ABSTRACT

Metabolism and Mechanistic Toxicity of Environmental Pollutants in Fish Models: Integrating *In Vitro* and *In Vivo* Systems for Ecotoxicological Studies

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The presence of legacy and emerging contaminants in the aquatic environment represents a significant threat to aquatic biota, often leading to significant declines of biodiversity. This issue is further aggravated by the influence of abiotic environmental factors, such as climate change, which could potentially modify organisms' exposure and responses to pollution. Historically, ecotoxicological studies have relied on the use of live animals and endpoints such as mortality, growth, and reproduction to determine whether exposed organisms and populations are at risk. However, these approaches are often hindered by cost, ethic, and scientific limitations, making them unable to provide a thorough representation of exposure conditions and resulting adverse effects. Recently, significant research efforts have highlighted the need to understand pollutant-driven alterations at different levels of biological organization. In this context, new approach methodologies (NAMs), such as *in vitro* systems, have emerged as robust bioanalytical tools to mechanistically describe chemical-organism interactions, predict potential adverse effects from exposure, and support comprehensive assessments of risk, while reducing animal use. The scope of this dissertation relies on the applicability of NAMs,

specifically cell-based bioassays, and their integration with more traditional approaches (e.g. *in vivo* systems) to address research gaps associated with the biotransformation of legacy compounds by fish populations with different exposure history, the endocrine disruption potential of wastewater effluents, and the description of mechanistic toxicity of natural and anthropogenic pollutants, while considering the influence of different environmental stressors. This work demonstrated that descriptions of adverse effects from exposure to pollutants are significantly facilitated by *in vitro* systems, but that overall characteristics of the species and areas of interest must be accounted for when selecting appropriate cell-based models, as their improper selection could significantly mislead observations and subsequent environmental management strategies. The integration of cell-based and whole-animal approaches showed that the sensitivity and specificity of *in vitro* systems are significant limitations for their implementation, and that their value in ecotoxicological studies relies on their integration with more complex experimentation through weight of evidence (WoE) approaches.

Metabolism and Mechanistic Toxicity of Environmental Pollutants in Fish Models: Integrating *in vitro* and *in vivo* Systems for Ecotoxicological Studies

by

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A Dissertation

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May this accomplishment be a motivation for always going after our biggest dreams.

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CHAPTER ONE

Introduction

Background and Significance

Chemical pollution continues to be a matter of significant concern given the continuous introduction of different natural and anthropogenic compounds into the environment, where aquatic ecosystems are among the most significantly compromised. This has resulted in the loss of important species and major declines in aquatic biodiversity (Maasri, Jähnig, Adamescu, Adrian, & al, 2021; Seehausen, Van Alphen, & Witte, 1997). The issue is further aggravated by rapid changes in other environmental factors (e.g. climate change) that could contribute to increasing the impact of pollution in already-threatened ecosystems. While some species have shown the ability to undergo rapid evolutionary change to adapt to contaminated environments (Jayasundara et al., 2017; Reid et al., 2016) and maintain ecological fitness, other more-sensitive species could experience severe adverse effects, leading to major changes at the population, community, and ecosystem levels. Furthermore, the unprecedented emergence and introduction of new and, potentially, more severe chemicals bring significant obstacles to identify which substances are the most concerning and to what extent negative effects that could arise from exposure. This represents a major challenge in the field of ecotoxicology, as not only legacy contaminants (e.g. polychlorinated biphenyls and polycyclic aromatic hydrocarbons) continue to be present in many environmental compartments, but also emerging and less-studied contaminants (e.g. endocrine disruptors, pharmaceuticals) now contribute to the need of investigating potential adverse

effects associated with them and the need of developing new and novel approaches to evaluate the risk associated with different classes of chemicals.

In the last two decades, the field of ecotoxicology has been supported by advances in new technologies, as other fields such as molecular biology, toxicogenomics, bioinformatics, and computational biology have evolved to support evaluations of the toxicity associated with environmental pollutants. These advances have enhanced the ability to mechanistically evaluate and predict cause-effect relationships of xenobiotics and biomarkers of interest (Krewski et al., 2010; NRC, 2007), and have illustrated new approach methodologies (NAMs) aimed to replace, reduce and refine (3R principles) animal testing. Historically, experimentation with live animals has been effectively used to assess the effects and dose-response relationships of chemical compounds. This is despite *in vivo* testing presenting economic, ethic, and scientific limitations (Yoon, Campbell, Andersen, & Clewell, 2012). NAMs, as proposed alternatives to *in vivo* testing, include the use of *in vitro* systems, where whole cells or cellular components are used to evaluate chemical-organism relationships (Brinkmann, Preuss, & Hollert, 2016). However, as on-going research continues to support the development of new techniques in the field of ecotoxicology, significant attention has been given to the implementation of multi-approach assessments, where the integration of computational (*in silico*), *in vivo,* and *in vitro* systems can elucidate mechanisms of toxicity as well as subsequent adverse effects at different levels of biological organization, and that could be otherwise difficult to identify by relying on a single approach.

Even though it is well established that comparisons between NAMs and whole animal models are limited by differences in exposure to xenobiotics, toxicokinetics and

toxicodynamics, and overall biological responses, significant experimentation has highlighted the applicability of different mechanistic, non-animal approaches to describe exposure scenarios, and components of absorption, distribution, metabolism and excretion (ADME) (Blaauboer, 2015). This, in combination with the characterization of adverse effects at the *in vivo* level, has been advantageous in evaluating how organisms respond to chemical exposure. The integration of multiple lines of evidence with the application of whole animals and NAMs to advance the field of ecotoxicology is a matter of intensive research and requires extensive investigations to support the establishment of frameworks to enhance and strengthen ecotoxicological studies.

One specific scenario that can be significantly benefited from the implementation and integration of NAMs with whole-animal systems is populations inhabiting heavily polluted environments and/or with different exposure and evolutionary histories. Fish communities that are chronically exposed to chemical compounds represent a unique opportunity to explore how these species maintain ecological fitness under stressful environments. The adaptations allowing exposed organisms to thrive usually correspond to modifications at the molecular level, making certain *in vitro* systems that facilitate mechanistic evaluations fundamental in research projects aimed to describe such adaptations. However, the continuous implementation of traditional approaches (e.g. *in vivo* experimentation) results necessary to consider how these mechanisms of adaptation influence whole-animal physiology and overall population responses. In the context of evolutionary, molecular, and environmental toxicology, one of the most suitable scenarios to explore adaptation mechanisms is shown by the industrial pollution in the Houston Ship Channel (HSC), Texas, USA, and specifically, with the Gulf killifish

(*Fundulus grandis*), which has been recently described to be pollution-adapted (Oziolor et al., 2019). This type of pollution represents a human-driven force that introduces selective pressure and adverse effects on different ecological aspects of aquatic ecosystems (Oziolor, Bigorgne, Aguilar, Usenko, & Matson, 2014; Oziolor et al., 2019), allowing for the opportunity to use this species as a model to understand and predict adaptation to pollution not only in other fish species but also in a wide variety organisms that experience long-term exposure to environmental pollutants.

In a different scenario, and with the environment facing new challenges with emerging natural and anthropogenic contaminants, including pharmaceuticals, endocrine disrupting compounds (EDCs), and algal toxins from harmful blooms, multi-approach evaluations also result useful in describing potential environmental hazard to aquatic organisms. The emergence of these contaminants has been shown to be a direct result of the growing human populations and increasing urbanization rates. In terms of EDCs, this increase in urbanization has serious implications for water management, as larger discharges of wastewater and untreated sewage often result in impacted surface waters, altering the chemical and biological composition of aquatic systems (Brooks, 2018). Furthermore, the effects of climate change on global temperatures and altered weather dynamics inherently modify the complexity of aquatic ecosystems, especially those that receive a direct impact of external environmental factors, such as streams that receive input from seasonal snowmelt. Because of this complexity, assessing the effects of EDCs in aquatic ecosystems has been challenging as responses are significantly variable among types of EDCs, fish species, life stages and sex, and specific environmental factors.

Similarly, with global climate change becoming more significant than ever and with continuous introduction of nutrients from e.g. agricultural lands into aquatic ecosystems, the emergence of natural pollutants such as cyanotoxins has received significant attention. Under specific conditions, harmful microalgae are able to cause significant water quality issues, leading, in many cases, to major ecosystem impairment and damage to public and environmental health (Haddad et al., 2019). Unfortunately, there is a significant lack of information for some of the most damaging harmful microalgae. One of this species is the golden algae *Prymnesium parvum,* as large ecological impacts have been the result of the ichthyotoxicity exerted by this haptophyte in different areas around the world. *P. parvum* toxins, which are most commonly referred as prymnesins (Igarashi, Satake, & Yasumoto, 1999), have been suggested to compromise the integrity of cell membranes, leading to a malfunction of cell permeability (Johansson & Granéli, 1999; Shilo, 1981). However, other secondary metabolites contributing to the deleterious effects of *P. parvum* remain an active area of research (Bertin, Voronca, Chapman, & Moeller, 2014; Bertin, Zimba, Beauchesne, Huncik, & Moeller, 2012; Blossom et al., 2014; Henrikson et al., 2010). One of the major research gaps corresponds to specific mechanisms of toxicity by *P. parvum,* as they have not been fully described given that analytical standards of prymnesins are not readily available (Brooks et al., 2010).

Under these circumstances and considering these scenarios, testing strategies and biological models that facilitate fast, yet detailed mechanistic evaluations of toxicity and adverse outcomes become paramount. For example, the implementation of NAMs could support traditional bioassays employed to examine exposure scenarios that include

endpoints that are not sufficiently sensitive for biologically active substances that elicit sublethal toxicity through various molecular initiation events (Ankley, Brooks, & Hugget, 2007). In this case, bioanalytical tools, such as *in vitro* bioassays and specific gold-standard biomarkers have facilitated comprehensive evaluations of potential water quality alterations (Brion et al., 2019; Pawlowski et al., 2003; Sumpter & Jobling, 1995). Implementing well-characterized testing strategies and bioanalytical tools, such as cell-based assays, is critical in describing negative effects of environmental pollutants in exposed populations (Huggett et al., 2003), and provide valuable information to support environmental management decisions. However, the identification and applicability of this type of tools requires extensive investigation on its own and significant research gaps exists as to describe simple, yet useful tools that can facilitate the measurement of adequate biomarkers and the description of species-specific sensitivity to chemicals and complex environmental samples. In recent years, one major advancement in (eco)toxicology has been the establishment of cell lineages as strong alternative toxicological assays (Castaño et al., 2003).

Several studies have reported valuable data using cell lines, yet only a few have focused on characterizing molecular and biochemical properties of existing lineages. The applicability of cell-based models requires the collection of data from *in vivo* studies, previously used *in vitro* models, preliminary experimentation and information about compounds of interest. Model selection also requires exposure information to simulate environmentally relevant scenarios, and the integration of suitable biomarkers of exposure and effect as shown in figure 1.1. Thus, there is an imminent need to continue

the search for suitable cell-based systems to appropriately select the best models, while incorporating environmental factors that influence exposure assessments.

Scope of Dissertation

As aquatic environments continue to experience negative impacts from legacy and emerging contaminants, and in an effort to establish NAMs as an important avenue for ecotoxicological studies, this dissertation addresses significant research gaps related to molecular adaptations to polluted environments and adverse biological effects caused by emerging contaminants in complex environmental scenarios, through the integration of *in vitro* and whole-animal experimentation. The first research chapter included in this dissertation describe, for the first time, population- and sex-related differences in the biotransformation of legacy contaminants by Gulf killifish (*Fundulus grandis*) with different exposure and evolutionary histories through the implementation of a modified standardized *in vitro* methodology (OECD 319B). Chapter three presents novel observations regarding differential *in vitro* and *in vivo* biotransformation of the common legacy compound benzo[a]pyrene through comparisons of enzyme activity, tissue concentrations, and metabolite profiles between pollution-adapted and non-adapted *F. grandis*. Chapter four presents a multi-approach evaluation of estrogenicity in fish inhabiting effluent-dominated surface waters impacted by seasonal changes in streamflow and highlights the applicability of a cell-based model and its integration with field- and laboratory-based experimentation. Furthermore, based on the initial stage of this assessment, chapter five presents a subsequent evaluation of estrogenicity from *on-site* wastewater treatment systems and pointed out the strengths and limitations of the implemented techniques, contributing valuable knowledge to establish useful and logical

paths when assessing endocrine disruption in aquatic ecosystems. Chapters six and seven advance the applicability of fish cell lines through the characterization of biomarkers of biotransformation and oxidative stress upon exposure to single chemicals and harmful algal toxins, respectively. Both chapters describe species- and organ-specific responses *in vitro*, suggesting the need to appropriately select useful models depending on research needs. Altogether, these assessments represent a path forward for considering NAMs and their integration with whole-animal assessments as major tools in experimentation aimed to advance ecotoxicological studies while reducing and refining animal use.

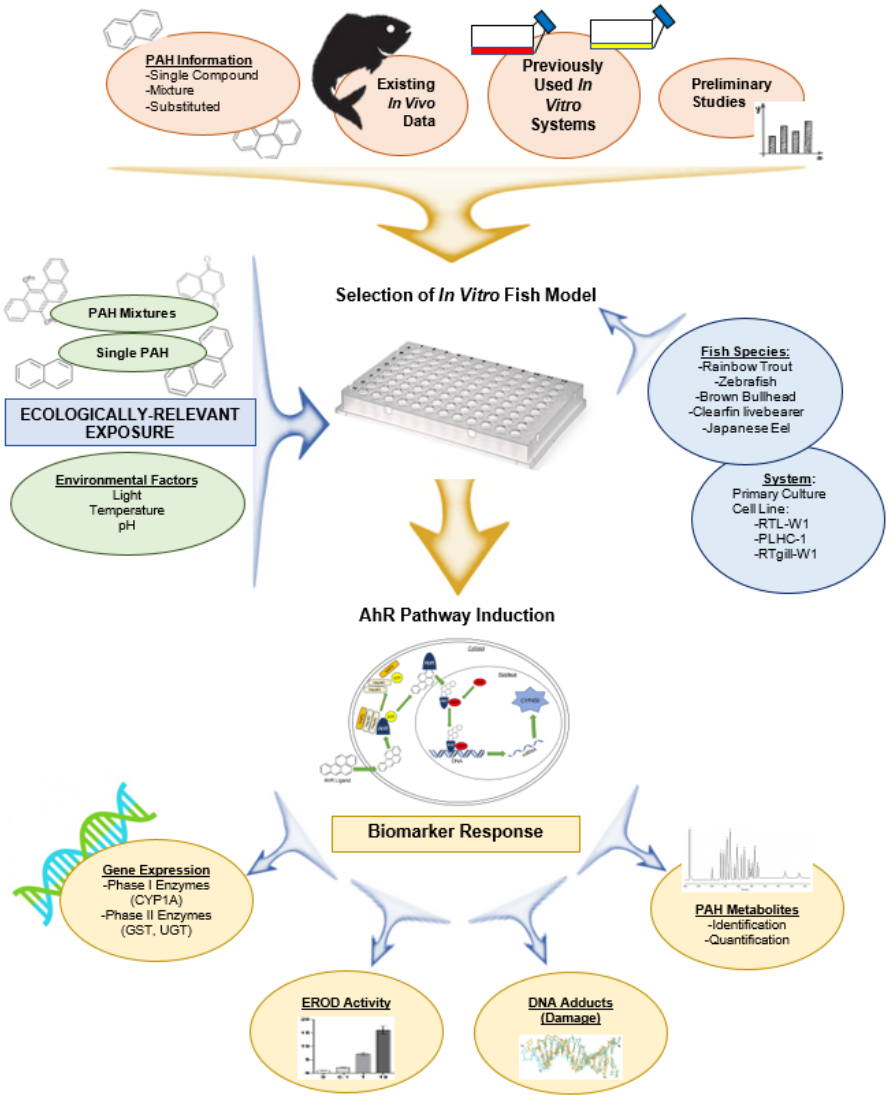

Figure 1.1. Conceptual model for the appropriate selection of *in vitro* fish models to evaluate biomarker responses. The model shows a framework for PAH biotransformation assessments, though it can be applied to other exposure assessments conducted *in vitro*. From Franco and Lavado (2019).

CHAPTER TWO

Reduced Biotransformation of Polycyclic Aromatic Hydrocarbons (PAHs) in Pollution-Adapted Gulf Killifish (*Fundulus grandis)*

Abstract

Anthropogenic pollution represents a significant source of selection, potentially leading to the emergence of evolutionary adaptations in chronically exposed organisms. A recent example of this scenario corresponds to Gulf killifish *(Fundulus grandis)* populations inhabiting the Houston Ship Channel (HSC), Texas, USA, which have been documented to have adapted to this heavily contaminated environment. Although not fully elucidated, one particularly important aspect of their adaptation involves the reduced inducibility of the aryl hydrocarbon receptor (AhR) and, potentially, the alteration of major biotransformation pathways. In the present study, we employed a modified Organization for Economic Cooperation and Development (OECD) 319-B test guideline to explore population and sex-related differences in the hepatic biotransformation of six polycyclic aromatic hydrocarbons (PAHs) in *F. grandis* populations with different exposure histories. Pollution-adapted *F. grandis* showed significantly lower hepatic clearance of PAHs than non-adapted fish, especially for high molecular weight PAHs (chrysene, benzo[k]fluoranthene, and benzo[a]pyrene), with pollution-adapted females presenting the lowest clearance. The characterization of different phase I biotransformation enzymes revealed that the basal activity of CYP1A,

fundamental in the biotransformation of PAHs, was significantly lower in pollution-adapted fish, especially in females, which showed the lowest activity. Contrarily, basal CYP2C9-like activity was significantly higher in pollution-adapted fish. These results demonstrate the importance of exposure and evolutionary histories in shaping organisms' responses to pollution and provide significant evidence of sex-specific biotransformation differences in *F. grandis* populations.

Introduction

Industrial pollution has been described as one of the most frequent human-driven forces that introduces selective pressure and adverse effects on different ecological aspects of aquatic ecosystems (Jayasundara et al., 2017; Oziolor et al., 2019). These forces shape physiological factors, such as modified biochemical pathways, in response to chronic exposure to different classes of contaminants. Classic examples of pollution-driven selection in the United States include several populations of Atlantic killifish (*Fundulus heteroclitus*) (Nacci, Champlin, & Jayaraman, 2010; Reid et al., 2016), and more recently, Gulf killifish (*Fundulus grandis*) populations inhabiting the Houston Ship Channel (HSC) and Galveston Bay, TX (Oziolor, Bigorgne, Aguilar, Usenko, & Matson, 2014; Oziolor et al., 2019). Point and non-point sources of pollution have introduced industrial contaminants from which polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), and polychlorinated dibenzo-dioxins and -furans (PCDD/Fs) are highly abundant. Due to their persistence, bioaccumulative, and toxic properties, aquatic communities in the HSC have suffered from chronic exposure. Thus, aquatic organisms inhabiting these areas are important for ecotoxicological studies addressing the potential for adaptation to such extreme conditions (Oziolor et al., 2018).

In the last decade, extensive research focusing on *F. grandis* inhabiting the HSC has suggested that these populations display reduced inducibility of the aryl hydrocarbon receptor (AhR) pathway (Oziolor et al., 2019) and the potential downregulation of enzymes involved in xenobiotic biotransformation. However, the precise biochemical mechanisms for these observations remain unclear (Reid et al., 2016). In comparison to *F. grandis* populations living in less contaminated areas and with minimal anthropogenic activity, *F. grandis* from the HSC do not show a significant magnitude of adverse effects from exposure to AhR agonists (e.g. PCBs and PAHs), such as cardiac teratogenesis and embryotoxicity (Oziolor, Dubansky, Burggren, & Matson, 2016). These observations have led to classify these populations as pollution-adapted. This proposed adaptation has significant implications for the bioaccumulation potential of industrial, organic chemicals to which *F. grandis* are chronically exposed. Despite being from the same species, *F. grandis* populations in the HSC may not respond in the same manner as populations living in less impacted areas, and assessments of risk in non-adapted populations may not reflect the same responses. In this context, the applicability of rapid and informative methodologies, such as substrate depletion bioassays, provide a significant advantage to explore factors related to absorption, distribution, metabolism, and excretion (ADME) of chemicals. Indeed, evaluating ADME in fish populations displaying biochemical alterations in response to selective pressure could depict important physiological responses that have not been previously characterized.

In vitro biotransformation approaches have been fundamental in supporting bioaccumulation assessments and describing different aspects of ADME of different environmental pollutants. Besides these methods being reliable for the measurement of

biotransformation and clearance for the regulation of chemicals (J. Nichols et al., 2018), they also provide an excellent approach for other exposure and toxicological assessments and to evaluate potential adverse effects in organisms simultaneously exposed to different pollutants (Nichols, Ladd, Hoffman, & Fitzsimmons, 2019). One of the most commonly applied methodologies for biotransformation assessments has been the determination of *in vitro* intrinsic clearance using liver S9 subcellular fractions or cryopreserved hepatocytes (when available) (Fay et al., 2015; Johanning et al., 2012). Indeed, post-mitochondrial S9 fractions, including the microsomal and cytosolic components of cells, contain several phase I and II biotransformation enzymes and have been advantageous in biotransformation and bioaccumulation studies (Embry et al., 2010). However, for assessments focusing on fish, standardization of this approach only exists for one species, rainbow trout (*Oncorhynchus mykiss*) (OECD, 2018b). Furthermore, measurements of kinetic parameters (Nichols, Ladd, & Fitzsimmons, 2018), the establishment of bioconcentration and bioaccumulation models (Laue et al., 2020), and extrahepatic biotransformation models (Saunders, Fitzsimmons, Nichols, & Gobas, 2020) have also focused on this single species.

Recently, Grimard et al. (2020) reported an *in vitro-in vivo* extrapolation (IVIVE) model for uptake and biotransformation of benzo[a]pyrene in the fathead minnow, providing a major step in the description of biotransformation and applicability of the S9 *in vitro* clearance methodology for another fish species. However, the environmental and evolutionary history of model species are factors not often considered in the development of biotransformation and bioaccumulation models, despite the need to acknowledge evolution-driven characteristics of the species of interest during evaluations of risk.

Considering current and potential future exposure does not likely provide a comprehensive analysis of risk, as previous events can significantly alter population responses to environmental stressors.

In the present study, we adapted the OECD 319B methodology for estimating hepatic intrinsic clearance using liver S9 subcellular fractions derived from male and female Gulf killifish. We evaluated phase I biotransformation rates of six PAHs classified as priority pollutants by the US Environmental Protection Agency (EPA). Furthermore, we characterized the basal activity of four different phase I biotransformation enzymes in both adapted and non-adapted *F. grandis*, under the rationale that reduced inducibility of the AhR pathway would result in reduced activity of AhR-dependent enzymes, such as CYP1A, and the likelihood of alterations to other CYP isoforms to support biotransformation processes. This study provides evidence of the role of environmental and evolutionary history in shaping biochemical processes and physiological responses associated with the biotransformation of chemicals in fish and presents evidence of differential responses between male and female fish. Furthermore, the present study highlights another domain of applicability for *in vitro* bioassays using S9 sub-cellular fractions.

Materials and Methods

Chemicals and Reagents

Phenanthrene (PHE), pyrene (PYR), chrysene (CHR), and benzo[a]pyrene (BaP) were obtained from Sigma-Aldrich (St. Louis, MO); fluoranthene (FLU), and benzo[k]fluoranthene (BkF) were from AccuStandard (New Haven, CT), and BaP-D12

(97%) was obtained from Cambridge Isotope Laboratories, Inc. (Andover, MA). Resorufin sodium salt, 7-ethoxyresorufin (7-ER), 7-pentoxyresorufin (7-PR), 7-methoxy-4-(trifluoromethyl)coumarin (MFC), and 7-benzyloxy-4-(trifluoromethyl)coumarin (BFC) were obtained from Sigma-Aldrich (St. Louis, MO). 7-Hydroxy-4-(trifluoromethyl)coumarin was from AAT Bioquest (Sunnyvale, CA). Reduced β-nicotinamide adenine dinucleotide 2'-phosphate, tetrasodium salt (NADPH; 96%) was obtained from ACROS Organics (Fair Lawn, NJ). Acetone and acetonitrile were obtained from Fisher Scientific (Pittsburgh, PA) and were of HPLC-grade.

Fish Collection and Care

Pollution-adapted and reference, non-adapted Gulf killifish were collected from Vince Bayou, along the HSC (29°43'03" N, 95°13'00" W), and Smith Point, in Galveston Bay (29°32'02" N, 94°45'58" W), Texas, respectively, following previous assessments of these areas and populations (Oziolor et al., 2019). All fish used in experimentation were collected during June 2018 and between August – October 2020. Baited minnow traps were deployed for ~5 h prior to their removal from the water. Individuals were immediately transferred to aerated coolers for transport to the laboratory, where they were maintained in 114 – 151 L glass tanks at ~10 ppt salinity and 23.0 – 25.0 °C, with a 14:10 h light cycle. Fish were fed TetraMin® Tropical Fish flakes and Purina AquaMax Fry Starter 200 pellets twice a day. Fish were maintained in the laboratory for > 5 months prior to initiation of experiments and to ensure depuration of compounds from the field.

S9 Subcellular Fractions

S9 fractions were isolated from a total of 18 fish from each population and combined in four different S9 pools as follows: one pool consisted of three males, another included five males, and two pools consisted of five females each. Female fish were all non-gravid, considering previous reports suggesting that egg bearing females may display reduced biotransformation ability (Hansson et al., 2006). All fish were processed individually following standard S9 preparation guidelines (Johanning et al., 2012; OECD, 2018a). Briefly, each individual was desensitized by immersion in ice-cold water (4-5 °C) for approximately 5 minutes. Individuals were then measured for length and weight (Table A2.1) and dissected in accordance with an approved Institutional Animal Care and Use Committee (IACUC) protocol at Baylor University. The liver was located and perfused through the hepatic vein with ~30 mL of ice-cold potassium phosphate buffer (100 mM, pH = 7.8), excised, and weighed. The livers from each fish pool were placed in a petri dish with two volumes of homogenization buffer, minced, and transferred to 50 mL conical tubes for homogenization prior to centrifugation at 13,000 *g* for 20 minutes at 4 °C. The supernatant was then transferred in aliquots of 0.5 mL to 1.5 mL cryogenic vials and stored at -80 °C until assays were performed. S9 subcellular fractions were also subject to protein quantification via the colorimetric Coomassie blue (Bradford) assay and by using bovine serum albumin (BSA) as a standard (Bradford, 1976).

Characterization of Phase I Biotransformation Enzymes

Phase I enzymatic activity was measured for four different CYP isoforms (CYP1A, CYP2B, CYP2C9, and CYP3A4) in liver S9 fractions via standard fluorescence

bioassays. The determination of CYP1A activity was done via the ethoxyresorufin-*O*-dealkylase (EROD) activity bioassay, measuring the dealkylation of 7-ethoxy resorufin. Similarly, the pentoxyresorufin-*O*-dealkylase (PROD) activity bioassay was used to quantify CYP2B-like activity. Both EROD and PROD bioassays were conducted at substrate concentrations of 1.0 μM, selected based on the lowest concentration that produced detectable activity on preliminary experimentation. CYP2C9-like and CYP3A4-like activities were measured via the dealkylation of 7-methoxy-4-trifluorocoumarin (MFC) and of 7-benzyloxy-4-trifluoromethylcoumarin (BFC), respectively. MFC and BFC dealkylation were conducted at a substrate concentration of 100 μM, selected based on previous studies and preliminary experimentation (Smith & Wilson, 2010). All enzyme activity bioassays were conducted in eight technical replicates for each S9 pool, at a S9 protein concentration of 1 mg/mL and at 24 °C. Enzyme activities were measured kinetically by fluorescence spectroscopy (Synergy™ H1, BioTek, VT).

Substrate Depletion Bioassays

A modified version of the OECD TG 319-B was employed for the measurement of phase I *in vitro* intrinsic hepatic clearance in adapted and non-adapted, male and female *F. grandis*. The modifications consisted in the use of NADPH but not the rest of cofactors usually employed for full biotransformation (phase I and II) assays (e.g., UDPGA, GSH, and PAPS), following the hypothesis of reduced AhR inducibility and subsequent reduction of CYP1A activity in adapted fish. Additionally, depletion bioassays were conducted at 25°C for *F. grandis*, contrary to the 12°C suggested for rainbow trout in the OECD 319B TG. The depletion of the six PAHs was conducted in 1

mL reactions composed of 1 mg/mL liver S9 protein, 100 mM potassium phosphate (K-PO_4) buffer at pH 7.8, and the cofactor β-nicotinamide adenine dinucleotide 2'-phosphate (NADPH; 2.0 mM). After a 10 min pre-incubation period, reactions were started by the addition of the test PAHs using acetone as the carrier (0.5% [v/v] final concentration). Initial PAH concentrations in depletion assays are shown in Table A2.2. In preliminary experimentation, PAH concentrations ten times lower than the selected ones for the present study presented values below limits of detection, mainly due to the rapid depletion, and were below K_m. Contrarily, first-order Michaelis-Menten kinetics and K_m constants were observed at the PAH concentrations reported in this study. Thus, these concentrations were selected for subsequent depletion assays. For each S9 pool, depletion assays for all PAHs were performed in triplicate and run simultaneously with depletion assays using denatured S9 protein.

All reactions were conducted for 10 min, with 100 µL aliquots taken at 2, 4, 6, 8, and 10 min. Reactions were terminated by placing aliquots in 400 µL of a chilled stopping solution containing 5.0 nM benzo[a]pyrene-D12, used as internal standard, in acetonitrile. The samples were then placed on a vortex mixer followed by centrifugation at 2000 *g* for 10 min at 4 °C. Approximately 300 µL of the supernatant were then transferred to 1.5 mL autosampler vials.

Instrumental Analyses

Samples were analyzed by high-performance liquid chromatography (HPLC) coupled with a RS fluorescence detector (UltiMate 3000, Thermo Fisher Scientific, Waltham, MA). A Kinetex® C-18 LC column (1.7 µm, 150 x 2.1 mm; Phenomenex, Torrance, CA) was used for chromatographic separation. The selected solvent (80%

acetonitrile and 20% MQ water) was maintained at an isocratic flow rate of 0.5 mL/min and at a temperature of 40 °C. Excitation and emission wavelengths (nm), and limits of detection (LOD) for each PAH are shown in Table A2.2.

In Vitro Hepatic Intrinsic Clearance Calculation

Measured concentrations (C) from substrate depletion bioassays were Log_{10}-transformed and plotted against time (t; h). The slopes of the linear equations were then multiplied by -2.3 to determine the first-order depletion rate constant (k_{DEP}; 1/h) for each PAH. To calculate intrinsic clearance ($CL_{INT,S9}$; mL/h/mg protein)), k_{DEP} values were divided by the concentration of S9 protein in the depletion assays (C_{S9}, 1 mg/mL).

Statistical Analyses

Differences between fractional liver weights and condition factors from fish used in the preparation of S9 fractions, basal activities of all phase I enzymes, as well as the $CL_{IN\ VITRO}$ of each PAH, were determined between adapted and non-adapted *F. grandis* populations, and between male and females using two-sample *t*-tests. Furthermore, two-way analysis of variance (ANOVA) followed by Tukey post-hoc tests were employed to describe differences in basal enzyme activity and $CL_{IN\ VITRO}$ from the population-sex interaction. For the analyses of enzyme activity, the two S9 pools for each group (population * sex) were analyzed in 7-8 replicates each, and these replicates were then combined for statistical analyses. $CL_{IN\ VITRO}$ was analyzed using the resulting measurements of triplicate experiments for each S9 pool, for a total of 6 measurements when the data from the two S9 pools for each group were combined. Significance was

determined at an $\alpha = 0.05$, unless otherwise stated. The statistical output for enzyme activity and $CL_{IN\ VITRO}$ comparisons are shown in tables A2.3 and A2.4, respectively.

Linear regression analyses were used to determine the log-linearity of substrate depletion over time and to corroborate that the chemical concentrations followed first-order kinetics. A regression analysis was also employed to evaluate the relationship between *in vitro* hepatic clearance and log K_{ow} values of all PAHs tested. All statistical analyses were performed using the DBI and agricolae packages in R (version 3.5.1) and through the R-studio platform.

Results

Morphometrics of Fish Used in S9 Isolation

Measured lengths, weights, and liver weights of each individual used in the preparation of S9 sub-cellular fractions were used to determine fractional liver weights (g liver g $fish^{-1}$) and condition factors (K; Table A2.1). Neither fractional liver weights (p = 0.6341) nor K (p = 0.4753) were significantly different between pollution-adapted and non-adapted fish. Between males and females, fractional liver weights did not present differences (p = 0.2743), though the mean K for males (1.67 ± 0.07) was significantly higher (p = 0.0395) than for females (1.50 ± 0.04).

Phase I Biotransformation Enzymes

Basal CYP1A activity, as measured by the EROD bioassay, was significantly lower in pollution-adapted *F. grandis*, with pollution-adapted females showing the lowest activity ($p = 3.317e^{-5}$; Fig. 2.1A). Even though CYP2B-like activity in the PROD bioassay was higher in males than in females when populations were combined (p =

1.009e^{-5}, Fig. 2.1B), no significant differences were observed between adapted and non-adapted populations, nor from the population-sex interaction. Contrarily, although no sex-specific differences were observed, pollution-adapted fish showed significantly higher basal CYP2C9-like activity in the MFC bioassay compared to non-adapted fish (p = 0.0019; Fig. 2.1C). CYP3A4-like basal activities in the BFC bioassay did not present significant differences among *F. grandis* populations, sexes, nor from their interaction (Fig. 2.1D). CYP activities for each population and sex are shown in table A2.5.

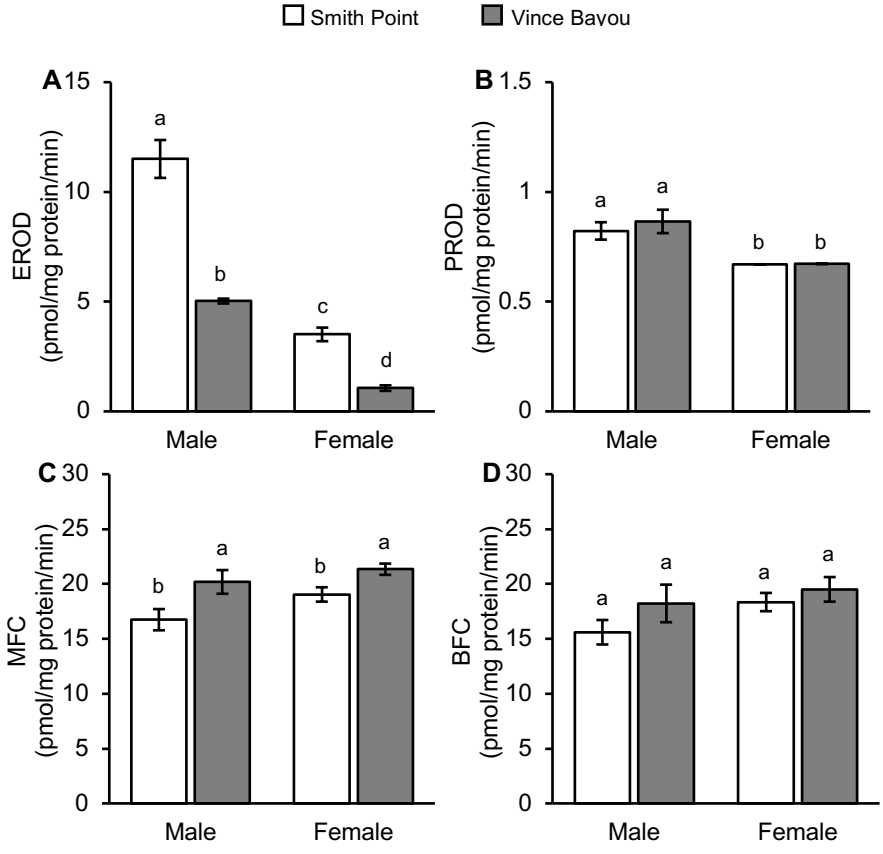

Figure 2.1. Basal A) CYP1A (EROD), B) CYP2B-like (PROD), CYP2C9-like (MFC), and D) CYP3A4-like activity in liver S9 fractions derived from non-adapted (Smith Point) and pollution-adapted (Vince Bayou) male and female *F. grandis*. Data are reported as mean ± SEM, with each bar representing combined data from two S9 pools with 7-8 technical replicates for each pool. Different lower-case letters denote significant differences ($p < 0.05$) from comparisons between populations and sexes.

PAH Depletion and Intrinsic Hepatic $CL_{IN\ VITRO}$

$CL_{IN\ VITRO}$ of the six evaluated PAHs were significantly lower for pollution-adapted *F. grandis*, with high molecular weight PAHs presenting the most significant differences among populations (Fig. 2.2, Table 2.6). In pollution-adapted fish, $CL_{IN\ VITRO}$ for these high molecular weight PAHs was ~2.6x, 2.2x, and 2.3x lower for CHR, BkF, and BaP, respectively, than the observed clearance for non-adapted *F. grandis*. In general, females from both populations presented the lowest $CL_{IN\ VITRO}$, relative to their male counterparts for all PAHs tested. Results from the substrate depletion bioassays for each PAH and from individual S9 pools are shown in Figures A2.3-2.8.

$CL_{IN\ VITRO}$ for PHE (Fig. 2.2A) was not significantly different between adapted and non-adapted fish, though males from both populations depleted PHE at a significantly faster rate than females ($p = 0.0015$). For PYR (Fig. 2.2B), pollution-adapted fish presented the lowest $CL_{IN\ VITRO}$, when considering both sexes ($p = 0.0032$). There were no significant differences in the depletion of PYR between sexes nor from the population-sex interaction. $CL_{IN\ VITRO}$ for FLU (Fig. 2.2C) presented significant differences among both populations and sexes; adapted females showed clearance approximately 2x, 1.2x, and 1.1x lower than non-adapted males, non-adapted females, and adapted males, respectively.

The most significant differences in $CL_{IN\ VITRO}$ occurred for CHR (Fig. 2.2D), which was, in general, depleted at faster rates than the other PAHs across both populations and sexes. Pollution-adapted fish depleted CHR approximately 2.5x slower than non-adapted fish ($p = 0.0003$), and males showed significantly higher $CL_{IN\ VITRO}$ than females ($p = 0.0019$). The population-sex interaction analysis revealed that

pollution-adapted females presented the lowest $CL_{IN\ VITRO}$, with values ~6.7x lower than the ones observed for non-adapted males, which were the ones with the highest $CL_{IN\ VITRO}$ for CHR.

Observations for both BkF (Fig. 2.2E) and BaP (Fig. 2.2F) were generally similar, even though their $CL_{IN\ VITRO}$ were lower than for CHR. For BkF, $CL_{IN\ VITRO}$ was lower for pollution-adapted fish ($p = 1.375e^{-5}$), with adapted females presenting rates of ~4.2x, 2.6x, and 2.1x lower than non-adapted males, non-adapted females, and adapted males, respectively. Similarly, $CL_{IN\ VITRO}$ for BaP was lower in females regardless of population, in pollution-adapted fish when sexes were combined, and also presented significant differences from the population-sex interaction analysis. Pollution-adapted females presented the lowest clearance among all groups, with $CL_{IN\ VITRO}$ being of ~4.0x, 2.1x, and 1.6x lower than non-adapted males, non-adapted

Table 2.6. *In vitro* hepatic clearance (mL/h/mg protein ± std. error) of six priority PAHs by non-adapted (Smith Point) and pollution-adapted (Vince Bayou) male and female *F. grandis.*

PAH	Smith Point $CL_{IN\ VITRO}$		Vince Bayou $CL_{IN\ VITRO}$	
	Males	**Females**	**Males**	**Females**
Phenanthrene	3.46 ± 0.34	1.90 ± 0.25	3.10 ± 0.61	1.77 ± 0.24
Pyrene	5.09 ± 0.51	3.84 ± 0.33	3.07 ± 0.25	3.11 ± 0.35
Fluoranthene	6.37 ± 0.54	3.46 ± 0.19	3.17 ± 0.38	2.96 ± 0.42
Chrysene	26.27 ± 2.30	12.79 ± 0.26	11.52 ± 1.08	3.91 ± 0.15
Benzo[k]fluoranthene	11.88 ± 0.65	7.51 ± 0.22	5.98 ± 0.69	2.86 ± 0.15
Benzo[a]pyrene	15.15 ± 1.17	8.11 ± 0.24	6.22 ± 0.48	3.82 ± 0.32

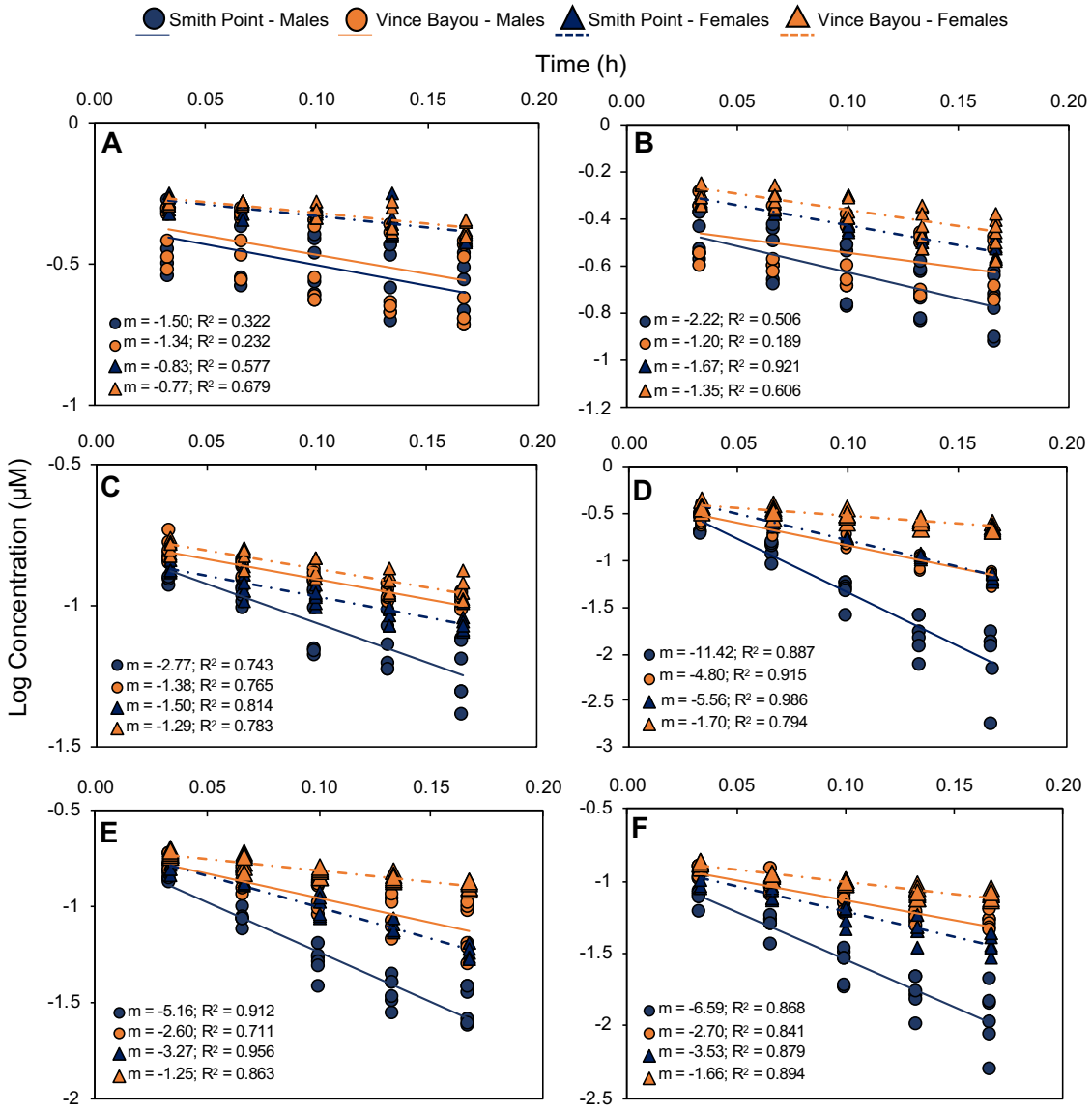

Figure 2.2. Phase I depletion of A) phenanthrene, B) pyrene, C) fluoranthene, D) chrysene, E) benzo[k]fluoranthene, and F) benzo[a]pyrene by liver S9 sub-cellular fractions of non-adapted (Smith Point) and pollution-adapted (Vince Bayou) male and female *F. grandis.* Data points correspond to individual Log_{10} concentrations resulting from triplicate experiments with each S9 fish pool.

PAHs Log K_{ow} vs $CL_{IN\ VITRO}$

A comparison between log K_{ow} values for each PAH tested and their $CL_{IN\ VITRO}$ for both populations and sexes showed that, in general, hepatic clearance increased for PAHs with higher log K_{ow} values (Fig. 2.9). Females from both populations presented the strongest correlation between Log K_{ow} and $CL_{IN\ VITRO}$; however, for males and females of both populations R^2 values ranged from 0.31 to 0.57.

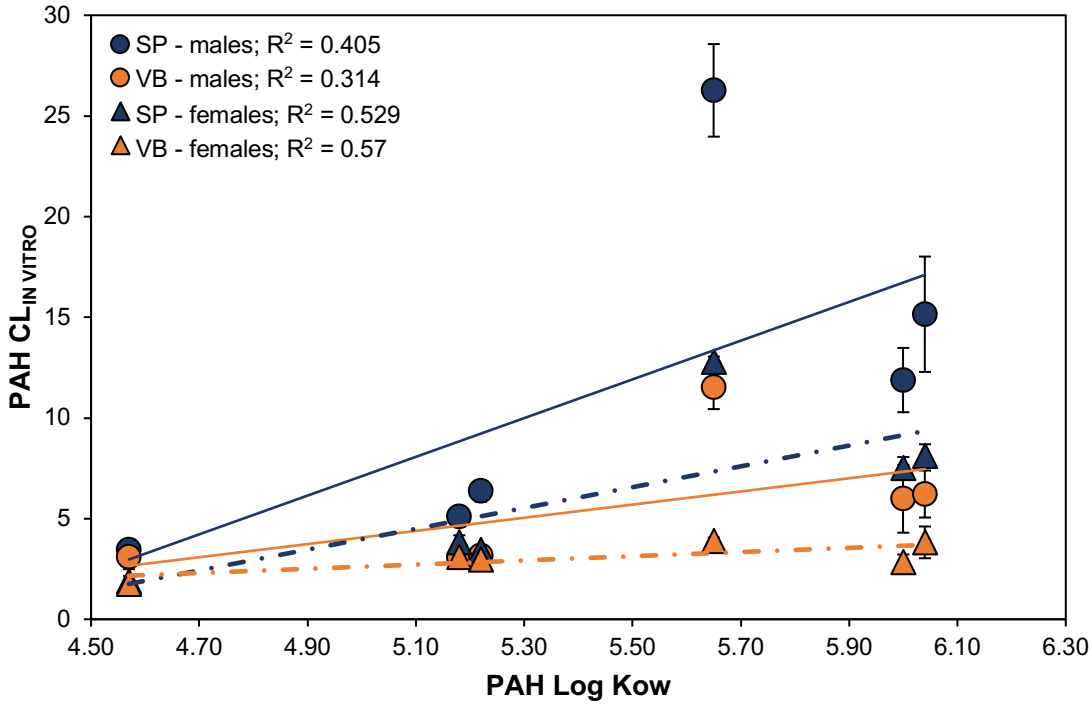

Figure 2.9. Relationship between Log K_{ow} of PAHs (Table A2.2) and their corresponding mean hepatic $CL_{IN\ VITRO}$ (mL/h/mg protein) for non-adapted (Smith Point; SP) and adapted (Vince Bayou; VB) male and female *F. grandis*. Data points correspond to mean $CL_{IN\ VITRO}$ for each population and sex ± SEM.

Discussion

Chronic exposure to different groups of pollutants, coupled with the non-migratory nature of *Fundulus grandis*, have acted as a driving mechanism of selection, allowing populations in the HSC to undergo evolutionary rescue and to be classified as pollution-adapted. This evolutionary adaptation was first documented by Oziolor et al. (2014), who described reduced CYP1A activity and resistance to cardiac teratogenesis in fish embryos. While the characterization of the mechanisms of adaptation shown by *F. grandis* has not been fully described, previous studies have suggested that this adaptation arises from reduced inducibility of the aryl hydrocarbon receptor (AhR) pathway, similar to the observations for the sister species *Fundulus heteroclitus* (Clark & Di Giulio, 2012; Clark, Matson, Jung, & Di Giulio, 2010). In accordance with these observations, the present study represents a step forward towards the description of biotransformation processes in adapted populations and, more specifically, into how these individuals process contaminants like PAHs in the HSC.

The selection of PAHs in the present study was based on the rationale that the estuarine ecosystems found in close connection with the HSC have been heavily polluted for more than five decades, as these areas contain a significant proportion of the oil refineries in the United States (Camargo et al., 2021; Howell, Rifai, & Koenig, 2011; Willett et al., 1997). Furthermore, PAH biotransformation in aquatic organisms has been investigated for many years, and while there are significant research gaps in the available literature (e.g., biotransformation of heterocyclic PAHs), the existing knowledge on this topic is rather extensive, allowing for thorough evaluations involving species that have not been evaluated in the past or that do not represent common models in ecotoxicology.

