126. https://doi.org/10.1021/es00025a012

Krahn, M. M., Myers, M. S., Burrows, D. G., Malins, D. C., Krahn, M. M., Myers, M. S., Burrows, D. G., & Malins, D. C. (1984). Determination of metabolites of xenobiotics in the bile of fish from polluted waterways. *Xenobiotica*, *14*(8), 633–646. https://doi.org/10.3109/00498258409151461

Magnusson, B.; Örnemark, U. (2014). The Fitness for Purpose of Analytical Methods. In *Eurachem Guide*.

Nahrgang, J., Brooks, S. J., Evenset, A., Camus, L., Jonsson, M., Smith, T. J., Lukina, J., Frantzen, M., Giarratano, E., & Renaud, P. E. (2013). Seasonal variation in biomarkers in blue mussel (Mytilus edulis), Icelandic scallop (Chlamys islandica) and Atlantic cod (Gadus morhua)-Implications for environmental monitoring in the Barents Sea. *Aquatic Toxicology*, *127*(9037), 21–35. https://doi.org/10.1016/j.aquatox.2012.01.009

Parrott, J. L., Raine, J. C., McMaster, M. E., & Hewitt, L. M. (2019). Chronic toxicity of oil

sands tailings pond sediments to early life stages of fathead minnow (Pimephales promelas). *Heliyon*, *5*(9), e02509. https://doi.org/10.1016/j.heliyon.2019.e02509

Robidoux, P. Y., Virginie, B., Judith, L., & Marc, D. (2018). Assessment of acute and chronic toxicity of unweathered and weathered diluted bitumen to freshwater fish and invertebrates. *Ecotoxicology and Environmental Safety*, *164*, 331–343. https://doi.org/10.1016/j.ecoenv.2018.08.010

Sarasquete, C., & Segner, H. (2000). Cytochrome P4501A CYP1A in teleostean fishes. *The Science of the Total Environment*, *247*, 313–332.

Singer, M. M., Aurand, D. V., Coelho, G. M., Bragin, G. E., Clark, J. R., Sowby, M., & Tjeerdema, R. S. (2001). Making, measuring, and using water-accommodated fractions of petroleum for toxicity testing. *International Oil Spill Conference Proceedings*, *2001*(2), 1269–1274. https://doi.org/10.7901/2169-3358-2001-2-1269

Sturve, J., Balk, L., Liewenborg, B., Adolfsson-Erici, M., Förlin, L., & Carney Almroth, B. (2014). Effects of an oil spill in a harbor assessed using biomarkers of exposure in eelpout. *Environmental Science and Pollution Research*, *21*(24), 13758–13768. https://doi.org/10.1007/s11356-014-2890-z

Tierney, K. B., Kennedy, C. J., Gobas, F., Gledhill, M., & Sekela, M. (2013). Organic Contaminants and Fish. In K. B. Tierney, A. P. Farrell, & C. J. Brauner (Eds.), *Organic Chemical Toxicology of Fishes* (First Edit, Vol. 33, pp. 1–52). Elsevier Inc. https://doi.org/10.1016/B978-0-12-398254-4.00001-7

Tilghman Hall, A., & Oris, J. T. (1991). Anthracene reduces reproductive potential is maternally transferred during long-term exposure in fathead minnows. *Aquatic Toxicology*, *19*(3), 249–264. https://doi.org/10.1016/0166-445X(91)90022-2

van den Hurk, P., Edhlund, I., Davis, R., Hahn, J. J., McComb, M. J., Rogers, E. L., Pisarski, E., Chung, K., & DeLorenzo, M. (2020). Lionfish (Pterois volitans) as biomonitoring species for oil pollution effects in coral reef ecosystems. *Marine Environmental Research*, *156*(February), 104915. https://doi.org/10.1016/j.marenvres.2020.104915

Weinstein, J. E., Oris, J. T., & Taylor, D. H. (1997). An ultrastructural examination of the mode

of UV-induced toxic action of fluoranthene in the fathead minnow, Pimephales promelas. *Aquatic Toxicology*, *39*(1), 1–22. https://doi.org/10.1016/S0166-445X(97)00018-0

Zahl, I. H., Kiessling, A., Samuelsen, O. B., & Hansen, M. K. (2011). Anaesthesia of Atlantic halibut (Hippoglossus hippoglossus) Effect of pre-anaesthetic sedation, and importance of body weight and water temperature. *Aquaculture Research*, *42*(9), 1235–1245. https://doi.org/10.1111/j.1365-2109.2010.02711.x

Chapter 3: Assessing exposure of small-bodied fish to polycyclic aromatic compounds following crude oil spills using biliary metabolites

Abstract

Knowledge gaps pertaining to the remediation of freshwater lakes impacted by oil spills have persisted despite recent record highs for oil production and transportation across vulnerable regions in North America. The Freshwater Oil Spill Remediation Study (FOReSt), conducted at the IISD-Experimental Lakes Area in Canada, is focusing on the efficacy of minimally invasive methods for remediating oil spills in freshwater boreal lakes. In this study, the impacts and remediation of diluted bitumen (dilbit) and conventional heavy crude oil (CHV) spills were investigated. Two common small-bodied fish, fathead minnows (*Promephales promelas*) and finescale dace *(Chrosomus neogaeus*), were used to assess exposure to oil constituents in model enclosed shoreline ecosystems impacted by spills. Exposure to polycyclic aromatic compounds (PACs), the most toxicologically relevant compounds in oil, were assessed in fish using biliary metabolite concentrations. Finescale dace and fathead minnows residing in oil treated enclosures each had biliary pyrene metabolite concentrations that were positively correlated with pyrene concentrations in the water of the enclosures. Three months after the initial spills, fish in the enclosure receiving dilbit were significantly more exposed to PACs than fish in reference enclosures that did not receive oil. Overall, PAC exposure in experimental fish was not always related to aqueous PAC concentrations which may have implications for future remediation efforts and assessments of future freshwater oil spills.

3.1 Introduction

In 2018 Canada produced almost 1.06 billion barrels of oil sands bitumen and 465 million barrels of crude oil – volumes that are projected to increase over the next decade (CAPP, 2022a, 2022b; CER, 2021). Pipelines, the safest mode of transportation for oil, are already at capacity and record amounts of petroleum were being transported by rail prior to Covid-19's economic disruption (CER, 2022; Furchtgott-Roth, 2013). These transportation routes travel over, and near, many of North America's freshwater resources, allowing the possibility of spills. There is currently debate regarding the efficacy of methods for remediation, reclamation, and restoration of freshwater habitats following an oil spill, as well as the environmental impact of current invasive remediation techniques (Lee et al., 2015). The IISD-Experimental Lakes Area initiated the Freshwater Oil Spill Remediation Study (FOReSt) to examine the utility of using minimally invasive remediation methods to degrade oil not removed by initial oil spill cleanup methods (i.e. residual oil). FOReSt was comprised of a series of contained model oil spills conducted in a pristine boreal lake at the IISD-Experimental Lakes Areas from 2018-2021. Shoreline enclosures were treated with model spills of crude oil and, following a primary cleanup, were remediated using a minimally invasive method. Unlike lab studies, lake enclosures encompass entire ecosystems while still allowing for a semi-controlled experimental environment. This study will help inform remediation efforts of shorelines affected by future unintentional oil releases.

Here, spills of diluted bitumen (dilbit) and conventional heavy crude oil (CHV) were compared. Diluted bitumen is created by adding diluents to lower the viscosity of bitumen (which does not readily flow through pipelines) and allow for practical transportation (Banerjee, 2012). Following the introduction of oil into the enclosures, monitored natural recovery (MNR) was used for remediation. MNR relies upon microbial break down of petrochemicals following initial clean-up of surface oil (Lee et al., 2015; Magar et al., 2009). The affected enclosures, as well as two references enclosures, were monitored to assess breakdown of oil constituents and to determine potential impacts to flora and fauna and their recovery. Although perception of passive techniques can be poor, MNR is utilized in situations where human intervention negatively impacts the spill location more than the oil itself (i.e. the trampling of plants and habitats) or in ecosystems where natural remediation is expected to proceed quickly (Lee et al.,

2015). Implementing MNR is best accompanied by increased monitoring and recovery trajectory modeling (Magar et al. 2009).

Determining exposure and recovery of oil spill impacted sites is challenging. While chemical recovery can be readily determined, biological and ecological recovery is also important but more difficult to assess. Measuring biliary polycyclic aromatic compound (PAC) metabolites in fish is a powerful tool for assessing short-term exposure and metabolism of oil constituents (Beyer et al., 2010; Dearnley et al., 2020). PACs are the compounds of greatest concern with respect to toxicity of oil to fish. In fish, PACs are generally metabolized by enzymes of the cytochrome P4501A family (Collier et al., 2013). These enzymes are found in various locations within fish but are at their highest concentration in the liver. As a result, many PAC metabolites are excreted and accumulated in the gallbladder and can be quantified in the bile. Numerous studies have linked biliary PAC concentrations to short-term PAC exposure so they are often measured in monitoring studies (Collier et al., 2013; Dearnley et al., 2020; Sarasquete & Segner, 2000). Thus, biliary metabolite concentrations in resident fish are an excellent metric for determining the efficacy of remediation methods. This short-term indicator of PAC exposure not only provides important information about the efficacy of remediation efforts, but when combined with measured PAC concentrations from the water and sediment can indicate routes of uptake for wild fish and elucidate on potential mechanisms of fish exposure. This in turn can aid in risk assessment.

Small bodied fish are especially useful for monitoring studies because they tend to have small home ranges, exhibit higher site fidelity and feed within localized foodwebs. They are often abundant and have widespread geographical distributions, making them even more useful for comparative studies. Two small-bodied fish species were used as exposure vectors for the current study. Fathead minnows (*Pimephales promelas*) are used widely for laboratory-based toxicity research and have a broad geographic range. Their natural territory spans between the Rocky Mountains and Appalachians, from northern Mexico to the Alberta/Northwest Territories border where they reside in creeks, headwaters, small rivers, lakes, and ponds (Gidmark & Simons, 2014; Wakefield, n.d.). They are likely to be found at a high proportion of potential spill sites in North American, a useful attribute for PAC exposure monitoring. Dose-dependent responses to PACs have been demonstrated in fathead minnows (Grimard et al., 2020; Chapter 2). Finescale

dace (*Chrosomus neogaeus*) were not included in the original study design but either infiltrated the sealed enclosures or hatched from eggs trapped inside the enclosures during setup (finescale dace spawn in early spring during the time that enclosures were constructed). In Canada the species is found from British Columbia in the west to New Brunswick in the east and as far north as the Northwest Territories. In the United States, they are widely distributed along the northern portions of the St. Lawrence, Mississippi, and Missouri River drainages inhabiting slow moving waters in streams, small lakes and ponds (especially those with dense shoreline vegetation). (Stasiak & Cunningham, 2006). A literature search found no published PAC or oil-related research involving finescale dace.

In this study, four shoreline enclosures were erected and stocked with fathead minnows. Two enclosures were unoiled and used as references, one received a model dilbit spill, and one received a model CHV spill. Primary clean-up of surface oil was initiated 72 hours after oil introduction to reflect realistic response times and the sole secondary remediation activity was MNR. Fish were retrieved at two different time points to assess ongoing PAC exposure using bile metabolites which tested the hypothesis that fish exposed to simulated oil spills will show significantly different exposure to PACs than unexposed fathead minnows. Biliary metabolite concentrations were also compared to parent PAC concentrations in the water and sediment to delineate routes of exposure. Bile metabolites from both time collection periods indicated ongoing exposure to PACs. This study's approach mirrors the historic and ongoing use of biliary PAC metabolites in assessing exposure from freshwater and marine oil spills globally.

3.2 Methods and materials

3.2.1 Location and enclosures

The study was conducted at the International Institute for Sustainable Development-Experimental Lakes Area (IISD-ELA) in the boreal forest of Northwest Ontario, Canada. IISD-ELA encompasses 58 small lakes and their watersheds, which are set aside for scientific research. The region is sparsely populated so lakes are largely unaffected by anthropogenic impacts and are representative of pristine boreal lakes at-risk for oil spills across the country.

Experimental work took place on Lake 260, a hydrologically isolated bedrock lake with a maximum depth of 14.4 m, a volume of 1.764 × 10^6 m^3, and a surface area of 34 hectares (Brunskill & Schindler, 1971).

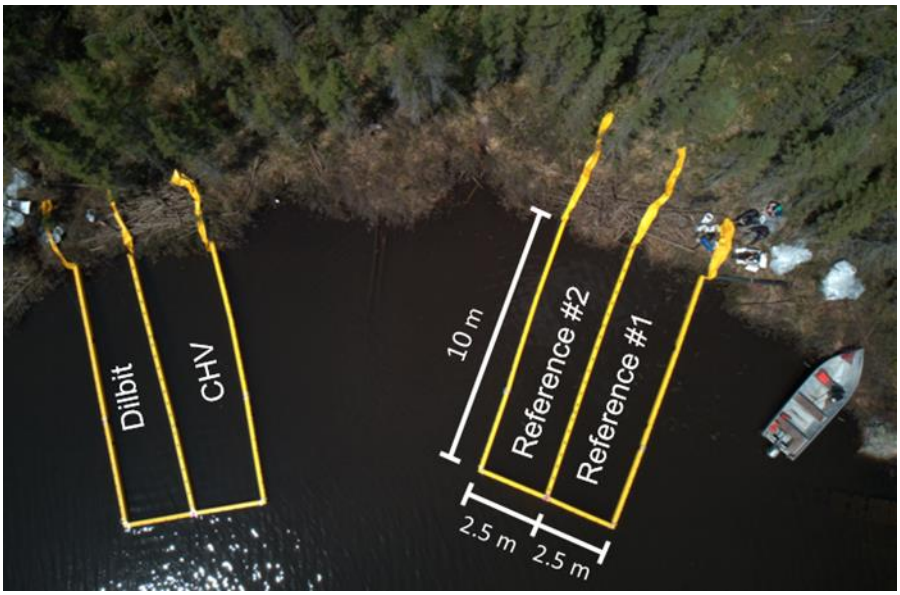


Figure 3.1: The four (under construction) enclosures used in the study. (S. Higgins, IISD-ELA)

This study commenced in spring 2018 with two enclosures (Curry Industries, Winnipeg, Canada) deployed on sand/organic shorelines in Lake 260. Each enclosure extended 10 m from the shoreline into the lake with a width of 5 m (Figure 3.1). The enclosures were comprised of floating collars from which impermeable polypropylene curtains extended down to the lake bottom where they were sealed to the underlying sediments using sandbags to prevent the exchange of water between the cells or the general lake environment. Each enclosure was divided down the middle by another curtain to create two side by side enclosure cells, each 2.5m wide × 10m long (note: from this point forward, any use of the word "enclosure" refers to these half-enclosure cells). A skirt protruding from the end of the floating collar extended 5 m onto the shoreline and was also pinned into place with sandbags. Each enclosure was protected with fine grade mesh to prevent unwanted interactions with native fauna beyond the scope of the experiment.

3.2.2 Oil weathering and application

Two oil products were used in the study: Western Canadian Select (CHV) and Cold Lake Blend (dilbit). To simulate realistic spill scenarios of oil emanating from a point source on the water, traveling across a lake, and eventually being deposited on the shoreline, both oil products used were weathered over lake water in stainless steel evaporation pans with diameter ~1 m prior to application. After two pans were filled with 220 L of lake water, CHV was poured into one, and

dilbit was poured into the other. The evaporation pans were contained within a fenced perimeter with predator mesh on the top. A tarp was also placed overtop of the pans overnight to protect them from any rain. Each oil product was weathered for 36 hours, after which they were retrieved with stainless steel spoons and placed into amber glass bottles for transport to the enclosure site. The oil was weathered from June 14-16 and applied to enclosures on June 16. With a weathering period of 36 hours, the partial-to-total evaporation from the oil of volatile compounds such as benzene, alkyl-benzenes, toluene, xylenes, 2-3 ring PAHs and <C17 alkanes can be expected, leading to an increased viscosity of the product (Fingas, 2015; Gros et al., 2014; Lee et al., 2015). Application of the oil proceeded by pouring the designated oil product onto the water approximately 1 meter from the shoreline and evenly distributed over the full width of its enclosure. In total, 1460 g of CHV and 1563 g of dilbit were applied to their respective enclosures. In accordance with realistic response times to oil spill scenarios, no remediation action was taken for 72 hours. After this time had elapsed, free floating oil was recovered from the water surface using oleophilic sorbent pads (Spill Ninja Oil Only Pads, MEP Brothers, Winnipeg, Canada). No attempt was made to remove oil from vegetation, the enclosure walls or from shoreline substrates. Following the sorbent pad cleaning, monitored natural recovery (MNR) was applied as the sole remediation method to address the residual oil (Maga et al. 2009). The enclosures were monitored for 3 months, and the study concluded fifteen weeks on October 4 later when oiled shoreline substrates were removed and the mesocosms were dismantled.

3.2.3 Water and sediment sampling and PAC testing

Water to be analyzed for alkanes, PACs, and alkylated-PACs was sampled 3 days prior to the introduction of oil to determine baselines, and then on days 1, 2, 3, 5, 9, 13, 18, 53, and 80 after oil application. Water samples were pumped from a depth of approximately 10 cm, three meters from the shoreline. Samples were stored in glass amber bottles with head space minimized and stored at 4 °C prior to transport to Winnipeg where extraction occurred within seven days of collection. Surface grab samples of sediment and soil were collected monthly, stored in amber glass bottles at -80 °C until analysis, and analyzed for the same oil constituents as the water. PAC concentrations were measured using GC-MS/MS at the Centre for Oil and Gas Research and Development in Winnipeg.

3.2.4 PAC Analysis of water and sediment

PAC concentrations were measured using GC-MS/MS at the Centre for Oil and Gas Research and Development in Winnipeg using validated methods for sediment and water. Briefly, lyophilized sediment was extracted by accelerated solvent extraction (ASE) using a Dionex™ ASE™ 350 Accelerated Solvent Extractor. Samples were weighed and loaded into extraction cells along with dispersant and Ottawa sand and were spiked with 10 µL of a 50 ng/µL reference internal standard (RIS) to assess compound loss in the extraction and clean up process. Two cycles of extractions were conducted at 1500 psi with oven temperature at 100 °C, and dichloromethane (DCM) used as the solvent. Following extraction by ASE, excess water was removed with 5 g of sodium sulfate and the extract volume was reduced to approximately 1 mL by rotary evaporation. The extracts were then cleaned using adsorption chromatography. Columns were prepared by adding a slurry of 11 g of silica gel in DCM, followed by 1 g of 5% deactivated alumina, followed by 1 g of anhydrous sodium sulfate to a pre-rinsed 30 cm × 1 cm glass column plugged with glass wool. Before transferring the extract, the column was pre-conditioned with 25 mL of hexane. Once added, the extract was eluted with 25 mL DCM/hexane (1:1, v/v). Eluted extracts were reduced to approximately 5 mL by rotary and nitrogen evaporation

For water, 250 mL of each sample was first filtered by Whatman GF/C filters and added to a separatory funnel. They were then spiked with 20 µL of a 5 ng/µL RIS (d8-naphthalene, d8-acenaphthylene, d10-acenaphthene, d10-fluorene, d10-phenanthrene, d10-pyrene, d12-benz(a)anthracene, d12-chrysene, d12-benzo(b)fluoranthene, d12-benzo(k)fluoranthene, d12-benzo(a)pyrene, d12-indeno(1,2,3-c,d)pyrene, d14-dibenzo(a,h)anthracene, and d14-benzo(g,h,i)perylene) and had 10 g sodium chloride added and dissolved to aid in extraction. They were then twice extracted using 50 mL DCM which was gently swirled with the water for a minimum of one minute before being collected in a round bottom flask. DCM volume was reduced using rotary evaporation, excess water was removed with sodium sulfate, and the extract volume was further reduced to approximately 1 mL.

Reduced extracts of sediment and water were fortified with an instrument performance internal standard of d10-anthracene (IPIS; 10 µL of 50 ng/µL for sediment, 20 µL of 5 ng/µL for water) and stored at 4°C in amber GC vials until instrumental analyses. Extracts were analyzed on an Agilent 7890 gas chromatograph coupled with a triple quadrupole mass spectrometer fitted with

electron ionization source using an Agilent J&W HP-5ms ultra inert column (30 m × 0.25 mm × 0.25 μm), with carrier gas helium at a constant flow rate of 1.2 mL/min. The PAL RSI 85 autosampler injected 1 μL of sample in spitless mode. The oven temperature was held at 60°C for 1 min then raised to 120°C at 35°C/min, further ramped up to 220°C at 14°C/min, 260°C at 5°C/min and held for 5 min, to 300°C at 10°C/min and finally to 310°C at 50°C/min. Transfer line and source temperatures were both set to 320°C and UHP nitrogen was used as the collision gas at 60 psi. Multi-reaction monitoring ion transitions were used to quantify PACs based on the average relative response factors of PAC standards at nominal concentrations of 10, 200, 400, 600, 800, 1000 pg/μL. Final concentrations were quantitated by correcting for RIS and IPIS recoveries.

3.2.5 Fish stocking, recovery, and gall bladder collection

Baited minnow traps were used to remove any native fish trapped inside the enclosures during their installation. Prior to oil application, each enclosure was stocked with 12 fathead minnows (6 male, 6 female) captured from Lake 260. Fish were anesthetized with MS-222, weighed and measured, had their sex determined, and had their caudal fin clipped as an identifying mark. They were allowed to recover in fresh lake water before being introduced into their randomly assigned enclosure. The fish were released into their enclosures in Lake 260 on the day of oil introduction. A first round of fish harvesting occurred July 17–Aug 9, beginning one month after oil introduction. Fish were collected using baited minnow traps set overnight. Recovered fish were transported to the on-site laboratory (~6 km drive) in oxygen enriched lake water, where they were sacrificed using an overdose of pH buffered MS-222 (0.4g/L), measured, weighed, and had their gall bladders excised. Gall bladders were stored on dry ice in 0.5 ml Eppendorf tubes and transported back to Winnipeg where they were stored in the dark at -80 °C with exposure to light minimized during transportation. Following this first collection period, the enclosures were restocked with fish in the same method as they were in June. A final round of fish harvesting occurred September 11-28 prior to the decommissioning of the mesocosms. Experimental work was permitted by animal use protocol #F18-007.

3.2.6 Preparation of bile samples

Preparation and HPLC-F analysis of bile followed methods of Jonsson et al (2003) and Grung et al (2016), each acknowledging Krahn et al (1987, 1992) as providing an initial analytical

framework. Due to the small size of each gallbladder, bile was determined by weight. For analysis, gallbladders were allowed to thaw and were weighed on an analytical balance (0.0001g) while still in the microcentrifuge tube used for storage. Once weighed, each microcentrifuge tube had 50 μL of 0.5% ascorbic acid buffer (pH: 5.0) added, the contents were vortexed, and then centrifuged for 5 min at 9,600 g. Gallbladders were then minced with a pipette tip in their tube to ensure full extraction of bile and the resulting bile/buffer mixture was transferred to a 1.5 mL microcentrifuge tube containing 5 μL of β-glucuronidase solution, vortexed and incubated at 37°C in the dark for 2-5 hours. Following incubation, 50 μL of methanol containing 0.5% ascorbic acid was added, the tubes were briefly vortexed and then centrifuged for 5 min at 9,600 g with the supernatant analyzed. Gallbladders that appeared to have larger volumes of bile (>10 μL) were further diluted with additional buffer and 0.5% ascorbic acid methanol (in a 1:1 ratio). During incubation, tubes used for gall bladder storage were dried and re-weighed to determine the mass of the empty gallbladders and to allow calculation of bile mass.

3.2.7 HPLC-F analysis

The HPLC system consisted of a Varian ProStar 410 Autosampler, Varian ProStar 210 pumps, and a Varian ProStar 363 fluorescence detector. Optimum excitation/emission (ex/em) wavelength pairs for each analyte were determined by direct injection into the fluorescence detector with a syringe pump while scanning through a range of emission or excitation wavelengths. The optimal ex/em pairs used were 275/361 nm for 2-naphthol, 300/383 nm for 1-phenanthrol, 340/384 nm for 1-pyrenol, 265/380 nm for 2-chrysenol, and 381/435 nm for 3-benzo[a]pyrenol. Ultrapure water (A) and methanol (B) comprised the mobile phase for the elution. The elution profile was as follows: 0-1 minute, isocratic 38% B; 1-35 min, 38-95% B linear gradient; 35-37 min, isocratic 95% B; 37-45 min, 95-38% B linear gradient; 45-50 min, isocratic 38% B. The flow rate was 0.30 mL/min. The chromatography column was held at 40°C during analysis and the autosampler delivered 10 μL for each injection. The samples were run on ZORBAX RR Eclipse XDB-C18, 80Å, 2.1 x 50 mm, 3.5 μm columns.

3.2.8 Calculations

The concentrations of 1-pyrenol were calculated from an average response factor obtained from a 10 ng/mL mixed standard containing all analytes run at the beginning, middle, and end of each

sample run. The concentration of 2-chrysenol was calculated from the recovery of the metabolite in a reference sample spiked to 10 ng/mL with each analyte. In some samples, run on a newer chromatographic column, 2-chrysenol could not be quantified in experimental samples due to a changed elution pattern and interference. In these samples, a consistent peak was observed 30 seconds after the 2-chrysenol peak. This was presumed to be a different isomer of chrysenol, was dubbed x-chrysenol, and was quantified in the same manner as 2-chrysenol. The concentration of phenanthrols was estimated based on the average response factor of 1-phenanthrol in the 10 ng/mL mixed standard. When other isomers were detected, the response factor of 1-phenanthrol was corrected with the relative response factor of the isomer in question. When isomers coeluted, their concentrations were estimated based on the relative amount of each co-eluting isomer in fully resolved samples. For calculations, the density of bile was assumed to be 1.00 g/mL, which is slightly higher than the 0.975 g/mL reported for sheepshead minnow (*Cyprinodon variegatus*; G. Jonsson et al., 2003)

3.2.9 Statistical Analysis

Statistical tests were performed using RStudio software with statistical significance accepted at $p < 0.05$. The parametric least squares regression and Welch's 2-sided t-test were run as appropriate. The assumptions of normality and homogeneity of variance were confirmed graphically using histograms and quantile-quantile plots of the residuals and a heteroscedasticity plot of the residuals, respectively. Metabolite data was natural log-transformed as necessary to meet the assumptions of the models and achieve the best possible fit. When group comparisons were required, the assumptions of the parametric ANOVA were not met by the data of this study, thus the non-parametric Kruskal-Wallis test was run instead in conjunction with a post-hoc Holm corrected Mann-Whitney-Wilcoxon test. When t-test assumptions were not met, the non-parametric Mann-Whitney U test was used.

3.3 Results

Between the two sampling periods, only 10 (of a combined 96) fin-clipped fish were recovered from the experimental enclosures. However, many unexpected finescale dace were also captured, having either infiltrated the enclosures or hatched from eggs laid prior to enclosure construction. All fish with a body length > 37 cm were subject to biliary metabolite analysis, regardless of

species (see Supplemental Information II for body sizes). In total, bile from 75 individual fish was analyzed, though statistical analysis was hampered by uneven recoveries among the different enclosures and time periods. Nevertheless, significant differences in PAC metabolite concentrations were observed among different enclosures and aqueous PAC concentrations were found to predict fish exposure.

3.3.1 Method Validation

The analytic method for PAC metabolites in bile was validated according to Eurachem guidelines (Magnusson, B.; Örnemark, 2014). The determined limits of detection (LOD) and limits of quantitation (LOQ) are shown in Table 2.1. Both were obtained from replicate measurements of each analyte in reference material (archived bile from walleye (*Sander vitreus*) collected from Lake Winnipeg) after spiked reagent blanks yielded poor, unrepresentative recoveries in comparison. A high concentration of 1-pyrenol and non-detect of 2-chrysenol in the reference material prevented accurate determination of their LOD and LOQ. For each analyte, linearity was demonstrated over the range 1-250 ng/mL (see Supplemental Information I). The method was shown to be robust to changes in incubation times (1-8 hours) and bile volume (3-10 µL; 5 µL was the default volume for method development). The method was also robust to chromatography column changes (Waters XSelect HSS T3 XP Column, 100Å, 2.5 µm,

Analyte	Limit of detection (ng/mL)	Limit of detection (pg)	Limit of quantitation (ng/mL)	Limit of quantitation (pg)
1-Phenanthrol	0.14	1.45	0.48	4.82
3-BaPol	0.05	0.54	0.18	1.81

Table 3.1: Limits of detection and quantitation (LOD/LOQ) for the bile metabolites analyzed in the FOReSt study. These quantities reflect concentrations found in the bile/buffer/methanol solution run on the column. LOD/LOQ in bile will vary from sample to sample based on the volume of bile extracted. Under the method, the reported LOQs correspond to concentrations of 9.54 ng/mL (1-phenanthrol) and 3.58 ng/mL (3-benzo[a]pyrenol) in a 5 µL sample of bile.

2.1 mm X 50 mm, specifically) with respect to analytic standards, though slight changes in elution time of other compounds in bile samples sometimes led to interference of 2-chrysenol. While 2-naphthol standards demonstrated excellent potential for analysis, interference in bile

samples precluded their quantitation in this study. Triphenylamine, often used as an internal standard in HPLC-F biliary metabolite analysis (Holth et al., 2008; Nahrgang et al., 2013), was tested for use in this method, but demonstrated unsuitably low recoveries. In its absence, each sample run contained a sample of reference bile and a sample of reference bile spiked to achieve an additional 10 ng/mL of each metabolite, which assessed metabolite recoveries and the efficiency of the enzymatic decoupling of parent metabolites.

3.3.2 Metabolite concentrations

With the exception of 3-benzo[a]pyrenol, which was not reliably detected in any sample, the targeted metabolites were generally detected in samples, and at concentrations above the determined limits of quantitation when applicable. 2-chrysenol was not quantified for some of the fish due to a change in the chromatographic column; for these fish a presumed chrysenol isomer, x-chrysenol was measured. The fish from the two different collection periods are shown separately and the disparity in fish collections across the different enclosures are readily apparent (Figures 2.2-7, Tables 2.2-7). No documentation was available for several fish collected in late July/early August and these are labelled as unknown, though they are likely finescale dace or fathead minnows. Additional information regarding fish size, sex, and bile mass recoveries of experimental fish are compiled in Supplemental Information II.

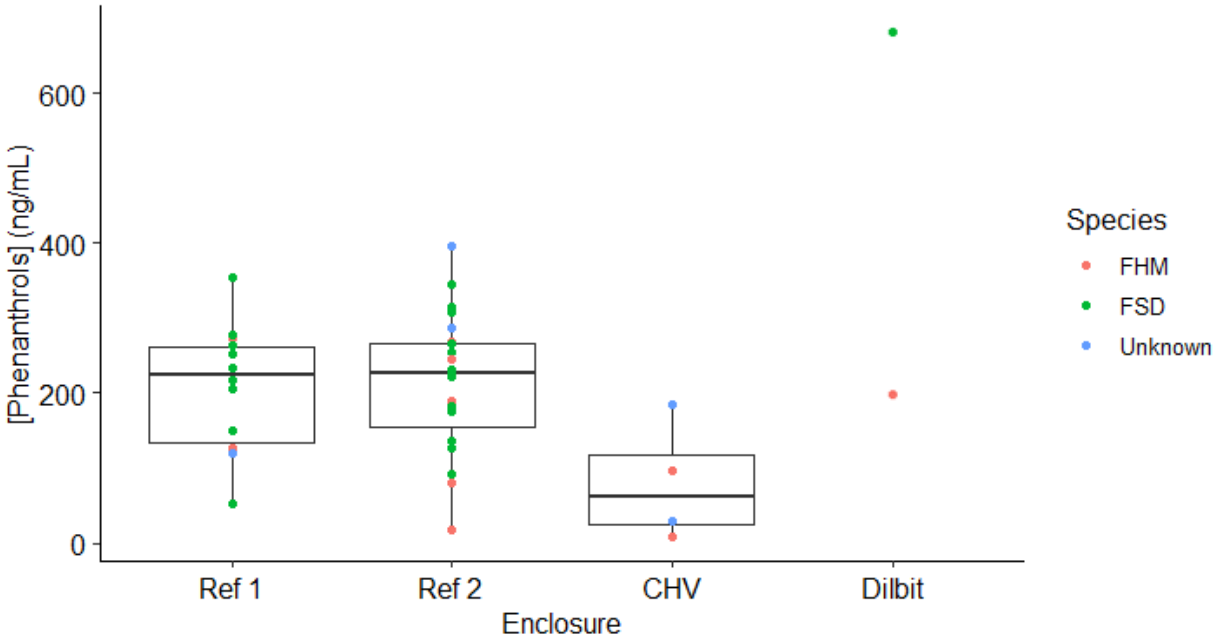


Figure 3.2: Concentrations of phenanthrol metabolites in fish from the first collection period (late July/early Aug). Box plots show the 25th percentile (bottom hinge), median (centre line), 75th percentile (top hinge), and whiskers extending to 1.5 interquartile range. No significant differences ($p<0.05$) between treatments were detected. FHM ≡ fathead minnow, FSD ≡ finescale dace, CHV ≡ conventional heavy crude oil, dilbit ≡ diluted bitumen, ref ≡ reference.

			Phenanthrols (ng/mL)				
Enclosure	**Species**	**Fish**	**Mean**	**Median**	**Std dev**	**Min**	**Max**
Ref 1	FHM	2	200.6	-	-	127.3	274.0
	FSD	10	225.9	242.7	80.6	53.4	355.1
	Unknown	2	119.1	-	-	118.8	119.4
	All	14	207.1	224.9	82.4	53.4	355.1
Ref 2	FHM	5	160.0	189.6	107.9	17.3	267.4
	FSD	16	216.1	226.8	78.7	81.4	345.7
	Unknown	2	342.1	-	-	287.5	396.8
	All	23	214.8	226.9	93.6	17.3	396.8
CHV	FHM	2	52.1	-	-	8.58	95.6
	Unknown	2	106.7	-	-	67.7	184.7
	All	4	79.4	62.1	79.5	8.58	184.7
Dilbit	FHM	1	199.3	-	-	-	-
	FSD	1	682.2	-	-	-	-
	All	2	440.7	-	-	199.3	682.2

Table 3.2: Summary data for phenanthrol metabolites by enclosure and species for the first collection period (late July/early Aug). FHM ≡ fathead minnow, FSD ≡ finescale dace, CHV ≡ conventional heavy crude oil, dilbit ≡ diluted bitumen, ref ≡ reference**.**

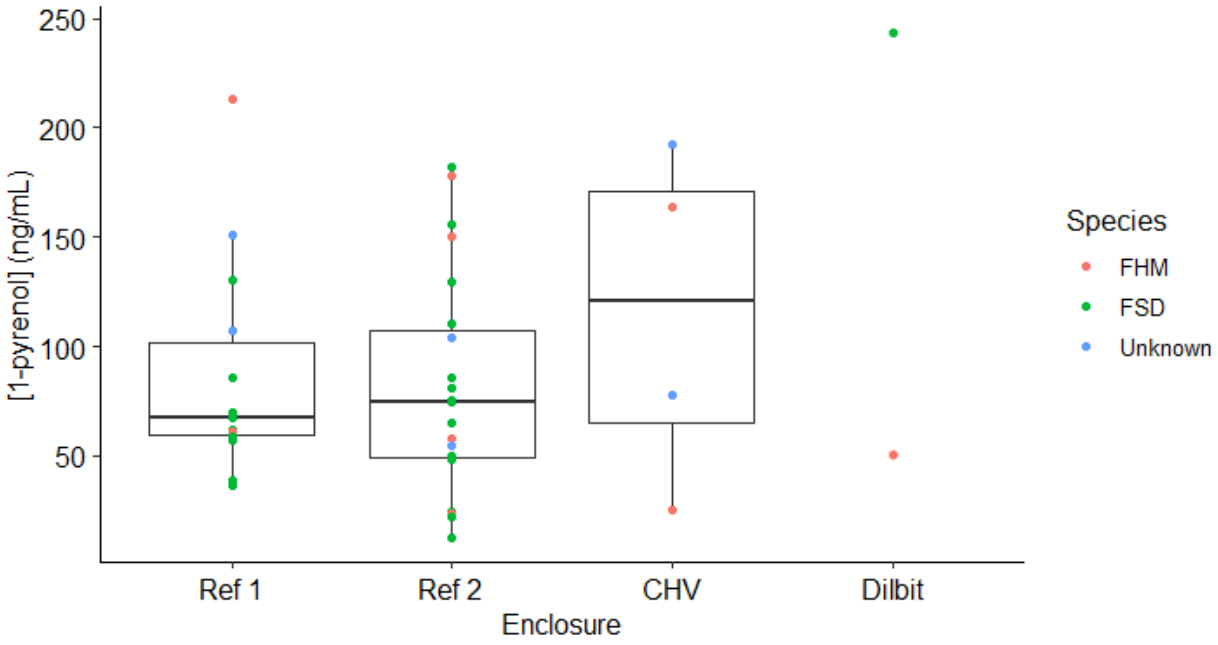


Figure 3.3: Concentrations of 1-pyrenol in fish from the first collection period (late July/early Aug). Box plots show the 25th percentile (bottom hinge), median (centre line), 75th percentile (top hinge), and whiskers extending to 1.5 interquartile range. No significant differences ($p<0.05$) between treatments were detected. FHM ≡ fathead minnow, FSD ≡ finescale dace, CHV ≡ conventional heavy crude oil, dilbit ≡ diluted bitumen, ref ≡ reference.

