Non-lethal analysis of F_2-isoprostanes in the epidermal mucus of freshwater fish species as an indicator of diluted bitumen toxicity

by

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A thesis submitted in partial fulfillment of the requirements for the degree

Master of Science

Department of Chemistry

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Abstract

Canada is among the world's leading producers and exporter of diluted bitumen (dilbit). Despite this, there is a lack of understanding surrounding the effects of dilbit spills in freshwater ecosystems. F_2-isoprostanes (F2-IsoPs) are reliable biomarkers of oxidative stress, one of dilbit's toxicity pathways in fish, however they are seldom studied in fish. This thesis describes the development of a non-invasive method for measuring F_2-IsoPs in the epidermal mucus of fish to measure oxidative stress in fathead minnows (Pimephales promelas) exposed to dilbit. An HPLC-MS/MS method was first developed and validated using Northern pike mucus (Esox lucius), and used to successfully detect F_2-IsoPs in lake trout (Salvelinus namaycush) angled at the IISD-Experimental Lakes Area in Northwestern Ontario, proving these compounds are present at detectable concentrations in wild fish mucus. The relative concentrations of F_2-IsoPs were then examined in tissues of rainbow trout (Onchorynchus mykiss) exposed to acute physical stress. F_2-IsoP concentrations were also compared to total glutathione (GSH) and the GSH redox status in this study, finding a possible link between F2-IsoPs and GSH in gills and evidence of a mild oxidative stress response in physically stressed trout. The effect of dilbit on epidermal mucus and liver F_2-IsoP concentrations was then examined in juvenile fathead minnows exposed for 7 days to 0-30% dilbit solutions, or water accommodated fraction (WAF). F_2-IsoP concentrations did not differ significantly between any dilbit treatments: however this lack of response may have been caused by low uptake of polycyclic aromatic compounds. Liver and mucus F_2-IsoPs were positively correlated in the control group and negatively correlated in the 30% WAF treatment. Finally, the developed method was field-tested in the Freshwater Oil Spill Remediation Study (FOReSt), a collaborative project aiming to determine the fate and behaviour of dilbit in a freshwater shoreline environment. Adult fathead minnows were exposed to environmentally

relevant concentrations of weathered dilbit for ~75 days in shoreline enclosures at the IISD-ELA. Mucus F_2-IsoPs were quantified in fathead minnows and finescale dace (*Phoxinus neogaeus*). Concentrations were not significantly different between treatments, suggesting dilbit exposure did not cause a lasting oxidative stress response in either species.

Preface

This thesis is an original work written in its entirety by Patrique Bulloch. This thesis is written using the "sandwich method" and due to the style of this format there may be redundancies in the introductory paragraphs of the two manuscripts as well as the introductory chapter.

Chapter 2 in this thesis is a manuscript published in Chemosphere. I was responsible for writing this manuscript and most of the data processing and analyses. Drs. Guanyong Su and Robert Letcher provided expertise and high-resolution mass spectrometry data for this manuscript. Zhe Xia, Wesley Johnson, Mitchell Chu, Sara Schur, Dhasni Muthumuni provided assistance in method development conceptualization. Dr. Vince Palace reviewed the document and provided much expertise. Dr. Gregg Tomy was the primary investigator of this study, and provided invaluable input during experiment conceptualization and manuscript review. Lake trout were angled by Fisheries staff at the IISD-Experimental Lakes Area. Northern pike mucus was provided by Wesley Johnson.

Chapter 3 of this thesis was conducted at the Aquatic Toxicology Research Centre at the University of Saskatchewan. The experimental design was a collaboration between Dr. Jason Raine, Dr. Gregg Tomy, and myself. Fish were cared for by ATRF staff Dr. Azadeh Hatef and Dr. Dale Jefferson. I performed sample collection with their help and assistance from Emily Kennedy, a M.Sc. candidate now at the University of Saskatchewan. Glutathione assays were performed using the microplate reader in Dr. Steve Whyard's laboratory. The analysis of data and creation of all figures was my responsibility.

Chapter 4 of this thesis was conducted at the Aquatic Toxicology Research Centre at the University of Saskatchewan by Dan April, a M.Sc. candidate at the U of S. He designed the experiment and performed all data collection for my study on my behalf. Biliary metabolite processed data was provided by Jamie Dearnley, a M.Sc. candidate at the University of Manitoba. I was responsible for all data analysis, writing, and figure creation for this chapter.

Chapter 5 of this thesis was conducted at the IISD-Experimental Lakes Area. Ethics approval for this project was granted through the University of Manitoba Animal Care and Use Committee under animal use protocol AUP F18-007. I and several staff members, graduate students, and undergraduate students were responsible for experimental setup and data collection tasks including fish deployment, collection, and dissection. The FOReSt project was designed and led by Drs. Vince Palace, Mark Hanson, Valerie Langlois, and Gregg Tomy in collaboration with Drs. José-Luis Rodríguez-Gil and Heather Dettman. Chemistry analysis was performed by Dr. Lisa Peters, with some assistance from myself and Jamie Dearnley during sample extraction at COGRAD, University of Manitoba. Dr. Jose-Luis Rodriguez performed the tritium data processing. I was responsible for all writing and figure creation, and data analysis performed my raw data and processed data provided by the aforementioned people.

Acknowledgements

With this thesis now complete, I'm left with the impossible task of trying to express in a few short pages all the gratitude I feel for the legions of people who helped make this possible. Thank you to all the graduate and undergraduate students at the IISD-ELA that I've met and with whom I've become lifelong friends. It has been an absolute honour to have worked with you all. Special thanks to Joey Tonin and Justin Budyk for becoming the best squash partners I could have asked for. I'd also like to single out the students of Tox Crew, a very special group of which I'm proud to have been a part: Adam Scott, Aidan Guttormson, Alyssa Ball, Cody Jackson, Danielle Desrochers, Jamie Dearnley, Katarina Djordjevic, Kenzie Perry, Lauren Timlick, Madeline Stanley, Nic Blandford, Pepe Rodriguez-Ril, Sonya Michaleski, and Tyler Black. Though the work was at times gruelling, it was never not fun, and that's all thanks to you fine people (and Scatman John).

Thank you also to the staff at IISD-ELA: Chandra Rogers, Cyndy Desjardins, John Neall, Jesse Coelho, Ken Sandilands, Lauren Hayhurst, Lee Hrenchuk, Mike Rennie, Michael Paterson, Paul Fafard, Pauline Gerrard, Roger Mollot, Sarah Warrack, Sonya Havens, and Scott Higgins. Your dedication to this special research station is inspiring. It would not be the same without you. Thank you to the principal investigators of the FOReSt project for including me: Drs. Gregg Tomy, Lyle White, Mark Hanson, and Valerie Langlois. I consider myself lucky to have taken part in this landmark study.

From the University of Saskatchewan, I must first thank Dr. Jason Raine, who found me available fish at the ATRF, helped me design a study on very short notice after plans for a different study abruptly shifted, and granted me access to the wonderful facility that is the Aquatic Toxicology Research Laboratory. A huge thank you to Drs. Azateh Hatef and Dale Jefferson for setting up the experimental groups and lending me their time to collect samples amidst their busy schedules, and to Emily Kennedy for volunteering her time to help me and lodging me. I could not have done it without

you all. Thank you to Dr. Lynn Weber for including me in the diluted bitumen exposure study, and thank you to Dan April for taking it upon himself to add my sample collection into his already busy schedule when I was unable to join him in Saskatoon due to COVID lockdowns.

From the University of Manitoba, I must absolutely thank the staff at COGRAD for their assistance over the years: Ifeoluwa Idowu, Lisa Peters, Olga Fransisco, Thor Halldorson, Wesley Johnson, and Zhe Xia. Thank you for sharing your workspace with me and providing me with expertise and insight when I needed it. My fellow tenants at the office in Parker 517 deserve a special mention: I couldn't have asked for better people to share that limited square footage with. I'm also appreciative of the feedback and review provided by my committee members Drs. Gregg Tomy, Sabine Kuss, and Vince Palace. A special thank you to my advisor Gregg Tomy, who first welcomed me into his lab as an undergraduate research assistant in 2016 after I expressed interest in freshwater ecosystems chemistry and has supported me ever since. You set this chapter of my life in motion, and I'm deeply grateful.

I am thankful for the financial support that gave me this opportunity through making the FOReSt project possible: the MITACS Accelerate grant awarded to Gregg Tomy, Mark Hanson, Valerie Langlois, and the IISD-ELA, and an NSERC grant (CRDPJ 532225-18) awarded to Gregg Tomy, Lyle White, Mark Hanson, and Judit Smits. I also am grateful for the funding support awarded to me through the IISD-ELA Graduate Fellowship and the NSERC CGS-M.

Finally, I am grateful for those outside the realm of academia who were just as important to me over these last few years. My family, whose unconditional love and support upon which I can always rely. My friends, who are always good for a pint and a laugh. Lastly, I owe so very much to Lauren, who has been at my side throughout this whole process and was always there to lend a hand or an encouraging word whenever I was struggling.

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Chapter 1: Introduction & Background

1.1 Introduction

Canada possesses the world's third largest proven oil reserves, 97% of which is located in the Alberta oil sands region (AOSR). Despite mounting societal pressures to reduce our dependence on fossil fuels, crude oil production increased from 3.8 to 4.3 million barrels per day between 2014 and 2018, and is expected to rise to 6.0 million by 2030 [1–3]. The majority of the AOSR production is unconventional crude oil, extracted as bitumen. Bitumen is a thick, viscous substance that must be diluted with natural gas condensates to achieve lower viscosity prior to transportation in pipelines and requires upgrading prior to use as feedstock in oil refineries. The vast majority of diluted bitumen (dilbit) is transported in North America via pipelines, the four main pipelines being the Keystone, Trans Mountain, Express, and Enbridge Line 9. Pipelines are recognized as the safest transportation method for delivering crude oil to refineries and foreign markets, however several ruptures in the last decade have led to millions of liters of oil being released into the environment and heightened awareness among regulatory agencies and the public regarding the potential for environmental damage [4–6].

The Royal Society of Canada (RSC) identified several key knowledge gaps concerning the fate and toxicity of spilled crude oil in their 2015 report on the environmental impacts and behaviour of crude oil spills [7]. Chief among the high-priority research needs is to better understand the environmental impacts of oil spills in freshwater environments, from population to ecosystem level [7]. While it is recognized that volumes released into freshwater environments from pipeline and rail spills are small relative to marine incidents, they remain a major concern. Pipelines span approximately 825,000 km of Canadian territory, potentially endangering large

areas of pristine wilderness [8]. Additionally, the smaller spill volumes are offset by the limited dilution capacity of freshwater ecosystems [7].

Few studies have been performed regarding the effects of spilled crude oil on freshwater ecosystems, and these have relied solely on lab exposures and opportunistically studying accidental oil spills. These spills of opportunity provide valuable data, but quantifying effects and mapping out recovery objectives is difficult because baseline data at these locations is usually lacking. Furthermore, though the toxicity of dilbit has been established under laboratory conditions, the complexity of the bitumen-diluent mixture makes it difficult to predict its behaviour and toxicity in equally complex aquatic environments where interactions can occur between oil, water, sediment, and microbes. The Freshwater Oil Remediation Study project (FOReSt) was performed at the International Institute for Sustainable Development - Experimental Lakes Area (IISD-ELA) to address some of the existent knowledge gaps, by simulating dilbit and conventional heavy crude (CHV) spills in a boreal lake shoreline environment. Fate and toxicity of both heavy oils were examined, as well as the efficacy of different minimally invasive remediation techniques. The IISD-ELA is permitted by provincial (Ontario Regulation 60/14: Experimental Lakes Area) and federal (*Fisheries Act*, Section 36(5.2) *Experimental Lakes Area Research Activities Regulations*) legislation to carry out whole-ecosystem manipulations on 58 carefully selected freshwater lakes. Extensive data sets have been collected for their reference lakes over their 50 years of operation, making it an ideal location for this project, which addresses a major point of concern stated in the RSC report regarding the lack of background data in water bodies exposed to spilled oil.

This thesis focuses on the effects of dilbit only, as CHV studies were delayed until 2021. My project studies the toxicological effects of diluted bitumen components on fish, using a non-

invasive sampling method that I developed to quantify oxidative stress biomarkers in dermal mucus. Fathead minnows (*Pimephales promelas*) were selected for the field study, as they are a model small bodied fish that has been extensively characterized [9,10]. This species is also native to the IISD-ELA region, as well as large swathes of territory in North America, making it an ideal test subject. The field study was complemented with two laboratory studies using juvenile fathead minnows and rainbow trout (*Onchorynchus mykiss*), respectively. The former complements the FOReSt results by providing data on the toxicity of different dilbit doses to fathead minnows in a more controlled environment. The latter study focused on determining how the F_2-isoprostanes respond in different tissues and how they compare to other established chemical biomarkers of oxidative stress.

1.2 Diluted Bitumen

1.2.1 Composition

Bitumen is defined as heavy crude oil with an American Petroleum Institute (API) gravity of < 10°. The API scale is inversely proportional to the density of the crude oil: crudes of API gravity <10° are denser than water, causing them to sink. For comparison with other crudes, the threshold for heavy crudes is < 22° API [11]. Bitumen is a complex hydrocarbon mixture that is viscous to the point of being a semisolid at ambient temperature. Its viscosity is a product of the extensive degradation to which the bitumen was subjected over geological time periods: contact with sea water during transportation and bacterial degradation are thought to have depleted the low molecular weight (LMW) hydrocarbons (< C_{20} n-alkanes), leaving only the heavier fractions in the reservoir. Bitumen is typically diluted with LMW fractions to facilitate its transport through pipelines. Common diluents are natural gas condensates or naptha at a diluent to bitumen ratio of ~30:70 for pipelines [12], however 50:50 mixture of bitumen with partially upgraded heavy crude

– known as synthetic bitumen or "synbit" – is increasingly used [7]. As an added complexity, diluent ratios are varied seasonally meaning that different stocks from the same sources may differ in overall composition.

The complexity of dilbit is such that it is prohibitively expensive and analytically intensive to determine its exact chemical composition. Furthermore, dilbit chemical composition is generally proprietary information. Dilbit is mainly comprised of hydrocarbons, but also contains small amounts of heteroatoms, mainly nitrogen, oxygen, and sulfur, and trace levels of metals [7], The bulk properties of dilbit derive from its four constituent chemical fractions, which are common to all types of crude: saturates, aromatics, resins, and asphaltenes, or SARA. The heaviest of these four fractions – the resins and asphaltenes – are characterized by the solvent conditions that are used to extract them for chromatography while the lighter fractions are categorized according to chemical structure.

Saturates mainly consist of hydrocarbons with C-H and C-C single bonds, namely n-alkanes, iso-alkanes, and cycloalkanes (also known as paraffins). Unsaturated hydrocarbons are mostly non-detectable in crude oil [13], owing to the relative lability of the double carbon bonds. n-alkanes and iso-alkanes have straight-chain and branched-chain structures, respectively, while cycloalkanes contain ring or fused-ring substructures. Aliphatic chains range from gaseous methane (CH_4) to crystalline waxy solids ($C_{>20}$). In general, straight-chain saturates are easily biodegraded by microbes, and are therefore considered to be of little toxicological concern due to their lack of persistence in the environment [7]. Cycloalkanes and iso-alkanes are more resistant to biodegradation owing to their complex structures, and pose more danger of being integrated into biological membranes where they can induce toxicity [14]. A small number of these compounds are so persistent that they are used as biomarkers for environmental forensics: their concentrations

are unique to their reservoirs and can thus be traced to specific sources in the event of a spill [15]. Examples of these are hopane and prystane (iso-alkanes), and adamantane (cyclo-alkane) [7].