The reduced activity of CYP1A in pollution-adapted *F. grandis* shown in the present study provides strong evidence for the AhR pathway to be a main target of selection, as suggested in previous embryo studies (Oziolor et al., 2014; Oziolor et al., 2016). It is known that, upon the nuclear translocation of the PAH-AhR complex, upregulation of CYP1A enzymes takes place, which in turn target PAHs. However, with reduced inducibility of the AhR pathway, there is significant potential for the downregulation of major biotransformation enzymes from the cytochrome P450 complex, mainly from the CYP1A family. In the absence of significant CYP1A activity, an increased time period for PAH excretion may occur, followed by an increased potential for bioaccumulation if clearance rates are significantly low. Additionally, under normal conditions, CYP1A-mediated hydroxylation during phase I biotransformation would lead to the formation of reactive metabolites, which have the ability to interact with major biomolecules (Franco & Lavado, 2019; Pampanin et al., 2016; Zhou, Wang, Yang, & Liu, 2010) and cause toxicity. Nonetheless, pollution-adapted populations may experience reduced magnitude and frequency of adverse effects (e.g., DNA adducts and oxidative stress) due to the absence of active metabolites. Based on the observations from the present study regarding the low CYP1A-mediated clearance occurring in pollution-adapted populations, it is possible for chemical compounds to remain accumulated for extended time, while minimizing key molecular events associated with PAH metabolite toxicity.

It is also pertinent to establish that CYP1A enzymes, while often considered in charge of PAH biotransformation, are not the only CYP isoforms with the ability to process these compounds. For example, previous experimentation has shown elevated

activity of CYP3A in response to benzo[a]pyrene and fluoranthene exposure (Oziolor, Carey, & Matson, 2017). Despite the observations in the present study showing measurable CYP3A4-like activity, this did not present differences between adapted and non-adapted populations, nor between males and females. However, the significantly higher CYP2B-like activity in male fish compared to females may indicate a potential role of this isoform in supporting biotransformation of certain compounds, such as PHE, which presented higher biotransformation in male fish. A major highlight of the present study is the significant CYP2C9-like activity in pollution-adapted fish, which may also support phase I biotransformation of PAHs in the absence of CYP1A. Compensatory mechanisms have been previously described in fish (Ankley et al., 2007), where the upregulation of different CYP isoforms occurred in response to the inhibition of other CYP enzymes in the presence of a fungicide. Although possible, the information presented in our study does not provide sufficient evidence to conclude that CYP-related compensatory mechanisms took place in pollution-adapted fish. Therefore, the role of different CYP isoforms, potential modifications to metabolite profiles, and the activity of metabolites in driving PAH toxicity represents an active area of research.

In the present study, it is fundamental to highlight the evolutionary adaptation suggested for *F. grandis*, and how a potential reduction of AhR pathway inducibility and CYP1A biotransformation could represent a selective advantage for pollution-adapted populations. These alterations to the physiology of adapted fish populations may have significant implications regarding PAH toxicity. PAH metabolites are known genotoxic chemicals, with the ability to cause direct damage to nucleic acids, usually by the formation of adducts (Pampanin et al., 2017; Pampanin et al., 2016), and to cause

subsequent mutagenicity and teratogenicity (Clark et al., 2010; Oziolor et al., 2014). In this scenario, it could be suggested that reduced PAH biotransformation may result in less toxicity. However, while CYP1A expression and subsequent activity often result from the induction of the AhR pathway, some adverse effects may only be associated with the AhR but not necessarily with CYP activity. For example, in *F. heteroclitus*, Clark et al. (2010) described that embryonic cardiac malformations were primarily mediated by AhR2, one of the two AhR pathways described in *Fundulus*, yet the observed teratogenesis, or lack thereof, was independent of CYP1A biotransformation. When CYP1A is inhibited but the AhR pathway is active, previous studies have also shown elevated PAH embryotoxicity (Wills, Zhu, Willett, & Di Giulio, 2009), which demonstrates that some PAH-driven adverse effects are likely not associated with CYP1A-activated metabolites following the induction of AhR pathways.

In terms of the differences in hepatic clearance for high molecular weight PAHs, our observations are in line with the literature, suggesting that, while low molecular weight PAHs undergo biotransformation to some degree, high molecular weight PAHs are associated with significantly higher phase I biotransformation activity (Varanasi, Stein, & Nishimoto, 1989). Similarly, the hydrophobicity of PAHs plays a fundamental role in increasing their biotransformation. Thus, PAHs that are more hydrophobic usually have shorter half-lives due to rapid excretion by phase I and II biotransformation (De Maagd & Vethaak, 1998). In the present study, PAHs with relatively high Log K_{ow} also showed the highest hepatic clearance, with this being evident for both populations and sexes. Nonetheless, in the case of adapted *F. grandis*, hepatic clearance of high molecular weight PAHs was rather low, not only suggesting lower biotransformation ability but also

an elevated potential for these fish to increase PAH body burdens. In such a scenario, the bioaccumulation of PAHs in pollution-adapted fish represents a factor of concern, as it has been shown that *F. grandis* in the HSC have significant bioaccumulation of PCBs (Oziolor et al., 2018). Nonetheless, it is important to point out that PCBs do not undergo biotransformation and that, for PAHs, this possibility must be further investigated.

When exploring PAH biotransformation at the level of enzyme-substrate interaction, previous reports have also suggested that the PAH binding site in CYP1A is of hydrophobic nature, providing a high affinity for compounds with higher partitioning coefficients (De Maagd & Vethaak, 1998). Therefore, the affinity of more polar PAHs could be a rate-limiting step for PAH biotransformation since, based on our observations and those of previous studies (J. W. Nichols et al., 2018; Nichols et al., 2019), PAHs with 4 or more aromatic rings are more rapidly biotransformed than PAHs with 3 or fewer rings. Furthermore, the molecular structure of PAHs also influences the degree of biotransformation. In PAHs, different "molecular regions" have been established, including the "K", "bay", and "L" regions. For biotransformation, the "bay" region has been highly associated with the production of active metabolites and PAH mutagenic capabilities (Ramesh et al., 2004). It is also likely that PAHs displaying the "bay" region have a higher affinity for CYP enzymes, facilitating their biotransformation. In the present study, CHR, BkF, and BaP, all displaying "bay" regions, presented the highest hepatic clearance rates in both adapted and non-adapted *F. grandis*. CHR is of special interest, as this PAH displays two "bay" regions and showed significantly higher hepatic clearance in male and female *F. grandis* from both pollution-adapted and non-adapted populations, even with reduced CYP1A activity.

Despite PYR having relatively higher clearance in non-adapted fish, it is unclear why this 4-ring PAH did not present elevated hepatic clearance as shown with other high molecular weight PAHs. Given that previous studies employing rainbow trout S9 substrate depletion bioassays have reported significant *in vitro* clearance of PYR (Nichols, Hoffman, ter Laak, & Fitzsimmons, 2013), the absence of significant PYR biotransformation could be directly related to *F. grandis*. In addition, the observed hepatic clearance for FLU in both adapted and non-adapted populations is of special interest, as previous studies have pointed out the ability of FLU to reduce CYP1A activity as a non-competitive inhibitor (Willett, Wassenberg, Lienesch, Reichert, & Di Giulio, 2001). In this context, it is possible that other CYP isoforms are responsible for FLU biotransformation, given the potential inhibition of CYP1A by this PAH. However, as pointed out earlier, mechanisms of CYP interaction and compensation deserve further analysis.

Besides the significant reduction of biotransformation rates shown for pollution-adapted *F. grandis*, the present study also describes significantly lower biotransformation ability by female fish, with the lowest PAH clearance occurring in pollution-adapted females. In general, female fish of different species have been shown to modify their biotransformation ability depending on different stages of their reproduction cycle (Figueiredo-Fernandes, Fontainhas-Fernandes, Rocha, & Reis-Henriques, 2006; Hansson et al., 2006; Solé, Raldua, Barcelo, & Porte, 2003). For example, Hansson et al. (2006) reported that CYP1A-like activity in several fish species is the highest in non-gravid females and in females that have recently spawned. Contrarily, females bearing eggs display the lowest biotransformation activity. The female individuals used in the

preparation of S9 sub-cellular fractions were all non-gravid, suggesting minimal interference of their reproductive cycle in their biotransformation ability. Based on these observations, it appears that female *F. grandis* do indeed present lower biotransformation ability than males, regardless of their reproductive cycle. Therefore, the potential biological effects that pollution-adapted males could experience from reduced biotransformation could be even worse in females, leading to significant population and ecological changes arising from the inability of these fish to process pollutants. If the observed reduction in biotransformation translates into higher body burdens, it is also possible that accumulated pollutants could be transferred to developing eggs when females become gravid, as it has been shown in other fish species (Cazan & Klerks, 2014; Chen et al., 2016), though the specific implications from reduced biotransformation in female *F. grandis* must be further explored.

As shown in the present study, the significance of our observations relies on improving our understanding of how differential biotransformation of xenobiotics influences the adaptability of *F. grandis* to cope with elevated exposure to pollutants in the HSC. Furthermore, continuing to study these *F. grandis* population could elucidate how other populations inhabiting heavily polluted environments and experiencing strong selective pressure modify important biochemical and physiological processes to maintain ecological fitness. In this study, we provide significant evidence illustrating the importance of exposure history in shaping organisms' responses to xenobiotic exposure. Populations that have been chronically exposed to pollution and that seem to be adapted to such conditions are of relevance as they provide the baseline to investigate biochemical and physiological mechanisms driving such adaptation. Comparative studies

that consider population- and sex-specific differences regarding the ability to metabolize chemicals may lead to novel discoveries of the mechanisms used by organisms to cope with chronic exposure to different environmental pollutants.

Further, while *in vivo* toxicity tests using fish models have been important tools in assessing the effects of environmental contaminants on living organisms (Powers, 1989) and in conducting effective extrapolations to the natural environment, the use of animals comes with some ethical and animal welfare issues (Fent, 2001; Thibaut, Schnell, & Porte, 2009). Thus, efforts have been devoted to developing novel approaches that integrate different testing strategies, such as *in vitro* bioassays supported by robust analytical chemistry approaches. Although the OECD 319-B test guideline we employed is a standardized methodology established for rainbow trout, primarily for evaluating the bioaccumulation potential of chemicals, we demonstrated that this method is still robust for other species and that modifications to this approach are advantageous to explore biological processes that may otherwise be only possible through complex field and *in vivo* laboratory studies. In retrospect, however, it is important to point out that a full characterization of the biotransformation-related adaptation suggested for *F. grandis* may indeed require the establishment of large-scale *in vivo* studies, which represents a path forward in our research efforts. In such case, the integration of different lines of evidence (e.g. *in vitro* and *in vivo* systems) to investigate important aspects of adaptation to pollution and sex-specific responses, is promising to advance our ability to identify and predict potential evolutionary adaptations of populations living in polluted ecosystems.

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CHAPTER THREE

In Vitro-In Vivo Biotransformation and Phase I Metabolite Profiling of Benzo[*a*]pyrene in Wild Gulf Killifish (*Fundulus grandis*) Populations with Different Exposure History

Abstract

Chronic exposure to pollution may allow populations to display evolutionary adaptations associated with cellular and physiological mechanisms of defense against xenobiotics. This could lead to differences in the way organisms of the same species but inhabiting different areas cope with chemical exposure. In the present study, we explore two wild Gulf killifish (*Fundulus grandis*) populations with different exposure history for potential differences in the biotransformation of benzo[*a*]pyrene (BaP), and conduct a comparative evaluation of *in vitro* and *in vivo* approaches to describe the applicability of new approach methodologies (NAMs) for biotransformation assessments. Pollution-adapted and non-adapted *F. grandis* were subject to intraperitoneal (IP) injections of BaP in time-dependent exposures, prior to measurements of CYP biotransformation activity, BaP liver concentrations, and the identification and quantification of phase I metabolites. Additionally, substrate depletion bioassays using liver S9 fractions were employed for measurements of intrinsic hepatic clearance and to evaluate the production of metabolites *in vitro*. Pollution-adapted *F. grandis*, suggested to have reduced inducibility of the aryl hydrocarbon receptor (AhR) pathway, presented significantly low CYP1A activity and intrinsic clearance rates that were 3 to 4 times lower than non-adapted fish. Furthermore, the metabolite profiling of BaP indicated that, while the metabolite 1-hydroxy-benzo[*a*]pyrene was identified in both populations and from the *in vitro* and *in vivo*

approaches, the metabolites 9-hydroxy-benzo[*a*]pyrene and benzo[*a*]pyrene-4,5-dihydrodiol, only identified through the *in vivo* approach, presented higher concentrations in the bile of pollution-adapted fish relative to non-adapted individuals. These observations further the understanding of the evolutionary adaptation of *F. grandis* inhabiting heavily polluted environments in the Houston Ship Channel, TX, USA, and highlight the need of considering the evolutionary history of species and populations of interest during the implementation of NAMs and in conducting ecological risk assessments.

Introduction

The cellular and molecular pathways involved in the biotransformation of chemical compounds correspond to one of an organism's major mechanisms of defense against xenobiotics, and represent a major component of the chemical defensome (Eide et al., 2021; Goldstone et al., 2006). It is well-established that the way organisms cope with chemical exposure is largely dependent on their ability to process, detoxify, and excrete compounds (Eide et al., 2021). However, biotransformation processes can also lead to the formation of active metabolites, often with a better ability to interact with important biomolecules and resulting in adverse outcomes (Schlenk et al., 2008). A well-known example of this scenario is the biotransformation of polycyclic aromatic hydrocarbons (PAHs) (Franco & Lavado, 2019; Santana et al., 2018). These legacy compounds are ubiquitous in the aquatic environment and their impact on aquatic organisms has been continuously studied due to their ability to compromise animal function at different levels of biological organization.

Significant advances have occurred in the last decade, where the development of new approach methodologies (NAMs), such as the use of *in vitro* bioassays have facilitated the description of important biotransformation pathways in fish. Additionally, while *in vitro-to-in vivo* extrapolation (IVIVE) continues to be the most significant challenge when implementing NAMs, recent experimentation has provided valuable information (e.g. advanced toxicokinetic models) that facilitate extrapolation (Laue et al., 2020; Mangold-Doring et al., 2021; Nichols, Fitzsimmons, & Burkhard, 2006; Stadnicka-Michalak, Tanneberger, Schirmer, & Ashauer, 2014). Unfortunately, a significant proportion of these efforts have focused on a reduced number of species (e.g. rainbow trout) and non-wild populations, reducing their environmental relevance. Indeed, fish populations in the wild may experience different biological impact resulting from a variety of exposure scenarios, leading to potential modifications in the expression of the chemical defensome and the emergence of intraspecific differences that could modify the way organisms process contaminants. Previous studies have pointed out that environmental factors, such as temperature and salinity, and anthropogenic impacts like pollution can affect biotransformation processes (Cerveny et al., 2021; Lavado, Maryoung, & Schlenk, 2011; Strobel, Burkhard-Holm, Schmid, & Segner, 2015). These observations highlight the importance of habitat characteristics as major influences of species sensitivity and overall responses to contaminant exposure resulting from potential adaptations.

In the United States, two important examples of wild populations that have modified important physiological processes to cope with pollution include the Atlantic killifish (*Fundulus heteroclitus*) from the Atlantic Wood Superfund Site on the Elizabeth

River, VA, USA (Clark & Di Giulio, 2012) and, more recently, the Gulf killifish (*Fundulus grandis*) inhabiting the Houston Ship Channel (HSC), TX, USA (Oziolor et al., 2019). The estuarine ecosystems found in close connection with the HSC have been heavily polluted for more than five decades, as these areas contain approximately 40% of the oil refineries in the country (Howell, Rifai, & Koenig, 2011). The U.S. Environmental Protection Agency (EPA) has reported elevated concentrations of industrial pollutants in sediments, including PAHs at concentrations close to 50 mg/kg, making some areas in the HSC part of the Superfund National Priority List (NPL) (Anchor, 2010). Furthermore, due to the close connection of the HSC to the Gulf of Mexico, natural phenomena (e.g. hurricanes and tropical storms) have been documented to redistribute PAHs (Camargo et al., 2021), increasing potential exposure to aquatic organisms.

For chronically exposed *Fundulus*, it has been suggested that their adaptation to pollution is partially mediated by a reduced inducibility of the aryl hydrocarbon receptor (Ahr) pathway and a subsequent downregulation of biotransformation enzymes, particularly CYP1A isoforms. In this context, and to comparatively assess potential Ahr-mediated biotransformation differences between *F. grandis* populations inhabiting areas with different levels of pollution, PAHs are of particular interest due to their well-documented ability to induce the Ahr pathway and the molecular transformation that they undergo in preparation for excretion. It is well-established that, upon absorption and induction of the Ahr pathway, several PAHs undergo phase I biotransformation mediated by enzymes from cytochrome P450 (CYP450) system, mainly CYP1A (Gelboin, 1980). Phase I biotransformation leads to the production of active metabolites, including phenols, diones and dihydrodiols, and these are often responsible for the adverse

biological effects resulting from their interaction with major macromolecules (e.g. nucleic acids) (Straif et al., 2005). In this context, there is logical reasoning to evaluate biotransformation in *F. grandis* populations with different exposure histories, through the integration of *in vitro*, *in vivo*, and analytical assessments, as this is key in understanding the mechanisms by which these populations cope with extreme pollution.

In this study, we take advantage of previously characterized *F. grandis* populations with different exposure and evolutionary histories (Oziolor et al., 2019) to 1) explore differential biotransformation sensitivity of these populations after exposure to the "prototypical" PAH benzo[*a*]pyrene (BaP), and through the quantification of four CYP450 isoforms, intrinsic hepatic clearance, and the production of BaP phase I metabolites, and 2) compare and contrast the selected end-points from *in vitro* bioassays and time-dependent *in vivo* experimentation in the context of *F. grandis* biotransformation responses. It is important to establish that this study does not correspond to an *in vitro-in vivo* extrapolation effort, but rather to a comparison of the resulting outcomes derived from *in vitro* assays and whole-animal assessments. The present study aims to contribute to the current understanding of the advantages and limitations of NAMs and their applicability to represent actual *in vivo* responses for biotransformation assessments. Additionally, this study provides further insight into the adaptation of *F. grandis* populations inhabiting heavily polluted ecosystems and provide a baseline for further experimentation with other industrial pollutants and organisms experiencing chronic exposure and the potential for evolutionary adaptation.

Materials and Methods

In Situ Processing of Collected Fundulus grandis

Pollution-adapted *F. grandis* were collected from Vince Bayou, along the HSC (29°43'03" N, 95°13'00" W), whereas non-adapted fish, used as reference, were collected from Smith Point, in Galveston Bay (29°32'02" N, 94°45'58" W), Texas. Further information about animal collection and maintenance is included as supplemental material (Appendix B). For *in situ* processing, fish were removed from traps and placed in an ethyl-3-aminobenzoate methanesulfonate (MS-222) solution prior to morphometric measurements (length and weight), dissection, and excision of the liver. Whole livers were placed in 1.5 mL cryovials and flash-frozen in liquid nitrogen. Fish carcasses were placed in whirl-pack bags and transported in ice.

S9 Substrate Depletion Bioassays

Liver S9 subcellular fractions were prepared according to the protocol by Johanning et al. (2012) with minor modifications; the detailed procedure is included as supplemental information (Appendix B). All fish were processed in accordance with an approved Institutional Animal Care and Use Committee (IACUC) protocol at Baylor University.

A modified version of the OECD TG 319 was employed for the estimation of *in vitro* intrinsic hepatic clearance of BaP (OECD, 2018). Depletion assays were conducted in triplicate and consisted in 1 mL reactions with 1 mg/mL S9 protein, 20 mM of NADPH, 2 mM of UDPGA, 5 mM of GSH, and 0.1 mM PAPS, used as cofactors for phase I and II biotransformation enzymes. Additionally, 25 µg/mL of alamethicin was

added to increase the availability of UGT enzymes from the endoplasmic reticulum (Johanning et al., 2012). The reaction was started by the addition of BaP in acetone (0.5% [v/v]) to a final concentration of 0.2 μM, selected from similar experiments reported in Franco, Johanning, Matson, and Lavado (2021). Depletion assays using basal, non-induced S9 were conducted for 10 min, with 100 μL aliquots taken at 2, 4, 6, 8, and 10 min. Assays using S9 fractions with βNF-induced Ahr were conducted for 2 min, with 100 μL aliquots taken at 20, 40, 60, 80, and 100 s. Aliquots were placed in 400 μL of chilled acetonitrile containing 5.0 nM benzo[a]pyrene-d_{12} as internal standard. The samples were then centrifuged at 2,000g for 10 min at 4°C, and 300 μL of the supernatant were transferred to autosampler vials for instrumental analyses.

For the hepatic intrinsic clearance calculation, measured concentrations (C) from the BaP depletion bioassays were Log_{10}-transformed and plotted against time (t; h). The first-order depletion rate constant (k_{DEP}; 1/h) was determined by multiplying the slopes of the resulting linear equations by -2.3. Intrinsic clearance was then calculated by dividing k_{DEP} values by the S9 protein concentrations in the assays (C_{S9}, 1 mg/mL).

An additional set of BaP depletion experiments were conducted to evaluate the production of phase I metabolites *in vitro*. For this purpose, triplicate experiments were conducted in 1 mL reactions, with 1 mg/mL non-induced S9 protein and 20 mM of NADPH. All other co-factors were not included as to prevent conjugation reactions. The production of metabolites was assessed at 30, 60, and 90 min after the addition of BaP (2 μM initial concentration) in acetone (0.5% [v/v]), following the procedure described above.

Intraperitoneal Injections of Benzo[a]pyrene

A total of four whole-animal experiments were conducted in the laboratory, where adult, male *F. grandis* were intraperitoneally (IP) injected with either 2.5, 5.0 mg/kg BaP or corn oil (used as carrier), using 50 µL glass syringes (Hamilton, Reno, NV). The doses were selected based on previous experiments with Fundulus heteroclitus (Willett, Wassenberg, Lienesch, Reichert, & Di Giulio, 2001). Before injections, fish were transferred to aerated 2.5-gallon glass aquaria, fed once, and acclimated for 48 h at room temperature and ~10 ppth salinity. In the first experiment, fish were exposed for 96 h and the injection treatments included corn oil, used as negative control, 2.5, and 5.0 mg/kg BaP. Each BaP treatment had n = 10 individuals from each population, whereas n = 5 individuals from each population were used as control. In the second experiment, fish were injected with a single dose of 5.0 mg/kg BaP and exposed for 24, 48, 72, and 96 h. At each time point n = 3 individuals were processed from each population; negative controls also had n = 3 individuals. In the third and fourth experiments, n = 4 fish from each population were again injected with a 5.0 mg/kg dose of BaP and processed at 24, 48, 72, 96, 120, and 144 h post injection. Fish were not fed during the times of exposure as to support bile accumulation. Average length, weight, and condition factor of individuals used in each *in vivo* experiment are shown in table B3.1.

In all four experiments and at the end of each exposure period, fish were euthanized and dissected. The bile was collected from the gallbladder and placed in 1 mL glass vials. Additionally, the liver was excised and placed in cryogenic vials. Both the bile and livers were stored at -80°C for no longer than two weeks prior to the start of chemical extractions and microsomal isolation.

Microsomal Activity of CYP Isoforms

For all *in vivo* experiments, a fraction of the collected livers was used in the isolation of microsomes (see supplemental information (Appendix B) for the microsome isolation procedure) and for subsequent measurements of CYP biotransformation activity, according to the protocols reported by Hahn, Lamb, Schultz, Smolowitz, and Stegeman (1993) and Smith and Wilson (2010) with modifications.

The microsomal activity of CYP1A was measured in all four experiments described in the previous section and via the ethoxyresorufin-O-dealkylase (EROD) activity bioassay, measuring the dealkylation of 7-ethoxy resorufin at a concentration of 10 μM. Additionally, CYP2B-, CYP2C9- and CYP3A4-like activities were measured in the samples from the third and fourth experiments only, and assays were done via the pentoxyresorufin-O-dealkylase (PROD) activity bioassay, the dealkylation of 7-methoxy-4-trifluorocoumarin (MFC), and the dealkylation of 7-benzyloxy-4-trifluoromethylcoumarin (BFC), respectively. Enzymes substrates were evaluated at 10 μM for PROD, and at 100 μM for both MFC and BFC. All enzyme activities were measured kinetically by fluorescence spectroscopy (Synergy™ H1, BioTek, VT), and normalized per mg of microsomal protein, measured via the Coomassie blue (Bradford) assay.

Fish Bile and Liver Extractions

The Bile and liver extraction of benzo[a]pyrene and its metabolites section in the supplemental material (Appendix B) includes further information about the bile and liver extraction protocols.

Briefly, bile extractions of BaP and five of its phase I metabolites: 1-hydroxybenzo[a]pyrene (1-OH-BaP), 6-hydroxybenzo[a]pyrene (6-OH-BaP), 9-hydroxybenzo[a]pyrene (9-OH-BaP), benzo[a]pyrene-4,5-dihydrodiol (BaP-4,5-diol), and benzo[a]pyrene-7,8-dihydrodiol (BaP-7,8-diol) were conducted based on the protocol described by Zhu et al. (2008), with minor modifications. The detailed information about extraction protocols are included as supplemental information (Appendix B).

For liver extractions of BaP, a small fraction of liver tissue (15-20 mg) was homogenized in phosphate buffer and subsequently extracted with a 4:1 hexane:methylene chloride solution. Homogenates were transferred to activated ISOLUTE® PLD+ protein and phospholipid removal columns (Biotage, Charlotte, NC) for cleanup, and were subsequently dried and resuspended in 500 µL acetonitrile with 5.0 nM benzo[a]pyrene-d_{12}, used as internal standard, and transferred to autosampler vials for instrumental analyses.

Instrumental Analyses

Concentrations of BaP in samples from the S9 depletion bioassays and from the liver tissue extractions were determined using high-performance liquid chromatography coupled with an RS fluorescence detector (HPLC-FLD; UltiMate 3000, Thermo Fisher Scientific, Waltham, MA). Chromatographic separation was achieved using a Kinetex® C-18 column (1.7 µm, 150 x 2.1 mm; Phenomenex, Torrance, CA). 80% aqueous acetonitrile was maintained at an isocratic flow rate of 0.5 mL/min and a column temperature of 40°C. BaP was detected at excitation and emission wavelengths of 295 and 403 nm, respectively, and its limit of detection (LOD) was approximately 0.5 nM (~130 ng/L).

The analysis of BaP and its metabolites in bile was done via ultra-performance liquid chromatography (UPLC; Dionex UltiMate 3000, Thermo Fisher Scientific, Waltham, MA) coupled with high resolution mass spectrometry (HRMS; Q Exactive™ Focus Quadrupole-Orbitrap™, Thermo Fisher Scientific, Waltham, MA), based on the method described by Zhu et al. (2008) with modifications. During method optimization procedures, the detection of 6-OH-BaP was highly variable and unstable; therefore, this metabolite was not considered in subsequent experimentation. For BaP and the rest of metabolites, chromatographic separation was achieved with the Kinetex® C-18 column described before at 45°C. The flow rate was maintained at 0.35 mL/min, with a mobile phase consisting of A) 100% acetonitrile and B) an aqueous 0.1% formic acid solution. The sample run time was 13 min, with a multi-step gradient of 0-3 min 40/60 (A/B), 3-6 min 45/55 (A/B), 6-8.5 min 98/2 (A/B), and 8.5-13 min 40/60 (A/B). Samples were ionized in positive-mode heated electrospray ionization (+HESI), with sheath and auxiliary gas flow rates of 15 and 5, respectively, spray voltage of 4 kV, and capillary temperature of 320 °C, followed by MS/parallel reaction monitoring (PRM) with 35,000 resolution, AGC target of $5x10^4$, and MS scan range of 50 – 310 m/z.

Analytical standards were used to quantify the concentrations of the target analytes in bile, through external 8-point calibration ranging from 0.05-50 ng/mL for BaP, 10-2000 ng/mL for 1-OH-BaP, 1-200 ng/mL for 9-OH-BaP, and 0.5-100 ng/mL for both BaP-4,5-dihydrodiol and BaP-7,8-dihydrodiol. A quality control (QC) sample, corresponding to a mid-range calibration point, was used to monitor the accuracy and precision of the instrumental analyses. Limits of detection (LOD) for BaP and each of its metabolites were determined to be three times the lowest detected peak area (Table B3.2)

and quantification was conducted for samples that presented peak areas within the analytes' linear range. For the bile extraction procedures, the percent (%) recoveries of BaP and its metabolites were determined in seven independent experiments by spiking 5 µL of bile collected from non-exposed fish with final, nominal concentrations of 5 ng/mL, 1 µg/mL, 100 ng/mL, 50 ng/mL, and 50 ng/mL of BaP, 1-OH-BaP, 9-OH-BaP, BaP-4,5-dihydrodiol, and BaP-7,8-dihydrodiol, respectively. Samples were subject to the full bile extraction procedure, and the percent recoveries were calculated based on the relative proportion of peak areas with respect to those of bile samples spiked after extraction (Table B3.2).

Statistical Analyses

In situ CYP1A (EROD) activity was compared among populations via a Student's t-test, and linear regression analyses were used to corroborate that BaP concentrations in S9 depletion bioassays followed first-order kinetics. A comparison of BaP metabolite concentrations resulting from substrate depletion bioassays was performed between population and incubation times via 2-way ANOVA. For the 96-h exposure assessment (first *in vivo* experiment), significant differences in CYP1A activity, BaP concentrations in bile and liver tissue, and metabolite concentrations in bile were evaluated via 2-way ANOVA, considering population and BaP dose as independent factors and the interaction between them. Student's t-tests were employed when treatment groups did not have quantifiable data (e.g. non-detected analytes in a given treatment group). Similarly, the data resulting from three independent *in vivo* experiments and corresponding to CYP1A activity, BaP concentrations in bile and liver tissue, and metabolite concentrations in bile were combined, and subsequently analyzed via 2-way ANOVA, considering population

and time of exposure as independent factors. Statistical significance was determined at $\alpha = 0.05$, and all statistical analyses were performed in R/R-studio (version 3.5.1).

Results

CYP Biotransformation Activity

The activity of four CYP isoforms was measured to explore potential differences in the biotransformation ability of both *F. grandis* populations, though CYP1A was of special interest due to its direct relationship with the differential inducibility of the Ahr pathway among the populations of interest. A direct comparison of *in situ* CYP1A activity, as measured by the EROD assay, between pollution-adapted and non-adapted *F. grandis* revealed no significant differences ($t = 0.34$, $p = 0.7352$; Fig. 3.1). However, all subsequent measurements from the four individual *in vivo* experiments showed significantly elevated EROD activity in non-adapted fish, whereas pollution-adapted individuals maintained lower and constant activity. First, a dose-response increase in EROD activity was observed in non-adapted fish upon exposure to corn oil (carrier control), 2.5 and 5 mg/kg BaP ($F = 7.697$, $p = 0.0014$, Fig. 3.2), and while a slight increase in activity was observed for pollution-adapted fish at the highest dose, this was significantly lower than the observed activity for the non-adapted population ($F = 12.349$, $p = 0.0010$). Furthermore, even though there was a noticeable population-dose interaction influencing EROD activity, the statistical analysis could not confirm this observation ($F = 3.129$, $p = 0.0536$).

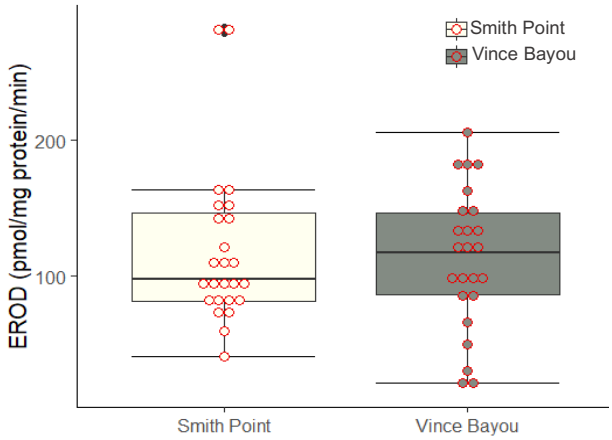

Figure 3.1. *In situ* EROD (CYP1A) activity (pmol/mg protein/min) in non-adapted (Smith Point, n = 25) and pollution-adapted (Vince Bayou, n = 24) *F. grandis.*

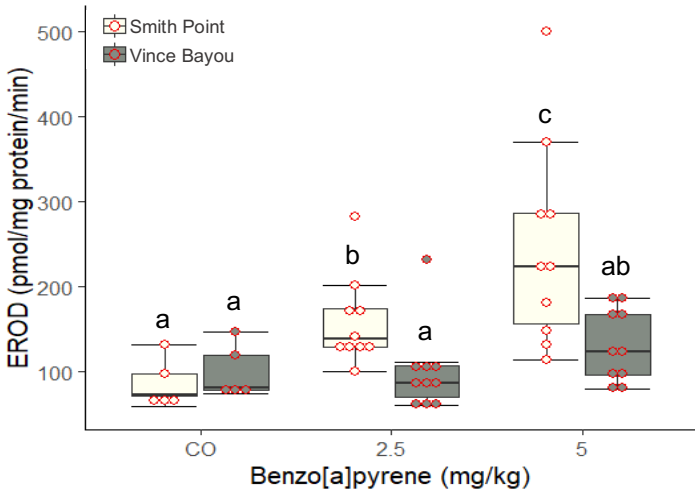

Figure 3.2. Microsomal EROD (CYP1A) activity (pmol/mg protein/min) in non-adapted (Smith Point) and pollution-adapted (Vince Bayou) *F. grandis* after a 96-h exposure following intraperitoneal injections of 2.5 and 5 mg/kg benzo[*a*]pyrene. CO: corn oil, used as carrier control. Different letters denote significant differences between populations and treatment groups (n = 5-10).

During *in vivo* experiments two, three, and four, EROD activity in no-adapted populations increased significantly upon exposure to 5 mg/kg BaP, relative to the control group (Fig. 3.3); however, EROD was significantly lower in pollution-adapted fish ($F = 79.277$, $p = 3.51e^{-15}$), with only slight increases with respect to their control group after 72 h of exposure. Additionally, the statistical analysis pointed out a significant time influence ($F = 3.072$, $p = 0.0076$) and time-population interaction ($F = 2.658$, $p = 0.0181$), though a specific time-dependent trend was not observed for either population. EROD activity measurements from the individual *in vivo* experiments followed the same trends, with significant differences among populations and are shown in Fig. B3.4.

Similar results were observed for CYP2B-like activity, measured through the PROD assay, though the magnitude of the activity was lower than the observed for EROD. PROD was significantly higher in non-adapted *F. grandis*, even though no specific time-dependent increases were observed (Fig. B3.5). Even though, for pollution-adapted fish, PROD activity increased significantly at 120 and 144 h with respect to the control group and the 24 – 96 h time points in the third experiment (Fig. B3.5A), though the highest PROD activity was still significantly lower than the observed activity in non-adapted fish.

Lastly, the activities of both CYP2C9 and CYP3A4, measured by the MFC and BFC assays, respectively, and during the two 144 h experiments did not show any population- nor time-dependent differences upon exposure to 5 mg/kg BaP (Fig. B3.6 and B3.7). While there was higher basal activity (control group) of these two CYP isoforms in non-adapted fish than in pollution-adapted fish, exposure to BaP did not appear to induce either isoform at any time point.

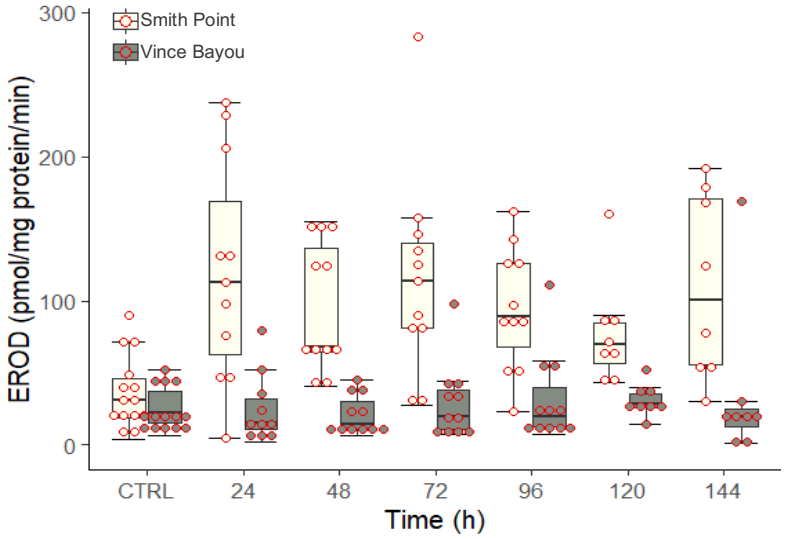

Figure 3.3. Microsomal EROD (CYP1A) activity (pmol/mg protein/min) in non-adapted (Smith Point) and pollution-adapted (Vince Bayou) *F. grandis,* measured at 24 h intervals following an intraperitoneal injection of 5 mg/kg benzo[*a*]pyrene. CTRL: corn oil, used as carrier control. Data represent the combined results of three independent *in vivo* experiments, with n = 8-11.

S9 Depletion of Benzo[a]pyrene and Production of Metabolites In Vitro

The depletion of BaP under both basal (Fig. 3.8A) and induced (Fig. 3.8B) Ahr pointed out a significant population difference, where pollution-adapted fish presented lower clearance rates ($CL_{IN\ VITRO}$) compared to non-adapted fish. $CL_{IN\ VITRO}$ in pollution-adapted *F. grandis* under basal conditions was 5.26 mL/h/mg protein, approximately 3.6X lower than non-adapted fish (18.92 mL/h/mg protein). Furthermore, while $CL_{IN\ VITRO}$ increased in both populations under induced Ahr conditions (83.08 and 19.51 mL/h/mg protein for non-adapted and pollution-adapted fish, respectively) pollution-adapted fish still showed a $CL_{IN\ VITRO}$ 4.3X lower than non-adapted *F. grandis*.

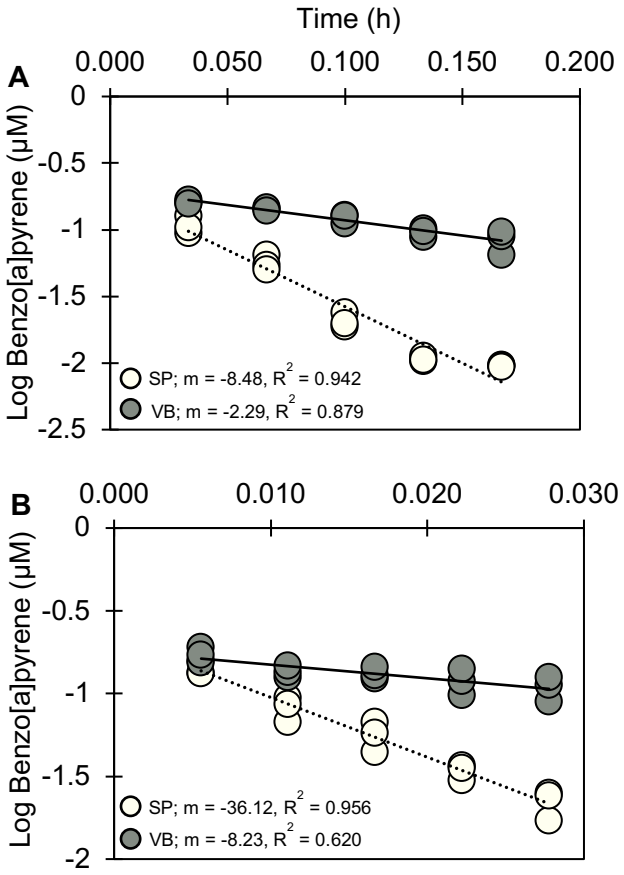

Figure 3.8. Depletion of benzo[*a*]pyrene by liver S9 sub-cellular fractions with A) basal, non-induced Ahr and B) βNF-induced Ahr of non-adapted (SP) and pollution-adapted (VB) *F. grandis.* Data points correspond to individual Log_{10} concentrations resulting from triplicate experiments.

Furthermore, a set of S9 substrate depletion bioassays were performed to evaluate the production of BaP metabolites *in vitro*. From these analyses, only 1-OH-BaP was detected and quantified after 30, 60, and 90 min of incubation (Fig. 3.9). A significant population difference was observed ($F = 5.055$, $p = 0.0441$), where pollution-adapted fish produced time-dependent increases ($F = 6.779$, $p = 0.0107$) of 1-OH-BaP, with the highest concentration at 90 min. Non-adapted populations also presented an elevated 1-OH-BaP concentration at 60 min relative to the 30 min time point, and even though the

concentration of this metabolite was, on average, lower at 90 mins, this was not significantly lower than the concentration observed at 60 min.

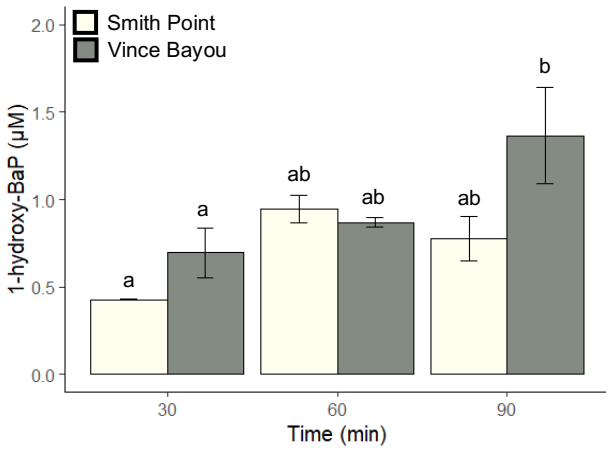

Figure 3.9. Production of 1-hydroxy-benzo[*a*]pyrene by liver S9 sub-cellular fractions from non-adapted (Smith Point) and pollution-adapted (Vince Bayou) *F. grandis* and at 30, 60, and 90 min from initial spiking with benzo[*a*]pyrene. Data correspond to the results of triplicate experiments (independent incubations), and different letters denote significantly different groups.

Concentrations of Benzo[a]pyrene in Liver

The concentrations of BaP were quantified in liver tissue collected during all IP-injection experiments. During the 96 h exposure to 2.5 and 5 mg/kg BaP, liver concentrations were the highest in the livers of individuals injected with the highest dose ($F = 29.360$, $p = 3.72e^{-6}$; Fig. 3.10A), yet there were no population differences at either BaP dose ($F = 0.245$, $p = 0.6235$) nor a dose-population interaction leading to differences in liver BaP concentrations ($F = 0.571$, $p = 0.4548$).

Similarly, BaP concentrations were not significantly different among populations ($F = 1.079$, $p = 0.3014$) nor were time-dependent ($F = 1.493$, $p = 0.1981$) during the second, third and fourth *in vivo* experiments (Fig. 3.10B). However, population comparisons at each time point showed higher average BaP concentrations in pollution-

adapted fish at 24 and 48 h of exposure, but a lower concentration at 144 h, relative to non-adapted fish. Average liver concentrations of BaP from the individual *in vivo* experiments are shown in table B3.3.

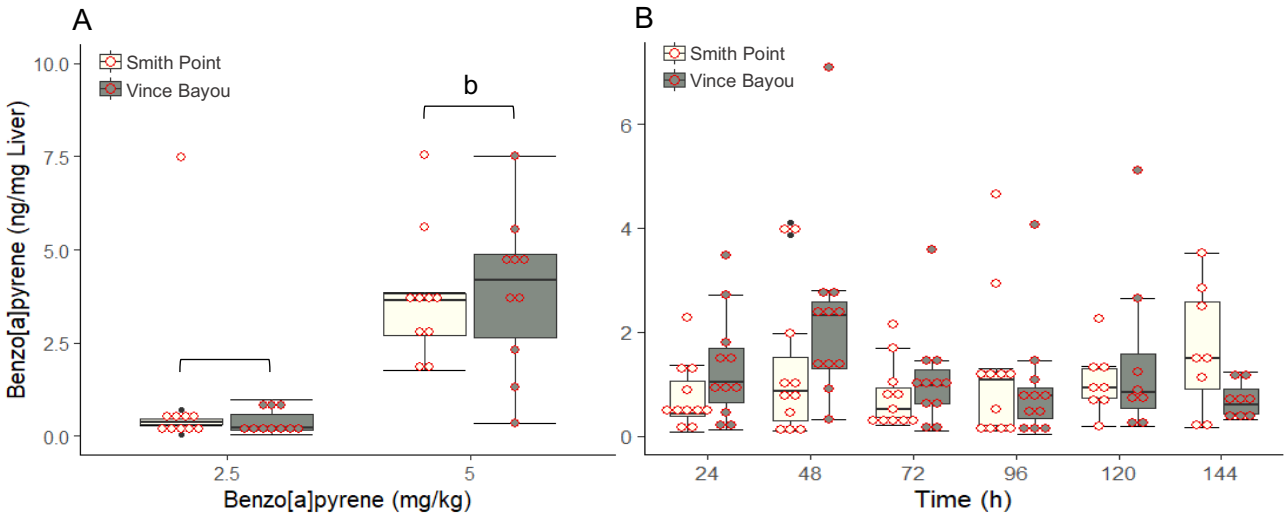

Figure 3.10. Concentrations of benzo[*a*]pyrene in liver tissue collected from non-adapted (Smith Point) and pollution-adapted (Vince Bayou) *F. grandis* intraperitoneally injected with A) 2.5 or 5 mg/kg benzo[*a*]pyrene and exposed for 96-h and B) injected with 5 mg/kg benzo[*a*]pyrene prior to measurements at 24 h intervals. Different letters denote significant differences between treatment groups.

Concentrations of Benzo[a]pyrene in Bile

As with liver concentrations in the 96 h exposure experiment, BaP concentrations were significantly higher in the bile of fish injected with the 5 mg/kg BaP dose ($F = 5.947$, $p = 0.0198$; Fig. 3.11A), and while on average, the bile concentration of BaP in non-adapted fish was higher at this dose, the rather variable distribution of measured concentrations did not account for a significant population difference within this treatment ($F = 2.569$, $p = 0.1177$). No dose-population interaction was observed either ($F = 2.325$, $p = 0.1361$).

In the time-dependent exposure assessments, BaP concentrations in bile were not significantly different among populations ($F = 1.628$, $p = 0.2049$), and while a time-

dependent trend was not observed for either population, significantly elevated BaP concentrations were measured in pollution-adapted fish processed at 72 h (Fig. 3.11B). This may have accounted for time ($F = 2.370$, $p = 0.0444$) and time-population interaction ($F = 2.699$, $p = 0.0247$) differences observed in the statistical analyses. The bile BaP concentrations measured for each individual, time-dependent *in vivo* experiment are shown in table B3.4.

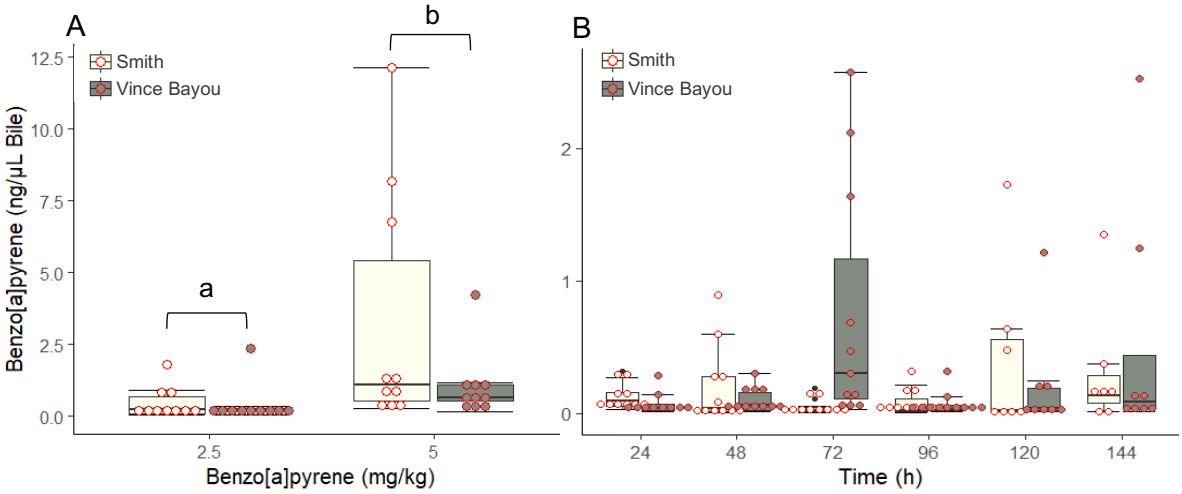

Figure 3.11. Concentrations of benzo[*a*]pyrene in bile collected from non-adapted (Smith Point) and pollution-adapted (Vince Bayou) *F. grandis* intraperitoneally injected with A) 2.5 or 5 mg/kg benzo[*a*]pyrene and exposed for 96-h and B) injected with 5 mg/kg benzo[*a*]pyrene prior to measurements at 24 h intervals. Different letters denote significant differences between treatment groups.

Metabolite Profiling of BaP

Even though the chemical analyses for fish bile targeted four BaP phase I metabolites, only 1-OH-BaP, 9-OH-BaP, BaP-4,5-dihydrodiol were detected in all *in vivo* experiments, and their average concentrations in each experiment are shown in table B3.4.

During the 96-h exposure assessment, 1-OH-BaP was present in individuals from both populations and exposed to both BaP doses. However, measured concentrations of

this metabolite appeared to be lower at the highest BaP dose, though their distribution was rather variable, which could have led to no dose-dependent differences ($F = 2.373$, $p = 0.1336$) nor differences among populations ($F = 0.422$, $p = 0.5207$; Fig. 3.12A). During the second, third, and fourth *in vivo* experiments, the concentration of 1-OH-BaP, although not quantified in all individuals from both populations and processed at each time point, did not appear to be time-dependent ($F = 1.106$, $p = 0.3699$) nor presented significant differences among populations ($F = 2.00$, $p = 0.1447$; Fig 3.12D). At specific time points, the concentration of 1-OH-BaP appeared to be higher in pollution-adapted fish processed at 48 and 144 h, though for the latter, the distribution of measured concentrations was significantly variable.