			1-pyrenol (ng/mL)				
Enclosure	**Species**	**Fish**	**Mean**	**Median**	**Std dev**	**Min**	**Max**
Ref 1	FHM	2	137.1	-	-	60.9	213.4
	FSD	10	67.3	64.3	26.4	36.6	130.2
	Unknown	2	129.2	-	-	107.4	151.0
	All	14	86.1	67.5	49.1	36.6	213.4
Ref 2	FHM	5	99.0	85.5	64.0	23.4	177.8
	FSD	16	75.9	69.9	47.8	12.6	181.6
	Unknown	2	79.2	-	-	54.6	103.8
	All	23	81.2	74.5	49.5	12.6	181.6
CHV	FHM	2	94.4	-	-	25.2	163.7
	Unknown	2	135.3	-	-	78.1	192.4
	All	4	114.8	120.9	77.0	25.2	192.4
Dilbit	FHM	1	50.7	-	-	-	-
	FSD	1	243.3	-	-	-	-
	All	2	147.0	-	-	50.7	243.3

Table 3.3: Summary data for 1-pyrenol by enclosure and species for the first collection period (late July/early Aug). FHM ≡ fathead minnow, FSD ≡ finescale dace, CHV ≡ conventional heavy crude oil, dilbit ≡ diluted bitumen, ref ≡ reference**.**

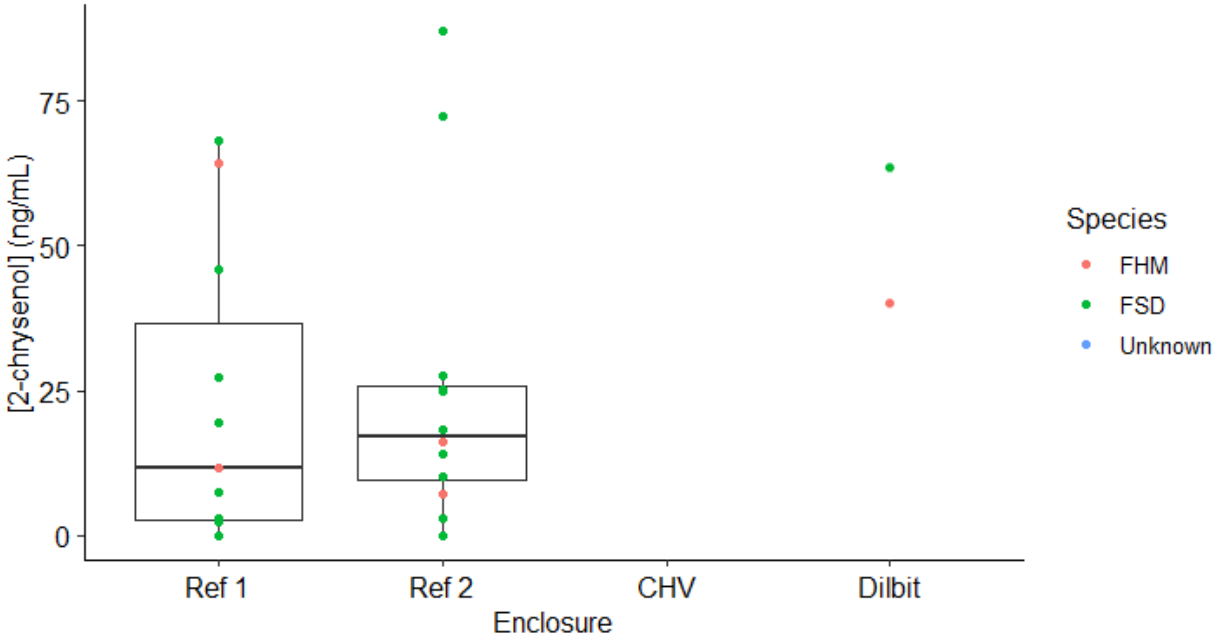


Figure 3.4: Concentrations of 2-chrysenol in fish from the first collection period (late July/early Aug). Box plots show the 25th percentile (bottom hinge), median (centre line), 75th percentile (top hinge), and whiskers extending to 1.5 interquartile range. No significant differences ($p<0.05$) between treatments were detected. FHM ≡ fathead minnow, FSD ≡ finescale dace, CHV ≡ conventional heavy crude oil, dilbit ≡ diluted bitumen, ref ≡ reference.

			2-chrysenol (ng/mL)				
Enclosure	**Species**	**Fish**	**Mean**	**Median**	**Std dev**	**Min**	**Max**
Ref 1	FHM	2	37.9	-	-	11.6	64.1
	FSD	9	19.6	7.6	23.7	ND	68.1
	All	11	22.9	11.6	25.4	ND	68.1
Ref 2	FHM	2	11.6	-	-	7.1	16.1
	FSD	10	28.3	21.7	28.8	ND	87.1
	All	12	25.5	17.2	26.9	ND	87.1
CHV	-	-	-	-	-	-	-
Dilbit	FHM	1	40.2	-	-	-	-
	FSD	1	63.8	-	-	-	-
	All	2	52.0	-	-	40.2	63.8

Table 3.4: Summary data for 2-chrysenol by enclosure and species for the first collection period (late July/early Aug). FHM ≡ fathead minnow, FSD ≡ finescale dace, CHV ≡ conventional heavy crude oil, dilbit ≡ diluted bitumen, ref ≡ reference, ND ≡ no detect.

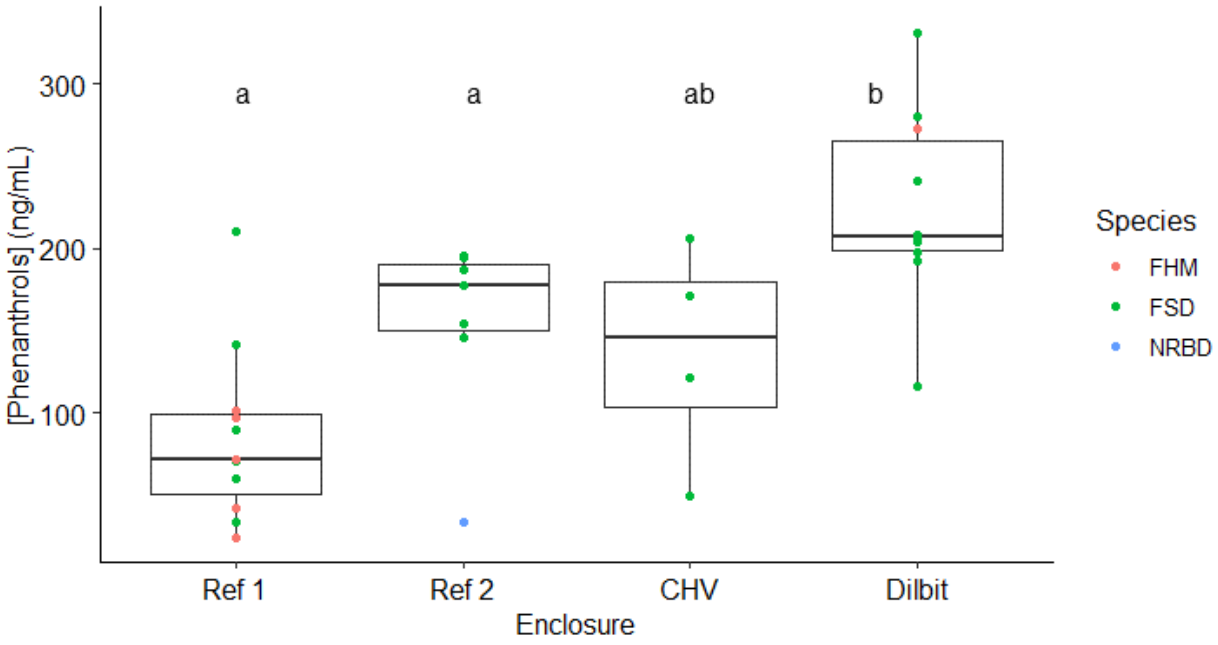


Figure 3.5: Concentrations of phenanthrols in fish from the second collection period (Sept). Box plots show the 25th percentile (bottom hinge), median (centre line), 75th percentile (top hinge), and whiskers extending to 1.5 interquartile range. Significant differences ($p<0.05$) between treatments are denoted by lower case letters. FHM ≡ fathead minnow, FSD ≡ finescale dace, NRBD ≡ northern redbelly dace, CHV ≡ conventional heavy crude oil, dilbit ≡ diluted bitumen, ref ≡ reference.

			Phenanthrols (ng/mL)				
Enclosure	**Species**	**Fish**	**Mean**	**Median**	**Std dev**	**Min**	**Max**
Ref 1	FHM	5	67.4	71.8	33.8	24.4	101.6
	FSD	6	101.2	80.5	64.6	33.9	210.5
	All	11	85.8	71.8	53.5	24.4	210.5
Ref 2	FSD	6	176.1	182.6	21.1	146.3	196.1
	NRBD	1	33.6	-	-	-	-
	All	7	155.8	178.1	57.2	33.6	196.1
CHV	FSD (all)	4	137.3	146.5	67.7	50.1	206.3
Dilbit	FHM	1	273.1	-	-	-	-
	FSD	9	219.7	205.4	60.3	116.1	331.2
	All	10	225.0	206.9	59.3	116.1	331.2

Table 3.5: Summary data for phenanthrols by enclosure and species for the second collection period (Sept). FHM ≡ fathead minnow, FSD ≡ finescale dace, NRBD ≡ northern redbelly dace, CHV ≡ conventional heavy crude oil, dilbit ≡ diluted bitumen, ref ≡ reference.

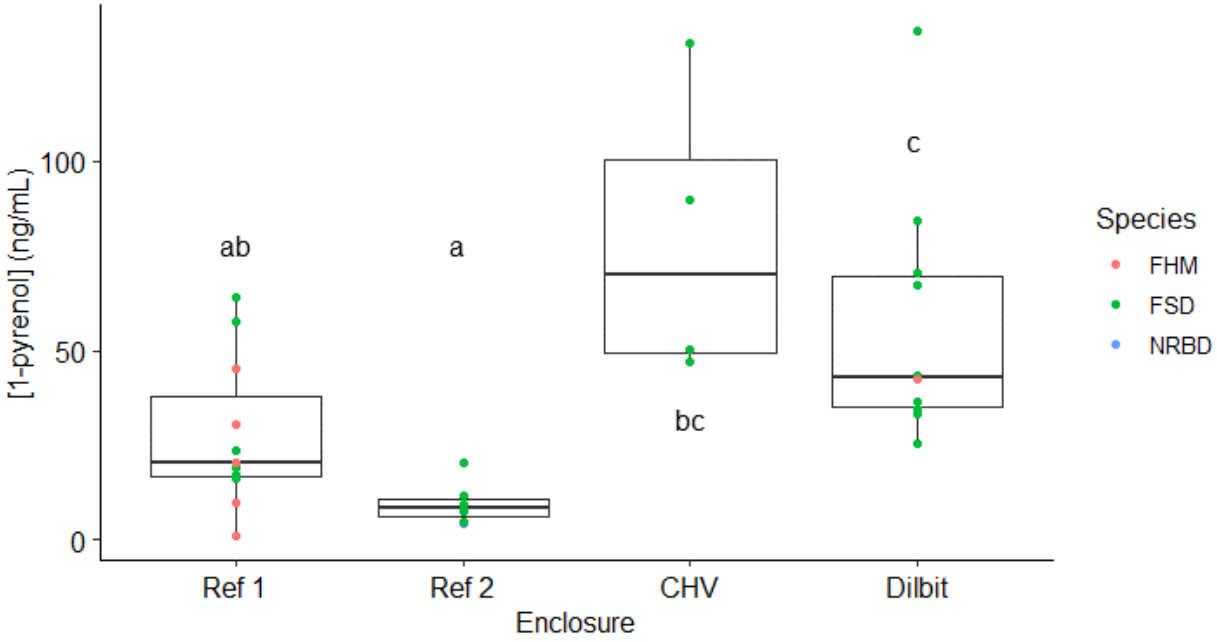


Figure 3.6: Concentrations of 1-pyrenol in fish from the second collection period (Sept). Box plots show the 25th percentile (bottom hinge), median (centre line), 75th percentile (top hinge), and whiskers extending to 1.5 interquartile range. Significant differences ($p<0.05$) between treatments are denoted by lower case letters. FHM ≡ fathead minnow, FSD ≡ finescale dace, NRBD ≡ northern redbelly dace, CHV ≡ conventional heavy crude oil, dilbit ≡ diluted bitumen, ref ≡ reference.

			1-pyrenol (ng/mL)				
Enclosure	**Species**	**Fish**	**Mean**	**Median**	**Std dev**	**Min**	**Max**
Ref 1	FHM	5	21.4	20.4	17.1	1.2	45.1
	FSD	6	33.0	21.3	21.9	16.3	64.0
	All	11	27.7	20.4	19.8	1.2	64.0
Ref 2	FSD	6	10.4	9.1	5.5	4.6	20.6
	NRBD	1	4.6	-	-	-	-
	All	7	9.6	8.6	5.5	4.6	20.6
CHV	FSD (all)	4	79.6	69.9	39.5	47.3	131.2
Dilbit	FHM	1	42.4	-	-	-	-
	FSD	9	58.9	43.3	34.7	25.5	134.3
	All	10	57.2	42.8	33.1	25.5	134.3

Table 3.6: Summary data for 1-pyrenol by enclosure and species for the second collection period (Sept). FHM ≡ fathead minnow, FSD ≡ finescale dace, NRBD ≡ northern redbelly dace, CHV ≡ conventional heavy crude oil, dilbit ≡ diluted bitumen, ref ≡ reference.

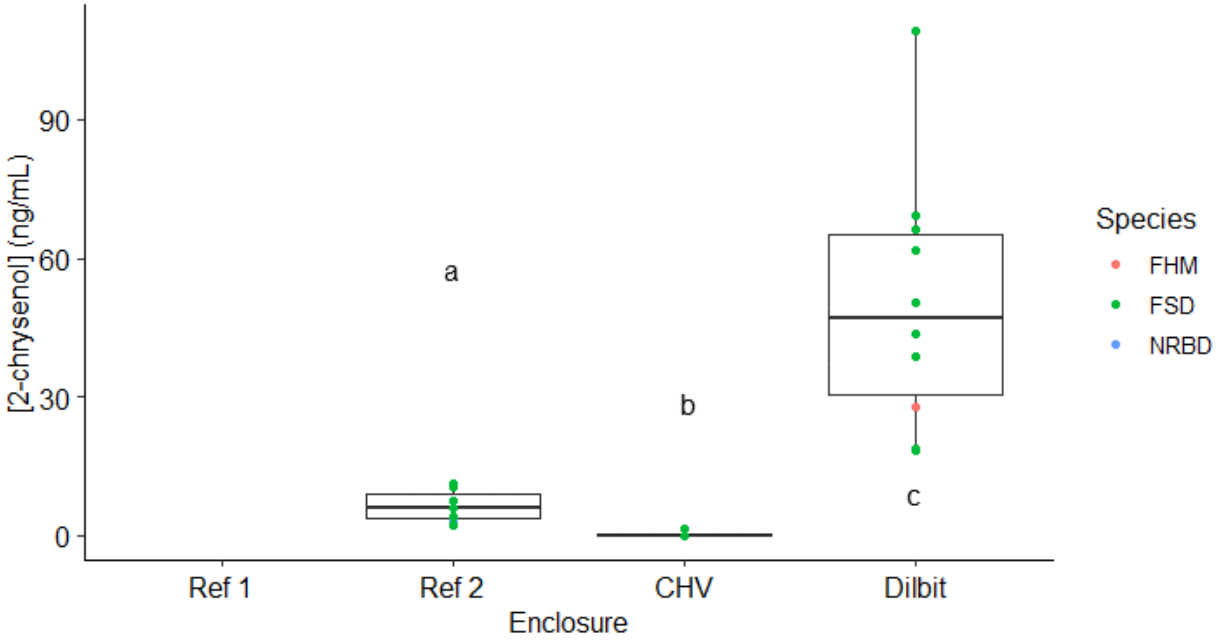


Figure 3.7: Concentrations of 2-chrysenol in fish from the second collection period (Sept). Box plots show the 25th percentile (bottom hinge), median (centre line), 75th percentile (top hinge), and whiskers extending to 1.5 interquartile range. Significant differences ($p<0.05$) between treatments are denoted by lower case letters. FHM ≡ fathead minnow, FSD ≡ finescale dace, NRBD ≡ northern redbelly dace, CHV ≡ conventional heavy crude oil, dilbit ≡ diluted bitumen, ref ≡ reference.

			2-chrysenol (ng/mL)				
Enclosure	**Species**	**Fish**	**Mean**	**Median**	**Std dev**	**Min**	**Max**
Ref 2	FSD	6	6.9	6.7	3.5	2.4	11.1
	NRBD	1	3.4	-	-	-	-
	All	7	6.4	5.9	3.5	2.4	11.1
CHV	FSD (all)	4	0.4	ND	0.7	ND	1.4
Dilbit	FHM	1	27.9	-	-	-	-
	FSD	9	52.9	50.4	28.2	18.3	109.2
	All	10	50.4	46.9	27.7	18.3	109.2

Table 3.7: Summary data for 2-chrysenol by enclosure and species for the second collection period (Sept). FHM ≡ fathead minnow, FSD ≡ finescale dace, NRBD ≡ northern redbelly dace, ND ≡ no detect, CHV ≡ conventional heavy crude oil, dilbit ≡ diluted bitumen, ref ≡ reference.

3.3.3 Comparisons between species

Uneven fish recoveries (with respect to both quantity and species) across the enclosures meant determining the equivalency of biliary metabolite concentrations between fathead minnows and finescale dace was of critical importance. Only Ref 2 in late July/early Aug (5 FHM, 16 FSD) and Ref 1 in September (5 FHM, 6 FSD) had sufficient quantities of both fish for meaningful comparison. While no significant differences in metabolite concentrations between the two species was found, the large confidence intervals indicate the differences between the two species could be large relative to the measured concentrations (Table 3.8). For each metabolite in each enclosure, one species may have a mean at least double the other. Thus, the two species are treated separately whenever possible. Statistical analysis using all fish are also presented, though conclusions from these models cannot be considered as robust as species-specific ones.

		Fathead minnow		Finescale dace			p-	
Enclosure	**Metabolite**	**Mean**	**SD**	**Mean**	**SD**	**Δ**	**value**	**95% CI**
Ref 2	Phenanthrols	160.0	107.9	216.1	78.70	-56.1	0.33	-187.1-74.86
Ref 1	Phenanthrols	67.4	33.8	101.2	64.6	-33.8	0.30	-104.2-36.7
Ref 2	1-pyrenol	99.0	64.0	75.9	47.8	23.1	0.49	-54.7-100.8
Ref 1	1-pyrenol	21.4	17.1	33.0	21.9	-11.5	0.35	-38.2-15.1
Ref 1	x-chrysenol	43.4	23.8	45.2	21.2	-1.8	0.90	-33.4-29.7

Table 3.8: Welch's 2-sided t-test between species in the same enclosures. All concentrations are ng/mL. Δ ≡ difference between the means, CI ≡ confidence interval

3.3.4 Comparisons between reference enclosures

Period	**Metabolite**	**Ref 1 Mean**	**Ref 2 Mean**	**p-value**
First	Phenanthrols	225.9	216.1	0.76
Second	Phenanthrols	101.2	176.1	0.035
First	1-pyrenol	67.3	75.9	0.56
Second	1-pyrenol	33.0	10.4	0.052
First	2-chrysenol	17.6	28.3	0.37

Table 3.9: Welch's 2-sided t-test between finescale dace in reference enclosures for each collection period. All concentrations are ng/mL.

Pooling fish from the two reference enclosures would be statistically advantageous for comparing enclosures, however this was not done due to differences detected in the second sampling period (Table 3.9).

3.3.5 Comparisons between enclosures for late July/early August

No significant effect of enclosure on metabolite concentrations was found for any of the analytes (phenanthrols: H(3)=6.88, p=0.08; 1-pyrenol: H(3)=1.45, p=0.69; 2-chrysenol: H(2)=2.22, p=0.33) for the first collection period. The CHV enclosure was omitted from the test for 2-chrysenol due to a lack of data.

3.3.6 Comparisons between enclosures for September

	Phenanthrols			**1-pyrenol**			**2-chrysenol**		
Enclosure	**Ref 1**	**Ref 2**	**CHV**	**Ref 1**	**Ref 2**	**CHV**	**Ref 1**	**Ref 2**	**CHV**
Ref 2	0.26	-	-	0.061	-	-	N/A	-	-
CHV	0.95	0.95	-	0.229	0.048	-	N/A	0.010	-
Dilbit	0.11	0.13	0.32	0.149	0.002	0.260	N/A	0.001	0.010

Table 3.10: p-values of Holm corrected Mann-Whitney-Wilcoxon pairwise comparison tests between finescale dace in enclosures for the second collection period. CHV ≡ conventional heavy crude oil, dilbit ≡ diluted bitumen, ref ≡ reference.

The quantities of fish in the second collection period allowed for comparison among finescale dace, in addition to all fish. For finescale dace, significant effects of enclosure on metabolite concentration were found for each analyte. In phenanthrols (H(3)=9.6, p=0.022), despite a Kruskal-Wallis analysis showing enclosures had an affect on metabolite concentration, pairwise comparison revealed no significant difference between any two enclosures (Table 3.10). For 1-pyrenol (H(3)=15.6, p=0.001), the Dilbit and CHV enclosures were found to have significantly higher concentrations than Ref 2 (p=0.002 and p=0.048) but only marginally higher than Ref 2 (p=0.061). For 2-chrysenol (H(2)=15.4, p<0.001) each of the three enclosures for which data was available were found to be significantly different from each other. Significant effects of enclosure on metabolite concentration were also found for each metabolite using all species. For phenanthrols (H(3)=15.6, p = 0.001), pairwise comparison indicated the Dilbit enclosure fish had higher metabolite concentrations than both references (p=0.002, p=0.034, Table 3.11). 1-pyrenol

(H(3)=19.1, p<0.001) concentrations were found to be significantly different between the dilbit enclosure and the two reference enclosures (p=0.040, p<0.001) as well as between the CHV enclosure and Ref 2 (p=0.030). Differences between the two references (p=0.053) and the CHV enclosure and Ref 1 (p=0.053) were marginally significant. For 2-chrysenol, the first reference enclosure was not included due to a lack of data, however metabolite concentrations were affected by enclosure (H(2)=17.0, p<0.001) in the other three. Metabolite concentrations of 2-chrysenol were found to be significantly different in each of the three enclosures.

	Phenanthrols			**1-pyrenol**			**2-chrysenol**		
Enclosure	**Ref 1**	**Ref 2**	**CHV**	**Ref 1**	**Ref 2**	**CHV**	**Ref 1**	**Ref 2**	**CHV**
Ref 2	0.176	-	-	0.053	-	-	N/A	-	-
CHV	0.451	0.788	-	0.053	0.030	-	N/A	0.009	-
Dilbit	0.002	0.034	0.228	0.040	<0.001	0.188	N/A	<0.001	0.009

Table 3.11: p-values of Holm corrected Mann-Whitney-Wilcoxon pairwise comparison tests between fish of all species in enclosures for the second collection period. CHV ≡ conventional heavy crude oil, dilbit ≡ diluted bitumen, ref ≡ reference.

3.3.7 Intraenclosure comparisons between July/August and September

Significant differences in concentrations of metabolites in finescale dace were observed for phenanthrol in Ref 1 and for 1-pyrenol in both reference enclosures, with fish captured in September having lower concentrations of metabolites in all three cases (Table 3.12). No significant differences were observed between collection periods for the CHV enclosure, though this may have been a consequence of small sample size (n=4 for both collection periods). When numbers permitted only one species was compared, otherwise all fish were used. The Dilbit enclosure was omitted from the analysis due to lack of fish in July/Aug, and 2-chrysenol was only available for both time periods for Ref 2.

Metabolite	Enclosure	Species	Jul/Aug Mdn	Sept Mdn	U	p
Phenanthrol	Ref 1	FSD	243.0	80.5	53	0.011
	Ref 2	FSD	227.0	183.0	65	0.231
	CHV	All	62.1	146.0	4	0.343
1-pyrenol	Ref 1	FSD	64.3	21.3	52	0.016
	Ref 2	FSD	69.9	9.1	95	<0.001
	CHV	All	121.0	69.9	10	0.686
2-chrysenol	Ref 2	FSD	21.7	6.6	47	0.073

Table 3.12: Mann Whitney U Test comparing metabolite concentrations between the two sampling periods in each enclosure (medians (mdn) in ng/mL). FSD ≡ finescale dace, CHV ≡ conventional heavy crude oil, dilbit ≡ diluted bitumen, ref ≡ reference.

3.3.8 Differences between sex

Only two enclosures, both references from the first sampling period, had a sufficient quantity and sex distribution of one species of fish to assess for differences in metabolite concentrations between sex using Welch's 2-sided t-test. In Ref 1, no differences between sex were found for any of the metabolites (p=0.265-0.918). In Ref 2, no differences between sex were found for 2-chrysenol (p=0.738), however male finescale dace (M=282.1 ng/mL) were found to have higher phenanthrol concentrations than females (M=186.1 ng/mL, p=0.002). Significant differences were also found between males (M=121.7 ng/mL) and females (M=55.1 ng/mL) for 1-pyrenol (p=0.026).

3.3.9 Metabolite concentrations and fish size

Finescale dace in Ref 1 and Ref 2 from the first collection period and in the Dilbit enclosure from the second collection period were investigated for a relationship between metabolite concentration and fish size (body length). For Ref 1 in July/August, the regression was not significant for any of the metabolites (phenanthrol: $R^2 = 0.044$, $F(1,8)=0.61$, $p=0.456$; 1-pyrenol: $R^2 <0.001$, $F(1,8)<0.01$, $p=0.965$; 2-chrysenol: $R^2 = 0.029$, $F(1,7)=0.78$, $p=0.408$). For Ref 2, however, the regression was significant ($R^2 = 0.294$, $F(1,14)=7.24$, $p=0.018$), as body size did predict phenanthrol metabolite concentration ($\beta=-10.1\pm8.0$ (95% CI), $p=0.018$), with smaller fish having larger metabolite concentrations. This significance was not observed for 1-pyrenol ($R^2 = 0.096$, $F(1,14)=2.60$, $p=0.129$) nor 2-chrysenol ($R^2 = 0.058$, $F(1,8)=0.49$, $p=0.502$). In the Dilbit enclosure in September a significant relationship ($R^2 = 0.510$, $F(1,7)=9.3$, $p=0.019$) was found

with fish size predicting phenanthrol concentration (β=6.1±4.7, p=0.019). Contrary to Ref 2 in July/August, here phenanthrol concentration increased with fish size. No such relationship was found for 1-pyrenol (R^2 = 0.056, F(1,7)=0.58, p=0.472) nor 2-chrysenol (R^2 = 0.075, F(1,7)=1.65, p=0.240).

3.3.10 Metabolite concentrations and sediment and water PAC concentrations

Least square regressions were performed to probe the relationship between water PAC concentrations and biliary metabolite concentrations across each of the enclosures. Water was collected monthly, though sample collection times did not always overlap with fish collections (see Supplemental Information-II). For fish collected within a week of a water sampling date, the sampling date concentration was used. Water concentrations were estimated for fish collected outside of this time range by interpolating between the two most recent sampling dates. Water PAC concentrations in September were an exception, as fish were caught 3 weeks after water sampling, but no later data was available for interpolation. No water data was available for Ref 2 fish in September and these fish were omitted from regressions. Separate regressions were run for each of finescale dace, fathead minnows, and all fish. Sediment PAC concentration data was only available for the first collection period, but models generated with it did not sufficiently meet the assumptions of a regression and have been omitted.

Concentrations of 1-pyrenol in finescale dace, fathead minnows, and all fish combined were significantly and positively predicted by aqueous pyrene concentrations (Table 3.13, Figure 3.10). Aqueous concentrations of phenanthrene and chrysene did not significantly predict biliary concentrations for their respective metabolites.

Metabolite	Species	β	95% CI	Int	R^2	DoF	F	p
1-pyrenol	FHM	3.536	±2.609	1.401	0.332	1,14	8.45	0.011
1-pyrenol	FSD	1.067	±0.797	3.304	0.123	1,44	7.28	0.010
1-pyrenol	All	1.475	±0.875	3.035	0.138	1,66	11.3	0.001

Table 3.13: Significant least squares regressions of aqueous PAC concentrations predicting biliary PAC metabolite concentrations of fish in all enclosures. All data was natural log-transformed. CI ≡ confidence interval of the predictor, p is for both the regression and the predictor, FHM ≡ fathead minnows, FSD ≡ finescale dace.

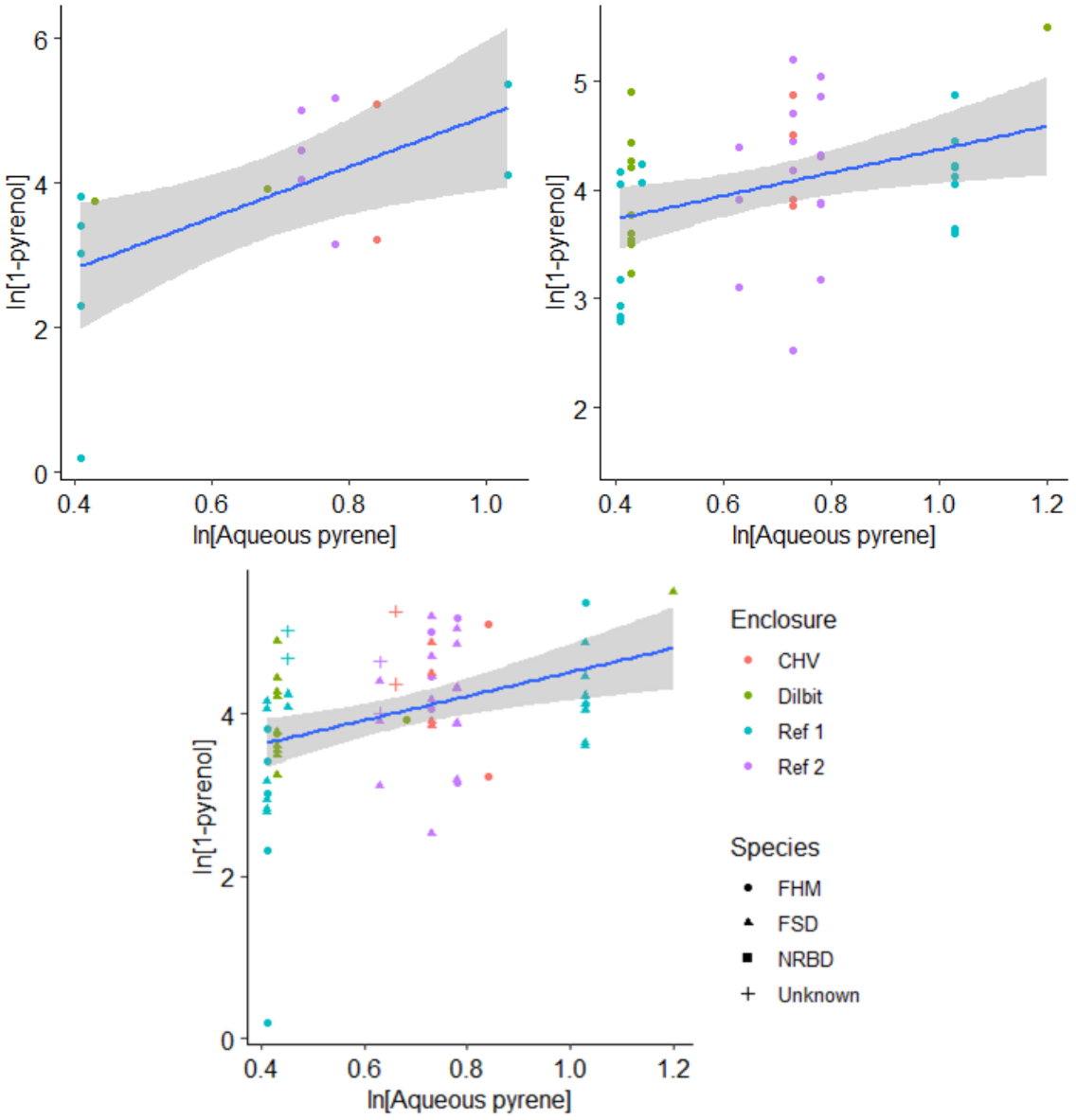


Figure 3.8: Least squares regression of natural log-transformed 1-pyrenol and water pyrene concentrations for (clockwise from top left) fathead minnow, finescale dace, and all fish (95% confidence interval shaded). CHV ≡ conventional heavy crude oil, dilbit ≡ diluted bitumen, ref ≡ reference, FHM ≡ fathead minnow, FSD ≡ finescale dace, NRBD ≡ northern redbelly

3.4 Discussion

Two and half months after model oil spills and their subsequent remediation, biliary PAC metabolite concentrations in small-bodied fish exposed to model oil spills in shoreline enclosures demonstrated a significant increase in PAC exposure compared to fish in reference enclosures. This was especially noticeable for fish exposed to dilbit, which had significantly higher concentrations of all three metabolites compared to reference fish. Aqueous pyrene concentrations predicted exposure in experimental fish, regardless of species. Statistical analysis in the study, especially for the first collection period, was hampered by low fish recoveries from oiled enclosures.

The correlation between aqueous pyrene and biliary pyrene concentrations is particularly notable in assessing spill remediation efforts, but also significant within the wider literature. Studies of biliary metabolites in wild fish, when using targeted analyses, often focus on 1-pyrenol, as this compound was found to account for 76% of biliary PAC metabolites in a study of European eels (*Anguilla, anguilla;* Kammann et al., 2017; Ruddock et al., 2003). Our results validate 1-pyrenol's use as a biomarker in wild fathead minnows and finescale dace, thereby permitting comparison of the concentrations of metabolite in the experimental fish to those measured in other studies of PAC pollution or future oil spills. If aqueous concentrations of pyrene can be lowered in an environment following remediation, whether in response to an oil spill or commercial pollution, fathead minnows should experience less uptake and less exposure. This has important implications for fish health. Pyrene itself has been linked to toxic outcomes in fish and threshold biliary 1-pyrenol concentrations have been identified in predatory marine fish, above which chronic health effects may occur (Davies & Vethaak, 2012; Kammann et al., 2017). Moreover, exposure to pyrene may be correlated with exposure to other PACs, particularly alkylated PACs, though alkylated metabolites were not quantified in this study. Nevertheless, alkylated PACs are characteristic of petrogenic PAC sources and are often more toxic than unalkylated ones (Abrajano et al., 2013; Hodson, 2017). In the case of fathead minnows, the model using aqueous pyrene concentrations to explain biliary 1-pyrenol concentrations explained much of the variance in the population (R^2=0.332). The models were less predictive in finescale dace and in all species combined. The difference may be an artifact of the sample size; a smaller population of fathead minnows (n=15) allows for less variation compared to the larger

population of finescale dace (n=45) across all enclosures. Alternatively, these differences may stem from species-species differences in behaviour, metabolism, and/or habitat preferences. Sex, size, and seasonal differences may also contribute non-PAC related variation for each species, none of which PAC modelling presented attempts to explain.