Aromatic hydrocarbons are planar, cyclic compounds with one or more delocalized carbon double-bond systems, also known as π-systems. The building block of aromatics is benzene (C_6H_6); larger polycyclic aromatics consist of fused benzene ring subunits. Like the saturates fraction, alkyl substituents are common on aromatic hydrocarbon, resulting in many possible isomeric confirmations. The most prevalent monoaromatics are known as BTEX (benzene, toluene, ethylbenzene, and xylene(s)). These compounds are on the United States Environmental Protection Agency's (USEPA) list of priority contaminants, as they are highly toxic [16]. They are also volatile and flammable, which poses additional risks to responders at an oil spill site during the first 48 hours following the incident.

Polycyclic aromatic compounds and their alkylated homologues (PACs and alkyl-PACs) of concern range from 2-5 fused benzene rings, and comprise 30-35% of the total hydrocarbon content of Alberta bitumen [11]. Their water solubility is inversely proportional to the number of benzene rings. Over 41 parent (non-substituted) PACs are on the US EPA's priority list, as they are persistent in the environment, are toxic, and often carcinogenic. Despite representing the majority of the total PACs in crude oil, fewer data are available regarding the alkyl-PACs, as the number of structural isomers can reach into the thousands [17]. However, it has been established that alkyl-PACs with 3-5 rings are responsible for most chronic toxicity effects elicited by exposure to crude oil. PACs also include the naphthenoaromatics, which contain both aromatic and non-aromatic cyclic structures and heterocycles. These compounds are more prevalent in heavier oils such as dilbit, perhaps the most notable of which is dibenzothiopene [13].

Resins are a complex fraction with molecular weights of approximately 500-1000 Da [18]. They differ from asphaltenes by being soluble in n-pentane and n-heptane, while the latter are soluble in toluene [19]. Resins contain one or more heteroatom, making resins more polar than hydrocarbons [19]. They are typically smaller and contain fewer aromatic moieties than asphaltenes. Asphaltenes are similar to resins, but have an average molecular weight of ~ 2000 Da [19]. There is still much debate as to the chemical constituents of resins and asphaltenes as they are poorly resolved by chromatography due to their complexity. These two fractions may constitute up to 50% of heavier crudes like dilbit, and are largely responsible for its density and viscosity [11].

Minor components of dilbit include inorganic sulfur, metals, and napthenic acids. Inorganic sulfur is commonly found at low levels as elemental sulfur and as gaseous H_2S. The gas is toxic, corrosive, and explosive, and is therefore removed prior to transportation for safety reasons. The most common metals are vanadium, nickel, copper, iron, and chromium. Metals are often part of organometallic structures named porphyrins [20], and are known to interfere with catalytic processes during refining [11]. Naphthenic acids (NAs) are thought to be the end products of cyclo-alkane biodegradation over geological time periods [21]. Due to the multitude of possible isomers, their exact structures are as controversial as those of asphaltenes.

1.2.2 Behaviour of spilled oil in aquatic environments: dispersion and weathering

The physical and chemical properties of crude oil change when it is subjected to environmental conditions. This process, known as weathering, affects properties such as viscosity, specific gravity, the mass of oil at the surface, and toxicity of the spilled oil [7]. Weathering is dependent on both the type of spilled oil as well as the type of environment. Therefore, remediation

and environmental toxicity assessments must take into consideration these physical and chemical changes in order to be effective.

Surface weathering processes that can affect oil behaviour include spreading, evaporation, photooxidation, and emulsification [7]. Spreading is dependent on factors such as oil viscosity, air temperature, and wave action. Sheens of only a few molecules in thickness can extend far beyond the spill site, greatly increasing the affected area. Volatilization of LMW hydrocarbons such as BTEX and $C_{<15}$ saturates occurs in the first 48 hours post-spill, increasing the viscosity and density of the oil [22]. Evaporation is controlled by oil slick thickness, which can trap LMW components away from the surface, and by ambient temperature; higher temperatures accelerate evaporative losses. For these reasons – and owing to their higher HMW concentrations – dilbits experience significantly lower evaporative losses (< 18% mass) than lighter crudes (30-50%) [23]. Dilbit becomes more susceptible to sinking after weathering, as its density can exceed 1.0 g/mL.

Other weathering processes can also affect toxicity and bioavailability of dilbit. Photooxidation is a poorly understood process that can increase the solubility of aromatic compounds. The oxidized products are potentially more toxic by becoming simultaneously more bioavailable and less biodegradable [7]. Emulsions are created through the incorporation of oil with large volumes of water, extending the affected oiled site several fold [24]. Suspended droplets and mousses can greatly impede biodegradation and hamper recovery efforts. However, a study conducted by the Government of Canada and subsequently verified by Zhou et al (2015) observed that two weathered dilbits did not form stable emulsions [25].

In the water column itself, spilled crude can weather through several different processes. Dissolution of the more soluble LMW hydrocarbons can enrich the remaining oil in larger PACs

[7]. Natural dispersion involves the formation of small oil droplets that can remain suspended in the water column or be transported by currents. Dispersion results in greater surface area of oil to volume ratios, which can promote biodegradation by microbial communities, but also increase bioavailability to other aquatic organisms [26]. Dispersed oil particles may eventually sink if biodegradation or agglomeration to solid particulates increases their density to > 1.0 g/cm^3. These can accumulate in the sediment, and be re-suspended under turbulent conditions. Dilbits are prone to the formation of tar mats and tar balls due to their elevated HMW concentrations. These are generally thought to be less toxic due to the lower surface area to volume ratio.

1.3 Toxic effects of dilbit on fish

Whereas terrestrial organisms are susceptible to direct contact to the spilled oil, the most common exposure route for fish, and other aquatic organisms, is interacting with the water accommodated fraction (WAF) of oil. The WAF represents those compounds in oil that can dissolve or be suspended in the water column. Crude oil toxicity can be characterized into two broad categories – acute and chronic. Acute toxicity is of most concern in the first 48 hours of exposure. During this period, soluble LMW hydrocarbons (e.g BTEX) are rapidly lost from the oil, resulting in water column concentrations elevated enough to result in fish kills [27]. Their acute toxic effects derive from a process known as narcosis, which is dependent on the ability of oil constituents to partition into biological membranes, a property that can be approximated to their hydrophobicity (measured with the octanol-water partition coefficient, K_{OW}) [28]. In dilbits, LMW compounds are mostly present in the diluent fraction. Acute lethality is generally a concern for only the first 48 hours post-spill, after which environmental concentrations have sufficiently lowered by dispersion, evaporation, and dilution processes [27].

Sublethal chronic effects are attributed to medium to large PACs and alkyl-PACs containing 3-5 rings that are too insoluble to be acutely toxic [29]. Alkyl-PACs are of special concern, as they remain bioavailable for extended periods of time due to their resistance to biodegradation. Biomagnification does not occur because fish rapidly metabolize many PACs. However, the phenolic metabolites from the CYP1A detoxification pathways are often more toxic than the parent compounds. Deleterious effects include cardiac abnormalities, lower reproductive rates, developmental abnormalities, and oxidative damage, as discussed below [30–32].

Fish in their early life stages are highly vulnerable to PAC toxicity: chronic exposure leads to deleterious effects at ΣPAC concentrations as low as 1μg/L in developing embryos [33]. For example, Madison et al (2017) noted an increased prevalence in blue sac disease and elevated molecular indicators of oxidative stress in Japanese medaka (*Oryzias latipe*) embryos exposed to Access Western Blend dilbit [34]. Pacific herring (*Clupea pallasi)* embryos exposed to oiled sites from the Cosco Busan oil spill near San Fransisco, CA, developed cardiac deformities after 3 months [35]. Embryos are more susceptible because several genes are only upregulated during early development. PACs can bind to protein receptors that disrupt these gene pathways, resulting in the broad range of negative effects alluded to previously [36,37].

Photo-enhanced toxicity of PACs is an additional concern for early life stages of fish, Ultraviolet radiation (UV) can interact with the π electron systems of PACs, creating highly energetic radical species that readily oxidize into more toxic quinone derivatives [38]. This is especially relevant for the embryos of many species, which are transparent. *In vivo,* photo-oxidation produces elevated concentrations of reactive oxygen species (ROS) such as -OH radicals that react rapidly with lipids, proteins, and nucleic acids, leading to necrosis and death [39,40]. Embryo mortality has been shown to occur at far lower PAC concentrations when paired with

exposure to UV light [35]. More transparent embryos, as well as embryos from environments with clearer water are more at risk of photo-enhanced toxicity due to greater UV penetration into the water column [7].

1.3.1 Oxidative stress and biomarkers of exposure

Biochemical responses are promising early indicators of crude oil exposure in fish. Several studies have observed biochemical responses indicative of PAC biotransformation and oxidative stress, such as elevated antioxidant gene expression and lipid peroxidation [31,34,41]. Accordingly, measurements of biomarkers are now often included in studies of crude oil's effects on fish health parameters.

Oxidative stress is recognized as a mechanism of action for PAC and alkyl-PAC toxicity alongside DNA and protein adduct formation [42], and is best described as an increased concentration of ROS that overwhelms an organism's antioxidant capacity leading to metabolic and regulatory pathway alterations [43]. Large amounts of ROS are generated during the biotransformation of PACs by phase I detoxification enzymes, the most notable family being the membrane-bound cytochrome P450 (or CYP) monooxygenases [44]. The CYP1A1 enzyme – also known as aryl hydrocarbon hydroxylase – is induced by PACs in teleost fish to oxygenate the parent compounds, making them more hydrophilic and thus easier to excrete in aqueous media like urine and bile. *cyp1a* gene upregulation and CYP1a activity are measured with a simple assay that characterizes an operationally defined activity, ethoxyresorufin-O-deethylase (EROD), based on the metabolism of an artificial substrate to a fluorescent product [45]. They are not however direct indicators of oxidative damage, though increased *cyp1a* translation and EROD activity correlate to oxidative stress.

Several antioxidant enzymes are currently used as biomarkers of PAC-induced oxidative stress in fish. Superoxide dismutase (SOD) converts superoxide ($O_2^{\cdot-}$) anions to hydrogen peroxide, which is subsequently converted into water by catalase (CAT). Together, these two enzymes prevent the formation of highly reactive hydroxyl radicals. Their activities are commonly used as biomarkers of oxidative stress, though van der Oost et al (2003) note conflicting results between field and laboratory studies [38]. The most robust of the antioxidant enzyme is glutathione-S-transferase (GST), a multifunctional enzyme family with the critical role of conjugating glutathione (GSH) to contaminants to facilitate excretion during phase II detoxification, including oxidative stress products like lipid peroxides [46]. GSH itself is not an enzyme but a low-molecular weight thiol-containing tripeptide whose redox activity make it an effective antioxidant. It reduces ROS by cycling between its reduced and oxidized dimeric form (GSSG:GSSG) in the cytosol via a process mediated by glutathione reductase (GR) and glutathione peroxidase (GPx). Smaller ROS such as H_2O_2 are thus converted to water, preventing oxidative stress [47]. The GSH detoxification system can also conjugate GSH with larger ROS such as lipid peroxides using the GST pathway, as previously mentioned. Total GSH and ratios of reduced-to-oxidised GSH are therefore commonly used to measure oxidative stress in fish, as are activities of GST, GR, and GPx [46]. However, these assays are indirect measures of oxidative stress and therefore do not quantify damage caused by ROS.

Measures of lipid peroxidation (LPO) are also used as biomarkers of oxidative stress. LPO encompasses a wide array of chain reactions triggered by radicals reacting with fatty acids. The products of these chain reactions are often toxic aldehydes that can damage DNA and proteins by forming adducts. A common measurement technique is the thiobarbituric acid reactive species (TBARS) assay. LPO measurements in the literature are highly inconsistent due to variation in test

species, environment, and experimental methodology [38]. The TBARS assay suffers from the additional setback of being non-specific to its intended target molecule, malondialdehyde (MDA). High-performance liquid chromatography methods specific to MDA have more recently been developed but are not prevalent in the literature, though they are likely more robust than standard TBARS assays [48–50]. Such indirect measures are used because ROS are extremely short-lived chemical species, making them difficult to quantify.

1.4 Isoprostanes: potential biomarkers of PAC exposure in fish

Prostaglandin F_2-like derivatives, specifically the F_2-isoprostanes (F_2-isoPs), have emerged as reliable biomarkers for oxidative stress in visceral tissues of mammals, but have seldom been studied in fish – and in long term studies of oxidative stress [51–54]. To date, F_2-isoP have primarily been used as biomarkers of lipid peroxidation to investigate the pathogenesis of human diseases [55–57]. F_2-isoPs are the free radical catalyzed products of non-enzymatic lipid peroxidation of arachidonic acid (AA) [58], a fatty acid found abundantly in brain tissue and cell membranes of all animals. Several factors make F_2-isoPs advantageous as biomarkers of oxidative stress. They are chemically stable, and have been detected at relatively constant low levels in all biological tissues and in the fluid of healthy vertebrates, namely urine, plasma, and cerebrospinal fluid [58,59], F_2-isoPs are also more reliable indicators of lipid peroxidation than TBARS, as the latter is susceptible to interactions that reduce the assay's accuracy [48]. High performance liquid chromatography/tandem mass spectrometry (HPLC-MS/MS) has become the preferred method of analysis for F_2-isoPs, as it achieves the desired separation and sensitivity without time-consuming derivatization and yields more reproducible results than enzyme-linked immunoassay (ELISA)

kits [52,60–62]. F_2-isoprostanes have potential as robust biomarkers of oxidative stress in fish, but methods must first be developed and validated prior to implementation.

1.4.1 F_2-Isoprostane structure and formation

F_2-isoprostanes were first observed by GC-MS in human plasma by Morrow et al (1990) [63], and have since become "the gold standard" for assessing oxidative stress *in vivo* in mammalian models [54,64]. F_2-isoprostanes are secondary end products of lipid peroxidation, a process by which ROS react with labile moieties of lipids, thereby oxidating them. Structurally, isoprostanes are very similar to prostaglandins – a class of biologically active compounds formed during the peroxidation of arachidonic acid. However, isoprostanes are formed through a non-enzymatic free-radical mediated process, whereas prostaglandin formation involves the cyclooxygenase enzymes COX1 and COX2 [54]. The formation mechanism proposed by Morrow et al involves formation of an endoperoxide intermediate followed by a second oxygenation and subsequent reduction to form isoprostanes [65]. As a result, the hydroxyl groups bonded to the prostane ring are exclusively in the *cis* configuration in isoprostanes, while they are generally *trans* in prostaglandins.

Since arachidonic acid has four double bonds available for allylic hydrogen abstraction during radical attack, the initial oxygenation – leading to the formation of the 5-carbon prostane ring by endocyclization – can result in four possible regioisomers. As shown in Figure 1, the placement of the prostane ring results in variable side chain lengths. The possible orientations of the alkyl chains, prostane hydroxyls, and side-chain hydroxyl further result in eight pairs of diastereromers, for a total of 64 possible F_2-IsoPs. To date the most studied of these compounds is 8-iso-$PGF_{2\alpha}$, a 15-series isomer [62].