For 9-OH-BaP, even though most individuals from both populations presented concentrations in bile that were below limits of detection at the 2.5 mg/kg BaP dose, a significantly high concentration of this metabolite was measured in pollution-adapted *F. grandis* injected with 5 mg/kg BaP, compared to non-adapted fish ($t = -5.082$, $p = 0.0003$; Fig. 3.12B). Due to the detection issues at the 2.5 mg/kg dose, no dose-dependent nor dose-population interaction were assessed. The combined data from the second, third, and fourth experiment revealed a similar trend from the 96 h exposure, as for this metabolite there were both a significant difference among populations ($F = 8.239$, $p = 0.0060$), where pollution-adapted fish showed higher concentrations, and also a time-dependent concentration increase ($F = 2.566$, $p = 0.0384$), though this was only observed for pollution-adapted fish (Fig. 3.12E). No time-population interactions were observed ($F = 1.594$, $p = 0.1790$). Specifically, for pollution-adapted individuals, the bile concentration of 9-OH-BaP in this population remained similar during the first 72 h of exposure, but

presented continuous, time-dependent increases at 96, 120, and 144 h. While 9-OH-BaP showed slight, continuous increases in non-adapted *F. grandis* between 24 and 72 h, its concentrations remained relatively unchanged for the remaining time.

BaP-4,5-dihydrodiol also presented quantification limitations at the 2.5 mg/kg BaP dose during the 96-h exposure assessment, with only two individuals from the non-adapted population presenting measurable concentrations (Fig. 3.12C). However, the analysis performed for the 5 mg/kg BaP dose revealed a significant population difference, where the concentration of BaP-4,5-dihydrodiol was higher in pollution-adapted fish ($t = -6.345$, $p = 2.453e^{-5}$). This population difference was also observed for the time-dependent exposure assessments ($F = 4.138$, $p = 0.0453$), where pollution-adapted fish presented higher average BaP-4,5-dihydrodiol concentrations than non-adapted fish at the 48, 96, and 144 h time points (Fig. 3.12F). This was also shown by a significant time-population interaction ($F = 4.639$, $p = 0.0009$), although no particular time-dependent trend was observed for either population.

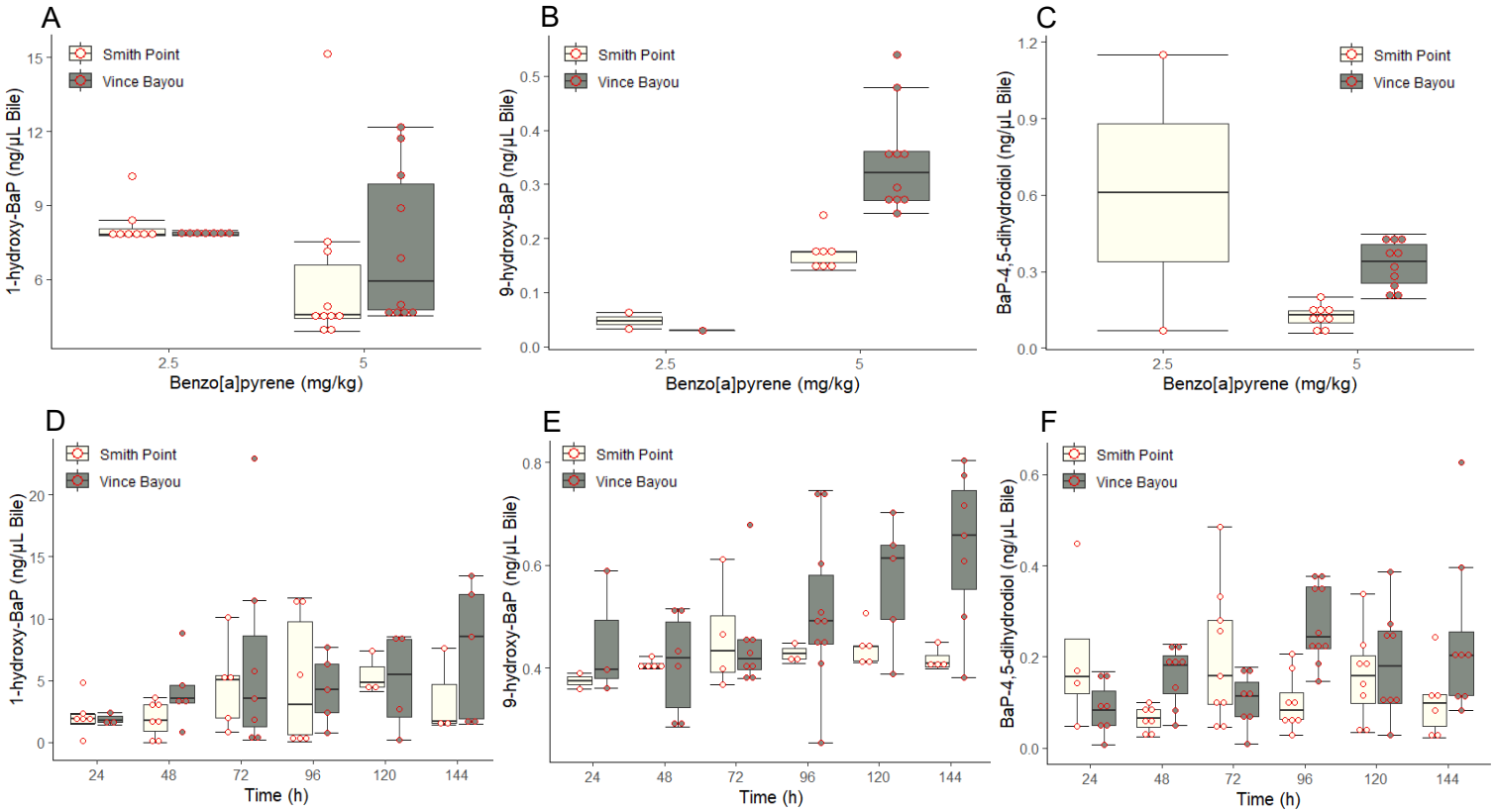

Figure 3.12. Concentrations of the benzo[*a*]pyrene metabolites A/D) 1-hydroxy-BaP, B/E) 9-hydroxy-BaP, and C/F) BaP-4,5-dihydrodiol in bile collected from non-adapted (Smith Point) and pollution-adapted (Vince Bayou) *F. grandis* intraperitoneally injected with A/B/C) 2.5 or 5 mg/kg benzo[*a*]pyrene and exposed for 96-h and D/E/F) injected with 5 mg/kg benzo[*a*]pyrene prior to measurements at 24 h intervals.

Discussion

As shown in previous studies on *F. grandis* (Franco et al., 2021; Oziolor et al., 2019), populations inhabiting areas connected to the Houston Ship Channel (HSC) display reduced inducibility of the Ahr pathway and reduced activity of associated CYP enzymes, for which CYP1A is of special attention given its role in the biotransformation of PAHs. In the present study, we provide further evidence of this reduced Ahr inducibility through both measurements of microsomal CYP1A activity and *in vitro* intrinsic clearance of BaP. In all experiments presented here, CYP1A, as measured by the EROD assay, was significantly lower in pollution-adapted individuals, and it is likely that this reduced activity is directly responsible for the lower hepatic clearance rates observed for these populations. These observations in pollution-adapted *F. grandis* were also shown in a previous assessment by our team (Franco et al., 2021), where significantly low clearance rates were measured for high-molecular weight PAHs, including BaP, under basal phase I biotransformation activity. Additional to these results, the induction of the Ahr pathway and associated CYP enzymes by βNF prior to the isolation of S9 sub-cellular fractions and subsequent depletion assays was advantageous in confirming these population differences, as while the BaP clearance rates increased for both groups, pollution-adapted *F. grandis* still showed significantly lower clearance than the non-adapted population.

However, from these *in vitro* approaches, it is difficult to determine what are the implications of the reduced biotransformation of BaP in pollution-adapted *F. grandis*. Since the S9 depletion approach has been effectively implemented in bioaccumulation assessments (Embry et al., 2010), it is likely that an immediate consequence would be

higher bioaccumulation of this BaP or, at least, elevated body burdens for longer time periods. The measurement of BaP in liver tissue was conducted with the purpose of depicting time-dependent trends in its distribution and hepatic biotransformation, under the rationale that the observed reduced hepatic clearance in pollution-adapted fish could result in elevated liver BaP concentrations. Hypothetically, measurements at different time intervals could show these differences among populations. However, besides the elevated liver concentration in pollution-adapted fish at 48 h, no other population nor time-dependent differences were observed. These results suggest no major differences in the distribution of BaP between pollution-adapted and non-adapted *F. grandis*, though more specific toxicokinetic evaluations and further assessments of absorption, distribution, metabolism, and excretion (ADME) could be beneficial.

Based on these observations, the construction of *in vitro-to-in vivo* extrapolation (IVIVE) models may represent the best alternative to depict whether the observations *in vitro* are maintained when considering whole-animal models (Brinkmann, Preuss, & Hollert, 2016; Grimard et al., 2020; Saunders, Fitzsimmons, Nichols, & Gobas, 2020). Unfortunately, IVIVE remains as the biggest challenge in *in vitro* (eco)toxicology and current approaches to derive models from *in vitro* data are hindered by the complexity of whole animals and the environmental factors that contribute to their biological responses. As shown in our results, the evolutionary and exposure histories of organisms and populations of interest significantly influence physiological responses, making them a necessary consideration when evaluating populations at risk and implementing NAMs. In terms of the measured BaP concentrations in fish bile, it is unclear why free BaP would be present in this matrix, as, in theory, compounds like PAHs that reach the bile

are ready for excretion after undergoing biotransformation and they are often present as biotransformation products, making them a dependable biomarker of PAH exposure (Baali, Kammann, Hanel, El Qoraychy, & Yahyaoui, 2016; Snyder, Olin, Pulster, & Murawski, 2020). The presence of the parent compound in bile could be the result of direct transport across tissue membranes or direct partitioning into this compartment. While no population nor time-dependent differences were observed regarding BaP concentrations in bile, one particularly interesting observation was the elevated BaP in the bile of pollution-adapted fish at 72 h post-injection, which was 24 h after the significantly high concentration in the liver described above. However, a critical aspect of the experimental design employed in these assessments and that could hinder measurements of parent compounds and biotransformation products in bile is the uncertainty associated with excretion, as even when attempting to control this factor (e.g. through avoiding feeding to support bile accumulation), any released chemicals in between sampling procedures would influence measured concentrations. Therefore, further experimentation is required to explore these issues.

An important contribution of this study is the phase I metabolite profiling of BaP in both *F. grandis* populations. On-going discussions related to biotransformation in fish (e.g. the Health and Environmental Sciences Institute (HESI) Bioaccumulation Committee workshop on fish biotransformation in bioaccumulation) have shown interest in the identification and quantification of biotransformation products to identify biochemical pathways at play and potential adverse effects associated with these products. Understanding what metabolites are likely to be produced by species the of interest and how they can affect the integrity of exposed organisms represents an

important research avenue that could help in chemical screening procedures and in describing species sensitivity to legacy and emerging contaminants. While no population differences were observed for the presence of 1-OH-BaP in bile, our results indicated a significantly high production of 9-OH-BaP in pollution-adapted fish, as well as of BaP-4,5-dihydrodiol at some time points during the exposure assessments. Under reduced inducibility of the Ahr pathway and lower activity of CYP1A isoforms, other biotransformation enzymes or additional cellular components may compensate for this activity, as was shown for ketoconazole in the fathead minnow (Ankley et al., 2007). Given the similar microsomal CYP1A and CYP2B-like biotransformation activity in the present study, CYP2B isoforms may, to some extent, support the biotransformation of BaP in *F. grandis* and potentially modify its metabolite profile. Furthermore, in Franco et al. (2021), the characterization of CYP isoforms in S9 sub-cellular fractions suggested elevated CYP2C9-like activity in pollution-adapted *F. grandis*, and while this observation was not confirmed by microsomal CYP2C9-like activity in the present study, this provides evidence for the presence and activity of other cellular factors; for example, NADPH-independent enzymes (e.g. esterases) (Saunders, Fontanay, Nichols, & Gobas, 2018). These factors could support biotransformation processes when major pathways are compromised, and they are likely to influence the type of metabolites being produced and their role in allowing exposed organisms to maintain ecological fitness in heavily polluted environments.

Both the *in vitro* and *in vivo* approaches implemented in this study suggest 1-OH-BaP to be a major biotransformation product of BaP in *F. grandis*. For comparative purposes, the studies by Strobel et al. (2015) and Grimard et al. (2020) on rainbow trout

and fathead minnow, respectively, suggested that the metabolite 3-Hydroxy-benzo[a]pyrene was a major BaP metabolite. Unfortunately, the analytical standard for such metabolite was not available in our study, and it is unclear whether this biotransformation product is also a primary BaP metabolite in *F. grandis*. Nonetheless, these observations represent evidence of interspecific differences in the biotransformation of PAHs and could be potential indicators of species sensitivity to BaP toxicity. Furthermore, during the chemical analyses, an unknown peak corresponding to a 268.09 mass fragment at a retention time of 4.93 min was observed in fish bile and S9 depletion assays – in some cases, with time-dependent increases, particularly for pollution-adapted *F. grandis* (Fig. B3.13 and B3.14). Because of the chromatographic characteristics of this analyte, it may correspond to a BaP-dihydrodiol, potentially BaP-9,10-dihydrodiol, another of the most common secondary phase I metabolites of BaP (Hatfield & Ramos, 2001). However, similar studies using Fundulus heteroclitus did not detect this metabolite in bile either (Zhu et al., 2008). Upon availability of such analytical standard, it would be of particular interest to confirm whether this unknown/potential BaP metabolite corresponds to BaP-9,10-dihydrodiol or to a different compound, given that BaP-7,8-dihydrodiol was not detected.

In terms of the identified BaP metabolites, both 1-OH-BaP and 9-OH-BaP are major primary metabolites of BaP that have a reduced likelihood of being further processed into secondary metabolites, such as quinones and diols (Hatfield & Ramos, 2001). Unlike 6-OH-BaP, 1-OH-BaP and 9-OH-BaP appear to have a lower ability to become oxo-radicals and, subsequently, quinones, which have been directly associated with elevated oxidative damage to major macromolecules (e.g. nucleic acids and

phospholipids) (Pampanin et al., 2016; Verma, Pink, Rettenmeier, & Schmitz-Spanke, 2012) and potential genotoxicity (Pampanin et al., 2017). Furthermore, BaP-4,5-dihydrodiol, despite being a secondary metabolite arising from the transformation of BaP-4,5-epoxide, is also an ending product of phase I biotransformation of BaP. PAH-dihydrodiols are highly reactive and have a significant affinity for DNA molecules, where they form adducts – a main effect leading to genotoxicity, mutagenicity, and carcinogenicity (Pampanin et al., 2016; Ramesh et al., 2004; Stiborova et al., 2014). However, the relative toxicity of BaP-4,5-dihydrodiol has been suggested to be lower than that of e.g. BaP-7,8-dihydrodiol (Verma et al., 2012), a major player driving the carcinogenicity of BaP. Giving the absence of this compound in our metabolite profiling for *F. grandis*, it is possible that this species, and specially pollution-adapted individuals, may allocate resources to the production of less toxic metabolites, though further evaluations are necessary to explore this possibility.

Lastly, one particularly important observation from the present study was some of the differential results from the single time-point *in vivo* assessment (96 h) and the continuous, time-dependent assessments for 144 h. Traditionally, evaluations of toxicity focus on one specific time of exposure where data collection occurs. During the *in vivo* experimentation, we observed that although certain patterns were maintained between the single 96 h exposure and the 144-h exposure with 24 h measurement intervals (e.g. higher concentration of 9-OH-BaP in the bile of pollution-adapted fish than in non-adapted fish), the continuous measurement approach provided a better representation of the time-dependent biotransformation in the populations of interest and demonstrated that having considered an exposure time less than 96-h for a single measurement could have

potentially resulted in an incorrect depiction of differences among *F. grandis* populations. Therefore, our results highlight the advantages of time-dependent experimentation in ecotoxicological studies, rather than traditional one-point assessments. Additionally, besides the population-related differences described above, the differential observations from the S9 *in vitro* assessment, where only 1-OH-BaP was identified, and the *in vivo* experimentation, where additional metabolites were observed, reflects the difficulty of conducting IVIVE and confirms the need for further research efforts to establish NAMs as robust alternatives to *in vivo* experimentation.

Conclusions

It is well-known that aquatic ecosystems continue to be threatened by anthropogenic pollution, and its short- and long-term effects on aquatic biota pose significant risk to their ecological integrity. Our findings reflect that, for chronically exposed organisms, the continuous exposure to xenobiotics represents a significant force of selection, potentially leading to evolutionary adaptations and allowing these organisms to cope with multiple environmental stressors (Jayasundara et al., 2017; Reid et al., 2016), through modifications of their chemical defensome. In this study, we demonstrate that the exposure history *Fundulus grandis* populations inhabiting estuaries from the Gulf of Mexico with different levels of pollution, represent a key factor in mediating biochemical and physiological responses to contaminant exposure, particularly those associated with biotransformation. The integration of *in vitro* and *in vivo* approaches allowed for a thorough description of major biotransformation differences between pollution-adapted and non-adapted *F. grandis*, as shown by significant differences in the activity of major biotransformation enzymes, intrinsic hepatic clearance, and production

of phase I metabolites. Furthermore, these results highlight the need of considering habitat characteristics and potential evolutionary adaptation of wild populations when conducting assessments of risk. There is no doubt that studies considering species that do not represent traditional models in toxicology and those aiming to reduce animal testing through the development of IVIVE models are significant for ecological risk assessment and of outstanding merit. However, current approaches to conduct comprehensive assessments of risk do not often consider specific characteristics of the species and areas of interest, as multiple physical, chemical, biological, and environmental factors have a direct influence over how organisms respond to pollution. The evolutionary adaptation of *F. grandis* populations in the HSC, Texas, is a clear example on how organisms in the wild and suffering from chronic contaminant exposure differ from those inhabiting less polluted areas and/or from those raised under controlled conditions (e.g. hatcheries). Therefore, research efforts that aim to describe and/or extrapolate observations from the use of NAMs and traditional model species must provide clear assumptions about the applicability of their results, as such observations may or may not reflect the actual status of species and populations in different environmental settings.

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CHAPTER FOUR

Multi-Approach Assessment for the Evaluation of Spatio-Temporal Estrogenicity in Fish from Effluent-Dominated Surface Waters Under Low Instream Flow

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Abstract

Current practices employed by most wastewater treatment plants (WWTP) are unable to completely remove endocrine disrupting compounds (EDCs) from reclaimed waters, and consistently discharge these substances to receiving systems. Effluent-dominated and dependent surface waters, especially during low instream flows, can increase exposure and risks to aquatic organisms due to adverse biological effects associated with EDCs. Given the ecological implications that may arise from exposure to such compounds, the present multi-approach study examined spatio-temporal estrogenic potential of wastewater effluent to fish in East Canyon Creek (ECC), Utah, USA, a unique urban river with instream flows seasonally influenced by snowmelt. Juvenile rainbow trout (*Oncorhynchus mykiss*) were caged at different upstream and downstream sites from an effluent discharge during the summer and fall seasons. In the summer, where approximately 50% of the streamflow was dominated by effluent, fish from the upstream and a downstream site, located 13 miles away from the effluent discharge, presented significantly elevated concentrations of plasma vitellogenin (VTG). Similarly, significantly high β-estradiol to 11-keto testosterone ratios were measured in the summer

across all sites and time points, compared to the fall. In the laboratory, juvenile fish and primary hepatocytes were exposed to concentrated effluent and surface water samples. Quantification of VTG, although in significantly lower levels, resembled response patterns observed in fish from the field study. Furthermore, analytical quantification of common EDCs in wastewater revealed the presence of estriol and estrone, though these did not appear to be related to the observed biological responses, as these were more significant in sites were no EDCs were detected. These combined observations suggest potential estrogenicity for fish in ECC under continuous exposures and highlight the advantages of following weight-of-evidence (WoE) approaches for environmental monitoring, as targeted analytically-based assessments may or may not support the identification of causative contaminants for adverse biological effects in effluent-impacted waters.

Introduction

The human population continues to increase at unprecedented rates and is predicted to reach approximately 9.8 billion people in the next 30 years (UN, 2017). With this population growth, urban areas will become more prominent, housing approximately 70% of the world population by 2050 (Sims et al., 2020; UN, 2017). This increase in urbanization has serious implications for water management, as larger discharges of wastewater and untreated sewage will result in effluent-dominated and dependent surface waters, altering the chemical and biological composition of urban streams (Brooks, 2018). Furthermore, the effects of climate change on global temperatures and altered weather dynamics will inherently modify the complexity of stream ecosystems, especially those for which instream flows are influenced by these urban discharges and

other external sources (i.e. snowmelt). In fact, an urban water cycle (resulting from the reuse of effluent) (Sowby, 2014) results from locally-scaled processes that contribute to the collection, use, distribution, treatment and reuse of water, as highlighted in effluent-dominated systems. Thus, direct alterations to water resources in urbanizing regions requires sustainable management of water quality to minimize adverse outcomes to public health and the environment.

Increasing urbanization leads to more human consumption, including many consumer products (e.g. pharmaceuticals and personal care products) referred to as environmental contaminants of emerging concern (CECs). These contaminants have been a matter of intensive research, and several studies have identified the presence of CECs in surface waters (Campbell et al., 2006; Haddad et al., 2018; D. W. Kolpin et al., 2002; D W Kolpin, Skopec, Meyer, Furlong, & Zaugg, 2004; Vidal-Dorsch et al., 2012); with a large proportion of comprehensive assessments being conducted in North America (Huggett et al., 2003), Europe (Tousova et al., 2017) and Asia (Yan, Lu, Liu, & Jin, 2012; Zhao et al., 2011). Unfortunately, most wastewater treatment plants (WWTPs) are not equipped with the technology to effectively remove CECs, of which pharmaceuticals, personal care products, and endocrine disrupting compounds (EDCs) can present important risks to water quality. This translates to scenarios where direct input of wastewater effluent to surface waters results in exposure of aquatic organisms to CECs, and such exposures are often elevated in effluent-dominated and dependent surface waters in which effluents comprise the majority of or all instream flows, respectively (Brooks, Riley, & Taylor, 2006).

Fish populations in urban surface waters are commonly at risk from EDCs (Denslow, Lee, Bowman, Hemmer, & Folmar, 2001; Ramon Lavado, Thibaut, Raldua, Martin, & Porte, 2004; Snyder et al., 2004). While acute responses from exposure to EDCs have been observed in fish, these are more prominent at concentrations in the μg/L and mg/L ranges – far from environmentally relevant levels (Moreman et al., 2017; Won, Woo, & Yum, 2014); thus, acute responses associated to EDCs are relatively uncommon. However, chronic effects are highly prominent in EDC-contaminated waters, especially for early life stages. These effects range from induction of the protein vitellogenin, reduced gonad size, and disruption of ovarian and testicular morphology and histopathology, to more severe effects such as complete alteration of sex ratios and feminization of male fish (R. Lavado et al., 2009; Niemuth & Klaper, 2015; A. M. Vajda et al., 2008; A M Vajda et al., 2011). Biological changes resulting from endocrine disruption have consequences for both the individual and population levels of biological organization, affecting the complexity of stream ecosystems and associated ecosystem services.

Unfortunately, traditional analytical approaches are not intended to describe adverse biological effects and only provide information about the presence of chemical compounds that may cause such effects in exposed organisms. Therefore, the primary objective of this multi-approach study was to comparatively examine potential spatio-temporal biological (estrogenic) responses in fish downstream of a WWTP in East Canyon Creek (ECC), UT, USA, a semi-arid stream, at two different seasons, under the hypothesis that elevated exposure to wastewater effluent would increase the levels of selected biomarkers of endocrine disruption, especially under low instream flows, and

that this would be evident across all of lines of investigation taken. This type of integrated assessments have been primordial in the description of estrogenic contamination in aquatic environments (Vethaak et al., 2005). ECC is a semi-arid river influenced by instream flow fluctuations resulting from seasonal snowmelt, receiving approximately 60% of annual precipitation in the fall and winter, and effluent discharge from the East Canyon Water Reclamation Facility. Because CEC exposure in such urban systems influenced by snowmelt are poorly understood, Du, Perez-Hurtado, Brooks, and Chambliss (2012) initially evaluated potential bioaccumulation of 15 pharmaceuticals in fish from ECC, including carbamazepine, diphenhydramine, diltiazem, and fluoxetine. Haddad et al. (2018) further observed bioaccumulation and trophic dilution of several ionizable pharmaceuticals, across seasons in which instream flows fluctuated from the melting snowpack. However, in addition to pharmaceuticals, EDCs include many other chemical compounds from both synthetic and biogenic origins, and these CECs are consistently discharged from WWTPs to surface waters (A. M. Vajda et al., 2008).

In the present study, juvenile rainbow trout (*Oncorhynchus mykiss*) were individually caged *in situ* at one site upstream and three sites downstream of the effluent discharge for 1, 3 or 7 days prior to blood and liver collections. In the laboratory, concentrated ECC effluent and surface water samples from the same locations were used for 7-day exposures in intraperitoneally-injected fish and 48-h exposures in primary hepatocytes. Endocrine responses were assessed through measurements of expression and presence of vitellogenin (VTG) in plasma, and quantification of β-estradiol (E2) and 11-keto testosterone (11KT). The description of E2/11KT ratios is useful in the evaluation of potential hormonal changes and sex differentiation associated with exposure to estrogenic

and/or androgenic compounds (Guiguen, Fostier, Piferrer, & Chang, 2010); thus, elevated E2/11KT ratios would be indicative of estrogenicity and vice versa. Furthermore, common EDCs were analytically determined in effluent and surface waters to potentially identify causative chemical compounds if estrogenic activity was observed. This combination of analytical chemistry, biomarkers of exposure, and field- and laboratory-based bioassays provided multiple lines of evidence during evaluations of estrogenic potential of effluent-dominated surface waters and may contribute to the development of advanced strategies for water and wastewater management in urban areas.

Materials and Methods

Study site

East Canyon Creek is a semi-arid stream, initiating north of Wasatch County and spreading over the western stretch of Summit and Morgan Counties (Fig. 4.1). The creek is located in a mountainous region, receiving approximately 60% of annual precipitation in the winter (Haddad et al. 2018), which result in elevated stream discharges due to snow melt in the spring but reduced flows in the summer. The effluent discharge from the Snyderville Basin Water Reclamation District (SBWRD) WWTP is located near Park City, UT at the coordinates 40°45’28.4” N and 111°33’49.9” W, and at an elevation of ~1,907 m. This WWTP has a design capacity of approximately 15,000 m^3 day^{-1}, with mean daily loads of approximately 11,500 m^3 day^{-1} (Haddad et al., 2018). As noted above, the selected study sites included one upstream site and three downstream locations at incremental distances (0.13, 1.4, and 13 miles) from the WWTP. Coordinates and elevation of the sites are shown as supplemental information (Appendix C) in table C4.1.

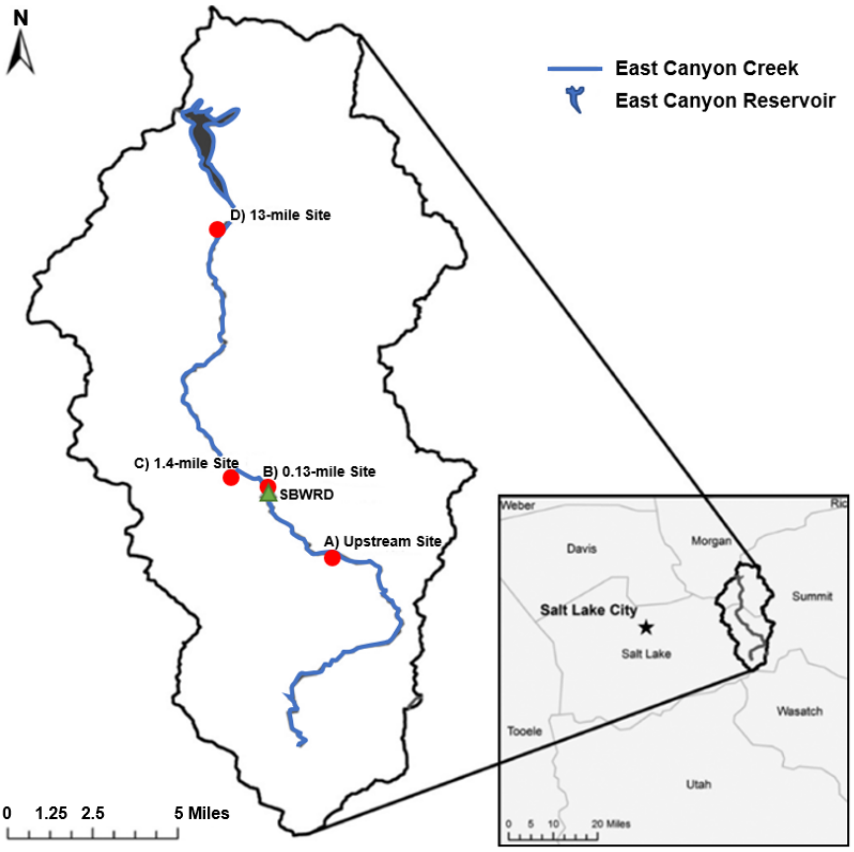

Figure 4.1. Location of sampling sites and the Snyderville Basin Water Reclamation District (SBWRD) wastewater treatment plant in East Canyon Creek, Park City, UT, USA. Modified from Haddad et al. (2018)

Water sampling and streamflow measurements

To test our primary hypothesis, water samples were collected in duplicates during the summer and fall seasons in 2018, using sterilized 4.0 L amber glass bottles. Water quality parameters (pH, specific conductivity, DO, and temperature) were measured every 15 min using pre- and post-calibrated multiparameter sondes (YSI Incorporated, OH, USA) deployed for the entirety of the sampling period in each season.

Streamflow data was obtained from USGS flow-gage stations located at the 0.13- and 13- mile sites. However, due to the lack of stations at the upstream and the 1.4-mile

sites, streamflow was manually measured on days 0, 1, 3 and 7, using standard methods with a Marsh McBirney Flo-Mate sensor (Hach, Loveland, CO, USA). Detailed methodology is shown as supplemental information (Appendix C).

Fish caging and deployment

Juvenile rainbow trout (*Oncorhynchus mykiss*) were obtained from a local hatchery (Cold Springs Trout Farm, North Ogden, UT, USA). Fish were individually placed in PVC tubes (cages), with length and diameter dimensions of 25.0 cm and 11.5 cm, respectively, and sealed with 2.80-mm flexible mesh and adjustable stainless-steel clamps on both ends. In the summer, cage sets consisted of seven PVC tubes attached to each other and tied to two 4.5-Kg weights. In the fall, cage sets consisted of nine PVC cages, arranged in two rows and positioned with stream flow. For both seasons, three cage sets were deployed at the upstream site and each of the three sites downstream of the WWTP, corresponding to exposures of 1, 3 and 7 days. Average length, weight, and condition factor of the deployed fish in both the summer and fall seasons are shown in supplemental information (Appendix C; Table C4.2).

Plasma and liver collection

After receiving the fish in the summer and following a 24-h acclimation period to creek-like conditions, three individuals were processed for plasma and liver collections - used as negative (non-exposed fish) controls. In the fall, six individuals were used as controls given we encountered the opportunity to access a larger number of fish, thus increasing the sample size as to provide more robustness to the study. For both seasons and on days 1, 3 and 7, cage sets were unassembled, and fish were placed in 5-gallon

buckets with creek water. First, fish were anesthetized by immersion in 2-4°C water and 1 mL plastic syringes with 23G needles were used to draw blood through caudal venous punctures; this procedure was conducted on-site. Collected blood was transferred to 1.5 mL microcentrifuge tubes and spun at 1,300*g* for 10 min at 4 °C. Plasma was collected, transferred to 1.5 mL cryovials, and placed in dry ice for transport. In the laboratory, plasma was stored at -80°C until bioassays were conducted. After blood collection, fish were euthanized by cervical dislocation, dissected and the liver was removed and placed in 1.5 mL cryovials containing 500 µL of a mRNA-Later solution; the tissue was kept at 5°C until mRNA extractions. Fish care and handling was conducted in accordance to an approved Institutional Animal Care and Use Committee (IACUC) protocol at Baylor University.

Intraperitoneal (IP) injections

Juvenile rainbow trout obtained from Westover Farms (Steelville, MO, USA) were maintained in a living stream system (Frigid Units Inc., OH, USA) prior to a 1-week acclimation to ~12 °C and 0.8 PSU water in 0.01-m^3 glass aquaria. After acclimation, fish were anesthetized by immersion in 2-4°C water, injected with 2.5 µL/g of extract-ethanol suspensions (preparation methodology is shown as supplemental information (Appendix C)) and returned to their assigned aquarium; three fish were used per treatment. After 72 h, fish received a second injection. Plasma and livers were collected after 7 days from the first injection following the methodology described in the previous section.

In vitro exposure

Primary hepatocytes were isolated from juvenile rainbow trout, also obtained from obtained from Westover Farms (Steelville, MO, USA), and seeded in 12-well plates as described by R. Lavado et al. (2009). Briefly, livers were homogenized in sterile PBS with a Trypsin-EDTA solution (10x, 0.05% trypsin and 0.02% EDTA), homogenates were filtered, centrifuged at 300 *g* for 10 min at 4 °C, and the resulting pellet was re-suspended in sterile PBS with 10% inactivated fetal bovine serum (FBS) (Atlas Biologicals, Fort Collins, CO, USA). The cell suspension was then added to a Percoll solution with 200 mM NaCl and centrifuged at 900 *g* for 25 min at 4 °C. The interface was collected and placed in L-15 cell culture media, prior to a clean-up step by centrifugation at 300 *g* for 10 min at 4 °C. The resulting pellet (hepatocytes) was re-suspended in L-15 medium with 10% FBS and seeded at a density of $1.0x10^6$ cells per cm^2.

After 24 h from seeding, cells were exposed to 1% of extract-DMSO suspensions (preparation methodology is shown as supplemental information (Appendix C)) and exposed for 48 h in a humidified incubator at 19 °C and ambient air, prior to cell collection and mRNA purification. A total of three biological and two technical replicates were used, with DMSO-exposed cells used as control.

Biomarkers

The egg yolk precursor protein VTG (Arukwe & Goksøyr, 2003) was selected as a gold standard biomarker of endocrine disruption, and was measured through commercially available rainbow trout standardized bioassays. The expression of VTG was quantified via RT-qPCR using the *Power* SYBR® Green RNA-to CT™ *1-Step* Kit

(Applied Biosystems, CA, USA), and the detail protocol is shown as supplemental information (Appendix C). For *in vitro* assessments, VTG expression is reported as estradiol equivalency quotients (EEQs) calculated from a standard curve (Fig. C4.2). Furthermore, concentrations of VTG in rainbow trout plasma were measured using an enzyme-linked immunosorbent assay (ELISA; Biosense Laboratories, Bergen, Norway), and concentrations of VTG were calculated as ng/mL of plasma and, for IP-injected fish, also reported as EEQs calculated from a standard curve (Fig. C4.3). The specific ELISA methodology is included as supplemental information (Appendix C).

Concentrations of E2 and 11KT in plasma were also quantified using an AchE competitive ELISA (Cayman Chemical, MI, USA), and concentrations of E2 and 11KT, followed by the E2/11KT ratio, were calculated as pg/mL of plasma. The ELISA protocol is also included as supplemental information (Appendix C).

Endocrine disrupting compounds (EDCs) in water

A targeted analysis using liquid chromatography – tandem mass spectrometry (LC-MS/MS; Agilent Technologies, Santa Clara, CA, USA) was performed for eight common EDCs potentially present in samples collected from whole effluent and creek water from each of the sites where fish were deployed. The EDCs included in the analysis were: 17α-estradiol, 4-nonylphenol, 17β-estradiol, 17α-ethinyl estradiol, equilenin, equilin, estriol, and estrone. The selection of these analytes was also based on previous assessments in ECC by the East Canyon water reclamation facility and our research group. The methodology for solid-phase extraction, sample preparation, analytical instrumentation and instrument calibration are presented in detail as supplemental

material (Appendix C). Method detection limits (MDLs), linear ranges, and extraction efficiencies (% recovery) are shown in Table C4.3.

Data analyses

Expression of liver VTG, concentrations of plasma VTG in field deployed fish, EEQs, and E2/11KT ratios were evaluated among seasons, sites, time points, and relative to the interaction between the site and day factors using multifactor generalized linear models (GLM) following a Gamma distribution. For statistical analyses, values below the minimum detected level (MDL) in bioassays were considered to be ½ of the minimum detected level for each biomarker (Cohen & Ryan, 1989). P-values less than 0.05 were considered statistically significant. If significance was detected, specific differences among sites were identified via Tukey (HSD) post-hoc pairwise comparisons. Furthermore, regression analyses were conducted for plasma VTG concentrations in field-deployed and IP-injected fish to investigate the degree at which laboratory analyses were representative of field observations. All statistical analyses were carried out using the R statistical software (version 3.5.3) and through the R-Studio platform for Windows.

Results

Streamflow (Q)

Mean streamflow (Q) was lower during the summer than in the fall, for all sampling sites and time points (Fig. C.4.4). Across all sampling locations and time points, total mean (±SD) streamflow was 0.212 ± 0.067 m^3 s^- in the summer and 0.368 ± 0.123 m^3 s^- in the fall. In the summer, the effluent discharge accounted for 49.9% of the total

flow downstream of the WWTP, whereas in the fall the effluent discharge accounted for 24.85% of the total streamflow.

Vitellogenin in field-deployed fish

The comparison between seasons showed no significant differences regarding VTG expression ($p = 0.3663$), However, specifically for the summer, and relative to time and among all sites, the highest levels of VTG expression were observed in fish from the 0.13-mile site ($p = 0.0354$), with day 3 representing an approximate 28-fold increase in expression relative to unexposed fish (Fig. C4.5A). Fish processed on day 7 from the 1.4-mile site also presented expression ~20 times higher than levels in controls, although the variability among samples and other time points was rather large. Furthermore, there were no statistical differences related to time (days; $p = 0.9629$) and no interaction between factors, yet average expression appeared to be more prominent on day 7.

In the fall, the highest VTG expression also occurred in fish from the 0.13-mile site with more than a 20-fold increase on day 3 relative to control fish, though the probability of the combined responses from each site being different was not significant ($p = 0.0536$; Fig. C4.5B). In general, the fish from this site, located immediately downstream from the effluent discharge, presented elevated expression compared to individuals from the other sites. The only exception was the expression in fish from the 1.4-mile site on day 1, as VTG was expressed approximately 15 times higher relative to unexposed fish. As with the summer, there was no statistical evidence for VTG differences among time points ($p = 0.9120$) and from the interaction of factors.

Considering all sites and time points, plasma VTG was higher in fish deployed in the summer (Fig. 4.6A) than in fish from the fall assessment (Fig. 4.6B; $p = 0.0109$).

Specifically, for the summer, the highest concentrations of plasma VTG were observed in fish deployed at the upstream and furthest downstream sites ($p = 0.0001$), with the highest levels being 990.9 ± 198.0 and 1125.3 ± 250.6 ng/mL for the upstream and 13-mile sites, respectively; this represented increases of approximately 23 and 30 times higher relative to VTG levels in unexposed fish. Among time points, the concentration of VTG was generally and significantly higher on day 7 ($p = 0.0224$) for the upstream, 0.13- and 13-mile sites, whereas in fish deployed at the 1.4 site, VTG levels were higher in all time points, with the highest level being approximately 14 times higher than levels in control fish. No interaction of site and day appeared to influence VTG levels. Plasma VTG in fish processed during the fall season presented significant differences among sites, with the highest levels the 0.13-mile site ($p = 0.0379$), representing a 9.6-fold increase on day 3 relative to control fish (Fig. 30B). Fish from all other sites presented elevated VTG concentrations at least on one time point, even though these levels remained lower than the observed for the summer assessment. No differences were observed among time points and from the interaction of factors.

17β-estradiol /11-ketoTestosterone ratios in field-deployed fish

As with VTG, ratios of E2/11KT, representing concentrations of plasma E2 relative to 11KT, were significantly higher in the summer compared to the fall season ($p = 1.527e^{-6}$). In the summer, the highest ratio, relative to controls, were observed in fish deployed at the 13-mile site on days 3 and 7, yet these levels were marginally significant among all sites ($p = 0.0538$; Fig. 4.6C). With the exception of fish from the 13-mile site on day 1, all other individuals from the other sites presented relatively high ratios. There was no evidence that suggested significant differences among time points ($p = 0.7528$). In

contrast, fish deployed in the fall from all sites presented significantly low ratios, with some values being below the average ratio of unexposed individuals (Fig. 4.6D). E2 to 11KT ratios increased in fish from the upstream, 0.13- and 1.4-mile sites on day 7 compared to the other time points ($p = 0.0144$), though these levels were not significantly different from control fish.

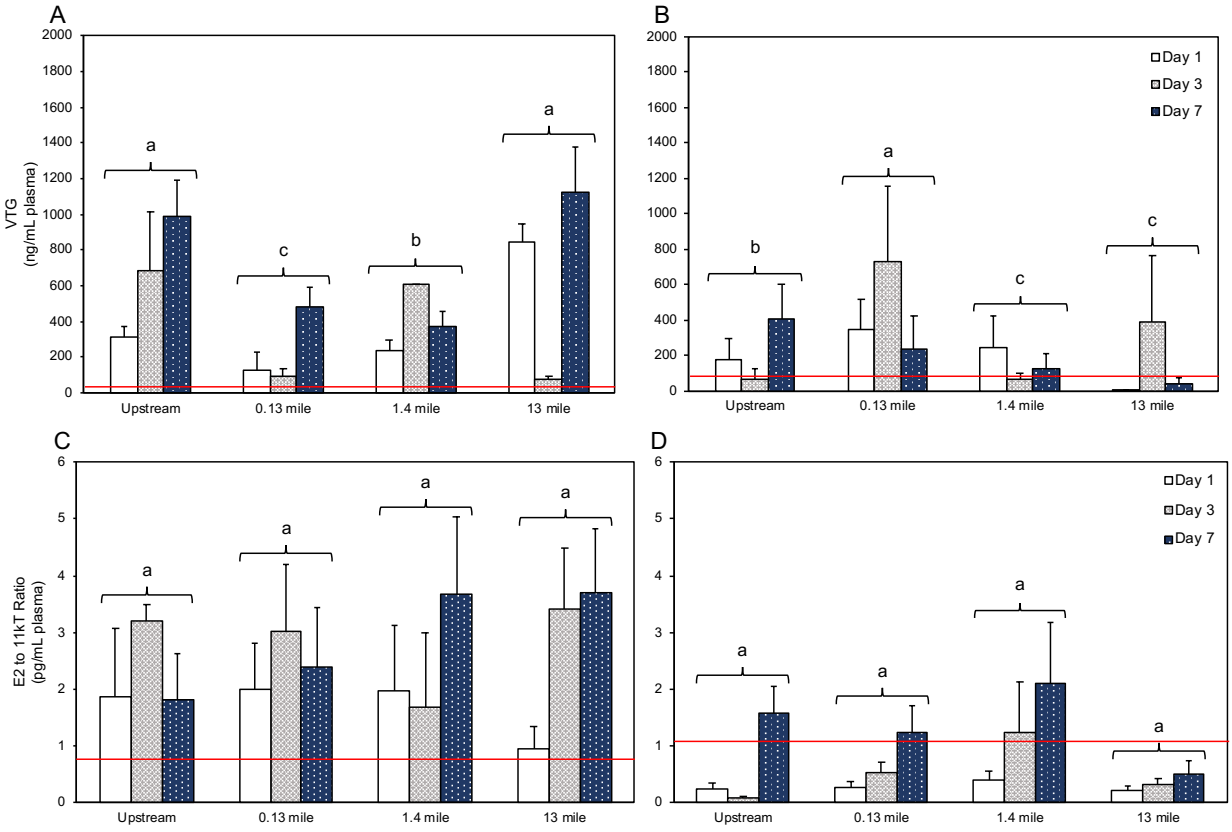

Figure 4.6. Mean vitellogenin (VTG) concentration in plasma (A and B) and mean plasma 17β-estradiol (E2) to 11-keto testosterone (11kT) ratios (C and D) of rainbow trout deployed at different sites and at three different time points during Summer (A and C) and Fall 2018 (B and D). Average VTG in control fish were 42.18 ± 4.80 and 75.63 ± 57.66 in the Summer and Fall, respectively, and are represented by horizontal lines. Average E2/11kT ratio from control fish were 0.72 ± 0.11 and 1.13 ± 0.52 in the Summer and Fall, respectively, and are also represented by horizontal lines. Data are presented as mean ± standard error (SEM). Different letters denote significant differences among sites (post-hoc analysis was based on site differences).

Vitellogenin in IP-injected fish

Plasma VTG concentrations (ng/mL) in IP-injected fish were further standardized as EEQs based on the plasma VTG levels in fish injected with different doses of E2 (Fig. C4.3). Despite the observations for the summer injections resembling the estrogenicity observed in field-deployed fish, no seasonal (summer vs fall) differences in plasma VTG were apparent when all sites and time points were combined ($p = 0.6045$; Fig. 4.7A and 4.7B). In both the summer and fall, the potency of effluent extracts to induce plasma VTG was significantly higher than in all other sites. However, extracts from water samples collected at the upstream and 13-mile sites during the summer season presented elevated EEQs ($p = 0.0093$), strengthening the observations of plasma VTG in field-deployed fish during the summer study. Furthermore, a significant ($r^2 = 0.52$; $p = 0.0124$) relationship was observed between plasma VTG concentrations of field-deployed and IP-injected fish in the laboratory (Fig. 4.8A) during these lower flow conditions. No significantly different EEQs appeared to be driven neither by the time factor nor from the site-time interaction.

In contrast, given the low and rather variable plasma VTG concentrations in the fall, the regression analysis did not highlight a relationship for VTG between field-deployed and IP-injected fish during this period ($r^2 = 0.07$ and $p = 0.4451$; Fig. 4.8B). The time factor appeared to induce marginal differences ($p = 0.0502$), yet the interaction between factors did not appear to influence EEQs.

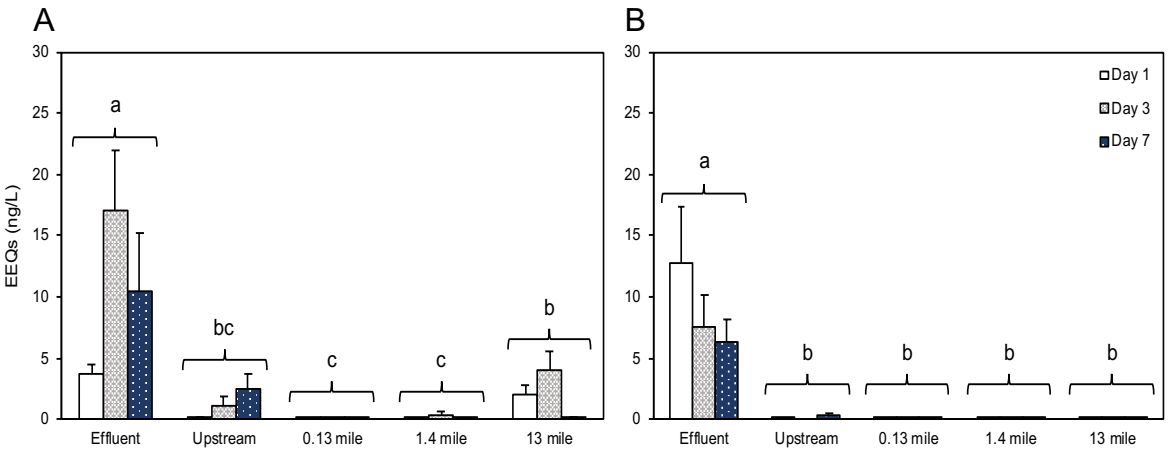

Figure 4.7. Mean estradiol equivalency quotients (EEQs) ± standard error (SEM) derived from plasma VTG levels in rainbow trout injected with extracts from effluent and water samples collected at different sites and at three different time points during the A) Summer and B) Fall 2018. Ethanol-injected fish (negative controls) presented VTG levels below detection limits and EEQs could not be quantified by the EEQ calibration curve (Fig. C4.3). Different letters denote significant differences among sites (post-hoc analysis was based on site differences).

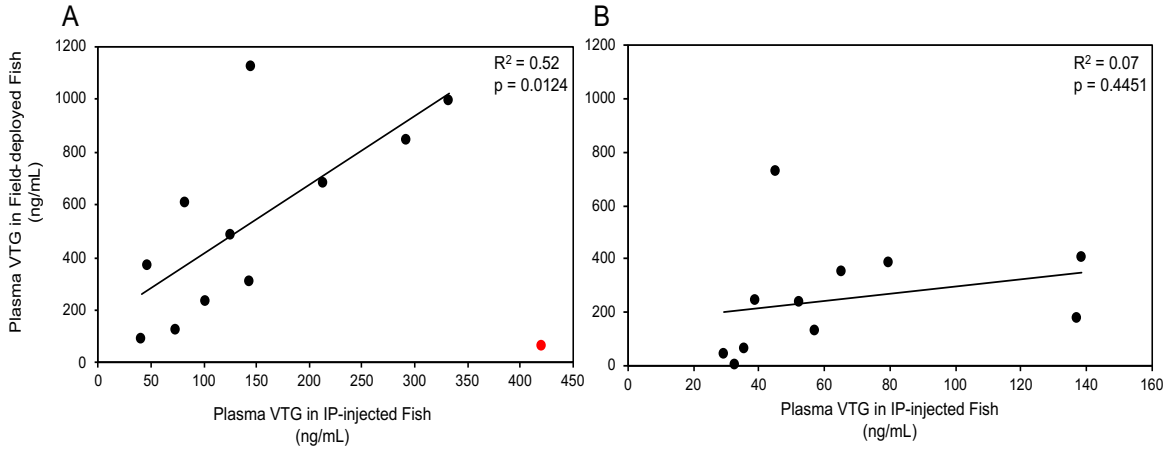

Figure 4.8. Fitted regression of plasma VTG concentrations in IP-injected and in field-deployed fish during the A) Summer and B) Fall 2018. Individual points represent average VTG concentrations per site and time points (days). The colored point in the summer analysis was not representative of the site when compared to data from the same location and was therefore not included in the analysis.