Unlike pyrene, aqueous concentrations of phenanthrene and chrysene did not predict biliary metabolite concentrations. This lack of relationship may be attributed to several factors. For one, aqueous exposure may simply be a more prominent route of uptake for pyrene compared to the other PACs studied. In a seven day exposure of juvenile fathead minnows to different concentrations of water accommodated fractions of dilbit (0%, 3%, 10%, 30%), a nominal concentration range of 1.72-157.4 ng/L for phenanthrene was required to observe a significance difference in phenanthrol between exposure groups (Dearnley, 2022). This is much narrower than the range of aqueous phenanthrene concentrations in this study (Table 3.14), though literature has also shown aqueous exposure to a mixture of PACs with a pyrene range of 0.6-6.0 ng/L was sufficient to observe significant differences in exposure for Atlantic cod (*Gadus morhua;* Grung et al., 2009). Alternatively, aqueous uptake, compared to other routes of exposure, may have been less prominent for other PACs than for pyrene in this study. Dermal uptake of PACs through sediment and ingestion through diet are other routes of exposure for fish (Logan, 2007) and these were not fully quantified in this study. Diet was found to be an important route of exposure for experimental fish in a subsequent year of the FOReSt study, where model spills of dilbit were examined and PACs in periphyton were quantified. In that study, sediment and water parent PACs were not found to consistently predict biliary metabolite concentrations (Dearnley, 2022). Nevertheless, lab studies have observed uptake of benzo[a]pyrene from sediment in fathead minnows and noted fish disturbance of the sediment led to higher aqueous concentrations of benzo[a]pyrene in aquaria (McCarthy et al., 2003). The PACs in the dynamic ecosystem of the enclosures may also differ in their bioavailability, driven by differences in their K_{OW} (Achten & Andersson, 2015). Lastly, lack of correlations between metabolite concentrations and parent PACs may simply be a product of fluctuating experimental conditions. The variance in aqueous concentrations, even within a collection period is readily apparent (Table 3.14) and limited the study's ability to assess exposure concentrations for the fish. A study with a larger exposure range would be more resilient to this margin of error, but this study's relatively narrow concentration gradient renders its predictive usefulness less robust

to fluctuations in concentrations. The lack of correlation between some metabolites in this study, however, is not a failure but rather an important finding. Fluctuations are part of natural systems and illustrate the difficulties presented in modelling real world exposure. While this study has demonstrated that modelling can be at times be an effective tool for predicting fish exposure to PACs after an oil spill, it has also shown that direct measurement can be the most reliable tool in assessing fish exposure.

PAC	Collection Period	Water concentration (ng/L)				Sediment concentration (ng/L)			
		Ref 1	Ref 2	CHV	Dilbit	Ref 1	Ref 2	CHV	Dilbit
Phenanthrene	July/Aug	3.33-2.64	5.48-1.68	1.51-1.29	3.03-1.60	2.42-2.63	1.59-1.28	29.4-33.4	11.1-7.67
Pyrene	July/Aug	1.79-0.57	1.19-0.88	1.33-0.94	2.32-0.97	1.70-8.49	1.46-3.02	30.0-44.3	6.83-22.1
Chrysene	July/Aug	0.71-0.31	0.58-0.17	0.42-0.32	2.03-0.31	0.37-0.27	0.10-0.09	9.83-15.9	2.84-0.67
Phenanthrene	Sept	1.88	N/A	4.75	1.60	N/A	N/A	N/A	N/A
Pyrene	Sept	0.51	N/A	1.07	0.53	N/A	N/A	N/A	N/A
Chrysene	Sept	0.23	N/A	0.49	0.18	N/A	N/A	N/A	N/A

Table 3.14: Parent PAC concentrations in the enclosures. Though modelling with sediment was not feasible, the concentrations are presented for readers' interest. CHV ≡ conventional heavy crude oil, dilbit ≡ diluted bitumen, ref ≡ reference. A breakdown of concentration ranges are given in Supplemental Info II.

Direct comparison of metabolite concentrations between fish in enclosures aids interpretation of the results of modelling. In September, all species of Dilbit enclosure fish were significantly more exposed to all three parent PACs than the reference enclosure fish. When only finescale dace were considered, these differences still held for pyrene and chrysene, despite aqueous PAC concentrations being nearly identical in Ref 1 and the Dilbit enclosure during fish collection (Ref 2 concentrations were not available; Table 3.14). For chrysene, the Dilbit enclosure fish (all species or finescale dace) only had significantly higher metabolite concentrations than the CHV enclosure, despite aqueous parent PAC concentrations being, at minimum, double in the CHV enclosure than in the Dilbit or Ref 1 enclosures. Similarly, CHV 1-pyrenol concentrations were only significantly higher than the Ref 2 fish, with no differences observed for phenanthrols. For 2-chrysenol, concentrations in the CHV fish were significantly lower than those of Ref 2. This data demonstrates that while aqueous PAC concentrations can help predict some exposure to fish, other environmental factors play an important role in governing fish uptake, as evidenced

by the significant differences between populations with recent exposure to nearly identical aqueous PAC concentrations in this study. Dietary inputs, which were not measured in this study, are a probable candidate to explain some of these differences. Whereas fish can quickly metabolize PAC compounds, lower trophic level organisms unable to process PACs may serve as continual sources of exposure after water concentrations return to baseline concentrations. In assessing fish exposure to PACs in the wake of an oil spill and subsequent remediation, measuring water concentrations is not enough. Though they predicted some PAC exposure here, this study indicates that fish must be sampled directly to fully assess ongoing exposure to PACs.

In addition to aforementioned predictors of PAC exposure, other factors relevant to this study can influence biliary concentrations of PAC metabolites. Variables such as species, size, and sex each have the potential to contribute to variability. Previous research on biliary metabolites has identified both size and sex as potentially influencing measured concentrations. We recently reviewed two studies that reported a significant impact of sex on metabolite concentrations and another six studies that did not (Dearnley et al., 2020). While most of these studies were on marine fish, no difference between sexes was found among five species of freshwater fish within the Athabasca/Slave River system (Ohiozebau et al., 2016). Correlations, or lack thereof, between fish size are less commonly reported but have been identified in several studies (Davies & Vethaak, 2012; Vethaak et al., 2016). Many studies on wild fish selectively sample their fish based on size and sex and these factors may have contributed variation to the data in the current study. While species were treated independently whenever possible, sex and size were not considered in analyses as they did not consistently demonstrate significant regressions or differences between populations in this study.

Seasonal variation in biliary PAC metabolite concentrations are frequently reported in wild fish (Dearnley et al., 2020) and may have been observed in this study. Significant differences were noted for several metabolites between July/Aug and September in finescale dace from the two reference enclosures. Finescale dace breed in the spring from April to June while fathead minnows breed in July and August at our study site (Gidmark & Simons, 2014; Stasiak & Cunningham, 2006, V. Palace, IISD-ELA, unpubl. Observ.). Reproductive efforts may have affected the uptake and metabolism of PACs in late July/August compared to late September. Alternatively, lower parent PAC concentrations in the enclosures for the later collection period

may be responsible (see Table 3.14) or significant differences in metabolite concentrations between the two collection periods may simply be attributable to a combination of both factors.

Due to low recaptures of tagged fish, the residence time for many experimental fish is unknown but this is not expected to have influenced results. A widely cited study on sheepshead minnow suggest peak PAC metabolite concentrations are reached within 2-6 days of exposure (Davies & Vethaak, 2012; Grung et al., 2016; Jonsson et al., 2004). Similarly, at PAC concentrations relevant to this study, exposed fathead minnows reached peak metabolite concentrations within 7 days (Grimard et al., 2020). Thus, it is assumed fish collected have been in the enclosures long enough to yield PAC metabolite concentrations reflective of the conditions, despite the unknown residence times.

3.5 Conclusion

Analysis of PAC metabolites in bile of small-bodied fish can provide information regarding their exposure after an oil spill, but additional work is required to fully understand routes of exposure. After remediating model oil spills in shoreline enclosures, fish were collected and analyzed for biliary PAC metabolites to assess their exposure to PACs. Between all four experimental enclosures, be them reference or oiled, aqueous pyrene concentrations significantly predicted biliary 1-pyrenol levels in both species studied. The study also found that additional factors influencing fish exposure to PACs can supersede modelling based on water and sediment concentrations of PACs and create significant differences between populations. Although each species is apt to serve as a monitoring species for PACs, simply measuring the water concentrations of PACs in the wake of an oil spill and its remediation is insufficient to assess exposure to resident minnows.

3.6 Connection to later chapters

2018 served as the "pilot" year of the four year FOReSt project. Although the study design was not significantly altered the following year (beyond adding replication), 2018's experimental work helped identify logistical issues around the experiment. In the following year the enclosures were subject to additional methods of minimally invasive spill remediation and replication of enclosures was increased. Overall, there were three main changes to the study design the following year. First, fish were only collected in September, instead of the two

collection periods used in this year of the study. Second, periphyton was collected and analyzed to try to assess dietary exposure for the experimental fish. Finally, only diluted bitumen was used for the spills, and two different shoreline substrates were investigated. Otherwise, bile was still analyzed in the same method, for the same PAC metabolites, and the data set was then subsequently studied for the same type of relationships reported in this chapter.

3.7 References

Abrajano, T. A., Yan, B., & O'Malley, V. (2013). High Molecular Weight Petrogenic and Pyrogenic Hydrocarbons in Aquatic Environments. In *Treatise on Geochemistry: Second Edition* (2nd ed., Vol. 11). Elsevier Ltd. https://doi.org/10.1016/B978-0-08-095975-7.00913-X

Achten, C., & Andersson, J. T. (2015). Overview of Polycyclic Aromatic Compounds (PAC). *Polycyclic Aromatic Compounds*, *35*(2–4), 177–186. https://doi.org/10.1080/10406638.2014.994071

Banerjee, D. K. (2012). Oil Sands, Heavy Oil and Bitumen - From Recovery to Refinery. In *PennWell*.

Beyer, J., Jonsson, G., Porte, C., Krahn, M. M., & Ariese, F. (2010). Analytical methods for determining metabolites of polycyclic aromatic hydrocarbon (PAH) pollutants in fish bile: A review. *Environmental Toxicology and Pharmacology*, *30*(3), 224–244. https://doi.org/10.1016/j.etap.2010.08.004

Brunskill, G. J., & Schindler, D. W. (1971). Geography and Bathymetry of Selected Lake Basins, Experimental Lakes Area, Northwestern Ontario. *Journal of the Fisheries Research Board of Canada*, *28*(2), 139–155. https://doi.org/10.1139/f71-028

CAPP. (2022a). *Canadian crude oil production 1947-2020*. https://www.capp.ca/resources/statistics/

CAPP. (2022b). *Canadian oil sands production 1967-2020*. https://www.capp.ca/resources/statistics/

CER. (2021). *Canada's Energy Future 2021*. https://www.cer-rec.gc.ca/en/data-analysis/canada-

energy-future/2021/index.html

CER. (2022). *Canadian Crude Oil Exports by Rail – Monthly Data*. https://www.cer-rec.gc.ca/en/data-analysis/energy-commodities/crude-oil-petroleum-products/statistics/canadian-crude-oil-exports-rail-monthly-data.html

Collier, T. K., Anulacion, B. F., Arkoosh, M. R., Dietrich, J. P., Incardona, J. P., Johnson, L. L., Ylitalo, G. M., & Myers, M. S. (2013). Effects on Fish of Polycyclic Aromatic Hydrocarbons (PAHS) and Naphthenic Acid Exposures. In K. B. Tierney, A. P. Farrell, & C. J. Brauner (Eds.), *Organic Chemical Toxicology of Fishes* (First Edit, Vol. 33, pp. 195–255). Elsevier Inc. https://doi.org/10.1016/B978-0-12-398254-4.00004-2

Davies, I. M., & Vethaak, D. (2012). *Integrated marine environmental monitoring of chemicals and their effects* (Issue November 2012).

Dearnley, J. M. (2022). *Assessing exposure of small-bodied fish to experimental crude oil spills in contained shoreline environments using biliary polycyclic aromatic compound metabolites*. University of Manitoba.

Dearnley, J. M., Killeen, C., Davis, R. L., Palace, V. P., & Tomy, G. T. (2020). Monitoring polycyclic aromatic compounds exposure in fish using biliary metabolites. *Critical Reviews in Environmental Science and Technology*, *0*(0), 1–45. https://doi.org/10.1080/10643389.2020.1825895

Fingas, M. (2015). Handbook of Oil Spill Science and Technology. In *Handbook of Oil Spill Science and Technology*. https://doi.org/10.1002/9781118989982

Furchtgott-Roth, D. (2013). *Pipelines are safest for transportation of oil and gas* (Issue 23).

Gidmark, N. J., & Simons, A. M. (2014). Cyprinidae: Carps and minnows. In W. L. J. Melvin & B. M. Burr (Eds.), *Freshwater Fishes of North America: Volume 1: Petromyzontidae to Catostomidae* (Vol. 1, pp. 354–450).

Grimard, C., Mangold-Döring, A., Schmitz, M., Alharbi, H., Jones, P. D., Giesy, J. P., Hecker, M., & Brinkmann, M. (2020). In vitro-in vivo and cross-life stage extrapolation of uptake and biotransformation of benzo[a]pyrene in the fathead minnow (Pimephales promelas). *Aquatic Toxicology*, *228*(May). https://doi.org/10.1016/j.aquatox.2020.105616

Gros, J., Nabi, D., Wu, B., Wick, L. Y., Brussaard, C. P. D., Huisman, J., Meer, J. R. Van Der, Reddy, C. M., & Arey, J. S. (2014). First Day of an Oil Spill on the Open Sea: Early Mass Transfers of Hydrocarbons to Air and Water. *Environmental Science & Technology*, *48*(16), 9400–9411. https://doi.org/10.1021/es502437e

Grung, M., Holth, T. F., Jacobsen, M. R., & Hylland, K. (2009). Polycyclic aromatic hydrocarbon (PAH) metabolites in Atlantic cod exposed via water or diet to a synthetic produced water. *Journal of Toxicology and Environmental Health - Part A: Current Issues*, *72*(3–4), 254–265. https://doi.org/10.1080/15287390802539210

Grung, M., Petersen, K., Fjeld, E., Allan, I., Christensen, J. H., Malmqvist, L. M. V., Meland, S., & Ranneklev, S. (2016). PAH related effects on fish in sedimentation ponds for road runoff and potential transfer of PAHs from sediment to biota. *Science of the Total Environment*, *566–567*(0349), 1309–1317. https://doi.org/10.1016/j.scitotenv.2016.05.191

Hodson, P. V. (2017). The Toxicity to Fish Embryos of PAH in Crude and Refined Oils. *Archives of Environmental Contamination and Toxicology*, *73*(1), 12–18. https://doi.org/10.1007/s00244-016-0357-6

Holth, T. F., Nourizadeh-Lillabadi, R., Blaesbjerg, M., Grung, M., Holbech, H., Petersen, G. I., Aleström, P., & Hylland, K. (2008). Differential gene expression and biomarkers in zebrafish (Danio rerio) following exposure to produced water components. *Aquatic Toxicology*, *90*(4), 277–291. https://doi.org/10.1016/j.aquatox.2008.08.020

Jonsson, G., Bechmann, R. K., Bamber, S. D., & Baussant, T. (2004). Bioconcentration, biotransformation, and elimination of polycyclic aromatic hydrocarbons in sheepshead minnows (Cyprinodon variegatus) exposed to contaminated seawater. *Environmental Toxicology and Chemistry*, *23*(6), 1538–1548. https://doi.org/10.1897/03-173

Jonsson, G., Beyer, J., Wells, D., & Ariese, F. (2003). The application of HPLC-F and GC-MS to the analysis of selected hydroxy polycyclic hydrocarbons in two certified fish bile reference materials. *Journal of Environmental Monitoring*, *5*(3), 513–520. https://doi.org/10.3233/978-1-60750-938-7-19

Kammann, U., Akcha, F., Budzinski, H., Burgeot, T., Gubbins, M. J., Lang, T., Le Menach, K.,

Vethaak, A. D., & Hylland, K. (2017). PAH metabolites in fish bile: From the Seine estuary to Iceland. *Marine Environmental Research*, *124*, 41–45. https://doi.org/10.1016/j.marenvres.2016.02.014

Krahn, M. M., Burrows, D. G., Ylltalo, G. M., Brown, D. W., Wlgren, C. A., Collier, T. K., Sin-Lam, C., & Usha, V. (1992). Mass Spectrometric Analysis for Aromatic Compounds in Bile of Fish Sampled after the Exxon Valdez Oil Spill. *Environmental Science and Technology*, *26*(1), 116–126. https://doi.org/10.1021/es00025a012

Lee, K., Boufadel, M., Chen, B., Foght, J., Hodson, P., Swanson, S., Venosa, A., Lee, K., Boufadel, M., Chen, B., Foght, J., Hodson, P., Swanson, S., & Venosa, A. (2015). The Behaviour and Environmental Impacts of Crude Oil Released into Aqueous Environments. In *Royal Society of Canada* (Issue November). http://www.rsc.ca/en/expert-panels/information-about-expert-panels%0Ahttp://www.rsc.ca/sites/default/files/pdf/OIW_Executive Summary_1.pdf%0Ahttps://www.rsc-src.ca/sites/default/files/pdf/OIW Report.pdf%0Ahttps://www.rsc-src.ca/sites/default/files/pdf/OIW

Logan, D. T. (2007). Perspective on ecotoxicology of PAHs to fish. *Human and Ecological Risk Assessment*, *13*(2), 302–316. https://doi.org/10.1080/10807030701226749

Magar, V. S., Chadwick, D. B., Bridges, T. S., Fuchsman, P. C., Conder, J. M., Dekker, T. J., Steevens, J. A., Gustavson, K. E., & Mills, M. A. (2009). Monitored Natural Recovery at Contaminated Sediment Sites. In *Environmental Security Technology Certification Program*. http://oai.dtic.mil/oai/oai?verb=getRecord&metadataPrefix=html&identifier=ADA512822%5Cnhttp://citeseerx.ist.psu.edu/viewdoc/download?doi=10.1.1.364.6282&rep=rep1&type=pdf

Magnusson, B.; Örnemark, U. (2014). The Fitness for Purpose of Analytical Methods. In *Eurachem Guide*.

McCarthy, J. F., Burrus, L. W., & Tolbert, V. R. (2003). Bioaccumulation of benzo(a)pyrene from sediment by fathead minnows: Effects of organic content, resuspension and metabolism. *Archives of Environmental Contamination and Toxicology*, *45*(3), 364–370.

https://doi.org/10.1007/s00244-003-2148-0

Nahrgang, J., Brooks, S. J., Evenset, A., Camus, L., Jonsson, M., Smith, T. J., Lukina, J., Frantzen, M., Giarratano, E., & Renaud, P. E. (2013). Seasonal variation in biomarkers in blue mussel (Mytilus edulis), Icelandic scallop (Chlamys islandica) and Atlantic cod (Gadus morhua)-Implications for environmental monitoring in the Barents Sea. *Aquatic Toxicology*, *127*(9037), 21–35. https://doi.org/10.1016/j.aquatox.2012.01.009

Ohiozebau, E., Tendler, B., Hill, A., Codling, G., Kelly, E., Giesy, J. P., & Jones, P. D. (2016). Products of biotransformation of polycyclic aromatic hydrocarbons in fishes of the Athabasca/Slave river system, Canada. *Environmental Geochemistry and Health*, *38*(2), 577–591. https://doi.org/10.1007/s10653-015-9744-6

Ruddock, P. J., Bird, D. J., McEvoy, J., & Peters, L. D. (2003). Bile metabolites of polycyclic aromatic hydrocarbons (PAHs) in European eels Anguilla anguilla from United Kingdom estuaries. *Science of the Total Environment*, *301*(1–3), 105–117. https://doi.org/10.1016/S0048-9697(02)00292-9

Sarasquete, C., & Segner, H. (2000). Cytochrome P4501A CYP1A in teleostean fishes. *The Science of the Total Environment*, *247*, 313–332.

Stasiak, R., & Cunningham, G. R. (2006). *Finescale Dace (Phoxinus neogaeus): a technical conservation assessment*. https://www.fs.usda.gov/Internet/FSE_DOCUMENTS/stelprdb5206787.pdf

Vethaak, A. D., Baggelaar, P. K., van Lieverloo, J. H. M., & Ariese, F. (2016). Decadal Trends in Polycyclic Aromatic Hydrocarbon (PAH) Contamination Assessed by 1-Hydroxypyrene in Fish Bile Fluid in the Netherlands: Declining in Marine Waters but Still a Concern in Estuaries. *Frontiers in Marine Science*, *3*(November), 1–13. https://doi.org/10.3389/fmars.2016.00215

Wakefield, N. (n.d.). *Pimephales promelas (fathead minnow)*. Centre for Agriculture and Bioscience International Invasive Species Compendium. Retrieved January 25, 2022, from https://www.cabi.org/isc/datasheet/69889

Chapter 4: Assessing exposure of small-bodied fish to experimental spills of diluted bitumen in contained shoreline environments using biliary polycyclic aromatic compound metabolites

Abstract

Knowledge gaps pertaining to the remediation of freshwater lakes impacted by oil spills have persisted despite recent record rates of oil production and transportation across vulnerable regions in North America. The Freshwater Oil Spill Remediation Study (FOReSt), conducted at the IISD-Experimental Lakes Area in Canada, focused on the efficacy of minimally invasive methods for remediating oil spills in freshwater ecosystems. In this study, impacts and remediation of model diluted bitumen (dilbit) spills in two shoreline types, rock-cobble and organic-wetland, were investigated. Two common small-bodied fish, fathead minnows (*Promephales promelas*) and finescale dace *(Chrosomus neogaeus*) were used to assess exposure to petrogenic polycyclic aromatic compounds (PACs) in model enclosed shoreline ecosystems impacted by spills and remediated using three different techniques (enhanced monitored natural remediation, shoreline cleaning agent, and engineered floating wetlands). Short-term exposure to PACs, the most toxicologically relevant compounds in oil, was assessed in fish by measuring biliary metabolites of phenanthrene, pyrene, and chrysene. Both finescale dace and fathead minnows residing in oil-treated enclosures, regardless of shoreline substrate, showed increased exposure to PACs compared to fish in reference enclosures and the pristine lake environment two and a half months after the spills. No significant differences were observed among the remediation treatments. Biliary PAC metabolite concentrations were positively predicted by PAC concentrations in periphyton, a dietary item for both species. PACs in periphyton two and a half months after oil introduction were positively correlated with PACs in the enclosures one week after spills. This correlation was especially apparent when only alkylated-PACs (aPACs) were considered, suggesting fish also had increased exposure to aPACs through periphyton, though metabolites for these compounds were not quantified in this study.

4.1 Introduction

In 2019, Canada produced a record 1.08 billion barrels of oil sands bitumen, volumes that are projected to increase over the next decade (CAPP, 2022; CER, 2021). Bitumen is too viscous to flow so diluted bitumen (dilbit) is created using diluents which lower bitumen's viscosity and ease transport (Banerjee, 2012). Pipelines, the safest mode of transportation for oil are already at capacity and record amounts of petroleum were being transported by rail prior to Covid-19's economic disruption (CER, 2022; Furchtgott-Roth, 2013). These transportation routes travel over and adjacent to many of North America's freshwater resources, allowing the possibility of spills. There is currently debate regarding the efficacy of methods for remediation, reclamation, and restoration of freshwater habitats following an oil spill, as well as the environmental impact of contemporary remediation techniques (Lee et al., 2015). The IISD-Experimental Lakes Area (IISD-ELA) initiated the Freshwater Oil Spill Remediation Study (FOReSt) to examine the utility of using minimally invasive remediation methods to degrade oil not removed by initial oil clean up methods (i.e. residual oil). FOReSt was comprised of a series of contained model oil spills conducted in a pristine boreal lake at the IISD-ELA from 2018-2021. Shoreline enclosures were treated with model spills of crude oil and, following a primary clean-up, were remediated using one of three different minimally invasive methods. Unlike lab studies, lake enclosures encompass entire model ecosystems while still allowing for a semi-controlled experimental environment. This study was designed to inform remediation efforts of shorelines affected by future unintentional oil releases.

In 2019 model spills of dilbit were studied on two different shoreline substrates: rock-cobble and organic-wetland. After primary clean up of surface oil, each enclosure was remediated using either enhanced monitored natural recovery (eMNR), a shoreline cleaning agent (SC), or an engineered floating wetland (EFW). eMNR relies upon microbial break down of petrochemicals, with nutrients added to supplement the microbial community following the influx of carbon (Lee et al., 2015). SCs are designed to remove oil from flora and rocks in affected shorelines so that, after a brief soaking interval following application, oil is rinsed onto the surface of the water where the liberated oil can be manually recovered (Fingas, 2013). Engineered floating wetlands comprise of vegetation mounted on floating mats with roots extending below the water's surface where the plants nourish rhizospheric microbes and create hospitable conditions for the microbial

community breakdown of petrochemicals (Afzal et al., 2019; Lee et al., 2015). Implementation of theses techniques are best accompanied by increased monitoring and recovery trajectory modelling (Magar et al., 2009). The SC and EFW represent relatively novel approaches to shoreline remediation; EFW is a recently developed treatment, while SC was not registered for freshwater use in Canada at the time of the study.

While chemical recovery of oil-impacted shoreline environments can be readily determined, biological and ecological recovery is often more difficult to assess. Polycyclic aromatic compound (PAC) metabolites in bile are a powerful metric for assessing short term exposure in fish. PACs are the compounds of greatest concern with respect to oil exposure and toxicity to fish and many are readily metabolized in fish. Enzymes of the cytochrome P4501A family are responsible for metabolism and are at their highest concentration in the liver (Collier et al., 2013; Dearnley et al., 2020; Sarasquete & Segner, 2000). Consequently, many PAC metabolites are excreted into the gallbladder where they accumulate. Numerous studies have linked biliary PAC concentrations to short-term PAC exposure, leading to their widespread use in monitoring studies (Beyer et al., 2010; Dearnley et al., 2020). Here, biliary metabolite concentrations in fish are used to assess the efficacy of the different remediation methods and to delineate the routes of PAC exposure from the environment using parent PAC concentrations in enclosure water, sediment, and periphyton.

Small-bodied fish are especially useful for monitoring studies as they tend to have small home ranges, exhibit high site fidelity, and feed within localized food webs. The species examined in this study, fathead minnows (*Promephales promelas*) and finescale dace (C*hrosomus neogaeus*), have broad geographic ranges across North America and therefore are likely to be found at a high proportion of potential spill sites (Gidmark & Simons, 2014; Stasiak & Cunningham, 2006; Wakefield, n.d.). Dose-dependent responses to PACs have been identified in fathead minnows, while a literature search found no published PAC or oil-related research involving finescale dace (Grimard et al., 2020; Chapter 2).

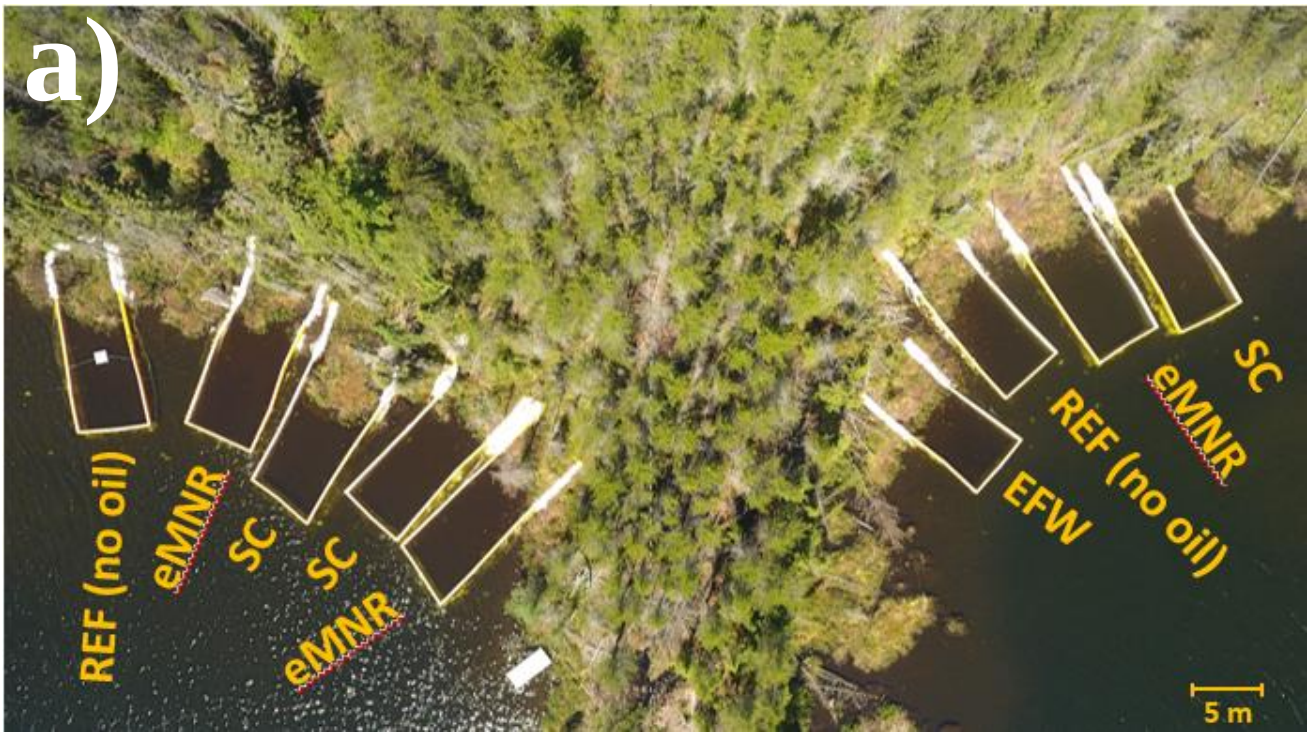
This study was comprised of two sets of nine shoreline enclosures; one set was erected over rock-cobble lake-bottom substrate, the other over wetland-organic. Within each set of enclosures, SC and eMNR were studied in triplicate, EFW was employed as a remediation method in one, and two unoiled enclosures were used as references. Two and a half months after

oil introduction, primary clean up, and remediation, all fish in the enclosures were retrieved and had their bile analyzed for PAC metabolites to test the hypothesis that fish exposed to simulated oil spills have significantly different exposure to PACs than unexposed fathead minnows. Biliary metabolites indicated elevated exposure of fish in oiled enclosures and this metric was used to determine the routes of exposure for environmental PACs. This approach mirrors the historic and ongoing use of biliary PAC metabolites in the assessment of freshwater and marine oil spills globally.

4.2 Methods and materials

4.2.1 Location and enclosures

The study was conducted on Lake 260 (volume: 1.764×10^6 m^3, max depth: 14.4 m, area: 34 ha; Brunskill & Schindler, 1971) at the International Institute for Sustainable Development-Experimental Lakes Area (IISD-ELA) in Northwest Ontario. IISD-ELA, situated in the boreal forest, encompasses 58 small lakes and their watersheds which are set aside for scientific research. The region is sparsely populated meaning the lakes are largely unaffected by anthropogenic impacts and are representative of pristine boreal lakes at-risk for oil spills across Canada. In spring 2019, eighteen enclosures (Curry Industries, Winnipeg, Canada) were



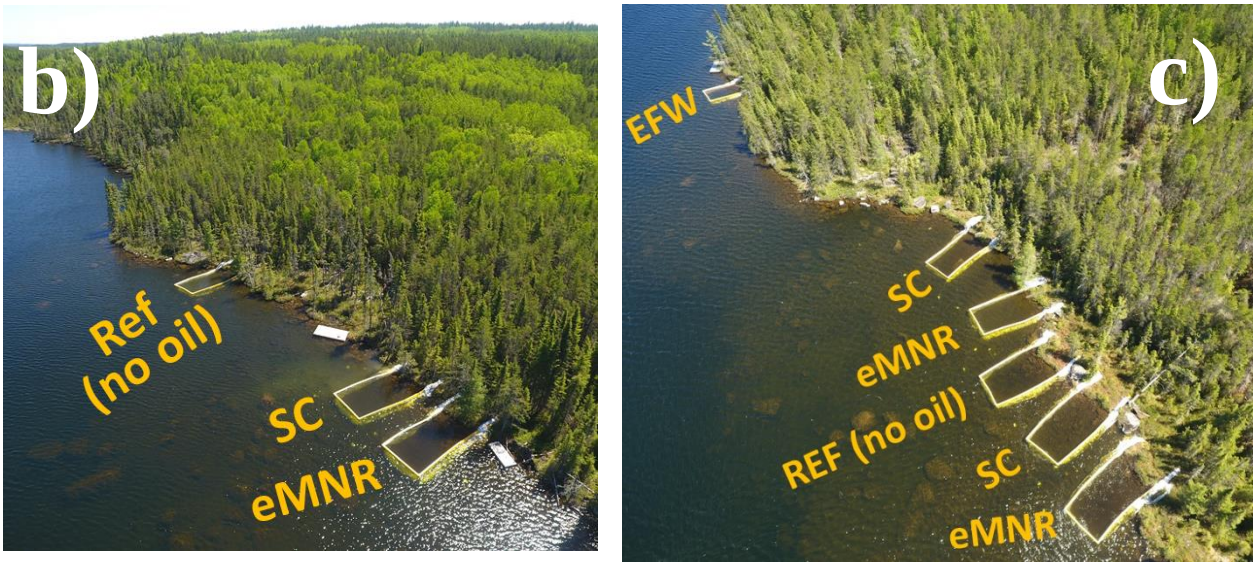


Figure 4.1: Enclosures during installation and their remediation techniques over organic-wetland (a) and rock-cobble (b,c) substrates. A legend for size is omitted for rock-cobble enclosures due to the photos' perspective, though all untruncated enclosures in the two substrates have the same dimensions. EFW ≡ engineered floating wetland, eMNR ≡ enhanced monitored natural recovery, SC ≡ shoreline cleaning agent, REF ≡ reference.

deployed in two sets of nine with each set erected over a different shoreline substrate, rock-cobble or wetland-organic (abbreviated as wetland henceforth). Each enclosure extended 10 m from the shoreline into the lake with a width of 5 m and was comprised of floating collars from which impermeable polypropylene curtains extended down to the lake bottom (see Figure 4.1). The polypropylene curtains were sealed to the underlying sediments using sandbags to prevent the exchange of water between the general lake environment. Two enclosures (EFW for each shoreline substrate) were truncated to 7.5 m due to rapid increases in shoreline depth which would have prevented curtain sealing and increased enclosure volume significantly over the others used for the study. A skirt protruding from the end of each floating collar extended 5 m onto the shoreline and was also pinned into place with sandbags. Of the nine enclosures for each substrate, seven were subject to oil spills. Of these seven, three were remediated using enhanced monitored natural recovery, three with a shoreline cleaning agent, and one with an engineered floating wetland. Two enclosures were used as references and were not subject to oil introduction. A two-week acclimation period allowed for the establishment and characterization of baseline enclosure conditions prior to oil addition.

4.2.2 Oil weathering and application

To simulate realistic spill scenarios of oil emanating from a point source on the water, traveling across a lake, and eventually being deposited on the shoreline, diluted bitumen (dilbit) was weathered over lake water in stainless-steel evaporation pans. Dilbit (~19 kg) was poured into pans (~1.1 m diameter) containing 220L (25 cm depth) of Lake 260 water and left to weather from being exposed to the elements outdoors for 36 hours (June 19-21 for the wetland enclosures and June 20-22 for the rock-cobble enclosures). Weathered dilbit was then spooned into amber vials using metal utensils and immediately transported to the lake for application. Oil was applied to the wetland enclosures (Mean mass applied = 1284 ± 43g) on June 21 and to the rock-cobble enclosures on June 22 (Mean mass applied = 1456 ± 19g). The oil was applied to the surface of the water within 50 cm of the shoreline and distributed evenly along the width of the enclosure. The initial deposition of oil onto the shoreline was simulated by waves generated through manual oscillation of the rear enclosure wall and collar. Wetland enclosures subject to oil received 1079-1444 g and rock-cobble enclosures received 1408-1525 g (see Supplemental Information III).

4.2.3 Spill remediation

In accordance with realistic response times to oil spills, remediation commenced 72 hours after oil introduction. For wetland substrate enclosures, primary clean-up was performed Jun 24 & 25 and for wetland enclosures Jun 25 & 26. All enclosures, regardless of treatment, first received a primary clean-up which consisted of flushing the shorelines of each enclosure. Over a 12 minute period, 1200 L of enclosure water was rinsed over the 5 m of the enclosure's shoreline in a low pressure stream at 10 cm intervals to rinse oil off the shoreline and back into the water. Surface dilbit was then recovered using oleophilic sorbent pads (Spill Ninja Oil Only Pads, ME Brothers, Winnipeg, MB). No attempt was made to remove oil from vegetation, the enclosure walls, or shoreline substrates. Secondary remediation methods commenced 24 hours after primary recovery (Jun 26 for wetland, Jun 27 for rock-cobble). Enhanced monitored natural recovery enclosures received a one-time supplement of ~60 g of a granular nutrient fertilizer (Scott's Osmocote Classic Slow Release Pro™19:6 Nitrogen (N): Phosphorus (P) by weight) within 1 m of the shoreline. For SC treated enclosures, Corexit EC9580A™ (Nalco Environmental Solutions LLC, USA) was used. The product was applied using a pesticide grade hand-held applicator and was distributed for ~45 seconds per enclosure on the shoreline vegetation and

riparian zone such that each enclosure received ~250 g. The SC was allowed to soak for 5 minutes before being flushed with enclosure water from a hand-held applicator. Liberated oil was captured using the same model of sorbent pads used for primary treatment. The EFW enclosures contained a floating platform whose wells were stocked with local plants that were selected for their potential to aid in remediation.