Another notable difference between formation processes of F_2-isoPs and their prostaglandin analogues is that they are formed *in situ* on phospholipids, where arachidonic acid esterified to phospholipids are peroxidized [66]. Free F_2-isoPs are then released, presumably by phospholipases. This contrasts $PGF_{2\alpha}$, which is formed exclusively from free AA molecules. It is therefore important to consider both esterified and free isoprostanes when measuring these compounds in tissues.

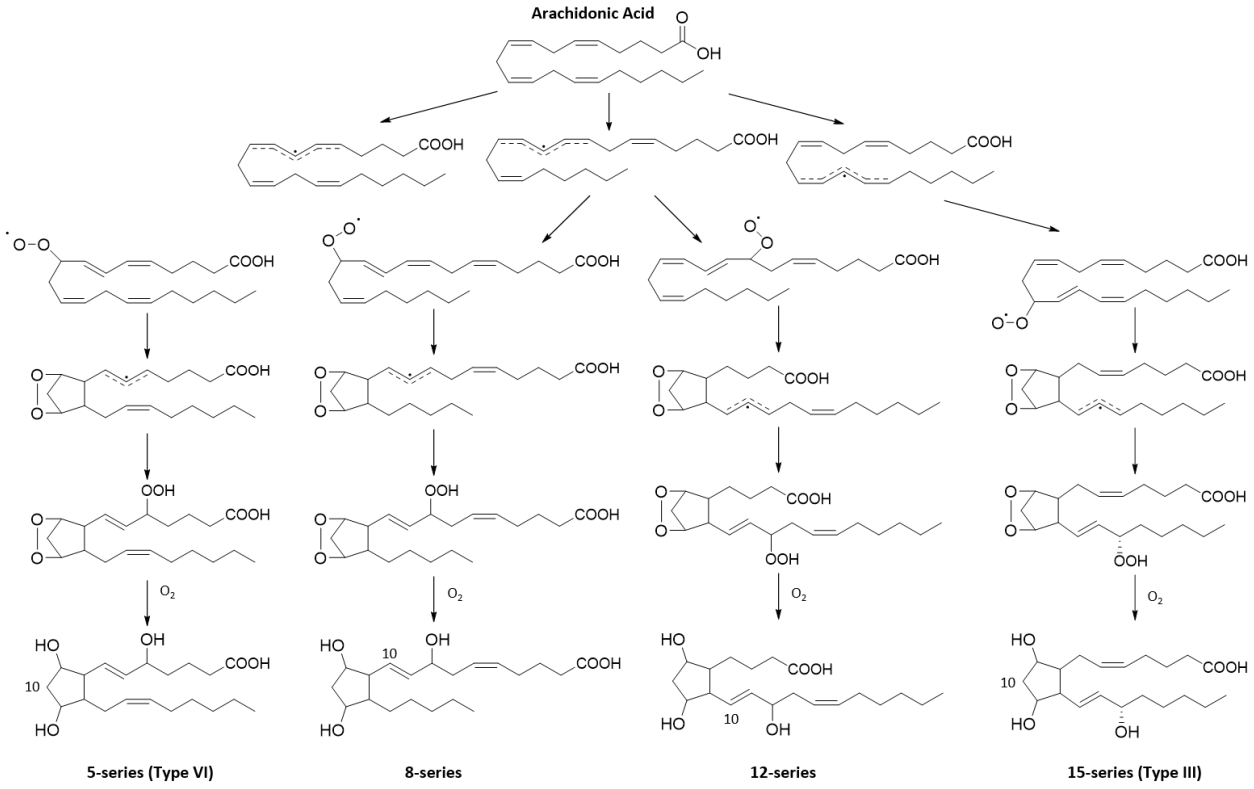

Figure 1.1 Free-radical mediated formation of the F2-isoprostanes resulting in four regioisomer classes. Each class can form eight racemic pairs of diastereomers, for a total of 64 (Reproduced from Milne et al (2005).

1.4.2 Evidence that F_2-isoprostanes are indicators of oxidative stress

As lipid peroxidation is a well-known mechanism of oxidative stress, F_2-isoprostanes were immediately considered as potential biomarkers of oxidant injury. Morrow et al (1992)

demonstrated that levels rose 55-fold in rats four hours after being exposed to CCl_4, a known hepatotoxic inducer of lipid peroxidation, and remained 21-fold higher after 24 hours, indicating continued LPO [66]. Importantly, they also showed that F_2-isoP levels were mostly specific to agents causing LPO, rather than being general markers of hepatotoxicity: dosing the rats with hepatotoxic compounds not causing LPO resulted in only small increases in F_2-isoP levels relative to the severity of induced liver damage – likely a secondary effect not causally related to toxicity. This study also demonstrated that F_2-isoP concentrations increased 5- to 8-fold when rats were pre-treated with chemical agents that induce cytochrome P450 enzymes prior to CCl_4 administration, and that this was directly related to the rate of CCl_4 metabolism. Finally, the relation of F_2-isoP formation to oxidative stress was further confirmed by showing that levels increased when glutathione (a prevalent antioxidant that mitigates oxidative stress) was chemically suppressed.

Since this seminal study, F_2-isoPs have been correlated to several human diseases thought to cause oxidative stress. Studies of atherosclerotic plaque revealed that levels were elevated four-fold, indicating greater peroxidation relative to normal vascular tissue [67]. In post-mortem analyses of patients with Alzheimer's disease, F_2-isoP concentrations in cerebrospinal fluid (CSF) were correlated to the extent of neurodegeneration, providing more evidence that oxidative stress is part of the pathophysiology of this disease [68,69]. A similar link was also established between cigarette smoking and risk of cardiovascular and pulmonary disease: plasma F_2-isoP levels were significantly elevated in heavy smokers relative to non-smokers, and individuals who successfully abstained from smoking for two weeks experienced statistically significant decreases [70,71]. As a result of these findings and many others, F_2-isoPs have become widely regarded as the one of the most reliable indicators of oxidative stress *in vivo*.

1.4.3 Relevance of F_2-isoprostanes to oil spill research

As discussed in section 1.3.1, phase I metabolism of PACs by CYP450 enzymes can result in toxic effects in crude oil-exposed fishes. CYP enzymes are monooxygenases that are responsible for detoxifying drugs and xenobiotics, which they achieve mainly by oxidation of their substrates. The resulting metabolites are more hydrophilic, which facilitates excretion through the kidneys. A heme group in the CYP450 active site is responsible for activating O_2: upon reduction, a hydroxyl group can be transferred to the xenobiotic substrate [72].

The interaction between the CYP450 isoform CYP1A and the aryl hydrocarbon receptor (AhR) has been the focus of many studies investigating the toxicity mechanisms for PACs and other planar xenobiotics compounds [73–76]. AhR, a cytosolic protein with a binding affinity for planar compounds, is a potent inducer of CYP1A [77]. Several experiments have demonstrated that chemically stable polyhalogenated aromatic hydrocarbons (pHAHs) with strong binding affinities for AhR – known as AhR agonists – such as PCBs can uncouple electron transfer in CYP450 [73–76], resulting in the release of ROS such as hydrogen peroxide and superoxide anion. While smaller parent PACs are rapidly metabolized and excreted, larger alkylated congeners such that are found in dilbit could potentially cause similar uncoupling, as their steric bulk renders them less labile, while retaining their strong affinity for AhR. Furthermore, a growing body of research is reporting the presence of halogenated PACs in Oils Sands bitumen and local biota [78]. Such compounds could exhibit similar properties to PCBs in uncoupling electron transfer in the CYP450 detoxification pathway.

CYP metabolism of PACs can also result in increased production of ROS through redox cycling of phase I metabolites [79]. Compounds commonly found in dilbit such as phenanthrene and benzo[a]pyrene are hydroxylated by CYP1A, usually forming *trans* diols at energetically

favourable sites (e.g. BaP-7,8-dihydrodiol) [80]. Amplification of ROS production is associated with the aldo-keto reductase (AKR1A1) pathway, whereby the diols are oxidized further by a dihydrodiol dehydrogenase and the resulting ketols tautomerize to a catechol intermediate [81]. Catechols are readily oxidized to form quinones through a two-electron oxidation process, generating ROS at each step. When coupled to CYP450, these quinones can repeatedly redox cycle by using NADPH from the CYP1A1 pathway to regenerate the catechol intermediate, thus creating a continuous cycle of ROS production. Several studies have linked these catechol-mediated ROS to oxidative DNA damage [80–83].

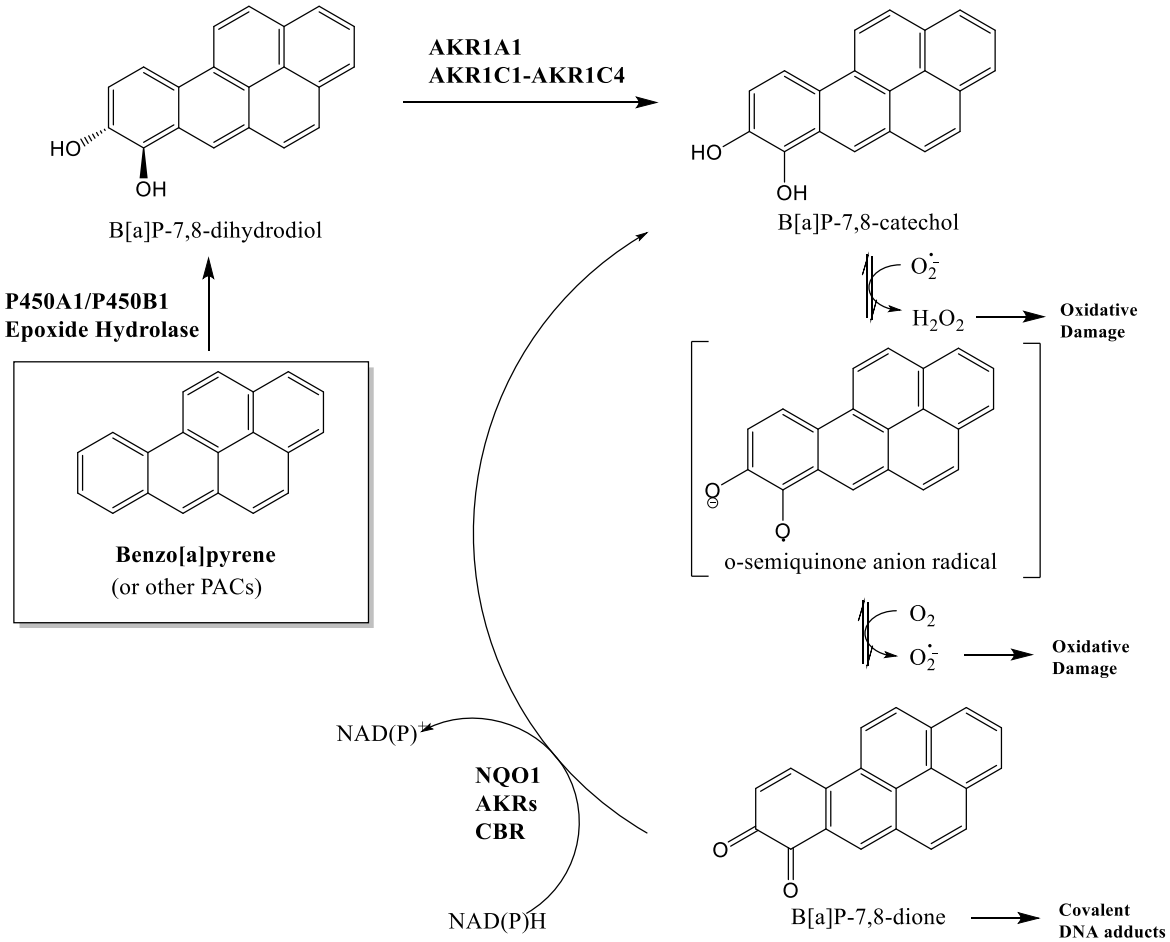

Figure 1.2 PAC-induced production of reactive oxygen species via generation of quinones in the AKR1 pathway. Figure was recreated using Figure 2 of Penning et al (2014) [80]. P450A1/B1 epoxide hydrolase and P450 peroxidase pathways not shown in this figure.

Given the ubiquity of these compounds in dilbit and other crude oils, it is apparent that there is high potential for oxidative damage in aquatic organisms exposed to these water soluble PACs. As a reliable class of oxidative stress biomarkers *in vivo*, F_2-isoprostanes are strong potential candidates for quantifying the deleterious effects that dilbit spills can effect on fishes.

1.5 Variables confounding the interpretation of oxidative stress biomarker response in wild fishes

Though the practice of measuring biomarkers of oxidative stress in fish has become ubiquitous in toxicological studies, contradictory findings are common when comparing laboratory and field exposures [38]. Field studies suffer from having the difficult task of accounting for multitudes of environmental factors that are otherwise controlled in a laboratory study. An increasing number of studies have demonstrated that extrinsic factors such as water temperature, changes in salinity and oxygen availability contribute to fishes' oxidative stress response [84]. Furthermore, life histories and other intrinsic factors such as diet, reproductive strategy, and senescence have also been shown to affect antioxidant/pro-oxidant balance in fish [84]. These variables must be considered when interpreting oxidative stress responses of wild fish species and are discussed in more detail below.

1.5.1 Effects of water temperature on oxidative status of fishes

According to thermodynamic principles, temperature changes in an organism are usually accompanied by a change in metabolic rate. As ROS are generated by all aerobic organisms during metabolism – a result of electron leakage in complexes I and III of the mitochondrial electron transport chain (ETC) – changes in temperature should result in a corresponding change in the rate of ROS production and therefore oxidative status.

Fish species have adapted to an exceptionally diverse range of thermal environments, ranging from the glacial depths of the Arctic Ocean to warm, tropical coastal waters, lakes, and streams. Appropriately, their metabolisms have become optimized to the temperature ranges in the environments they populate. However, according to the 2013 IPCC report, climate change will cause global water temperatures to increase by an average of 2°C by the end of the century, which

will potentially alter the metabolic rates and oxidative status of fish populations everywhere [83]. Therefore, understanding the confounding effects of water temperature on oxidative status is essential if biomarkers of oxidative stress are to be used accurately as indicators of exposure to contaminants.

Though it seems logical that a higher water temperature should positively correlate to accelerated metabolic rates and greater ROS production, some studies show that this is not necessarily the case. Vinagre et al (2012) observed that catalase activity and MDA concentration was elevated in muscle samples of European sea bass (*Dicentrarchus labrax*) exposed to temperatures above and below their optimal temperature range of 20-25°C, rather than following a linear trend [85]. These findings are consistent with two studies conducted on North Sea eelpout (*Zoarces viviparus*) that exposed these fish to acute cold and heat exposures, observing elevated responses of oxidative stress biomarkers in both experiments [86,87].

Additionally, another study conducted by Vinagre et al (2014) demonstrated that in rock goby (*Gobius paganellus*), CAT, SOD, GST, and LPO responses increased concomitantly with water temperature in liver and muscle but not in the gills, where they observed higher basal antioxidant defenses [88]. This suggests that certain tissues may be preferentially protected from oxidant injury in order to maximize survival and fitness, and may be more useful for analysis of oxidative stress biomarkers, as such tissues' responses might not be confounded by extrinsic factors such as water temperature, due to their higher resilience to environmental variability. Tissue-dependence of oxidative response should therefore also be considered when analyzing biomarkers of oxidative stress.