In vitro expression of VTG

Expression of VTG in primary hepatocytes exposed to extracts from water samples collected in the summer pointed out elevated estrogenicity from the effluent and

samples from the 13-mile site ($p = 7.23x10^{-11}$; Fig. C4.9A), while expression in all other sites remained significantly lower. A similar trend was observed in hepatocytes exposed to extracts from samples collected in the fall, where the effluent and water samples from the 13-mile site caused elevated VTG expression ($p = 0.0167$); however, site differences were driven by only one time point in the 13-mile site, where EEQs were above 40 ng/L (Fig. C4.9B). Among seasons, VTG expression was shown to be significantly high in the fall compared to the summer ($p = 0.0279$), yet again this was likely the result of the elevated EEQ at the 13-mile site in the fall. Lastly, no influences of time nor from the interaction of site and time factors were observed in the statistical analyses.

Endocrine disrupting compounds (EDCs) in water

Targeted EDCs are not commonly detected above MDLs in the current study. Results from analytical quantification of these targeted EDCs across all sites, time points and sampling season are shown in Table C4.5. Among all target EDCs evaluated in the study, only estrone was detected during the summer season at the 1.4-mile site and with a mean concentration, across all time points, of 4.86 ± 0.15 ng/L. Similarly, estriol was the only compound measured in whole effluent samples across all time points during both the summer and fall seasons, with mean concentrations of 4.19 ± 1.71 and 2.37 ± 0.78 ng/L, respectively.

Discussion

Environmental monitoring of chemical, physical and biological indicators represents a common approach to examine the status of surface water quality (Tousova et al., 2017; Vethaak et al., 2005). Different types of information can contribute to weight-of-evidence (WoE) approaches (Lowell, Culp, & Dubé, 2000; Mumtaz & Durkin, 1992) during assessments of

complex environments, where multiple stressors can contribute to ecosystem degradation. Perhaps the most common WoE approach for environmental assessment is the sediment quality triad (Long & Chapman, 1985), in which information from analytical measures, ambient toxicity assays and biological surveys are performed from potentially contaminated sites. More recent guidance has been provided for employing WoE in ecological risk assessments (USEPA, 2016). Because traditional assays employed to examine effluent discharges (e.g., whole effluent toxicity assays) included endpoints that were not sufficiently sensitive for EDCs, pharmaceuticals and other biologically active substances that elicit sublethal toxicity through various molecular initiation events (Ankley, Brooks, & Hugget, 2007), bioanalytical tools, such as *in vitro* bioassays and specific, gold-standard biomarkers have facilitated comprehensive evaluations of potential water quality alterations (Brion et al., 2019; Pawlowski et al., 2003; Sumpter & Jobling, 1995). For example, specific testing strategies such as whole animal and cell-based assays are critical in describing negative effects of EDCs for exposed populations (Ashby et al., 1997; Huggett et al., 2003), and provide valuable information to support environmental management decisions.

In semi-arid and arid regions, effluent-dominated surface waters are presenting unique management opportunities, particularly in the face of climate change (Luthy, Sedlak, Plumlee, Austin, & Resh, 2015). Such effluent-dominated and dependent systems appear to represent worse case scenarios for exposure to down the drain chemicals (Brooks et al., 2006), including the EDCs examined in the present study, because effective exposure duration is increased by decreased instream dilution (Ankley et al., 2007). For example, Rice and Westerhoff (2017) recently found that wastewater discharges contribute to 50% or more of total instream flows for more than 900 lotic systems streams in the United States. Thus, in these effluent-dominated systems, much like summer conditions of ECC, fish exposure to EDCs can exceed associated guidance values and toxicological benchmark concentrations (Rice & Westerhoff, 2017). In the current study, we observed significant expression and concentrations of liver and plasma VTG and E2/11KT ratios in rainbow trout during the summer season, which provides evidence for the

direct influence of lower instream flows as an indirect contributing factor to the enhanced potential endocrine disrupting effects observed in ECC.

Furthermore, observations from the present study strongly highlight the importance of selecting appropriate, but most importantly, complementary tools, such as well-established biological models to augment analytical chemistry measures when investigating bioactivity profiles and potential impacts of wastewater to aquatic organisms. Integrated evaluations with *in vivo, in vitro*, analytical, and OMICS approaches continue to be fundamental in assessments of estrogenicity (Burki et al., 2006). While the targeted analysis for common EDCs in whole effluent and water samples from different locations in ECC identified estriol in the effluent during both summer and fall, and estrone at the 1.4-mile site in the summer, it is fundamental to point out that wastewater effluent contains complex mixtures of chemicals with diverse biological activities, including endocrine disruptors that can trigger significant estrogenic responses but that were not identified. Though we targeted common endogenous and exogenous EDCs in the current study, these target analytes were not consistently identified nor quantitated among study sites or time periods. For example, the detection of estriol in the effluent occurred on sampling days 0, 1, and 7 but not on day 3. Subsequently, observations of both estriol and estrone did not appear to be directly related to biomarker responses measured in the present study, as significant estrogenic responses were observed at different locations and time points, especially during the summer where the effluent discharge accounted for approximately half of the total creek streamflow downstream of the WWTP (Fig. C4.4).

Here we selected juvenile rainbow trout because it is a well-characterized fish model and is representative of brown trout in ECC. The rainbow trout model has been extensively used in endocrine disruption assessments primarily by measurements of VTG (Arukwe & Goksøyr, 2003; Copeland, Sumpter, & Croft, 1986; Mouchel, Trichet, Betz, LePennec, & Wolff, 1996). Furthermore, it is well established that estrogenicity is highly dependent on sex, with females often presenting significantly higher levels of VTG than males. However, previous comparisons

of VTG levels in male and female rainbow trout have pointed out that these differences are only pronounced when individuals attain sexual maturity, and that immature fish, such as the ones used in the present study, do not present major differences in VTG (Copeland et al., 1986). Under normal conditions, the presence of endogenous estrogens leads to VTG synthesis in the liver of mature females, where it is secreted to the bloodstream and sub-sequentially taken up by oocytes (Mouchel et al., 1996). However, in juvenile fish, detectable VTG in the blood is often the result of external factors influencing VTG production (Ackermann, Schwaiger, Negele, & Fent, 2002; Thorpe, Hutchinson, Hetheridge, Sumpter, & Tyler, 2009), being VTG the most sensitive biomarkers of estrogenicity in trout (Donohoe & Curtis, 1996).

In the context of VTG expression and plasma concentration differences among sites, we believe this corresponds to a combination of factors related to different modes of action of EDCs and time of exposure. The relatively elevated VTG expression at the two sites immediately after the effluent discharge may suggest that the potency of estrogenic factors directly associated with the WWTP may have been sufficient to induce expression yet not strong enough to induce vitellogenesis and elevated protein content within the 7-day period. This would be in line with the observations by Le Guellec, Lawless, Valotaire, Kress, and Tenniswood (1988), who demonstrated that the appearance and accumulation of serum VTG is lagged by approximately 2 days following the first induction of VTG mRNA, and that this expression was short-lived in individuals that experienced a first exposure to estrogenic compounds. Therefore, it is likely that low estrogenic potency in these two areas was responsible for the observed VTG expression but lack of VTG content in fish plasma. Furthermore, it is unclear why mRNA expression was not significantly elevated in fish deployed at the upstream and 13-mile site, as they presented elevated plasma VTG; this may deserve further evaluation. However, we believe that the estrogenic factors in these areas, while not directly related to the WWTP, were significant, as illustrated by plasma VTG and previous studies (Haddad et al., 2018). It is likely that the compounds responsible for these estrogenic responses may have acted over different VTG mRNA

and rRNA sequences, as was also suggested in Le Guellec et al. (1988), and that this was not quite illustrated by the RT-qPCR bioassays.

In the case of rainbow trout deployed in ECC, it is likely that the observed increases in VTG during the summer season is attributable to a mixture of diverse EDCs in the water, rather than or at least in addition to additive effects of common, individual estrogen agonists that were routinely below MDLs. Similar observations were pointed out in studies by Harries et al. (1997) and R. Lavado et al. (2009), where endocrine disruption was observed in feral fish but the identity of specific compounds causing such estrogenic activity could not be determined. The detection of EDCs in surface waters is a complex procedure, often involving different collection volumes, numerous extractions and different instrumental analyses (Trenholm, Vanderford, Holady, Rexing, & Snyder, 2006). However, a large portion of the literature reports that extraction is often performed for 1 L samples, a standard volume that has provided successful results in the quantification of EDCs and other chemicals in water (Ahrer, Scherwenk, & Buchberger, 2000; Liu, Zhou, & Wilding, 2004; Trenholm et al., 2006). Furthermore, previous studies have suggested LC-MS/MS as one of the best analytical approaches for the identification and quantification of EDCs, following solid phase extraction, and with MDLs ranging between 1-10 ng/L (Trenholm et al., 2006; Vanderford, Pearson, Rexing, & Snyder, 2003), with some reporting MDLs above 15 ng/L (Gentili et al., 2002). MDLs in our study ranged from 0.1 to 5 ng/L. On this context, the sample preparation, extraction and analytical methods employed in our study are in line with protocols and guidelines reported in the literature. However, It is possible that the chemical analyses, specifically during solid phase extractions, were hindered by the physicochemical properties of many EDCs, as their hydrophobicity may play an important role in reducing the soluble fraction after each filtration step prior to extraction (Chang, Choo, Lee, & Choi, 2009). The analytical identification and quantification of the estrogenic compounds included in this study were performed for the waterborne fractions; thus, compounds that were adsorbed to organic matter and other suspended solids may have been removed during the

filtration steps, and subsequently not detected. It is also important to point out that solid phase extraction procedures for estrogenic compounds reported in the literature are rather diverse, with studies employing filtration steps using a wide range of filters (1.5 – 0.2 μm) for the removal of solids, yet identification of EDCs in water has been successful (Gentili et al., 2002; Liu et al., 2004; Zhang et al., 2012). Additionally, while all water samples were processed within 24 h of collection, it is possible that degradation of some target analytes took place before acidification of the samples, yet with processing activities happening in a matter of hours, we do not believe this had a significant impact on the chemical analyses.

Currently, the Snyderville Basin Water Reclamation District employs primary, secondary and tertiary treatments. Secondary and tertiary treatments involve biological nutrient removal, and chemical removal of phosphorus by the addition of alum to coagulate remaining solids, filtration and UV disinfection, respectively. Tertiary filtration systems may also account for some target chemicals being below MDLs, again due to their partitioning to suspended solids and organic particles that are removed prior to effluent discharge. Based on these observations, future studies are therefore necessary to identify causative estrogenic agents in ECC. Specifically, toxicity identification evaluations or effect-directed analyses should be performed, and further supported by non-targeted analytical methods.

In terms of the spatial evaluation for estrogenic activity in ECC, the highest levels of biomarker responses in fish plasma were observed at the upstream and the furthest downstream (13-mile) site from the WWTP. These interesting results were contrary to expectations as the upstream site was initially selected for non-effluent influenced reference conditions, as it is located within the Swaner Preserve and EcoCenter, a nature preserve in Park City, UT. Haddad et al. (2018) previously observed low levels of several pharmaceuticals and effluent tracers in ECC at a location upstream from the WWTP discharge, but downstream from the reference site examined in the present study. As noted previously, such observations may have resulted from diffuse contributions by septic systems (Haddad et al., 2018). Furthermore, the observations at the

upstream site limit the ability to clearly determine whether the WWTP pose a significant risk to fish populations downstream. In this context, establishing further control locations upstream of the selected control site or in an adjacent stream not impacted by WWTP could have been advantageous, as this could have potentially helped in further illustrating the risk of effluent discharges. However, it is important to point out that *in situ* evaluations, as the ones conducted in the present study, inevitably present influences from non-WWTP-related factors that could contribute to estrogenicity and potentially lead to similar observations like the ones from the selected control site. In the case of the 13-mile site, it appears likely that the elevated VTG and E2/11KT ratios were the result of direct human impact, as this site was located adjacent to a resort for recreational vehicles and campground with significant human activity in the summer but no centralized WWTP or point source discharge. In the fall, biomarker levels were lower, apparently reflecting slightly higher instream flows and relatively limited anthropogenic influences in the area.

Whether the observed *in situ* biomarker responses are indicative of significant endocrine disruption in fish inhabiting ECC presents an important question for water quality management. Herein, it is useful to compare our results with observations from studies with similar experimental design. Harries et al. (1997) reported plasma VTG concentrations in caged, adult male trout as low as 10 ng/mL prior to deployment, and concentrations between 30 to approximately 52,000 μg/mL following exposure to effluent discharges. In the present study, the highest concentration of plasma VTG was measured in the summer and corresponded to 1125.3 ± 250.6 ng/mL. Basal levels in immature male trout have been reported to be between 1.0 – 53 ng/mL and as low as 82 ng/mL in immature females (Copeland et al., 1986), approximately 10-times lower relative to the highest VTG values in our study. Compared to adult, unexposed fish from Harries et al. (1997), plasma VTG in juvenile rainbow trout deployed in ECC was induced approximately 100 times higher, which represents a significant response of estrogenic activity.

However, these observations were only evident when instream flows of ECC were effluent-dominated and may only present significant risk in the long term. Furthermore, while the magnitude of estrogenic responses at the upstream location was generally higher than in the downstream sites, this does not necessarily suggest that risks associated with the WWTP are absent for aquatic organisms. While estrogenicity in the two sites immediately downstream of the WWTP may not require immediate attention, long-term exposures to effluent-dominated waters may represent a significant threat; thus, continuous monitoring assessments should be considered.

Field studies are often preferred given the ability to consider the complexity of environmental factors that are difficult to simulate in a laboratory setting, yet they often come with elevated costs and onerous logistics. In such cases, having additional approaches that strengthen and complement field observations are advantageous. In the present study, while the responsiveness of the IP-injections approach may sometimes hinder its applicability (as it was for fall assessments), it was shown that under scenarios of interest (effluent-dominated waters) the system provided valuable information that could be extrapolated to field observations. However, the relationship between these field and laboratory IP-injection approaches requires further experimentation and validation, with the goal of strengthening the information obtained from laboratory-based experimentation and its potential use to draw conclusions for environmentally relevant scenarios. Furthermore, the use of primary hepatocytes have been widely applicable in environmental research (Baksi & Frazier, 1990; R. Lavado et al., 2009), yet their use has been mostly limited to single-chemical assessments (Bols, Dayeh, Lee, & Schirmer, 2005; Franco, Sutherland, & Lavado, 2018) and the evaluation of complex environmental samples using these models continues to be of intensive research (Brion et al., 2019). The major limitation of these systems arises from their sensitivity to respond to relevant environmental exposures. In the case of primary cell cultures for EDC-associated effects, the method may require further evaluation and optimization to obtain valuable data, representative of whole animal assessments.

Conclusions

As noted above, effective exposure duration of EDCs and many other contaminants is increased in these semi-arid systems because effluent introduction rates exceed chemical degradation rates (Ankley et al., 2007). Thus, such observations may represent future conditions associated with the intersections of increased population growth, which may increase effluent discharge and the likelihood of prolonged effluent-dominated conditions, particularly if instream flows are influenced to a lesser degree by reduced snowmelt in response to climate change. In the current effort, we selected a unique study system that is seasonally influenced by snowmelt, becomes increasingly effluent-dominated, and during droughts, can become effluent-dependent for base flows. Based on the results from the present study, the effluent discharge did not have detectable levels of major estrogenic compounds. However, other contaminants appear to be responsible for the estrogenic effects observed here, which may be of concern only during dry seasons when snowpack is not pronounced and thus snowmelt provides more limited dilution. Furthermore, besides the observations of estrogenicity in ECC and potential implications for fish populations inhabiting these areas, this study contributes major information about the advantages of integrating multiple approaches in the evaluation of estrogenic activity. In this context and based on the multiple lines of evidence approach assessment employed in this study, water reclamation facilities should consider the use of well-established bioassays for monitoring, and toxicity identification evaluations with targeted and non-targeted analysis when biological activity is elevated, to support sustainable management of effluent-dominated and dependent surface waters.

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CHAPTER FIVE

Plasma Vitellogenin Reveals Potential Seasonal Estrogenicity in Fish from On-Site Wastewater Treatment Systems in Semi-Arid Streams Influenced by Snowmelt

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Abstract

Effluents from on-site wastewater treatment systems can influence surface water quality, particularly when infrastructure is aging, malfunctioning, and improperly installed. Municipal wastewater often contains chemical compounds that can lead to adverse biological effects, such as reproductive impairment, in organisms that are chronically exposed. A significant number of these compounds are endocrine-disrupting chemicals. Water quality influences of on-site systems are poorly studied in semi-arid regions where instream flows are seasonally dependent on snowmelt, and when instream dilution of wastewater effluents is minimal during other times of the year. Here we examined surface water estrogenicity in low order tributaries of two unique semi-arid streams with on-site wastewater treatment systems, for which seasonal instream flow fluctuations occur in Park City, UT, USA. Water samples were collected from a total of five locations along two lotic systems downstream from active on-site treatment systems. Samples were extracted for targeted chemical analyses and to perform *in vivo* and *in vitro* bioassays with juvenile rainbow trout. Estrogenic activity was measured by quantifying the concentration and expression of vitellogenin (VTG) in plasma and liver, respectively.

Plasma VTG presented elevated levels in fish exposed to water samples collected at the two sites in close proximity to on-site systems and during seasons with low stream discharge, though the levels observed did not suggest severe endocrine disruption. However, long-term exposure to these surface water could compromise the fish populations. While the sensitivity of *in vitro* bioassays was low and targeted chemical analyses did not identify causative compounds, the use of complementary lines of evidence (e.g., *in vivo* biological models) was advantageous in identifying estrogenic activity in waters influenced by effluents from on-site wastewater systems.

Introduction

On-site wastewater treatment systems are commonly used in rural and peri-urban regions for municipal wastewater management. For example, in the United States, it has been estimated that at least 25% of households and 33% of new homes are serviced by on-site systems (Otis et al., 2002; USEPA, 2005). Despite the emergence of new wastewater treatment technologies, many urban and suburban regions around the globe continue to rely on septic systems for wastewater management (Lambert, Giller, Skelly, & Bribiescas, 2016). However, on-site wastewater treatment can result in groundwater and surface water contamination (Robertson, Cherry, & Sudicky, 1991), particularly when these systems are malfunctioning, aging, or improperly installed. Surface water pollution from on-site wastewater has significant implications for environmental quality, as sewage often contains diverse contaminants (Schaider, Rodgers, & Rudel, 2017) that represent threats to aquatic ecosystems, including reproductive impairment.

Endocrine-disrupting compounds (EDCs) can present important risks to aquatic organisms, particularly in surface waters influenced by reclaimed wastewater (Vajda et

al., 2008). Although exposure to EDCs and their effects in surface waters have been extensively studied, exposure and effects of EDCs and other contaminants of emerging concern (CECs) have received much less attention in rural and peri-urban watersheds affected by on-site wastewater treatment systems (Du et al., 2014; Garcia et al., 2013; Schaider et al., 2017). For example, Scott et al. (2016) and Scott et al. (2019) identified aquatic hazards of CECs in a watershed heavily influenced by on-site systems to exceed several urban bayous with major centralized effluent discharges. In addition, the use of biological models has been paramount in evaluations of EDC-associated effects, as they are able to identify the magnitude of estrogenicity in fish populations inhabiting polluted environments (Franco et al., 2020; Huggett et al., 2003). Though previous studies (Guyader et al., 2018) reported exposure to and effects of EDCs in fish from lakes influenced by on-site wastewater treatment systems, these diffuse influences of EDCs on water quality are less defined than for urban systems influenced by point source discharges.

The estrogenic potential of wastewater becomes even more concerning in systems influenced by marked weather dynamics, where instream flows and effluent dilution are subject to seasonal fluctuations (Brooks, Riley, & Taylor, 2006). This scenario is illustrated by McLeod Creek (MC) and East Canyon Creek (ECC) in Park City, Utah, USA, as these streams are directly influenced by seasonal snowmelt and represent important systems for recreationally-important fish species. Given urbanization occurring in the watersheds of these two streams and the potential influences of on-site wastewater treatment systems, we aimed to identify and measure common EDCs and the degree of estrogenicity in fish populations using rainbow trout (*Oncorhynchus mykiss*) as a model

species. Efforts in the present study were specifically informed by a major field study conducted in East Canyon Creek (Franco et al., 2020). We employed previously described laboratory methods (Franco et al., 2020), including gold-standard biomarkers of endocrine disruption in fish that were exposed *in vivo* (intraperitoneally-injected) and *in vitro* (primary hepatocytes) with extracted water samples from different locations along MC and ECC. The present study identifies EDC risks for fish to potentially experience long-term reproductive effects and supports the use of complementary analytical and biological approaches for comprehensive assessments of surface waters influenced by diffuse and decentralized sources of EDCs.

Materials and methods

Sample collection and preparation

Water samples were collected in duplicate during the months of March, May and August 2019, from a total of five sites, two (sites 4 and 5) on MC and three (sites 1, 2 and 3) on ECC (Fig. 5.1) using acetone-cleaned 4.0 L glass bottles. The selection of sites was based on the density of parcels with on-site wastewater treatment systems and, thus, the potential to impact these streams. We included an upstream site, considered to be a negative field control, and four sites downstream from this location, strategically selected to investigate potential estrogenicity associated with on-site effluents. Sampling events also accounted for the influence of seasonal snowmelt on streamflow, with lower instream flows in August, followed by higher flows in March and the highest in May. Stream discharge data were obtained from USGS flow-gage stations located in MC (USGS 10133600; Fig. 5.2A) and ECC (USGS 10133650; Fig. 5.2B).

Extraction of estrogenic compounds and intraperitoneal (IP) injections were conducted in the laboratory following methods previously described in (Franco et al., 2020). Briefly, extraction was conducted by passing 700 mL of unfiltered water through methanol-activated Empore™ 47 mm C18 extraction disks (Dyneon LLC, Oakdale, MN, USA). Disks were then eluted with 10 mL of methanol, and eluents were dried under nitrogen and resuspended in 700 µL of ethanol. A 200 µL aliquot was further dried and resuspended in 200 µL of dimethyl sulfoxide (DMSO). The resulting extracts were used to conduct intraperitoneal (IP) injections in juvenile rainbow trout and to expose primary hepatocytes.

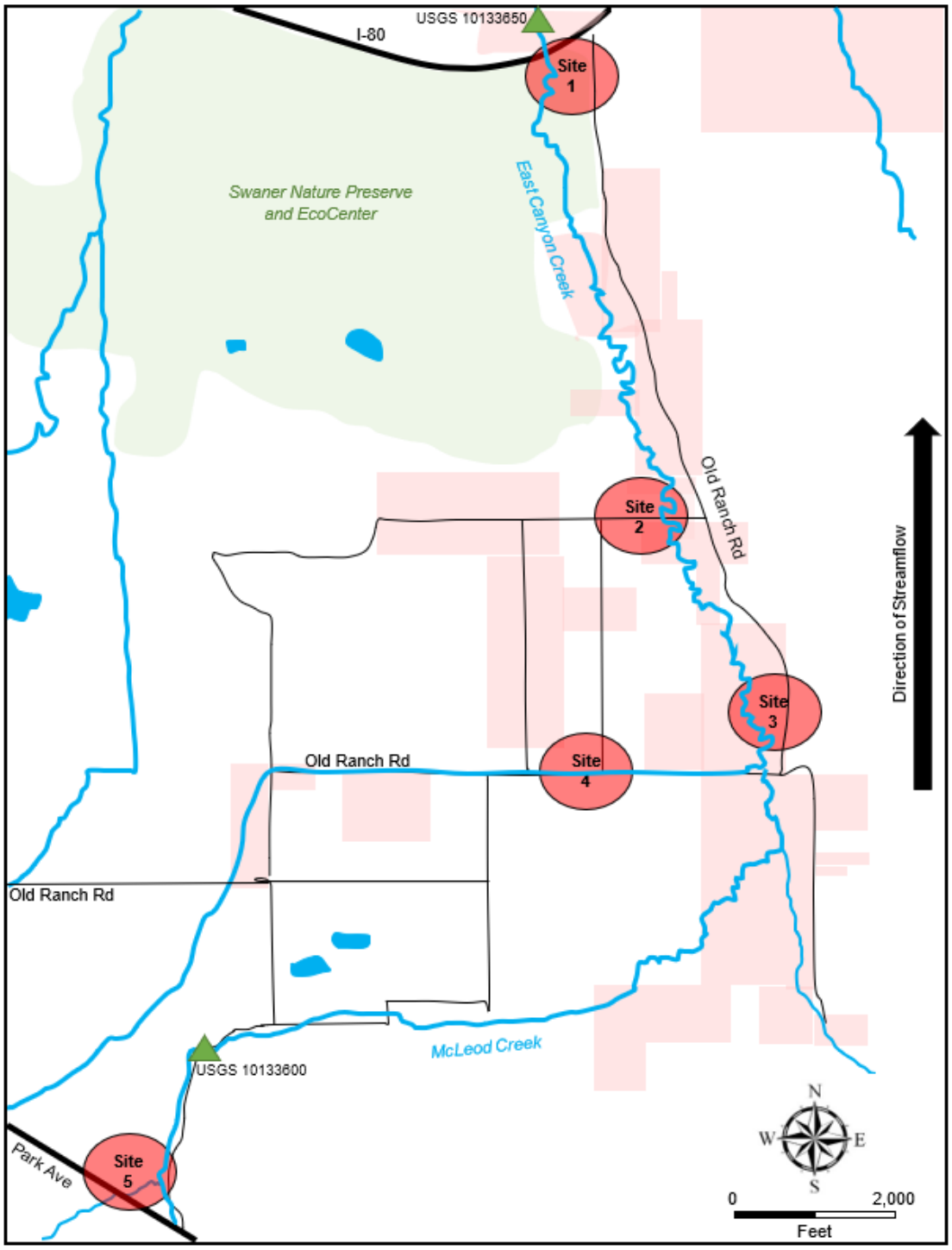

Figure 5.1. Location of sampling sites on McLeod Creek and East Canyon Creek (blue lines), Park City, UT, USA. Black lines represent major roads, and colored areas represent active septic parcels serving the nearby urban and suburban areas (pink) and the Swaner Nature Preserve (green).

Fish, vitellogenin (VTG) expression and quantification

Juvenile rainbow trout were obtained from Westover Farms (Steelville, MO, USA) and maintained in a living stream system (Frigid Units Inc., OH, USA) acclimated to ~12 °C and 1.3-1.5 mS/cm water. Gonadal development in these fish was in early stages and clear differentiation between males and females was not possible. However, the selection of juvenile fish was based on the rationale that VTG differences are significant and more prominent when individuals attain sexual maturity, and that elevated concentrations of VTG in immature fish are often the result of external factors associated with VTG induction (Copeland, Sumpter, & Croft, 1986; Franco et al., 2020). For IP injections, fish with mean length (cm), weight (g) and condition factor of 13.12 ± 1.35, 21.33 ± 5.50, and 0.94 ± 0.14, respectively, were anesthetized by immersion in 2-4°C water, injected with 2.5 µL g- of ethanol suspensions and returned to 2.5-gallon glass aquaria. After 72 h, fish received a second IP injection. Negative controls consisted of fish injected with 95% ethanol (v/v). After a 7-day exposure, 1 mL plastic syringes were used to draw blood through caudal venous punctures. Collected blood was transferred to microcentrifuge tubes and spun at 1,300 g for 10 min. Plasma was collected, transferred to 1.5 mL cryovials, and stored at -80°C until plasma VTG levels were quantified. Concentrations of VTG in rainbow trout plasma were measured using an enzyme-linked immunosorbent assay (ELISA; Biosense Laboratories, Bergen, Norway).

For *in vitro* bioassays, primary hepatocytes were isolated from juvenile fish and homogenized as described by (Lavado et al., 2009). Three biological replicates and two technical replicates were cultured in 12-well plates using L-15 medium supplemented with 10% inactivated fetal bovine serum (FBS). Cells were maintained in a humidified

incubator at 19°C and ambient air. After 24 h from seeding, hepatocytes were exposed to 1% of the extract-DMSO suspensions for 48 h. Cells were then collected for mRNA purification via the SV Total RNA Isolation System (Promega Corporation, WI, USA), and 80 µmol of purified mRNA were transferred to a 96-well qPCR plate. Expression of liver VTG was quantified via the Power SYBR® Green RNA-to CTTM 1-Step Kit (Applied Biosystems, CA, USA). The housekeeping genes β-actin and 18S rRNA were run in parallel, though 18S was used for final comparison. RT-qPCR reactions were conducted in a StepOneTM thermocycler (Thermo Fisher Scientific, MA, USA). Detailed procedures for ELISA and RT-qPCR bioassays are reported in (Franco et al., 2020).

Targeted chemical analyses

A targeted analysis using liquid chromatography-tandem mass spectrometry (LC-MS/MS; Agilent Technologies, Santa Clara, CA, USA) was performed for eight common EDCs potentially present in water samples: 17α-estradiol, 4-nonylphenol, 17β-estradiol, 17α-ethynylestradiol, equilenin, equilin, estriol, and estrone. Analytical instrumentation and instrument calibration, limits of detection, linear ranges, and extraction efficiencies (% recovery) are fully described in (Franco et al., 2020).

Data analyses

Concentrations of plasma VTG and liver expression were analyzed for significant differences among sampling seasons and sites via generalized linear models (GLM) followed by Tukey pairwise comparisons if significance was detected ($\alpha = 0.05$). VTG values below limits of detection, similar to analytical measures, were considered to be ½

of the minimum detected level. Analyses were carried out using the R statistical software (version 3.5.3) and through the R-Studio platform for Windows.

Results and Discussion

Among sampling seasons, instream flows of both streams were highest in May, with mean flows of 1.44 ± 0.26 and 2.05 ± 0.33 m^3 s^- for MC (Fig. 5.2A) and ECC (Fig. 5.2B), respectively. This directly resulted from seasonal snowmelt, as accumulated snowpack in the surrounding areas led to significant inflows of water in both systems. Contrarily, in August, where temperatures were higher, and the snowpack was absent, both MC and ECC registered the lowest discharge, with less than 0.3 m^3 s^- for both streams.

Plasma VTG was significantly higher in fish exposed to extracts from surface water samples during low instream flows (August), whereas the lowest VTG concentrations were observed when stream flows were highest (May; $p = 0.0008$, Fig. 5.3). Significantly elevated VTG levels also corresponded to the most downstream location (Site 1; 467.5 ± 188.3 ng mL^-; $p = 1.62e^{-7}$), which was located within the Swaner Nature Preserve and EcoCenter, but downstream from a number of parcels with on-site treatment systems. Furthermore, water extracts from site 2 led to moderately elevated VTG concentrations of 337.3 ± 158.8 ng mL^-. Under intermediate instream flows (March), the highest VTG levels were 181.1 ± 138.7 and 209.1 ± 101.9 ng mL^- in fish exposed to water extracts from sites 1 and 2, respectively. VTG induction from extracts derived from the remaining sites was significantly lower. Finally, while extracts from water samples collected under higher stream flows (May) at site 1 caused elevated VTG

concentrations relative to observations in control fish, these levels were significantly lower than VTG in fish exposed to extracts from the two other seasons.

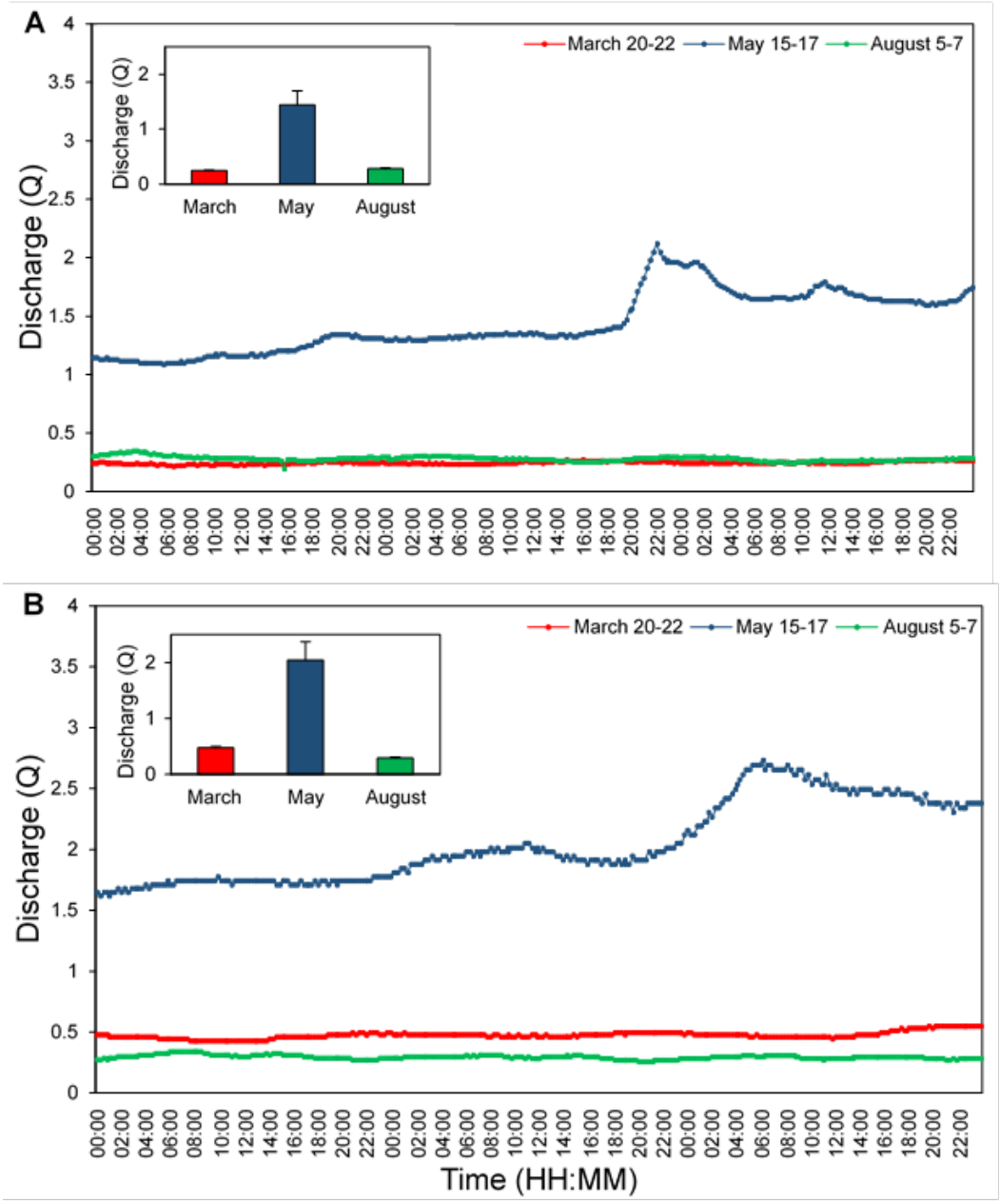

Figure 5.2. Mean and continuous stream discharge (m^3 s^-) in A) McLeod Creek (USGS 10133600) and B) East Canyon Creek (USGS 10133650) 24-h before and after the day of sampling in March, May, and August 2019.

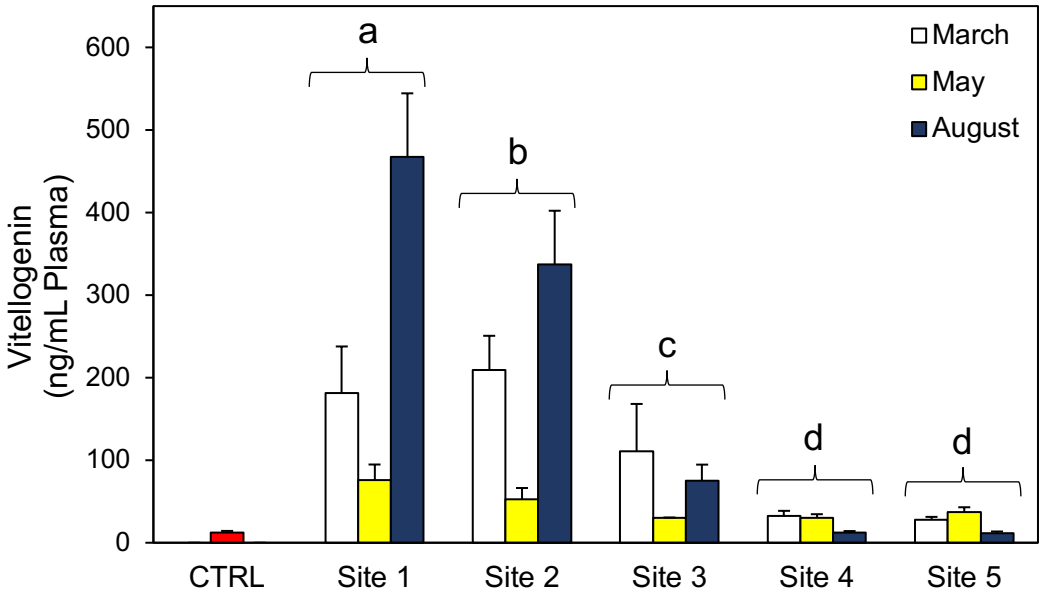

Figure 5.3. Mean plasma vitellogenin (ng mL^{-}) ± SEM in juvenile rainbow trout intraperitoneally-injected with extracted water samples collected in McLeod Creek (sites 4 and 5) and East Canyon Creek (sites 1-3). Different letters denote significant differences among sites (Tukey post-hoc; $p < 0.05$).

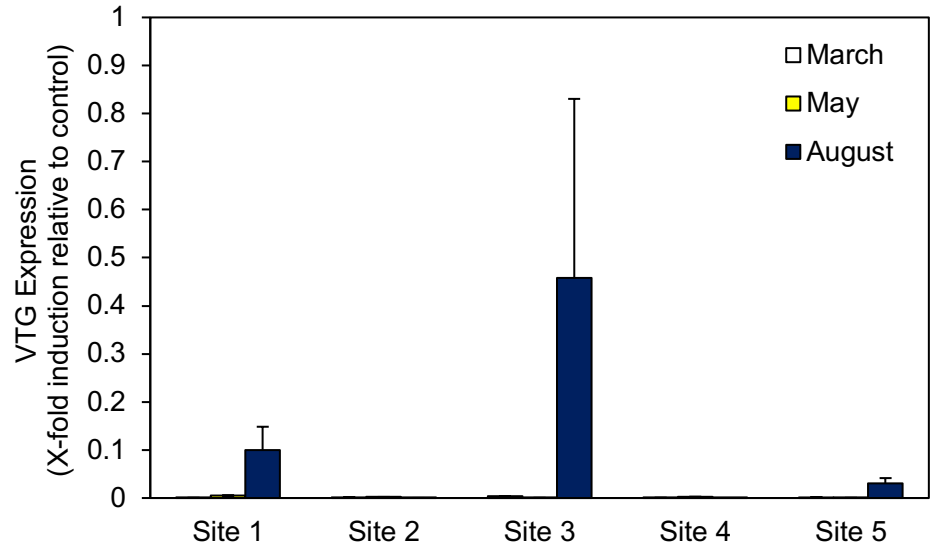

Figure 5.4. Mean liver VTG expression ± SEM in rainbow trout primary hepatocytes exposed to extracted water samples collected in McLeod Creek (sites 4 and 5) and East Canyon Creek (sites 1-3).

Based on these observations, estrogenicity to fish, apparently driven by active on-site treatment systems, significantly differed over space and time in the present study. Gradients of VTG increases from the upstream to the most downstream location were observed in all seasons, with the highest VTG induction caused by extracts from sites 1 and 2. Surface waters from sampling locations near dense on-site activity appeared to induce the highest VTG in juvenile rainbow trout. Similarly, water samples collected in the season with the lowest instream flows also induced more plasma VTG. In this case, seasonal instream dilution dynamics of these systems appear to play a fundamental role in modifying exposure to EDCs. For semi-arid streams, such as the ones evaluated in the present study, effluent-impacted waters may require unique management strategies (Luthy, Sedlak, Plumlee, Austin, & Resh, 2015), and during dry seasons, they may represent worse-case scenarios for exposure to EDCs and other chemicals often found in wastewater (Brooks et al., 2006).

Estrogenicity was not evident from the experimentation with primary hepatocytes, as VTG expression was rather low and did not point out major differences among sites nor seasons (Fig. 5.4). VTG expression appeared to be higher in fish exposed to extracts collected in August from sites 1 and 3, yet the high variability among replicates impairs the ability to draw conclusions. The *in vitro* bioassays included in this study, despite their wide application in environmental research (Baksi & Frazier, 1990), presented low sensitivity from exposure to environmental samples, as it was also shown in Franco et al. (2020). However, assessments of estrogenicity must include integrated evaluations covering different experimental scales, from traditional analytical approaches to more complex whole-animal models (Burki et al., 2006). Thus, the applicability of *in vitro*

systems to evaluate environmental samples must continue to be explored and may require further optimization to successfully extrapolate *in vitro* data to whole animal responses. Furthermore, in the present study, by quantifying the presence of plasma VTG in whole animals, we provide evidence of potential estrogenicity beyond the gene expression level.

Juvenile fish have been successfully used in endocrine disruption assessments, most commonly by measuring the expression and presence of plasma VTG (Copeland et al., 1986). In the present study, biological responses in IP-injected rainbow trout are indicators of potential endocrine disruption in both MC and ECC. Whether fish in these recreationally important sub-watersheds will experience adverse effects at the population levels is not known but presents an important consideration for water quality management and wastewater practitioners. It is also important to point out that, in the case of rainbow trout and other trout species, spawning takes place in the spring and the beginning of the summer, which corresponded to seasons where more significant impact from the on-site treatment systems was observed. Therefore, in effluent-impacted streams, a combined effect of estrogenic substances and increasing temperature cues for spawning may alter reproductive processes, even in individuals that have not fully attain sexual maturation.

Based on previous assessments where VTG concentrations in rainbow trout reached levels in the μg and mg per mL plasma (two orders of magnitude higher than levels observed in the present study) (Harries et al., 1997), estrogenicity in MC and ECC may not require immediate attention. However, continuous exposure under low instream flow conditions may present more significant long-term risks for fish populations. Furthermore, plasma VTG concentrations in IP-injected fish may be representative of

impacted individuals living in effluent-dominated waters when the sensitivity of bioassays is sufficient to provide informative data, as was also demonstrated in (Franco et al., 2020).

The targeted chemical analyses did not identify any of the target EDCs considered in the study. However, while neither of the target EDCs appeared to be causative of the observed biological effects, the significant VTG concentrations in fish exposed to water samples collected near and downstream from areas with on-site activity may suggest the presence of other compounds that were not identified in this study. This represents further evidence highlighting the importance of including non-targeted analysis, which could provide more detailed information about the presence of different EDCs, and employing bioassays with well-established biological models for surface assessments of EDCs, where traditional targeted chemical analysis can be enhanced by multiple lines of evidence. Specific testing strategies, such as whole animal approaches, are fundamental in assessing the effects of EDCs (Huggett et al., 2003) and provide useful information to support environmental management decisions in rural and peri-urban watersheds with elevated on-site wastewater treatment systems.

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CHAPTER SIX

Xenobiotic Metabolism in the Fish Hepatic Cell Lines Hepa-E1 and RTH-149, and the Gill Cell Lines Rtgill-W1 and G1B: Biomarkers of CYP450 Activity and Oxidative Stress

This chapter published as: Franco, M. E., Sutherland, G. E., Lavado, R. (2018). Xenobiotic metabolism in the fish hepatic cell lines Hepa-E1 and RTH-149, and the gill cell lines RTgill-W1 and G1B: biomarkers of CYP450 activity and oxidative stress. *Comparative Biochemistry and Physiology, Part C: Toxicology and Pharmacology*, 206, 32-40.

Abstract

The use of fish cell cultures has proven to be an effective tool in the study of environmental and aquatic toxicology. Valuable information can be obtained from comparisons between cell lines from different species and organs. In the present study, specific chemicals were used and biomarkers (e.g. 7-Ethoxyresorufin-O-deethylase (EROD) activity and reactive oxygen species (ROS)) were measured to assess the metabolic capabilities and cytotoxicity of the fish hepatic cell lines Hepa-E1 and RTH-149, and the fish gill cell lines RTgill-W1 and G1B. These cell lines were exposed to β-naphthoflavone (BNF) and benzo[a]pyrene (BaP), the pharmaceutical tamoxifen (TMX), and the organic peroxide tert-butylhydroperoxide (tBHP). Cytotoxicity in gill cell lines was significantly higher than in hepatic cells, with BNF and TMX being the most toxic compounds. CYP1-like associated activity, measured through EROD activity, was only detected in hepatic cells; Hepa-E1 cells showed the highest activity after exposure to both BNF and BaP. Significantly higher levels of CYP3A-like activity were also observed in Hepa-E1 cells exposed to TMX, while gill cell lines presented the lowest levels. Measurements of ROS and antioxidant enzymes indicated that peroxide levels were

higher in gill cell lines in general. However, levels of superoxide were significantly higher in RTH-149 cells, where no distinctive increase of superoxide-related antioxidants was observed. The present study demonstrates the importance of selecting adequate cell lines in measuring specific metabolic parameters and provides strong evidence for the fish hepatocarcinoma Hepa-E1 cells to be an excellent alternative in assessing metabolism of xenobiotics, and in expanding the applicability of fish cell lines for *in vitro* studies.

Introduction

Increased attention has been directed towards the necessity of identifying known and emerging environmental contaminants, and the ecotoxicological effects associated with them. Examples of these efforts include the Registration, Evaluation, Authorization, and Restriction of Chemicals (REACH) initiated by the European Union, and the U.S. Toxic Substances Control Act (TSCA) (Schmidt, 2016; Williams, Panko, & Paustenbach, 2009). For several years, toxicity tests using fish models have been important tools in assessing the effects of chemicals on living organisms (Powers, 1989). It has been established that the aquatic ecosystem is one of the most diverse ecosystems and comprise a significant percentage of vertebrates used in models for toxicity assays in environmental and biomedical studies (N.C. Bols, Dayeh, Lee, & Schirmer, 2005; Goswami, Dubey, Yadav, Sharma, & Lakra, 2015; Lakra, Swaminathan, & Joy, 2011; Taju, Abdul Majeed, Nambi, & Sahul Hameed, 2017). Aquatic organisms are often exposed to contaminants such as pharmaceuticals and polycyclic aromatic hydrocarbons (PAHs), which often result from anthropogenic activities (N.C. Bols et al., 2005). Toxicity tests are often based on *in vivo* assays, and although the results from these whole

organism assays can be effectively extrapolated to the natural environment, the use of animals has been debated for ethical reasons, as well as for animal welfare issues (Fent, 2001; Schirmer et al., 2008; Thibaut, Schnell, & Porte, 2009). Initiatives such as the "3 Rs", standing for Refinement, Reduction and Replacement, have been implemented to find suitable alternatives to the use of animals (Guhad, 2005), increasing the need for appropriate *in vitro* systems for toxicity testing. One of such alternatives to *in vivo* studies is the use of cell cultures. Cell lines have become a cost-efficient and effective *in vitro* tool in aquatic toxicology as they allow for the determination of the interference with biological processes by contaminant exposure (Castano & Gomez-Lechon, 2005; H. Segner & Braunbeck, 2003).

Segner (1998), Bols et al. (2005), Nichols et al. (2006), Goswami et al. (2015), and Taju et al. (2017) have shown fish cell lines to accurately represent *in vivo* metabolic capability. Recently, Lakra et al. (2011) reported 283 cell lines derived from fish and their use in life sciences – this number continues to increase. For example, the cell line PLHC-1, a hepatocellular carcinoma cell line derived from the desert topminnow (*Poeciliopsis lucida*), has been widely used to evaluate cytotoxicity and metabolic activity, as it has the capacity to express the cytochrome P4501A system as well as containing the aryl hydrocarbon receptor (AhR) (Fent, 2001). While important and novel information has been obtained from using fish cell lines in toxicological assays (H. Babich, Rosenberg, & Borenfreund, 1991; Caminada, Escher, & Fent, 2006; Castano & Gomez-Lechon, 2005; Fent, 2001; Huuskonen, Hahn, & Lindstrom-Seppa, 1998), some limitations have also been described in terms of substance specificity (local vs systemic toxicity), loss of biotransformation abilities by cells, and dose-response relationships that

cannot be readily extrapolated to environmentally realistic scenarios in the same manner as it has been done for *in vivo* assays (Thibaut et al., 2009). These issues have led to the constant development of new cell lines originating from different fish species, organs, and regions of the world (J. L. Fryer & Lannan, 1994; Lakra et al., 2011). The use of cell lines in ecotoxicology has been increasing in recent years, and while several publications have reported valuable data using *in vitro* models, few studies have focused on characterizing molecular and biochemical properties of existing cell lines. Thus, it is fundamental to continue the search for more suitable cell lines as models to select appropriate alternatives to address specific environmental issues and for effective ecological risk assessments.