4.2.4 Water and sediment sampling

Water to be analyzed for alkanes, PACs, and alkylated PACs was sampled at 14 timepoints during the study: 3 days before oil addition, and 1, 2, 3, 4, 5, 6, 7, 8, 13, 21, 39, 67, and 88 days after oil addition. Water was pumped from a depth of approximately 10 cm and was collected from two locations in the enclosures, one near the shoreline and one far from the shoreline (~4.5 m out). Samples were stored in glass amber bottles with head space minimized and stored at 4 °C prior to transport to Winnipeg where filtration and extraction occurred within seven days of collection. Surface grab samples of sediment and soil were taken monthly, stored in amber glass bottles at -80°C until analysis, and analyzed for the same oil constituents as the water.

4.2.5 Periphyton

Periphyton was an abundant food source due to its growth on the walls of the enclosures and was collected to assess dietary PAC exposure to experimental fish. Periphyton was collected from 10 cm × 95 cm strips cut from excess enclosure wall material (polypropylene). A small pouch was created at the bottom of the strips using staples and filled with rocks to keep the otherwise buoyant strips submerged. The top of the strips were affixed ~10 cm above the water's surface and were deployed 12 days prior to oil application to allow colonization prior to oil introduction and best replicate the periphyton of the enclosure walls. Five strips were placed in each enclosure and in two reference locations in the lake for each substrate. Wetland periphyton strips were collected Sept 4 and rock-cobble periphyton strips were collected Sept 11. The strips were cut 8 cm below the lowest visible markings of oil to avoid contamination, placed in resealable plastic bags, and placed on dry ice before being stored at -20 °C after transport back to the lab. Prior to analysis, the strips were lyophilized to remove any residual water and the dry periphyton was then collected by scraping each strip with a scoopula, which was cleaned with acetone in between each strip. The collected periphyton was homogenized prior to analysis using a mortar and pestle. Dry mass of recoveries were 0.34 – 2.80 g.

4.2.6 PAC Analysis of water, sediment, and periphyton

PAC concentrations were measured using GC-MS/MS at the Centre for Oil and Gas Research and Development in Winnipeg using validated methods for periphyton (Idowu et al., 2018), sediment, and water. Briefly, lyophilized sediment or periphyton were extracted by accelerated solvent extraction (ASE) using a Dionex™ ASE™ 350 Accelerated Solvent Extractor. Samples were weighed and loaded into extraction cells along with dispersant and Ottawa sand and were spiked with 10 µL of a 50 ng/µL reference internal standard (RIS) with a mix of deuterated PACs to assess compound loss in the extraction and clean up process. Two cycles of extractions were conducted at 1500 psi with oven temperature at 100 °C, and dichloromethane (DCM) used as the solvent. Following extraction by ASE, excess water was removed with 5 g of sodium sulfate and the extract volume was reduced to approximately 1 mL by rotary evaporation. Sediment and/or periphyton extracts were then cleaned using adsorption chromatography. Columns were prepared by adding a slurry of 11 g of silica gel in DCM, followed by 1 g of 5% deactivated alumina, followed by 1 g of anhydrous sodium sulfate to a pre-rinsed 30 cm × 1 cm glass column plugged with glass wool. Before transferring the extract, the column was pre-conditioned with 25 mL of hexane. Once added, the extract was eluted with 25 mL DCM/hexane (1:1, v/v). Eluted extracts were reduced to approximately 1 mL for periphyton or 5 mL for sediment by rotary and nitrogen evaporation

For water, 250 mL of each sample was first filtered by Whatman GF/C filters and added to a separatory funnel. Samples were then spiked with 20 µL of a 5 ng/µL RIS (d8-naphthalene, d8-acenaphthylene, d10-acenaphthene, d10-fluorene, d10-phenanthrene, d10-pyrene, d12-Benz(a)anthracene, d12-chrysene, d12-benzo(b)fluoranthene, d12-benzo(k)fluoranthene, d12-benzo(a)pyrene, d12-indeno(1,2,3-c,d)pyrene, d14-dibenzo(a,h)anthracene, and d14-benzo(g,h,i)perylene) and had 10 g sodium chloride added and dissolved to aid in extraction. They were then twice extracted using 50 mL DCM which was gently swirled with the water for a minimum of one minute before being collected in a round bottom flask. DCM volume was reduced using rotary evaporation, excess water was removed with sodium sulfate, and the extract volume was further reduced to approximately 1 mL.

Reduced extracts of water, periphyton, and sediment were fortified with an instrument performance internal standard of d10-anthracene (IPIS; 10 µL of 10 ng/µL standard solution for

periphyton, 10 µL of 50 ng/µL for sediment, 20 µL of 5 ng/µL for water) and stored at 4°C in amber GC vials until instrumental analyses. Extracts were analyzed on an Agilent 7890 gas chromatograph coupled with a triple quadrupole mass spectrometer fitted with electron ionization source using an Agilent J&W HP-5 ms ultra inert column (30 m × 0.25 mm × 0.25 µm), with carrier gas helium at a constant flow rate of 1.2 mL/min. The PAL RSI 85 autosampler injected 1 µL of sample in spitless mode. The oven temperature was held at 60°C for 1 min then raised to 120°C at 35°C/min, further ramped up to 220°C at 14°C/min, 260°C at 5°C/min and held for 5 min, to 300°C at 10°C/min and finally to 310°C a 50°C/min. Transfer line and source temperatures were both set to 320°C and UHP nitrogen was used as the collision gas at 60 psi. Multi-reaction monitoring ion transitions were used to quantify PACs based on the average relative response factors of PAC standards at nominal concentrations of 10, 200, 400, 600, 800, 1000 pg/µL. Final concentrations were quantitated by correcting for RIS and IPIS recoveries.

4.2.7 Fish

Baited minnow traps were used to remove any native fish trapped inside the enclosures during their installation. Prior to oil application, each enclosure was stocked with 10 fathead minnows (5 male, 5 female) captured from Lake 260. Fish were anesthetized with MS-222, weighed, measured, had their sex determined, and were marked with a fluorescent dye. They were allowed to recover in fresh lake water before being introduced to their randomly assigned enclosure. Two and a half months later, fish were retrieved from the enclosures using baited minnow traps set overnight. Recovered fish were transported in oxygen enriched lake water to the on-site laboratory (~6 km drive) where they were sacrificed using an overdose of pH buffered MS-222 (0.4 g/L), measured, weighed, and had their gall bladders excised. Gall bladders were stored on dry ice in 0.5 mL Eppendorf tubes and transported back to Winnipeg where they were stored in the dark at -80 °C with exposure to light minimized during collection and transportation. Wetland enclosure fish were collected Sept 3-6 and rock-cobble fish were collected Sept 5-6, 17. Additionally, fish from Lake 260 were collected Sept 23 & 25 and were processed in the same manner as the enclosure fish. This work was permitted by animal use protocol #F18-007.

4.2.8 Preparation of bile samples

Preparation and HPLC-F analysis of bile followed the methods of Jonsson et al (2003) and Grung et al (2016), with each acknowledging Krahn et al (1987, 1992) as providing an initial analytical framework. Due to the small size of each gallbladder, bile was determined by weight. For analysis, gallbladders were allowed to thaw and were weighed on an analytical balance (0.0001g) while still in the microcentrifuge tube used for storage. Once weighed, each microcentrifuge tube had 50 µL of 0.5% ascorbic acid buffer (pH: 5.0) added and the contents were vortexed, and then centrifuged for 5 min at 9,600 g. Gallbladders were then minced in the tube to ensure full extraction of bile and the resulting bile/buffer mixture was transferred to a 1.5 mL microcentrifuge tube containing 5 µL of β-glucuronidase solution, quickly vortexed, and incubated at 37°C in the dark for 2-5 hours. Following incubation, 50 µL of methanol containing 0.5% ascorbic acid was added, and the tubes were briefly vortexed then centrifuged for 5 min at 9,600 g with the supernatant analyzed. Gallbladders with larger volumes of bile (>10 µL) were further diluted with additional buffer and 0.5% ascorbic acid methanol (in a 1:1 ratio). During incubation, tubes used for gall bladder storage were dried and re-weighed to determine the mass of the empty gallbladders and to allow for calculation of bile mass.

4.2.9 HPLC-F analysis

The HPLC system consisted of a Varian ProStar 410 Autosampler, Varian ProStar 210 pumps, and a Varian ProStar 363 fluorescence detector. Optimum excitation/emission (ex/em) wavelength pairs for each analyte were determined by direct injection into the fluorescence detector with a syringe pump while scanning through a range of emission or excitation wavelengths. The optimal ex/em pairs used were 275/361 nm for 2-naphthol, 300/383 nm for 1-phenanthrol, 340/384 nm for 1-pyrenol, 265/380 nm for 2-chrysenol, and 381/435 nm for 3-benzo[a]pyrenol. Ultrapure water (A) and methanol (B) comprised the mobile phase for the elution. The elution profile was as follows: 0-1 minute, isocratic 38% B; 1-35 min, 38-95% B linear gradient; 35-37 min, isocratic 95% B; 37-45 min, 95-38% B linear gradient; 45-50 min, isocratic 38% B. The flow rate was 0.30 mL/min. The chromatography column was held at 40°C during analysis and the autosampler delivered 10 µL for each injection. The samples were run on ZORBAX RR Eclipse XDB-C18, 80Å, 2.1 x 50 mm, 3.5 µm columns.

4.2.10 Biliary metabolite calculations

The concentrations of 1-pyrenol were calculated from an average response factor obtained from a 10 ng/mL mixed standard containing all analytes run at the beginning, middle, and end of each sample run. The concentration of 2-chrysenol was calculated from the recovery of the metabolite in a reference sample spiked to 10 ng/mL with each analyte. In some samples, run on a newer chromatographic column, 2-chrysenol could not be quantified in experimental samples due to a changed elution pattern and interference. In these samples, a consistent peak was observed 30 seconds after the 2-chrysenol peak. This was presumed to be a different isomer of chrysenol, was dubbed x-chrysenol, and was quantified in the same manner as 2-chrysenol. The concentration of phenanthrols was estimated based on the response factor of 1-phenanthrol in the 10 ng/mL mixed standard. When other isomers were detected, the response factor of 1-phenanthrol was corrected with the relative response factor of the isomer in question. When isomers coeluted, their concentrations were estimated based on the relative amount of each co-eluting isomer in fully resolved samples. For calculations, the density of bile was assumed to be 1.00 g/mL, which is slightly higher than the 0.975 g/mL reported for sheepshead minnow (*Cyprinodon variegatus*; G. Jonsson et al., 2003).

4.2.11 Statistical Analysis

Statistical tests were performed using RStudio software with statistical significance accepted at $p < 0.05$. Parametric least squares regression and Welch's 2-sided t-test were run as appropriate, with the Shapiro-Wilk test performed as necessary to assess the normality of data for t-tests. For regressions, the assumptions of normality and homogeneity of variance were confirmed graphically using histograms and quantile-quantile plots of the residuals and a heteroscedasticity plot of the residuals, respectively. Data was natural log-transformed as necessary to meet the assumptions of the models and achieve the best possible fit. The assumptions of the parametric ANOVA for group comparisons were not met by the data of this study, thus the non-parametric Kruskal-Wallis test was run instead in conjunction with a post-hoc Holm corrected Mann-Whitney-Wilcoxon test. When t-test assumptions were not met, the non-parametric Mann-Whitney U test was used instead.

4.3 Results

Of the 180 tagged fathead minnows introduced to the enclosures (i.e. 10/enclosure), a total of only 11 were recovered from all 18 enclosures. However, a viable sample size for both sets of enclosures was achieved from the recovery of many untagged fish, mostly finescale dace or fathead minnows. A small quantity of northern pearl dace (*Margariscus nachtriebi*), northern red belly dace (*Chrosomus eos*), and lake chub (*Couesius plumbeus*) were also captured (Tables 4.1-4.6). These fish either infiltrated the sealed enclosures or hatched from eggs trapped inside the enclosure during set-up. All fish with a fork length ≥ 43 mm were subject to biliary PAC metabolite analysis regardless of species (see Supplemental Information III for body sizes). In total, bile from 256 individual fish was analyzed for PAC metabolites. Fish from oil exposed enclosures showed increase exposure to PACs compared to fish from reference enclosures and the pristine lake environment. Biliary metabolite concentrations in fish were consistently correlated to parent PAC concentrations in the periphyton. No significant differences in PAC exposure to fish from the different remediation techniques were detected.

4.3.1 Method Validation

Refer to section 3.3.1.

4.3.2 Metabolite concentrations

With the exception of 3-benzo[a]pyrenol, which was not reliably detected in any sample, the targeted metabolites were generally detected in samples and were, when applicable, at concentrations above the determined limits of quantitation. 2-chrysenol was not quantified in some fish due to a change in the chromatographic column. For these fish a presumed chrysenol isomer, x-chrysenol was measured. Fish were recovered from each enclosure but were not always evenly distributed among them (Tables 4.1-4.6). Additional information regarding fish size, sex, and bile mass recoveries of experimental fish are available in Supplemental Information III.

			Phenanthrols (ng/mL)				
Enclosure	**Species**	**Fish**	**Mean**	**Median**	**Std dev**	**Min**	**Max**
Lake	FHM	17	75.4	71.2	36.0	27.8	123.3
	FSD	18	252.3	242.7	107.9	76.0	519.3

wRef 1	FSD	19	141.1	142.8	51.5	51.3	230.0
	PD	2	139.3	-	-	104.5	174.1
wRef 2	FSD	7	135.6	104.9	88.3	21.2	259.5
wEFW	FSD	5	88.1	72.2	51.3	52.7	177.8
	PD	4	152.4	139.3	45.3	113.3	217.8
wEMNR 1	FHM	5	179.9	144.1	104.7	66.1	324.8
	FSD	4	114.4	120.5	31.3	74.8	142.0
	LC	1	155.9	-	-	-	-
	PD	3	119.3	105.6	57.7	69.8	182.6
wEMNR 2	FHM	4	171.2	187.1	44.4	106.2	204.4
	FSD	2	337.9	-	-	332.1	343.7
wEMNR 3	FSD	5	177.4	147.3	142.4	14.4	351.2
	PD	1	59.9	-	-	-	-
wSC 1	FHM	4	319.7	284.1	105.9	236.9	473.5
	FSD	2	631.9	-	-	175.4	1088.4
	PD	2	357.6	-	-	261.9	453.3
wSC 2	FHM	15	215.2	171.1	153.5	28.4	486.1
	FSD	1	87.7	-	-	-	-
	PD	20	43.7	-	-	26.7	60.8
wSC 3	FHM	15	272.7	191.2	177.5	29.6	540.4
	FSD	3	123.4	112.7	20.0	110.9	146.5

Table 4.1: Summary data for phenanthrols in wetland (w) enclosures by species. FHM ≡ fathead minnow, FSD ≡ finescale dace, PD ≡ pearl dace, LC ≡ lake chub, Ref ≡ reference, EFW ≡ engineered floating wetland, EMNR ≡ enhanced monitored natural recovery, SC ≡ shoreline cleaning agent

			1-pyrenol (ng/mL)				
Enclosure	**Species**	**Fish**	**Mean**	**Median**	**Std dev**	**Min**	**Max**
Lake	FHM	17	10.2	7.37	7.32	4.50	27.5
	FSD	18	6.69	6.03	3.03	1.78	12.2
wRef 1	FSD	19	11.1	10.9	4.86	1.43	20.1
	PD	2	20.1	-	-	6.73	33.4
wRef 2	FSD	7	14.7	11.1	10.7	2.01	29.9
wEFW	FSD	5	75.7	45.8	52.9	26.5	150.6
	PD	4	109.8	116.4	30.0	70.0	136.3
wEMNR 1	FHM	5	52.7	75.2	39.0	0.47	83.7
	FSD	4	151.7	123.6	115.3	52.8	306.7
	LC	1	40.5	-	-	-	-
	PD	3	67.1	78.5	45.4	17.1	105.6
wEMNR 2	FHM	4	95.4	91.6	22.4	72.4	126.0

	FSD	2	151.6	-	-	135.0	168.3
wEMNR 3	FSD	5	49.1	28.1	41.5	7.94	95.1
	PD	1	7.74	-	-	-	-
wSC 1	FHM	4	91.4	86.9	36.9	56.9	134.9
	FSD	2	200.5	-	-	33.6	367.5
	PD	2	73.2	-	-	32.5	113.8
wSC 2	FHM	15	52.5	57.8	27.3	13.8	90.4
	FSD	1	64.2	-	-	-	-
	PD	2	21.7	-	-	8.26	35.1
wSC 3	FHM	15	81.9	87.5	54.7	0.61	172.6
	FSD	3	94.8	100.3	68.3	23.9	160.2

Table 4.2: Summary data for 1-pyrenol in wetland (w) enclosures by species. FHM ≡ fathead minnow, FSD ≡ finescale dace, PD ≡ pearl dace, LC ≡ lake chub, Ref ≡ reference, EFW ≡ engineered floating wetland, EMNR ≡ enhanced monitored natural recovery, SC ≡ shoreline cleaning agent

			x-chrysenol (ng/mL)				
Enclosure	**Species**	**Fish**	**Mean**	**Median**	**Std dev**	**Min**	**Max**
Lake	FHM	12	13.1	10.9	9.81	2.64	33.1
wRef 1	FSD	19	40.6	32.7	26.6	7.60	120.0
	PD	2	59.8	-	-	35.2	84.5
wRef 2	FSD	7	20.1	14.6	16.4	7.14	53.4
wEFW	FSD	5	14.2	11.9	8.50	3.62	24.3
	PD	4	15.1	14.2	8.89	5.46	26.7
wEMNR 1	FHM	5	20.6	28.0	16.0	ND	37.0
	FSD	4	8.08	8.17	5.48	2.30	13.7
	LC	1	124.8	-	-	-	-
	PD	2	24.4	-	-	12.7	36.1
wEMNR 2	FHM	4	27.8	28.1	8.62	18.1	37.0
	FSD	2	21.4	-	-	18.1	24.6
wEMNR 3	FSD	5	22.1	25.9	17.0	3.20	41.2
	PD	1	12.8	-	-	-	-
wSC 1	FHM	4	31.5	31.1	14.2	15.2	48.4
	FSD	2	76.1	-	-	8.54	143.6
	PD	2	7.81	-	-	3.62	12.0
wSC 2	FHM	15	9.62	7.76	7.43	ND	29.0
	FSD	1	8.99	-	-	-	-
	PD	2	3.01	-	-	1.15	4.87
wSC 3	FHM	15	26.3	14.6	29.3	0.66	92.6
	FSD	3	53.6	36.8	60.3	3.41	120.4

Table 4.3: Summary data for x-chrysenol in wetland (w) enclosures by species. FHM ≡ fathead minnow, FSD ≡ finescale dace, PD ≡ pearl dace, LC ≡ lake chub, Ref ≡ reference, EFW ≡ engineered floating wetland, EMNR ≡ enhanced monitored natural recovery, SC ≡ shoreline cleaning agent

			Phenanthrols (ng/mL)				
Enclosure	**Species**	**Fish**	**Mean**	**Median**	**Std dev**	**Min**	**Max**
Lake	FHM	17	75.4	71.2	36.0	27.8	123.3
	FSD	18	252.3	242.7	107.9	76.0	519.3
rRef 1	FHM	1	117.3	-	-	-	-
	FSD	4	243.6	244.0	24.9	218.5	267.9
	LC	1	143.1	-	-	-	-
rRef 2	FHM	2	151.8	-	-	83.8	219.8
	FSD	7	217.2	220.9	75.2	131.3	310.4
	LC	1	179.3	-	-	-	-
rEFW	FSD	13	194.0	198.0	75.9	60.2	363.1
rEMNR 1	FSD	11	137.7	119.2	156.8	66.9	559.2
rEMNR 2	FSD	18	247.5	242.7	67.1	147.6	409.7
	LC	1	243.7	-	-	-	-
	NRBD	1	223.9	-	-	-	-
rEMNR 3	FSD	19	190.0	188.1	78.7	81.3	410.5
rSC 1	FSD	8	181.3	170.5	57.8	110.4	295.9
rSC 2	FHM	1	198.8	-	-	-	-
	FSD	9	149.8	132.2	80.3	78.3	333.6
	NRBD	1	191.0	-	-	-	-
rSC 3	FSD	14	188.6	160.0	118.7	65.2	568.6
	LC	1	198.4	-	-	-	-

Table 4.4: Summary data for phenanthrols in rock-cobble (r) enclosures by species. FHM ≡ fathead minnow, FSD ≡ finescale dace, PD ≡ pearl dace, LC ≡ lake chub, NRBD ≡ northern redbelly dace, Ref ≡ reference, EFW ≡ engineered floating wetland, EMNR ≡ enhanced monitored natural recovery, SC ≡ shoreline cleaning agent

			1-pyrenol (ng/mL)				
Enclosure	**Species**	**Fish**	**Mean**	**Median**	**Std dev**	**Min**	**Max**
Lake	FHM	17	10.2	7.37	7.32	4.50	27.5
	FSD	18	6.69	6.03	3.03	1.78	12.2
rRef 1	FHM	1	17.7	-	-	-	-
	FSD	4	4.12	3.99	1.14	2.90	5.60
	LC	1	3.68	-	-	-	-
rRef 2	FHM	2	26.7	-	-	11.2	42.3
	FSD	7	6.39	6.46	1.98	3.31	8.79
	LC	1	6.24	-	-	-	-
rEFW	FSD	13	37.9	33.4	24.8	13.5	99.6
rEMNR 1	FSD	11	80.4	63.2	45.3	21.0	155.8
rEMNR 2	FSD	18	65.4	52.3	34.4	12.6	141.1
	LC	1	102.5	-	-	-	-
	NRBD	1	46.2	-	-	-	-
rEMNR 3	FSD	19	88.0	31.2	131.3	11.6	539.5
rSC 1	FSD	8	33.3	31.3	13.1	20.6	61.2
rSC 2	FHM	1	21.3	-	-	-	-
	FSD	9	45.7	44.3	22.4	10.2	79.8
	NRBD	1	20.3	-	-	-	-
rSC 3	FSD	14	31.0	30.5	15.6	4.11	64.8
	LC	1	16.2	-	-	-	-

Table 4.5: Summary data for 1-pyrenol in rock-cobble (r) enclosures by species. FHM ≡ fathead minnow, FSD ≡ finescale dace, PD ≡ pearl dace, LC ≡ lake chub, NRBD ≡ northern redbelly dace, Ref ≡ reference, EFW ≡ engineered floating wetland, EMNR ≡ enhanced monitored natural recovery, SC ≡ shoreline cleaning agent

			¦x-chrysenol /*2-chrysenol (ng/mL)				
Enclosure	**Species**	**Fish**	**Mean**	**Median**	**Std dev**	**Min**	**Max**
Lake	FHM¦	12	13.1	10.9	9.81	2.64	33.1
	FHM*	5	1.21	1.58	0.83	ND	2.07
	FSD*	18	2.10	0.78	3.29	ND	11.2
rRef 1	FHM¦	1	3.36	-	-	-	-
	FSD*	4	1.49	1.76	1.05	ND	2.45
	LC*	1	ND	-	-	-	-
rRef 2	FHM¦	2	7.23	-	-	5.14	6.18
	FSD*	7	8.93	0.74	18.3	ND	49.9
	LC*	1	1.16				
rEFW	FSD*	13	31.1	21.6	26.7	3.92	95.7
rEMNR 1	FSD*	11	39.7	44.2	12.0	11.6	53.5
rEMNR 2	FSD*	18	27.0	28.1	19.2	3.0	69.9
	LC*	1	19.7	-	-	-	-
	NRBD*	1	40.5	-	-	-	-
rEMNR 3	FSD*	19	26.9	6.04	46.6	ND	156.6
rSC 1	FSD*	8	21.3	15.4	17.5	3.67	58.3
rSC 2	FHM*	1	31.1	-	-	-	-
	FSD*	9	34.9	27.7	25.0	2.34	82.3
	NRBD*	1	9.21	-	-	-	-
rSC 3	FSD*	14	9.33	5.86	7.99	0.81	28.0
	LC*	1	2.34	-	-	-	-

Table 4.6: Summary data for chrysenol metabolites in rock-cobble (r) enclosures by species. *2-chrysenol, ¦x-chrysenol FHM ≡ fathead minnow, FSD ≡ finescale dace, PD ≡ pearl dace, LC ≡ lake chub, NRBD ≡ northern redbelly dace, Ref ≡ reference, EFW ≡ engineered floating wetland, EMNR ≡ enhanced monitored natural recovery, SC ≡ shoreline cleaning agent

4.3.3 Comparisons between species

Uneven fish recoveries among the enclosures with respect to species necessitated determination of whether the species can be considered equivalent with respect to PAC metabolite concentrations. A robust population of finescale dace and fathead minnows for statistical comparison was available from the lake itself though only one enclosure had sufficient recoveries of both species for comparison. A third enclosure had sufficient population to investigate differences between finescale dace and pearl dace. Data with normal distributions (Table 4.8) were assessed differently than non-normal distributions (Table 4.7).

		Fathead minnow		Finescale dace			
Enclosure	**Metabolite**	**Median**	**IQR**	**Median**	**IQR**	**W**	**p-value**
Lake	Phenanthrols	71.2	70.3	242.7	123	16	<0.001
Lake	1-pyrenol	7.37	5.36	6.03	4.65	197	0.153
Lake	2-chrysenol	1.58	0.92	0.78	1.99	46.5	0.939
		Pearl dace		**Finescale dace**			
WL6	Phenanthrols	139.3	29.5	72.2	21.2	3	0.111

Table 4.7: Mann-Whitney test results comparing biliary metabolite concentrations in different species of fish in the same enclosures. All concentrations are ng/mL. IQR ≡ interquartile range

		Fathead minnow		Finescale dace			p-	
Enclosure	**Metabolite**	**Mean**	**SD**	**Mean**	**SD**	**Δ**	**value**	**95% CI**
WL8	Phenanthrols	179.9	104.7	114.4	31.3	65.5	0.243	-62.5-193.5
WL8	1-pyrenol	52.7	39.0	151.7	115.3	-99.0	0.185	-274.9-77.0
WL8	x-chrysenol	20.6	16.0	8.08	5.48	12.6	0.162	-7.06-32.2
		Pearl dace		**Finescale dace**				
WL6	1-pyrenol	109.8		75.7		34.1	0.265	-33.2-101.5
WL6	x-chrysenol	15.1		14.2		0.9	0.881	-13.2-15.0

Table 4.8: Welch's 2-sided t-test comparing biliary metabolite concentrations in different species of fish in the same enclosures. All concentrations are ng/mL. Δ ≡ difference between means, CI ≡ confidence interval

Comparisons of fathead minnows and finescale dace indicated impactful species-species differences with respect to biliary phenanthrol concentrations; the populations were significantly different in the lake and had a large potential mean difference among fish captured in the enclosures. Meaningful differences in 1-pyrenol and chrysenol metabolite concentrations were not apparent in the lake given the low biliary concentrations for each species. Differences were more pronounced in an enclosure with the mean metabolite concentrations in one species being at least double the other for both metabolites. Moreover, there were large confidence intervals for the difference between the means. Thus, finescale dace and fathead minnows are analyzed independently in this study. Statistical significance in enclosures may have been hampered by sample sizes (n=4 and 5). Significant differences between pearl dace and finescale dace are not present, though consequential differences may exist for 1-pyrenol and phenanthrols. Once again, such determinations are hampered by sample sizes (n=4 and 5). These species would ideally be analyzed separately but are necessarily grouped for analysis of wetland enclosures due to low captures of finescale dace in some of the enclosures. While statistical analysis did not unequivocally indicate major differences between metabolite concentrations between finescale dace and pearl dace, any findings derived from their pooling cannot be considered as robust as species-specific analyses.

4.3.4 Differences in metabolite concentrations based on sex

Populations of fish in the general lake and the only enclosure with a balanced population were analyzed for significant differences between sexes. Species were analyzed independently and data with normal distributions (Table 4.9) were assessed differently than non-normal distributions (Table 4.10). Most fish in the enclosures were female.

			Female			Male				
Enclosure	**Metabolite**	**Species**	**n**	**Mean**	**SD**	**n**	**Mean**	**SD**	**t**	**p-value**
wSC 2	Phenanthrols	FHM	9	221	176	5	210	141	0.134	0.896
Lake	1-pyrenol	FHM	8	14.5	8.79	8	6.39	2.29	2.52	0.036
wSC2	1-pyrenol	FHM	9	50.2	26.6	5	55.6	34.0	-0.308	0.767
Lake	x-chrysenol	FHM	6	16.6	12.0	6	9.65	6.22	1.25	0.247
wSC2	x-chrysenol	FHM	9	11.5	8.97	5	6.93	3.64	1.33	0.210

Table 4.9: Welch's 2-sided t-test comparing biliary metabolite concentrations in fish of different sex in the same enclosures. All concentrations are ng/mL. FHM ≡ fathead minnows

Enclosure	Metabolite	Species	Female n	Female Med	Female IQR	Male n	Male Med	Male IQR	W	p-value
Lake	Phenanthrols	FHM	8	52.9	72.3	8	81.0	52.7	25	0.505
Lake	Phenanthrols	FSD	10	267	98.2	8	196	49.6	57	0.146
Lake	1-pyrenol	FSD	10	5.25	2.78	8	8.20	4.61	26	0.237
Lake	2-chrysenol	FSD	10	0.21	0.79	8	1.85	2.66	23	0.120

Table 4.10: Mann-Whitney test results comparing biliary metabolite concentrations in fish of different sex in the same enclosures. All concentrations are ng/mL. IQR ≡ interquartile range, FHM ≡ fathead minnow, FSD ≡ finescale dace

Sex generally did not significantly affect metabolite concentrations in fathead minnows nor finescale dace. Only lake fathead minnows showed a significant difference in metabolite concentrations between sexes and only for one metabolite, 1-pyrenol. Though this difference was significant, the difference between the means was small, and disappeared in fish with greater exposure to pyrene in wSC2. Overall, the differences in PAC metabolite concentrations between the groups are unlikely to be consequential from either a toxicological perspective nor a statistical one when enclosures with imbalanced sex populations are compared.

4.3.5 Metabolite concentrations and fish size

A linear regression was run between fish size (body length) and metabolite concentrations for each species in enclosures where numbers permitted (WL3 and 4 for fathead minnows, WL7, RC1, 2, and 9 for finescale dace) and the lake. No significant relationship was found between size and metabolite concentration for either species in any environment for any metabolite.

4.3.6 Comparisons between finescale dace in rock-cobble shorelines

For finescale dace in wetland shorelines, enclosures had an effect on biliary phenanthrol ($H(9) = 28.98$, $p < 0.001$), 1-pyrenol ($H(9) = 74.71$, $p < 0.001$), and 2-chrysenol ($H(9) = 62.20$, $p < 0.001$) concentrations. For phenanthrols, the only significant difference was between eMNR1 and eMNR2, with no differentiation between oiled and unoiled enclosures (Figure 4.2 and Table 4.11).

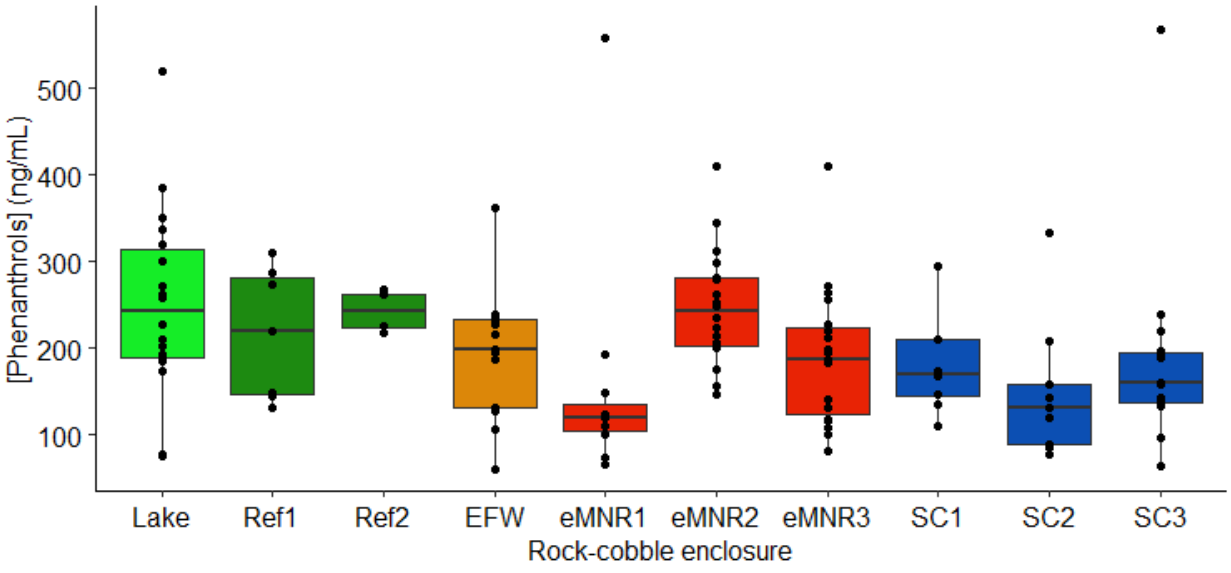


Figure 4.2: Concentrations of phenanthrols in finescale dace in rock-cobble enclosures. Boxplots show the 25th percentile (bottom hinge), median (centre line), 75th percentile (top hinge), and whiskers extending to 1.5 interquartile range. Ref ≡ reference, EFW ≡ engineered floating wetland, eMNR ≡ enhanced monitored natural recovery, SC ≡ shoreline cleaning agent

	Lake	**rRef1**	**rRef2**	**rEFW**	**reMNR1**	**reMNR2**	**reMNR3**	**rSC1**	**rSC2**
rRef1	NSD	-	-	-	-	-	-	-	-
rRef2	NSD	NSD	-	-	-	-	-	-	-
rEFW	NSD	NSD	NSD	-	-	-	-	-	-
reMNR1	0.182	0.616	0.668	NSD	-	-	-	-	-
reMNR2	NSD	NSD	NSD	NSD	0.011	-	-	-	-
reMNR3	NSD	NSD	NSD	NSD	NSD	0.492	-	-	-
rSC1	NSD	NSD	NSD	NSD	NSD	0.693	NSD	-	-
rSC2	0.667	NSD	NSD	NSD	NSD	0.102	NSD	NSD	-
rSC3	0.720	NSD	0.869	NSD	NSD	0.090	NSD	NSD	NSD

Table 4.11: Holm corrected p-values of Mann-Whitney-Wilcoxon pairwise comparison tests of phenanthrol metabolite concentrations between finescale dace in rock-cobble (r) enclosures. NSD ≡ No significant difference (corrected p-values are >1.000), Ref ≡ reference, EFW ≡ engineered floating wetland, eMNR ≡ enhanced monitored natural recovery, SC ≡ shoreline cleaning agent

Concentrations of 1-pyrenol differed significantly among enclosures (Figure 4.3 and Table 4.12). Most notably, fish in the enclosures receiving oil all had significantly higher concentrations than

the unpolluted lake and Ref1 enclosure. Ref 2, with only 4 fish recovered, had significantly lower concentrations than the EFW and eMNR enclosures, and qualitatively lower 1-pyrenol concentrations than the SC enclosures. In comparing the different remediation methods, no differences were found for 1-pyrenol concentrations between the EFW enclosure and any of the SC enclosures. Conversely, two eMNR enclosures had fish with higher 1-pyrenol concentrations than an SC enclosure. Additionally, several other eMNR enclosures approached statistical significance with higher metabolite concentrations than several EFW and SC enclosures. No significant differences were observed between replicate enclosures of any remediation strategy.