Further, the effect of water temperature on oxidative status is also potentially affected by population-specific adaptations. This was demonstrated by Nikinmaa et al (2013), who showed

that three different Finnish populations of three-spine stickleback (*Gasterosteus aculeatus*) exposed to acute temperature increase had statistically different expression rates of genes linked to oxidative stress response [89].

1.5.2 Oxygen availability

Though the majority of studies focus on marine species, the effect of oxygen availability is still a relevant variable for freshwater species in stratified lakes, where oxygen concentrations decrease at greater depths. Deep, boreal lakes such as those found at the IISD-ELA are examples of stratified lakes. These lakes turn over in the spring and fall, resulting in uniform temperature and oxygen profiles throughout the entire lake, and stratify in the summer and winter seasons. Lake trout (*Salvelinus namaycush*) are commonly found in these lakes: this species is both commercially important and considered to be a sentinel species for climate change due to its cold and narrow optimal temperature range of 8-12°C. Lake trout spend the majority of their time in the colder, more anoxic depths of the lakes during the summer while enjoying higher dissolved oxygen concentrations throughout the entire lake during the other seasons. Additionally, increasing temperatures expected with climate change have begun to force these fish deeper into less productive, more anoxic areas. Though studies on the effect of variable oxygen availability on this species are lacking, hypoxia and hyperoxia have both been linked to increased oxidative stress biomarker concentrations in several freshwater fish species including the fathead minnow, silver catfish (*Rhamdia quelen*) and common carp (*Cyprinus carpio*) [90–92]. Luschak et al (2005) further observed what appeared to be anticipatory induction of antioxidant defenses in the brain of common carp exposed to hypoxic conditions [92]. These findings demonstrate that like water temperature, variable oxygen availability can have a confounding effect on baseline data and post-exposure analysis of oxidative stress in freshwater species if it is not properly accounted for.

1.5.4 Life history and reproductive strategy

Life history theory acknowledges that energetically costly behaviours and physiological changes – such that are often observed in during fish spawning – necessarily involve the diversion of energetic resources, at the expense of other physiological functions. Despite the knowledge that reproduction often results in extended periods at higher metabolic rates, relatively few studies have explored the cost to fish in terms of oxidative stress caused by increased ROS generation. To further complicate matters, reproductive strategies are highly variable. Secondary sexual characteristics, migration, aggressive nest-guarding behaviours involving self-imposed fasting, and egg development are examples of energetically demanding reproductive strategies that could cause oxidative damage through excessive ROS production or the inability to replenish antioxidant defenses [93–95]. This underscores the importance of longitudinal studies for species of interest that are at risk of contaminant exposure, as their oxidative status can fluctuate during the year.

To date, clear relationships have not been established between apparent invested effort and oxidative damage in spawning fish. Contrary to expectations, a study of the semelparous Pink Salmon (*Oncorhyncus gorbushca*) found that oxidative stress in the brain was lower at the spawning grounds, while oxidative DNA damage was much higher in the heart tissue [93]. This once again underlines that fish appear to preferentially protect certain tissues in order to maximize fitness, and that strategies will differ.

In smallmouth bass (*Micropterus dolomieu*), vigorous paternal care did not result in oxidative stress, however during the most active stages of brood protection, antioxidant defenses were significantly lower [94]. These findings are corroborated by other studies who show that during the egg and embryonic phases of development, male smallmouth bass are more aggressively defending and maintaining their offspring, undergoing voluntary anorexia: as the

offspring enter the larval stage, the paternal fish have more opportunity to feed and replenish antioxidants through diet. On the other hand, male sticklebacks were shown to invest carotenoids so heavily into their mating displays as to cause themselves oxidative injury [95]. Clearly, understanding the reproductive strategy of a studied species is critical since they are more vulnerable to oxidative stress at specific – and sometimes very narrow – periods of time. In field studies or ecological monitoring programs, sampling time points should be selected with life history and reproductive strategy in mind.

1.5.5 Diet

Diet plays a role in maintaining the oxidative balance in fish. Certain antioxidants, such as vitamin E and other fat-soluble molecules, cannot be synthesized by fish and are therefore only available through their diet. Likewise, certain micronutrients are essential in the functioning of antioxidants such as GPX, for which selenium is essential [96]. Long periods of food deprivation, even partial, have been linked to oxidative stress – seen as increased MDA, GSH oxidation, and antioxidant enzyme activity – in several fish species. Further, Radi et al (1985) observed different trends in antioxidant enzyme activity of omnivorous fish compared to herbivorous [97]. Along these lines, Ruedo-Jasso (2004) demonstrated that Senegalese sole (*Solea senegalensis*) fed a high-lipid diet were more susceptible to OS than those fed low-lipid diets, which shows a possible link between dietary PUFA intake and OS [98]. This suggests that environmental incidents could result in greater oxidative stress to native fish species whose food sources are made scarce or absent due to toxicity, by forcing either prolonged fasting or a switch to alternate food sources. Diet can therefore confound efforts to directly assess toxicity in wild fishes exposed to contaminants if toxicity is measured using OS biomarkers.

1.5.6 Ageing and senescence

Age-related decline in antioxidant production and consequent increased ROS production are thought to be the main drivers of senescence, as mounting oxidative damage gradually impedes physiological function more and more [99]. This theory – known as the free radical theory of aging – has not been well studied in fish: as a result, it is unclear if fish experience a similar age-dependent degeneration of antioxidant capacity leading to senescence. Though the studies on this subject are scarce, age-dependent decline of antioxidant defenses has been observed. Wdzieczak et al (1982) observed higher antioxidant capacity in younger individuals of eight species native to both freshwater and marine environments [100]. In two other studies, antioxidant enzyme activity were lower in older cohorts of rainbow trout, black bullhead, and freshwater murrel [101,102], though the enzymes in question differed by species, and decline appeared to be tissue dependent. In contrast, Sanz et al (2001) observed increased GPX and CAT activity in the plasma of older sturgeon, suggesting that their antioxidant defenses actually increase with age. It is therefore possible that the expected trend could be reversed in long lived species with indefinite growth in order to maintain fitness during their considerable lifespans. However, the lack of longitudinal studies has been identified as a main problem in accurately establishing these trends [84].

1.6 Conclusion and objective of this thesis

There is a pressing need for research into the potential toxic effects of dilbit spills on freshwater fishes. Measuring oxidative stress biomarkers in fish tissues has shown promise in assessing the impact to fish health, however the smaller scale of freshwater ecosystems calls for a change in sampling methodology. Complex interactions between environmental factors and antioxidant enzymes are also liable to confound results of field studies, which has resulted in

contradictory findings in the literature. Furthermore, the F_2-isoprostanes – considered the gold standard for quantifying oxidative stress in humans – have not been utilized to a significant extent in fish-related studies. This thesis attempts to address these gaps in the literature, by applying a non-invasive sampling protocol to measure F_2-isoprostanes in fish mucus.

1.7 Thesis structure

Chapter 1 (this section) provides background on the state of research regarding the effects of crude oil spill on fish and the role of oxidative stress in PAC toxicity pathways. Through discussion of F_2-isoprostane formation and release in response to toxicants, we establish the underutilization of F_2-isoprostanes as a biomarker of oxidative stress, and their potential in crude oil exposures. The complexities of measuring oxidative stress in complex aquatic environments are also discussed.

Chapter 2 presents the first iteration of the extraction protocol and the proof of concept for measuring F_2-isoprostanes in fish mucus. The presence of F_2-IsoPs is confirmed in two freshwater species. This methodology is then refined in the following chapters, eventually employing swabs to collect the epidermal mucus and solid phase extraction of the F_2-IsoPs.

Chapter 3 expands upon these findings: in this laboratory based study, F_2-IsoPs were quantified in different tissues of immature rainbow trout. The effect of physical stress on rainbow trout's F_2-IsoP concentrations are examined. Concentrations of four F_2-IsoP are compared between tissues, namely liver, plasma, and gill. Levels of F_2-IsoPs are compared to glutathione – another other well-established biomarkers of oxidative stress – and cortisol in plasma.

In Chapter 4, a complementary laboratory study to the FOReSt study (Chapter 5) is presented, where juvenile fathead minnows were exposed to a range of dilbit concentrations in a

controlled setting. The objectives of this study were to examine the oxidative stress response of fathead minnows in a controlled setting and to determine if a dose-response relationship exists between WAF concentration and F_2-IsoP concentration.

In Chapter 5, the results of a simulated dilbit spill in real-world conditions are presented, as part of the FOReSt project at the IISD-ELA. The FOReSt study explored the effects of dilbit on the freshwater ecosystems using concentrations of dilbit typical of pipeline spills. Fathead minnows were used as a model organism to study the oxidative impact of dilbit on fishes. The effects of different secondary remediation methods (nutrient addition and Corexit 9580™ shoreline cleaner) on the toxicity of spilled dilbit are also considered.

Finally, in Chapter 6 a synthesis of findings is presented along with recommendations for future research.

1.8 Hypotheses

The hypotheses tested in this thesis are as follows:

Chapter 2:

H_1: F_2-IsoPs are present at detectable concentrations in the epidermal mucus of wild rainbow trout

Chapter 3:

H_1: Acute physical stress will cause a change in F_2-IsoP concentrations in the epidermal mucus and internal tissues of rainbow trout

H_2: F_2-IsoP concentrations in internal tissues and epidermal mucus are correlated.

H_3: The F_2-IsoP response is correlated to the response of glutathione and are both indicative of an oxidative stress response.

Chapter 4:

H_1: F_2-IsoP concentrations in epidermal mucus and liver will increase in a dose-dependent manner to the 7-day dilbit exposure for the control, low, medium, high exposure groups

H_2: Concentrations of F_2-IsoPs in the mucus will be correlated to concentrations in the liver.

Chapter 5:

H_1: There will be statistically significant differences in FHM mucus F_2-isoP concentrations between fish the in the control and dilbit-exposed enclosures.

H_2: There will be statistically significant differences in FHM mucus F_2-isoP concentrations between dilbit treatments subjected to different secondary remediation methods.

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Chapter 2: Development and validation of a non-invasive method to analyze F2-Isoprostanes in the epidermal mucus of fish.

2.1 Introduction

Contaminants are introduced daily into aquatic ecosystems through a variety of anthropogenic activities such as wastewater discharge, industrial effluents, agricultural runoff, and environmental incidents (e.g. oil spills) [1–4]. Many of these contaminants have toxic effects on aquatic organisms: notable examples are polycyclic aromatic hydrocarbons (PACs), pesticides, pharmaceuticals, and transition metals. Oxidative stress has been shown to be a mechanism of toxicity for the previously mentioned classes of environmental contaminants [5–8]. PACs have been shown to induce oxidative stress responses at aqueous concentrations of 10μg/L [9]. Significant increases in lipid peroxidation was demonstrated in channel catfish (*Clarias gariepinus*) by Amin et al (2012) upon exposure to low concentrations of the pyrethroid pesticide deltamethrin [10]. It has also been described as an important mechanism of mercury toxicity in fish [11]. Accordingly, studies routinely include analyses of oxidative stress biomarkers, such as thiobarbituric acid reactive substances (TBARS) and antioxidant enzymes activity assays as determinants of fish health post-exposure [8,12–14].

Prostaglandin F_2-like derivatives, specifically the F_2-isoprostanes (F_2-isoPs), have emerged as reliable biomarkers for oxidative stress in visceral tissues of mammals. To date, F_2-isoP have primarily been used as biomarkers of lipid peroxidation to investigate the pathogenesis of human diseases [15–17], but have seldom been studied in fish [18–21]. However, orally administered high molecular weight PAC exposures of 7.82μg/g fish resulted in increased muscle concentrations of F_2-isoPs in rainbow trout (*Oncorhyncus mykiss*) after 50 days [22]. Chung et al (2013) also demonstrated that F_2-isoP levels in medaka rise when exposed to a classic oxidant, hydrogen peroxide [23]. F_2-isoPs are the free radical catalyzed products of non-enzymatic lipid

peroxidation of arachidonic acid (AA) [24], a fatty acid found abundantly in brain tissue and cell membranes of all animals. Several factors make F_2-isoPs advantageous as biomarkers of oxidative stress. They are chemically stable, and have been detected at relatively constant low levels in all biological tissues and in the fluid of healthy vertebrates, namely urine, plasma, and cerebrospinal fluid [24,25]. F_2-isoPs are also more reliable indicators of lipid peroxidation than commonly used TBARS as biomarkers of oxidative stress, as the latter is a non-specific assay and is therefore susceptible to interactions that reduce the assay's accuracy [26]. High performance liquid chromatography/tandem mass spectrometry (HPLC-MS/MS) has become the preferred method of analysis for F_2-isoPs, as it achieves the desired separation and sensitivity without time-consuming derivatization and yields more reproducible results than enzyme-linked immunoassay (ELISA) kits [19,27–29].

Many established methods for analyzing fish health, including isoprostanes, involve lethal tissue sampling prior to chemical analysis.[23,30–33] To minimize the impact of repeated sampling on ecosystems – often with small fish populations – monitoring programs assessing the health of wild populations are increasingly moving toward non-destructive and non-invasive methods [34]. Current techniques for F_2-isoP quantification make use predominantly of blood and urine as matrices, and while these fluids can be collected non-lethally from fish, current methods are still invasive and stressful. More recently, fish skin mucus has been investigated as a potential biological matrix for the analysis of fish health [35–37]. Mucus has important biological functions for fish, ranging from communication and reproduction to osmotic regulation [38]. It is composed mainly of glycoproteins, but also contains immunoglobulins, pheromones, lysozyme, and proteolytic enzymes [38]. Previous analyses using this matrix include hemoglobin, metabolomics studies, and antioxidant activity assays[35–37], but mucus is also known to contain proteins, large

molecules such as egg yolk precursors, [35] and RNA and DNA material [39–41]. Here we describe the development and optimization of a HPLC-MS/MS method to isolate and quantify F_2-isoPs isomers in fish mucus from several lakes in northwestern Ontario and Manitoba, Canada [42]. Sampling was non-invasive, and processing was simple and rapid as few modifications to the matrix were needed prior to analysis.

2.2 Materials and Methods

2.2.1 Materials

Methanol (Optima grade), hydrochloric acid (HCl), and water (HPLC grade) were purchased from Fisher (Fair Lawn, NJ). (±) 5-iso-prostaglandin-$F_{2\alpha}$-VI, (±) 5-iso-prostaglandin-$F_{2\alpha}$-VI-d_{11} (± indicates a racemic mixture of two enantiomers), 8-iso-prostaglandin-$F_{2\alpha}$, and 8-iso-prostaglandin-$F_{2\alpha}$-d_4, ent-iso-15(S)-prostaglandin- $F_{2\alpha}$ and ent-iso-15(S)-prostaglandin- $F_{2\alpha}$-d_9 (all >98%) were procured from Cayman Chemicals (Ann Arbor, MI). The chemical structures can be found in Figure S1.