In the present study, four fish cell lines, two originating from the liver (Hepa-E1 from *Anguilla japonica* and RTH-149 from *Oncorhynchus mykiss*), and two from the gills (RTgill-W1 from *Oncorhynchus mykiss* and GIB from *Clarias batrachus*) were assessed for their metabolic capabilities after being exposed to selected xenobiotics: the polycyclic aromatic hydrocarbons β-naphthoflavone (BNF) and benzo[*a*]pyrene (BaP), the pharmaceutical tamoxifen (TMX), and the industrial oxidizer *tert*-butylhydroperoxide (*t*BHP). BNF and BaP are both potent agonists of the aryl hydrocarbon receptor, and as such, are inducers of detoxification enzymes such as cytochromes P450 (CYPs) (Carlson, Li, & Zelikoff, 2004; Frasco & Guilhermino, 2002; Wilson, Vijayan, Kennedy, Iwama, & Moon, 1998). The pharmaceutical TMX is known to block estrogen receptors in cancer cells, which is why it is highly used in estrogen receptor-positive breast cancer treatments (Lumachi et al., 2011). However, TMX has been found in surface water of urban effluents and other important water sources at significant concentrations, and while

information on cytotoxicity of TMX is limited, previous studies have identified the induction of CYP3A and CYP1B by TMX (Desai et al., 2002). *t*BHP, an organic peroxide, is described as a strong inducer of reactive oxygen species (ROS) and the consequent induction of oxidative stress in fishes (Ploch, Lee, MacLean, & Di Giulio, 1999).

The goal of the present study is to provide a thorough biochemical characterization of fish cell lines; specifically, on the metabolic capabilities of Hepa-E1, RTH-149, RTgill-W1, and G1B to respond to contaminants. A better knowledge of the metabolic capabilities of fish liver cell lines should help to select the most adequate ones for both toxicity and metabolism studies. The cell lines selected are evaluated in terms of their ability to show biomarker responses such as CYP1A-like activity (assessed as ethoxyresorufin-*O*-deethylase (EROD) activity), CYP3A4-like activity (assessed as testosterone 6β-hydroxylase activity), generation of ROS (hydrogen peroxide and oxygen superoxide production), and activity of antioxidant enzymes (catalase, superoxide dismutase and glutathione peroxidase). These cell lines were also evaluated in terms of cytotoxicity (resistance to the selected compounds). Moreover, comparisons between these cell lines are provided as a reference to determine the best alternatives when assessing particular contaminants and/or biochemical responses after exposure.

Materials and Methods

Chemicals and solutions

Eagle's minimum essential medium, Leibovitz's L-15, L-glutamine, penicillin G, streptomycin, phosphate-buffered saline (PBS) and trypsin–EDTA were obtained from

Gibco Life Technologies (ThermoFisher Scientific, Waltham, MA). Ham's F-12K (Kaighn's) medium was obtained from ATCC (Manassas, VA). Fluorescamine, H_2-DCFDA (2',7'-dichlorodihydrofluorescein diacetate), DHE (dihydroethidium), and HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) were obtained from Sigma-Aldrich (St. Louis, MO). Fetal bovine serum was obtained from Atlas Biologicals (Fort Collins, CO). Acetonitrile and isopropanol were of analytical grade (ThermoFisher Scientific, Waltham, MA). Deionized water (DI water) was obtained using a Milli-Q water purification system (Millipore, Billerica, MA).

Cell culture

The cell lines used in this study are shown in Table 6.1. All media was supplemented with 2 mM L-glutamine, 50 U/mL penicillin G, and 50 μg/mL streptomycin.

Experiments were carried out on confluent cell monolayers obtained after seeding cells ($1x10^5$ cells/cm^2) in 96-well plates or 12-well plates (antioxidant enzymes) and allowing them to grow for 24 h (Hepa-E1), 36 h (RTgill-W1), and 48 h (RTH-149 and G1B). The culture medium was then changed and replaced by medium containing the selected xenobiotics or solvent control medium. Plates were returned to the incubator for a 48-h exposure period. The experiments were performed with biological (plates) and technical (wells) replicates. Four to five biological with six technical replicates were used to determine cytotoxicity, CYP1-like and CYP3A-like activities. To determine radical oxygen species (ROS) production, five biological with five technical replicates were used. For the antioxidant enzymes determination, two biological with three technical replicates were used.

Table 6.1. Fish cell lines used in this study (ATCC: American Type Culture Collection. ECACC: European Collection of Authenticated Cell Cultures).

Cell line	Fish species	Tissue	Provider	Culture conditions	Reference
RTH-149	Rainbow trout (*Oncorhynchus mykiss*)	Liver	ATCC (CRL-1710)	Eagle's minimum essential medium + 10% fetal bovine serum, in a humidified incubator with 5% CO_2 at 21°C	Lannan et al. (1984)
Hepa-E1	Japanese eel (*Anguilla japonica*)	Liver	ECACC (99072812)	E-RDF medium* + 5% heat-inactivated fetal bovine serum, in a humidified incubator 5% CO_2 at 26°C	Riken Cell Bank, Japan
RTgill-W1	Rainbow trout (*Oncorhynchus mykiss*)	Gill	ATCC (CRL-2523)	Leibovitz's L15 medium + 10% fetal bovine serum, in a humidified incubator with atmospheric air at 19°C.	Bols et al. (1994)
G1B	Walking catfish (*Clarias batrachus*)	Gill	ATCC (CRL-2536)	Ham's F-12K (Kaighn's) medium + 10% fetal bovine serum, in a humidified incubator with 5% CO_2 at 26°C.	Noga and Hartmann (1981)

* E-RDF medium was obtained from Kyokuto Pharmaceuticals, Tokio, Japan.

Cytotoxicity

The viability of the cells was assessed after the exposure to the selected compounds by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (Lavado, Loyo-Rosales, et al., 2009). The MTT assay is a colorimetric assay based on the ability of NAD(P)H-dependent cellular oxidoreductase enzymes of reducing the tetrazolium dye MTT to its insoluble formazan form, which has a purple color. The color was measured by absorption spectroscopy (BioTek Synergy™ H1) at 595 nm and it reflected the number of viable cells present.

EROD (7-Ethoxyresorufin-O-deethylase) activity (CYP1A-like associated activity)

7-ethoxyresorufin-O-deethylase (EROD) activity gave a measurement of CYP1A-mediated metabolism. This activity was measured by the deethylation of 7-ethoxyresorufin in the presence of endogenous NADPH to resorufin, a strongly fluorescent molecule emitting at 590 nm. The measurement of EROD activity was made in intact, live cells as described by Heinrich et al. (2014). Briefly, the cells were seeded in 96-well plates at different densities (depending on the cell line) and allowed to grow to confluence, before the 48-h xenobiotic exposure. Cells were washed with PBS and then exposed to 200 μL of 7-ethoxyresorufin solution. The plates were read every minute for 20 minutes to measure the formation of resorufin (excitation/emission wavelength 530/590 nm). The detection limit of the assay was 0.1 pmol/min/mg protein.

Preparation of S9 fractions from cell cultures

S9 fractions were prepared as described by Thibaut et al. (2009) with minor modifications. Cells were detached from culture plates with 0.05% (w/v) trypsin and 0.5 mM ethylenediaminetetraacetic acid (EDTA) and washed with PBS. Then, cells were homogenized in cold 100 mM KH_2PO_4/K_2HPO_4 buffer pH 7.4, containing 100 mM KCl, and 1 mM EDTA. S9 fractions were obtained after centrifugation at 12,000g for 20 min at 4°C and immediately stored at -80°C. Protein concentrations were determined by the Coomassie Blue method using a commercial kit (Pierce Inc., Rockford, IL) and bovine serum albumin (BSA) as a standard.

Testosterone hydroxylase activity (CYP3A-like associated activities)

Testosterone hydroxylase activity was measured in S9 fractions as described by Lavado et al. (2009) with minor modifications. Briefly, cells were incubated with 50 μM

testosterone and 500 μM NADPH in a final volume of 150 μL of 50 mM Tris-HCl, 10 mM $MgCl_2$, pH 7.4. Samples were incubated for 2 h at 25°C. Incubations were stopped by adding 100 μL of acetonitrile, and after centrifugation (12,000g for 10 min), 10 μL of supernatant were injected into a reverse-phase ultra-high-performance liquid chromatography (UHPLC) system. Negative controls consisted of identical additives but excluding NADPH or the acetonitrile to denature proteins before the start of the reaction. UHPLC analyses were performed on a Thermo Dionex Ultimate 3000 UHPLC system equipped with a 150 mm × 2.1 mm Vanquish C18 (2.2 μm) reverse-phase column (ThermoFisher Scientific). Separation of testosterone metabolites employed an HPLC gradient system elution at a flow rate of 0.5 mL/min with a mobile phase composed of (A) 75% water and 25% acetonitrile and (B) 45% water and 55% acetonitrile. The run consisted of a 10-min linear gradient from 100% A to 100% B. Chromatographic peaks were monitored by a diode-array detector (DAD) at 254 nm. Metabolites (6β- and 16β-hydroxytestosterone) were identified by co-chromatography with authentic standard compounds and quantified by integrating the area under the peaks (the detection limit was 0.5 pmol/min/mg protein).

Protein measurement in cell culture wells (for EROD and CYP activities)

The amount of protein in cell culture wells was determined with fluorescamine as described by Thibaut et al. (2009), and using BSA as standard. Briefly, the culture medium was removed from the wells and the cell monolayer rinsed with PBS. The plates were then frozen at -80°C for 24 h. The plates were thawed at room temperature to disrupt the cells; PBS and 250 μL of fluorescamine solution (0.3 mg/mL in acetonitrile)

were added to each well. After 5 min of shaking in the dark, the plates were read at excitation and emission wavelengths of 360 and 460 nm, respectively.

Measurement of reactive oxygen species (ROS)

Superoxide anions were detected by dihydroethidium (DHE) staining according to Antherieu et al. (2013) with modifications. Briefly after exposure, cells were incubated with 5 μM DHE for 30 minutes at 19°C (RTgill-W1 cells), 21°C (RTH-149 cells), and 26°C (Hepa-E1 and G1B cells). They were then washed with chilled phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde, and fluorescence intensity was determined using excitation/emission wavelengths of 535/635 nm.

Hydrogen peroxide generation was determined by the 2',7'-dichlorodihydrofluorescein diacetate (H_2-DCFDA) assay according to Antherieu et al. (2013), with minor modifications. Briefly, cells were incubated for 2 h with 5 μM H_2-DCFDA; then they were washed with cold PBS and scraped in 1:1 potassium buffer (10 mM, pH 7.4):methanol (v/v) completed with 0.1% Triton X-100. Fluorescence intensity of cell extracts were determined using excitation/emission wavelengths of 498/520 nm.

Measurement of antioxidant enzymes

Measurements of the antioxidant enzymes catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPX) were performed in S9 fractions, and by using 96-well plate bioassay kits (Cayman Chemical, Ann Arbor, MI). CAT was measured from its reaction with methanol in presence of hydrogen peroxide; the formaldehyde produced was measured colorimetrically with the chromogen 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole at 540 nm (Johansson & Borg, 1988). Total SOD was measured

by the detection of superoxide radicals generated by the enzymes xanthine oxidase and hypoxanthine in presence of a tetrazolium salt. After a 30-min incubation period, plates were read at 450 nm. Lastly, GPX activity was indirectly measured with a coupled reaction with glutathione reductase, where endogenous NADPH was oxidized to $NADP^+$; the decreasing rate of GPX activity was measured kinetically at 340 nm for 5 minutes.

Data analysis

Statistical significance was assessed using one-way ANOVA tests to evaluate differences between cell lines, with the use of GraphPad Prism version 7.00 for MacOS (GraphPad Software, San Diego, CA, USA) and the R-studio statistical software (R version 3.4.1). A p-value of less than 0.05 was considered statistically significant unless otherwise indicated. If an overall significance was detected, Tukey's and Bonferroni's multiple range tests were performed for comparing exposure groups. Samples showing levels below the detection limits were considered as having 50% of the minimal values detectable for statistical comparisons. All data were analyzed prior to statistical analysis to meet the homoscedasticity and normality assumptions of parametric tests.

Results

Cytotoxicity

Hepatic fish cell lines (Hepa-E1 and RTH-149) were significantly less susceptible to the exposure to selected xenobiotics than gill cell lines (RTgill-W1 and G1B), as higher cytotoxicity, reported as mean % survival ± standard deviation (SD), was observed for the latter (Fig. 6.1). In hepatic cell lines, significantly higher mortality was observed for RTH-149 exposed to BNF and BHP at a 100 μM concentration ($p<0.05$); the lowest

survival was observed for cells exposed to BHP (64.4% ± 1.7) (Fig. 6.1A). For Hepa-E1 cells, significantly higher toxicity was observed for TMX and BNF at 100 μM ($p<0.05$); at this concentration, survival for cells exposed to TMX was the lowest (49.9% ± 0.3) followed by BNF (80.3% ± 3.4) (Fig. 6.1B).

Fish gill cell lines were more sensitive to these xenobiotics, as higher levels of cytotoxicity were observed in both RTgill-W1 and G1B for all of them. The survival of RTgill-W1 cells exposed to TMX began to be compromised at concentrations as low as 100 nM (77.8% ± 3.1) (Fig. 6.1C) ($p<0.05$). RTgill-W1 survival continued to decrease at higher concentrations for all xenobiotics. The survival for BNF, BaP, BHP, and TMX at 100 μM were 9.4% ± 0.3, 35.4% ± 1.0, 42.7% ± 1.7, and 12.5% ± 0.6, respectively; BNF and TMX were the two compounds with significantly higher cytotoxicity at high concentrations ($p<0.01$). Similarly, G1B cells showed low survival at high xenobiotic concentrations (Fig. 6.1D). Significant survival reductions were observed for G1B cells exposed to 1 μM of BNF (63.6% ± 3.2) and TMX (55.8% ± 3.9). Higher xenobiotic concentrations led to even lower survival rates, being TMX the most cytotoxic compound to G1B cells, followed by BNF and BHP. Percent survival for all cells was normalized based on a 100% survival in control groups.

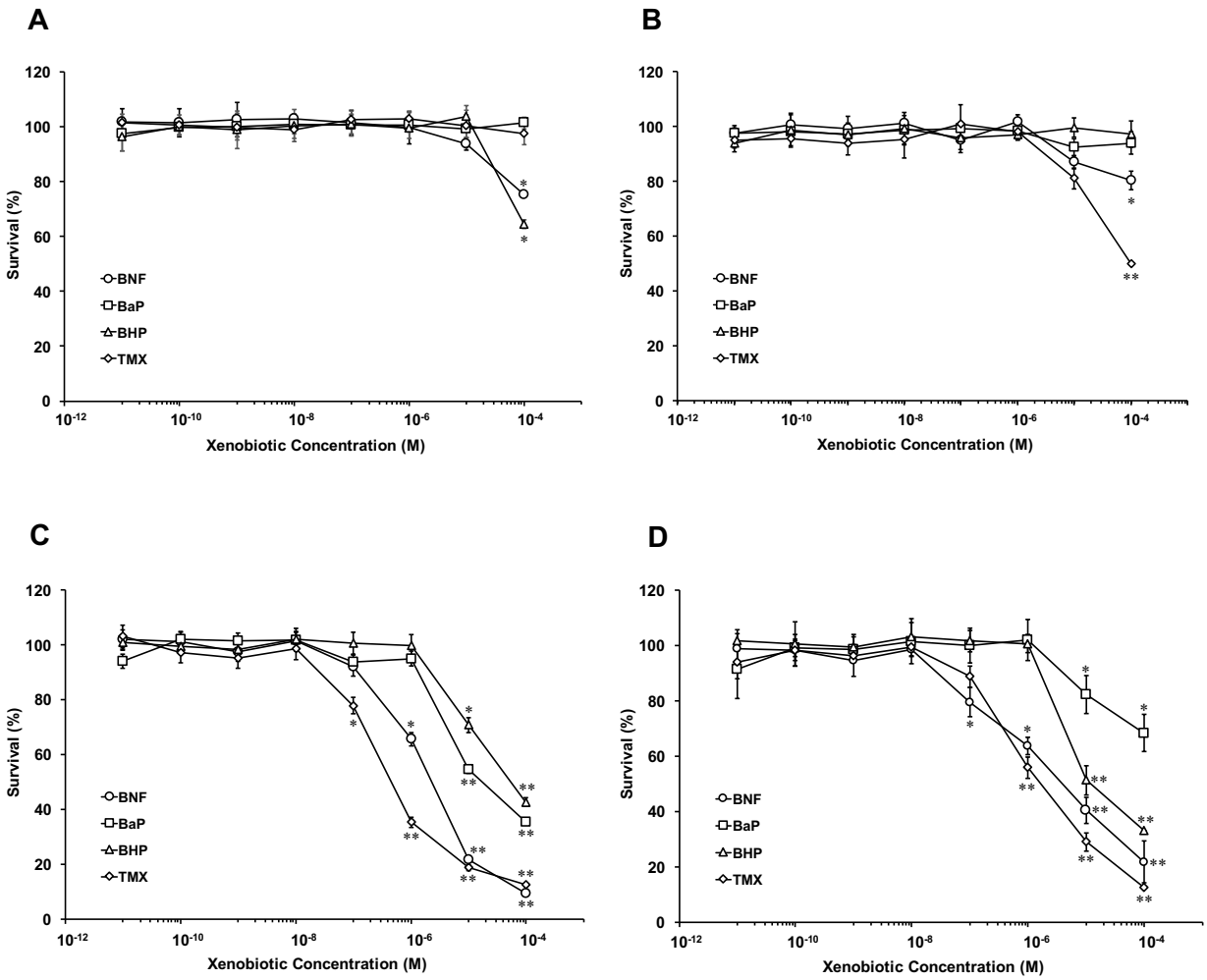

Figure 6.1. Cytotoxic response of the different cell lines RTH-149 (**A**), Hepa-E1 (**B**), RTgill-W1 (**C**), and G1B (**D**) exposed to selected compounds (BNF: β-Naphthoflavone; BaP: benzo[a]pyrene; BHP: *tert*-butylperoxide; and TMX: tamoxifen). Data are presented as mean ± SD (n=5 biological experiments of 6 replicates per experiment). Significant differences with control and solvent control are shown as * (p<0.05; One-way ANOVA) and ** (p<0.01; One-way ANOVA).

EROD (7-Ethoxyresorufin-O-deethylase) activity (CYP1A-like associated activity)

EROD activity was measured in all cell lines exposed to BNF and BaP, and reported as mean pmol/min/mg protein ± SD (Fig. 6.2). Hepatic cell lines exposed to BNF showed significantly higher levels of EROD activity compared to gill cell lines (Fig. 6.2A). Hepa-E1 cells showed the highest activity, with a distinctive spike at 10 nM BNF. The maximum EROD activity in Hepa-E1 cells was 14.0 ± 1.4, at 1 μM BNF, with a slight decrease in activity at the next highest concentration. RTH-149 cells also presented relatively high levels of EROD activity, with a steadier dose-response relationship to BNF concentrations; the maximum activity was 6.0 ± 1.8 at 10 μM. Both RTgill-W1 and G1B showed activity below the detection limits (<0.1 pmol/min/mg protein).

Hepa-E1 cells were the only cell line for which EROD activity was above the detection limits after BaP exposure (Fig. 6.2B). Relatively low activity is observed in the low BaP concentrations; however, activity levels significantly increased at 100 nM and above. The maximum activity value recorded was 12.2 ± 0.9 at 10 μM of BaP. Both gill cell lines and the hepatic RTH-149 cell line showed EROD activity below the detection limits.

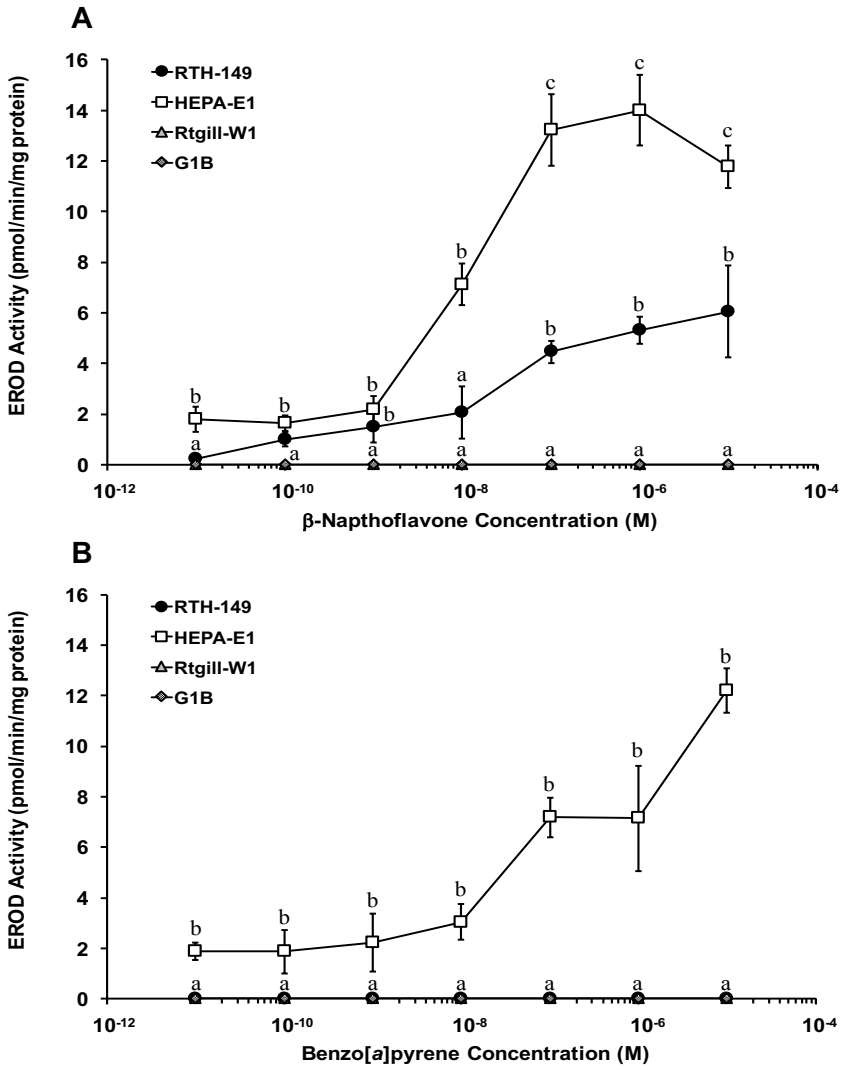

Figure 6.2. CYP1A-like activity, shown as 7-ethoxyresorufin (EROD) activity of the different cell lines RTH-149, Hepa-E1, RTgill-W1, and G1B exposed to β-Naphthoflavone (**A**) and benzo[a]pyrene (**B**). Data are presented as mean ± SD (n=4 biological experiments of 6 replicates per experiment). Different letters show significant differences between different cell lines (p<0.05; One-way ANOVA).

Testosterone hydroxylase activity (CYP3A-like associated activities)

Testosterone 6β-hydroxylase activity, evaluated in S9 fractions isolated from cells, was found to be significantly higher in hepatic cell lines than in gill cell lines (Fig. 6.3). Both hepatic cell lines (Hepa-E1 and RTH-149) showed a dose response relationship with TMX concentrations. Hepa-E1 cells showed the highest activity at

concentrations of 1 μM (54.6 ± 5.8) and 10 μM (53.2 ± 5.7). RTH-149 cells followed Hepa-E1 cells, showing the highest activity at a TMX concentration of 10 μM (48.3 ± 4.8). Relative to gill cell lines, both hepatic cell groups showed a spike in CYP3A-like activity at approximately 100 nM; enzymatic activity stayed relatively low and steady in all cell lines for concentrations of 1 nM and lower. While CYP3A-like activity in both RTgill-W1 and G1B experienced gradual increases, with maximum values between 10 nM and 100 nM, this activity was nearly absent as compared to hepatic cell lines. The metabolite 16β-hydroxytestosterone was not found in any of the testosterone incubations with S9 fractions, suggesting that low or no CYP2-like activity was present in any of the cells characterized (Maldonado et al., 2016).

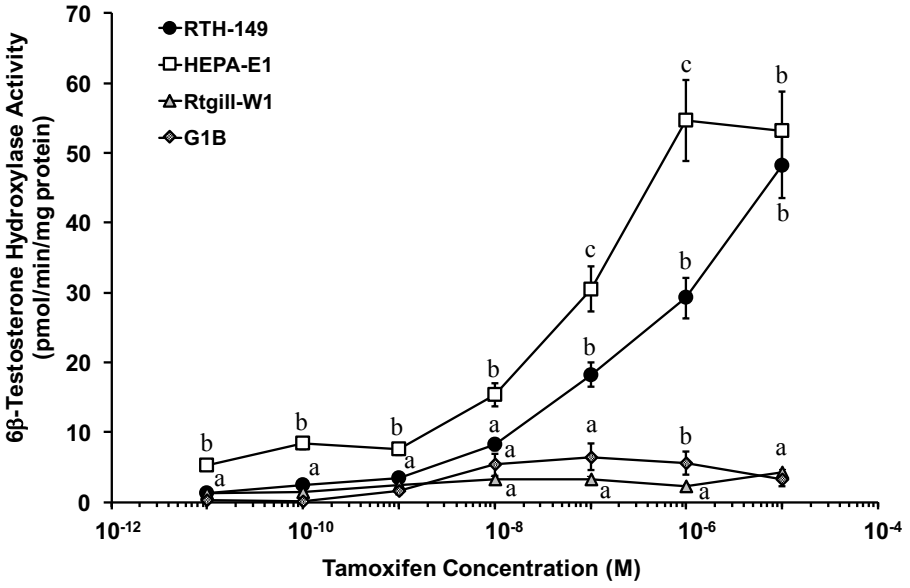

Figure 6.3. CYP3A-like activity, shown as 6β-hydroxytestosterone hydroxylase activity of the different cell lines RTH-149, Hepa-E1, RTgill-W1, and G1B exposed to tamoxifen. Data are presented as mean ± SD (n=4 biological experiments of 6 replicates per experiment). Different letters show significant differences between different cell lines (p<0.05; One-way ANOVA).

Reactive Oxygen Species (ROS)

Peroxide (H_2O_2) and superoxide (O_2^-) production as a result of tert-butyl hydroperoxide (tBHP) exposure was measured in both hepatic and gill cell lines, and it is reported as mean fold induction ± SD with respect to control groups (control and solvent control exposure) (Fig. 6.4). In terms of peroxide production (Fig. 6.4A), RTgill-W1 cells showed significantly higher levels at concentrations of 100 nM and above, with a drastic increase from the closest, lower concentration of 10 nM. The maximum peroxide production of 13.3-fold by RTgill-W1 cells was observed at 10 µM tBHP. G1B cells also showed significantly higher levels of peroxide production as compared to both hepatic cell lines ($p<0.05$). The maximum peroxide level of 10.2-fold by G1B cells was also observed at 10 µM of tBHP. On average, the hepatic cell line RTH-149 showed lower levels of peroxide production compared to both gill cell lines. However, peroxide production started to increase at concentrations of 100 nM and above. The maximum peroxide production by RTH-149 cells was 7.5-fold, recorded at 10 µM tBHP. Contrarily to the other cell lines, peroxide production by Hepa-E1 cells remained particularly low and approximately constant at all tBHP concentrations.

Among all cell lines, hepatic RTH-149 cells showed significantly higher levels of superoxide production at tBHP concentrations of 1 µM and above, with a distinct spike observed at 100 nM (Fig. 6.4B). Both RTgill-W1 and G1B cells showed relatively low and constant levels of superoxide production in low and middle tBHP concentrations. However, significant increases in superoxide levels were observed at 1 µM for RTgill-W1, and at 100 nM for G1B cells. Maximum superoxide production for RTH-149, RTgill-W1, and G1B, all recorded at 10 µM tBHP, were 6.5-, 3.4-, and 3.7-fold,

respectively. Hepa-E1 cells produced the lowest levels of superoxide. Although superoxide production is slightly higher compared to RTH-149 and RTgill-W1 in low and middle concentrations, superoxide levels remain low and steady at all tBHP concentrations in Hepa-E1 cells.

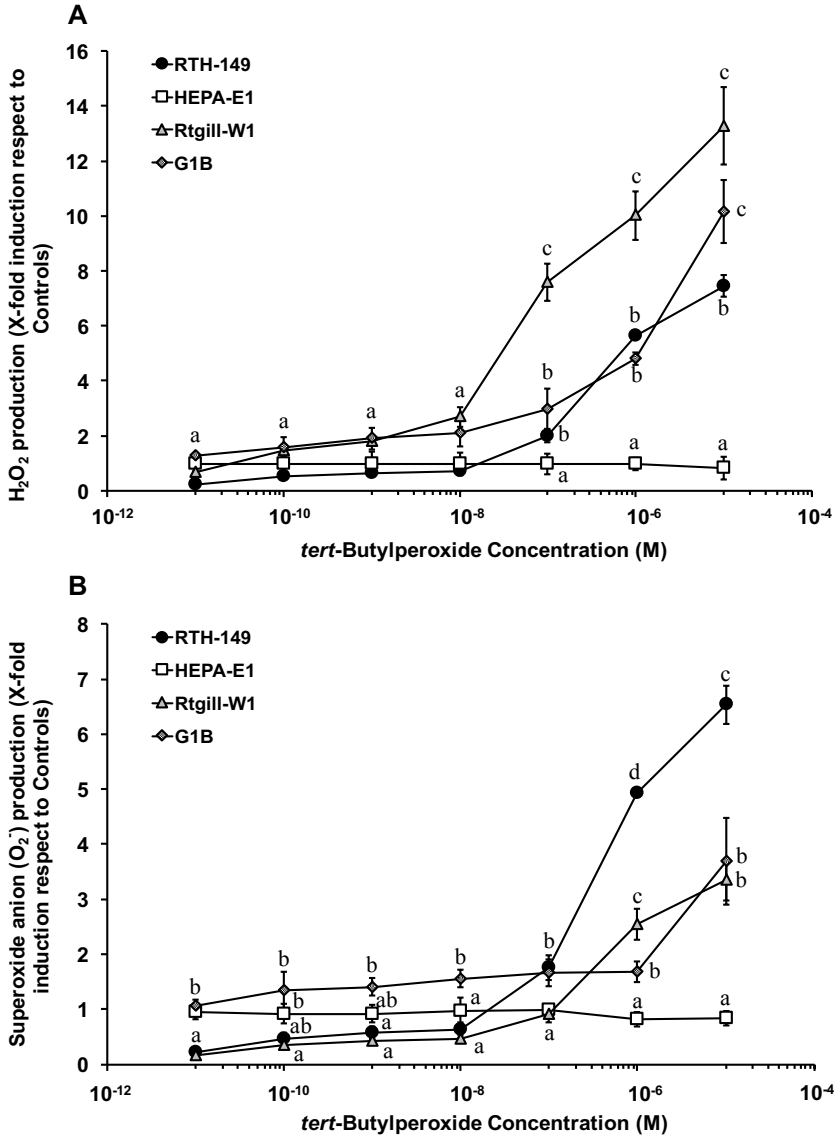

Figure 6.4. Radical Oxygen Species (ROS) production in the different cell lines RTH-149, Hepa-E1, RTgill-W1, and G1B exposed to *tert*-butylperoxide. Data are shown as hydrogen peroxide H_2O_2 production (**A**) and superoxide O_2^- generation (**B**). Data are presented as mean ± SD (n=5 biological experiments of 5 replicates per experiment). Different letters show significant differences between different cell lines (p<0.05; One-way ANOVA).

Antioxidant enzymes

High concentrations of tBHP led to significantly higher CAT activity in both gill cell lines, whereas no significant differences were observed for hepatic cell lines (Table 6.2). The levels of CAT activity were the highest for G1B cells at tBHP concentrations of 10 nM and above ($p<0.01$); the highest level was 6.87 ± 1.09 nmol/min/mg protein at 100 nM of tBHP. Similarly, RTgill-W1 cells showed maximum CAT activities of 2.89 ± 1.46 and 1.68 ± 0.67 at 1 and 10 μM, respectively ($p<0.01$). SOD levels in rainbow trout gills (RTH-149 and RTgill-W1) were relatively constant at tBHP concentrations lower than 1 μM, but significantly decreased at the two highest concentrations ($p<0.01$; Table 6.3). In contrast, Hepa-E1 and G1B cell lines showed the highest levels of SOD between 1 and 100 nM of tBHP, though decreasing SOD levels were observed at 1 μM ($p<0.01$). No distinctive patterns were observed for GPX, as all cell lines tested showed no clear activity across all tBHP concentrations tested.

Table 6.2. Catalase activity reported as nmol/min/mg protein ± SD as a function of *tert*-Butylhydroperoxide concentration in fish gill and hepatic cell lines. Significant differences assessed among concentrations with one-way ANOVA and n=3.

	t-BHP (M)																								*P*-value
	SC[a]			10^{-11}			10^{-10}			10^{-9}			10^{-8}			10^{-7}			10^{-6}			10^{-5}			
RTH-149	0.50	±	0.14	0.63	±	0.14	0.37	±	0.41	0.88	±	1.46	1.94	±	1.84	1.05	±	1.01	4.28	±	6.81	1.54	±	0.97	NS*
HEPA-E1	0.74	±	1.23	0.04	±	0[b]	1.30	±	1.28	1.61	±	1.37	1.83	±	0.82	3.68	±	5.89	4.68	±	4.34	3.65	±	5.86	NS*
RTgill-W1	0.23	±	0.31	0.53	±	0.22	0.49	±	0.29	0.68	±	0.2	0.47	±	0.38	0.37	±	0.32	2.89	±	1.46	1.68	±	0.67	< 0.01
G1B	0.58	±	0.43	3.70	±	0.18	3.70	±	3.37	2.46	±	2.02	5.56	±	1.38	6.87	±	1.09	6.06	±	0.91	5.86	±	1.66	< 0.01

*No statistical significance among treatment groups. [a]Solvent Control (DMSO). [b]Values below limit of detection.

Table 6.3. Levels of superoxide dismutase reported as Units/mg protein ± SD as a function of *tert*-Butylhydroperoxide concentration in fish gill and hepatic cell lines. Significant differences assessed among concentrations with one-way ANOVA and n=3.

	t-BHP(M)																								*P*-value
	SC[a]			10^{-11}			10^{-10}			10^{-9}			10^{-8}			10^{-7}			10^{-6}			10^{-5}			
RTH-149	1.3	±	0.16	1.38	±	0.28	1.09	±	0.37	1.24	±	1.16	1.47	±	0.37	1.44	±	0.13	0.27	±	0.17	0.36	±	0.03	< 0.01
HEPA-E1	0.01	±	0.0005	0.01	±	0.001	0.01	±	0.004	0.59	±	0.37	0.94	±	0.21	1.05	±	0.56	0.55	±	0.1	0.98	±	0.28	< 0.01
RTgill-W1	0.78	±	0.28	0.93	±	0.0805	1.03	±	0.1	1.23	±	0.2	1.11	±	0.04	1.12	±	0.32	0.08	±	0.07	0.08	±	0.04	< 0.01
G1B	0.09	±	0.10	0.65	±	0.05	0.25	±	0.21	1.17	±	0.23	1.19	±	0.3	1.07	±	0.3	0.8	±	0.53	0.95	±	0.51	< 0.01

[a]Solvent Control (DMSO).

Discussion

Originally, fish cell lines were almost exclusively used for the isolation of viruses and in infectious diseases research, but there has been rapid expansion in the derivation and application of these in exposure assessments (Lakra et al., 2011). The philosophy of the 3 Rs has been fundamental in the discovery of suitable *in vitro* approaches to reduce the use of animals (Guhad, 2005). For example, PLHC-1 and RTL-W1 cells are two commonly used fish liver cell lines for toxicity screening of chemicals and environmental samples, and although they both have been widely used as *in vitro* models, they exhibited lower phase I and phase II biotransformation activities than those reported in primary cultures of fish hepatocytes (Thibaut et al., 2009). Consequently, a better knowledge of the metabolic capabilities of fish liver cell lines should help to select the most adequate ones for both toxicity and metabolism studies, and to support programs of global importance such as REACH and TSCA.

In the present study, the high survival rates shown by hepatic cell lines across a broad range of xenobiotic concentrations may be the result of the abilities of this group of cells to metabolize chemical compounds, as toxic responses are highly dependent on xenobiotic biotransformation in cells (Thibaut et al., 2009); thus, cells that are equipped with more sensitive and effective metabolic mechanisms may be less prone to high toxicity. The combination of low metabolism in gill cells and the high rates of compound absorption by gills (Srivastav, Srivastava, & Srivastav, 1997) may have compromised the survival of gill cell lines. Xenobiotics may also be more toxic to gill cells due to the sensitivity of the lysosomal membrane (Connolly et al., 2015). In terms of xenobiotic specificity, the highest concentration of BNF decreased survival by 20 to 25% in hepatic

cell lines, more than what was observed from exposure to BaP. It is likely that metabolites of BNF metabolism may induce more toxicity to hepatic cells than the ones emerging from BaP metabolism at the concentrations tested. BaP dihydrodiols, 3-hydroxy BaP and BaP diones have been described to be common BaP metabolites and potential toxic compounds (Moller, Hermsen, Floehr, Lamoree, & Segner, 2014; Stiborova et al., 2016; Zhu et al., 2008). However, limited information about toxic BNF metabolites is available for fish species. Teles, Gravato, Pacheco, and Santos (2004) found that sea bass' genotoxic responses induced by BNF was significantly higher that other compounds after 24 hours of exposure. In rats, Vyas et al. (1983) described that the metabolite BNF-5,6-dihydrodiol presented a pseudo-diaxial conformational change, which is important for genotoxicity as it influences to what extent the toxic diol epoxides are formed (Vyas et al., 1983).

At its highest concentration, tBHP was the most cytotoxic for RTH-149 cells, decreasing survival by approximately 35%. tBHP toxicity studies such as Toussaint, Houbion, and Remacle (1993) have concluded that the potent induction of ROS by this compound leads to cell degeneration, increasing the likelihood of cell death. Among the hepatic cell lines used in this study, RTH-149 showed elevated levels of ROS, giving further evidence of the cytotoxic properties of tBHP on this cell line. TMX only resulted significantly cytotoxic at the highest concentration, suggesting that Hepa-E1 cells cope efficiently with TMX exposure by increasing the expression or the activity of CYP450 proteins, until TMX surpasses the levels that can be managed by these hepatic cells. In gill cells, TMX may have been easily transported inside the cell, where it exhibited its toxicity due to reduced biotransformation (lack of CYP3A-like activity). TMX has a

solubility limit in water of approximately 0.01%, sufficient to dissolve readily into aqueous biological compartments (e.g. plasma, cytosol) (Berube et al., 2006). Moreover, gills represent the first barrier for uptake of chemical compounds, and transport of these compounds to the cytosol is moderated by both lipid membrane permeability and aqueous diffusion (McKim, Schmieder, & Veith, 1985).

Both BaP and BHP were highly cytotoxic for RTgill-W1 and G1B cells; however, the lowest survival rates were induced by BNF and TMX. These results suggested that, in gills, the mechanisms of toxicity of BNF are AhR-independent. As a potent agonist of the AhR, BNF induces the expression of CYP450 enzymes that catalyze the formation of active metabolites with genotoxic properties. However, the lack of metabolic capabilities of gill cells points out that the AhR pathway may not be the primary driver of BNF metabolism, as other biochemical pathways related to ROS production may be favored. In rats, BNF has been shown to induce high levels of oxidative stress (Kuwata et al., 2011), which may have played an important role in increasing cytotoxicity in gill cells. Dewa et al. (2008) provided evidence to reveal the capability of BNF to enhance oxidative damage and lipid peroxidation via increased production of ROS.

In regards to EROD activity in gill cells, the observations from the present study may be the result of exposure times, as Kienzler, Tronchère, Devaux, and Bony (2012) observed significant EROD induction in RTgill-W1 after 8 hours of exposure to BaP but activity decreased after 16 and 24 hours; the present study assessed activity after a 48-hour exposure. On the other hand, the high rates of EROD activity by hepatic cell lines after being exposed to BNF and BaP suggest high rates of CYP1A1 expression. Extensive research has been done assessing the metabolic mechanisms of the hepatic cell

line RTH-149 (H Babich, Martin-Alguacil, & Borenfreund, 1989; J L Fryer, McCain, & Leong, 1981; Lannan et al., 1984). Particularly, the observations in the present study agree with the findings by (H Babich et al., 1989), where it was shown that BaP exposure to RTH-149 cells led to slight toxicity due to insufficient P450 enzyme activity of these cells to induce the formation of cytotoxic compounds from BaP metabolism. Contrarily to BaP, BNF did elicit an increase in EROD activity in RTH-149 cells. Previous studies support the observations here presented regarding BNF cytotoxicity. According to Vijayan, Pereira, Forsyth, Kennedy, and Iwama (1997) and Tintos, Gesto, Miguez, and Soengas (2008), BNF represents the most potent inducer of the cytochrome P450 system in rainbow trout, leading to increases in phase I and phase II enzymes after exposure. Additionally, metabolic alterations by BNF may favor certain pathways where some enzymes are more up-regulated than others (Vijayan et al., 1997). Limited information is available for Hepa-E1 cell lines regarding expression of CYP1A1 genes. However, the increased EROD activity shown by Hepa-E1 cells after exposure to both BNF and BaP, makes this cell line a suitable candidate for *in vitro* experimentation of xenobiotic metabolism and CYP expression. PLHC-1 has been the most used cell line to evaluate CYP450 activity (Caminada et al., 2006; Fent, 2001; Huuskonen et al., 1998). While excellent results may be obtained with it, effective alternatives must be available to expand the applicability of *in vitro* studies. The present study has shown strong evidence to consider the Hepa-E1 cell line as one of these alternatives.

Limited knowledge exists about the metabolic mechanisms to process TMX by aquatic organisms. Previous studies in other animals have shown that TMX is metabolized differently for activation and detoxification, and that these processes depend

on the expression of CYP450 enzymes (Krueger, VanDyke, Williams, & Hines, 2006). In rats, evidence for increased CYP3-like activity, specifically 3A1 proteins, was shown after animals were treated with TMX (White, Davies, Smith, Dawson, & de Matteis, 1993). As for EROD activity, Hepa-E1 cells could be equipped with more sensitive mechanisms to metabolize TMX and similar compounds. While RTH-149 cells also showed enzymatic activity, the smaller levels, as compared to the ones shown by Hepa-E1 cells, could be the result of differences in the reduced binding affinity of the teleost fish to estrogen receptor with compounds like TMX (Kitano, Yoshinaga, Shiraishi, Koyanagi, & Abe, 2007). The low levels of enzymatic activity observed in gill cell lines could be attributed to less metabolic capabilities, and higher susceptibility for cytotoxicity. Our results suggest that the activity CYP2-like enzymes in the 4 cells lines tested is very low, as it was found for other fish cell lines (Thibaut et al., 2009). These authors did not find any CYP3-like activity; however, in the present study, 6β-OH-testosterone hydroxylase activity was observed in S9 fractions isolated from both hepatic cell lines. As suggested in previous studies, it is possible that widely used cell lines, such as PLHC-1, lost the capacity to respond to CYP3A inducers, as compared to RTH-149 and Hepa-E1 cell lines (Celander, Hahn, & Stegeman, 1996).

Oxidative stress results from the formation of ROS, enhancing adverse biological processes such as lipid peroxidation, DNA damage and protein carbonylation (Bopp, Abicht, & Knauer, 2008; Parvez & Raisuddin, 2005; Rau, Whitaker, Freedman, & Di Giulio, 2004). Different species may differ in ROS production, and they may possess different mechanisms to tackle oxidative stress induced by exposure to pollutants (Rau et al., 2004). The antioxidant mechanisms of both the liver and gills have been proven to

remove ROS, and reports by Slaninova and Modra (2009) suggested that adaptation to ROS generation may be tissue-specific. Parvez and Raisuddin (2005) suggested that the gills are highly sensitive to contaminants with more probability of presenting oxidative damage. However, in the present study, G1B cells showed elevated CAT and SOD activity when exposed to tBHP. Even though the high generation of peroxide by gill cells may be due to the high rates of absorption observed in this organ (Sayeed et al., 2003; Srivastav et al., 1997), antioxidant mechanisms may be expressed differently in certain fish species as shown in the present study. The high metabolic activities shown by Hepa-E1 cells and RTH-149 cells may explain the lower amounts of peroxide, as the biotransformation capacity of cells is key in determining toxic responses (Thibaut et al., 2009). Both hepatic cell lines also presented increasing levels of CAT activity, which explains the low levels of peroxide observed.

Contrarily to the findings for peroxide production, RTH-149 cells generated the highest levels of superoxide. SOD levels in RTH-149 cells remained relatively constant, which would explain the inability of rainbow trout cells to cope with superoxide production. While Toussaint et al. (1993) found an inverse relationship between oxidative damage in cells and GPX activity, the present study did not find distinctive patterns of GPX activity at the tBHP concentrations tested, suggesting both rainbow trout cell lines (RTH-149 and RTgill-W1) may be more vulnerable to oxidative damage from superoxide. Contrarily, Hepa-E1 and G1B cells showed increasing SOD levels in presence of high tBHP concentrations, allowing these cell lines to be more efficient in managing superoxide production. All cell lines tested, especially the rainbow trout cells,

presented low levels of SOD at the highest tBHP concentrations, which could be the result of high cytotoxicity and cell mortality.

Conclusions

Fish cell culture has recently offered many unique contributions to environmental physiology and toxicology (Fent, 2001; Mazon Ade, Nolan, Lock, Wendelaar Bonga, & Fernandes, 2007). Cultured cells and tissues offer the advantage of not requiring the sacrifice of live animals, and can also provide easier linkage between cause and effect due to controlled manipulation of experimental variables. Consequently, the demand for *in vitro* systems continues to increase, and the integration of *in vitro* and *in vivo* model results promising in attempts to extrapolate experimental data to realistic environmental scenarios. This study not only corroborates the usefulness of fish cell cultures to assess metabolism of xenobiotics, but it also shows that the biomarkers measured not only differ between species but also between specific organs. Thus, the metabolic characterization of cell lines represents strong evidence of the advantages of using certain cell lines to measure specific parameters, depending on the researcher's goals. The observations in the present study will aid in better selecting *in vitro* tools for future experimentation, and to increase the effectiveness of *in vitro* studies. These recent findings should help and encourage researchers to develop new fish cell lines that could be used as alternative *in vitro* tools not only for studying xenobiotic metabolism and toxicity in fish, but to also implement *in vitro* systems in programs designed for screening of chemicals and in conducting comprehensive risk assessments.

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CHAPTER SEVEN

Prymnesium parvum Differentially Triggers Sublethal Fish Antioxidant Responses *In Vitro* Among Salinity and Nutrient Conditions

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Abstract

Significant fish kills have been attributed to Prymnesium parvum in coastal and inland waters around the world. However, specific mechanisms responsible for adverse outcomes resulting from this harmful algal bloom (HAB) species remain unclear, though the gill has previously been identified as an important target organ. In the present study, an *in vitro* approach was used to examine cytotoxicity and antioxidant responses in fish liver (Hepa-E1 and PLHC-1) and gill (G1B and RTgill-W1) cell lines, following exposure to P. parvum grown at different salinities and nutrient concentrations, which can influence the magnitude of acute toxicity. Cultures from high salinity compromised survival of hepatic cell lines exposed to high dilutions, whereas no significant cytotoxicity was observed for gill cell lines. With respect to control groups, catalase showed significant activity in both gill cell lines, especially RTgill-W1, following exposure to high salinity cultures. High levels of superoxide dismutase were measured in Hepa-E1 cells exposed to all experimental treatment combinations and in RTgill-W1 cells following exposure to high salinity conditions, with respect to non-exposed cells Glutathione peroxidase activity was also detected at significant levels in Hepa-E1 cells after exposure to cultures from high salinity and the low salinity X low nutrients. Slight

GPx increases were only observed in PLHC-1 and G1B exposed to P. parvum grown at high salinity. These results suggest that: 1. specific combinations of salinity and nutrient levels may contribute to production and potency of P. parvum toxins resulting in sub-lethal effects, and 2. sub-lethal responses are more prominent than cytotoxicity, and that oxidative stress may be a significant adverse effect of toxins produced by P. parvum.

Introduction

Prymnesium parvum Carter has been responsible for major harmful algal blooms (HABs) affecting the integrity of aquatic ecosystems (Brooks, Grover, & Roelke, 2011; Brooks et al., 2010; Roelke et al., 2016; Schug et al., 2010; Shilo, 1981). Large ecological impacts, such as massive fish kills, have been the result of the ichthyotoxicity exerted by this haptophyte in different areas around the world. P. parvum toxins, which are most commonly referred as prymnesins (Igarashi, Satake, & Yasumoto, 1999), possess hemolytic and cytotoxic properties that are suggested to compromise the integrity of cell membranes, leading to a malfunction of cell permeability (N. Johansson & Granéli, 1999; Shilo, 1981). However, other secondary metabolites contributing to the deleterious effects of P. parvum remain an active area of research (Bertin, Voronca, Chapman, & Moeller, 2014; Bertin, Zimba, Beauchesne, Huncik, & Moeller, 2012; Blossom et al., 2014; Henrikson et al., 2010). Thus far, it appears that gill-breathing organisms are primarily affected by this group of toxins (Granéli & Johansson, 2003b; Shilo, 1981), but it remains unclear whether mortality results from gill cell damage or breathing complications due to erythrocyte lysis. Specific mechanisms of toxicity by P. parvum have not been fully described, as analytical standards of prymnesins are not yet available (Brooks et al., 2010).

A large proportion of the bioassays that have been performed aiming to evaluate the effects of *P. parvum* toxins on aquatic organisms have used field samples, which may contain diverse compounds with potential to cause toxicity. While this presents complications in terms of understanding the potential toxicity of specific toxins for which standards are not available, laboratory experimentation has been useful to develop predictive models of *P. parvum* blooms in the field (Grover, Crane, Baker, Brooks, & Roelke, 2011; Grover, Roelke, & Brooks, 2012, 2017). Additionally, several studies (Granéli & Johansson, 2003a, 2003b; N. Johansson & Granéli, 1999; Roelke et al., 2007) have demonstrated influences of nutrients on growth, development, and the apparent production of toxins by *P. parvum* in both coastal and inland waters. Baker et al. (2007) and Baker et al. (2009) further identified influences of other environmental factors, including salinity, light, and temperature on growth of this alga across freshwater to marine gradients.