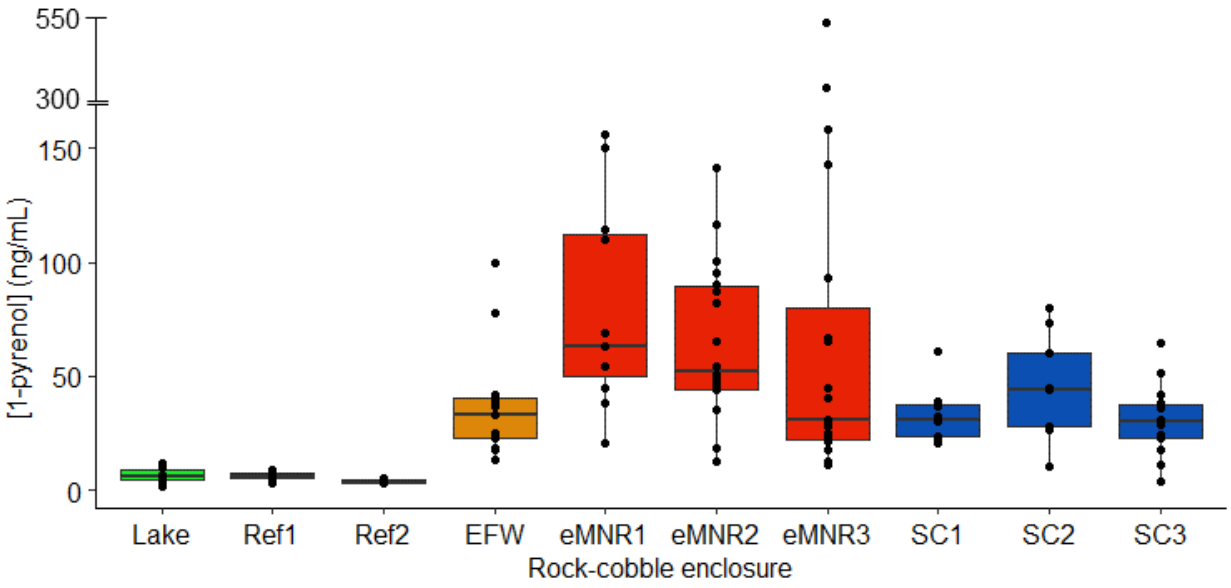


Figure 4.3: Concentrations of 1-pyrenol in finescale dace in rock-cobble enclosures. Boxplots show the 25th percentile (bottom hinge), median (centre line), 75th percentile (top hinge), and whiskers extending to 1.5 interquartile range. Ref ≡ reference, EFW ≡ engineered floating wetland, eMNR ≡ enhanced monitored natural recovery, SC ≡ shoreline cleaning agent

	Lake	**rRef1**	**rRef2**	**rEFW**	**reMNR 1**	**reMNR 2**	**reMNR 3**	**rSC1**	**rSC2**
rRef1	NSD	-	-	-	-	-	-	-	-
rRef2	NSD	NSD	-	-	-	-	-	-	-
rEFW	<0.001	0.001	0.024	-	-	-	-	-	-
reMNR1	<0.001	0.002	0.038	0.119	-	-	-	-	-
reMNR2	<0.001	<0.001	0.009	0.117	NSD	-	-	-	-
reMNR3	<0.001	<0.001	0.007	NSD	NSD	NSD	-	-	-
rSC1	<0.001	0.010	0.093	NSD	0.109	0.175	NSD	-	-
rSC2	<0.001	0.006	0.067	NSD	NSD	NSD	NSD	NSD	-

rSC3	<0.001	0.015	0.065	NSD	0.024	0.023	NSD	NSD	NSD

Table 4.12: Holm corrected p-values of Mann-Whitney-Wilcoxon pairwise comparison tests of 1-pyrenol concentrations between finescale dace in rock-cobble (r) enclosures. NSD ≡ No significant difference (corrected p-values are >1.000), Ref ≡ reference, EFW ≡ engineered floating wetland, eMNR ≡ enhanced monitored natural recovery, SC ≡ shoreline cleaning agent

Biliary metabolite concentrations of 2-chrysenol (Figure 4.4 and Table 4.13) displayed similar trends to 1-pyrenol, though with less statistical significance. Once again, all enclosures receiving oil contained fish with significantly higher 2-chrysenol concentrations than the lake. However, unlike 1-pyrenol, no oiled enclosure fish had significantly higher 2-chrysenol concentrations than Ref1 and only the EFW and two eMNR enclosures significantly eclipsed Ref2. In comparing the different remediation strategies, no consistent trends were observed. SC3 had significantly lower concentrations than eMNR1 and fell just outside of significance for eMNR2, but the other SC enclosures were no different than the eMNR enclosures. The EFW enclosure fish were equivalent to fish of all oiled enclosures. No statistical differences were observed between enclosures with identical remediation techniques.

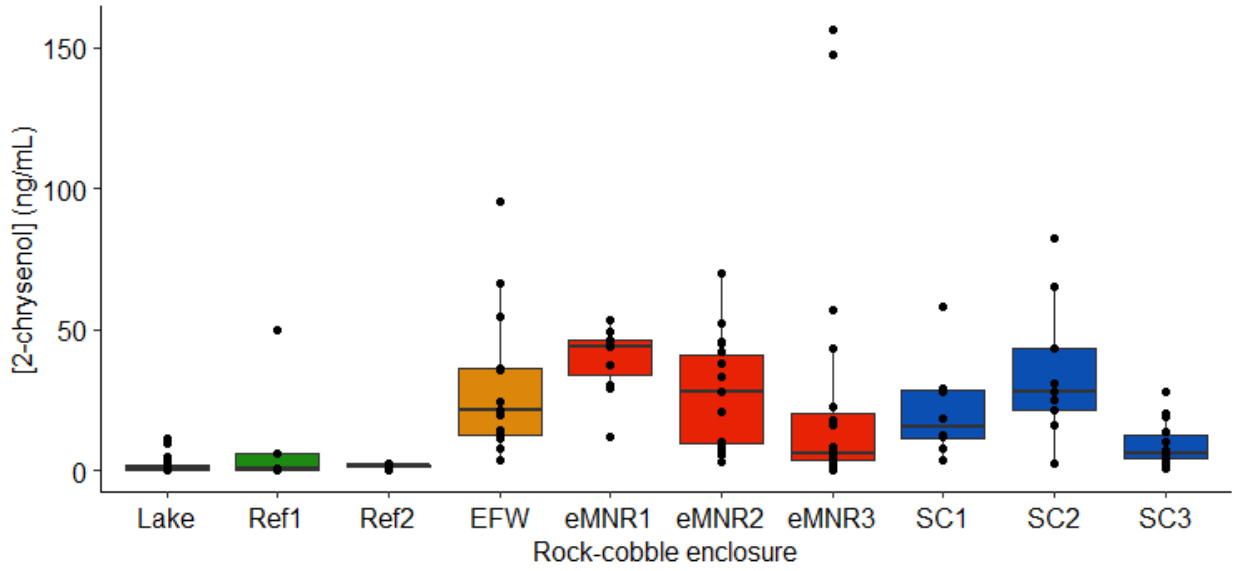


Figure 4.4: Concentrations of 2-chrysenol in finescale dace in rock-cobble enclosures. Boxplots show the 25th percentile (bottom hinge), median (centre line), 75th percentile (top hinge), and whiskers extending to 1.5 interquartile range. Ref ≡ reference, EFW ≡ engineered floating wetland, EMNR ≡ enhanced monitored natural recovery, SC ≡ shoreline cleaning agent

	Lake	**rRef1**	**rRef 2**	**rEFW**	**reMNR 1**	**reMNR 2**	**reMNR 3**	**rSC1**	**rSC2**
rRef1	NSD	-	-	-	-	-	-	-	-
rRef2	NSD	NSD	-	-	-	-	-	-	-
rEFW	<0.001	0.238	0.030	-	-	-	-	-	-
reMNR1	<0.001	0.265	0.051	NSD	-	-	-	-	-
reMNR2	<0.001	0.251	0.010	NSD	0.578	-	-	-	-
reMNR3	0.006	NSD	0.245	0.888	0.136	0.867	-	-	-
rSC1	0.007	0.633	0.125	NSD	0.571	NSD	NSD	-	-
rSC2	0.002	0.449	0.162	NSD	NSD	NSD	NSD	NSD	-
rSC3	0.011	NSD	0.220	0.091	<0.001	0.051	NSD	NSD	0.109

Table 4.13: Holm corrected p-values of Mann-Whitney-Wilcoxon pairwise comparison tests of 2-chrysenol concentrations between finescale dace in rock-cobble (r) enclosures. NSD ≡ No significant difference (corrected p-values are >1.000), Ref ≡ reference, EFW ≡ engineered floating wetland, **e**MNR ≡ enhanced monitored natural recovery, SC ≡ shoreline cleaning agent

4.3.7 Comparisons between dace in wetland shorelines

For wetland analysis, finescale dace and pearl dace were considered equivalent due to low recoveries of finescale dace in several enclosures. Enclosures had a significant effect on biliary concentrations of phenanthrols ($H(9) = 32.59$, $p < 0.001$), 1-pyrenol ($H(9) = 54.19$, $p < 0.001$), and x-chrysenol ($H(8) = 20.76$, $p = 0.007$). Compared to the rock-cobble fish, recoveries for dace among wetland enclosures were notably lower, especially among oiled enclosures from which 3 to 7 fish were captured. These recoveries impaired detection of significant differences in pairwise comparisons between some enclosures.

Pairwise comparison of enclosures with respect to phenanthrol yielded no clear trends (Figure 4.5 and Table 4.14). There was no clear distinction between oiled and unoiled enclosures, nor differences between treatments. The only statistical differences detected were between the lake and three enclosures (rRef1, rEFW, rEMNR1) with the lake being higher in all three incidences.

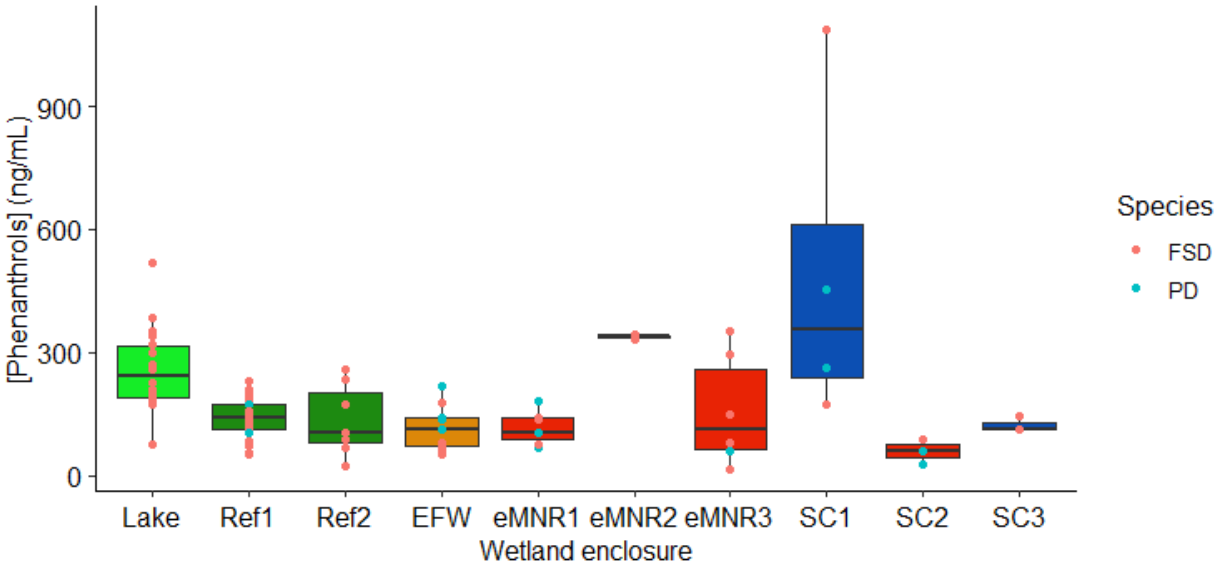


Figure 4.5: Concentrations of phenanthrols in dace in wetland enclosures. Boxplots show the 25th percentile (bottom hinge), median (centre line), 75th percentile (top hinge), and whiskers extending to 1.5 interquartile range. Ref ≡ reference, EFW ≡ engineered floating wetland, eMNR ≡ enhanced monitored natural recovery, SC ≡ shoreline cleaning agent, FSD ≡ finescale dace, PD ≡ pearl dace

	Lake	**Ref1**	**Ref2**	**EFW**	**eMNR1**	**eMNR2**	**eMNR3**	**SC1**	**SC2**
Ref1	0.006	-	-	-	-	-	-	-	-
Ref2	0.760	0.006	-	-	-	-	-	-	-
EFW	0.033	NSD	NSD	-	-	-	-	-	-
eMNR1	0.033	NSD	NSD	NSD	-	-	-	-	-
eMNR2	NSD	0.316	NSD	NSD	NSD	-	-	-	-
eMNR3	NSD	NSD	NSD	NSD	NSD	NSD	-	-	-
SC1	NSD	0.120	0.848	0.436	0.461	NSD	NSD	-	-
SC2	0.247	0.818	NSD	NSD	NSD	NSD	NSD	NSD	-
SC3	NSD	NSD	NSD	NSD	NSD	NSD	NSD	NSD	NSD

Table 4.14: Holm corrected p-values of Mann-Whitney-Wilcoxon pairwise comparison tests of phenanthrol metabolite concentrations between dace in wetland enclosures. NSD ≡ No significant difference (corrected p-values are >1.000), Ref ≡ reference, EFW ≡ engineered floating wetland, eMNR ≡ enhanced monitored natural recovery, SC ≡ shoreline cleaning agent

Biliary metabolite concentrations of 1-pyrenol in wetland dace showed some differences between oiled and unoiled enclosures but there was no distinction between the remediation techniques (see Figure 4.6 and Table 4.15). Three enclosures, one from each remediation method, had significantly higher 1-pyrenol concentrations than the lake and Ref1. The EFW enclosure was also significantly higher 1-pyrenol concentrations than Ref2. No significant differences were observed between any of the oiled enclosures nor between any of the unoiled enclosures.

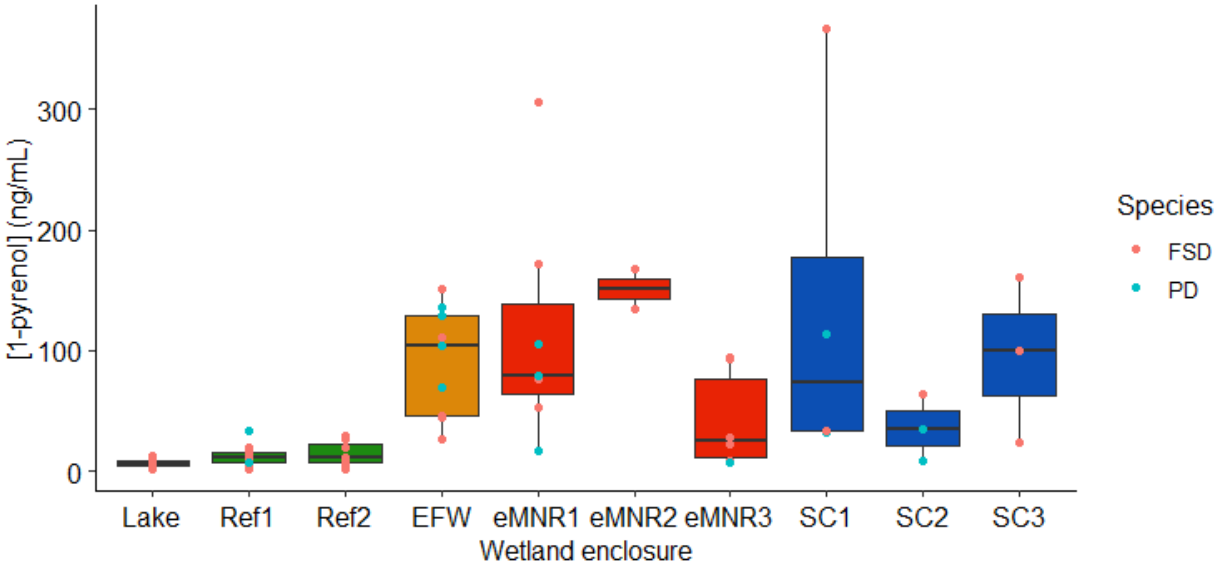


Figure 4.6: Concentrations of 1-pyrenol in dace in wetland enclosures. Boxplots show the 25th percentile (bottom hinge), median (centre line), 75th percentile (top hinge), and whiskers extending to 1.5 interquartile range. Ref ≡ reference, EFW ≡ engineered floating wetland, eMNR ≡ enhanced monitored natural recovery, SC ≡ shoreline cleaning agent, FSD ≡ finescale dace, PD ≡ pearl dace

	Lake	**Ref1**	**Ref2**	**EFW**	**eMNR1**	**eMNR2**	**eMNR3**	**SC1**	**SC2**
Ref1	0.089	-	-	-	-	-	-	-	-
Ref2	NSD	NSD	-	-	-	-	-	-	-
EFW	<0.001	<0.001	0.014	-	-	-	-	-	-
eMNR1	<0.001	<0.001	0.139	NSD	-	-	-	-	-
eMNR2	0.326	0.252	NSD	NSD	NSD	-	-	-	-
eMNR3	0.069	NSD	NSD	NSD	NSD	NSD	-	-	-
SC1	0.011	0.013	0.200	NSD	NSD	NSD	NSD	-	-
SC2	0.722	NSD	NSD	NSD	NSD	NSD	NSD	NSD	-
SC3	0.057	0.071	NSD	NSD	NSD	NSD	NSD	NSD	NSD

Table 4.15: Holm corrected p-values of Mann-Whitney-Wilcoxon pairwise comparison tests of 1-pyrenol concentrations between dace in wetland enclosures. NSD ≡ No significant difference (Corrected p-values are >1.000), Ref ≡ reference, EFW ≡ engineered floating wetland, eMNR ≡ enhanced monitored natural recovery, SC ≡ shoreline cleaning agent

The only significant pairwise difference in x-chrysenol concentrations observed was between EFW and Ref1, with EFW being significantly lower (Figure 4.7 and Table 4.16). There was no difference between oil-treated enclosures and reference enclosures for this metabolite. Lake fish were not included in the x-chrysenol analysis, as this isomer was not quantified in them.

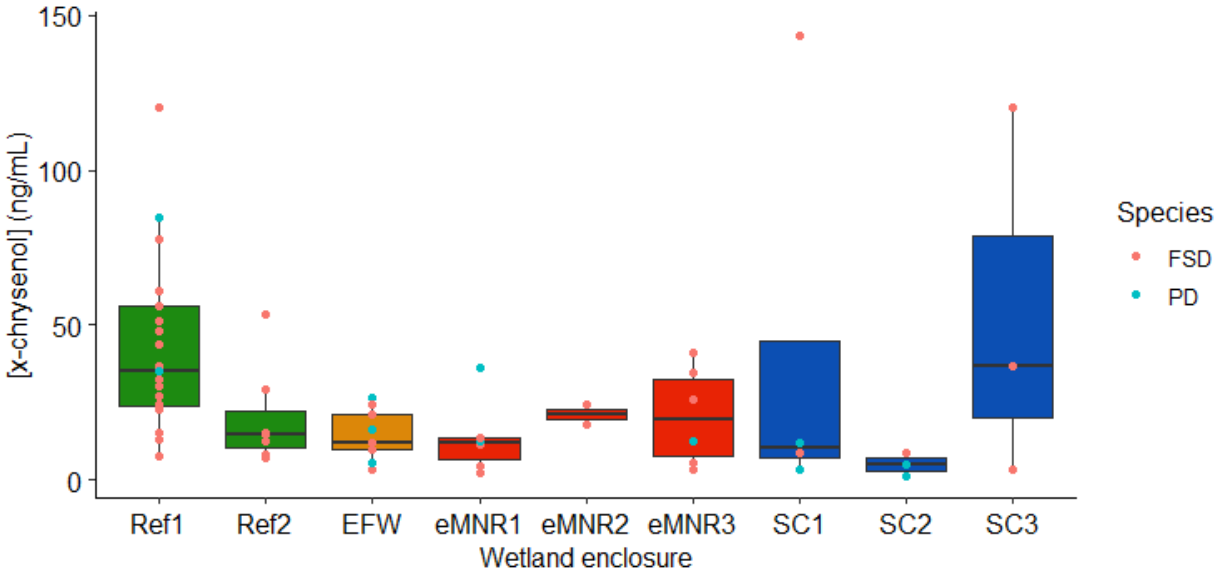


Figure 4.7: Concentrations of x-chrysenol in dace in wetland enclosures. Boxplots show the 25th percentile (bottom hinge), median (centre line), 75th percentile (top hinge), and whiskers extending to 1.5 interquartile range. Ref ≡ reference, EFW ≡ engineered floating wetland, eMNR ≡ enhanced monitored natural recovery, SC ≡ shoreline cleaning agent, FSD ≡ finescale dace, PD ≡ pearl dace

	Ref1	**Ref2**	**EFW**	**eMNR1**	**eMNR2**	**eMNR3**	**SC1**	**SC2**
Ref2	0.657	-	-	-	-	-	-	-
EFW	0.018	NSD	-	-	-	-	-	-
eMNR1	0.114	NSD	NSD	-	-	-	-	-
eMNR2	NSD	NSD	NSD	NSD	-	-	-	-
eMNR3	NSD	NSD	NSD	NSD	NSD	-	-	-
SC1	NSD	NSD	NSD	NSD	NSD	NSD	-	-
SC2	0.069	NSD	NSD	NSD	NSD	NSD	NSD	-
SC3	NSD	NSD	NSD	NSD	NSD	NSD	NSD	NSD

Table 4.16: Holm corrected p-values of Mann-Whitney-Wilcoxon pairwise comparison tests of x-chrysenol concentrations between dace in wetland enclosures. NSD ≡ No significant difference (corrected p-values are >1.000), Ref ≡ reference, EFW ≡ engineered floating wetland, eMNR ≡ enhanced monitored natural recovery, SC ≡ shoreline cleaning agent

4.3.8 Comparisons between fathead minnows in wetland shorelines

Fathead minnows were found in five of the nine wetland enclosures. There was a significant effect of enclosure on biliary concentrations of phenanthrol ($H(5) = 22.72$, $p < 0.0001$), 1-pyrenol ($H(5) = 28.56$, $p < 0.001$), and x-chrysenol ($H(5) = 12.60$, $p = 0.027$) for these fish. Pairwise comparisons were better able to detect significant differences among enclosures SC1 and SC2 as these enclosures had more fish recovered (n=15) than the other three enclosures (n = 4 or 5).

Biliary phenanthrol concentrations were significantly higher in the SC enclosures than in the lake, while no significant differences were observed between the EMNR enclosures and the lake (Figure 4.8 and Table 4.17). No significant differences were observed between the two remediation strategies.

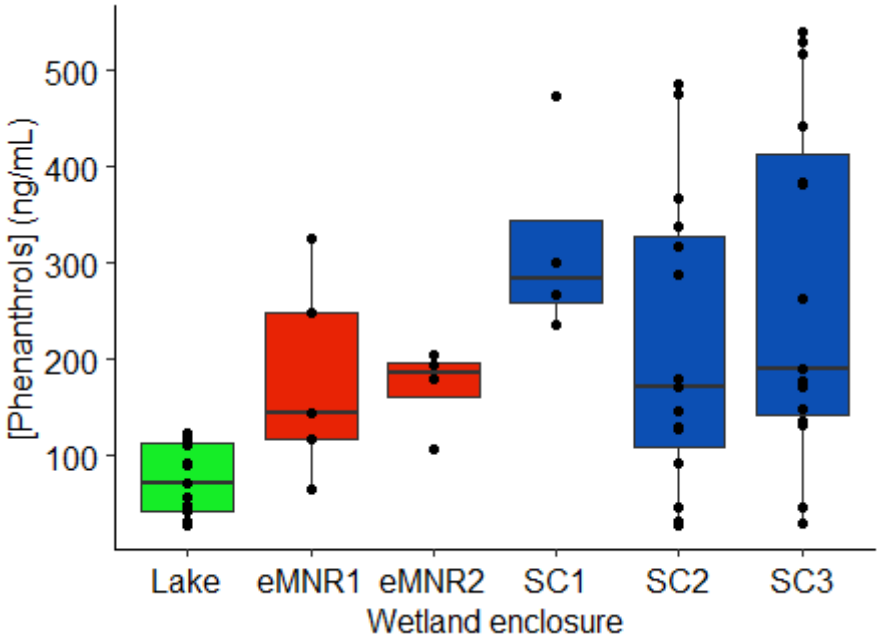


Figure 4.8: Concentrations of phenanthrol metabolites in fathead minnows in wetland enclosures. Boxplots show the 25th percentile (bottom hinge), median (centre line), 75th percentile (top hinge), and whiskers extending to 1.5 interquartile range. Ref ≡ reference, eMNR ≡ enhanced monitored natural recovery, SC ≡ shoreline cleaning agent

	Lake	wEMNR1	wEMNR2	WSC1	WSC2
wEMNR1	0.125	-	-	-	-
wEMNR2	0.108	NSD	-	-	-
wSC1	0.005	NSD	0.286	-	-
wSC2	0.035	NSD	NSD	NSD	-
wSC3	0.001	NSD	NSD	NSD	NSD

Table 4.17: Holm corrected p-values of Mann-Whitney-Wilcoxon pairwise comparison tests of phenanthrol metabolite concentrations between fathead minnows in wetland (w) enclosures. NSD ≡ No significant difference (corrected p-values are >1.000), eMNR ≡ enhanced monitored natural recovery, SC ≡ shoreline cleaning agent

Pairwise comparison of 1-pyrenol concentrations in fathead minnows showed clear differentiation between enclosures receiving oil and the lake itself, with four of the five oiled enclosures having significantly higher concentrations of 1-pyrenol than the lake (Figure 4.9 and Table 4.18). No significant differences were identified between any of the enclosures subject to oil spills.

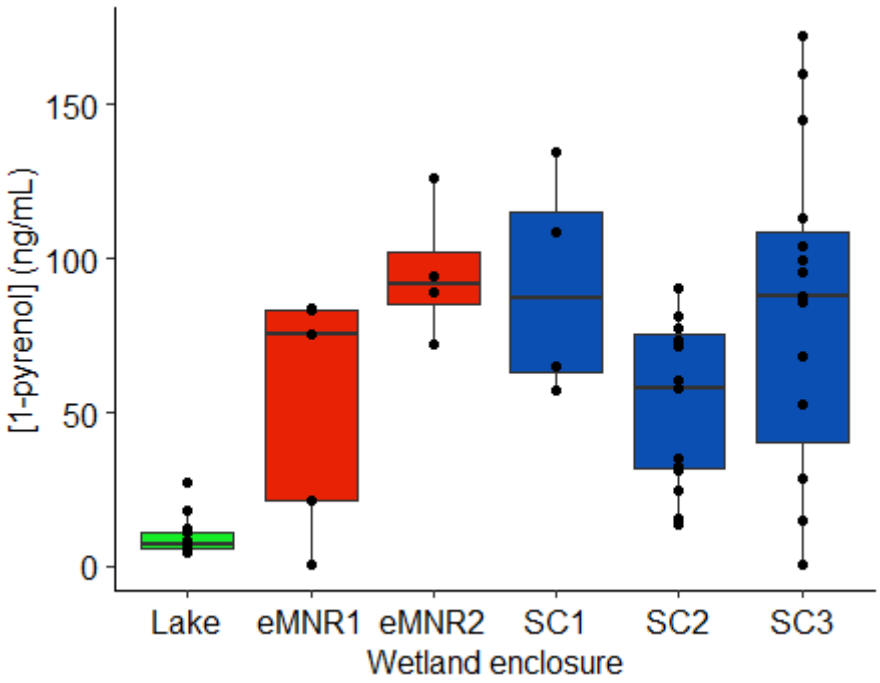


Figure 4.9: Concentrations of 1-pyrenol in fathead minnows in wetland enclosures. Boxplots show the 25th percentile (bottom hinge), median (centre line), 75th percentile (top hinge), and whiskers extending to 1.5 interquartile range. eMNR ≡ enhanced monitored natural recovery, SC ≡ shoreline cleaning agent

	Lake	wEMNR1	wEMNR2	WSC1	WSC2
wEMNR1	0.706	-	-	-	-
wEMNR2	0.005	NSD	-	-	-
wSC1	0.005	NSD	NSD	-	-
wSC2	<0.001	NSD	0.216	NSD	-
wSC3	0.005	NSD	NSD	NSD	NSD

Table 4.18: Holm corrected p-values of Mann-Whitney-Wilcoxon pairwise comparison tests of 1-pyrenol concentrations between fathead minnows in wetland (w) enclosures. NSD ≡ No significant difference (corrected p-values are >1.000), eMNR ≡ enhanced monitored natural recovery, SC ≡ shoreline cleaning agent

Despite the significant effect of enclosure, no significant differences were detected between any enclosures or the lake with resect to biliary concentrations of x-chrysenol (Figure 4.10 and Table 4.19)

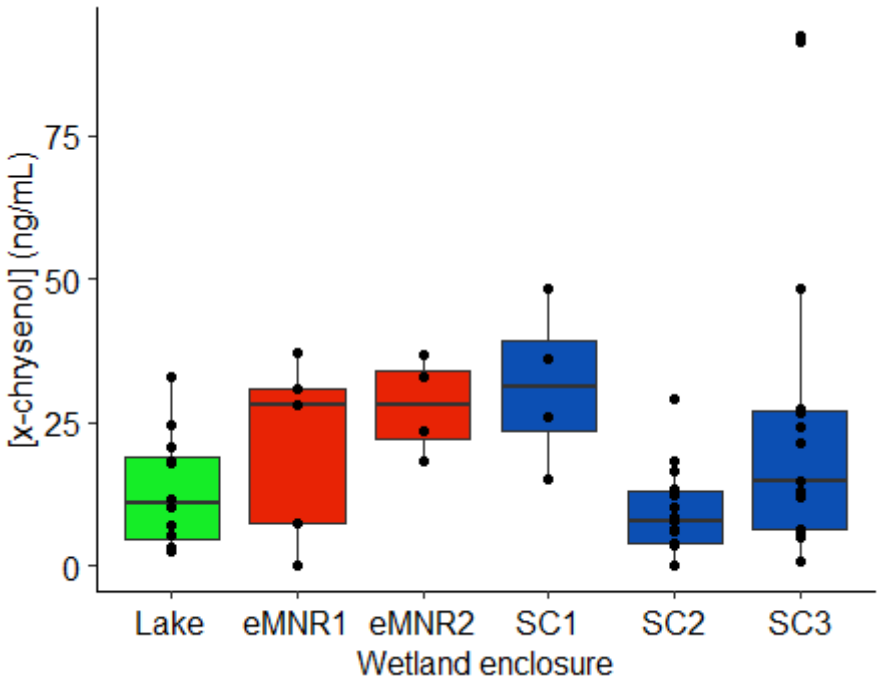


Figure 4.10: Concentrations of x-chrysenol in fathead minnows in wetland enclosures. Boxplots show the 25th percentile (bottom hinge), median (centre line), 75th percentile (top hinge), and whiskers extending to 1.5 interquartile range. eMNR ≡ enhanced monitored natural recovery, SC ≡ shoreline cleaning agent

	Lake	wEMNR1	wEMNR2	WSC1	WSC2
wEMNR1	NSD	-	-	-	-
wEMNR2	0.501	NSD	-	-	-
wSC1	0.386	NSD	NSD	-	-
wSC2	NSD	NSD	0.054	0.087	-
wSC3	NSD	NSD	NSD	NSD	0.674

Table 4.19: Holm corrected p-values of Mann-Whitney-Wilcoxon pairwise comparison tests of x-chrysenol concentrations between fathead minnows in wetland (w) enclosures. NSD ≡ No significant difference (corrected p-values are >1.000), eMNR ≡ enhanced monitored natural recovery, SC ≡ shoreline cleaning agent

4.3.9 Periphyton PAC concentrations and comparisons with aqueous concentrations

Periphyton grew on all enclosure walls and was a readily available food source for fish as well as lower trophic organisms. Periphyton (collected at the same time as the fish, Sept 4 & 11) lack the ability to metabolize PACs (Moermond et al., 2007) and their PAC concentrations were compared to aqueous PAC concentrations one day after the model oil spills, one week after the oil spills (which is also 4 days post-clean up), and the closest water sampling date to periphyton collection (exact dates are given below). Separate analysis was done based on the different PAC sizes and the full list of PAC concentrations are available in Supplemental Information III. Alkylated and non-alkylated PACs were considered both separately and together. As the periphyton was collected near the far-shore wall of the enclosures, their PAC concentrations were compared to the far shore water concentrations (available by ring size in Supplemental Information III).

In the wetland enclosures (Table 4.20), concentrations of PACs with more than two rings were higher in periphyton from oil-exposed enclosures compared to reference enclosures. This was especially noticeable for alkylated compounds. Oil-exposed enclosures often had comparable levels of unalkylated PACs compared to reference enclosures. None of the remediation methods consistently led to lower periphyton PAC concentrations compared to the others.

Rings	Typ	Ref1	Ref2	EFW	EMR 1	EMR 2	EMR 3	SC1	SC2	SC3
2	Unal	92.7	107.3	29.3	73.4	21.2	69.8	10.4	53.7	62.3
2	Alk	1320	632.7	1153	217.7	669.7	5848	717.6	1454	7088
2	All	1413	740.0	1182	291.2	690.9	5918	728.0	1508	7150
3	Unal	318.9	206.1	457.6	181.9	318.1	784.7	309.8	597.5	856.9
3	Alk	6559	2302	18810	2078	9969	20280	14420	18927	32086
3	All	6878	2509	19268	2260	10287	21065	14730	19524	32943
4	Unal	253.8	78.2	1107	71.7	392.0	315.2	658.2	937.1	305.9
4	Alk	314.7	262.3	1071	247.3	1704	1211	1524	1167	2798
4	All	568.6	340.5	2178	319.0	2096	1526	2182	2104	3104
5+	Unal	5.57	28.8	50.3	39.9	71.1	56.8	86.8	77.5	98.0
5+	Alk	19.7	3.20	126.9	55.6	154.4	58.2	180.0	121.8	150.0
5+	All	25.3	32.0	177.2	95.5	225.5	115.0	266.8	199.3	248.0

Table 4.20: PAC concentrations in periphyton from wetland enclosures. All concentrations are ng/g of dry sediment. Typ ≡ type, Unal ≡ unalkylated PACs, Alk ≡ alkylated PACs, EFW ≡ engineered floating wetland, EMR ≡ enhanced monitored natural recovery, Ref ≡ reference, SC ≡ shoreline cleaning agent

In the rock-cobble enclosures (Table 4.21), concentrations of PACs of all size were higher in periphyton from oil-exposed enclosures compared to reference enclosures. As in the wetland enclosures, this disparity was largely driven by alkylated compounds. The engineered floating wetland enclosure had among the highest concentrations of each class of compounds, but no remediation method consistently demonstrated lower periphyton PAC concentrations than any of the others.

Rings	Typ	Ref1	Ref2	EFW	EMR 1	EMR 2	EMR 3	SC1	SC2	SC3
2	Unal	62.6	92.3	30.5	38.6	41.3	33.0	30.5	37.2	59.0
2	Alk	231.9	493.7	4950	1838	3353	531.8	6230	3131	1863
2	All	294.5	586.0	4980	1877	3394	564.8	6260	3168	1922
3	Unal	130.3	282.8	708.5	328.0	532.9	122.1	690.9	348.6	389.2
3	Alk	1179	6209	25200	6409	17296	3018	32764	8580	13514
3	All	1309	6492	25908	6737	17828	3140	33455	8929	13903
4	Unal	50.7	548.8	442.2	157.4	477.2	61.3	323.7	144.4	328.8
4	Alk	52.0	167.4	653.2	260.9	510.3	333.4	1048	300.6	552.0
4	All	102.6	716.2	1095	418.3	987.5	394.7	1372	445.0	880.8
5+	Unal	0.20	1.68	27.2	27.3	16.5	24.7	62.9	23.3	40.5
5+	Alk	0.00	2.84	42.4	26.0	30.5	18.3	101.9	16.8	74.0
5+	All	1.51	5.13	69.6	53.3	46.9	43.0	164.9	40.1	114.5

Table 4.21: PAC concentrations in periphyton from rock-cobble enclosures. All concentrations are ng/g of dry sediment. Typ ≡ type, Unal ≡ unalkylated PACs, Alk ≡ alkylated PACs, EFW ≡ engineered floating wetland, EMR ≡ enhanced monitored natural recovery, Ref ≡ reference, SC ≡ shoreline cleaning agent

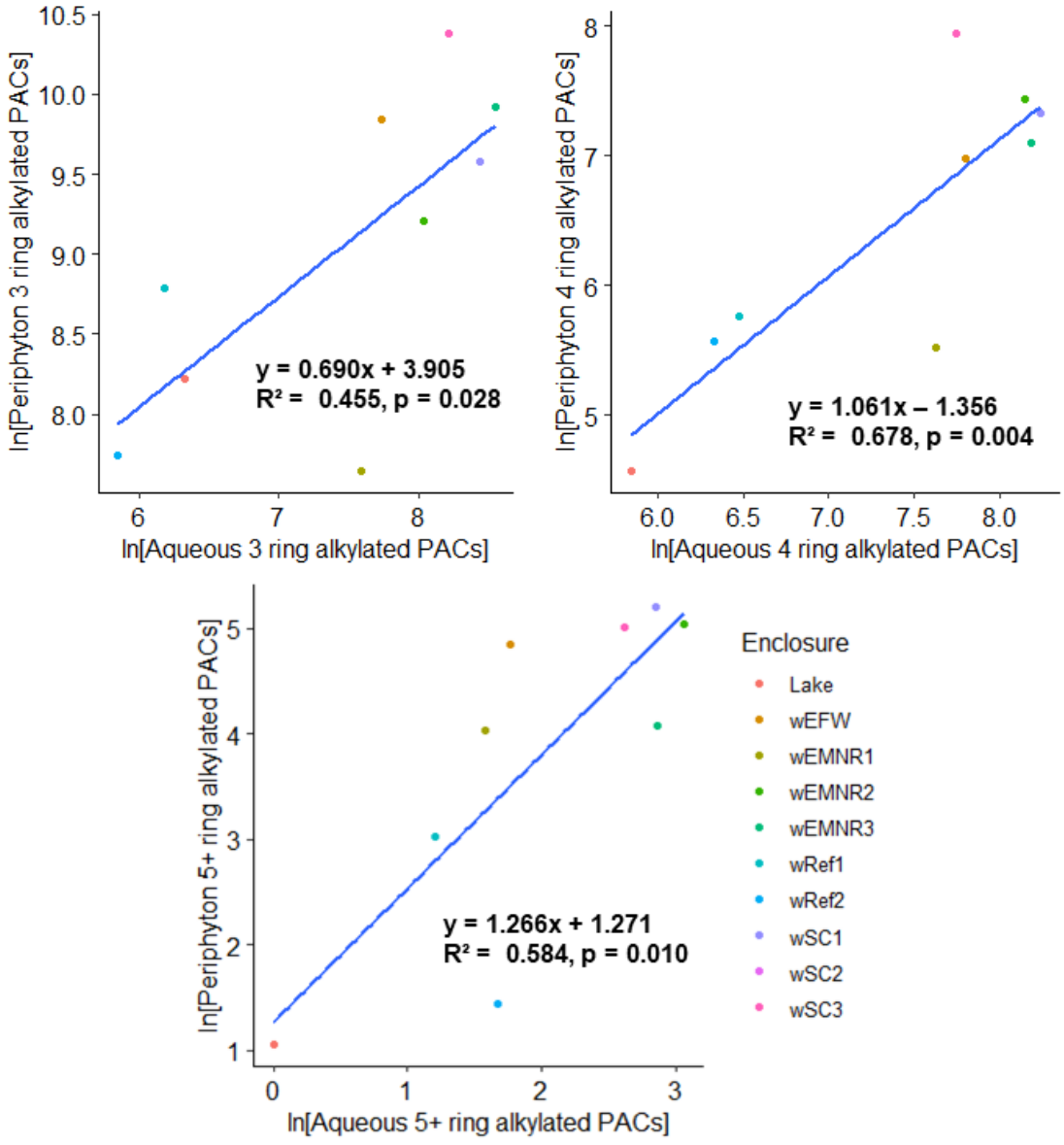


Figure 4.11: Aqueous alkylated PACs predicting periphyton alkylated PACs by ring size in wetland (w) enclosures. All data was natural-log transformed. EFW ≡ engineered floating wetland, EMNR ≡ enhanced monitored natural recovery, Ref ≡ reference, SC ≡ shoreline cleaning agent

In the wetland enclosures, no significant relationships were observed between unalkylated PAC concentrations in the periphyton and water. Similarly, no significant relationships were observed between aqueous and periphyton concentrations for any class of 2 ring PACs. However, for alkylated PACs and all PACs of 3, 4, and 5≤ rings, significant relationships were observed. For each of these, periphyton PAC concentrations (collected Sept 4) were significantly predicted by aqueous PAC concentrations on June 27 (4 days post-clean up; Table 4.22 and Figure 4.11). The concentrations of alkylated 4 ring PACs periphyton and water on Aug 26 were also significantly correlated. No other significant relationships were identified. All concentrations were log transformed for analysis.