2.2.2 Sample collection

Lake trout (*Salvelinus namaycush)* were angled from five experimental lakes at the International Institute for Sustainable Development – Experimental Lakes Area (IISD-ELA): lake 223 (49.698194°, -93.707952°), lake 224 (49.688778°, -93.719360°), lake 260 (49.694108°, -93.767919°), lake 373 (49.743863°, -93.799417°), and lake 626 (49.753069°, -93.796672°). More experimental lake parameters can be found in Table S2. Individuals of mixed sex (n=7) were sampled from each of the five lakes (35 total) at over a period of two weeks in autumn (September 26 – October 10, 2018) to attempt preliminary measurements of baseline F_2-isoP concentrations across a spectrum of lakes. Care was taken to minimize handling and to reduce the surface area

denuded of mucus, to minimize impact on each individual fish. Fish were anaesthetized in lake water containing pH buffered MS-222 (0.4g/L), until all fin movement ceased (~3min), and then measured, weighed and their sex was determined using external characteristics. A small volume of mucus (10-50mg, but variable among individuals) was scraped from the skin using methanol-rinsed forceps and aspirated using a sterile syringe. The sample was then dispensed into a 1.5 ml microcentrifuge tube and immediately frozen on dry ice for transport to the laboratory where they were stored at -80C until processing. The lake trout sampling procedures were conducted according to standards established by the Canadian Council on Animal Care and were approved under AUP #F16-018. Northern pike (*Esox lucius*) mucus samples collected from specimens angled in Rice River, MB (Latitude: 51.3364°N, Longitude: 96.4025°W), were provided by a recreational angler, though sample storage practices were still observed. These samples were used for method development and validation due to the large quantity available.

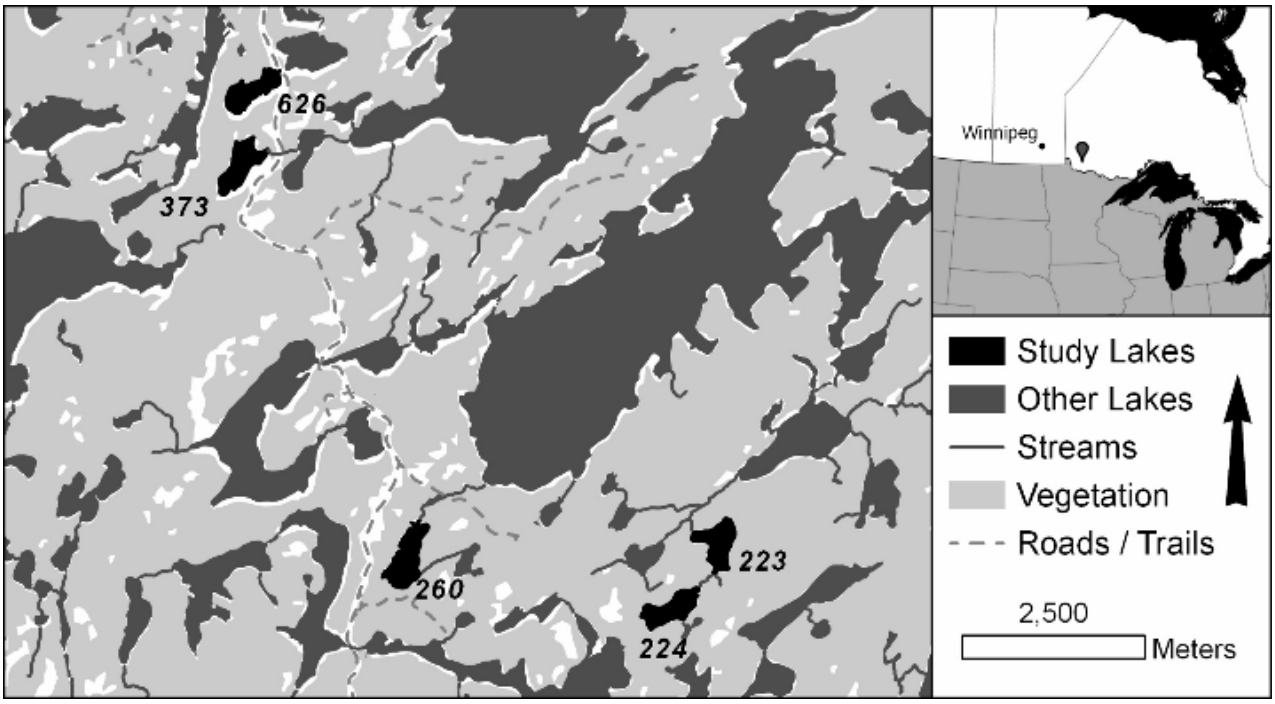

Figure 2.1 Location of IISD-Experimental Lakes Area research station in Northwestern Ontario, Canada (top right) and research lakes sampled for this study (main body). Credit: G. Gunn, IISD-ELA. Data sources: NRCAN and IISD-ELA.

2.2.3 Sample preparation: fish mucus

Northern pike mucus samples prepared from undiluted mucus (45mL total) were used for the development of the extraction protocol. Lake trout mucus samples were prepared using the same method, but were not pooled. Samples were centrifuged at 5000rpm for 15min. 50µL of the supernatant was aliquoted into microcentrifuge tubes. Aliquots of 50µL (n = 10) were weighed, and found to have an average density of ~1.00g/L. Deuterated isoprostanes (5µL of 1 ng/µL stock) were spiked directly into the mucus. Sample volume was adjusted to 0.5mL with methanol, acidified to pH 3 with 0.1M HCl, and vortexed. Solid mucus components were removed by centrifuging for 5min at 14,800rpm. The supernatant was then transferred to 2mL plastic microcentrifuge tubes, with two rinses of methanol. Samples were centrifuged again to assure full exclusion of particulates and precipitates, and transferred to fresh 2mL vials to be blown down to 100µL under a nitrogen stream, using an N-EVAP 111 (Organomation, Berlin, MA).

2.2.4 Separation and quantitation of isoprostanes by triple quadrupole tandem MS

All sample analyses were performed using a Varian 1100HPLC system (Palo Alto, CA) equipped with a CTC PAL autosampler. A Phenomenex (Torrance, CA) Zorbax Eclipse XDB-C18 column (2.1mm x 50mm x 3.5µm particle size) was used and maintained at room temperature. The mobile phase consisted of methanol (A) and water (B), both containing 0.1% formic acid (v/v). Flow rate was 300µL/min. A solvent gradient was used to resolve the F_2-isoP isomers: 30% B for 1min, increased to 60% B by 7min, 80% B at 15min, to 100% B at 15.5min, held for one minute, then back to initial conditions by 17min. Injection volume was 5µL. The HPLC was coupled to a Sciex API 365 triple quadrupole mass spectrometer (QqQ-MS) equipped with an ESI source. The source was a custom designed hot source induced desolvation (HSID) source with orthogonal injection. The instrument was operated in negative ionization mode, with the source temperature set to 500°C. Spray voltage was maintained at -3500V. Samples were analyzed using multiple reaction monitoring (MRM) mode. Each compound was monitored using two MRM transitions; one for quantitation and one for confirmation. The eight chemical standards (four native F_2-isoP and four deuterated F_2-isoP) were optimized individually. Declustering potential (DP), focusing potential (FP), collision energy (CE), and cell exit potential (CXP) were optimized manually for each compound. Entrance potential was -10V. The quantitative transition for 8-isoP and its enantiomer ent-8-iso-15(S)-PG was *m/z* 353/193. The *m/z* 357/197 and *m/z* 362/300 transitions were monitored for their respective internal standards. The *m/z* 353/115 and *m/z* 364/115 transitions were monitored for (±) 5-isoPG-VI and its deuterated internal standard. Confirmation transitions can be found in Table S3.

2.2.5 Confirmation of isoprostanes by high resolution time of flight MS

An Agilent 1200 LC system, consisting of a degasser, binary high-pressure gradient pump, and autosampler coupled to an Agilent 6520A Q-ToF-MS system (Agilent Technologies, Mississauga, ON, Canada), was used to confirm the identity of isoprostanes in fish mucus extracts. LC separation was carried out on a Luna C18(2) column (2.0 mm × 50 mm, 3 μm particle size) (Phenomenex, Torrance, CA, USA). The LC mobile phases were water (A) and methanol (B), and both contained 0.1 % of formic acid. The mobile phase flow rate was 0.3 mL/min and the gradient was as follows: 0 min, 5% B; 0-5 min, 30 % B; 1-7 min, 60 % B; 7-15 min 80 % B; 15-15.5 min 100 % B; post run, 10 min. A volume of 20 μL of isoprostane standards (50 ng/mL) or fish mucus extract was injected into the LC system, respectively. The Q-ToF was tuned and calibrated with tuning calibration solution (G1969-85000) provided by Agilent Technologies. The resolution of the MS was >20 000 at both *m/z* 301.9981 and 601.9790 when the ESI interface was operated in the negative mode and the capillary voltage was 3500 V. The fragmentor and skimmer voltages were 180 and 80 V, respectively. Nitrogen was used as the drying and nebulizing gas. The gas temperature was 350 °C, dry gas 10 L/min, and nebulizer 40 psi. Full-scan data acquisition was performed by scanning from *m/z* 50 to 1700. For each run, the reagent containing TFA anion (m/z 112.9855) and HP-0921 formate adduct (m/z 966.0007) were consistently introduced into the Q-ToF-MS as reference masses. Potential F_2-isoP isomers in fish mucus extract were confirmed by monitoring its theoretical $[M-H]^-$ ion (*m/z* 353.2333); *m/z* values within 5 ppm of its theoretical value were considered as a positive identification.

2.3 Results and Discussion

2.3.1 Method Development

The LC-MS/MS method parameters were validated according to the Eurachem guidelines for analytical methods [42]. Matrix effect, recoveries, limits of detection, linearity, and repeatability were assessed and found to comply within the accepted ranges (Note: as previously mentioned, the concentrations of of 5-isoPG-VI, 5-*epi*-isoPG-VI and their d-analogs are halved due to being racemic mixtures). Only Northern pike mucus was used for method development.

2.3.1.1 Linearity

The linear dynamic range was determined by injecting standards prepared in methanol from stock mixture solutions containing four F_2-isoP and their d-analogs, at concentrations of 5, 10, 25, 50, 100, 500, and 1000pg/µL. The concentrations of 5-isoPG-VI, 5-*epi*-isoPG-VI, and their deuterated internal standards are halved (2.5, 5, 12.5, 25, 50, 250, 500 pg/µL), since they are 50:50 in their racemic standard solution. All compounds tested showed linearity within this concentration range ($R^2 > 0.99$). A matrix matched approach was additionally carried for the deuterated F_2-isoPs, using extracted pike mucus samples. Linearity was observed over this range of concentrations ($R^2 > 0.999$). Matrix matched external standard (MMES) linearity was not attempted for non-deuterated native F_2-isoPs due to interfering endogenous F_2-isoP.

2.3.1.2 Matrix effect

Interference from matrix components is common in HPLC-ESI-MS analysis, and may result in addition or suppression of the signal. Matrix effect was therefore assessed to determine the impacts on this F_2-isoP protocol. Matrix matched standards were prepared by spiking 8-isoP-d_4, ent-iso-15(S)-isoPG-d_9, and (±) 5-isoPG-VI-d_{11} at concentrations of 5, 10, 25, 50, and 100 pg/µL into final mucus extracts. Integrated areas were then compared to those of d-F_2-isoP

standards in methanol to determine the matrix effect, by taking the ratio of MMES and solvent calibration curves. Both MMES and standards in solvent were run in triplicates at each concentration.

As shown in Table 2.1, matrix interference was relatively small using 50µL mucus, ranging from 1-7 %. R^2 values were all > 0.993. Matrix effects were consistent for each of the four isomers.

Table 2.1 Matrix effect calibration lines of four deuterated F2-isoprostanes in Northern pike mucus.[a]

Analyte	Curve equation Matrix matched	Curve equation Solvent matrix	% difference in matrix
8-isoPG-d_4	$y = 986.8.5x + 1735$	$y = 1012.4x - 166$	97 ± 2
5-isoPG-VI-d_{11}	$y = 1024.2x + 405.8$	$y = 986.4x - 381$	104 ± 3
5-*epi*-isoPG-VI-d_{11}	$y = 1481.8x + 1840$	$y = 1595x - 1453$	93 ± 5
ent-iso-15(S)-isoPG-d_9	$y = 713.3x + 399.4$	$y = 723.7x - 1129$	99 ± 4

[a] Matrix effect is determined by dividing the slopes of a matrix-matched standard calibration curve and a standard curve from analyte prepared in a pure solvent, methanol.

2.3.1.4 Recoveries

Acceptable extraction efficiency from the mucus matrix was achieved for all four isoprostane isomers. Determination of analyte recoveries was conducted using matrix matched standards, to account for any possible signal interference from the matrix constituents. Peak areas of mucus samples (n = 6) spiked with d-isoPs at three different concentrations (5, 25 and 100pg/µL) were compared to matrix matched standards of equivalent concentrations.

Recoveries of the four isoprostane isomers ranged between 78-95 %, with RSD of ≤ 15% at all three concentration levels. Student t-tests were used to determine if statistically significant

differences in analyte recovery existed between concentrations: recoveries did not differ significantly for any of the four isomers. Recovery of Type III isomers was slightly higher than Type VI isomers. Detailed results can be found in Table 2.2.

Table 2.2 Internal standard recoveries at varying IS concentrations in Northern pike mucus.

Sample	Spiked	Measured (mean + SD)[a]	Recovery	RSD
	pg/μL		*%*	*%*
8-isoPG-d_4	5	4.5 ± 0.7	90.5	14.7
	25	21.6 ± 1.6	86.3	6.3
	100	89.6 ± 11.7	89.6	13.1
5-isoPG-d_{11}	2.5	2.1 ± 0.2	82.2	8.2
	12.5	10.2± 0.9	81.7	7.6
	50	39.0 ± 1.8	78.1	4.6
5-*epi*-isoPG-d_{11}	2.5	2.1 ± 0.4	84.7	9.6
	12.5	10.1 ± 0.8	81.1	6.6
	50	41.7 ± 3.3	83.3	7.8
ent-iso-15(S)-isoPG-d_9	5	4.6 ± 0.2	92.1	5.1
	25	22.3 ± 2.2	89.0	8.8
	100	95.4 ± 10.4	95.4	10.3

[a] mean and standard deviation (n = 6)

RSD: Relative standard deviation, in %

2.3.1.5 Limits of detection

Low limits of detection were achieved for all four isoprostane isomers, ranging between 0.44-2.0 ng/g. Water blanks (n = 10) were spiked with 0.5 ng of each F_2-isoP and extracted according to the protocol, giving a final concentration of 5pg/μL in the injected sample. These samples were prepared in distilled water because endogenous isoprostanes observed in the mucus

samples would interfere with the signal. The internal standards were not used, as they showed lower responses than the native isoprostanes. Better detectability of the native isoPs, paired with the small matrix effect, justified the use of water as a sample matrix. Limit of detection (LOD) calculations were based on the Eurachem specifications ($LOD = 3\sigma * \sqrt{\frac{1}{n} + \frac{1}{n_{blank}}}$, where n is the number of replicates), and converted to concentration in ng/g [42]. Similarly, limits of quantitation (LOQs) were determined using a 10-fold multiplication in the above equation. These results are tabulated below.

Table 2.3 Limits of detection and quantitation of four F_2-isoprostane isomers

Analyte	LOD (ng/g)	LOQ (ng/g)
8-isoPG	1.4	4.7
5-isoPG-VI	0.44	1.5
5-*epi*-isoPG-VI	0.63	2.1
ent-iso-15(S)-isoPG	2.0	6.5

LOD: Limit of detection

LOQ: Limit of quantitation

2.3.1.6 Repeatability

Northern pike mucus samples showed acceptable variability in isoprostane signal (RSD < 20 %) for same-day and inter-day batch precision analysis. Method precision was measured through repeatability measurements. Same-day and inter-day repeatability were measured using Northern pike (n = 6). Pike samples were analyzed with IS concentrations of 5, 25 and 100pg/µL. Day-to-day repeatability tests were carried out for 3 days.