Though most of the toxicity studies with *P. parvum* have been performed descriptively with hemolysis assays or various *in vivo* models (Brooks et al., 2010), it is fundamentally important to understand sublethal responses in fish. Mechanistic studies can be aided by *in vitro* models, which further provide benefits to reduce the use of animals, given the ethical and economic difficulties associated with *in vivo* bioassays (Dayeh, Bols, Tanneberger, Schirmer, & Lee, 2013; Thibaut, Schnell, & Porte, 2009). Developments such as the 3Rs and landmark publications such as the U.S. National Research Council's "Toxicity Testing in the 21st Century" (NRC, 2007) have advanced the use of *in vitro* systems for diverse environmental and biomedical studies. While cell-based systems are limited by factors related to exposure to xenobiotics, and components

of absorption, distribution, metabolism and excretion (ADME) observed *in vivo* (Blaauboer, 2015), these assays continue to be advantageous for mechanistic evaluations of the toxicity of chemicals and environmental samples (Dayeh et al., 2013). Consequently, cell lines are increasingly employed as alternative toxicological assays, while representing cost-efficient tools to explore biological responses using a mechanistic approach (Castaño et al., 2003).

In the present study, we hypothesized that salinity and nutrient conditions would differentially influence the production and potency of *P. parvum* toxins, identified by the magnitude of sublethal fish toxicity associated with them. Additionally, due to the lack of information regarding mechanisms by which this haptophyte elicits adverse outcomes in aquatic organisms, the suitability of four fish cell lines was evaluated to describe potential modes and mechanisms of action (MOA) of *P. parvum* toxins. For this purpose, antioxidant enzyme activity was used as a proxy for sub-lethal responses, given previous reports (Dorantes-Aranda, Seger, Mardones, Nichols, & Hallegraeff, 2015) suggesting the formation of reactive oxygen species (ROS) and the subsequent potential for oxidative stress in fish gill cells exposed to algal toxins. The rationale for investigating these responses also arises from the ability of many chemical compounds to undergo redox cycling and generate ROS (Di Giulio, Washburn, Wenning, Winston, & Jewell, 1989). *In vitro* assays using gill cells have previously been demonstrated useful for studies of different harmful marine microalgae (Dorantes-Aranda et al., 2015; Dorantes-Aranda et al., 2011). These experiments showed induction of oxidative stress in liver, kidney, and gills, through the measurement of lipid peroxidation and expression of antioxidant enzymes. These fish cell lines selected to conduct the experiments described

in this study have also been previously evaluated for their ability to express several biomarkers of interest. Franco, Sutherland, and Lavado (2018) characterized responses of the hepatic cell line Hepa-E1, and the gill cell lines G1B and RTgill-W1 after exposure to an oxidative stress-causing compound, whereas the liver fish cell line PLHC-1 was used by Pichardo et al. (2005, 2007) during comparisons of toxic effects of different microcystins to fish. Also, because of the increased attention to advance alternatives to animal testing, the present study provides a useful comparison among these fish cell lines for investigating MOA of algal toxins, and for appropriate selection of *in vitro* fish models.

Materials and Methods

Prymnesium parvum culture

A Texas strain was obtained from the University of Texas at Austin Culture Collection of Algae (UTEX LB 2797, Austin, TX, USA). Culture conditions were selected to resemble environmental conditions when *P. parvum* blooms have occurred in Texas inland waters and were based off previous methods by our laboratory group (Brooks et al., 2010; James et al., 2011). Briefly, media for salinity treatment levels were prepared by diluting artificial seawater (ASW) (Instant Ocean, Spectrum Brands, Blacksburg, VA) and Nanopure water (818.2 megohm ionic purity; Barnstead, ThermoFisher, Wilmington, DE) using Nanopure water to 2.4 and 5 ppt. Water was then enriched with either f/2 (882 µM $NaNO_3$, 36.2 µM NaH_2PO_4 H_2O) or f/8 220.5 µM $NaNO_3$, 9.125 µM NaH_2PO_4 H_2O) nutrients (f/8 is one fourth the concentration of f/2), with trace metals and vitamins for both nutrient conditions (Guillard, 1975). Each culture

condition was cross-classified in a 2 x 2 experimental design. ASW and all media stocks were autoclaved before use and acclimated to 15 °C. Approximately 100 cells/mL were introduced to 15 L for each experimental unit (20 L glass carboys). Experimental units were incubated (VWR International, West Chester, PA) at 15 °C, on a 12:12 light:dark cycle at 4,000 Lux, and each experimental unit was swirled and rotated daily. Every second day, chlorophyll-a fluorescence was determined using a handheld fluorometer (Turner Designs, San Jose, CA) to identify growth status of each experimental unit. These subsamples collected for chlorophyll-a fluorescence were preserved with 200 µL of 25% aqueous glutaraldehyde for cell counts. Cell counts (Table 7.1) were determined using a haemocytometer following previously published methods (Southard, 2005). Whole cell cultures were frozen at -20 ℃ after stationary growth phase was observed. This growth phase was targeted for sublethal work due to markedly decreased fathead minnow acute mortality observed in previous studies (Hill et al., 2020). For these assays, pH was titrated to 8.5 (Valenti, Taylor, Back, King, & Brooks, 2011) and conducted in the dark due to potential photodegradation of toxins (James et al., 2011). Approximately four months after freezing, cultures were thawed in the dark to include both intra- and extracellular toxins. Cell debris were removed by filtration (0.45 µm GN Metricel® Membrane Disc Filters, Pall Laboratory) in the dark to prevent toxin photodegradation (James et al., 2011).

Chemicals and solutions

Leibovitz's L-15 and Eagle's minimum essential media, L-glutamine, penicillin G, streptomycin, phosphate-buffered saline (PBS) and trypsin–EDTA were obtained from Gibco Life Technologies (ThermoFisher Scientific, Waltham, MA). Ham's F-12K

(Kaighn's) medium was obtained from ATCC (Manassas, VA). HEPES salt (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), sterile RPMI culture medium, Triton™ X-100 and sodium heparin were obtained from Sigma-Aldrich (St. Louis, MO). Fetal bovine serum (FBS) was obtained from Atlas Biologicals (Fort Collins, CO). Deionized water (DI water) was obtained using a Milli-Q water purification system (Millipore, Billerica, MA).

Hemolysis

Because the ability to cause hemolysis by *P. parvum* toxins has been commonly described in the literature as an effect of *P. parvum* (Brooks et al., 2010; Schug et al., 2010), hemolytic activity was measured as a toxicity endpoint, and thus for the presence of toxins in each of the samples from cultures. Hemolysis was measured following the protocol described by Brooks et al. (2010) with minor modifications. Sterile 100% packed sheep erythrocytes (Innovative Research Inc., Southfield, MI) were diluted by 1% (approximately $1x10^7$ cells/mL) in sterile RPMI culture medium diluted by 10% in PBS (pH=7.2), containing 0.005 mg/mL sodium heparin. Samples from cultures and control solutions dilutions of 5, 10, 25, and 50% of total volume were evaluated for hemolysis. For the assay, 200 µL of the erythrocyte suspension were transferred to microcentrifuge tubes, and specific volumes of toxin cultures or media control solutions, depending on specific treatments, were added; PBS was used to compensate for the remaining volume to reach a total of 400 µL in each tube. Cell suspensions were incubated for 1 hour at room temperature, then centrifuged at 3,500 *g* for 5 min, and 150 µL of supernatant were transferred to a 96-well plate in duplicates. Absorbance was read at 425 nm (Synergy™ H1, BioTek, VT), and converted to percent lysis using a linear calibration curve from a

100% hemolysis standard, prepared by mixing erythrocyte suspension and 40% Triton™ X-100 from laboratory grade.

Cell lines culture and dosing

In vitro cell lineages used in the present study were selected based on the characterization of antioxidant responses for Hepa-E1, G1B, and RTgill-W1 conducted by Franco et al. (2018), and for PLHC-1 by Pichardo et al. (2007). Culture conditions of the cell lines are described in Table 7.2. All culture media was supplemented with 2mM L-glutamine and antibiotics (50 U/mL penicillin G, and 50 µg/mL streptomycin). Cells were seeded and allowed to grow for 24 h (PLHC-1 and G1B), or 48 h (Hepa-E1 and RTgill-W1) to reach confluence before dosing. Exposure experiments were performed in cell monolayers (1×10^5 cells/cm^2) seeded in 96-well plates for cytotoxicity assays and in 12-well plates for measurements of antioxidant enzymes. Experimental treatment levels were 5, 10, 25, 50, and 75% dilutions of *P. parvum* filtrates from experimental units or cell-free filtrate media (used as controls), with four technical replicates for each cell line. Media controls contained the same nutrient proportions and salinity. Before dosing, the cell culture medium was carefully removed and replaced by specific volumes of fresh medium without FBS. Specific volumes of samples from the four cultures or media controls were then added to total volumes of 100 µL and 1 mL in 96-well plates and 12-well plates, respectively. Plates were returned to incubators for a 48-h exposure period.

Preparation of S9 fractions

Preparation of S9 fractions followed the protocol described by Thibaut et al. (2009) with minor modifications. S9 fractions were selected as the antioxidant enzyme bioassays required the use of cell lysates. Briefly, plates were washed with PBS and cells were detached with 0.05% (w/v) trypsin and 0.5 mM ethylenediaminetetraacetic acid, and placed in microcentrifuge tubes for centrifugation at 300 *g* for 5 min at 4°C. The supernatant was then discarded, and cells were resuspended in cold 100 mM KH_2PO_4/K_2HPO_4 buffer (pH=7.4), containing 100 mM KCl, and 1 mM EDTA. The cell suspension was centrifuged again at 12,000 *g* for 20 min at 4°C, and stored at -80°C until assays were performed. Protein content was measured using the Coomassie Blue method (Pierce Inc., Rockford, IL) and bovine serum albumin (BSA) as standard.

Cytotoxicity

Cytotoxicity assays were conducted in 96-well plates, and cell viability was measured though the application of a slightly modified MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-d-phenyltetrazolium bromide] assay, as described by Lavado et al. (2009). The MTT method measures the ability of NAD(P)H-dependent cellular oxidoreductase enzymes to reduce the tetrazolium dye to insoluble formazan, which has a purple color. This color was measured by absorption spectroscopy (BioTek Synergy™ H1) at 595 nm.

Activity of antioxidant enzymes

Activity of the antioxidant enzymes catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx) was measured in S9 fractions using commercial 96-well plate bioassay kits (Cayman Chemical, Ann Arbor, MI). These bioassays are

designed for the evaluation of cell lysates, hence the selection of S9 fractions to evaluate enzymatic activity. CAT activity was measured colorimetrically from the production of formaldehyde with the chromogen 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole at 540 nm, after 20 min of reaction (L. H. Johansson & Borg, 1988). Activity is reported as nmol formaldehyde per minute per mg of protein. Total amounts of SOD were quantified by the detection of superoxide radicals, formed by the activity of xanthine oxidase and hypoxanthine, after 30 mins of incubation; absorbance was read at 450 nm, and data are reported as units of SOD per mg of protein. GPx activity was measured kinetically for 5 min, from a coupled reaction with glutathione reductase, in which NADPH was oxidized to $NADP^+$; absorbance was read at 340 nm, and activity is reported as nmol per minute per mg of protein.

Data analysis

All statistical analyses were conducted in the R statistical software (version 3.5.1), and through the R-studio platform. *P*-values of less than 0.05 were considered statistically significant. Hemolytic activity of the different dilutions of the four *P. parvum* culture extracts was evaluated through a one-way ANOVA, and the interaction between the four culture extracts and the four dilutions was evaluated through a two-way ANOVA. The ability of each culture extract to cause cytotoxicity was also evaluated through a two-way ANOVA, assessing the differences between cell lines and extract dilutions. Antioxidant responses (CAT, SOD and GPx) were all analyzed to compare responses between cell lines and culture dilutions, for each of the four extract cultures, using a two-way ANOVA. If the models resulted significant, Tukey post-hoc analyses were conducted for each dataset. For statistical comparisons, antioxidant enzymes that

showed activity below detection limits were considered as having ½ of the minimum values detected by the assays. Normality of the data was evaluated with the Shapiro-Wilk normality test to meet the assumptions of parametric tests.

Table 7.1: *Prymnesium parvum* cell densities (cells/mL) grown to stationary phase at 2.4 (low) and 5 (high) ppt on study days 60 and 67, respectively, at 15 °C with a 12:12 light:dark cycle, under high (f/2) and low (f/8) nutrient conditions. Cultures were frozen, thawed and filtered prior to performing *in vitro* experiments.

Low Salinity/High Nutrients	Low Salinity/Low Nutrients	High Salinity/High Nutrients	High Salinity/Low Nutrients
154,000	126,000	580,000	374,000

Table 7.2. Fish cell lines used to conduct experiments (ATCC: American Type Culture Collection. ECACC: European Collection of Authenticated Cell Cultures.

Cell Line	Fish Species	Tissue	Provider	Culture Conditions	Reference
Hepa-E1	Japanese eel (*Anguilla japonica*)	Liver	ECACC (99072812)	E-RDF medium[a] + 5% FBS, in a humidified incubator w/ 5% CO_2 at 26°C	Riken Cell Bank, Japan
PLHC-1	Top minnow (*Poeciliopsis lucida*)	Liver	ATCC (CRL-2406)	Eagle's minimum essential medium + 5% FBS in a humidified incubator w/ 5% CO2 at 30°C	Hightower and Renfro (1988)
G1B	Walking catfish (*Clarias batrachus*)	Gill	ATCC (CRL-2536)	Ham's F-12 K (Kaighn's) medium + 10% FBS, in a humidied incubator w/ 5% CO_2 at 26°C	Noga and Hartmann (1981)
RTgill-W1	Rainbow trout (*Oncorhynchus mykiss*)	Gill	ATCC (CRL-2523)	Leibovitz's L15 medium + 10% FBS, in a humidified incubator w/ atmospheric air at 19°C	Bols et al. (1994)

[a] E-RDF medium was obtained from Kyokuto Pharmaceuticals, Tokio, Japan.

Results

Hemolysis

Hemolysis of sheep erythrocytes was evaluated to corroborate the potential toxicity of the different *P. parvum* culture conditions and solvent controls used in this study. For all four combinations of salinity and nutrient levels, hemolytic activity was significantly high for erythrocytes exposed to experimental extracts than their corresponding solvent controls at dilutions of 25 and 50%. Among culture extracts, both high salinity extracts (Figure 7.1 A, B) caused > 40% hemolysis at the 25 and 50% dilutions, and hemolysis of erythrocytes exposed to the high salinity extracts was higher than their respective solvent controls at all dilutions. For 25 and 50% dilutions, low salinity extracts (Figure 7.1 C, D) caused < 40% of erythrocyte lysis (approximately 1.5-fold relative to their controls), but no significant hemolysis was observed for dilutions of 5 and 10%.

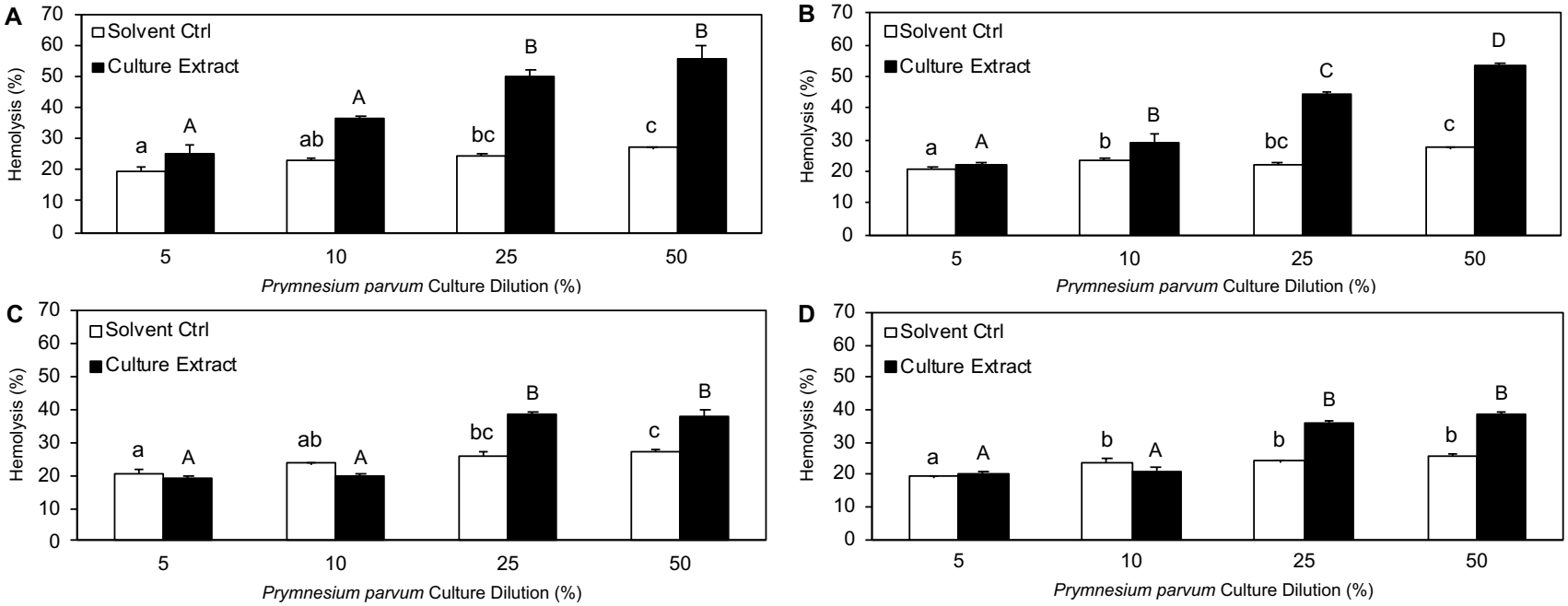

Figure 7.1. Hemolysis of sheep erythrocytes after exposure to *P. parvum* cultures under conditions of A) high salinity-low nutrients, B) high salinity-high nutrients, C) low salinity-low nutrients, and D) low salinity-high nutrients. Different lower-case letters denote significant differences between dilutions of solvent controls, whereas different upper-case letters denote significant differences between toxin extract dilutions. Data is reported as mean % hemolysis ± std. deviation.

Cytotoxicity

Variable responses were observed among the four cell lines exposed to solvent controls of both high (Figure 7.2C, D) and low (Figure 7.3C, D) salinity, though none of the culture extracts caused survival of cells to fall below 80%, at any dilution. Hepa-E1 cells appeared to slightly proliferate from the 5% to the 25% dilution, but survival was lower at the 50 and 75% dilutions. The G1B cell line presented relatively higher mortality compared to the other three cell lines, though it remained above 80% survival in all treatments.

For all cell lines, levels of cytotoxicity were relatively low (> 80% survival) for most of the culture extract dilutions at which cells were exposed. However, among all four cell lines, the high salinity/low nutrients culture extract (Figure 7.2A) significantly reduced cell viability of the Hepa-E1 liver cell line by half (48.23 ± 2.46%) at the highest (75%) dilution ($p<0.05$). This was not observed neither for PLHC-1 cells nor for gill cells as, while survival did decrease with increasing dilutions, these cell lines remained above 80% viability in all of them. Similar results were observed for the high salinity/high nutrients culture extract (Figure 7.2B), where survival of Hepa-E1 cells was compromised at the 75% dilution (51.52 ± 1.85%; $p<0.05$). Survival of PLHC-1 cells and both gill cell lines was not significantly reduced at any dilution (cell viability > 80%). While differences in survival between the four cell lines and culture extract dilutions (such as an apparent proliferation of Hepa-E1 cells at low and mid dilutions) were observed at the low salinity-low nutrients culture extract (Figure 7.3A; $p<0.05$) and low salinity-high nutrients culture extract (Figure 7.3B; $p<0.05$), none of the cell lines presented viability below 80%.

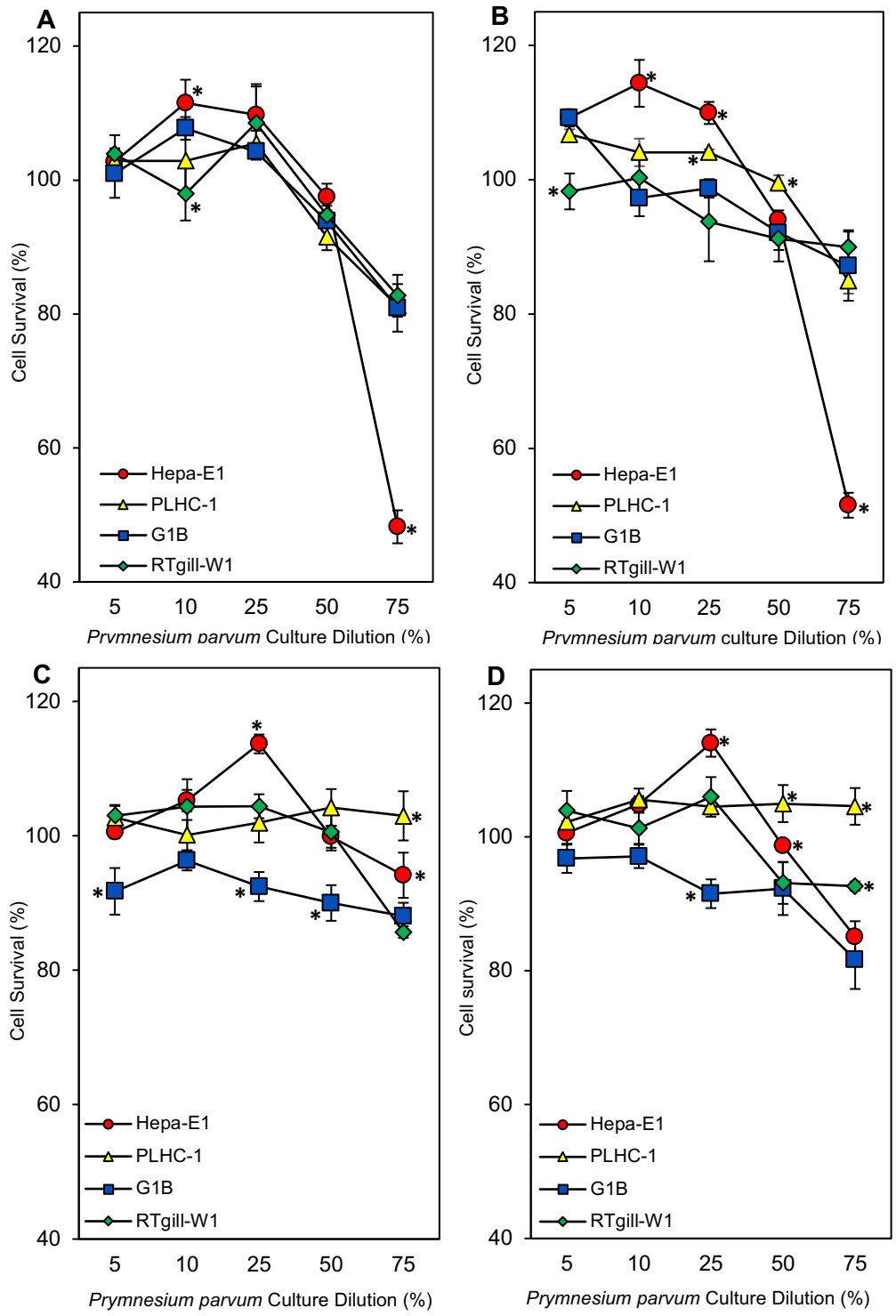

Figure 7.2. Cytotoxicity of *P. parvum* extracts cultured at high salinity (5 ppt) and A) low nutrients or B) high nutrients, and their corresponding solvent controls (C and D) in all four fish cell lines. Data are presented as mean % survival ± std. error. (*) denote significantly different groups (cell lines) at specific culture dilutions.

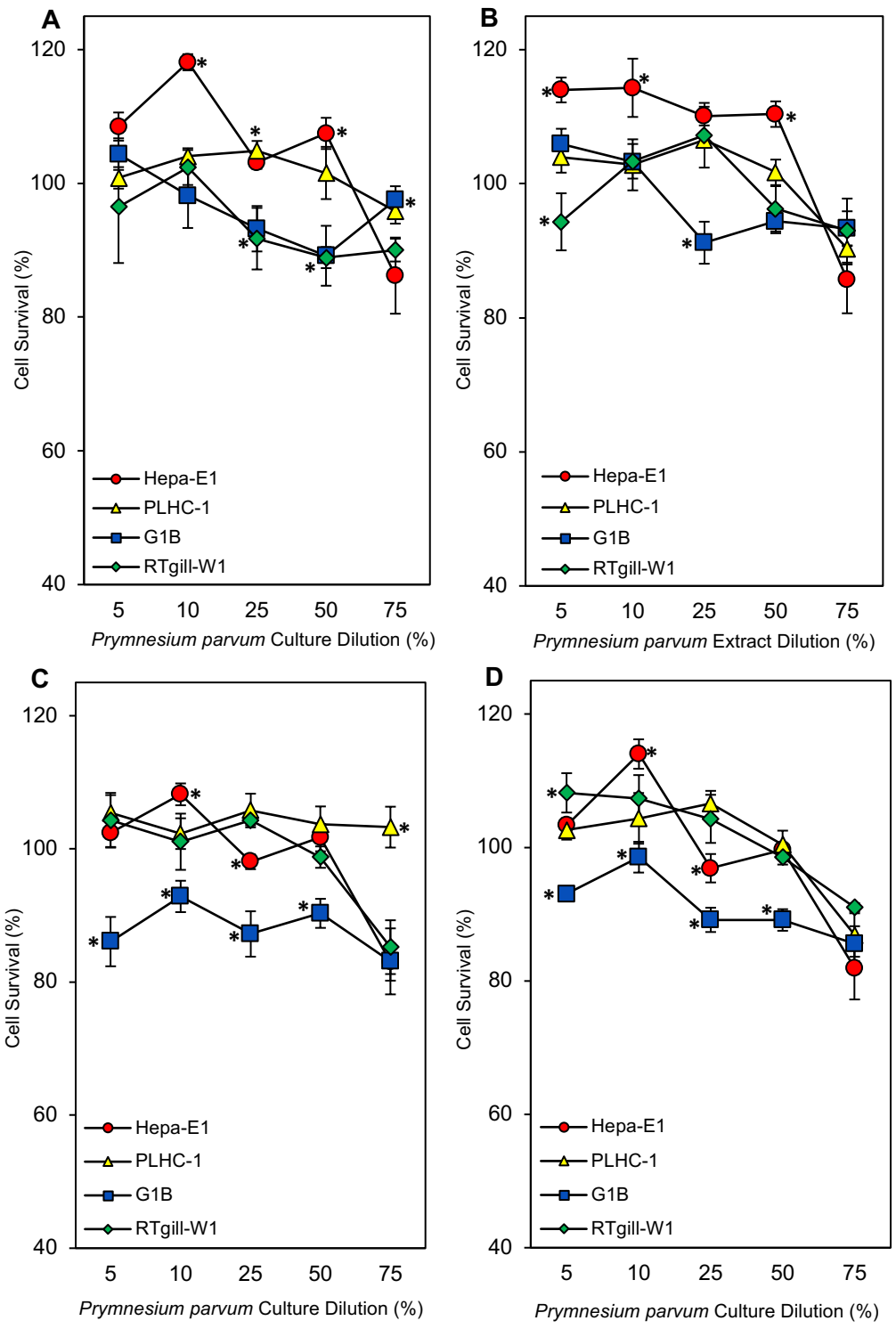

Figure 7.3. Cytotoxicity of *P. parvum* extracts cultured at low salinity (2.5 ppt) and A) low nutrients or B) high nutrients, and their corresponding solvent controls (C and D) in all four fish cell lines. Data are presented as mean % survival ± std. error. (*) denote significantly different groups (cell lines) at specific culture dilutions.

Antioxidant Reponses

Catalase (CAT)

When exposed to extracts from algae cultured in high salinity and low nutrient conditions (Figure 7.4A), PLHC-1 cells showed the highest activity among all cell lines; however, the gill cell line RTgill-W1 showed the highest activity of all four cell lines with respect to its corresponding control ($p<0.05$). CAT activity for this gill cell line remained relative constant at 5, 10, and 25% culture dilutions, with values between 2- to 3-fold higher than its control. The highest activity was observed at a 50% dilution, with a value of 2.32 ± 0.74, representing more than a 4-fold increase in activity relative to the control. The G1B cell line had slight dose-response increases in activity, with the highest value being approximately twice as high as its control (5.49 ± 0.89). Both liver cell lines, Hepa-E1 and PLHC-1, showed slight dose-response increases at dilutions of 5, 10, and 25%., with the highest activity for Hepa-E1 and PLHC-1 being twice as high as their controls at the 25% dilution. However, CAT activity significantly decreased for these two cell lines at the 50 and 75% culture extract dilutions, showing less than half the activity observed in control groups.

A similar trend for CAT activity was observed in cells exposed to high salinity and high nutrient culture extracts (Figure 7.4B), as the cell lines PLHC-1 and RTgill-W1 showed the highest activity among all cell lines, with respect to their corresponding controls ($p<0.05$). The highest activity of PLHC-1 cells was observed at the 25% dilution (66.56 ± 12.98), while the highest for RTgill-W1 was observed at dilutions of 10 and 25%, with values between 3- and 4-fold higher than controls. However, enzyme activity decreased at the 50 and 75% dilutions. G1B cells presented slightly high activity at the

50% dilution, but no significantly different activity at the other dilutions compared the control group. The Hepa-E1 cell line presented the lowest activity of all cells relative to its control and was particularly low at the two highest dilutions with values close to the limits of detection.

Culture extracts from low salinity led to lower CAT activity in all cell lines, with no major increases relative to control groups. The liver cell line PLHC-1, exposed to the low salinity and low nutrients culture (Figure 7.4C) showed the highest activity at all dilutions, being this activity almost twice as high as the activity in non-exposed cells. The highest activity when exposed to the low salinity X high nutrients culture extract (Figure 7.4D) occurred at a dilution of 75%. RTgill-W1 cells had the lowest activity among all cell lines, though it remained higher at most culture dilutions relative to its control. Neither G1B nor Hepa-E1 presented significantly high CAT activity relative to the controls, with the exception of G1B at the 75% dilution of the low salinity X high nutrient treatment combination.

Superoxide dismutase (SOD)

Among all culture conditions examined, the high salinity X low nutrient culture caused the highest levels of SOD primarily for the Hepa-E1 and RTgill-W1 cell lines ($p<0.05$; Figure 7.5A). RTgill-W1 presented significantly high levels of SOD that were approximately twice as high as control groups for the 5, 10, 25, and 50% culture dilutions, and approximately 4-fold higher than controls at the 75% dilution level (6.19 ± 0.88). On the other hand, and contrarily to the observations for CAT activity, the liver cell line Hepa-E1 showed significant levels of SOD at low and middle dilutions, but levels decreased nearly to basal levels at the 75% dilution. The gill cell line G1B only

presented significant SOD levels at the 25% culture dilution; enzyme levels in the other four dilutions remained approximately the same as in control groups. Similar to G1B, SOD levels in PLHC-1 cells remained nearly the same as the observed in non-exposed cells, but significantly decreased at the highest two dilutions, where levels were less than half of the basal SOD levels.

Compared to cells exposed to the high salinity X low nutrient culture, SOD levels in cells exposed to the high salinity X high nutrient culture conditions decreased in most treatments (Figure 7.5B). An exception was observed at the 25% dilution, where both liver cell lines showed levels approximately three times higher than their respective controls, but with the high variability within this treatment, SOD levels were not significantly different among all cell lines ($p>0.05$). Moreover, Hepa-E1 cells showed dose-response increases from the 5% to the 25% dilution, but enzyme levels decreased at the two highest culture dilutions, though SOD levels remained above basal levels. PLHC-1 cells presented their highest enzyme levels at the 25% dilution, while levels at the 50 and 75% dilutions were similar observed levels in non-exposed cells. Both gill cell lines showed slightly higher activity than their respective control groups but nearly constant across all culture dilutions.

Among all cell lines, Hepa E-1 represented the cell line with the highest SOD induction after exposure to P. parvum cultured in low salinity X low nutrients ($p<0.05$; Figure 7.5C). At all dilution levels, SOD levels in Hepa-E1 cells were two to three times higher than non-exposed cells – the highest enzyme levels were observed at the 25% dilution. SOD levels in all other cell lines remained low, and nearly the same as basal levels. PLHC-1 cells presented slight increases in SOD levels at the 75% culture dilution,

but not enough to be significantly different from the levels shown by Hepa-E1. SOD in G1B presented slight increases at the 50 and 75% dilutions, and in RTgill-W1 cells, it remained relatively low and constant across all dilutions.

Finally, while the Hepa-E1 cell line was again the group with the highest SOD levels after exposure to the low salinity X high nutrients culture ($p<0.05$; Figure 7.5D), these SOD levels were lower compared to observations for the other three culture conditions. PLHC-1 showed slight increases at the two highest dilutions, and both gill cell lineages presented low and nearly constant SOD levels across all dilutions.

Glutathione peroxidase (GPx)

The Hepa-E1 cell line had significantly higher levels of GPx at the 5, 10, and 25% dilutions of the high salinity X low nutrient levels, compared to the other cell lines ($p<0.05$; Figure 7.6A). However, enzyme levels dropped below basal levels at the two highest culture dilutions. No major differences in GPx activity was observed for the other three cell lines.

Similar results were observed in cells exposed to the high salinity X high nutrients culture (Figure 7.6B). Among all cell lines, Hepa-E1 showed significantly higher GPx activity at the first three dilution levels; activity at the 25 % dilution was more than four times the basal activity (51.48 ± 7.80). As observed for the other high salinity culture condition, GPx levels in Hepa-E1 dropped near basal levels at the 50% and 75% dilutions. PLHC-1 cells presented slight dose-response increases of GPx activity from the 5% to the 25% dilutions, but nearly absent activity at the 50 and 75% culture dilutions. Besides the slight increase at the 10% dilution, G1B cells did not showed significant GPx

activity at any other dilution level. RTgill-W1 cells showed close to absent activity at all dilutions.

GPx activity increased by three-fold in Hepa-E1 cells at the 5, 10, and 25% dilutions following exposure to the low salinity X low nutrients toxin culture (Figure 7.6C), making this liver cell line the only model in which P. parvum elicited significant GPx activity ($p<0.05$). All other cell lines did not show GPx activity above basal levels. Lastly, while statistical differences were observed between cell lines after being exposed to the low salinity X high nutrients culture ($p<0.05$; Figure 7.6D), mainly due to the relatively high GPx activity in Hepa-E1 cells, none of the remaining cell lines presented evidence of GPx induction. Measurements were, in most cases, below levels shown by non-exposed cells and, in some cases, close to undetectable activity.

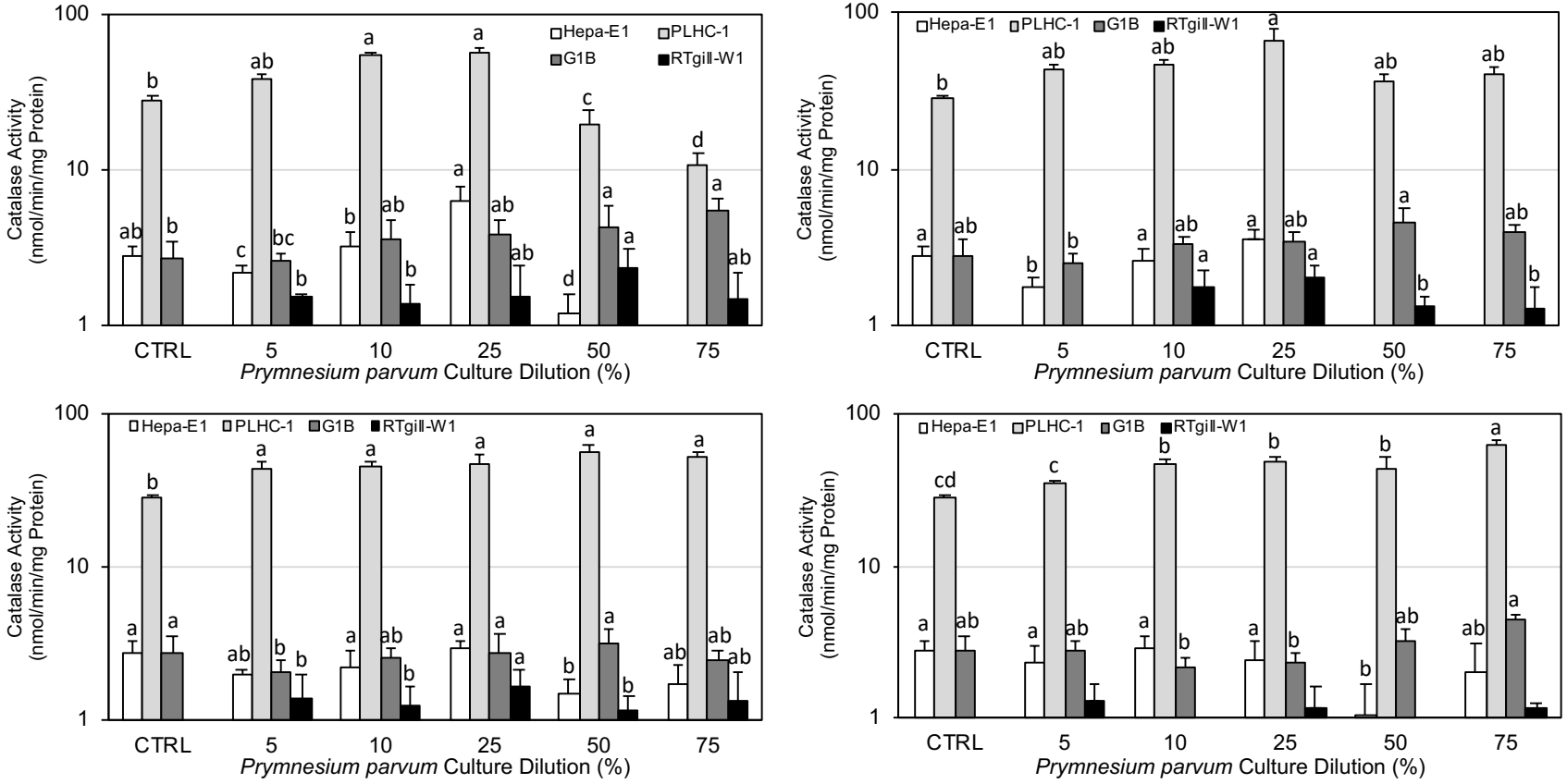

Figure 7.4. Activity of catalase (CAT), reported as mean nmol/min/mg protein ± std. error, in all four cell lines exposed to A) high salinity-low nutrient, B) high salinity-high nutrient, C) low salinity-low nutrient, and D) low salinity-high nutrient *P. parvum* culture extracts. Control values for each cell line are as follows: Hepa-E1: 2.74 ± 0.48; PLHC-1: 28.05 ± 1.25; G1B: 2.72 ± 0.76; and RTgill-W1: 0.52 ± 0.33. Different letters denote significantly different activity among culture extract dilutions for each cell line. Absence of letters denote no significant differences among treatment groups. Missing bars correspond to values below 1, which were not plotted for graphical purposes (logarithmic scale).

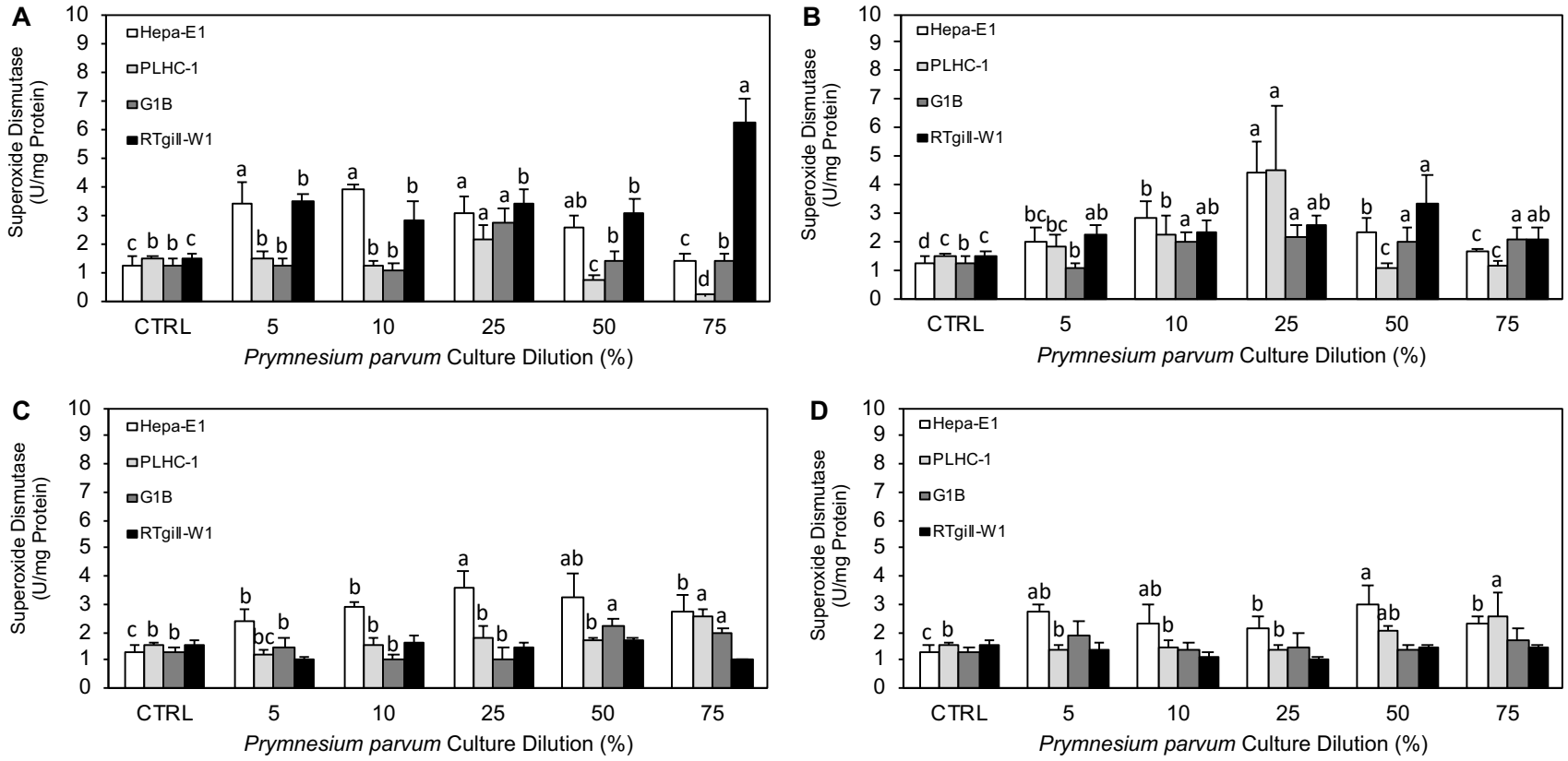

Figure 7.5. Levels of superoxide dismutase (SOD), reported as mean Units/mg Protein ± std. error, in all four cell lines exposed to A) high salinity-low nutrient, B) high salinity-high nutrient, C) low salinity-low nutrient, and D) low salinity-high nutrient *P. parvum* culture extracts. Control values for each cell line are as follows: Hepa-E1: 1.28 ± 0.26; PLHC-1: 1.52 ± 0.07; G1B: 1.27 ± 0.21; and RTgill-W1: 1.53 ± 0.14. Different letters denote significantly different activity among culture extract dilutions for each cell line. Absence of letters denote no significant differences among treatment groups.

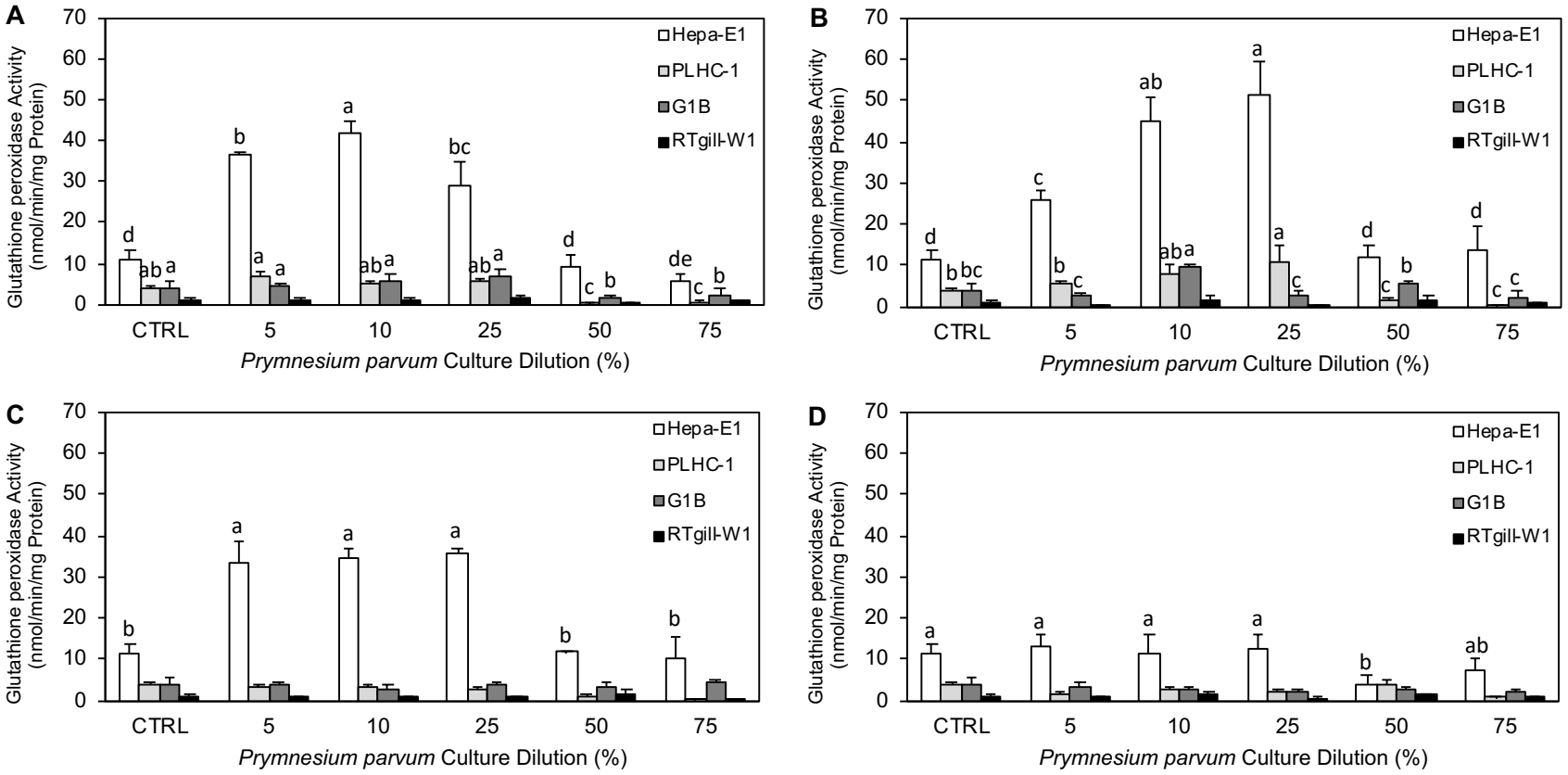

Figure 7.6. Activity of glutathione peroxidase (GPx), reported as mean nmol/min/mg protein ± std. error, in all four cell lines exposed to A) high salinity-low nutrient, B) high salinity-high nutrient, C) low salinity-low nutrient, and D) low salinity-high nutrient *P. parvum* culture extracts. Control values for each cell line are as follows: Hepa-E1: 11.01 ± 2.41; PLHC-1: 3.99 ± 0.69; G1B: 4.05 ± 1.62; and RTgill-W1: 0.82 ± 0.53. Different letters denote significantly different activity among culture extract dilutions for each cell line. Absence of letters denote no significant differences among treatment groups.

Discussion

Identification of MOAs of toxic agents, primarily through the elucidation of molecular initiation events and resultant adverse outcomes, represents a fundamental aspect in aquatic toxicology studies. This becomes even more important when the causes of major ecological impacts, such as the fish kills caused by P. parvum toxic blooms, are poorly understood and standards for known toxins are unavailable. The combination of the potential shown by many compounds to generate ROS and the ability of biological organisms to express antioxidant responses as a defense mechanism makes the measurement of antioxidant enzyme activity a suitable biomarker to explore when little knowledge about MOAs is available (Di Giulio et al., 1989). Thus, investigations of oxidative stress, in this case measured as the induction of antioxidant enzymes, represent an important step toward understanding sublethal mechanisms of toxicity caused by P. parvum blooms. Additionally, cytotoxicity, measured as cell survival, is often assessed during *in vitro* experimentation, to provide information on cellular toxicity, and whether lethal or sub-lethal responses are anticipated. A summary of the experimental design and major findings of this study, presented as a conceptual model, is given in Figure 7.7, and may facilitate the understanding of the potential mechanisms by which P. parvum may exert its toxicity in fish.

Hemolysis of sheep erythrocytes were used as validation of the presence of P. parvum toxins in the culture extracts, given previous studies elucidating the hemolytic potential of golden alga (Brooks et al., 2010; Svendsen, Andersen, Hansen, & Steffensen, 2018). The direct comparison of hemolytic potential across all dilutions between media controls and experimental cultures indicated the presence of hemolysis-causing

compounds in the latter. Furthermore, higher hemolysis observed for high-salinity culture conditions represents evidence of the differing potential of the euryhaline P. parvum to cause toxicity under different, and apparently stressful, environmental conditions.