Date	Rings	Class	β_{H2O}	95% CI	Int	R^2	DoF	F	p
Jun 27	3	Alkyl	0.690	±0.589	3.905	0.455	1,7	7.67	0.028
Jun 27	3	All	0.673	±0.574	4.062	0.455	1,7	7.68	0.028
Jun 27	4	Alkyl	1.061	±0.594	-1.356	0.678	1,7	17.8	0.004
Aug 26	4	Alykl	1.166	±0.958	3.622	0.433	1,8	7.87	0.023
Jun 27	4	All	0.858	±0.588	0.547	0.577	1,7	11.9	0.011
Jun 27	5+	Alkyl	1.266	±0.856	1.271	0.584	1,7	12.2	0.010
Jun 27	5+	All	1.927	±1.564	-1.470	0.484	1,7	8.49	0.023

Table 4.22: Significant least squares regressions of aqueous PAC concentrations predicting periphyton PAC concentrations in wetland enclosures. Date refers to water sampling date. All data was natural-log transformed. Alkyl ≡ alkylated PACs, EFW ≡ engineered floating wetland, EMNR ≡ enhanced monitored natural recovery, Ref ≡ reference, SC ≡ shoreline cleaning agent,

In the rock/cobble enclosures periphyton PAC concentrations were largely predicted by aqueous PAC concentrations on June 28 (4 days post-clean up; Figure 4.12 and Table 4.23). Significant relationships were noted among alkylated, unalkylated, and all PACs across the size spectrum. No significant relationships were found between the aqueous PAC concentrations on the date (Sept 17) nearest to periphyton collection (Sept 11). Similarly, periphyton PAC concentrations

were not predicted by aqueous concentrations one day post-oil introduction (Jun 23), with significant relationships only observed for classes involving alkylated naphthalenes.

Date	Rings	Class	β_{H2O}	95% CI	Int	R^2	DoF	F	p	ln
Jun 23	2	Alkyl	5.780	±4.512	-22.42	0.462	1,8	8.73	0.018	N
Jun 23	2	All	5.571	±4.377	15.73	0.458	1,8	8.62	0.019	N
Jun 28	3	Unalk	0.438	±0.330	4.277	0.481	1,8	9.35	0.016	Y
Jun 28	3	Alkyl	0.822	±0.420	3.028	0.683	1,8	20.4	0.002	Y
Jun 28	3	All	0.804	±0.416	3.180	0.677	1,8	19.9	0.002	Y
Jun 28	4	Unalk	1.013	±1.001	3.138	0.330	1,8	5.44	0.048	Y
Jun 28	4	Alkyl	0.255	±0.165	5.328	0.564	1,8	12.6	0.007	N
Jun 28	4	All	0.752	±0.418	0.929	0.643	1,8	17.2	0.003	Y
Jun 28	5	Alkyl	3.545	±2.435	-12.64	0.533	1,8	11.3	0.010	N

Table 4.23: Significant least squares regressions of aqueous PAC concentrations predicting periphyton PAC concentrations in rock-cobble enclosures. Date refers to water sampling date. ln indicates if data was natural log-transformed. Alkyl ≡ alkylated PACs, EFW ≡ engineered floating wetland, EMNR ≡ enhanced monitored natural recovery, Ref ≡ reference, SC ≡ shoreline cleaning agent

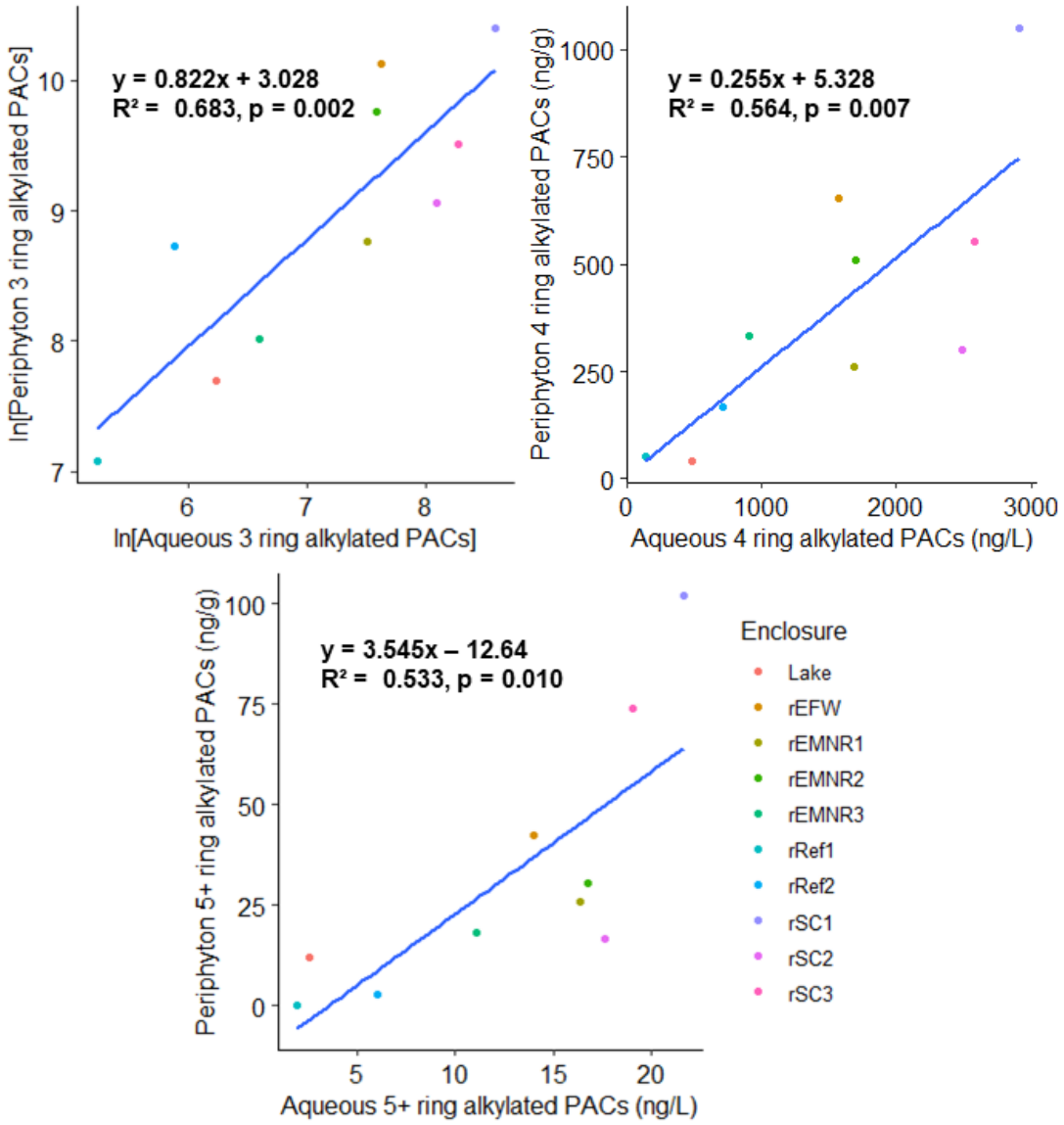


Figure 4.12: Aqueous alkylated PACs predicting periphyton alkylated PACs by ring size in rock-cobble (r) enclosures. EFW ≡ engineered floating wetland, EMNR ≡ enhanced monitored natural recovery, Ref ≡ reference, SC ≡ shoreline cleaning agent

4.3.10 Modelling biliary PAC metabolite concentrations to environmental PAC concentrations

PAC concentrations were measured in the water, sediment, and periphyton for each of the enclosures. Each of these represent a potential route of exposure to the experimental fish; uptake through the gills from the water, dermal uptake through the sediment, and dietary uptake through the periphyton (Logan, 2007). Parent PAC concentrations in each of these sources were

examined univariately to predict biliary PAC metabolite concentrations. Of these predictors, only periphyton consistently yielded coherent significant regressions (Table 4.24, Figures 4.12-14). While significant regressions of aqueous or sediment PAC concentrations were generated, they were inconsistent in their conclusions. Models of sediment or aqueous PAC concentrations negatively predicted biliary PAC concentrations almost as frequently as they positively predicted them. Moreover, these regressions were often contingent on one enclosure, the inclusion or omission of which would eliminate statistical significance. When examined altogether, these regressions were deemed incoherent and are not presented (but are shown in Supplemental Information III).

For finescale dace in the rock-cobble enclosures, periphyton parent PAC concentrations unequivocally predicted biliary metabolite concentrations for 2-chrysenol (Figure 4.12). For 1-pyrenol, no significant regression resulted when all enclosures were considered. The periphyton pyrene concentration in Ref 2, however, appeared to be an outlier having exceeded the concentrations of all other enclosures and was nearly triple the average of all oiled enclosures despite having relatively low concentrations of the other parent PACs. When this enclosure was disregarded, a significant relationship was observed between periphyton pyrene and biliary metabolites.

Significant regressions for each parent PAC and its metabolite were observed among finescale dace in wetland enclosures (Figure 4.13). Overall these models were not congruent, as periphyton pyrene positively predicted biliary metabolites while periphyton chrysene and phenanthrene negatively predicted biliary metabolite concentrations. The significance of the chrysene model was driven by high x-chrysenol concentrations and low chrysene concentrations in the reference enclosures.

Fathead minnows were recovered from five of the nine wetland enclosures (SC1-3, EMNR1,2), in addition to being sampled from the lake. Concentrations of each periphyton parent PAC positively predicted biliary metabolite concentrations, though two fish from SC3 were particularly influential on the periphyton regression (Figure 4.14) and the model lost significance with log-transformed data.

Metabolite	Subs	Species	β	95% CI	Int	R^2	DoF	F	p
1-pyrenol*	RC	FSD	0.286	±0.191	2.065	0.063	1,115	8.78	0.004

2-chrysenol	RC	FSD	0.671	±0.175	0.279	0.321	1,119	57.8	<0.001
Phenanthrols	WL	FSD	-0.350	±0.310	6.910	0.059	1,64	5.10	0.027
1-pyrenol[a]	WL	FSD	0.0021	±0.0011	2.501	0.153	1,64	12.7	0.001
x-chrysenol	WL	FSD	-0.209	±0.198	3.615	0.070	1,46	4.52	0.039
Phenanthrols	WL	FHM	0.532	±0.287	1.882	0.178	1,58	13.8	<0.001
1-pyrenol	WL	FHM	0.438	±0.249	1.261	0.162	1,58	12.4	0.001
x-chrysenol	WL	FHM	0.134	±0.124	11.19	0.063	1,53	4.64	0.036

Table 4.24: Significant least squares regressions of periphyton PAC concentrations predicting biliary metabolite concentrations. Subs ≡ substrate, RC ≡ rock-cobble, WL ≡ wetland, FSD ≡ finescale dace, FHM ≡ fathead minnow. All data was log-transformed except [a]periphyton data not transformed, [b]no data transformed. *Ref2 enclosure was omitted for this model

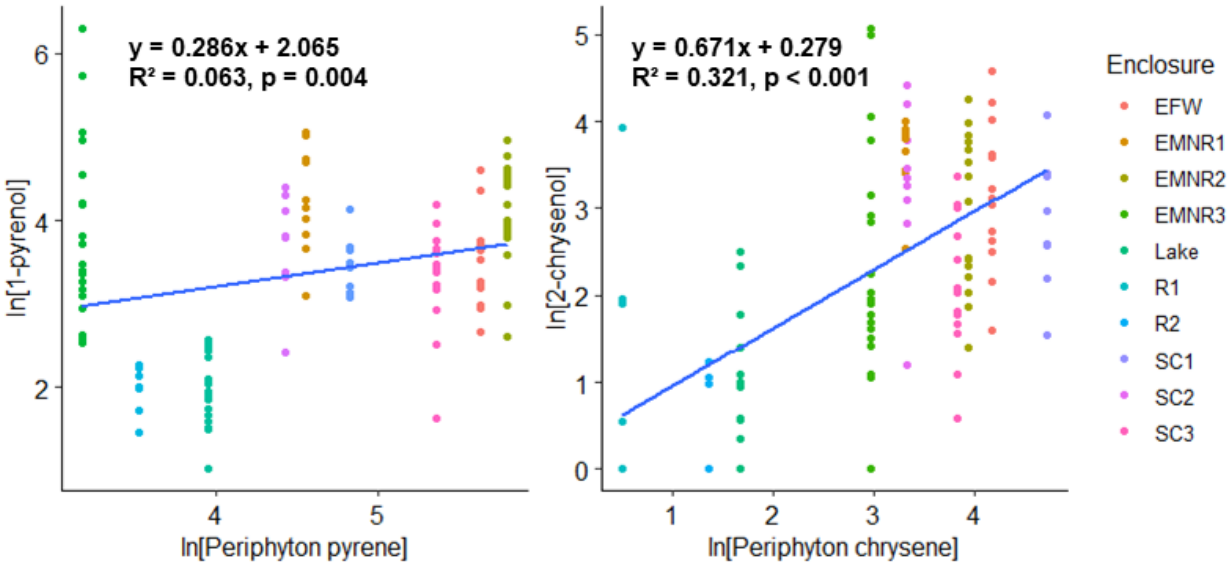


Figure 4.13: Periphyton parent PACs predicting biliary metabolites in finescale dace in rock-cobble enclosures. EFW ≡ engineered floating wetland, EMNR ≡ enhanced monitored natural recovery, R ≡ reference, SC ≡ shoreline cleaning agent. Ref2 was omitted from the pyrene model.

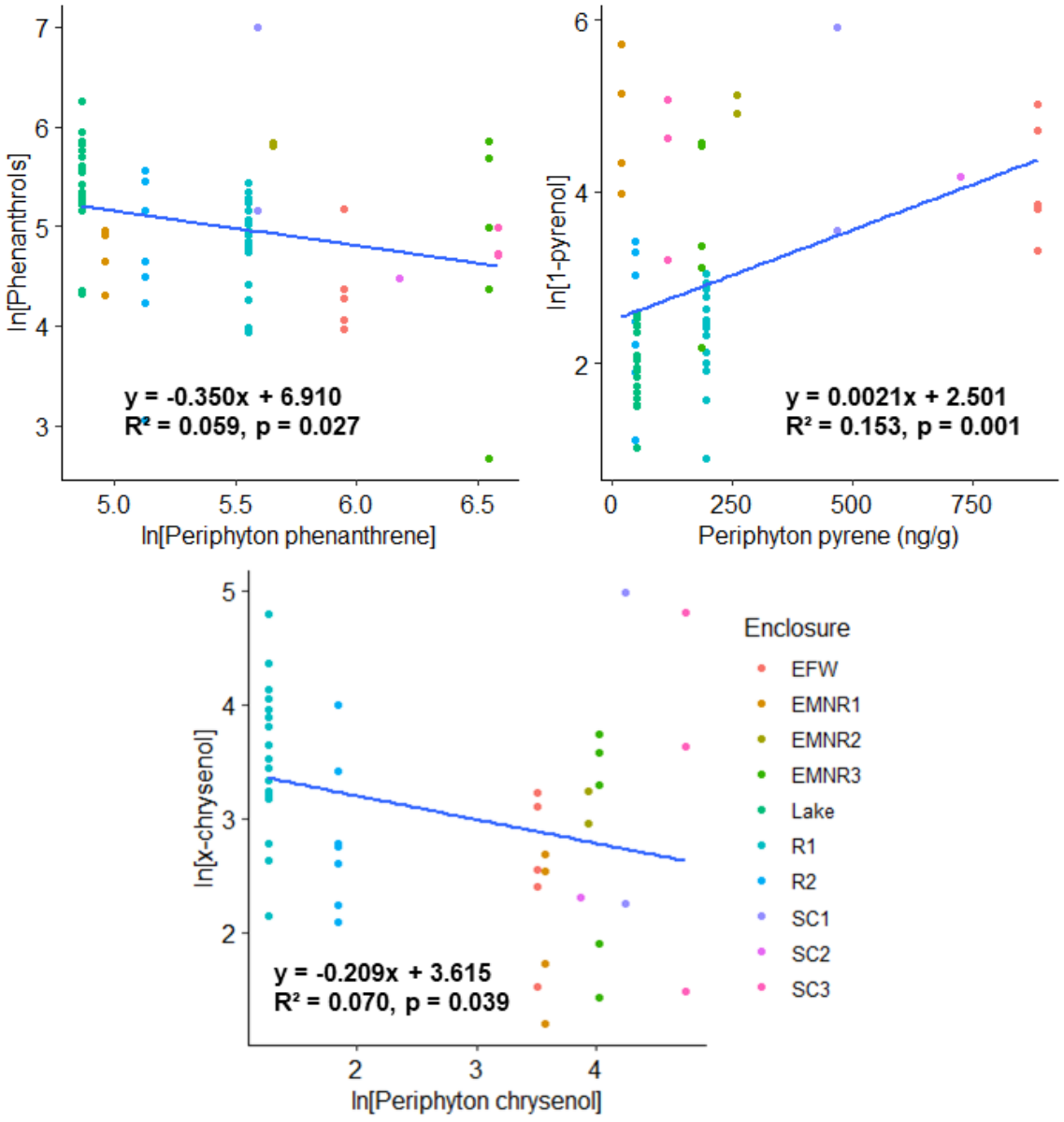


Figure 4.13: Periphyton parent PACs predicting biliary metabolites in finescale dace in wetland enclosures. EFW ≡ engineered floating wetland, EMNR ≡ enhanced monitored natural recovery, R ≡ reference, SC ≡ shoreline cleaning agent

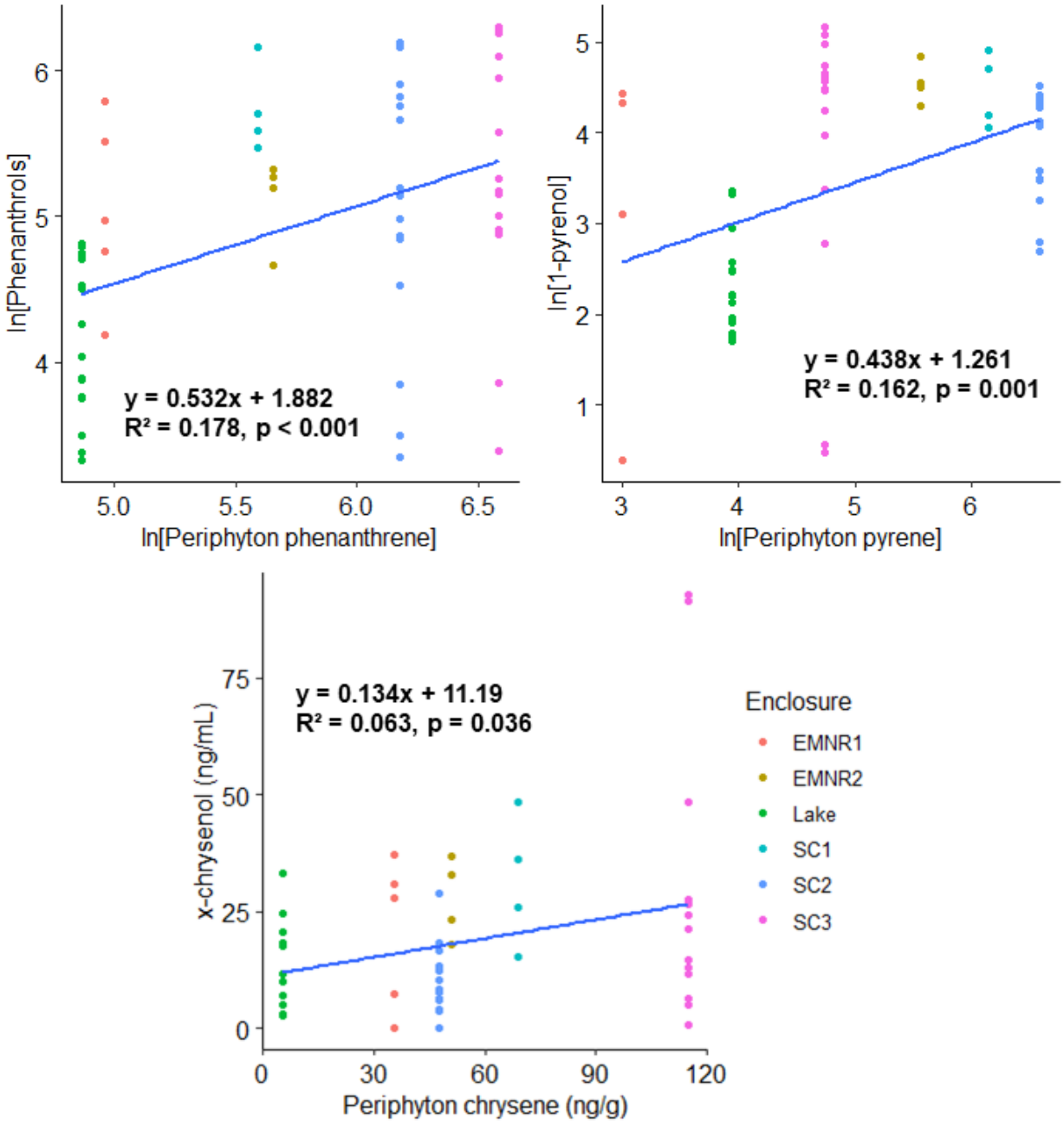


Figure 4.14: Periphyton parent PACs predicting biliary metabolites in fathead minnows in wetland enclosures. EFW ≡ engineered floating wetland, EMNR ≡ enhanced monitored natural recovery, SC ≡ shoreline cleaning agent

4.4 Discussion

Two and a half months after model oil spills and subsequent remediation, biliary PAC metabolite concentrations – a biomarker of short-term PAC exposure – in small-bodied fish exposed to model oil spills in shoreline enclosures demonstrated a significant increase in PAC exposure compared to fish in reference enclosures and the experimental lake at large, regardless of shoreline substrate. Periphyton was consistently shown to predict exposure to PACs in fathead minnows in wetland enclosures and finescale dace in rock-cobble enclosures. More than two and a half months after oil introduction, PAC concentrations in periphyton were correlated with water PAC concentrations from one week after the model spills. Periphyton in the enclosures may serve as a PAC repository and continue to expose fish to PACs after aqueous concentrations return to their baseline levels. Based on biliary PAC metabolite concentrations, none of the three different remediation techniques was superior to the others in reducing PAC exposure to fish.

In the rock-cobble enclosures, finescale dace were unequivocally more exposed to four ringed PACs than fish from reference enclosures and the general lake. This was especially apparent for pyrene, where all enclosures subject to oil spills contained fish with significantly higher 1-pyrenol concentrations than the lake and one of the reference enclosures. For chrysene, all oiled enclosures had fish with significantly higher 2-chrysenol concentrations than fish in the general lake. Conversely, phenanthrol concentrations in the rock-cobble finescale dace showed no relation to oil exposure, let alone different remediation methods. The only significant difference between phenanthrol concentrations in fish was found between two different eMNR enclosures. The lack of correlation between periphyton phenanthrene and its metabolites is likely a consequence of the parity of phenanthrol concentrations across finescale dace in rock-cobble enclosures. For the other PACs, dietary exposure was important in the rock-cobble enclosures, with chrysene and pyrene metabolite concentrations significantly and positively predicted by periphyton concentrations in the enclosures.

In the wetland enclosures, pearl dace and finescale dace were considered equivalent for comparing metabolite levels across each treatment. For these enclosures, only pyrene metabolites appeared to consistently differ between enclosures receiving oil compared to the reference enclosures and the lake, with four oiled enclosures having fish with significantly higher concentrations than lake fish and an additional two were marginally significant ($p < 0.07$).

Conversely, very few significant differences were found between the different enclosures for x-chrysenol and phenanthrol. Though low recoveries of fish affected the ability to detect differences in pairwise comparisons between some enclosures, visual inspection of metabolites (Figures 4.5 and 4.7) suggest the equivalency of exposure to phenanthrene and chrysene among the different wetland enclosures would persist with a larger data set. Concentrations of each metabolite were significantly predicted by at least one environmental factor, though not always positively; phenanthrol and x-chrysenol metabolite concentrations were negatively predicted by periphyton concentrations. The chrysene model was dictated by relatively high metabolite concentrations in reference enclosures. Contrarily, 1-pyrenol concentrations was positively predicted by periphyton and explained more variation ($R^2 = 0.153$) than the chrysene and phenanthrene models ($R^2 = 0.070$).

Fathead minnows were not captured from any of the wetland reference enclosures, nor the EFW one. Nevertheless, fish in oiled enclosures generally had increased exposure to phenanthrene and pyrene compared to those in the lake, though differences between the remediation strategies were not observed. In the enclosures they were found (as well as the lake), periphyton parent PAC concentration was a significant and positive predictor for biliary metabolite concentrations for each compound quantified, though the correlation with x-chrysenol was weak ($R^2 = 0.063$).

Though the inconsistent models of sediment and water predicting biliary metabolite concentrations were not presented, the factors that led to their inconsistent results are relevant to modelling in natural systems. The FOReSt study was not designed for regressions of dose-dependent responses in fish; each enclosure received roughly the same amount of dilbit and two of the three different minimally invasive remediation methods were executed in triplicate. Consequently, the range and variation within environmental PAC concentrations were often narrow. This was especially notable in aqueous PAC concentrations in the wetland enclosures. Between these nine enclosures, pyrene's concentration varied by 1.30 ng/L while chrysene's varied by 2.09 ng/L. The systems with enclosures were also dynamic, both temporally and spatially, which exacerbated difficulties in predicting exposure. In most enclosures, samples for water analysis were taken from two points; one near the shoreline and one far from the shoreline. In some cases, the discrepancy in parent PAC concentrations between the two different points exceeded the range in concentrations between all other enclosures. When probing relationships

between metabolite concentrations and aqueous parent PAC concentrations, the set of near shore values, far shore values, and average values between the two measurements were tested. This avoided a priori assumptions about where fish spent their time, nor about which measured concentration was best representative of the enclosure. Similarly, obtaining representative concentrations of sediment and periphyton PAC concentrations in enclosures was not assured. Periphyton was only sampled from one location, whereas sediment concentrations were averaged from three different locations. Temporal variation also introduced uncertainty into measured quantities, as sediment and water were sampled eight days prior to fish collection and their concentrations were prone to changing significantly in three week spans. In a designed regression study with a wide range of exposure concentrations, the data would be more resilient to reduced certainty in exposure concentrations but given the narrow range of concentrations of some PACs in this study, any variation in measured concentrations can be quite influential. Several regressions of sediment and water were noted to lose or gain significance when one enclosure was omitted. Numerous studies have demonstrated a dose-dependent response of fish to aqueous concentrations of PACs, including fathead minnows (Beyer et al., 2010; Collier & Varanasi, 1991; Grimard et al., 2020; Jonsson et al., 2004; Sturve et al., 2014; van den Hurk et al., 2020; Chapter 2). In Atlantic cod (*Gadus Morhua*), aqueous exposure to 0.6 ng/L and 6.0 ng/L of pyrene resulted in significantly different metabolite concentrations between exposure groups (Grung et al., 2009) and sediment PAC exposure has also been linked to increased PAC metabolite concentrations and related biomarkers in fish (Fragoso et al., 2006; Meier et al., 2013), lending merit to the modelling attempts of this study for those parameters which ultimately yielded inconsistent findings. Conversely, periphyton parent PAC concentration modelling was much more consistent, having positively predicted metabolite concentrations in finescale dace in rock-cobble enclosures and fathead minnows in wetland enclosures for both three and four ring PACs. Contradictory results were found for finescale dace in wetland enclosures, but these regressions, while significant, were less certain and explained less variation ($R^2 \leq 0.070$) than the ones finding a positive correlation, came from less populated enclosures, and for chrysene were a consequence of high metabolite concentrations in reference enclosure fish. The positive relationship found between periphyton parent PAC and metabolite concentrations in this study corroborate the dose-dependent response to dietary exposure found in previous studies (Grung et al., 2009).

Periphyton PAC concentrations in the enclosures showed a clear and unequivocal correlation to aqueous PAC concentrations in the enclosures one week after dilbit introduction (which is also four days post-primary recovery) despite being collected two and a half months after oil introduction. This has several important implications for remediation and biota exposure. For one, it demonstrates the importance of the primary clean up, as periphyton PAC concentrations did not correlate with aqueous concentrations one day after the spill and before remediation. It also suggests periphyton served as a repository of PACs that continually exposed fish even after aqueous concentrations had returned to concentrations comparable to reference enclosures, which was the case when experimental fish were collected. In this experiment metabolite concentrations for three non-alkylated PACs were measured. However, given that metabolite concentrations for each of these PACs were found to be predicted by periphyton parent PAC concentrations, other unmeasured PACs affiliated with the periphyton were likely also ingested by fish. Therefore, fish in oiled enclosures were likely exposed to the suite of alkylated PACs affiliated with the periphyton. Alkylated PACs are characteristic of petrogenic sources (Abrajano et al., 2013) and are a direct consequence of the model spills in the enclosures. This exposure is highly relevant toxicologically, as alkylated PACs have been observed to not only exert greater toxicity than non-alkylated components, but also to act synergistically (Hodson, 2017). The precise route of exposure to periphyton associated PACs to fish in this study is not necessarily direct. Fathead minnows are opportunistic omnivores and diets of adults is mostly comprised of detritus (Herwig & Zimmer, 2007). Finescale dace are more carnivorous, but in Ontario lakes have algae, detritus, and phytoplankton comprise a large portion of their diet (Litvak & Hansell, 1990; Stasiak & Cunningham, 2006). The abundance and accessibility of periphyton on the walls of the enclosures may have altered the diets of both species compared to free swimming lake fish. Regardless, based on this study, periphyton can still be expected to accumulate PACs and propagate exposure through the food web after freshwater oil spills.

Differences in biliary PAC metabolite concentrations based on sex and size have been sporadically reported in literature (Davies & Vethaak, 2012; Dearnley et al., 2020; Vethaak et al., 2016), but neither variable revealed significant effects in this study. In this study exposure lengths are generally unknown as very few fish recovered from the enclosures were tagged as being introduced prior to oil introduction. However, a widely cited study on sheepshead minnow suggests peak PAC metabolite concentrations are reached within 2-6 days of exposure (Davies &

Vethaak, 2012; Grung et al., 2016; Jonsson et al., 2004). Similarly, at PAC concentrations relevant to this study, exposed fathead minnows reached peak metabolite concentrations within 7 days (Grimard et al., 2020). Thus, fish collected are assumed to have been in the enclosures long enough to yield PAC metabolite concentrations reflective of the conditions and the unknown residence times are unlikely to have impacted this study's results.

4.5 Conclusion

Biliary PAC metabolites in small-bodied fish can provide information regarding their exposure after an oil spill and comparison to parent PACs in the environment can elucidate on the source of exposure. After remediating model oil spills using three different minimally invasive approaches in shoreline enclosures on rock-cobble and wetland substrates fish were collected and analyzed for biliary PAC metabolites to assess their short-term exposure. Two and a half months after model oil spills, experimental fish residing in oiled enclosures were found to be significantly more exposed to PACs than those in reference enclosures or the lake, regardless of substrate. The three different remediation techniques used (enhanced monitored natural remediation, shoreline cleaner, engineered floating wetland) did not result in significant differences in exposure. The biliary PAC metabolite concentrations in fish were predicted by their parent PAC concentrations in periphyton. The PAC concentrations in periphyton two and a half months after the spill were correlated with aqueous PAC concentrations in the enclosures one week after oil introduction. This suggests experimental fish had significant exposure to alkyl-PACs, though metabolites of these compounds were not quantified in this study.

4.6 References

Abrajano, T. A., Yan, B., & O'Malley, V. (2013). High Molecular Weight Petrogenic and Pyrogenic Hydrocarbons in Aquatic Environments. In *Treatise on Geochemistry: Second Edition* (2nd ed., Vol. 11). Elsevier Ltd. https://doi.org/10.1016/B978-0-08-095975-7.00913-X

Afzal, M., Rehman, K., Shabir, G., Tahseen, R., Ijaz, A., Hashmat, A. J., & Brix, H. (2019). Large-scale remediation of oil-contaminated water using floating treatment wetlands. *Npj Clean Water*, *2*(1), 1–10. https://doi.org/10.1038/s41545-018-0025-7

Banerjee, D. K. (2012). Oil Sands, Heavy Oil and Bitumen - From Recovery to Refinery. In *PennWell*.

Beyer, J., Jonsson, G., Porte, C., Krahn, M. M., & Ariese, F. (2010). Analytical methods for determining metabolites of polycyclic aromatic hydrocarbon (PAH) pollutants in fish bile: A review. *Environmental Toxicology and Pharmacology*, *30*(3), 224–244. https://doi.org/10.1016/j.etap.2010.08.004

Brunskill, G. J., & Schindler, D. W. (1971). Geography and Bathymetry of Selected Lake Basins, Experimental Lakes Area, Northwestern Ontario. *Journal of the Fisheries Research Board of Canada*, *28*(2), 139–155. https://doi.org/10.1139/f71-028

CAPP. (2022). *Canadian oil sands production 1967-2020*. https://www.capp.ca/resources/statistics/

CER. (2021). *Canada's Energy Future 2021*. https://www.cer-rec.gc.ca/en/data-analysis/canada-energy-future/2021/index.html

CER. (2022). *Canadian Crude Oil Exports by Rail – Monthly Data*. https://www.cer-rec.gc.ca/en/data-analysis/energy-commodities/crude-oil-petroleum-products/statistics/canadian-crude-oil-exports-rail-monthly-data.html

Collier, T. K., Anulacion, B. F., Arkoosh, M. R., Dietrich, J. P., Incardona, J. P., Johnson, L. L., Ylitalo, G. M., & Myers, M. S. (2013). Effects on Fish of Polycyclic Aromatic Hydrocarbons (PAHS) and Naphthenic Acid Exposures. In K. B. Tierney, A. P. Farrell, & C. J. Brauner (Eds.), *Organic Chemical Toxicology of Fishes* (First Edit, Vol. 33, pp. 195–255). Elsevier Inc. https://doi.org/10.1016/B978-0-12-398254-4.00004-2

Collier, T. K., & Varanasi, U. (1991). Hepatic activities of xenobiotic metabolizing enzymes and biliary levels of xenobiotics in english sole (Parophrys vetulus) exposed to environmental contaminants. *Archives of Environmental Contamination and Toxicology*, *20*, 462–473. http://download.springer.com/static/pdf/171/art%253A10.1007%252FBF01065834.pdf?originUrl=http%253A%252F%252Flink.springer.com%252Farticle%252F10.1007%252FBF01065834&token2=exp=1453740322~acl=%252Fstatic%252Fpdf%252F171%252Fart%2525253A10.1007%2525252FBF01

Davies, I. M., & Vethaak, D. (2012). *Integrated marine environmental monitoring of chemicals and their effects* (Issue November 2012).