The peak area ratios of quantitation: confirmation ion transitions were also monitored during batch analyses. Fragmentation patterns of stable molecules should remain constant during analyses if conditions are not changed. The average Q/C ratios of all four F_2-isoP isomers remained constant (< 10%) throughout the entirety of the method validation process, indicating the analyte underwent no degradation over the analysis period (data not shown).

2.3.2 Quantitation of F_{2-} isoprostanes in fish samples

2.3.2.1 Northern Pike

All four selected F_2-isoP isomers were observable and resolved chromatographically or by mass in Northern pike samples (see Figure S4). Isoprostane peak identities were confirmed with relative retention times of deuterated internal standards. Concentrations ranged from 5.3-28.8 ng/g – detailed results can be found in Table 2.4 below.

In addition, several other isoprostane peaks were fully or partially resolved, eluting up to 2min later than our compounds of interest. These were confirmed to be isoprostanes by high resolution LC-qTOF-MS to 2.5ppm accuracy (Table S1). During the F_2-isoP formation process, the multiple unsaturated sites of arachidonic acid result in 4 possible classes of regioisomers: Type III, IV, V, and VI, categorized by the location of the backbone hydroxyl group. Class-specific enantiomers bring up the total isomer count to 64. Resolving regioisomers *and* enantiomers is therefore essential since the accurate quantitation of selected isomers is dependent on freedom from interference from other stereoisomers with common quantitative m/z transitions. This study focused on two regioisomer classes: Type III and Type VI (shown in Figure S1). The presence of other unidentified isoprostane isomers could make this method more broadly applicable as additional commercial standards become available for other F_2-isoP isomers. 8-isoprostaglandin-

$F_{2\alpha}$, a Type III isomer, is the most researched of these compounds, and has been linked to several human diseases such as atherosclerosis [21]. However, other isomers – also lipid peroxidation endpoints – have the same functions and are more abundant [43]. A more comprehensive and accurate analysis of lipid peroxidation would be achieved by identifying and quantifying more isoprostanes.

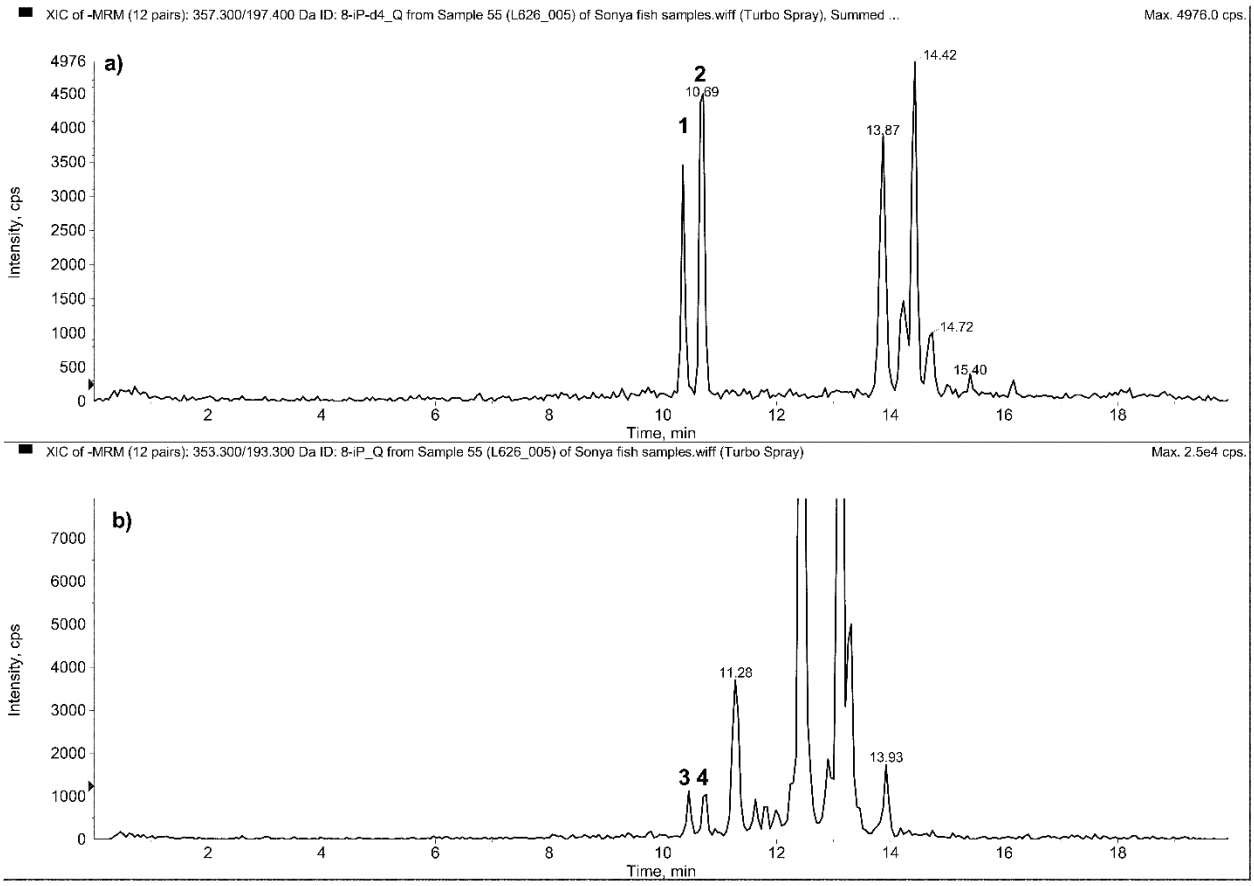

Figure 2.2 HPLC-MS/MS elution of **a)** deuterated standards *ent*-8-iso-15(S)- prostanglandin-$F_{2\alpha}$-d_9 (**Peak 1**) and 8-iso-prostanglandin-$F_{2\alpha}$-d_4 (**Peak 2**) and **b)** endogenous *ent*-8-iso-15(S)-prostanglandin-$F_{2\alpha}$ (**Peak 3**) and 8-iso-prostanglandin-$F_{2\alpha}$ (**Peak 4**) in a lake trout mucus sample. Other isoprostane peaks in the mucus are present but have yet to be positively identified.

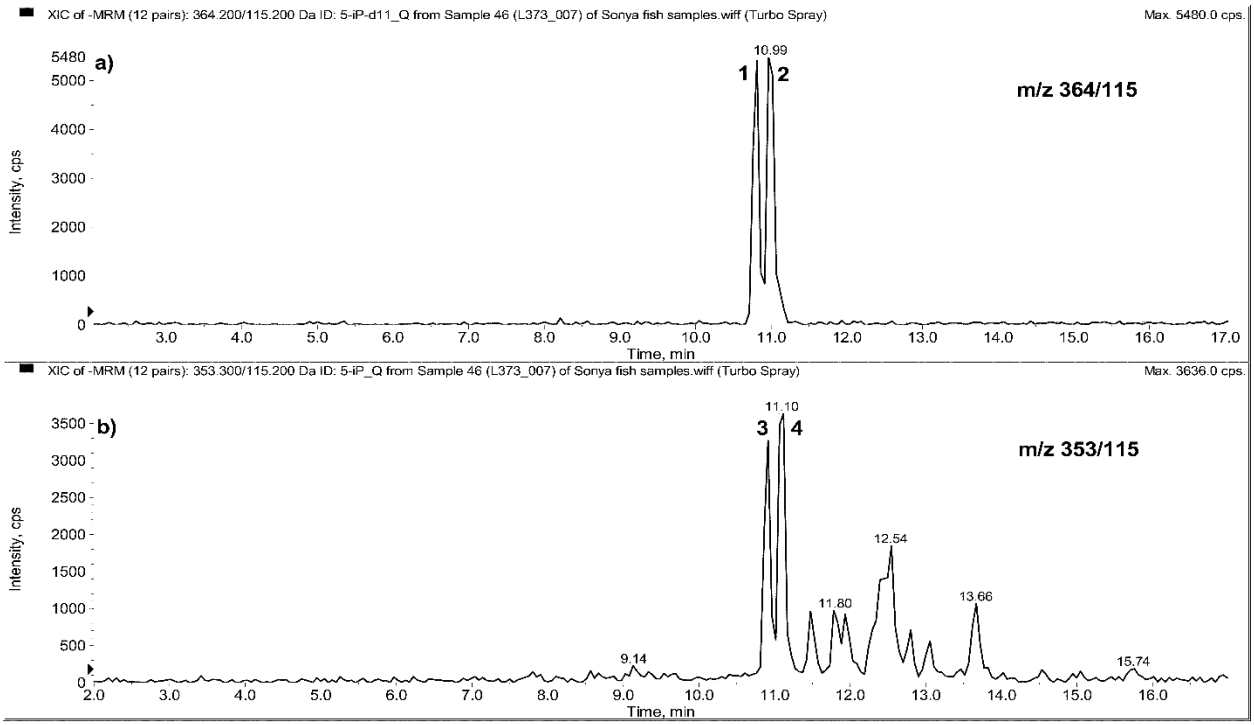

Figure 2.3. HPLC-MS/MS elution of **a)** endogenous 5-isoprostanglandin-$F_{2\alpha}$-VI-d_{11} (**Peak 1**) and 5-*epi*-isoprostanglandin-$F_{2\alpha}$-VI-d_{11} (**Peak 2**) and **b)** 5-iso-prostaglandin-$F_{2\alpha}$-VI (**Peak 3**) and 5-*epi*-iso-prostaglandin-$F_{2\alpha}$-VI in a lake trout mucus sample. Other isoprostane peaks in the mucus have not yet been posively identified.

2.3.2.2 Lake Trout

F_2-isoprostanes were detected in individual fish in four of the five lakes, the exception being Lake 223: an example chromatogram is shown in Figure 2.2. Measureable Type III isoprostane peaks were present in only one out of seven individuals in lake 224, and were detected but below the LOQ in one individual from Lake 260. In contrast, three and four individuals showed measurable concentrations in lakes 373 and 626 respectively with concentrations ranging from 6.3 to 132 ng/g (see Table 2.4). The variability was large: the concentrations of Type III isoprostanes in lake trout individuals from L373 with measurable levels ranged from 7.5-132ng/g. Similarly, in 4 individuals from L626, concentrations ranged from 6.3-70.7 ng/g, with an RSD of 91%. Parsons

et al (2009) demonstrated in rats and fathead minnows that natural variability is usually greater in biofluids such as mucus (~ 50%) than in tissues (~30%) [44]. The variability of metabolite concentrations in fathead minnow mucus was also shown to fall within the range of this natural variability typical of biofluids by Ekman et al (2015) [37]. The 91% variation observed in our results may indicate that the collection method requires modification: variable amounts of water or small particles in the mucus samples potentially skew the wet weight of the samples. This could be remedied by using dry weight, though the volume of mucus needed for this may prove impractical. Another solution could be to normalize isoprostane concentrations relative to protein and lipid content of the mucus, which may prove more accurate than wet sample mass. Such normalization is common for other biomarker assays of exposure such as for ethoxyresorufin-O-deethylase (EROD) and TBARS [8,12]. Protein is more common, but arachidonic acid or total lipid could alternatively be used. The applicability of these methods have yet to be verified for mucus.

Despite this, a qualitative comparison of isoprostanes can still be made on these preliminary results. A possible explanation for the higher isoprostane concentrations in trout from lakes 373 and 626 is the sampling dates, which coincide with the beginning of fall spawning season for lake trout [45]. Lake trout fall spawning typically begins when water temperature decreases closer to the species' optimal temperature range of 8-12°C. Spawning is a stressful, energetically demanding process [46], which could explain the heightened isoprostane concentrations observed in the samples from these lakes. Mean water temperatures (1m depth) for the five experimental lakes (Figure S5, Table S2) measured on the trout sampling days were within this range for the L373 sampling event (12.0°C), and just above for L626 sampling at 13.2°C. In comparison, the other three lakes range from 14.9-15.5°C – a significant difference of at least ~2°C. It is therefore

possible that higher F_2-isoprostane concentrations could be measured in the L223, L224, and L260 lake trout by sampling during spawning. However, further study is required to support this hypothesis, especially given the large variability observed in the data.

The two Type VI isomers measured in this study, 5- and 5-*epi*-isoprostanglandin-VI, were measurable only in one fish from L373 (see Table 2.4), though they were also present below LOQ in other individuals. Concentrations of both these compounds in the lake trout were within 2ng/g (or < 30%) of those measured in the pooled Northern pike samples. Though encouraging, this result must be treated with caution as conclusions cannot be drawn before several more samples are analyzed to properly compare F_2-isoP levels between the two species.

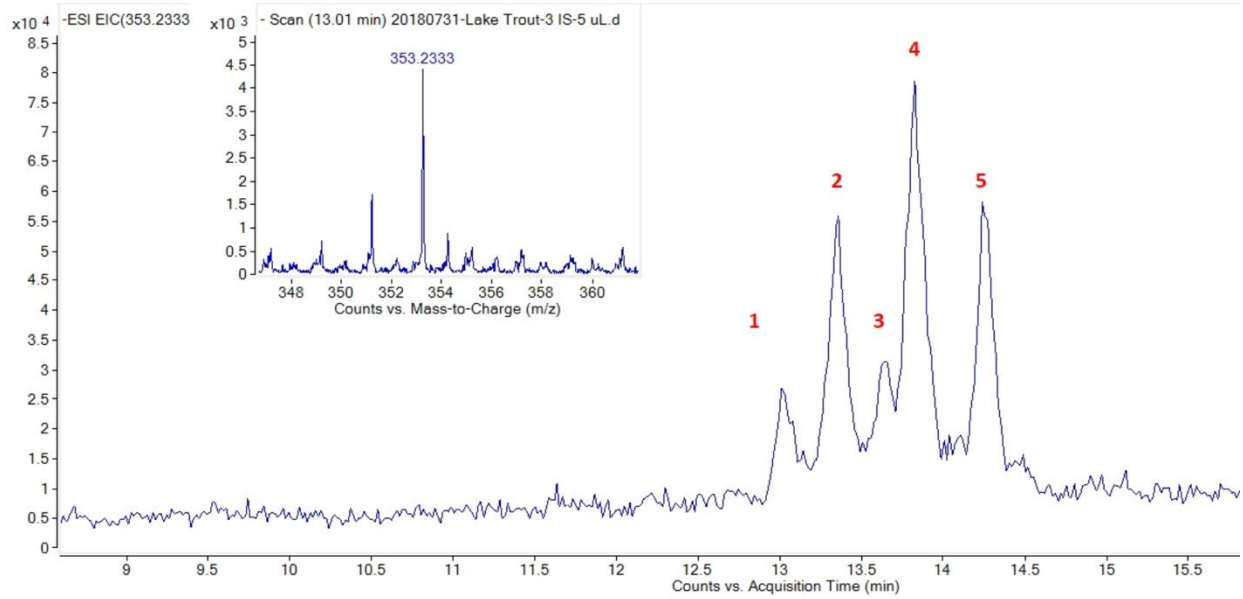

Figure 2.4. High-resolution LC-qTOF-MS mass chromatogram and mass spectrum at 13.01 min (inset) of endogenous F_2-isoprostane isomers in lake trout. The detailed information for all the peaks are provided in Table S1.