As observed in the present study, cell survival was not compromised following exposure to P. parvum cultures, with the exception of the hepatic cell line Hepa-E1 at the highest dilution tested - all other treatments did not cause significant cell mortality. This represents evidence that, besides the previously described hemolytic and gill toxicity properties of P. parvum toxins (Brooks et al., 2010; Schug et al., 2010), hepatotoxicity may be another adverse outcome of golden alga blooms. However, such observations may be more prominent at high levels of exposure. This would not be surprising as other algae-derived toxins, such as mycrocystins, are known to be hepatotoxic (Jos et al., 2005; Prieto, Jos, Pichardo, Moreno, & Camean, 2006). Additionally, antioxidant responses in the liver are often present at higher rates than in other organs, placing liver models as good alternatives to evaluate the production of ROS and the subsequent induction of antioxidant enzymes. Therefore, potential influences of P. parvum toxins on the liver deserves future attention.

Contrary to the cytotoxicity observed in liver cell lines, survival of gill cell lines was not affected. Though previous studies have visually reported P. parvum to affect gill-breathing organisms (Roelke et al., 2016), by apparently altering the integrity and dynamics of gill cell membranes, results from cytotoxicity assays with gill cells differed from previous studies. Such observations in this study may be explained by a lack of previous work examining sublethal toxicity. It is important to note that *in vitro* experiments in the present study were performed for 48 hours, a time period in which

rapid onset of mortality responses have been routinely observed (James et al., 2011; Prosser et al., 2012; Valenti et al., 2010). Given the results of antioxidant enzymes activity during these short-term studies, our observations suggest that sub-lethal responses are triggered by exposure to P. parvum, but that continuous alterations to biochemical pathways is required to cause cell death.

Induction of the antioxidant enzymes catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx) strengthens the importance of employing sub-lethal responses to understand P. parvum toxicity, and further facilitate the selection of appropriate cell lines for future research. Results from our current study are similar to observations by Dorantes-Aranda et al. (2015), where measurements of reactive oxygen species and antioxidant enzymes (SOD) were suggested to play a role in fish toxicity by harmful algae. However, the evaluation of CAT and GPx in the present study represent a step forward in the assessment of potential fish toxicity and biochemical alterations resulting from exposure to P. parvum. CAT, an antioxidant enzyme that protects against damage caused by hydrogen peroxide, in gill cell lines has been previously described (Franco et al., 2018), and results from the present study are in agreement with the RTgill-W1 cell line being a useful tool for the evaluation of this biomarker. The elevated levels of CAT after exposure to P. parvum cultures represents evidence of the potential of P. parvum toxins to generate ROS, potentially hydrogen peroxide, in gill cells. Induction of CAT, while with lower intensity, was also measured in G1B cells, and in hepatic cell lines at certain dilution levels. Prymnesins may possess a strong ability to produce hydroperoxide radicals, which will be responsible for the upregulation of CAT and other enzymes associated with hydrogen peroxide, such as GPx. Decreases in CAT activity in

liver cells at high doses may be due to the reduced survival of these cells, as was observed in the cytotoxicity assays. It is also important to recall that ROS generation in biological organisms may be tissue-specific (Slaninova & Modra, 2009), hence the differentially observed CAT activity intensity among the four cell lines evaluated. Moreover, based on the potential structural responses reported by Edvardsen and Imai (2006) in which membrane permeability is compromised due to disruptions in ionoregulation, and the high rates of absorption often seen in gill cells (Sayeed et al., 2003), it is possible that generation of peroxide, and the subsequent induction of CAT enzymes, is favored in gill cells exposed to P. parvum HAB events.

Li, Liu, Song, and Liu (2003) conducted measurements of superoxide dismutase (SOD), an enzyme that protects against superoxide radicals, and identified high activity of this enzyme in hepatocytes of common carp after exposure to microcystin-LR. Our results regarding this biomarker also point out significantly high levels of SOD in liver cells, specifically in Hepa-E1 cells. This cell line may be equipped with a more sensitive mechanism triggering expression of SOD upon formation of ROS, presumably from a hydroxyl (-OH) radical, and may be better suited to manage superoxide production (Franco et al., 2018). The observed levels of SOD may also point out that P. parvum toxins may have the ability of generating superoxide radicals in the liver, so not only liver cells may be better able to respond to such radicals but also exposure to P. parvum toxins may lead to high production of ROS of superoxide nature.

The activity of SOD represents a common mechanism that increases its activity to prevent oxidative damage (Di Giulio et al., 1989), and as in *in vivo* studies, this has also been shown in *in vitro* experimentation (Puerto, Pichardo, Jos, & Camean, 2009). RTgill-

W1 cells only presented significant SOD levels when exposed to high salinity culture conditions but not when exposed to low salinity cultures, and since these cells were observed to be less responsive in terms on SOD activity (Franco et al., 2018), strong inducers of hydroxyl radicals may be needed to trigger SOD in these gill cells. Low SOD induction was also observed in RTgill-W1 cells after exposure to purified toxins and crude extracts of P. parvum (Dorantes-Aranda et al., 2015)

As with SOD measurements, the activity of glutathione peroxidase (GPx), an enzyme that helps in the reduction of hydrogen peroxide and peroxide radicals, was more prominent in Hepa-E1 cells. According to Perez-Pertejo, Reguera, Ordoñez, and Balaña (2008), an overexpression of SOD is often followed by increases in GPx activity, which would explain both enzymes being significantly expressed in Hepa-E1 cells. Expression of these antioxidant enzymes provides further support to consider oxidative damage as a mechanism of toxicity of P. parvum toxins in fish. Furthermore, the differences observed among cell lines and the toxicity caused by the different culture conditions, and thus the toxins present within them, may be the result of changes in the assembly of enzyme subunits when concentration of toxins increase (Puerto et al., 2009; Yin et al., 2005), causing some cell lines to be able to respond better than others, even when such biological responses are taking place in all of these model systems.

We observed cytotoxicity and induction of antioxidant systems in fish cell lines to be higher from P. parvum cultured under high salinity conditions, and even more so, under low nutrient levels. Similar to previous observations (Baker et al., 2007, 2009), elevated salinity increased growth rates compared to lower salinity conditions, with cultures reaching stationary conditions 7 days earlier (Table 7.1). However, nutrient

limitation has been observed to increase toxicity apparently by influencing toxin production (Valenti et al. 2010), and thus potentially the composition of toxins. Based on the results obtained in this study, it appears that high salinity represents a major stressor for P. parvum, leading to the upregulation of the cellular mechanisms responsible of toxin production. Furthermore, as with most primary producers, nutrient limitation is an important factor in moderating major biological processes such as growth and reproduction. Thus, the production of toxins under low nutrient conditions may represent a response by which P. parvum influences its external environment and impact potential competitors. This was observed by Granéli and Johansson (2003b), where the apparent production of allelopathic substances, based on bioassay responses, in the genus Prymnesium increased in cultures grown with limited nitrogen and phosphorus. Furthermore, bioassays used to estimate the toxicity of P. parvum in fish pointed out an apparent reduction in the production of allelopathic compounds, again due to responses of invertebrate and vertebrate bioassays, where nutrients were sufficient (Roelke et al., 2007). Unfortunately, non-target organisms, such as fish, would also experience adverse effects, leading to serious ecological consequences during P. parvum HABs. Moreover, the fish cell lines used in the study are derived from freshwater fish, and high salinity may have influenced the potency of the extracts over the cells, even though we employed cell free media controls in the present study. Nonetheless, the combination of salinity and nutrients conditions apparently stressed P. parvum and differentially favored production of substances resulting in higher sublethal toxicity. This has implications for ecological effects that may be observed depending on gradients of environmental conditions where P. parvum blooms occur in both coastal/brackish and inland waters (Brooks et al., 2010).

It is also important to note that *in vitro* experimentation employed herein provided useful insights to mechanistically evaluate sublethal toxicity, but further experimentation is required to better integrate these *in vitro* results with *in vivo* studies.

The results obtained from this *in vitro* experimentation with P. parvum cultures and their associated MOAs in aquatic organisms represent a step forward in the description of sublethal toxicity associated with this alga when HAB events occur. Further evidence regarding the usefulness of fish cell lines in ecotoxicological studies of understudied HAB species is demonstrated, and the study should encourage researchers to continue to explore the suitability of cell-based models to investigate toxicity of poorly understood substances. Our observations of cytotoxicity and sub-lethal responses in liver and gill cells driven by P. parvum identify the importance of future work to elucidate toxins responsible for such sublethal responses.

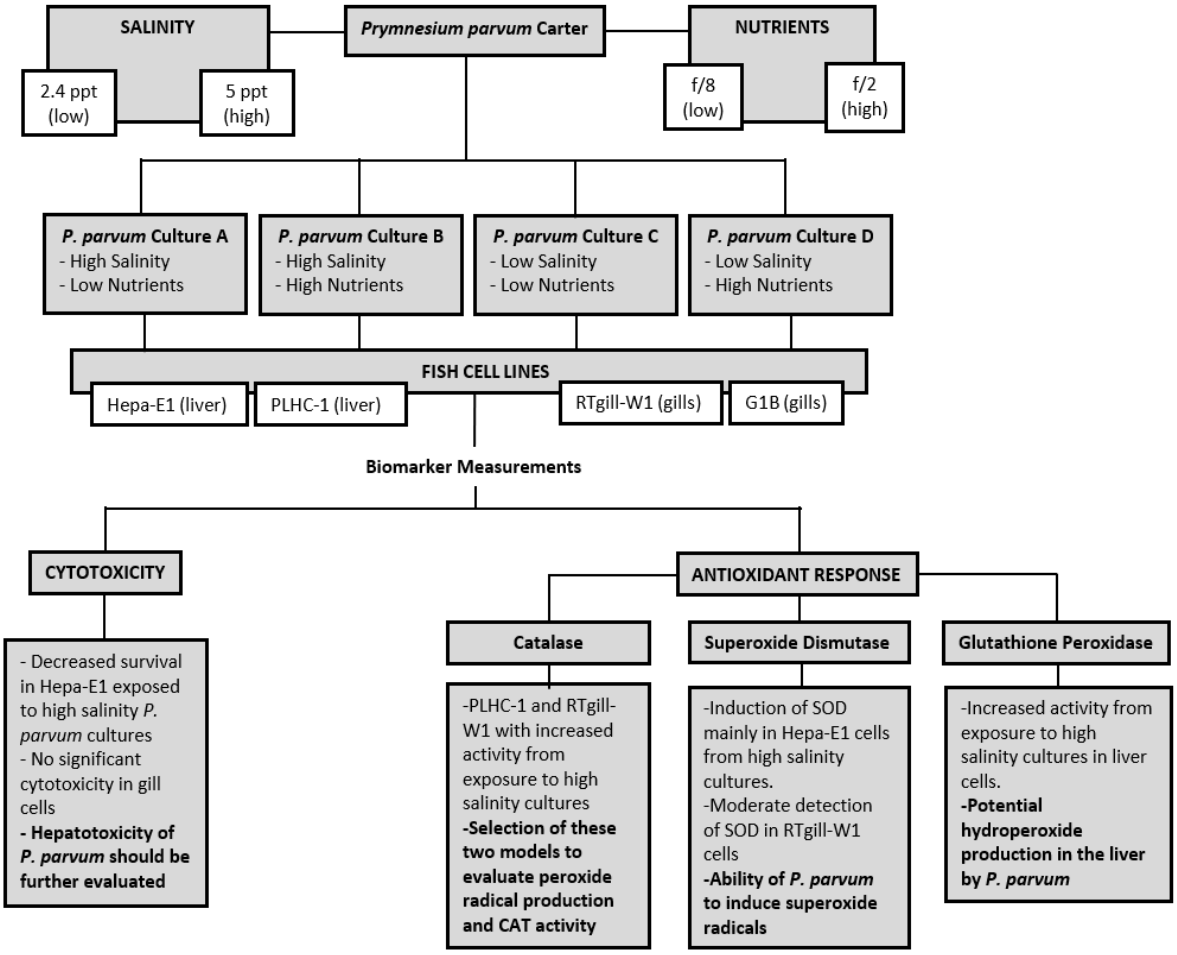

Figure 7.7. Conceptual model describing the ability of *Prymnesium parvum* to differentially induce cytotoxic and antioxidant responses in fish cell-based models. The model highlights the experimental design and major findings of the study, suggesting oxidative stress as a potential MOA of *P. parvum*, and the need to further evaluate the liver as a potential target of prymnesins.

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CHAPTER EIGHT

Summary and Conclusions

The overarching goal of this dissertation relies on the applicability of new approach methodologies (NAMs) and their integration with more traditional approaches (e.g. *in vivo* systems) to address research gaps associated with metabolism and mechanistic toxicity of legacy and emerging contaminants by piscine species, under the influence of different environmental stressors.

Comprehensive ecological risk assessments require the integration of well-established methodologies to assess the degree at which aquatic populations may be impaired from exposure to xenobiotics (Suter II, 2016). Unfortunately, for many years, hazard and risk assessments have relied on the implementation of whole-animal approaches and considered general endpoints such as mortality, growth, and reproduction to establish whether organisms and ecosystems are compromised. Furthermore, these evaluations of risk often focus on a reduced number of model species (e.g. rainbow trout) to derive data that can be then further extrapolated to predict adverse effects for other species. While these approaches continue to be fundamental in advancing assessments of risk (Mangold-Doring et al., 2021) and traditional model species in ecotoxicology facilitate evaluations of complex environmental scenarios, there is an imminent need to expand the current knowledge about exposure scenarios where important environmental factors could influence organisms' responses to pollution and that cannot be fully replicated in the laboratory. As demonstrated in the work presented in this dissertation, the selection, implementation, and integration of testing strategies must rely on the

specific characteristics of the areas, species, and populations of interest, and should consider the influence of other environmental factors with the ability to modify the risk associated with contaminants and the way organisms cope with chemical exposure.

Furthermore, traditional ecotoxicological endpoints may not be fully representative of the risks and hazards that aquatic populations pose upon exposure to environmental pollutants. Significant advances like the establishment of the adverse outcome pathway (AOP) framework (Ankley et al., 2010) have highlighted the need of considering more mechanistic approaches to describe chemical-organism relationships to further understand pollutant-driven alterations at different levels of biological organization. Due to the complexity of whole animal models, mechanistic studies are difficult to conduct with traditional approaches, as the influence of many molecular, cellular, and physiological processes often impair the ability to understand specific pathways of interest. In this context, NAMs are considered to be suitable alternatives to depict chemical-organism interactions and to predict potential adverse effects from exposure.

As shown in this dissertation, the applicability of NAMs allowed for the description of intraspecific and sex-specific differences in the biotransformation of polycyclic aromatic hydrocarbons (PAHs) by Gulf killifish populations with different exposure history. Through the implementation of liver S9 substrate depletion bioassays (OECD, 2018), it was possible to demonstrate that the suggested evolutionary adaptation involving a reduced inducibility of the aryl hydrocarbon receptor (AhR) pathway in pollution-adapted populations had a significant influence over how these populations processed common contaminants found in the ecosystems they inhabit. However, this

same experimentation also helped in elucidating some of the limitations associated with NAMs as, in the case of pollution-adapted Gulf killifish, while it was demonstrated that biotransformation enzymes associated with the AhR pathway did present lower activity and that this translated into lower hepatic clearance rates, the *in vivo* assessments provided further observations that were not possible to determine from the *in vitro* approach. Despite the lower AhR-related biotransformation ability shown by S9 depletion bioassays, the overall responses *in vivo* indicated the potential influence of extrahepatic factors, as demonstrated by the similar time-dependent concentrations of benzo[a]pyrene in liver tissue but different metabolite profile of this compound between adapted and non-adapted populations. If this type of assessment would have solely relied on the use of NAMs, the resulting observations would have not been representative of the actual responses of these populations. Thus, these assessments highlighted the value of NAMs in depicting specific pathways in response to contaminant exposure but also that their implementation relies on their integration with more complex techniques for more robust evaluations.

Besides the observations from comparing *in vitro* and *in vivo* approaches, evaluating the biotransformation ability of Gulf killifish populations in the Houston Ship Channel (HSC) led to valuable insight into biochemical and physiological modifications that chronically exposed individuals may display in order to maintain ecological fitness. As discussed in chapters two and three, there is an pressing need to include more specific aspects of the populations of interest, such as exposure history and potential evolutionary adaptations, in an attempt to reduce errors arising from intraspecific differences when relying on information derived from one area and species to others.

Furthermore, the work described in chapters four and five illustrated the advantages of weight of evidence (WoE) approaches and the necessity in integrating different lines of evidence for proper assessments of risk. While wastewater treatment technologies have advanced in the last decade, most treatment plants around the world still lack the infrastructure and technology to effectively remove micropollutants (e.g. pharmaceuticals, EDCs) (Huggett et al., 2003). These substances are not directly classified as persistent, yet their constant introduction from e.g. effluent discharges allow them to be continuously present in aquatic systems. This issue is further aggravated by other environmental factors that modify stream dynamics and impact the magnitude, frequency, and duration of exposure (Brooks, 2018). In the multi-approach evaluation to assess estrogenicity in surface waters included in this dissertation, it was demonstrated that in semi-arid streams that receive a direct influence of seasonal snowmelt and present varying streamflow, assessments of risk become more complex and that the selection of testing strategies is directly dependent on the characteristics of the study areas.

Despite the importance of implementing *in vitro* systems as part of a WoE approach, it was shown that the applicability of these cell-based systems is limited by the sensitivity of cells or cell components to environmentally relevant concentrations of contaminants. Indeed, Rehberger, Kropf, and Segner (2018) point out that evaluations of *in vitro* systems' sensitivity, specificity, and exposure conditions of bioassays are critical for proper selection of these models. This has been further hindered by new and emerging chemicals that cause adverse effects upon long-term exposure and that do not require high environmental concentrations to consider exposed populations at risk (Barceló & Petrovic, 2007). A clear example of this scenario is the presence of endocrine disrupting

compounds (EDCs) in surface waters. As exemplified by the use of rainbow trout primary hepatocytes, the performance of this type of technique was hindered by the significant changes in streamflow across seasons, and this *in vitro* approach was only informative when surface waters were effluent-dominated and environmental concentrations of EDCs were significant enough to trigger responses in cell-based bioassays. As with the observations for Gulf killifish described earlier, *in vivo* systems resulted necessary to identify the risk associated with effluent discharges and micropollutants during seasons of high streamflow. These limitations denote the need to continue to explore and advance the applicability of NAMs for their use in describing hazard and risk in complex environmental settings.

In general, NAMs aim to reduce, refine, and, to some extent, replace the use of laboratory animals. It is clear that the implementation of *in vitro* tools such as S9 subcellular fractions and primary hepatocytes presented advantages to evaluate adverse biological effects of exposed organisms, as it was shown in chapters 2-5. However, for these two *in vitro* systems in specific, while they do support a reduction of animal use, a constant supply of living animals must be maintained so that cells or cell fractions are available. This need, in turn, may have implications for system variability as the performance of these tools largely depends on the individuals from where they were derived. An *in vitro* alternative that reduces individual variability and that does not require a constant supply of live animals is the establishment of cell lines. While early in their application to be considered as the main alternative for *in vivo* toxicity testing and assessment of risks, cell lines are now widely employed in the screening of chemicals and environmental samples, and in the mechanistic evaluation of toxicity pathways

(Rehberger et al., 2018). These advances have led to major outcomes in ecotoxicology, such as the recent adoption of the OECD 249 Test Guideline on the use of a fish gill cell line for evaluations of acute toxicity (OECD, 2021).

Nonetheless. in the characterization of four different cell lines for their ability to show biomarkers of biotransformation and oxidative stress presented in chapter six, it was demonstrated that the selection of cell lines is species- and organ-specific, and they are limited by the type of biomarkers that can be measurable upon exposure. It is important to recall that this assessment was conducted with the purpose of evaluating cell responses to model chemicals through single exposures. This scenario is the simplest of the many applications that can be done with cell lines, though still reflects the advantages and limitations of these systems. A more complex scenario is the use of cell lines for the evaluation of environmental samples and chemical mixtures. In chapter six, it was shown that previously characterized cell lines were advantageous in describing potential modes of action of harmful algal toxins, even when a thorough characterization of these toxins continues to be limited. The observations from this *in vitro* approach were also reflected in other studies employing traditional *in vivo* approaches; specifically, for the alterations related to oxidative stress (Hill, Saari, Steele, Corrales, & Brooks, 2020). These observations provide significant evidence that, under relevant exposure conditions and by selecting appropriate models, the application of cell lines is promising in advancing systematic and predictive assessment for both single chemicals, laboratory-cultured samples, and environmental samples.

Altogether, the work presented in this dissertation advances the knowledge regarding the advantages and limitations of NAMs and reflects the need to continue to

investigate their suitability to provide valuable information for ecotoxicological studies. This work showed that assessments of biotransformation, endocrine disruption, and overall description of adverse effects from exposure to chemicals and environmental samples are significantly facilitated by *in vitro* systems, but that overall characteristics of the species and areas of interest must be accounted for when selecting appropriate cell-based models, as their improper selection could significantly mislead observations and subsequent environmental management strategies. Therefore, considering *in vitro* systems as valuable tools in ecotoxicology and assessments of risk must be under the rationale that they, in their current state, are only a component of complex evaluations and that their value comes from integrating them with other traditional as well as emerging approaches.

APPENDICES

APPENDIX A

Supplemental Information for Chapter Two

Table A2.1. Morphometrics of non-adapted (Smith Point; SP) and pollution-adapted (Vince Bayou; VB) *F. grandis* used in the preparation of S9 sub-cellular fractions.

Fish Pool	Fish No.	Population	Sex	Length (cm)	Weight (g)	Liver Weight (g)	Fractional Liver Weight (g liver g fish^{-1})	Cond Facto
A	1	Smith Point	Male	13.50	47.25	0.71	0.015	1.9
	2	Smith Point	Male	13.00	44.30	0.70	0.016	2.0
	3	Smith Point	Male	14.50	46.60	0.73	0.016	1.5
B	4	Vince Bayou	Male	14.00	54.60	1.07	0.020	1.9
	5	Vince Bayou	Male	14.00	61.00	1.19	0.020	2.2
	6	Vince Bayou	Male	13.50	43.15	1.28	0.030	1.7
C	7	Smith Point	Male	9.50	14.74	0.85	0.058	1.7
	8	Smith Point	Male	9.00	12.76	0.80	0.063	1.7
	9	Smith Point	Male	8.70	10.05	0.34	0.034	1.5
	10	Smith Point	Male	8.50	10.53	0.33	0.031	1.7
	11	Smith Point	Male	8.50	8.03	0.34	0.042	1.3
D	12	Vince Bayou	Male	9.50	12.28	0.41	0.033	1.4
	13	Vince Bayou	Male	8.60	9.23	0.17	0.018	1.4
	14	Vince Bayou	Male	9.80	14.03	0.55	0.039	1.4
	15	Vince Bayou	Male	8.70	10.06	0.47	0.047	1.5
	16	Vince Bayou	Male	8.50	8.17	0.22	0.027	1.3
E	17	Smith Point	Female	10.70	18.01	1.00	0.056	1.4
	18	Smith Point	Female	9.90	16.49	1.35	0.082	1.6
	19	Smith Point	Female	9.30	13.84	0.57	0.041	1.7
	20	Smith Point	Female	10.00	15.57	0.50	0.032	1.5
	21	Smith Point	Female	9.50	13.70	0.75	0.055	1.5
F	22	Vince Bayou	Female	10.50	17.66	0.81	0.046	1.5
	23	Vince Bayou	Female	11.00	20.90	0.63	0.030	1.5
	24	Vince Bayou	Female	9.40	11.78	0.61	0.052	1.4
	25	Vince Bayou	Female	8.50	11.16	0.69	0.062	1.8
	26	Vince Bayou	Female	8.80	9.87	0.68	0.069	1.4
G	27	Smith Point	Female	11.80	25.94	0.58	0.022	1.5
	28	Smith Point	Female	11.80	22.46	0.49	0.022	1.3
	29	Smith Point	Female	12.60	27.12	0.67	0.025	1.3
	30	Smith Point	Female	10.10	16.68	0.43	0.026	1.6
	31	Smith Point	Female	11.30	20.51	0.51	0.025	1.4
H	32	Vince Bayou	Female	11.10	18.22	0.27	0.015	1.3
	33	Vince Bayou	Female	11.20	19.37	0.37	0.019	1.3
	34	Vince Bayou	Female	11.40	16.97	0.49	0.029	1.1
	35	Vince Bayou	Female	11.50	24.64	0.94	0.038	1.6
	36	Vince Bayou	Female	11.20	20.18	0.32	0.016	1.4

Table A2.2. PAH molecular weight, partition coefficient (Log Kow), concentrations in substrate depletion incubations, limits of detection (LOD), and emission/excitation wavelengths for instrumental analyses.

PAH	Molecular Weight (g/mol)	Log K_{ow}*	Concentration in Reaction (µM)	LOD¥ (nM)	Fluorescence λ (nm)	
					Emission	Excitation
Phenanthrene	178.2	4.57	0.28	1.5	260	350
Pyrene	202.3	5.18	0.25	1.3	237	390
Fluoranthene	202.3	5.22	0.25	5.0	232	420
Chrysene	228.3	5.65	0.20	1.1	272	374
Benzo[k]fluoranthene	252.3	6.00	0.20	0.5	296	426
Benzo[a]pyrene	252.3	6.04	0.20	0.5	295	403

* PAH Log Kow values were obtained from the National Institute of Environmental Studies, Japan.
¥ Approximate limits of detection.

Table A2.3. Statistical output from the basal CYP enzyme activity comparisons between *F. grandis* populations, sexes, and the interaction of both. Test statistic corresponds to t-value or F-value for two-sample t-test and two-way ANOVA, respectively. Statistical significance is shown by * ($p < 0.05$) and ** ($p < 0.01$).

CYP Activity	Comparison	Test	Test Statistic*	*p*-value	
CYP1A	Population	Two sample t-test	4.675	$3.203e^{-5}$	**
	Sex	Two sample t-test	-7.612	$3.399e^{-9}$	**
	Population*Sex	Two-way ANOVA	20.197	$3.317e^{-5}$	**
CYP1B-like	Population	Two sample t-test	-0.58	0.5645	
	Sex	Two sample t-test	-5.263	$1.009e^{-5}$	**
	Population*Sex	Two-way ANOVA	0.378	0.5412	
CYP2C9-like	Population	Two sample t-test	-3.247	0.0019	**
	Sex	Two sample t-test	1.822	0.0743	
	Population*Sex	Two-way ANOVA	0.446	0.5068	
CYP3A4-like	Population	Two sample t-test	-1.486	0.1431	
	Sex	Two sample t-test	1.61	0.1132	
	Population*Sex	Two-way ANOVA	0.348	0.5574	

Table A2.4. Statistical output from the hepatic $CL_{IN\ VITRO}$ comparisons between *F. grandis* populations, sexes, and the interaction of both. Test statistic corresponds to t-value or F-value for two-sample t-test and two-way ANOVA, respectively. Statistical significance is shown by * ($p < 0.05$) and ** ($p < 0.01$).

PAH	Comparison	Test	Test Statistic*	*p*-value	
Phenanthrene	Population	Two sample t-test	0.516	0.6115	
	Sex	Two sample t-test	-3.816	0.0015	**
	Population*Sex	Two-way ANOVA	0.093	0.7642	
Pyrene	Population	Two sample t-test	3.414	0.0032	**
	Sex	Two sample t-test	-1.272	0.2193	
	Population*Sex	Two-way ANOVA	3.053	0.0960	
Fluoranthene	Population	Two sample t-test	3.186	0.0057	**
	Sex	Two sample t-test	-2.526	0.0238	*
	Population*Sex	Two-way ANOVA	11.412	0.0029	**
Chrysene	Population	Two sample t-test	4.488	0.0003	**
	Sex	Two sample t-test	-3.675	0.0019	**
	Population*Sex	Two-way ANOVA	5.278	0.0325	*
Benzo[a]fluoranthene	Population	Two sample t-test	5.646	$1.375e^{-5}$	**
	Sex	Two sample t-test	-3.051	0.0063	**
	Population*Sex	Two-way ANOVA	1.63	0.2163	
Benzo[a]pyrene	Population	Two sample t-test	5.134	0.0002	**
	Sex	Two sample t-test	-2.911	0.0105	*
	Population*Sex	Two-way ANOVA	12.314	0.0022	**

Table A2.5. Basal biotransformation activity of four CYP isoforms in liver S9 fractions derived from non-adapted (Smith Point) and pollution-adapted (Vince Bayou), and male and female *F. grandis*. Data are reported as mean pmol/mg protein/min ± SEM, with values representing combined data from all individuals from each population, and all males and females regardless of population.

CYP Activity	Population		Sex	
	Smith Point	Vince Bayou	Male	Female
CYP1A	7.38 ± 0.85	3.05 ± 0.37	8.16 ± 0.72	2.29 ± 0.27
CYP2B-like	0.75 ± 0.02	0.77 ± 0.03	0.84 ± 0.03	$0.67 \pm 5.4e^{-4}$
CYP2C9-like	17.89 ± 0.61	20.72 ± 0.62	18.46 ± 0.78	20.12 ± 0.47
CYP3A4-like	16.97 ± 0.73	18.84 ± 1.03	16.91 ± 1.03	18.91 ± 0.69

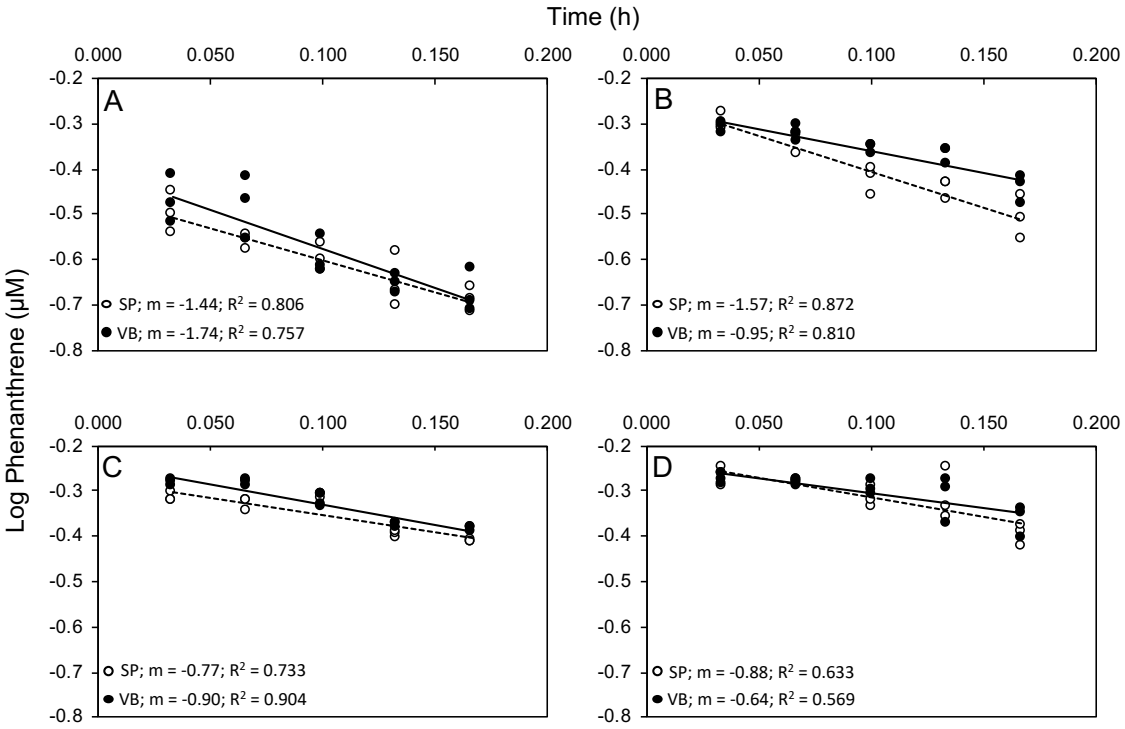

Figure A2.3: Depletion of phenanthrene by liver S9 sub-cellular fractions of non-adapted (SP) and pollution-adapted (VB) male (A and B) and female (C and D) *F. grandis.* Data points correspond to measurements of triplicate experiments with each fish S9 pool.

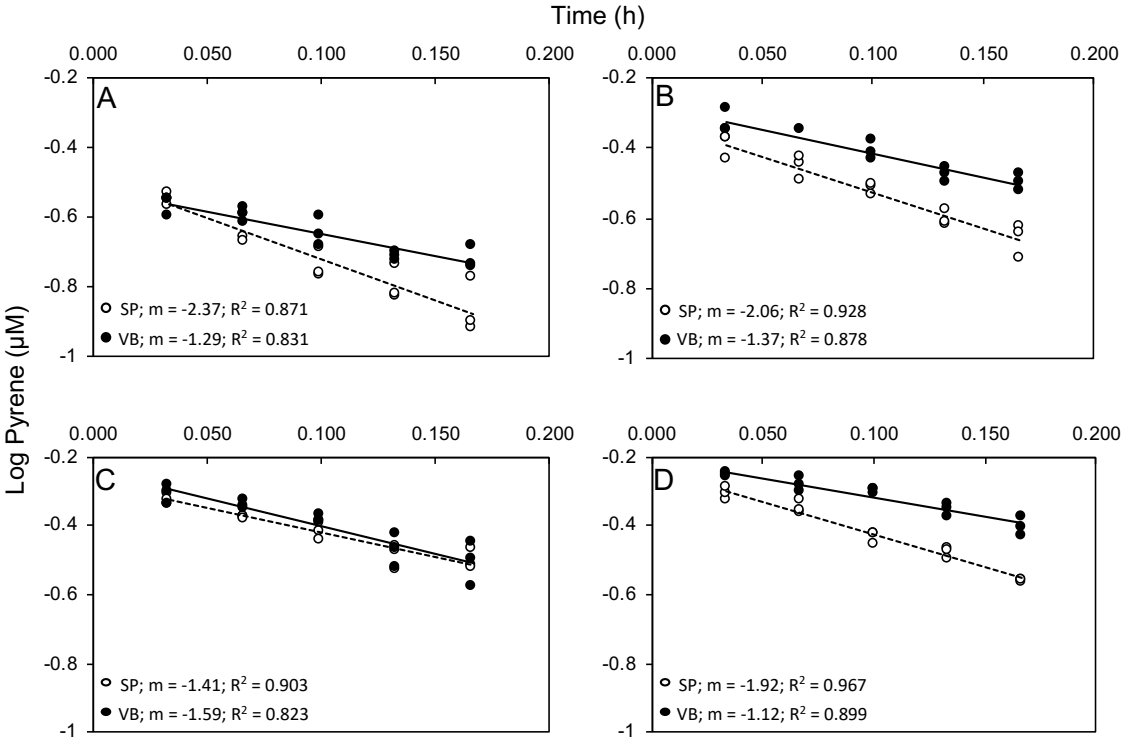

Figure A2.4: Depletion of pyrene by liver S9 sub-cellular fractions of non-adapted (SP) and pollution-adapted (VB) male (A and B) and female (C and D) *F. grandis*. Data points correspond to measurements of triplicate experiments with each fish S9 pool.

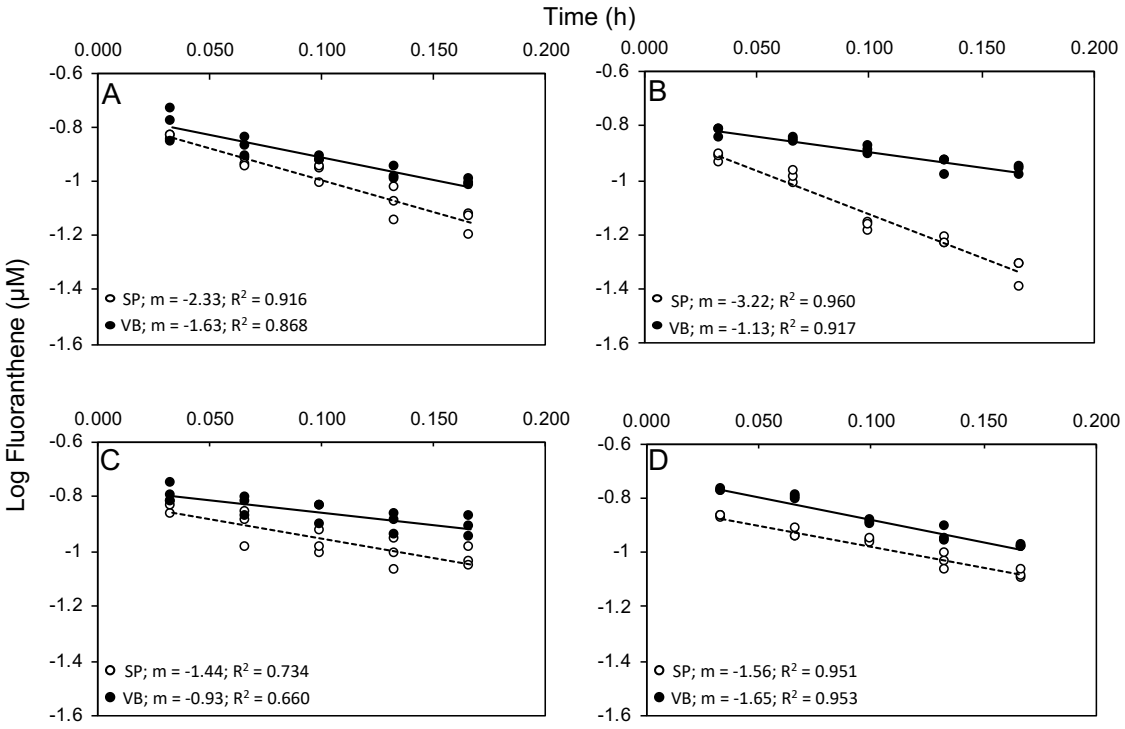

Figure A2.5: Depletion of fluoranthene by liver S9 sub-cellular fractions of non-adapted (SP) and pollution-adapted (VB) male (A and B) and female (C and D) *F. grandis*. Data points correspond to measurements of triplicate experiments with each fish S9 pool.

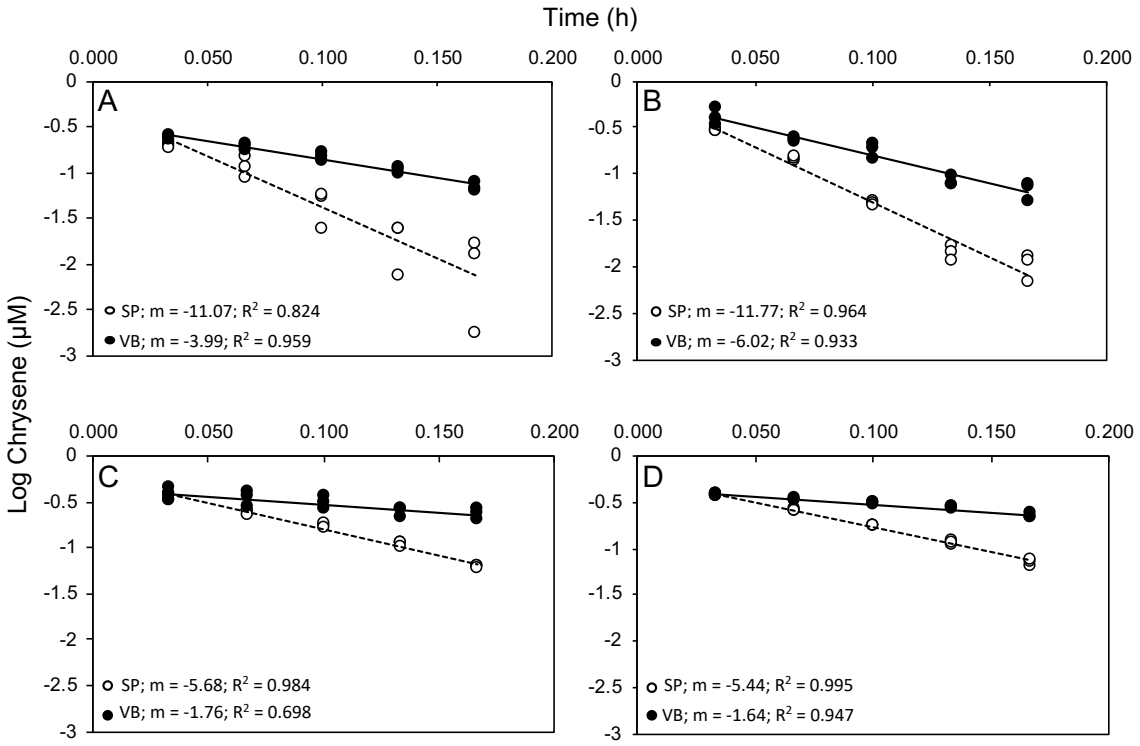

Figure A2.6: Depletion of chrysene by liver S9 sub-cellular fractions of non-adapted (SP) and pollution-adapted (VB) male (A and B) and female (C and D) *F. grandis*. Data points correspond to measurements of triplicate experiments with each fish S9 pool.

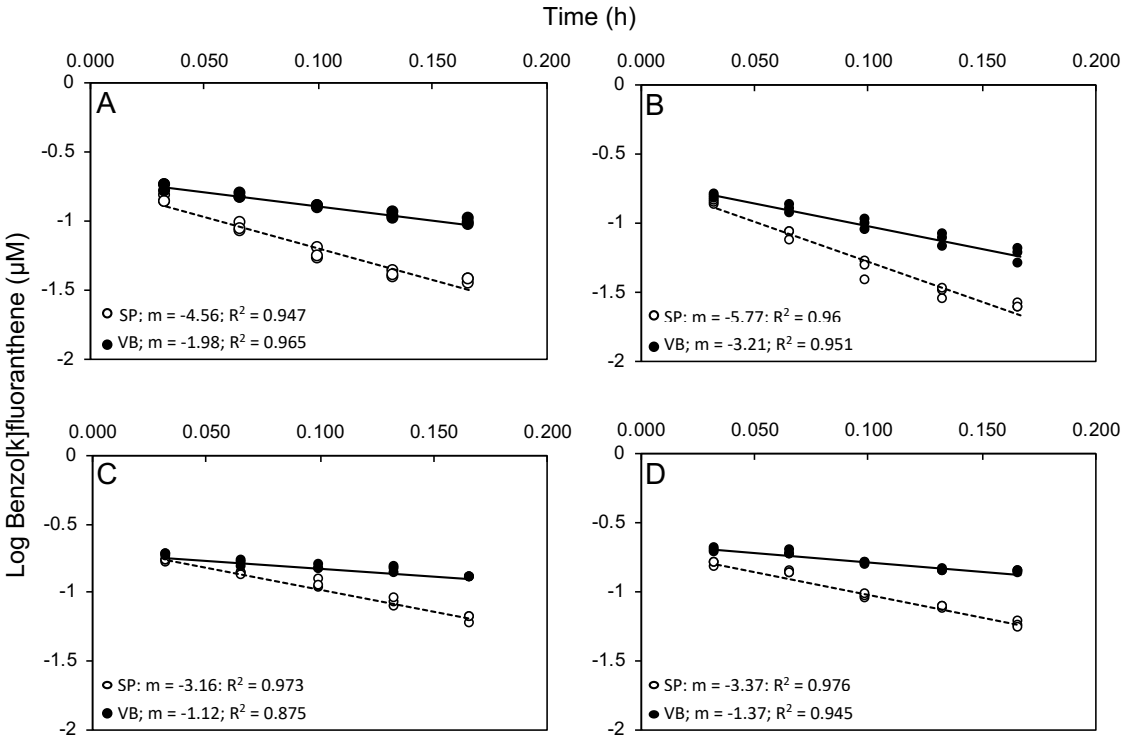

Figure A2.7: Depletion of benzo[k]fluoranthene by liver S9 sub-cellular fractions of non-adapted (SP) and pollution-adapted (VB) male (A and B) and female (C and D) *F. grandis*. Data points correspond to measurements of triplicate experiments with each fish S9 pool.

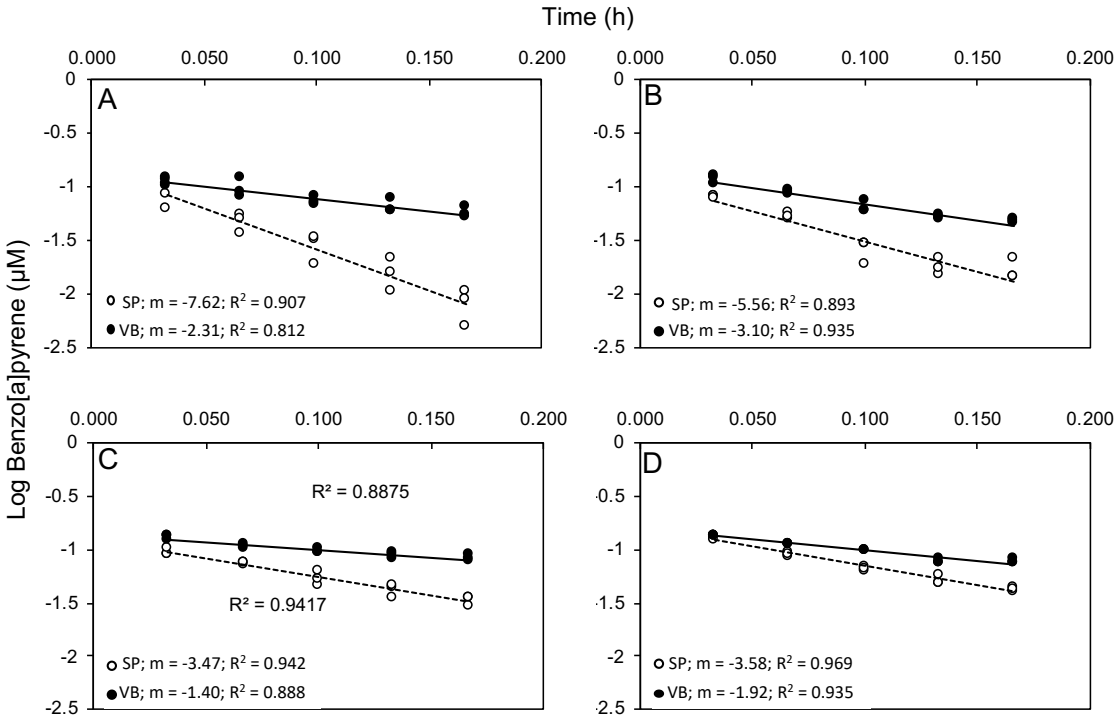

Figure A2.8: Depletion of benzo[a]pyrene by liver S9 sub-cellular fractions of non-adapted (SP) and pollution-adapted (VB) male (A and B) and female (C and D) *F. grandis*. Data points correspond to measurements of triplicate experiments with each fish S9 pool.

APPENDIX B

Supplemental Information for Chapter Three

Materials and Methods

Chemicals and solutions

Benzo[a]pyrene standards, resorufin sodium salt, 7-ethoxyresorufin (7-ER), 7-pentoxyresorufin (7-PR), 7-methoxy-4-(trifluoromethyl)coumarin (MFC), 7-benzyloxy-4-(trifluoromethyl) coumarin (BFC), alamethicin from Trichoderma viride, L-Glutathione reduced (GSH), uridine 5'-diphosphoglucuronic acid trisodium salt (UDPGA), adenosine 3'-phosphate 5'-phosphosulfate lithium salt hydrate (PAPS), and β-glucuronidase from Helix pomatia, Aryl sulfatase, and D-Saccharic acid-1,4-lactone monohydrate were obtained from Sigma-Aldrich (St. Louis, MO). Reduced β-nicotinamide adenine dinucleotide 2'-phosphate, tetrasodium salt (NADPH; 96%) was obtained from ACROS Organics (Fair Lawn, NJ). Benzo[a]pyrene metabolites (1-hydroxybenzo[a]pyrene, 6-hydroxybenzo[a]pyrene, 9-hydroxybenzo[a]pyrene, benzo[a]pyrene-4,5-dihydrodiol, and benzo[a]pyrene-7,8-dihydrodiol) were all obtained from MRI Global Chemical Carcinogen Repository (Kansas City, MO). Benzo[a]pyrene-D12 (97%) was obtained from Cambridge Isotope Laboratories, Inc. (Andover, MA). Deionized water (DI water) was obtained using a Milli-Q water purification system (Millipore, Billerica, MA). HPLC-grade acetonitrile, formic acid, ethyl acetate, and acetone were purchased from Thermo Fisher Scientific (Waltham, MA).

Animal collection and maintenance

At each collection site, baited minnow traps were deployed for approximately 5 h and individuals were immediately transferred to coolers for transport to the laboratory. Fish were maintained in 30- or 40-gallon glass tanks at ~10 ppt salinity and 23.0 – 25.0 °C, with a 14:10 h light cycle. Fish were fed TetraMin® Tropical Fish flakes and Purina AquaMax Fry Starter 200 pellets twice a day. All fish used in laboratory assessments were maintained in the lab for at least two months prior to beginning experiments.

Preparation of liver S9 and microsomal fractions

Three non-adapted, male *F. grandis* with average length (cm), weight (g), condition factor (K), and fractional liver weight (%) of 13.67 ± 0.76, 46.05 ± 1.55, 1.82 ± 0.26, and 1.55 ± 0.04, respectively, and three pollution-adapted males with average length of 13.83 ± 0.29, weight of 52.92 ± 9.04, K of 1.99 ± 0.23, and fractional liver weight of 2.29 ± 0.58 were used in the isolation of S9 sub-cellular fractions for subsequent bioassays measuring intrinsic clearance under basal, non-induced Ahr pathway. Additionally, three non-adapted, male *F. grandis* with average length, weight, K, and fractional liver weight of 12.50 ± 0.00, 35.37 ± 2.06, 1.81 ± 0.11, and 2.12 ± 0.07, respectively, and three pollution-adapted males with average length of 12.50 ± 0.87, weight of 35.57 ± 6.24, K of 1.81 ± 0.11, and fractional liver weight of 4.60 ± 2.65 were intraperitoneally injected with a 5 mg/kg dose of β-napthtoflavone (BNF) to induce the Ahr pathway and associated CYP enzymes 72 h prior to the isolation of S9 sub-cellular fractions.