Dearnley, J. M., Killeen, C., Davis, R. L., Palace, V. P., & Tomy, G. T. (2020). Monitoring polycyclic aromatic compounds exposure in fish using biliary metabolites. *Critical Reviews in Environmental Science and Technology*, *0*(0), 1–45. https://doi.org/10.1080/10643389.2020.1825895

Fingas, M. (2013). *Surface-washing agents: an update*. https://www.pwsrcac.org/wpfb-file/surfacewashing-agents-or-beach-cleaners-by-merv-fingas-2013-pdf-2/

Fragoso, N. M., Hodson, P. V., & Zambon, S. (2006). Evaluation of an exposure assay to measure uptake of sediment PAH by fish. *Environmental Monitoring and Assessment*, *116*(1–3), 481–511. https://doi.org/10.1007/s10661-006-7667-5

Furchtgott-Roth, D. (2013). *Pipelines are safest for transportation of oil and gas* (Issue 23).

Gidmark, N. J., & Simons, A. M. (2014). Cyprinidae: Carps and minnows. In W. L. J. Melvin & B. M. Burr (Eds.), *Freshwater Fishes of North America: Volume 1: Petromyzontidae to Catostomidae* (Vol. 1, pp. 354–450).

Grimard, C., Mangold-Döring, A., Schmitz, M., Alharbi, H., Jones, P. D., Giesy, J. P., Hecker, M., & Brinkmann, M. (2020). In vitro-in vivo and cross-life stage extrapolation of uptake and biotransformation of benzo[a]pyrene in the fathead minnow (Pimephales promelas). *Aquatic Toxicology*, *228*(May). https://doi.org/10.1016/j.aquatox.2020.105616

Grung, M., Holth, T. F., Jacobsen, M. R., & Hylland, K. (2009). Polycyclic aromatic hydrocarbon (PAH) metabolites in Atlantic cod exposed via water or diet to a synthetic produced water. *Journal of Toxicology and Environmental Health - Part A: Current Issues*, *72*(3–4), 254–265. https://doi.org/10.1080/15287390802539210

Grung, M., Petersen, K., Fjeld, E., Allan, I., Christensen, J. H., Malmqvist, L. M. V., Meland, S., & Ranneklev, S. (2016). PAH related effects on fish in sedimentation ponds for road runoff and potential transfer of PAHs from sediment to biota. *Science of the Total Environment*, *566–567*(0349), 1309–1317. https://doi.org/10.1016/j.scitotenv.2016.05.191

Herwig, B. R., & Zimmer, K. D. (2007). Population ecology and prey consumption by fathead

minnows in prairie wetlands: Importance of detritus and larval fish. *Ecology of Freshwater Fish*, *16*(3), 282–294. https://doi.org/10.1111/j.1600-0633.2006.00220.x

Hodson, P. V. (2017). The Toxicity to Fish Embryos of PAH in Crude and Refined Oils. *Archives of Environmental Contamination and Toxicology*, *73*(1), 12–18. https://doi.org/10.1007/s00244-016-0357-6

Holth, T. F., Nourizadeh-Lillabadi, R., Blaesbjerg, M., Grung, M., Holbech, H., Petersen, G. I., Aleström, P., & Hylland, K. (2008). Differential gene expression and biomarkers in zebrafish (Danio rerio) following exposure to produced water components. *Aquatic Toxicology*, *90*(4), 277–291. https://doi.org/10.1016/j.aquatox.2008.08.020

Idowu, I., Francisco, O., Thomas, P. J., Johnson, W., Marvin, C., Stetefeld, J., & Tomy, G. T. (2018). Validation of a simultaneous method for determining polycyclic aromatic compounds and alkylated isomers in biota. *Rapid Communications in Mass Spectrometry*, *32*(3), 277–287. https://doi.org/10.1002/rcm.8035

Jonsson, G., Bechmann, R. K., Bamber, S. D., & Baussant, T. (2004). Bioconcentration, biotransformation, and elimination of polycyclic aromatic hydrocarbons in sheepshead minnows (Cyprinodon variegatus) exposed to contaminated seawater. *Environmental Toxicology and Chemistry*, *23*(6), 1538–1548. https://doi.org/10.1897/03-173

Jonsson, G., Beyer, J., Wells, D., & Ariese, F. (2003). The application of HPLC-F and GC-MS to the analysis of selected hydroxy polycyclic hydrocarbons in two certified fish bile reference materials. *Journal of Environmental Monitoring*, *5*(3), 513–520. https://doi.org/10.3233/978-1-60750-938-7-19

Krahn, M. M., Burrows, D. G., Ylltalo, G. M., Brown, D. W., Wlgren, C. A., Collier, T. K., Sin-Lam, C., & Usha, V. (1992). Mass Spectrometric Analysis for Aromatic Compounds in Bile of Fish Sampled after the Exxon Valdez Oil Spill. *Environmental Science and Technology*, *26*(1), 116–126. https://doi.org/10.1021/es00025a012

Lee, K., Boufadel, M., Chen, B., Foght, J., Hodson, P., Swanson, S., Venosa, A., Lee, K., Boufadel, M., Chen, B., Foght, J., Hodson, P., Swanson, S., & Venosa, A. (2015). The Behaviour and Environmental Impacts of Crude Oil Released into Aqueous Environments.

In *Royal Society of Canada* (Issue November). http://www.rsc.ca/en/expert-panels/information-about-expert-panels%0Ahttp://www.rsc.ca/sites/default/files/pdf/OIW_Executive Summary_1.pdf%0Ahttps://www.rsc-src.ca/sites/default/files/pdf/OIW Report.pdf%0Ahttps://www.rsc-src.ca/sites/default/files/pdf/OIW

Litvak, M. K., & Hansell, R. I. C. (1990). Investigation of food habit and niche relationships in a cyprinid community. *Canadian Journal of Zoology*, *68*(9), 1873–1879. https://doi.org/10.1139/z90-267

Logan, D. T. (2007). Perspective on ecotoxicology of PAHs to fish. *Human and Ecological Risk Assessment*, *13*(2), 302–316. https://doi.org/10.1080/10807030701226749

Magar, V. S., Chadwick, D. B., Bridges, T. S., Fuchsman, P. C., Conder, J. M., Dekker, T. J., Steevens, J. A., Gustavson, K. E., & Mills, M. A. (2009). Monitored Natural Recovery at Contaminated Sediment Sites. In *Environmental Security Technology Certification Program*. http://oai.dtic.mil/oai/oai?verb=getRecord&metadataPrefix=html&identifier=ADA512822%5Cnhttp://citeseerx.ist.psu.edu/viewdoc/download?doi=10.1.1.364.6282&rep=rep1&type=pdf

Magnusson, B.; Örnemark, U. (2014). The Fitness for Purpose of Analytical Methods. In *Eurachem Guide*.

Meier, J. R., Snyder, S., Sigler, V., Altfater, D., Gray, M., Batin, B., Baumann, P., Gordon, D., Wernsing, P., & Lazorchak, J. (2013). An integrated assessment of sediment remediation in a midwestern U.S. stream using sediment chemistry, water quality, bioassessment, and fish biomarkers. *Environmental Toxicology and Chemistry*, *32*(3), 653–661. https://doi.org/10.1002/etc.2093

Moermond, C. T. A., Traas, T. P., Roessink, I., Veltman, K., Hendriks, A. J., & Koelmans, A. A. (2007). Modeling decreased food chain accumulation of PAHs due to strong sorption to carbonaceous materials and metabolic transformation. *Environmental Science and Technology*, *41*(17), 6185–6191. https://doi.org/10.1021/es0702364

Nahrgang, J., Brooks, S. J., Evenset, A., Camus, L., Jonsson, M., Smith, T. J., Lukina, J., Frantzen, M., Giarratano, E., & Renaud, P. E. (2013). Seasonal variation in biomarkers in blue mussel (Mytilus edulis), Icelandic scallop (Chlamys islandica) and Atlantic cod (Gadus morhua)-Implications for environmental monitoring in the Barents Sea. *Aquatic Toxicology*, *127*(9037), 21–35. https://doi.org/10.1016/j.aquatox.2012.01.009

Sarasquete, C., & Segner, H. (2000). Cytochrome P4501A CYP1A in teleostean fishes. *The Science of the Total Environment*, *247*, 313–332.

Stasiak, R., & Cunningham, G. R. (2006). *Finescale Dace (Phoxinus neogaeus): a technical conservation assessment*. https://www.fs.usda.gov/Internet/FSE_DOCUMENTS/stelprdb5206787.pdf

Sturve, J., Balk, L., Liewenborg, B., Adolfsson-Erici, M., Förlin, L., & Carney Almroth, B. (2014). Effects of an oil spill in a harbor assessed using biomarkers of exposure in eelpout. *Environmental Science and Pollution Research*, *21*(24), 13758–13768. https://doi.org/10.1007/s11356-014-2890-z

van den Hurk, P., Edhlund, I., Davis, R., Hahn, J. J., McComb, M. J., Rogers, E. L., Pisarski, E., Chung, K., & DeLorenzo, M. (2020). Lionfish (Pterois volitans) as biomonitoring species for oil pollution effects in coral reef ecosystems. *Marine Environmental Research*, *156*(February), 104915. https://doi.org/10.1016/j.marenvres.2020.104915

Vethaak, A. D., Baggelaar, P. K., van Lieverloo, J. H. M., & Ariese, F. (2016). Decadal Trends in Polycyclic Aromatic Hydrocarbon (PAH) Contamination Assessed by 1-Hydroxypyrene in Fish Bile Fluid in the Netherlands: Declining in Marine Waters but Still a Concern in Estuaries. *Frontiers in Marine Science*, *3*(November), 1–13. https://doi.org/10.3389/fmars.2016.00215

Wakefield, N. (n.d.). *Pimephales promelas (fathead minnow)*. Centre for Agriculture and Bioscience International Invasive Species Compendium. Retrieved January 25, 2022, from https://www.cabi.org/isc/datasheet/69889

5.1 Conclusion

Overall, this thesis demonstrated the utility of small-bodied fish for evaluating polycyclic aromatic compound (PAC) exposure in freshwater environments. The lab exposure of this thesis, having demonstrated a dose-dependent response of biliary PAC metabolites to aqueous PAC exposure (which is also supported by similar research (Grimard et al., 2020)), validated the use of fathead minnows for measuring PAC exposure. Then, in the dynamic ecosystem of the Freshwater Oil Spill Remediation Study (FOReSt) enclosures, the biliary metabolites of small-bodied fish, consistently showed differentiation between PAC exposure to fish in enclosures subject to model crude oil spills compared to those in unoiled reference enclosures. These biliary PAC metabolites, however, showed much less differentiation among enclosures receiving crude oil products. In year one of FOReSt, consistent differences in metabolite concentrations were not observed between fish in an enclosure receiving diluted bitumen (dilbit) and those in one receiving conventional heavy crude oil. Similarly, in year two of FOReSt none of the three different minimally invasive remediations led to differences in PAC exposure to fish. Therefore, for future unintentional oil releases affecting shorelines, the best remediation technique to employ should be determined by other endpoints of the broader FOReSt projects, such as impacts on invertebrates or early life stage fish, or the trajectory of PAC and alkane degradation and reestablishment of baseline concentrations within the enclosures. Nevertheless, fathead minnows or finescale dace should be monitored for PAC exposure using biliary PAC metabolites until (apparent) baseline concentrations have been achieved following oil releases.

This thesis also elucidated on routes of PAC exposure to small-bodied fish in the wake of model oil spills. Physical traits of the fish did not have a significant effect on PAC exposure. Though fish in year 1 FOReSt sporadically showed relationships between biliary PAC metabolite concentrations and size, these were dispelled by the larger sample sizes in year 2 of the study which identified no such relationships. Neither year of the study consistently found differences in metabolite concentrations between male and female fish. With respect to environmental sources of PACs, periphyton affiliated PAC concentrations consistently predicted biliary PAC metabolite concentrations in experimental fish, suggesting diet is an important route of exposure. Moreover, periphyton PAC concentrations were correlated with aqueous PAC concentrations one week after dilbit was introduced to each enclosure, despite being collected two and a half

months later. Therefore, periphyton may act as a repository for PACs even after aqueous concentrations have returned to baseline concentrations. This correlation between periphyton and aqueous PACs was especially apparent for alkylated PACs (aPAC), which are characteristic of petrogenic sources (Abrajano et al., 2013). Though alkylated PAC metabolites were not quantified in this study, given that non-alkylated PACs in periphyton predicted exposure in fish, experimental fish were likely also exposed to aPACs via periphyton. This may have implications for fish health, as alkylated PACs have been observed to not only exert greater toxicity than non-alkylated components, but to also act synergistically (Hodson, 2017). Based on year 1 of FOReSt, aqueous PAC concentrations may also predict PAC exposure in fish, but this relationship was much more ambiguous in year 2 and requires further study to confirm.

5.1.2 Future Directions

More research is needed to elucidate routes of exposure to small-bodied fish in natural systems. Lab studies have already demonstrated dose-dependent responses of biliary PAC metabolites to aqueous and dietary PAC exposure (Beyer et al., 2010; Grimard et al., 2020; Grung et al., 2009), but this thesis did not conclusively determine their prominence in entire ecosystems. While, dietary exposure was important in this thesis, the relative importance of aqueous exposure was indeterminate when compared across the two years of the FOReSt project. This would be best assessed with a regression study, where fish are exposed to oil spills over a range of magnitudes. Though periphyton was not anticipated to be a focal point when this thesis was designed, it played a pivotal role in the thesis. It appears that periphyton can act as a repository for PACs after aqueous concentrations return to baseline levels but this would be best assessed in a study with multiple collection time points to elucidate on temporal trends.

Finally, while this study was focused on HPLC/F measurement of specific PAC metabolites other methods have utility in future quantitation of biliary PAC metabolites. A critical benefit of HPLC/F was that it allowed for specific biliary PAC metabolites to be directly compared to parent PACs in sediment, water, and periphyton. However, for future monitoring of biliary PAC metabolites other, less labour intensive quantitation methods such as fixed wavelength fluorescence and synchronous fluorescence scanning are advisable if the desired outcome is only to track exposure spatially or temporally. Conversely, a study like FOReSt involving exposure to petrogenic PACs could also benefit from GC/MS or HPLC/MS analysis targeting alkylated

PACs to confirm uptake of these compounds. This would solidify the presumptive exposure of fish to these compounds from periphyton. Lastly, while this thesis focused on the exposure of small-bodied fish to petrogenic PACs, future monitoring programmes and assessments need not be limited to these sources. Small-bodied fish are just as suitable for assessing exposure from such diverse sources as urban pollution, commercial pollution, and forest fires.

5.2 References

Abrajano, T. A., Yan, B., & O'Malley, V. (2013). High Molecular Weight Petrogenic and Pyrogenic Hydrocarbons in Aquatic Environments. In *Treatise on Geochemistry: Second Edition* (2nd ed., Vol. 11). Elsevier Ltd. https://doi.org/10.1016/B978-0-08-095975-7.00913-X

Beyer, J., Jonsson, G., Porte, C., Krahn, M. M., & Ariese, F. (2010). Analytical methods for determining metabolites of polycyclic aromatic hydrocarbon (PAH) pollutants in fish bile: A review. *Environmental Toxicology and Pharmacology*, *30*(3), 224–244. https://doi.org/10.1016/j.etap.2010.08.004

Grimard, C., Mangold-Döring, A., Schmitz, M., Alharbi, H., Jones, P. D., Giesy, J. P., Hecker, M., & Brinkmann, M. (2020). In vitro-in vivo and cross-life stage extrapolation of uptake and biotransformation of benzo[a]pyrene in the fathead minnow (Pimephales promelas). *Aquatic Toxicology*, *228*(May). https://doi.org/10.1016/j.aquatox.2020.105616

Grung, M., Holth, T. F., Jacobsen, M. R., & Hylland, K. (2009). Polycyclic aromatic hydrocarbon (PAH) metabolites in Atlantic cod exposed via water or diet to a synthetic produced water. *Journal of Toxicology and Environmental Health - Part A: Current Issues*, *72*(3–4), 254–265. https://doi.org/10.1080/15287390802539210

Hodson, P. V. (2017). The Toxicity to Fish Embryos of PAH in Crude and Refined Oils. *Archives of Environmental Contamination and Toxicology*, *73*(1), 12–18. https://doi.org/10.1007/s00244-016-0357-6

Supplementary Information I: Additional metrics from the lab exposure study

S1.1 Additional metrics of experimental fish

		Bile mass (mg)					% Detect		
Dose	**Fish**	**Mean**	**Median**	**Std Dev**	**Min**	**Max**	**PHE**	**1-PYR**	**2-CHR**
Control	11	2.69	2.51	0.91	1.35	4.70	100	100	91
Low	9	4.28	3.13	2.19	1.44	7.51	100	100	100
Medium	10	4.24	3.05	2.87	1.52	11.34	100	100	100
High	13	3.78	3.65	1.62	1.31	6.28	100	100	100

Table S1.1: Summary statistics for mass of bile recovered from experimental and % detections of each metabolite. PHE ≡ Phenanthrols, 1-PYR ≡ 1-pyrenol, 2-CHR ≡ 2-chrysenol. For phenanthrol, any samples below the limit of quantitation are considered non-detects. For 1-pyrenol and 2-chrysenol, limits of quantitation and limits of detection are not available and only non-detects are reported.

S1.2 Benzo[a]pyrene exposure concentrations

	Control	**Low**	**Medium**	**High**
Benzo[a]pyrene (ng/L)	10.68	3.32	7.05	5.69

Table S1.2: Nominal aqueous benzo[a]pyrene concentrations in the different treatment groups

S1.3 Metabolite calibration curves

The calibration curves (using the average of three trials) validating the use of a single concentration response factor of each metabolite are shown below. Responses for 1-pyrenol and 2-chrysenol were off-scale at 250 ng/mL and were only assessed from 1-100 ng/mL.

S1.3.1 1-phenanthrol

Concentration (ng/mL)	**Trial 1**	**Trial 2**	**Trial 3**	**Average**	**Std Dev**
1	763.8	642.2	708.9	704.9667	60.89535
2	1563.2	1661.5	1627.5	1617.4	49.92224
5	4198.9	4398.2	4182.3	4259.8	120.145
10	8605.2	8532.7	8366.7	8501.533	122.2664
25	22157.4	22112.9	21144.4	21804.9	572.4424
50	45192.4	45832.8	42529.9	44518.37	1751.579
100	91486.4	90016.7	84711.9	88738.33	3563.584
250	242788.1	226498.2	211927.5	227071.3	15438.28
R^2	0.999448	0.999978	0.999999	0.999925	

Table S1.3: 1-phenanthrol concentration curve fluorescent responses (μV·Min)

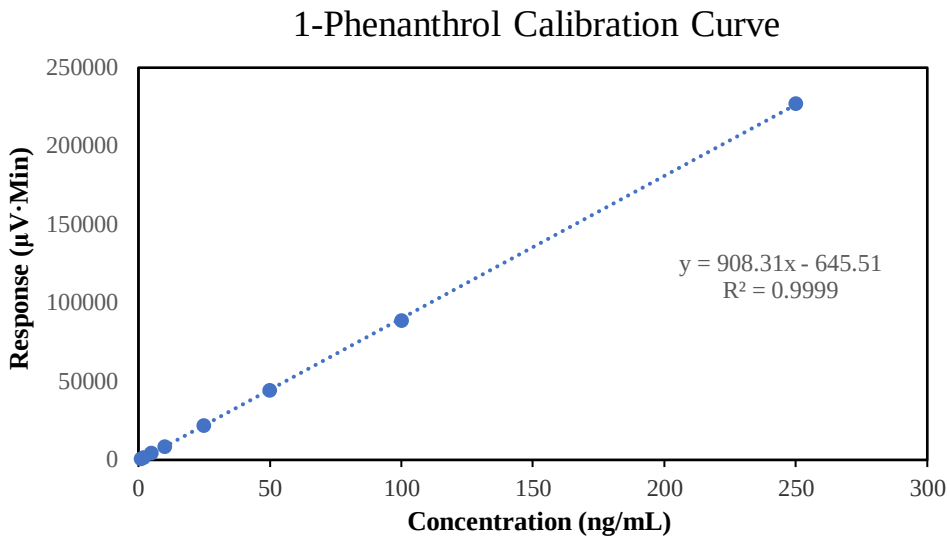


Figure S1.1: 1-phenanthrol calibration curve (µV·Min)

S1.3.2 1-pyrenol

Concentration (ng/mL)	Trial 1	Trial 2	Trial 3	Average	Std Dev
1	4466.2	4475.1	3946.4	4295.9	302.709
2	8781.6	9171	8687.9	8880.17	256.189
5	23205.8	23698	22841.6	23248.5	429.791
10	46390.3	46708.5	44827.8	45975.5	1006.62
25	120135	119511	115516	118387	2506.39
50	248315	255231	235416	246321	10056.9
100	508572	501195	484895	498221	12115.5
R^2	0.99978	0.99973	0.99977	0.99987	

Table S1.4: 1-pyrenol concentration curve fluorescent responses (µV·Min)

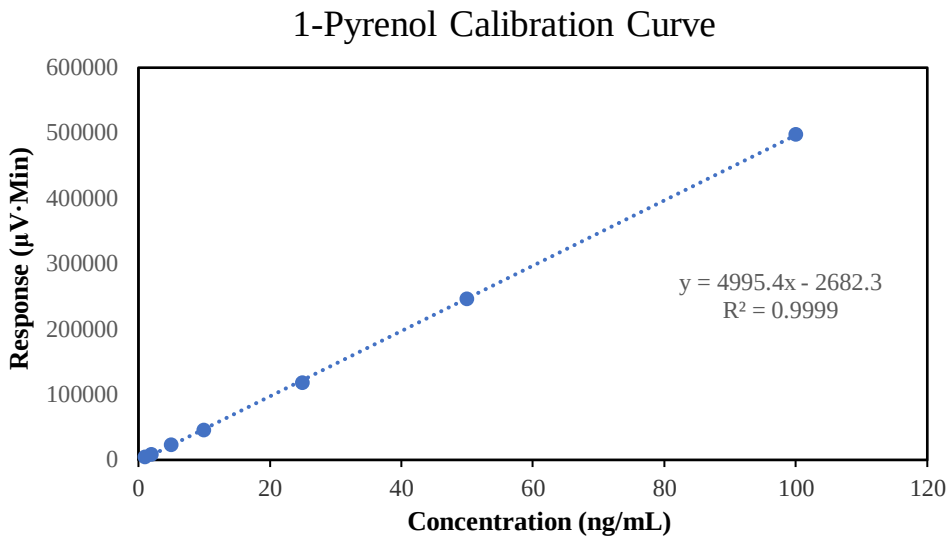


Figure S1.2: 1-pyrenol calibration curve (µV·Min)

S1.3.3 2-chrysenol

Concentration (ng/mL)	Trial 1	Trial 2	Trial 3	Average	Std Dev
1	2548.1	2917.5	2846.8	2770.8	196.0767
2	4430.4	5021.2	5232.6	4894.733	415.7843
5	12994.7	12402.1	12575.1	12657.3	304.7316
10	25852.2	26851.3	26224.6	26309.37	504.9151
25	67364	68962.2	67996.6	68107.6	804.8612
50	140569.1	147633.9	136270	141491	5737.768
100	296801.7	290947.6	281055.9	289601.7	7958.71
R^2	0.999069	0.999684	0.999736	0.999696	

Table S1.5: 2-chrysenol concentration curve fluorescent responses (µV·Min)

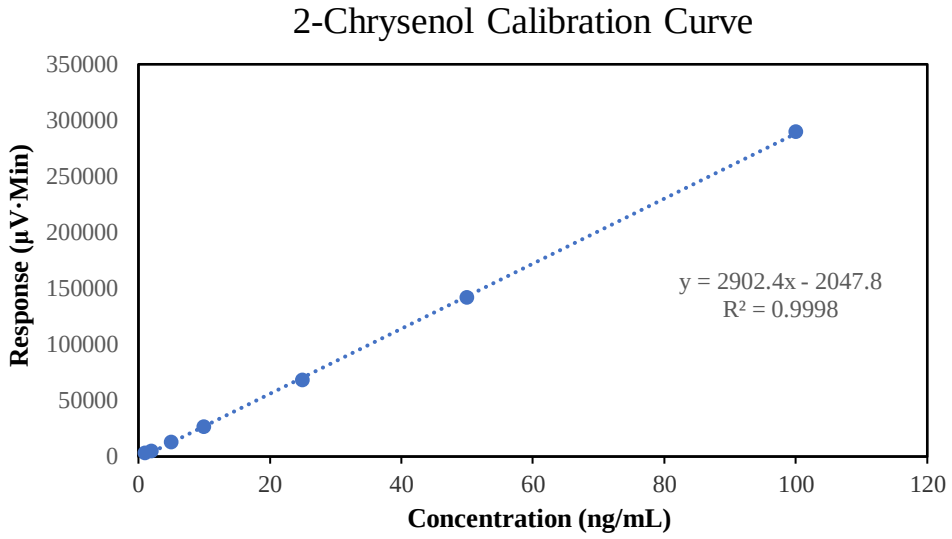


Figure S1.3: 2-chrysenol calibration curve (µV·Min)

S1.3.4 3-benzo[a]pyrenol

3-benzo[a]pyrenol calibration curves were affected by the retention of a small amount of compound on the chromatographic column after each standard. Standard curves with blanks after each injection were not evaluated after no 3-benzo[a]pyrenol was detected in experimental fish.

Concentration (ng/mL)	Trial 1	Trial 2	Trial 3	Average	Std Dev
1	78.2	502	463.2	347.8	234.285
2	273.5	714.3	642.8	543.5333	236.5725
5	1301.2	2064.4	1871.1	1745.567	396.784
10	3994.7	4862.4	4689.2	4515.433	459.208
25	14574.3	16080.7	15180.7	15278.57	757.9536
50	36947.2	39537.5	36080.9	37521.87	1798.528
100	89040	90671.5	85908.2	88539.9	2420.709
250	280199.2	258466.9	249431.6	262699.2	15814.42
R^2	0.991783	0.996572	0.995515	0.994752	

Table S1.6: 3-benzo[a]pyrenol concentration curve fluorescent responses (µV·Min)

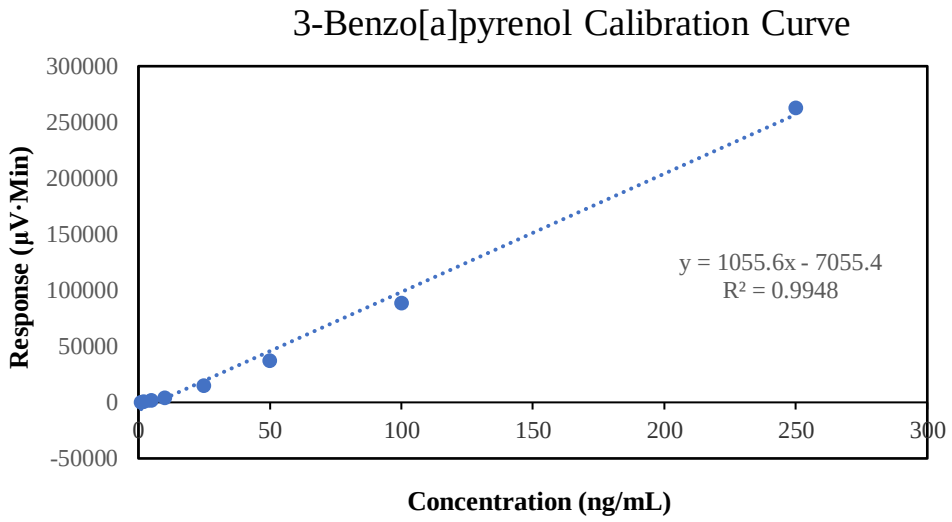


Figure S1.4: 3-benzo[a]pyrenol calibration curve (µV·Min)

Supplementary Information II: Additional metrics from year 1 (2018) of the Freshwater Oil Spill Remediation Study

S2.1 Aqueous and sediment parent PAC concentrations in the enclosures

			Water concentration (ng/L)			
PAC	**Fish collection period**	**Water sampling date**	**Ref 1**	**Ref 2**	**CHV**	**Dilbit**
Phenanthrene	N/A	03-Jul-18	4.02	9.29	1.95	4.46
Phenanthrene	July 17-19	Estimate	3.33	5.48	N/A	3.03
Phenanthrene	July 25-27	Estimate	N/A	4.22	1.51	N/A
Phenanthrene	Aug 1-9	07-Aug-18	2.64	1.68	1.29	1.60
Phenanthrene	Sept 27-28	04-Sep-18	1.88	ND	4.75	2.92
Pyrene	N/A	03-Jul-18	3.01	1.49	2.11	3.68
Pyrene	July 17-19	Estimate	1.79	1.19	N/A	2.32
Pyrene	July 25-27	Estimate	N/A	1.08	1.33	N/A
Pyrene	Aug 1-9	07-Aug-18	0.57	0.88	0.94	0.97
Pyrene	Sept 27-28	04-Sep-18	0.51	ND	1.07	0.53
Chrysene	N/A	03-Jul-18	1.10	0.99	0.61	3.75
Chrysene	July 17-19	Estimate	0.71	0.58	N/A	2.03
Chrysene	July 25-27	Estimate	N/A	0.44	0.42	N/A
Chrysene	Aug 1-9	07-Aug-18	0.31	0.17	0.32	0.31
Chrysene	Sept 27-28	04-Sep-18	0.23	ND	0.49	0.18

Table S2.1: Aqueous parent PAC concentrations in each enclosure for each fish collection period. N/A is listed when no fish were collected from that enclosure during the proscribed collection period. ND ≡ No data.

			Sediment concentration (ng/L)			
PAC	**Fish collection period**	**Sediment sampling date**	**Ref 1**	**Ref 2**	**CHV**	**Dilbit**
Phenanthrene	July 17-19	12-Jul-21	2.42	1.59	25.43	11.08
Phenanthrene	July 25-27	Estimate	N/A	1.44	29.43	N/A
Phenanthrene	Aug 1-9	09-Aug-21	2.63	1.28	33.42	7.67
Phenanthrene	Sept 27-28	ND	ND	ND	ND	ND
Pyrene	July 17-19	12-Jul-21	1.70	1.46	15.72	6.83
Pyrene	July 25-27	Estimate	N/A	2.24	30.00	N/A
Pyrene	Aug 1-9	09-Aug-21	8.49	3.02	44.29	22.08
Pyrene	Sept 27-28	ND	ND	ND	ND	ND
Chrysene	July 17-19	12-Jul-21	0.37	0.10	3.20	2.84
Chrysene	July 25-27	Estimate	N/A	0.09	9.53	N/A

Chrysene	Aug 1-9	09-Aug-21	0.27	0.09	15.86	0.67
Chrysene	Sept 27-28	ND	ND	ND	ND	ND

Table S2.2: Sediment parent PAC concentrations in each enclosure for each fish collection period. N/A is listed when no fish were collected from that enclosure during the proscribed collection period. ND ≡ No data.

S2.2 Additional metrics of experimental fish

			Sex			Bile mass (mg)				
Enclosure	**Species**	**Fish**	**Male**	**Female**	**Unk**	**Median**	**Mean**	**SD**	**Low**	**High**
Ref 1	FHM	2	2	-	-	-	7.54	-	6.99	8.10
	FSD	10	5	5	-	4.79	4.62	2.44	0.54	8.60
	Unknown	2	-	-	2	-	3.74	-	3.69	3.78
	All	14	7	5	2	4.79	4.91	2.35	0.54	8.60
Ref 2	FHM	5	2	3	-	2.31	4.73	2.74	2.31	7.86
	FSD	16	5	11	-	6.64	7.43	4.38	1.36	16.0
	Unknown	2	-	-	2	-	5.24	-	3.44	7.04
	All	23	7	14	2	6.64	6.65	4.02	1.36	16.0
CHV	FHM	2	1	1	-	-	9.00	-	7.95	10.0
	Unknown	2	-	-	2	-	9.57	-	8.22	10.9
	All	4	1	1	2	9.13	9.28	1.43	7.95	10.9
Dilbit	FHM	1	-	1	-	-	3.34	-	-	-
	FSD	1	-	1	-	-	11.8	-	-	-
	All	2	-	2	-	-	7.57	-	3.34	11.8

Table S2.3: The sex and bile mass of experimental fish from the first collection period (late July-early Aug). FHM ≡ fathead minnow, FSD ≡ finescale dace, Unk ≡ unknown; the sex of some fish was indeterminate at the time of collection, for others the information was lost.

			Body Length (mm)					Percent detection		
Enclosure	**Species**	**Fish**	**Mdn**	**Mean**	**SD**	**Low**	**High**	**PHE**	**1-PYR**	**2-CHR**
Ref 1	FHM	2	-	58.0	-	57	59	100	100	100
	FSD	10	52.5	51.7	7.1	40	63	100	100	89
	Unk	2	N/A	N/A	N/A	N/A	N/A	100	100	100
	All	14	54	52.8	6.9	40	63	100	100	91
Ref 2	FHM	5	50	52.4	3.4	50	57	100	100	100
	FSD	16	54	53.6	4.6	46	63	100	100	89
	Unk	2	N/A	N/A	N/A	N/A	N/A	100	100	N/A
	All	23	54	53.3	4.3	46	63	100	100	92
CHV	FHM	2	-	60.0	-	58	62	100	100	100
	Unk	2	N/A	N/A	N/A	N/A	N/A	100	100	N/A
	All	4	-	-	-	-	-	100	100	100
Dilbit	FHM	1	-	53.0	-	-	-	100	100	100
	FSD	1	-	56.0	-	-	-	100	100	100
	All	2	-	54.5	-	53	56	100	100	100

Table S2.4: Body length and metabolite detection rates of experimental fish from the first collection period (late July-early Aug). Unk ≡ unknown, mdn ≡ median; PHE ≡ phenanthrols; 1-PYR ≡ 1-pyrenol, 2-CHR ≡ 2-chrysenol, FHM ≡ fathead minnow, FSD ≡ finescale dace, Unk ≡ unknown. For phenanthrol, any samples below the limit of quantitation are considered non-detects. For 1-pyrenol and 2-chrysenol, limits of quantitation and limits of detection are not available and only non-detects are reported. 2-chrysenol was not quantified in all fish.

			Sex			Bile mass (mg)				
Enclosure	**Species**	**Fish**	**Male**	**Female**	**Unk**	**Median**	**Mean**	**SD**	**Low**	**High**
Ref 1	FHM	5	-	-	5	2.35	2.22	0.69	1.09	2.78
	FSD	6	2	3	1	1.8	1.93	1.23	0.4	3.78
	All	11	2	3	6	2.12	2.06	0.98	0.4	3.78
Ref 2	FSD	6	1	5	-	4.72	5.26	2.07	3.55	9.16
	NRBD	1	-	1	-	-	4.17	-	-	-
	All	7	1	6	-	4.2	5.11	1.94	3.55	9.16
CHV	FSD (all)	4	1	3	-	3.37	3.34	1.74	1.19	5.42
Dilbit	FHM	1	-	1	-	-	2.68	-	-	-
	FSD	9	2	7	-	4.74	4.24	1.57	1.11	6.32
	All	10	2	8	-	4.15	4.08	1.56	1.11	6.32

Table S2.5: The sex and bile mass of experimental fish from the second collection period (Sept). Unk ≡ unknown, FHM ≡ fathead minnow, FSD ≡ finescale dace, NRBD ≡ northern redbelly dace; the sex of some fish was indeterminate at the time of collection, for others the information was lost.