Several chromatographic peaks with the *m/z* 353 → 115 transition were in evidence in most of the sampled individuals listed above. These peaks eluted up to 2.5min later than the four isomers

selected for this study (see Figure 2.3). These peaks cannot be identified with certainty because commercial standards for most isoprostane isomers are unavailable, however their high resolution molecular weights correspond to those in the samples who were previously identified as isoprostane isomers by HR-LC-MS/MS (Figure 2.4, Table S1). Identifying and measuring these compounds is an important consideration for future studies.

Table 2.4 Endogenous F_2-isoprostanes measured in Northern pike and lake trout mucus[a]

Species/Lake	Concentration (ng/g)			
	8-isoPG	*ent*-8-iso-15(S)-PG	5-isoPF-VI	5-*epi*-isoPF-VI
Northern Pike [b]	<LOQ	28.8 ± 2.5	5.3 ± 0.8	9.9 ± 1.3
LT L224	<LOQ	11.3	ND	ND
LT L260	<LOQ	<LOQ	ND	ND
LT L373	11.0	7.5; 10.5; 132	7.2	8.0
LT L626	6.3	6.7; 24.0; 40.1; 70.7	< LOQ	<LOQ

[a] Concentrations for all individuals with detectable levels of F_2-isoPs, separated by " ; "

[b] Pike mucus was pooled, subsamples averaged (n = 12)

LT: Lake trout

ND: Not detected

<LOQ: Detected, but below the limit of quantitation listed in Table 2.3.

2.4 Conclusion

In this study, four F_2-isoprostane isomers were successfully separated and quantified in fish mucus of wild individuals from two species. Repeated F_2-isoP measurements in individuals of a population over time could eventually prove to be an effective method for monitoring fish population health during and after an exposure to contaminants, as *in vivo* concentrations have been shown to relate with oxidative stress [21]. Determining baseline levels of F_2-isoP in fish populations is therefore a crucial first step in the implementation of this method, as baseline concentrations of F_2-isoP likely vary between fish species and water bodies. Effects of water chemistry and temperature, temporal effects on physiology, and sexual dimorphism are all factors that require further study to better understand natural variability of F_2-isoPs in fish mucus.

Several further studies are required to validate this method before it is deemed applicable for measuring oxidative stress in wild fish. Though it demonstrated that the method can be extended to species other than Northern pike, preliminary results showed large variability in F_2-Isop concentrations of individual lake trout, both within and between lakes. Concentrations need to be validated against more typical sampling matrices, such as plasma, under controlled laboratory conditions to establish if there is a reliable, reproducible relation between F_2-isoP levels in these media. This will also provide a better understanding of natural variability. Additionally, the variability raises questions as to if normalizing F_2-isoP concentrations to total lipid and protein content in each sample would provide a more accurate measure of lipid peroxidation. Another important consideration would be to determine if F_2-IsoP concentrations differ when mucus is collected from different parts of the fish. Furthermore, determining a dose-response relationship to chemical stressors and comparing response to other established biomarkers such as ratios of oxidized to reduced glutathione and malondialdehyde will determine the usefulness of this method

as an ecological monitoring method. A study is currently underway to address these questions. Clearly, the validated method reported here demonstrates that quantifiable amounts of these robust oxidative stress biomarkers can be measured in very small mucus samples collected from fish in pristine environments and is a necessary prerequisite for future studies assessing the ecological significance of this new approach.

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Chapter 3: F_2-Isoprostane and glutathione response in rainbow trout (*Onchorynchus mykiss*) tissues after exposure to acute physical stress.

3.1 Introduction

Oxidative stress has been implicated in the mechanism of toxicity of several pollutant classes to which wild fish populations are exposed via anthropogenic activities: notable examples include the polycyclic aromatic compounds (PAC), metals including mercury and copper (review by Lushchak, 2011), and pesticides[1–3]. These compounds induce the formation of large amounts of reactive oxygen species (ROS) such as free radicals and peroxides. When produced in excess, ROS can overwhelm an organism's natural antioxidant capacity, resulting in damage to DNA bases, lipids, and proteins. In response, chemical monitoring programs have implemented the use of assays measuring biochemical indicators of oxidative damage to provide early warning of toxicity in wild fish populations. To date, the most commonly measured biomarkers of oxidative stress are the antioxidant enzymes catalase (CAT), superoxide dismutase (SOD), G-related enzymes [glutathione reductase (GR), glutathione-S-transferase (GST), glutathione peroxidase (GPx)], peroxidation endpoints such as TBARS and protein carbonyls, and small molecule antioxidants (vitamin E and glutathione) [1,4–6]. However, complex environmental factors can confound the interpretation of these assay results in wild fish, leading to a disparity between biological responses observed in different studies.

F_2-isoprostanes are a class of prostaglandin-like compounds derived from the non-enzymatic peroxidation of arachidonic acid by reactive oxygen species (ROS). Since their discovery by Morrow et al in 1990, F_2-isoPs have become the gold standard for measuring oxidative stress *in vivo* in mammalian models [7]. Though many isomeric variations are possible due to the multiple unsaturated sites of arachidonic acid, 8-isoprostaglandin-$F_{2\alpha}$ (8-isoP) is by far

the most studied F_2-IsoP isomer. Increased 8-isoP concentrations have been linked with several human diseases such as atherosclerosis and Alzheimer's disease [8–10]. Beyond being detectable at low concentrations in all biological fluids tested to date such as plasma [8,11], cerebrospinal fluid [12], and urine [13], they have the advantage of being produced specifically in response to ROS attack. Their production is not impacted by dietary lipid intake nor by variables affecting translational expression since it is mechanistically independent of cyclooxygenase enzymes [14]. Furthermore, high-performance liquid chromatography – tandem mass spectrometry (HPLC-MS/MS) of these compounds is highly selective compared to other traditional indices of lipid peroxidation such as TBARS and protein carbonyls, whose assays suffer from cross-reactivity which can lead to significant overestimation of concentrations [15]. Though F_2-isoPs have been successfully implemented in animal models of oxidative stress, their potential is of yet unfulfilled in assessing OS in fish models.

Due to their presence in biological fluids, F_2-isoPs have potential to be measured using minimally invasive sampling techniques. Currently, many of the assays listed above require lethal harvesting of tissues such as blood and liver. Epidermal mucus has more recently been studied as a non-invasive alternative [16]. In a previous study, we successfully developed a protocol for the extraction and HPLC-MS/MS quantification of F_2-isoPs in non-invasively collected epidermal mucus that was tested on freshwater fish species, Northern pike (*Esox lucius*) and lake trout (*Salvelinus namaycush*) [17]. However, we recognized the need to determine if relationships existed between F_2-isoP concentrations in mucus and other visceral tissues, and to compare levels to those of other biomarkers. The objectives of this study were to determine if stable baseline levels of F_2-isoprostane would be observed in the epidermal mucus of rainbow trout from a laboratory culture (*Oncorhynchus mykiss*) as well as traditionally sampled tissues (plasma, liver, and gill),

and to determine if concentrations were detectably affected by physical stressors. To more comprehensively assess any resulting oxidative stress, TBARS, SOD, and GSH were also measured in collected tissues in addition to F_2-isoPs.

3.2 Experimental

3.2.1 Materials and reagents

Hydrochloric acid (HCl), water (HPLC grade), metaphosphoric acid, phosphate buffered solution (PBS), butylated hydroxytoluene (BHT), chloroform, ethyl acetate, heparin sodium, and KOH were purchased from Fisher Scientific (Fair Lawn, NJ). (±) 5-iso-prostaglandin-$F_{2\alpha}$-VI, (±) 5-iso-prostaglandin-$F_{2\alpha}$-VI-d_{11} (± indicates a racemic mixture of two enantiomers), 8,12-isoPF-VI, 8,12-isoPF-VI-d_{11}, 8-iso-prostaglandin-$F_{2\alpha}$, and 8-iso-prostaglandin-$F_{2\alpha}$-d_4, ent-iso-15(S)-prostaglandin-$F_{2\alpha}$ and *ent*-8-iso-15(S)-prostaglandin-$F_{2\alpha}$-d_9 (all >98%) were procured from Cayman Chemicals (Ann Arbor, MI). The chemical structures can be found in Figure S1. These compounds will be referred to herein as 5-isoP, 8,12-isoP, 8-isoP, and *ent*-15-isoP

3.2.2 Experimental Design

Rainbow trout (n = 40) aged 18 months with a mean weight of 157 ± 32 grams were sourced from the Toxicology Centre, University of Saskatchewan cultured stock line. Fish were acclimated for 10 days prior to the experiment in large 800L rectangular tanks (Frigid Units Inc, model MT 700) with a continuous flow system (10 fish/tank, water temperature 13°C, photoperiod 8h:16h light/darkness, water flow 7 L/min). Fish were fed 2% of body weight daily at 10 am with a commercial salmon diet (CoRey Aquafeeds) until the day before the experiment.

Fish were divided into four experimental groups, according to the presence (or lack of) physical stress and time of sampling post-stress: 4 hours (control), 48 hours (control), 4 hours

(stressed), 48h stressed). Two fish from each tank (n = 8 total) were sampled immediately prior to the start of the experiment for baseline measurements. The remaining fish from two tanks (n = 8 each) were stressed by vigorously pursuing the fish with nets, catching and releasing them, and briefly lifting them out of the water, during a 5 minute period. To ensure that all trout were being continuously stressed during this period, a plastic divider was inserted to the tanks to corral them into a much smaller area, or approximately one quarter of total tank volume.

3.3.2 Sampling

At sampling time, fish were netted and anaesthetized in a buffered MS-222 bath until opercular movement had almost ceased. Prior to euthanasia, epidermal mucus was sampled from each side of the fish using sterile FloqSwabs (COPAN Diagnostics, Murrietta CA) until saturated and placed in pre-weighed 15ml Falcon tubes (the total mass of the dry swab and tube were weighed). Weight and length (total and fork) were then recorded for the determination of morphometric indices. Finally, blood was drawn from the caudal vein using 22-gauge heparinized syringes, after which fish were euthanized by cervical dislocation before further tissue sampling. Scales were collected by scraping against the scales with the blunt edge of a scalpel. Livers were perfused with Tris buffer (pH 7.4), blotted dry and weighed to determine the hepatosomatic index. The first gill arches from each side of the fish were excised. Liver and gill were snap frozen in liquid nitrogen. Plasma was obtained from whole blood by centrifuging for 10 minutes at 1500 rpm. All tissues were stored at -80°C pending chemical analysis.

3.2.4 Condition factor

To assess overall health of the fish, condition factors (CF) of each fish were calculated as follows:

$$CF = \frac{mass_{\,fish}}{TL^3}$$

Where TL is the total length of the fish in centimeters.

3.2.5 Free F_2-isoprostanes in mucus

Mucus samples were thawed and kept on ice until fully extracted. BHT was added (final concentration 0.05% v/v) to each sample to prevent auto-oxidation of the F_2-isoPs during sample preparation process. Samples were then spiked with an internal standard mixture of five deuterated F_2-isoPs (1ng), and vortexed 3 x 10 seconds to thoroughly mix. Swabs were then extracted using 0.1M PBS buffer (pH 7.4): samples were first vortexed for 2x1min in 500µL buffer, then rinsed with an additional 500µl and blotted dry on the walls of the Falcon tube before being discarded. Samples were then centrifuged at 10,000 x g for 20min at 4°C, after which 100µl supernatant was transferred to a 1.5mL centrifuge tube for protein content determination. The remaining supernatant was transferred to a clean 12ml glass test tube, then acidified to pH 3 using 0.1M HCl before being extracted using 2 x 1.5ml ethyl acetate. The organic layer was evaporated under a nitrogen stream and reconstituted to a final volume of 250µl mobile phase (30:70 methanol/water, pH 3).

3.2.6 Total F_2-isoprostanes in plasma

Plasma samples (200-800µl) were spiked with BHT (final concentration 0.05% v/v), after which base hydrolysis was carried out using 500µL 1M KOH. Samples were incubated at 45°C for 45min, then acidified to pH 3 using HCl and spiked with the F_2-isoP internal standard mixture. Solid phase extraction was then performed with Oasis HLB cartridges (60mg) pre-conditioned with 0.5ml MeOH and 0.5ml H_2O (pH 3). The cartridge was washed with 0.5mL H_2O (pH 3) and 0.5mL hexanes, then F_2-isoPs were eluted from the stationary phase using 1.5mL ethyl acetate.

The organic phase was then blown to dryness and reconstituted for HPLC-MS/MS in 250µl mobile phase.

3.2.7 Total F_2-isoprostanes in liver and gill tissue

Samples were thawed and homogenized. Homogenate (~0.25g liver, 40-100mg gill) was transferred to 30ml glass centrifuge tubes. The lipid fraction was extracted according to the Bligh-Dyer method. Briefly, a 2:1 chloroform : methanol mixture was added to each sample, followed by vortex mixing for 1 minute. Pure chloroform was then added to reach a chloroform: methanol: water ratio of 2:2:0.8, followed by centrifugation for 10 minutes at 800 x g at 4°C. The organic layer was transferred to a clean vial, blown down to dryness under a nitrogen stream and reconstituted in 2.0mL 1M NaOH. Hydrolysis and acidification were performed as with plasma. Extracts were diluted to 20mL with 1mM HCl prior to spiking with the internal standard mixture. Extraction was performed with 20cc cartridges: cartridge conditioning and washing was performed as above, but with volumes of 5mL. F_2-IsoPs were eluted with 8mL ethyl acetate and reconstituted in 250µl mobile phase.

3.2.8 GSH determination in liver and gill tissue

Reduced glutathione (GSH) and oxidized glutathione (GSSG) were determined as per the directions of the assay kit from Cayman Chemicals. Briefly, tissue was homogenized in cold MES (2-ethanesulfonic acid) buffer using a Precellys 24 homogenizer for 2 x 15s with a 30 second cooldown period. Samples were centrifuged at 10,000 x g for 15 minutes at 4°C, after which the supernatant was removed and kept on ice. A 50µl aliquot was removed at this time for protein content determination. The supernatant was deproteinated by mixing thoroughly with 10% metaphosphoric acid, followed by centrifugation for 2 minutes at 1500 x g. Samples were stored at -20°C until assayed.

Triethanolamine (TEAM, 1M) was added at a ratio of 200µl TEAM/ml supernatant to increase the pH of the samples. Liver and gill samples were diluted 20-fold and 5-fold respectively with MES buffer. Analysis of total GSH (tGSH) was performed on a 96-well microplate: DTNB was added to samples and absorbance at 412nm was measured at 5 minute intervals for 25 minutes, using a Biotek Synergy H1 microplate reader (Agilent Technologies, Santa Clara, CA). GSSG analyses were performed identically, with an additional step of derivatizing GSH using 2-vinylpyridine. Samples were then left to incubate for 1 hour prior to the addition of DTNB. In addition to tGSH and GSSG concentrations, the GSSG:tGSH ratio, a commonly used index of oxidative stress was then calculated as follows:

$$GSSG{:}\,GSH = \frac{GSSG}{tGSH} * 100$$

tGSH concentrations were normalized using protein concentrations determined from the Bradford assays.

3.2.9 Protein assays: total protein content and normalization

Aliquots of each tissue extract – with the exception of plasma – were analyzed using a modified Bradford method. After any necessary dilutions, absorbance was measured at 450nm and 590nm using a Varian Cary 100 UV-vis spectrophotometer (Agilent, Santa Clara CA). The ratio of A_{590} : A_{450} was used to determine concentrations. Total protein content in grams was used to normalize concentrations of glutathione and F_2-IsoPs. Concentrations of F_2-IsoP were protein normalized to more accurately compare levels with glutathione. Concentrations discussed in this study will be in the units of ng F_2-IsoP per gram protein.