The isolation of sub-cellular fractions followed standard preparation guidelines reported by OECD (2018) and Johanning et al. (2012). Briefly, individuals were

dissected, and the liver was perfused through the hepatic vein with 30-50 mL of ice-cold potassium phosphate buffer (100 mM, pH = 7.8), excised and weighed. Livers were pooled in 50 mL conical tubes with two volumes of homogenization buffer and homogenized. The resulting homogenates were centrifuged at 13,000 g for 20 min at 4 °C. The supernatant was then collected and transferred to cryogenic vials in aliquots of 0.5 mL and stored at -80 °C until assays were performed. The quantification of S9 protein was done via the Coomassie blue assay (Bradford, 1976), using bovine serum albumin (BSA) as a standard.

For all *in vivo* experiments, liver microsomes were isolated following the protocol described by Lavado, Thibaut, Raldua, Martin, and Porte (2004). Briefly, ~50 mg of liver tissue was placed in microcentrifuge tubes containing 200 µL of potassium phosphate buffer (100 mM, pH = 7.4) with 100 mM KCl, and 1 mM EDTA. A homogenization pestle was used to break the tissue, prior to a centrifugation step at 13,000 g for 20 min and at 4 °C. The resulting supernatant was collected, placed in 4 mL ultracentrifugation tubes (Beckman Coulter Life Sciences, Indianapolis, IN) and spun at 100,000 g for 90 min at 4 °C. The resulting pellet was resuspended in 1:10 (w/v) microsomal buffer (100 mM, pH = 7.4, 100 mM KCl, 1 mM EDTA, and 20% glycerol), transferred to a clean microcentrifuge tube, and stored at -80 °C until assays were performed.

Bile and liver extraction of benzo[a]pyrene and its metabolites

Bile extractions were conducted based on the protocols reported by Zhu et al. (2008) with modifications. 5 µL of bile were placed in borosilicate glass test tubes along with 0.5 mL sodium acetate buffer (200 mM, pH = 5.0) and 1 mL of a 2:1 ethyl acetate:acetone mix was added to the solution, vortexed, and the organic fraction was

transferred to a clean tube. An additional 1 mL ethyl acetate was added to the bile-sodium acetate solution and the organic fraction was collected. The latter step was repeated, and the combined organic fractions were evaporated under a gentle stream of nitrogen. The resulting material was resuspended in 250 µL of mobile phase (40% aqueous acetonitrile with 0.01% HCOOH) with 200 ng/mL of the internal standard (Benzo[a]pyrene-D12 97%).

After removing organic traces from the initial bile-acetate solution and to obtain metabolites that had been conjugated with glucuronic acid, 1,000 units of β-Glucuronidase were added and the resulting solution was incubated for 6 h at 37°C. The enzymatic reaction was stopped with the addition of 1 mL of 2:1 ethyl acetate:acetone, and the extraction of metabolites was conducted with two subsequent additions of ethyl acetate, as described before. The organic fractions were combined and evaporated, and the resulting material was resuspended in 125 µL mobile phase with internal standard. Lastly, after removing organic traces, the bile-acetate solution was treated with 20 units of aryl sulfatase and saccharic acid lactone (20 mM) and incubated for additional 6 h at 37 °C to obtain metabolites that had been conjugated with sulfates. After a triplicate round of extractions with ethyl acetate and acetone, and subsequent evaporation of the organic fractions, the resulting material was resuspended in 125 µL mobile phase with internal standard. All the resuspended fractions were then combined and transferred to autosampler vials for instrumental analyses.

During liver extractions of BaP, 15-20 mg of tissue were placed in glass vials with 150 µL of potassium phosphate buffer (100 mM, pH = 7.4) and homogenized with a pestle. 500 µL of a 4:1 hexane:methylene chloride solution were added to the

homogenate, followed by vigorous vortex and 5 mins of sonication. The homogenate was then centrifuged at 10,000 g for 5 min at 4 °C. The organic fraction was transferred to activated ISOLUTE® PLD+ protein and phospholipid removal columns (Biotage, Charlotte, NC) in an extraction system manifold for clean-up, and the clean solution was collected in borosilicate glass tubes. Additional 500 µL of the hexane:methylene chloride solution was added to the remaining tissue homogenate, and a second round of vortex, sonication, centrifugation, and column extraction was done. The clean organic fraction was collected and combined with the first collected fraction. The hexane:methylene chloride fraction was evaporated under a stream of nitrogen, and the remaining material was resuspended in 500 µL acetonitrile with 5.0 nM Benzo[a]pyrene-D12, used as internal standard, and transferred to autosampler vials for instrumental analyses.

Table B3.1. Morphometrics of male *F. grandis* used in each *in vivo* experiment and grouped by population. K: condition factor.

In Vivo Experiment No.	Smith Point			Vince Bayou		
	Length (cm)	**Weight (g)**	***K***	**Length (cm)**	**Weight (g)**	***K***
1	9.53 ± 0.82	13.97 ± 3.74	1.58 ± 0.15	9.10 ± 0.86	11.27 ± 3.64	1.46 ± 0.14
2	8.25 ± 0.90	9.20 ± 2.94	1.60 ± 0.17	8.54 ± 0.93	9.27 ± 3.30	1.45 ± 0.22
3	8.32 ± 0.72	8.60 ± 2.37	1.46 ± 0.13	8.38 ± 0.57	8.48 ± 1.78	1.43 ± 0.12
4	8.29 ± 0.68	8.06 ± 2.12	1.39 ± 0.10	9.32 ± 0.63	11.13 ± 2.18	1.36 ± 0.08

Table B3.2. Retention time, mass of selected fragment, % recovery (AVG ± SEM, n = 7), and limits of detection (LOD) of benzo[*a*]pyrene and its metabolites in fish bile.

Matrix	Analyte	Retention time (min	Selected fragment	% Recovery	LOD¥
Bile/S9	Benzo[*a*]pyrene	9.76	252.09	75.60 ± 5.23	0.03
	1-OH-BaP	8.58	268.09	73.07 ± 13.24	3.00
	9-OH-BaP	8.4	268.09	81.26 ± 6.62	0.95
	BaP-4,5-dihydrodiol	4.64	268.09	90.28 ± 3.24	0.10
	BaP-7,8-dihydrodiol	4.49	268.09	88.99 ± 2.49	< 0.50

¥ Limits of detection (LOD) represent approximate concentration (ng/mL), corresponding to three times the lowest detected peak.

Table B3.3. Concentrations of benzo[*a*]pyrene (ng/mg liver) in liver tissue collected from non-adapted (Smith Point) and pollution-adapted (Vince Bayou) *F. grandis,* measured at 24 h intervals following an intraperitoneal injection of 5 mg/kg benzo[*a*]pyrene. Data represent the individual results of three independent *in vivo* experiments.

In Vivo Experiment	Smith Point						Vince Bayou					
No.	**24 h**	**48 h**	**72 h**	**96 h**	**120 h**	**144 h**	**24 h**	**48 h**	**72 h**	**96 h**	**120 h**	**144 h**
2	0.23 ± 0.09	0.23 ± 0.11	0.73 ± 0.18	0.46 ± 0.35	n/a	n/a	0.29 ± 0.09	1.78 ± 0.74	0.73 ± 0.31	1.50 ± 1.29	n/a	n/a
3	0.90 ± 0.24	0.75 ± 0.21	0.84 ± 0.44	1.10 ± 0.65	0.91 ± 0.16	1.41 ± 0.53	1.31 ± 0.23	2.28 ± 0.28	0.86 ± 0.26	0.57 ± 0.13	0.73 ± 0.22	0.71 ± 0.18
4	0.99 ± 0.45	2.66 ± 0.81	0.78 ± 0.33	1.89 ± 0.94	1.16 ± 0.43	1.94 ± 0.71	2.15 ± 0.58	2.64 ± 1.48	1.61 ± 0.68	0.89 ± 0.25	2.21 ± 1.09	0.67 ± 0.18

Table B3.4. Concentrations of benzo[*a*]pyrene and three of its metabolites (ng/µL bile) in bile collected from non-adapted (Smith Point) and pollution-adapted (Vince Bayou) *F. grandis,* measured at 24 h intervals following an intraperitoneal injection of 5 mg/kg benzo[*a*]pyrene. Data represent the individual results of three independent *in vivo* experiments.

		Smith Point						Vince Bayo			
	Analyte	**24 h**	**48 h**	**72 h**	**96 h**	**120 h**	**144 h**	**24 h**	**48 h**	**72 h**	
Second Experiment	Benzo[*a*]pyrene	0.10 ± 0.04	0.12 ± 0.06	0.11 ± 0.05	0.02 ± 0.01	n/a	n/a	0.04 ± 0.01	0.02 ± 0.01	2.11 ± 0.27	0.(
	1-OH-BaP	0.17	0.95 ± 0.66	3.15 ± 2.29	0.64 ± 0.03	n/a	n/a	BLD	BLD	0.45 ± 0.27	1.(
	9-OH-BaP	BLD	BLD	BLD	BLD	n/a	n/a	BLD	0.29 ± 0.01	0.41 ± 0.01	0.4
	BaP-4,5-dihydrodiol	BLD	BLD	0.05	BLD	n/a	n/a	0.03 ± 0.02	0.08	0.01	0.2
Third Experiment	Benzo[*a*]pyrene	0.11 ± 0.05	0.02 ± 0.01	0.01 ± 0.00	0.01 ± 0.00	0.02 ± 0.00	0.14 ± 0.09	0.07 ± 0.03	0.09 ± 0.04	0.17 ± 0.05	0.(
	1-OH-BaP	3.19 ± 1.68	2.80	10.14	0.07	BLD	BLD	1.41	0.89	1.84	
	9-OH-BaP	0.38 ± 0.02	0.41 ± 0.01	0.48 ± 0.07	BLD	0.45 ± 0.02	0.43 ± 0.02	0.45 ± 0.07	0.48 ± 0.04	0.49 ± 0.10	0.(
	BaP-4,5-dihydrodiol	0.20 ± 0.09	0.07 ± 0.02	0.21 ± 0.05	0.07 ± 0.01	0.21 ± 0.05	0.14 ± 0.04	0.13 ± 0.02	0.21 ± 0.01	0.15 ± 0.02	0.2
Fourth Experiment	Benzo[*a*]pyrene	0.16 ± 0.06	0.45 ± 0.19	0.03 ± 0.01	0.19 ± 0.05	0.95 ± 0.39	0.56 ± 0.40	0.11 ± 0.09	0.17 ± 0.06	0.31 ± 0.16	0.1
	1-OH-BaP	2.07 ± 0.23	2.94 ± 0.59	3.53 ± 1.54	9.43 ± 1.98	5.46 ± 0.99	3.59 ± 2.02	2.12 ± 1.50	5.04 ± 1.30	10.91 ± 4.33	6.1
	9-OH-BaP	BLD	0.40 ± 0.00	0.4	0.43 ± 0.01	0.41	0.40 ± 0.00	BLD	0.43	0.46 ± 0.00	0.4
	BaP-4,5-dihydrodiol	BLD	0.06 ± 0.01	0.23 ± 0.10	0.13 ± 0.04	0.10 ± 0.04	0.03 ± 0.01	0.05	0.12 ± 0.04	0.07 ± 0.01	0.2

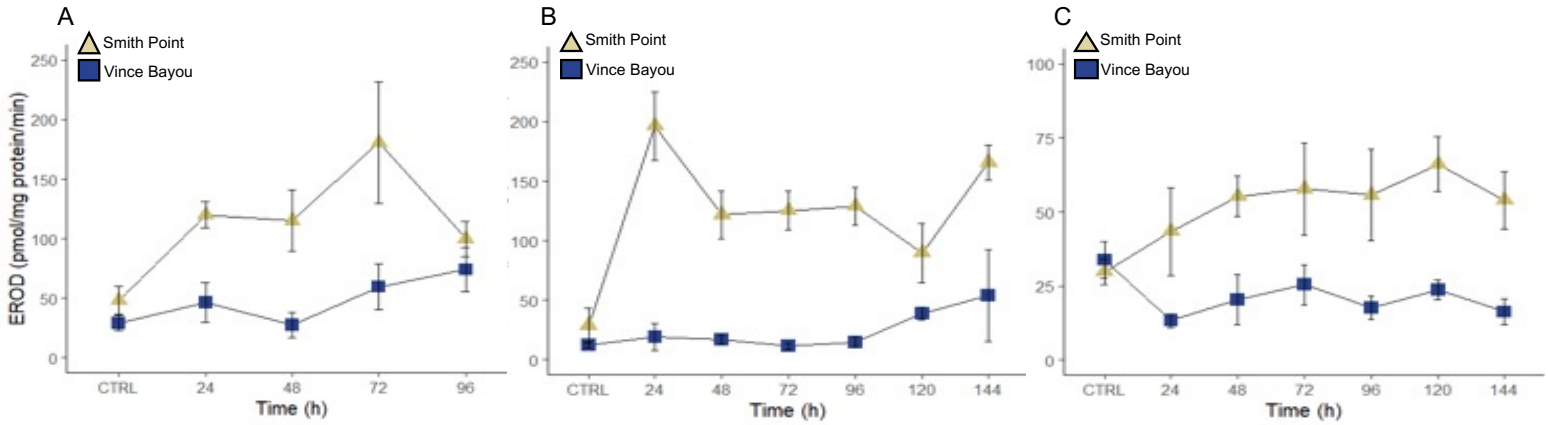

Figure B3.4. Microsomal EROD (CYP1A) activity (pmol/mg protein/min ± SEM) in non-adapted (Smith Point) and pollution-adapted (Vince Bayou) *F. grandis* measured at 24 h intervals following an intraperitoneal injection of 5 mg/kg benzo[*a*]pyrene. CTRL: corn oil, used as carrier control. Figures display the results from the A) second (n=3), B) third (n=4), and C) fourth (n=4) *in vivo* experiments.

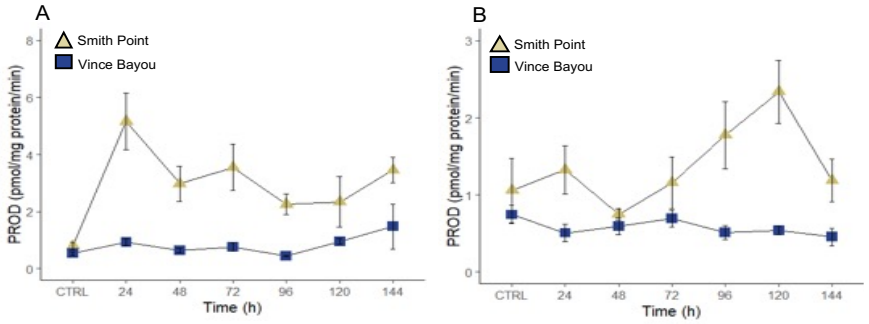

Figure B3.5. Microsomal PROD (CYP2B-like) activity (pmol/mg protein/min ± SEM) in non-adapted (Smith Point) and pollution-adapted (Vince Bayou) *F. grandis* measured at 24 h intervals following an intraperitoneal injection of 5 mg/kg benzo[*a*]pyrene. CTRL: corn oil, used as carrier control. Figures display the results from the A) third (n=4) and B) fourth (n=4) *in vivo* experiments.

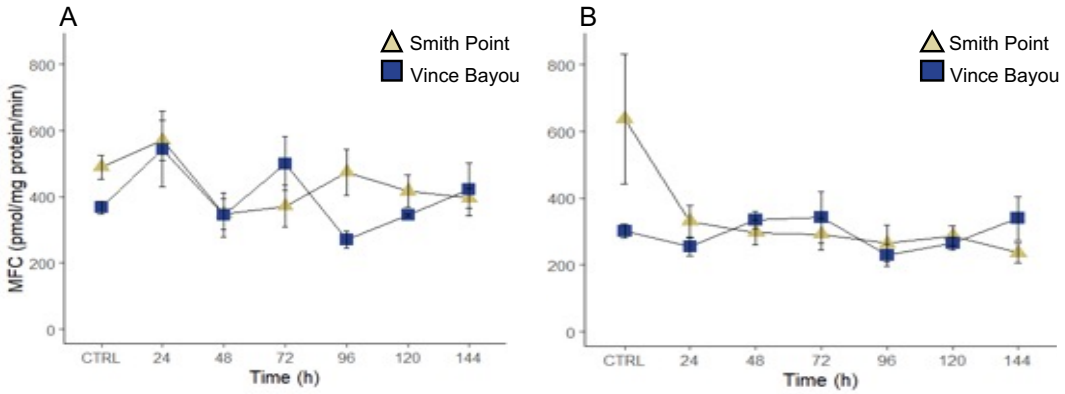

Figure B3.6. Microsomal MFC (CYP2C9-like) activity (pmol/mg protein/min ± SEM) in non-adapted (Smith Point) and pollution-adapted (Vince Bayou) *F. grandis* measured at 24 h intervals following an intraperitoneal injection of 5 mg/kg benzo[*a*]pyrene. CTRL: corn oil, used as carrier control. Figures display the results from the A) third (n=4) and B) fourth (n=4) *in vivo* experiments.

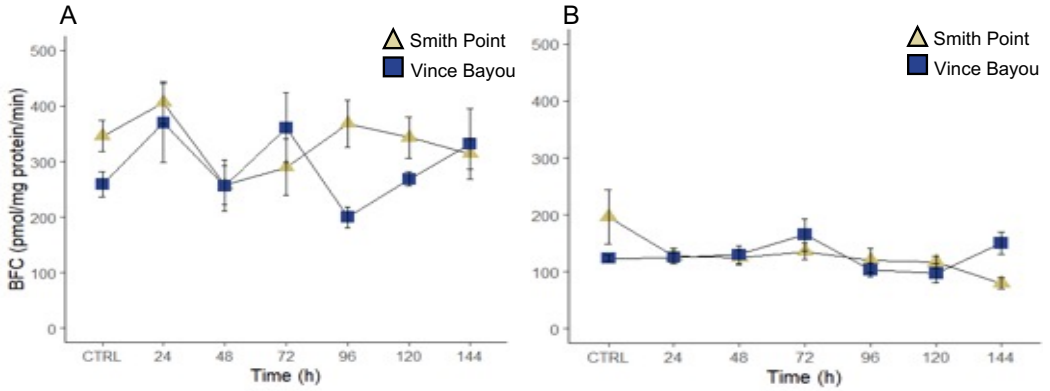

Figure B3.7. Microsomal BFC (CYP3A4-like) activity (pmol/mg protein/min ± SEM) in non-adapted (Smith Point) and pollution-adapted (Vince Bayou) *F. grandis* measured at 24 h intervals following an intraperitoneal injection of 5 mg/kg benzo[*a*]pyrene. CTRL: corn oil, used as carrier control. Figures display the results from the A) third (n=4) and B) fourth (n=4) *in vivo* experiments.

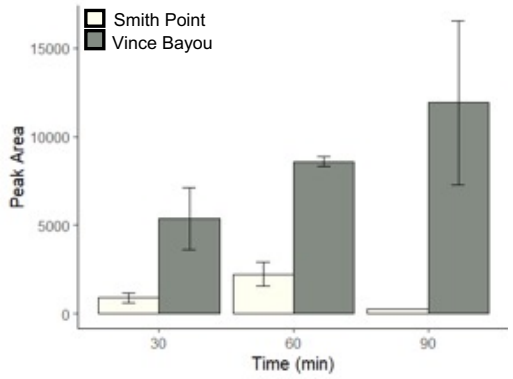

Figure B3.13. Peak area of an unknown/potential benzo[*a*]pyrene metabolite produced by liver S9 sub-cellular fractions non-adapted (Smith Point) and pollution-adapted (Vince Bayou) *F. grandis,* and at 30, 60, and 90 min from initial spiking with benzo[*a*]pyrene. Data correspond to the results of triplicate experiments (independent incubations).

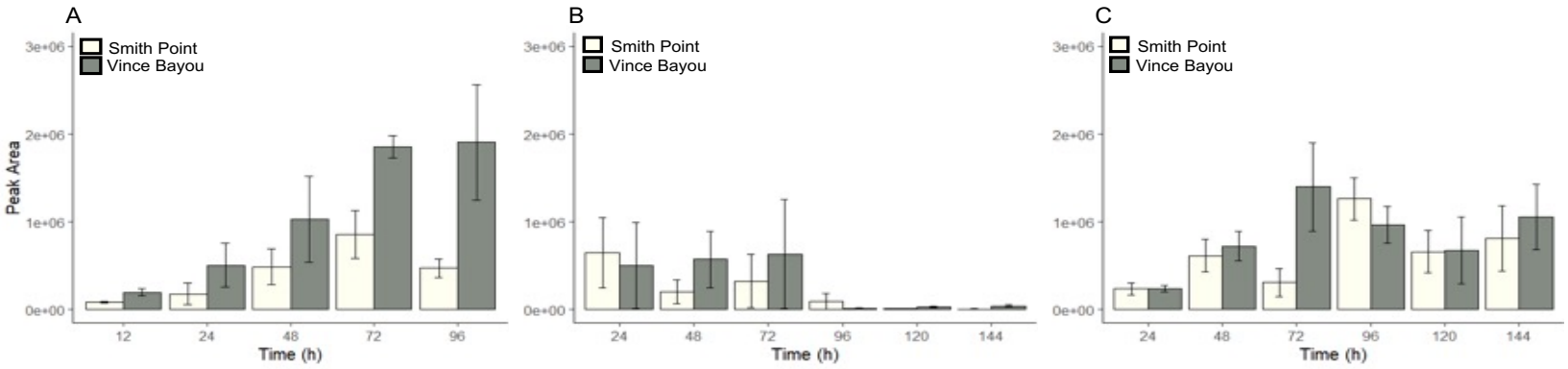

Figure B3.14. Peak area of an unknown/potential benzo[*a*]pyrene metabolite in bile collected from non-adapted (Smith Point) and pollution-adapted (Vince Bayou) *F. grandis,* measured at 24 h intervals following an intraperitoneal injection of 5 mg/kg benzo[*a*]pyrene. Figures display the results from the A) second, B) third, and C) fourth *in vivo* experiments.

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APPENDIX C

Supplemental Information for Chapter Four

Materials and Methods

Streamflow measurements

Manual stream discharge measurements were conducted at sites with no USGS gage and followed standard protocols from the Texas Commission of Environmental Quality (TCEQ). Briefly, a measuring tape was stretched perpendicularly across the stream, the stream width was recorded, and the number of flow cross-sections was determined (12 and 15 for the upstream and 1.4 mi sites, respectively). The depth of each of the cross-sections was measured using a wading rod, placed at the midpoint of each section. The flow sensor was then positioned at the midpoint of each section, parallel to the flow, and velocity was recorded. Discharge values were then calculated for each cross-section and added to obtain the total discharge of the stream (TCEQ, 2012).

EDC solid-phase extraction and sample preparation

For analytical quantification, water samples were filtered through three different filters to remove particulate. The three filters included a 1.0-µm glass fiber prefilter, a 0.45-µm nitrocellulose filter, and a 0.2-µm fine sanitation filter. The use of different filters is common in the literature regarding analysis of water samples, and has been previously employed by our team in work involving pharmaceuticals and other emerging contaminants (Du, Perez-Hurtado, Brooks, & Chambliss, 2012; Haddad et al., 2018).

Within 24 h of sampling, samples were measured to 1 L and spiked with 50 µL of deuterated standard [2000 ppb] mixture and 400 µL of concentrated formic acid to lower the pH to ~3. Samples were loaded onto a Strata C18E solid phase extraction cartridge at approximately 10 mL/min. Cartridges were previously conditioned with 5 mL of methanol and 5 mL of nanopure water (Barnstead™ Nanopure™). Samples were extracted via vacuum manifold with a flow rate of approximately 10 mL/min. Cartridges were blown dry under a vacuum and eluted using 5 mL of methanol. The resulting extracts were then dried in a test tube under a gentle stream of nitrogen.

After drying, 100 µL of 0.1M $NaCO_3/NaHCO_3$ buffer (pH =10.5) and 100 µL of 1 mg/mL dansyl chloride in acetone were added to the dried test tubes. Test tubes were vortexed for 30 seconds and heated to 60°C for 10 minutes. A solution of 800 µL 5:95 (v/v) methanol:water with 0.1 % formic acid was added to the sample to bring the final volume to 1 mL and neutralize the pH down to approximately ~7 (recommended for the use of a poroshell column). All samples were then filtered through a 0.2-µm syringe filter and placed in 2-mL sampler vials.

For intraperitoneal injections and primary hepatocyte exposure, 700 mL of unfiltered water were passed through methanol-activated Empore™ 47 mm C18 extraction disks (Dyneon LLC, Oakdale, MN, USA). Disks were subsequently eluted with 10 mL methanol, eluents were dried under nitrogen and resuspended in 700 µL of ethanol. A 200 µL aliquot was further dried and resuspended in 200 µL of dimethyl sulfoxide (DMSO).

Analytical Instrumentation

Chromatographic separation was carried out using an Agilent HPLC system consisting of an Agilent 1260 Quaternary Pump (G1311B), Agilent 1260 Infinity Standard Autosampler, and Agilent 1260 Infinity Thermostatted Column Compartment (G1316A). The HPLC system was interfaced with an Agilent G6420 Triple Quadrupole Mass Spectrometer.

Agilent MassHunter Data Acquisition for Triple Quadrupole Mass Spectrometer (version B.07.00) was used for data acquisition, while Agilent MassHunter Qualitative (version B.07.00) and Agilent MassHunter Quantitative software (version B.07.00) were used for data processing.

Instrument Calibration

The linear range reported in Table C4.3 for each analyte was confirmed from plots of sensitivity (i.e., response factor; RF) versus analyte concentration. Our criterion for linearity required that the relative standard deviation of RFs for standards spanning the noted range was ≤ 15%. Internal standard calibration curves were constructed for each analyte using a minimum of five standards that were within the corresponding linear range. Calibration data were fit to a linear regression, and correlation coefficients (r^2) for all analytes were ≥ 0.989.

Method limits of detection and extraction efficiency (recovery)

The EPA guideline (40 CFR Part 136, Appendix B) for generating method detection limits (MDLs) was followed to generate the current batch of MDLs. The experimental design used 8 replicates and the spiking level for each analyte was 1 ng/L.

After analysis, MDLs were calculated by multiplying the standard deviation resulting from 8 replicates by the one-sided student's *t* value for the corresponding number of samples.

Extraction efficiencies of the Strata C18E cartridge were determined from a total of eight cartridges. Four were marked 'Pre' and three were marked 'Post'. Pre samples were spiked with internal standard before extraction while post samples were spiked with internal standard after analytes were eluted from the cartridge. The samples were then analyzed, and the three replicates were averaged. Post samples were then divided by pre samples to yield the efficiency of the cartridge as a percent.

LC-MS/MS analytical methodology

A binary gradient method consisting of aqueous 0.1% formic acid as solvent B, and acetonitrile as solvent A, was used. First, the gradient was held at 50% B for 0.2 min, followed by a linear ramp up to 55% B until 6.2 min, where it was held until 7.2 min. Following this, the gradient was switched to 100% B until 10 min after which it was returned to 50% B until 12 min, re-equilibrating the column to the starting conditions of the next run. An Agilent Poroshell SB-C18 2.1 × 100 mm, 2.7 μm column was used, and flow rate was held constant at 0.5 mL min^{-}. The column temperature was maintained at 50°C. The injection volume was 10 μL. Analytes were ionized in positive mode using electrospray ionization. MRM transitions for the target analytes (table C4.4) and associated instrument parameters were automatically determined using MassHunter Optimizer Software by flow injection analysis. Optimized MS/MS parameters are presented in table C4.3. Cycle time was adjusted to 200 ms for the dynamic MRM acquisition mode.

Bioassays

RT-qPCR was conducted to measure the expression of liver VTG in IP- injected fish and primary hepatocytes. Briefly, liver tissues from field-deployed fish and stored in RNAlater, as well as primary hepatocytes from the *in vitro* assays, were used for the isolation and purification of mRNA using the SV Total RNA Isolation System (Promega Corporation, WI, USA). 80 µmol of purified RNA were transferred to a 96-well qPCR plate, and mixed with a reverse transcriptase-containing solution, forward and reverse VTG primers, and nuclease free water for a total volume of 50 µL. For every bioassay, the housekeeping genes β-actin and 18S rRNA were run in parallel, though 18S was used for final comparison. RT-qPCR reactions were conducted in a StepOne™ thermocycler (Thermo Fisher Scientific, MA, USA) and included reverse transcriptase activity for 30 min at 48°C, enzyme activation steps (95°C, 10 min) and 40 cycles of denaturation (95°C, 15 sec), annealing and extension (56°C, 1 min).

Concentrations of plasma VTG were measured via ELISA as follows: 100 µL of diluted plasma samples (1:10 or 1:20 in dilution buffer) were added in 96-well plates, pre-coated with a VTG capture antibody, and incubated overnight at 4°C. A detecting antibody, labeled with acetylcholinesterase (AChE), was then added to each well along with a binding substrate solution prior to incubation of the plates on an orbital shaker for 1 h at room temperature and in a dark environment. The absorbance of each well was then read at 105 nm (Synergy™ H1, BioTek, VT, USA)

Concentrations of E2 and 11KT in fish plasma were also conducted in each sample. For this purpose, 100 µL of plasma were extracted with 400 µL of diethyl ether, repeated three times. The resulting 1,200 µL were placed in glass vials and evaporated to

dryness under a gentle stream of nitrogen. Evaporated samples were resuspended in 100 µL ELISA buffer, and diluted 1:20 in dilution buffer. For both E2 and 11KT, 50 µL of the diluted extract were added into 96-well plates, pre-coated with either E2 or 11KT-specific rabbit antiserum binding sites. 50 µL ELISA antiserum and an AchE Tracer were then added, and plates were incubated for 18 h at 4°C. Plates were developed with the addition of 200 µL of Ellman's Reagent and 5 µL of a tracer dye and incubated on an orbital shaker for 2 h. The absorbance of each sample was measured at 405 nm (Synergy™ H1, BioTek, VT).

Table C4.1. Geographic location, distance from WWTP, and elevation of the selected sites upstream and downstream of the effluent discharge.

Site	Distance from WTTP (mi)	Coordinates	Elevation (ft)
Reference	Upstream	40°43'14.7" N; 111°31'04.1" W	6,394
Site 1	0.13	40°45'34.4" N; 111°33'52.4" W	6,257
Site 2	1.40	40°45'51.8" N; 111°34'47.9" W	6,227
Site 3	13.0	40°52'09.9" N; 111°35'11.5" W	5,757

Table C4.2. Mean length, weight and condition factor ± standard deviation (SD) of field-deployed and IP-injected juvenile rainbow trout (*Oncorhynchus mykiss*) used in summer and fall 2018 assessments. Condition factor (k) was calculated as Weight (g)/[Length (g)]^{3}x100.

Site	Length (cm)	Weight (g)	Condition Factor (k)
Field-deployed fish			
Summer 2018	12.9 ± 1.4	21.4 ± 7.3	0.971 ± 0.140
Fall 2018	13.9 ± 0.8	26.7 ± 4.2	0.996 ± 0.119
IP-injected fish			
Summer 2018	17.1 ± 1.3	42.3 ± 8.7	0.832 ± 0.072
Fall 2018	17.9 ± 1.1	50.2 ± 9.3	0.871 ± 0.083

Table C4.3. Method detection limits (MDLs), linear range, and extraction efficiencies (recovery) for target analytes. MDLs were quantified in nanopure water. The reporting limit in effluent is based on the visual inspection of peaks observed in fortified effluent samples. Concentrations reported for MDL and linear range represent native samples concentrations (i.e., before analytical sample preparation). Recovery is presented as mean ± standard deviation (SD).

Compound	MDL (ng/L)	Linear Range (ng/L)	Recovery (% ± SD)
4-nonylphenol	1.34	0.1-100	62 ± 8.6
17 alpha estradiol	5.00	5-500	108 ± 8.7
17 beta estradiol	2.25	1-500	102 ± 5.8
Estriol	0.58	0.1-500	103 ± 2.5
EE2	2.76	0.5-500	99 ± 6.9
Equilenin	0.10	0.1-500	104 ± 7.0
Equilin	1.59	1-100	105 ± 10.5
Estrone	3.86	0.5-100	103 ± 3.7

Table C4.4. A list of target analytes and MS/MS transition parameters for the Agilent 6420 MS/MS generated in MassHunter Optimizer Software.

Analyte	Precursor Ion (m/z)	Product Ion (m/z)	Fragmentor (V)	Collision Energy (V)	Retention Time
dansyl 4-nonylphenol	454.2	171	152	33	10.0
	454.2	156	152	61	10.0
dansyl 4-nonylphenol D6	460.3	171	103	33	10.0
dansyl 17 alpha estradiol	506.2	171	201	41	6.3
	506.2	156	201	69	6.3
dansyl 17 alpha estradiol D3	509.3	171	201	41	6.3
dansyl 17 beta estradiol	506.2	171.1	201	41	5.9
	506.2	156	201	69	5.9
dansyl estriol	522.2	171.1	201	45	1.9
	522.2	156	201	73	1.9
dansyl estriol D3	525.2	171	201	45	1.9
dansyl EE2	530.2	171.1	152	45	6.7
	530.2	156	152	77	6.7
dansyl EE2 D4	534.3	17.1	152	45	6.7
dansyl equilenin	500.2	171.1	152	41	6.1
	500.2	156	152	65	6.1
dansyl equilenin D5	504.2	171.3	152	37	6.1
dansyl equilin	502.2	171	201	41	6.6
	502.2	156	201	65	6.6
dansyl equilin D4	506.2	171	201	45	6.6
dansyl estrone	504.2	171	201	45	7.2
	504.2	156	201	69	7.2

Table C4.5. Concentration of target endocrine disrupting compounds (EDCs) and MDLs (ng/L) in samples from whole effluent and creek water from all sites, time points and sampling seasons.

			17-α estradiol	4-nonylphenol	17-β estradiol	EE2	Equilenin	Equilin	Estriol	Estrone
		MDLs	**5**	**1.34**	**2.25**	**2.76**	**0.1**	**1.59**	**0.58**	**3.86**
Month	**Site**	**Day**								
July	Effluent	0	ND	ND	ND	<MDL	<MDL	ND	**8.63**	<MDL
July	Effluent	0	ND	ND	ND	<MDL	<MDL	ND	**1.62**	<MDL
July	Effluent	1	ND	ND	ND	<MDL	<MDL	ND	**1.36**	<MDL
July	Effluent	3	ND	ND	ND	<MDL	<MDL	ND	<MDL	<MDL
July	Effluent	7	ND	ND	ND	<MDL	<MDL	ND	**5.16**	<MDL
July	Upstream	0	ND	ND	ND	ND	ND	<MDL	<MDL	<MDL
July	Upstream	1	ND	ND	ND	ND	ND	ND	<MDL	<MDL
July	Upstream	3	ND	ND	ND	ND	ND	ND	<MDL	<MDL
July	Upstream	7	ND	ND	ND	ND	ND	ND	<MDL	<MDL
July	0.13 mi	0	ND	ND	ND	ND	ND	ND	<MDL	<MDL
July	0.13 mi	1	ND	ND	ND	ND	ND	ND	<MDL	<MDL
July	0.13 mi	1	ND	ND	ND	ND	ND	ND	<MDL	<MDL
July	0.13 mi	3	ND	ND	ND	ND	ND	ND	<MDL	<MDL
July	0.13 mi	3	ND	ND	ND	ND	ND	ND	<MDL	<MDL
July	0.13 mi	7	ND	ND	ND	ND	ND	ND	<MDL	<MDL
July	1.4 mi	0	ND	ND	ND	ND	ND	ND	<MDL	**4.51**
July	1.4 mi	1	ND	ND	ND	ND	ND	ND	<MDL	**4.7**
July	1.4 mi	3	ND	ND	ND	ND	ND	ND	<MDL	**5.39**
July	1.4 mi	7	ND	ND	ND	ND	ND	ND	<MDL	**4.95**
July	1.4 mi	7	ND	ND	ND	ND	ND	ND	<MDL	**4.73**
July	13 mi	0	ND	<MDL	ND	<MDL	ND	ND	<MDL	<MDL
July	13 mi	1	ND	<MDL	ND	<MDL	ND	ND	<MDL	<MDL
July	13 mi	3	ND	ND	ND	<MDL	ND	ND	<MDL	<MDL
July	13 mi	7	ND	ND	ND	<MDL	ND	ND	<MDL	<MDL

			17-α estradiol	4-nonylphenol	17-β estradiol	EE2	Equilenin	Equilin	Estriol	Estrone
		MDLs	**5**	**1.34**	**2.25**	**2.76**	**0.1**	**1.59**	**0.58**	**3.86**
Month	**Site**	**Day**								
October	Effluent	0	ND	ND	ND	<MDL	<MDL	ND	**1**	<MDL
October	Effluent	1	ND	ND	ND	<MDL	<MDL	ND	**1.97**	<MDL
October	Effluent	3	ND	ND	ND	<MDL	<MDL	ND	**4.63**	<MDL
October	Effluent	7	ND	ND	ND	<MDL	<MDL	ND	**1.88**	<MDL
October	Upstream	0	ND	ND	ND	ND	ND	ND	<MDL	<MDL
October	Upstream	1	ND	ND	ND	ND	ND	ND	<MDL	<MDL
October	Upstream	3	ND	ND	ND	ND	ND	ND	<MDL	<MDL
October	Upstream	3	ND	ND	ND	ND	ND	ND	<MDL	<MDL
October	Upstream	7	ND	ND	ND	ND	ND	ND	<MDL	<MDL
October	0.13 mi	0	ND	ND	ND	ND	ND	ND	<MDL	<MDL
October	0.13 mi	1	ND	ND	ND	ND	ND	<MDL	<MDL	<MDL
October	0.13 mi	3	ND	ND	ND	ND	ND	<MDL	<MDL	<MDL
October	0.13 mi	7	ND	ND	ND	ND	ND	<MDL	<MDL	<MDL
October	0.13 mi	7	ND	ND	ND	ND	ND	<MDL	<MDL	<MDL
October	1.4 mi	0	ND	ND	ND	ND	ND	ND	<MDL	<MDL
October	1.4 mi	0	ND	ND	ND	ND	ND	ND	<MDL	<MDL
October	1.4 mi	1	ND	ND	ND	ND	ND	<MDL	<MDL	<MDL
October	1.4 mi	3	ND	ND	ND	ND	ND	<MDL	<MDL	<MDL
October	1.4 mi	7	ND	ND	ND	ND	ND	<MDL	<MDL	<MDL
October	13 mi	0	ND	ND	ND	<MDL	ND	ND	<MDL	<MDL
October	13 mi	1	ND	<MDL	ND	<MDL	ND	ND	<MDL	<MDL
October	13 mi	1	ND	<MDL	ND	<MDL	ND	<MDL	<MDL	<MDL
October	13 mi	3	ND	<MDL	ND	<MDL	ND	<MDL	<MDL	<MDL
October	13 mi	7	ND	ND	ND	<MDL	ND	<MDL	<MDL	<MDL

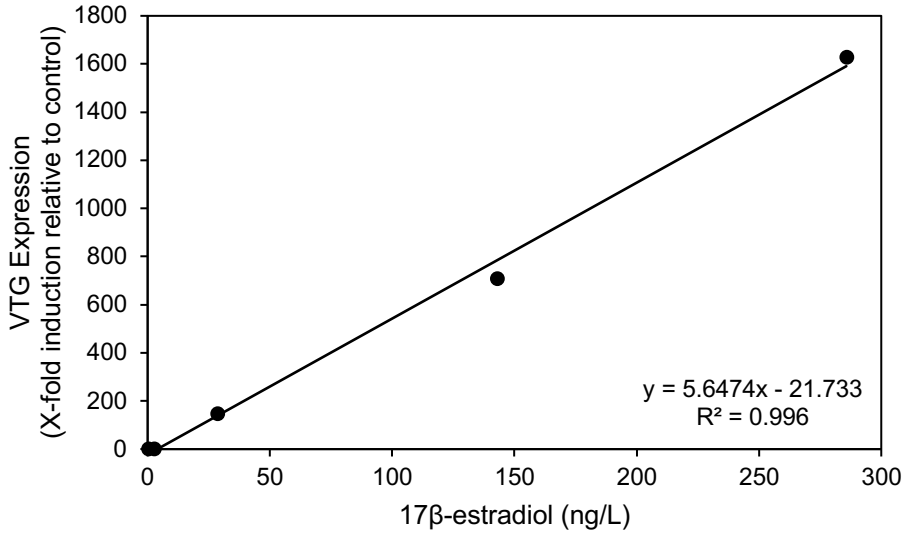

Figure C4.2. Calibration curve for EEQ determination based on VTG expression in rainbow trout primary hepatocytes.

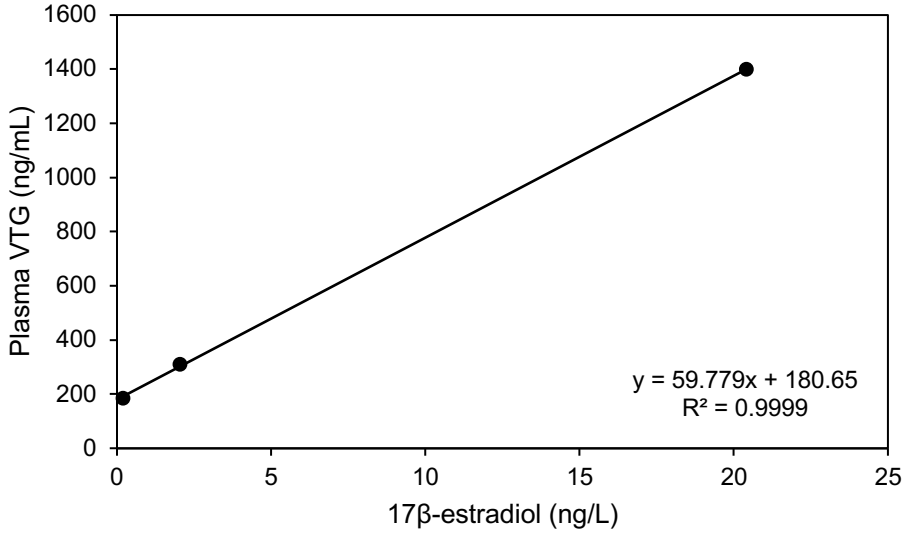

Figure C4.3. Calibration curve for EEQ determination based on plasma VTG concentrations in IP-injected rainbow trout.

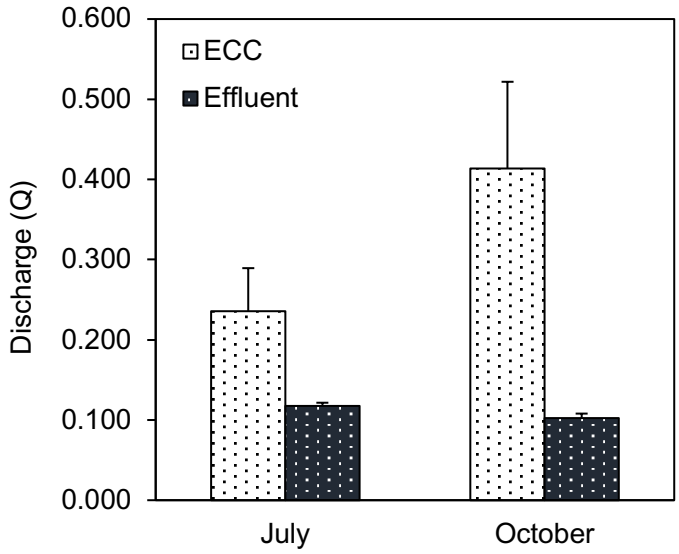

Figure C4.4. Mean East Canyon Creek (ECC) streamflow and effluent discharge (Q) ± standard deviation (SD), from days 0 to 7 in the Summer (July) and Fall (October) 2018. The effluent discharge accounted for approximately 49.90% and 24.85% of the total streamflow downstream of the WWTP in the summer and fall, respectively.

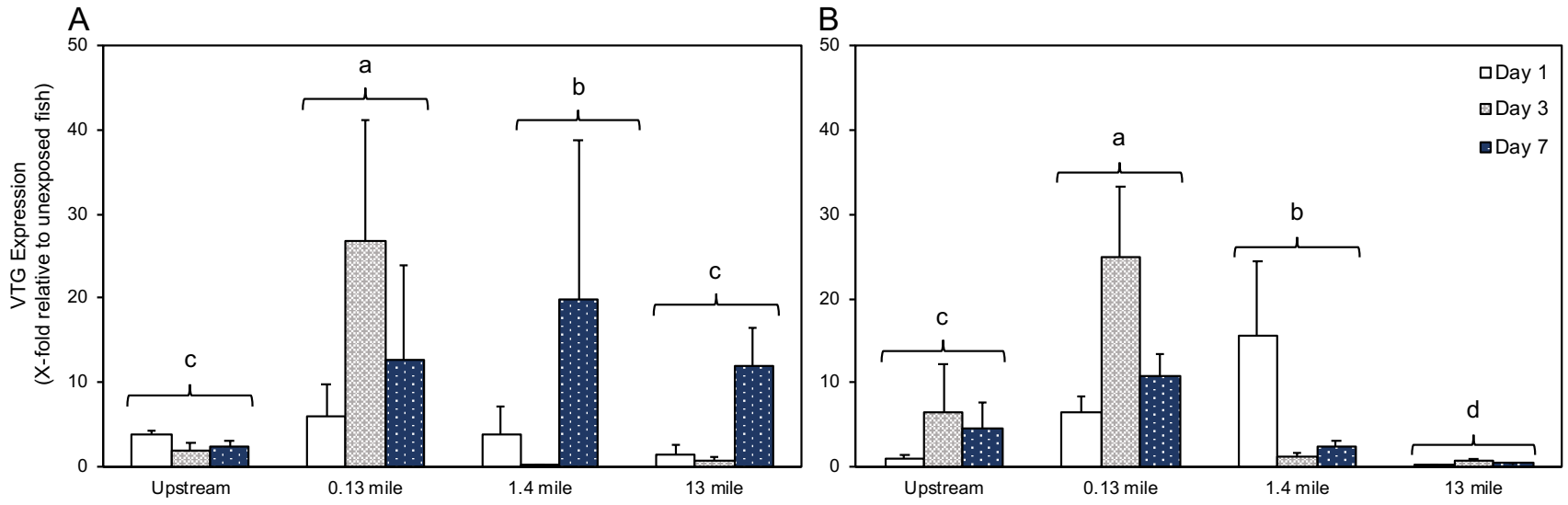

Figure C4.5. Mean expression of liver vitellogenin (VTG) presented as average X-fold expression ± standard error (SEM) in rainbow trout deployed at different sites and at three different time points during the A) Summer and B) Fall 2018. Different letters denote significantly different VTG expression among sites (post-hoc analysis was based on site differences).

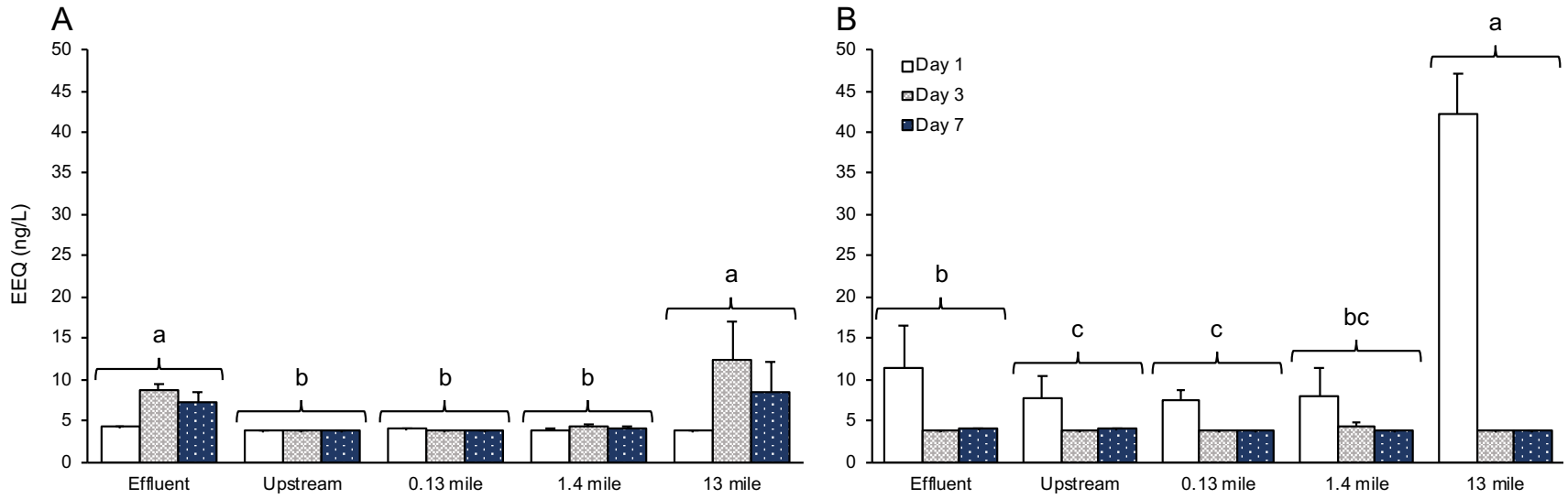

Figure C4.9. Mean estradiol equivalency quotients (EEQs) ± standard error (SEM) derived from expression of liver VTG in rainbow trout primary hepatocytes exposed to water extracts collected from the effluent and at different sites in ECC, and at three different time points during the A) Summer and B) Fall 2018. Different letters denote significant differences among sites (post-hoc analysis was based on site differences).

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