			Body Length (mm)					Percent detection		
Enclosure	**Species**	**Fish**	**Mdn**	**Mean**	**SD**	**Low**	**High**	**PHE**	**1-PYR**	**2-CHR**
Ref 1	FHM	5	38	38.0	1.0	37	39	100	100	N/A
	FSD	6	48	46.5	6.1	36	53	100	100	N/A
	All	11	39	42.6	6.2	36	53	100	100	N/A
Ref 2	FSD	6	53.5	52.8	4.1	46	57	100	100	100
	NRBD	1	-	45.0	-	-	-	100	100	100
	All	7	52	51.7	4.8	45	57	100	100	100
CHV	FSD (all)	4	47	47.3	10.3	37	58	100	100	25
Dilbit	FHM	1	-	55.0	-	-	-	100	100	100
	FSD	9	48	46.8	7.5	34	55	100	100	100
	All	10	48.5	47.6	7.5	34	55	100	100	100

Table S2.6: Body length and metabolite detection rates of experimental fish from the second collection period (Sept). Mdn ≡ median; PHE ≡ phenanthrols; 1-PYR ≡ 1-pyrenol, 2-CHR ≡ 2-chrysenol, FHM ≡ fathead minnow, FSD ≡ finescale dace, NRBD ≡ northern redbelly dace. For phenanthrol, any samples below the limit of quantitation are considered non-detects. For 1-pyrenol and 2-chrysenol, limits of quantitation and limits of detection are not available and only non-detects are reported. 2-chrysenol was not quantified in all fish.

Supplementary Information III: Additional metrics from year 2 (2019) of the Freshwater Oil Spill Remediation Study

S3.1 Diluted bitumen spill and recovery quantities

Substrate	Enclosure	Dilbit (g)	Amount recovered (g)	Residual dilbit (g)
WL	EFW	1444	49.4	1395
WL	EMNR1	1297	68.7	1228
WL	EMNR2	1234	79.2	1155
WL	EMNR3	1361	148	1213
WL	SC1	1263	73.4	1190
WL	SC2	1313	69.7	1243
WL	SC3	1079	39.0	1040
RC	EFW	1429	82.5	1347
RC	EMNR1	1525	40.4	1443
RC	EMNR2	1412	51.3	1361
RC	EMNR3	1471	70.0	1401
RC	SC1	1526	273	1253
RC	SC2	1408	34.0	1374
RC	SC3	1426	42.7	1383

Table S3.1: Masses of diluted bitumen spilled and recovered from primary cleaning with sorbent pads. EFW ≡ engineered floating wetland, EMNR ≡ enhanced monitored natural recovery, SC ≡ shoreline cleaning agent, WL ≡ wetland-organic, RC ≡ Rock-cobble

S3.2 Additional metrics of experimental fish

			Sex			Bile Mass (mg)				
Enclosure	**Species**	**Fish**	**Male**	**Female**	**Unk**	**Median**	**Mean**	**SD**	**Low**	**High**
Lake	FHM	18	8	10	-	4.74	5.07	1.71	2.37	8.98
	FSD	17	8	8	1	5.97	5.93	1.82	1.94	8.79
rRef 1	FHM	2	2	-	-	-	8.62	-	4.04	13.20
	FSD	7	2	4	1	5.17	5.36	2.27	2.53	8.84
	LC	1	1	-	-	-	5.58	-	-	-
rRef 2	FHM	1	-	-	1	-	7.54	-	-	-
	FSD	4	-	4	-	5.36	5.67	1.72	3.95	8.02
	LC	1	1	-	-	-	2.05	-	-	-
rEFW	FSD	13	3	8	2	4.54	5.33	2.51	2.50	9.97
rEMNR 1	FSD	11	1	10	-	6.84	7.97	3.70	4.64	16.65
rEMNR 2	FSD	18	2	15	1	5.41	6.33	2.32	3.66	11.18
	LC	1	1	-	-	-	7.84	-	-	-
	NRBD	1	-	1	-	-	6.86	-	-	-
rEMNR 3	FSD	19	2	16	1	6.05	7.43	3.78	1.51	16.51
rSC 1	FSD	8	3	5	-	6.04	6.28	2.97	2.48	11.10
rSC 2	FHM	1	1	-	-	-	5.96	-	-	-
	FSD	9	1	7	1	6.39	6.72	3.00	2.57	12.63
	NRBD	1	-	1	-	-	5.74	-	-	-
rSC 3	FSD	14	2	11	1	7.69	8.28	3.51	4.57	18.29
	LC	1	-	1	-	-	8.14	-	-	-

Table S3.2: The sex and bile mass of experimental fish from the rock-cobble (r) enclosures. Ref ≡ reference, EFW ≡ engineered floating wetland, EMR ≡ enhanced monitored natural recovery, SC ≡ shoreline cleaning agent, FHM ≡ fathead minnow, FSD ≡ finescale dace, NRBD ≡ northern redbelly dace, LC ≡ lake chub, Unk ≡ unknown; the sex of some fish was indeterminate at the time of collection

			Sex			Bile Mass (mg)				
Enclosure	**Species**	**Fish**	**Male**	**Female**	**Unk**	**Median**	**Mean**	**SD**	**Low**	**High**
Lake	FSD	18	8	10	-	4.74	5.07	1.71	2.37	8.98
	FHM	17	8	8	1	5.97	5.93	1.82	1.94	8.79
wRef1	FSD	19	3	16	-	7.46	8.40	4.25	2.53	22.71
	PD	2	1	-	1	-	3.72	-	3.42	4.01
wRef2	FSD	7	2	3	2	6.76	8.18	4.43	3.34	16.13
wEFW	FSD	5	1	-	4	3.13	7.10	6.84	1.71	17.75
	PD	4	3	-	-	6.53	5.81	2.22	2.67	7.51
wEMNR1	FHM	5	3	2	-	5.21	5.36	1.50	4.08	7.89
	FSD	4	1	3	-	6.47	6.43	2.48	3.94	8.85
	LC	1	-	1	-	-	3.83	-	-	-
	PD	3	-	1	2	6.65	6.48	2.50	3.91	8.89
wEMNR2	FHM	4	2	2	-	2.76	2.98	1.27	1.77	4.63
	FSD	2	-	2	-	-	8.94	-	7.64	10.23
wEMNR3	FSD	5	3	1	1	3.33	3.13	1.60	1.03	4.76
	PD	1	-	-	1	-	6.15	-	-	-
wSC1	FHM	4	2	2		2.78	2.93	2.00	1.12	5.05
	FSD	2	-	2	-	-	9.24	-	6.49	11.99
	PD	2	-	-	2	-	6.81	-	5.34	8.29
wSC2	FHM	15	5	9	1	2.50	3.46	1.86	1.21	6.37
	FSD	1	-	1	-	-	4.43	-	-	-
	PD	2	-	2	-	-	2.10	-	1.86	2.34
wSC3	FHM	15	3	10	2	3.86	3.96	3.09	1.01	13.14
	FSD	3	1	2	-	8.48	9.82	4.62	6.02	14.97

Table S3.3: The sex and bile mass of experimental fish from the wetland (w) enclosures. Ref ≡ reference, EFW ≡ engineered floating wetland, EMR ≡ enhanced monitored natural recovery, SC ≡ shoreline cleaning agent, FHM ≡ fathead minnow, FSD ≡ finescale dace, NRBD ≡ northern redbelly dace, LC ≡ lake chub, PD ≡ pearl dace, Unk ≡ unknown; the sex of some fish was indeterminate at the time of collection

			Fork length (mm)					% Detection			
Encl	**Species**	**N**	**Mdn**	**Mean**	**SD**	**Low**	**High**	**PHE**	**1PYR**	**2CHR**	**xCHR**
Lake	FHM	18	62.5	61.7	4.1	56	69	100	100	61	100
	FSD	17	56.0	55.7	5.8	49	69	100	100	80	100
rRef 1	FHM	2	-	53.5	-	50	57	100	100	-	100
	FSD	7	58.0	57.7	1.7	55	60	100	100	57	-
	LC	1	-	73.0	-	-	-	100	100	100	-
rRef 2	FHM	1	-	48.0	-	-	-	100	100	100	-
	FSD	4	56.0	56.2	1.3	55	58	100	100	75	-
	LC	1	-	73.0	-	-	-	100	100	0	-
rEFW	FSD	13	60.0	58.8	6.7	50	72	100	100	100	-
rEMR1	FSD	11	62.0	63.8	9.0	54	86	100	100	100	-
rEMR2	FSD	18	56.5	57.1	5.1	49	65	100	100	100	-
	LC	1	-	72.0	-	-	-	100	100	100	-
	NRBD	1	-	59.0	-	-	-	100	100	100	-
rEMR3	FSD	19	61.0	61.0	6.0	55	75	100	100	95	-
rSC 1	FSD	8	62.5	65.1	7.5	57	76	100	100	100	-
rSC 2	FHM	1	-	59.0	-	-	-	100	100	100	-
	FSD	9	60.0	59.6	5.1	51	69	100	100	100	-
	NRBD	1	-	52.0	-	-	-	100	100	100	-
rSC 3	FSD	14	61.5	63.4	8.8	50	83	100	100	100	-
	LC	1	-	76.0	-	-	-	100	100	100	-

Table S3.4: Body length and metabolite detection rates of experimental fish from rock-cobble (r) enclosures. Ref ≡ reference, EFW ≡ engineered floating wetland, EMR ≡ enhanced monitored natural recovery, SC ≡ shoreline cleaning agent, FHM ≡ fathead minnow, FSD ≡ finescale dace, LC ≡ lake chub, NRBD ≡ northern redbelly dace, PHE ≡ phenanthrols; 1PYR ≡ 1-pyrenol, 2CHR ≡ 2-chrysenol, xCHR ≡ x-chrysenol. For phenanthrol, any samples below the limit of quantitation are considered non-detects. For 1-pyrenol and 2-chrysenol and x-chrysenol, limits of quantitation and limits of detection are not available and only non-detects are reported.

			Fork length (mm)					% Detection			
Encl	**Species**	**N**	**Mdn**	**Mean**	**SD**	**Low**	**High**	**PHE**	**1PYR**	**2CHR**	**xCHR**
Lake	FSD	18	62.5	61.7	4.1	56	69	100	100	61	100
	FHM	17	56.0	55.7	5.8	49	69	100	100	80	100
wRef 1	FSD	19	61.0	61.9	5.1	56	78	100	100	-	100
	PD	2	-	56.5	-	53	60	100	100	-	100
wRef 2	FSD	7	54.0	55.1	6.0	43	61	100	100	-	100
wEFW	FSD	5	49.0	49.4	3.8	44	54	100	100	-	100
	PD	4	58.5	59.5	2.4	58	63	100	100	-	100
wEMR1	FHM	5	62.0	59.8	4.4	55	64	100	100	-	80
	FSD	4	57.5	59.2	5.3	55	67	100	100	-	100
	LC	1	-	85.0	-	-	-	100	100	-	100
	PD	3	52.0	52.7	2.1	51	55	100	100	-	100
wEMR2	FHM	4	65.0	65.0	2.9	62	68	100	100	-	100
	FSD	2	-	67.5	-	67	68	100	100	-	100
wEMR3	FSD	5	55.0	55.4	5.5	49	61	100	100	-	100
	PD	1	-	63.0	-	-	-	100	100	-	100
wSC 1	FHM	4	60.0	60.0	2.3	58	62	100	100	-	100
	FSD	2	-	58.0	-	52	64	100	100	-	100
	PD	2	-	52.0	-	49	55	100	100	-	100
wSC 2	FHM	15	55.0	55.4	5.2	49	65	100	100	-	93
	FSD	1	-	54.0	-	-	-	100	100	-	100
	PD	2	-	48.5	-	48	49	100	100	-	100
wSC 3	FHM	15	52.0	53.6	5.2	45	65	100	100	-	100
	FSD	3	57.0	57.3	5.5	52	63	100	100	-	100

Table S3.5: Body length and metabolite detection rates of experimental fish from rock-cobble (r) enclosures. Ref ≡ reference, EFW ≡ engineered floating wetland, EMR ≡ enhanced monitored natural recovery, SC ≡ shoreline cleaning agent, FHM ≡ fathead minnow, FSD ≡ finescale dace, LC ≡ lake chub, NRBD ≡ northern redbelly dace, PD ≡ pearl dace, PHE ≡ phenanthrols; 1PYR ≡ 1-pyrenol, 2CHR ≡ 2-chrysenol, xCHR ≡ x-chrysenol. For phenanthrol, any samples below the limit of quantitation are considered non-detects. For 1-pyrenol and 2-chrysenol and x-chrysenol, limits of quantitation and limits of detection are not available and only non-detects are reported.

S3.3 Univariate models of sediment or aqueous PACs predicting biliary metabolites

Sediment PAC concentrations were not available for rock-cobble enclosures, nor for the lake. Therefore, fish from the lake were omitted from sediment modelling, as were the rock-cobble enclosures. Similarly, the metabolite x-chrysenol was not quantified in finescale dace from the lake and were omitted from modelling.

Metabolite	Data Set	β	95% CI	Int	R^2	DoF	F	p
2-chrysenol	RC/H2O/FSD	1.197	±0.723	1.711	0.075	1,119	10.8	0.001
1-pyrenol[!]	WL/H2O/DAC	3.497	±1.098	1.058	0.460	1,46	41.1	<0.001
x-chrysenol	WL/H2O/DAC	-1.865	±1.048	3.825	0.201	1,46	12.8	0.001
Phenanthrols	WL/H2O/FHM	3.400	±2.359	-1.541	0.144	1,58	10.9	0.002
1-pyrenol	WL/H2O/FHM	-4.358	±1.59	7.050	0.328	1,58	29.7	<0.001
1-pyrenol	WL/SED/DAC	0.723	±0.347	1.466	0.261	1,46	17.6	<0.001
x-chrysenol	WL/SED/DAC	-0.305	±0.274	3.906	0.079	1,46	5.01	0.030
x-chrysenol	WL/SED/FHM	1.475	±1.227	-1.811	0.104	1,41	5.90	0.020

Table S3.6: Significant least squares regressions of aqueous or sediment PAC concentrations predicting biliary metabolite PAC concentrations. Data set ≡ substrate/medium/species, RC ≡ rock-cobble, WL ≡ wetland, FSD ≡ finescale dace, FHM ≡ fathead minnow, DAC ≡ finescale dace + pearl dace, H2O ≡ water, SED ≡ sediment. All data was log-transformed. [!]Lake data was omitted for this regression

The model of aqueous pyrene predicting biliary metabolite concentrations in finescale dace was only significant when fish from the lake were omitted. The lake had a higher concentration of pyrene than any of the enclosures, which may have indicated contamination was present in the sample. Moreover, estimating exposure concentrations to fish across an entire lake was thought to be more difficult than for those in enclosures. The negative correlations of chrysene models for dace in wetland enclosures were driven by high metabolite concentrations and low environmental chrysene concentrations in the reference enclosures. Regressions of aqueous concentrations for wetland fathead minnows lost significance when lake fish were omitted, illustrating the tenuous nature of some of the modelling.

S3.4 Periphyton PAC concentrations

In addition to strips inside each enclosure, periphyton was also collected from two sites in the lake on the outside of the enclosures.

Compound	wRef2	wEMR3	wSC2	wSC3	wEMR2	wEFW
Acenapthene	3.94	4.24	42.24	9.04	3.84	8.37
Acenapthylene	2.12	4.92	2.47	4.23	0.48	2.00
Anthracene	4.30	12.92	22.23	25.07	9.36	20.41
Benz[a]anthracene	1.82	6.16	3.60	10.55	4.28	1.85
Benzo[a]pyrene	1.37	3.20	1.54	5.17	4.57	1.52
Benzo[b]fluoranthene	7.11	14.47	21.77	24.39	20.85	14.11
Benzo[g,h,i]perylene	9.35	22.46	31.75	39.71	25.95	24.33
Benzo[k]fluoranthene	6.74	9.10	12.44	12.53	8.86	2.55
Chrysene	6.34	55.78	47.45	114.76	50.95	33.41
Dibenzo[a,h]anthracene	-3.02	-0.80	0.96	1.19	-0.76	0.03
Fluoranthene	22.22	66.82	160.22	65.65	74.94	184.99
Fluorene	27.54	67.67	49.09	95.97	18.54	44.14
Indeno[1,2,3-c,d]pyrene	4.18	7.60	10.01	16.20	10.89	7.79
Naphthalene	107.26	69.80	53.74	62.31	21.22	29.30
Phenanthrene	168.21	694.92	481.52	722.59	285.89	382.71
Pyrene	47.79	186.44	725.83	114.99	261.86	886.65

Table S3.7: EPA priority PAHs in periphyton in wetland (w) enclosures (part 1). All concentrations are ng/g. Compounds highlighted in red were not used. Parent PACs for metabolites are highlighted in green. EFW ≡ engineered floating wetland, EMR ≡ enhanced monitored natural recovery, Ref ≡ reference, SC ≡ shoreline cleaning agent

Compound	wRef1	wEMR1	wSC1	wLake1	wLake2
Acenapthene	4.70	3.82	3.04	2.74	4.23
Acenapthylene	2.83	2.24	1.36	2.14	1.93
Anthracene	12.26	14.34	11.80	6.75	9.53
Benz[a]anthracene	0.49	1.56	3.26	0.49	1.22
Benzo[a]pyrene	0.53	3.43	2.14	0.14	2.24
Benzo[b]fluoranthene	2.44	12.40	24.32	5.97	4.25
Benzo[g,h,i]perylene	1.75	14.26	42.27	4.41	4.17
Benzo[k]fluoranthene	0.85	5.41	8.84	2.84	3.15
Chrysene	3.54	35.50	68.91	8.08	5.08
Dibenzo[a,h]anthracene	-3.37	-9.70	2.95	-5.51	-1.99
Fluoranthene	53.55	14.59	116.82	17.79	66.59
Fluorene	41.70	19.14	25.11	26.81	24.91
Indeno[1,2,3-c,d]pyrene	ND	4.41	9.23	ND	0.05
Naphthalene	92.72	73.44	10.41	41.05	28.42
Phenanthrene	257.38	142.37	268.54	120.76	187.77
Pyrene	196.23	20.08	469.24	23.34	240.83

Table S3.8: EPA priority PAHs in periphyton in wetland (w) enclosures (part 2). All concentrations are ng/g. Compounds highlighted in red were not used. Parent PACs for metabolites are highlighted in green. EFW ≡ engineered floating wetland, EMR ≡ enhanced monitored natural recovery, Ref ≡ reference, SC ≡ shoreline cleaning agent, ND ≡ no detect

Compound	wRef2	wEMR3	wSC2	wSC3	wEMR2	wEFW
1,7-Dimethylphenanthrene	22.47	337.40	346.01	602.94	182.92	402.53
1,8-Dimethylphenanthrene	5.08	98.74	102.87	186.33	55.57	121.04
1-Methylnaphthalene	13.80	13.53	14.36	17.14	8.07	10.85
1-Methylphenanthrene	104.80	813.40	697.61	1034.44	379.35	726.47
2,6-Dimethylphenanthrene	10.50	123.46	133.54	216.26	75.16	156.97
2-Methylnaphthalene	28.30	20.01	25.26	23.79	14.41	19.48
2-Methylphenanthrene	109.24	973.88	826.54	1204.51	440.28	840.38
3,6-Dimethylphenanthrene	38.44	421.28	388.64	684.30	218.99	446.77
3-Methylphenanthrene	106.30	835.36	671.57	1001.39	359.77	661.00
9/4-Methylphenanthrene	113.61	1103.26	941.31	1391.03	470.23	905.17
C1 Chrysene	231.74	858.69	625.62	2148.02	1295.44	540.65
C2 Naphthalene	4.95	18.50	5.38	21.92	2.74	4.74
C2 Phenanthrene	196.77	2745.72	2753.11	4673.87	1476.49	3189.28
C3 Chrysene	13.94	171.78	276.36	308.82	219.93	278.60
C3 Naphthalene	282.00	3054.85	423.18	3107.08	226.94	369.47
C3 Phenanthrene	61.27	1047.33	1269.84	2130.85	779.10	1404.25
C4 Naphthalene (tetramethyl)	303.67	2741.02	985.82	3918.21	417.51	748.21
C4 Phenanthrene	20.60	205.40	300.57	471.14	236.59	370.38
C1 Benzo[a]pyrene	3.20	58.18	121.83	149.97	154.38	126.94
C1 Dibenzothiophene	204.01	2092.00	1434.75	2794.97	740.31	1194.72
C1 Fluorene	477.93	1562.58	1022.91	2319.81	420.46	840.48
C1 Pyrene	11.59	135.54	180.60	257.01	122.73	174.78
C2 Dibenzothiophene	296.32	4208.01	3813.13	7278.92	1932.71	3658.23
C2 Fluorene	419.44	1963.16	2467.17	2615.48	1074.30	2041.43
C2 Pyrene	5.08	44.97	84.02	83.99	66.27	77.41
C3 Dibenzothiophene	60.37	1505.12	1566.44	3208.85	1034.84	1704.41
Dibenzothiophene	55.50	243.85	190.58	270.72	91.93	146.91

Table S3.9: Alkyl PACs in periphyton in wetland (w) enclosures (part 1). All concentrations are ng/g. EFW ≡ engineered floating wetland, EMR ≡ enhanced monitored natural recovery, Ref ≡ reference, SC ≡ shoreline cleaning agent

Compound	wRef1	wEMR1	wSC1	wLake1	wLake2
1,7-Dimethylphenanthrene	98.90	42.73	317.69	13.99	81.46
1,8-Dimethylphenanthrene	26.63	13.63	96.29	3.01	26.87
1-Methylnaphthalene	14.69	ND	8.32	2.62	7.74
1-Methylphenanthrene	273.58	63.97	543.72	51.84	214.84
2,6-Dimethylphenanthrene	43.87	15.71	122.31	6.09	37.94
2-Methylnaphthalene	26.88	ND	12.09	ND	15.26
2-Methylphenanthrene	313.20	74.72	626.64	59.44	243.31
3,6-Dimethylphenanthrene	142.49	41.80	378.64	16.07	126.96
3-Methylphenanthrene	270.39	64.60	460.17	58.84	204.28
9/4-Methylphenanthrene	332.05	86.79	631.20	49.51	263.77
C1 Chrysene	192.10	55.55	843.57	34.62	70.71
C2 Naphthalene	7.32	0.54	4.00	2.87	2.92
C2 Phenanthrene	874.83	310.46	2466.80	104.53	787.27
C3 Chrysene	52.48	136.84	397.22	7.69	8.05
C3 Naphthalene	594.52	109.62	276.00	228.21	134.61
C3 Phenanthrene	288.52	189.06	1323.80	37.67	374.26
C4 Naphthalene (tetramethyl)	676.78	114.82	417.16	167.69	187.31
C4 Phenanthrene	52.87	74.60	395.68	10.93	47.65
C1 Benzo[a]pyrene	19.69	55.59	179.95	ND	3.72
C1 Dibenzothiophene	458.38	121.82	863.33	62.74	337.53
C1 Fluorene	971.53	213.99	390.43	1157.04	417.65
C1 Pyrene	48.46	28.01	200.36	8.29	45.50
C2 Dibenzothiophene	1072.18	336.97	2942.85	88.72	965.09
C2 Fluorene	928.14	201.39	1254.15	441.38	717.85
C2 Pyrene	21.71	26.92	82.85	1.82	12.99
C3 Dibenzothiophene	328.71	211.11	1514.93	16.46	302.07
Dibenzothiophene	82.61	15.15	91.82	19.69	50.07

Table S3.10: Alkyl PACs in periphyton in wetland (w) enclosures (part 2). All concentrations are ng/g. EFW ≡ engineered floating wetland, EMR ≡ enhanced monitored natural recovery, Ref ≡ reference, SC ≡ shoreline cleaning agent, ND ≡ no detect

Compound	rEMR3	rSC3	rRef2	rEMR4	rSC2	rRef1
Acenapthene	2.24	8.65	9.44	5.93	4.81	7.45
Acenapthylene	0.90	2.09	0.93	1.82	4.83	1.25
Anthracene	1.80	8.92	5.49	8.15	3.79	1.75
Benz[a]anthracene	1.79	3.83	0.12	3.59	0.72	0.12
Benzo[a]pyrene	1.68	2.03	0.10	0.46	0.23	0.00
Benzo[b]fluoranthene	7.45	10.13	2.18	5.84	6.61	1.51
Benzo[g,h,i]perylene	10.74	20.35	ND	8.86	10.74	ND
Benzo[k]fluoranthene	4.84	3.93	ND	1.29	1.74	ND
Chrysene	19.44	45.76	3.89	51.48	28.07	1.66
Dibenzo[a,h]anthracene	-6.37	-5.33	-11.30	-16.87	-9.04	-8.95
Fluoranthene	16.13	67.70	73.29	94.62	32.19	15.12
Fluorene	15.58	34.95	25.08	47.77	34.52	8.91
Indeno[1,2,3-c,d]pyrene	ND	4.02	ND	ND	3.96	ND
Naphthalene	33.02	59.02	92.28	41.27	37.17	62.63
Phenanthrene	101.57	334.54	241.85	469.24	300.61	110.98
Pyrene	23.90	211.56	471.52	327.49	83.45	33.75

Table S3.11: EPA priority PAHs in periphyton in rock-cobble (r) enclosures (part 1). All concentrations are ng/g. Compounds highlighted in red were not used. Parent PACs for metabolites are highlighted in green. EFW ≡ engineered floating wetland, EMR ≡ enhanced monitored natural recovery, Ref ≡ reference, SC ≡ shoreline cleaning agent

Compound	rEMR1	rSC1	rEFW	rLake1	rLake2
Acenapthene	3.61	4.03	5.35	1.12	1.75
Acenapthylene	1.02	2.58	2.69	0.26	0.73
Anthracene	5.63	6.24	9.03	0.87	5.21
Benz[a]anthracene	1.84	7.08	5.10	0.08	0.76
Benzo[a]pyrene	0.41	2.16	0.28	0.23	1.02
Benzo[b]fluoranthene	8.35	19.32	8.49	1.80	1.68
Benzo[g,h,i]perylene	11.39	27.81	13.40	1.62	2.13
Benzo[k]fluoranthene	3.86	6.49	5.02	1.20	0.60
Chrysene	27.67	112.43	64.88	3.33	4.77
Dibenzo[a,h]anthracene	-5.42	-4.61	-7.51	-9.54	-5.30
Fluoranthene	33.14	80.07	93.59	31.31	31.87
Fluorene	31.95	35.48	45.25	6.74	21.51
Indeno[1,2,3-c,d]pyrene	3.24	7.16	ND	ND	ND
Naphthalene	38.60	30.49	30.52	11.54	26.94
Phenanthrene	285.85	642.56	646.14	81.78	128.73
Pyrene	94.74	124.08	278.66	46.73	84.76

Table S3.12: EPA priority PAHs in periphyton in rock-cobble (r) enclosures (part 2). All concentrations are ng/g. Compounds highlighted in red were not used. Parent PACs for metabolites are highlighted in green. EFW ≡ engineered floating wetland, EMR ≡ enhanced monitored natural recovery, Ref ≡ reference, SC ≡ shoreline cleaning agent

Compound	rEMR3	rSC3	rRef2	rEMR2	rSC2	rRef1
1,7-Dimethylphenanthrene	92.40	320.07	137.51	388.38	185.90	15.21
1,8-Dimethylphenanthrene	25.68	89.48	43.52	118.14	49.68	2.93
1-Methylnaphthalene	4.52	12.22	16.31	9.83	9.08	12.21
1-Methylphenanthrene	109.20	462.88	284.37	625.41	339.22	53.23
2,6-Dimethylphenanthrene	29.56	106.94	59.95	132.99	64.01	7.38
2-Methylnaphthalene	6.90	21.43	29.16	16.02	12.82	22.07
2-Methylphenanthrene	136.10	590.21	297.75	715.62	438.56	65.11
3,6-Dimethylphenanthrene	95.10	360.56	168.16	423.77	226.44	25.68
3-Methylphenanthrene	107.00	413.24	250.86	555.92	322.70	59.90
9/4-Methylphenanthrene	133.17	533.70	324.14	761.03	419.44	51.72
C1 Chrysene	118.38	173.00	39.65	184.59	103.94	27.66
C2 Naphthalene	39.52	103.74	72.97	209.50	171.73	48.49
C2 Phenanthrene	636.86	2329.55	1166.90	2892.31	1427.52	131.27
C3 Chrysene	132.41	209.52	9.69	123.92	128.99	11.61
C3 Naphthalene	181.46	681.84	147.14	1423.11	1327.28	79.56
C3 Phenanthrene	378.06	1076.74	442.38	1303.31	509.63	59.11
C4 Naphthalene (tetramethyl)	299.38	1044.17	228.17	1694.43	1609.72	69.57
C4 Phenanthrene	103.66	220.65	110.91	268.03	102.86	22.70
C1 Benzo[a]pyrene	18.31	74.04	2.84	30.45	16.83	ND
C1 Dibenzothiophene	61.64	927.70	328.08	1356.33	707.33	84.93
C1 Fluorene	397.91	814.23	392.85	1142.99	762.74	278.62
C1 Pyrene	46.21	112.64	82.03	138.56	42.42	9.62
C2 Dibenzothiophene	211.53	2665.55	1006.22	3522.63	1540.44	131.79
C2 Fluorene	334.26	1280.30	775.43	1501.64	841.47	133.14
C2 Pyrene	36.39	56.80	36.02	63.26	25.23	3.09
C3 Dibenzothiophene	159.17	1229.52	369.34	1442.12	542.16	31.32
Dibenzothiophene	6.45	92.48	50.84	144.93	100.31	25.03

Table S3.13: Alkyl PACs in periphyton in rock-cobble (r) enclosures (part 1). All concentrations are ng/g. EFW ≡ engineered floating wetland, EMR ≡ enhanced monitored natural recovery, Ref ≡ reference, SC ≡ shoreline cleaning agent

Compound	rEMR1	rSC1	rEFW	rLake1	rLake2
1,7-Dimethylphenanthrene	147.01	895.84	654.69	30.05	56.19
1,8-Dimethylphenanthrene	41.00	248.29	179.71	6.11	12.07
1-Methylnaphthalene	5.72	7.61	11.95	3.53	6.45
1-Methylphenanthrene	245.76	1164.39	1010.69	83.67	129.42
2,6-Dimethylphenanthrene	52.64	295.85	216.19	14.67	21.82
2-Methylnaphthalene	8.77	12.34	20.27	3.27	9.79
2-Methylphenanthrene	316.61	1415.46	1258.39	99.35	147.85
3,6-Dimethylphenanthrene	167.44	949.58	679.29	43.14	71.37
3-Methylphenanthrene	231.78	1079.66	897.06	84.35	119.92
9/4-Methylphenanthrene	303.70	1538.41	1236.07	76.80	130.05
C1 Chrysene	110.58	523.09	259.82	31.58	12.76
C2 Naphthalene	121.39	269.45	275.46	44.02	70.45
C2 Phenanthrene	1100.68	6303.95	4567.54	239.34	432.69
C3 Chrysene	90.46	323.34	213.62	3.84	ND
C3 Naphthalene	821.46	2649.11	2260.24	76.26	194.94
C3 Phenanthrene	451.87	2957.44	1809.12	70.51	156.52
C4 Naphthalene (Tetramethyl)	881.08	3291.38	2381.97	64.66	154.01
C4 Phenanthrene	92.05	518.35	319.58	15.76	31.46
C1 Benzo[a]pyrene	26.01	101.93	42.37	24.00	0.00
C1 Dibenzothiophene	527.18	2555.23	1990.04	108.60	162.55
C1 Fluorene	465.05	1150.88	1419.20	280.54	426.48
C1 Pyrene	39.47	147.54	136.44	9.28	17.95
C2 Dibenzothiophene	1126.75	6879.67	4874.90	241.66	385.69
C2 Fluorene	635.71	1786.67	2169.51	205.49	281.24
C2 Pyrene	20.40	54.48	43.35	2.39	5.10
C3 Dibenzothiophene	428.71	2802.94	1694.41	56.36	119.93
Dibenzothiophene	75.31	221.19	223.36	17.11	27.29

Table S3.14: Alkyl PACs in periphyton in rock-cobble (r) enclosures (part 2). All concentrations are ng/g. EFW ≡ engineered floating wetland, EMR ≡ enhanced monitored natural recovery, Ref ≡ reference, SC ≡ shoreline cleaning agent

S3.5 Aqueous PAC concentrations for periphyton regressions

All data from June 27/28 are presented, as well as select values from Aug 26/27 and Jun 21/22 that resulted in significant regressions

Enclosure	Date	2All	3All	4All	5All	2Un	3Un	4Un	5Un
rEMNR3	28-Jun	469.4	749.3	914.0	25.40	39.04	15.29	4.79	14.33
rSC3	28-Jun	3541	3980	2603	33.48	473.5	70.09	19.75	14.42
rRef2	28-Jun	21.33	362.3	713.5	17.45	21.21	4.45	5.53	11.38
rEMNR2	28-Jun	717.2	1999	1709	28.15	24.79	34.37	11.20	11.38
rSC2	28-Jun	2930	3355	2495	30.76	429.2	67.78	10.23	13.09
rRef1	28-Jun	413.3	192.4	137.1	37.42	13.94	7.19	2.64	35.47
rEMNR1	28-Jun	1041	1885	1696	26.19	30.16	56.94	5.93	9.78
rSC1	28-Jun	5228	5478	2921	36.16	819.8	123.6	14.58	14.48
rEFW	28-Jun	802.8	2076	1583	27.74	20.00	36.35	7.20	13.75
rLake	28-Jun	NA	516.2	485.0	16.87	16.58	4.73	3.95	14.27
wRef2	27-Jun	27.90	348.9	564.3	19.13	18.14	5.87	4.62	14.79
wEMNR3	27-Jun	487.42	5226	3612	38.18	26.00	64.92	49.58	21.69
wSC2	27-Jun	NA	NA	NA	NA	NA	NA	NA	NA
wSC3	27-Jun	1341	3824	2313	29.27	441.4	104.8	13.49	16.58
wEMNR2	27-Jun	546.1	3181	3467	36.00	29.18	71.44	13.19	15.73
wEFW	27-Jun	535.7	2347	2443	17.10	31.70	63.29	6.09	12.22
wRef1	27-Jun	29.76	492.7	651.7	13.14	17.87	8.20	4.74	10.78
wEMNR1	27-Jun	469.4	2030	2056	16.39	32.37	54.20	4.97	12.55
wSC1	27-Jun	1548	4722	3777	25.76	420.4	101.9	13.35	9.37
wLake	27-Jun	31.26	565.1	355.0	11.81	19.99	7.32	11.46	11.81

Table S3.15: Aqueous PACs in rock-cobble (r) and wetland enclosures (part 1). All concentrations are ng/L. Numbers in the header refer to the number of rings. All ≡ all PACs, Un ≡ unalkylated PACs, EFW ≡ engineered floating wetland, EMR ≡ enhanced monitored natural recovery, Ref ≡ reference, SC ≡ shoreline cleaning agent

Enclosure	Date	2Alk	3Alk	4Alk	5Alk	Date	2Alk	2All	4Alk
rEMNR3	28-Jun	430.4	734.0	909.2	11.07	23-Jun	347.9	365.9	-
rSC3	28-Jun	3068	3910	2583	19.06	23-Jun	400.9	418.5	-
rRef2	28-Jun	0.12	357.9	708.0	6.08	23-Jun	12.59	19.57	-
rEMNR2	28-Jun	692.4	1964	1698	16.77	23-Jun	423.1	437.6	-
rSC2	28-Jun	2501	3287	2485	17.67	23-Jun	476.2	491.4	-
rRef1	28-Jun	399.4	185.2	134.5	1.95	23-Jun	26.62	29.65	-
rEMNR1	28-Jun	1011	1828	1690	16.42	23-Jun	771.4	797.3	-
rSC1	28-Jun	4408	5355	2907	21.68	23-Jun	630.6	656.4	-
rEFW	28-Jun	782.8	2040	1576	13.99	23-Jun	700.5	726.6	-
rLake	28-Jun	NA	511.5	481.0	2.60	23-Jun	217.0	225.6	-
wRef2	27-Jun	9.76	343.0	559.6	4.34	26-Aug	-	-	3.26
wEMNR3	27-Jun	461.4	5161	3563	16.48	26-Aug	-	-	9.19
wSC2	27-Jun	N/A	N/A	N/A	N/A	26-Aug	-	-	20.59
wSC3	27-Jun	899.5	3719	2300	12.69	26-Aug	-	-	23.51
wEMNR2	27-Jun	516.9	3110	3454	20.27	26-Aug	-	-	17.20
wEFW	27-Jun	504.0	2284	2437	4.88	26-Aug	-	-	17.04
wRef1	27-Jun	11.89	484.5	646.9	2.36	26-Aug	-	-	7.89
wEMNR1	27-Jun	437.0	1976	2051	3.83	26-Aug	-	-	20.14
wSC1	27-Jun	1127	4620	3764	16.39	26-Aug	-	-	10.46
wLake	27-Jun	11.26	557.7	343.5	ND	26-Aug	-	-	3.04

Table S3.16: Aqueous PACs in rock-cobble (r) and wetland enclosures (part 2). All concentrations are ng/L. Numbers in the header refer to the number of rings. All ≡ all PACs, Alk ≡ alkylated PACs, EFW ≡ engineered floating wetland, EMR ≡ enhanced monitored natural recovery, Ref ≡ reference, SC ≡ shoreline cleaning agent