3.2.10 Plasma cortisol

Plasma cortisol was measured with a 96-well microplate reader, as per the EIA kit instructions (Oxford Biomedical). Briefly, 100µl plasma was extracted with 1ml ethyl ether by vortexing for 30 seconds. The organic phase was transferred to a clean 12ml glass test tube, blown to dryness under a nitrogen stream, and reconstituted in the provided buffer solution. Samples were then diluted 100-fold in the provided buffer and vortexed. Cortisol was quantified by measuring the change in absorbance at 650nm resulting from the binding of cortisol to horseradish peroxidase on the microplate reader.

3.2.12 HPLC-MS/MS

Sample analyses were performed using a Varian 1100 HPLC system (Palo Alto, CA) equipped with a CTC PAL autosampler. Separation was achieved with a XSelect® HSS T3 C_{18} (3µm x 2.1 mm x 5cm) column maintained at 42°C temperature with an online column heater. The mobile phase consisted of methanol (A) and water (B), both containing 0.1% formic acid (v/v). Flow rate was 300µL/min. A solvent gradient was used to resolve the F_2-IsoP isomers: 30% B for 1min, increased to 60% B by 7min, 80% B at 15min, to 100% B at 15.5min, held for one minute, then back to initial conditions by 17min. Injection volume was 50µL.

Mass spectrometric analysis was performed in negative mode using a SCIEX API 365 triple quadrupole mass spectrometer (QqQ-MS) retrofitted with an HSID EP 10+ orthogonal ionization source. Source temperature was maintained at 500°C. Spray voltage was -3500V and entrance potential was -10V. Samples were analyzed using MRM m/z transitions specifically optimized to each F_2-IsoP. Declustering potential (DP), focusing potential (FP), collision energy (CE), and cell exit potential (CXP) were also optimized manually for each compound.

3.2.13 Statistics and correlation analyses

F_2-IsoPs were compared between liver and gill tissue of each fish, and between liver and plasma. There was insufficient overlap of tissue collection to compare gill and plasma F_2-IsoPs. The GSSG:GSH ratio was also compared between gill and liver tissue, and F_2-IsoP concentrations were correlated to GSSG:GSH ratio in both tissues. Data from each treatment was first assessed for normality using the Shapiro-Wilkes test, then Pearson's correlation were calculated to measure correlations and their statistical significance. This was done to determine if relationships exist between F_2-IsoP concentrations in different tissues under stressed and unstressed conditions, and to determine how glutathione and F_2-IsoP responses might differ.

Potential differences in biochemical responses between treatment groups and time points were analysed with student t-tests. In certain tissues where non-detects were more frequent, pairwise comparisons were performed using survival analysis statistics based on rank-base methods, using the R package NADA. Experimental groups were also compared to the baseline group using Dunnett tests. All statistical tests were performed using RStudio: statistical significance was assumed to be $P < 0.05$ in all cases.

3.3 Results

3.3.1 Condition factor

Condition factors did not differ significantly between any groups.

3.3.2 Plasma cortisol

Figure 3.1 depicts the observed responses of the five experimental groups. Notably, cortisol concentrations in the baseline group collected at time zero showed a tight distribution (mean cortisol: 19 ± 9 ng/ml), with the exception of one individual (141 ng/ml) that had a visibly

deteriorated tail fin (see corresponding discussion section below). A similarly high data point with disproportionate impacts on the treatment's mean cortisol was observed in the C-48h group (151 ng/ml), and so were removed as outliers. With the exclusion of these two data points, a statistically significant difference becomes observable between fish from the C-4h group (1.8-fold increase) and the baseline fish ($p = 0.51$ to $p < 0.01$).

Additionally, cortisol was lower in the E-4h and C-48h groups relative to the C-4h group ($P < 0.05$), but the opposite trend was observed between the C-48h and E-48h groups, with levels being elevated in fish from E-48h ($P = 0.11$). It should be noted that despite observing these differences between groups, only the C-4h group was significantly elevated relative to the baseline group.

To assess intra-group variability arising from possible sampling cohort effects, mean concentrations of cohorts 1 and 2 were calculated. Overall, cortisol was elevated in cohort 2 in all groups except E-48h, with mean cortisol concentrations ranging from 10-213% greater in the second cohort. Student t-tests showed the differences were not statistically significant ($P > 0.2$, results in Table S5).

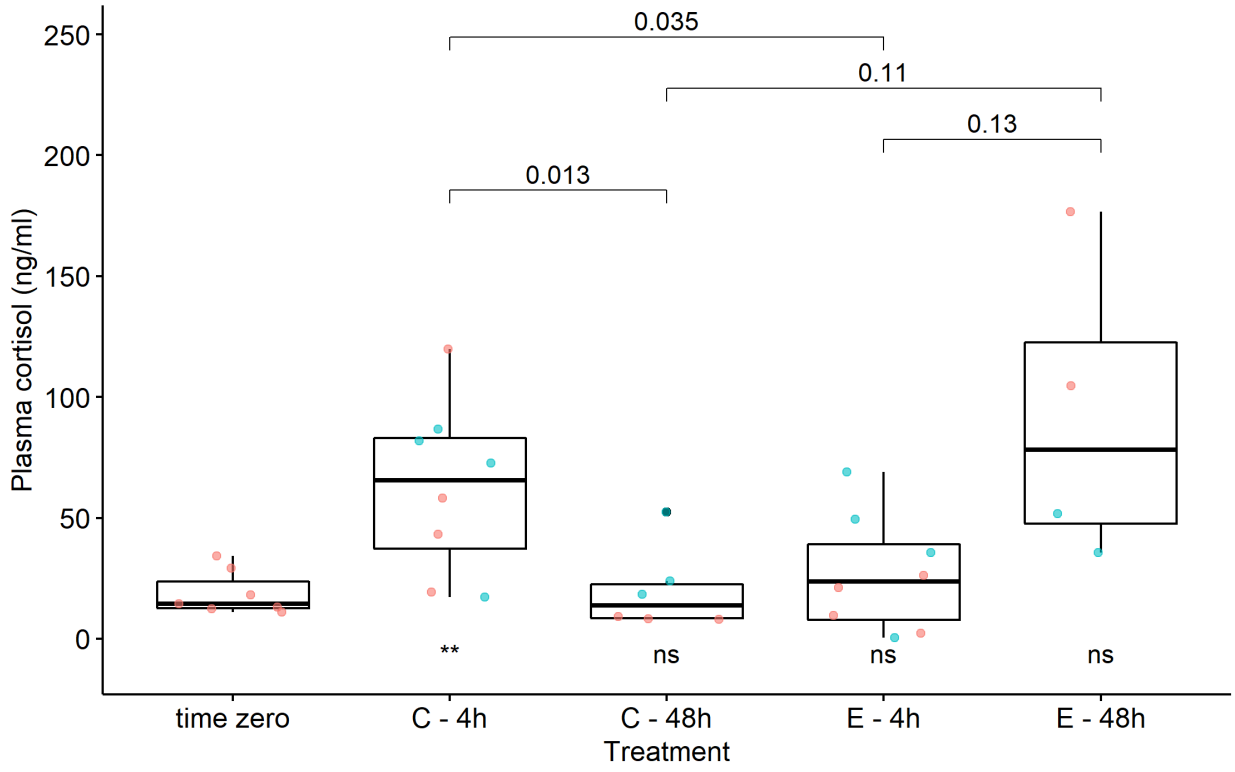

Figure 1.1 Plasma cortisol in rainbow trout. Sampling cohorts: red = cohort 1, blue = 2 (approximately 15min between sampling events). C = control, E = stressed. Pair-wise comparison significance level: * = $P < 0.05$, ** = $P < 0.01$.

3.3.3 F_2-isoprostanes in mucus

F_2-isoprostanes were below detection limits in all mucus samples. Due to an error on my part during a methods comparison experiment, an inadequate extraction methodology based on the detection of free F_2-IsoPs was used for the mucus samples collected in this experiment. Northern pike (*Esox lucius*) mucus samples were extracted for free F_2-IsoPs with liquid-liquid extraction and for total isoprostanes (SPE method similar to the methods described above for other tissues) to compare their extraction efficiency, with the intention of selecting the more accessible and time-efficient method if their efficacies were similar (i.e LLE method). Unfortunately, I performed these tests with mucus samples that had been previously thawed and refrozen, and thus subjected to auto-oxidation. Therefore, the results of this experiment unsurprisingly showed artefactual, large

and relatively equal concentrations of native F_2-IsoPs recovered by both methods: the free isoprostane method based on LLE was selected. This method proved to be inadequate to detect naturally occurring concentrations of F_2-isoprostanes in the mucus samples.

3.3.4 F_2-isoprostanes in plasma

Average concentrations were significantly different between type III and type VI isoprostanes, ranging from 11-57 pg mL^{-1} and 99-953 pg mL^{-1} respectively. 8,12-IsoP was the most abundant isomer measured. Variability within treatments was large (RSD: 36-106%), with group E-48h being the most variable in general. Mean F_2-IsoP concentrations in both control and stressed fish increased 1.2- to 1.9-fold from 4h to 48h for all isomers, however these changes were not statistically significant (see Figure 3.2). Treatment effects were not apparent at all: concentrations generally varied by less than 20%. Overall, F_2-IsoP concentrations did not change significantly and did not differ from baseline levels in any treatment group.

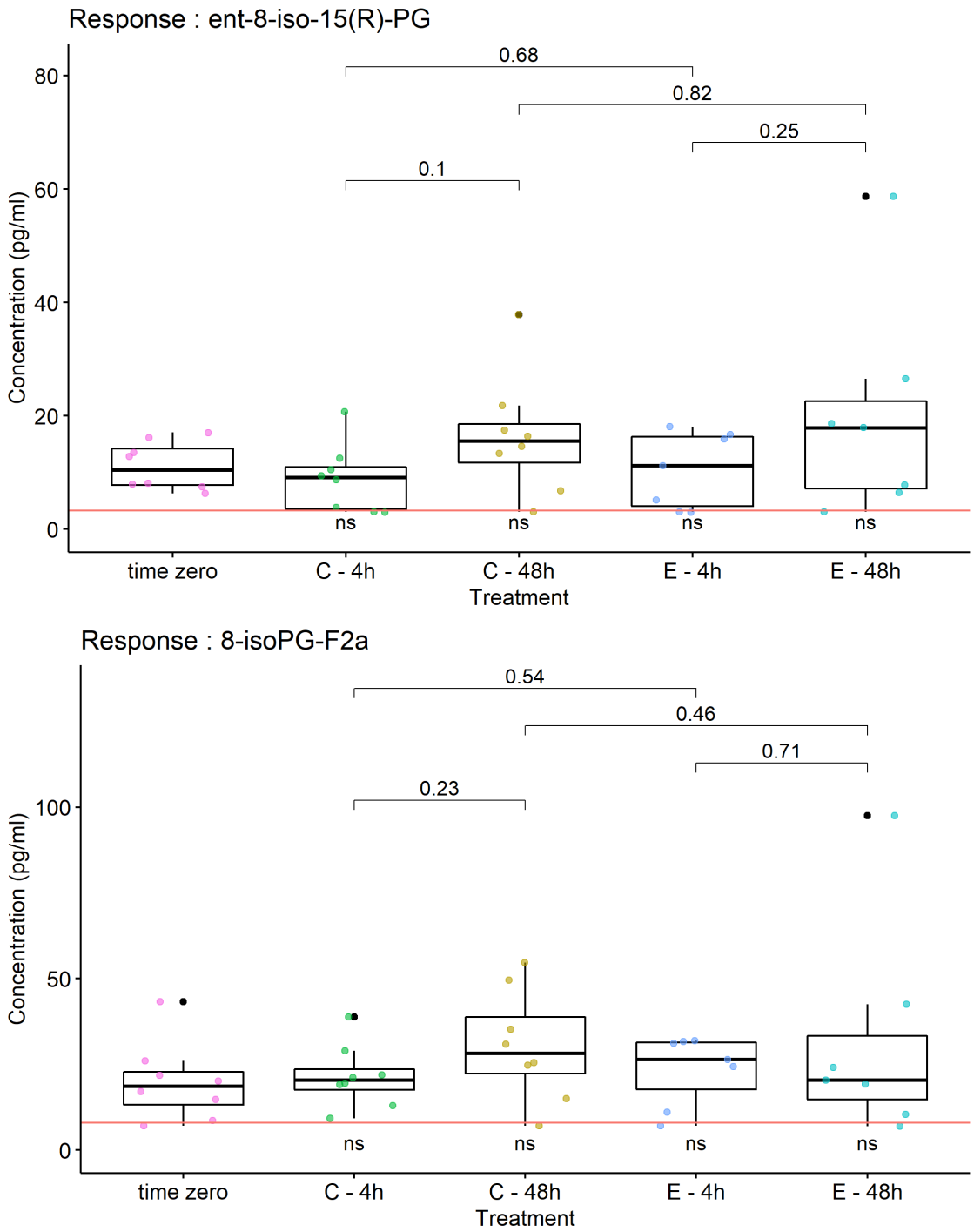

Figure 1.2 Type III F_2-isoprostane concentrations in rainbow trout plasma, with pair-wise comparisons shown. C = control, E = stressed. Pair-wise comparison significance level: * = P < 0.05, ** = P < 0.01, ns = not significant. Data points below the red line are under the detection limits

Table 1.1 Concentrations of four F_2-IsoP isomers in rainbow trout tissues.

	Type VI		Type III	
Treatment	5-isoP	8,12-isoP	8-isoP	ent-15-isoP
Liver [a]				
time zero	7.5(2.3)	11.7(3.7)	2.2(1.4)	1.6(0.9)
C - 4h	9.8(6.6)	24.4(21.8)	2.2(1.5)	1.3(0.6)
C - 48h	12.6(5.9)	22.8(13.9)	2.1(0.6)	2.2(0.7)
E - 4h	12(3.7)	19.9(11.5)	3.2(1.6)	2.7(0.9)
E - 48h	6.4(2.2)	10.5(4.2)	1.6(0.6)	1.4(0.7)
Gill [a]				
time zero	25.3(11.5)	41.4(14.4)	9.4(3.7)	4.8(2.6)
C - 4h	19.9(6.9)	45.6(13.9)	7.6(4.9)	3.3(0.8)
C - 48h	16.8(6.2)	25.9(10.5)	4.3(2.6)	3.5(0.9)
E - 4h	12(2.2)	31.2(12.2)	5.4(2.9)	3.4(0.5)
E - 48h	16.1(7)	16.7(6.1)	3.4(2.4)	3.9(1.7)
Plasma [b]				
time zero	79.3(28.8)	359.8(124.1)	20.0(11.3)	11.1(4.2)
C - 4h	82.3(26)	446.2(335.2)	21.4(9.2)	9.1(5.9)
C - 48h	154.1(95.7)	789.4(839.1)	31.3(14.8)	16.9(10)
E - 4h	105.3(42.6)	968(883.2)	25.6(11.0)	12.0(7.5)
E - 48h	205.6(175.9)	667.6(544.2)	36.9(28.8)	23.2(16.5)

[a] F_2-isoP concentration in ng/g protein (standard deviation)

[b] F_2-isoP concentration in ng/ml plasma (standard deviation)

3.3.5 F_2-isoprostanes in liver

Mean protein normalized F_2-IsoP in liver tissue are shown in Table 3.1: relative abundance of isomers was comparable to plasma, with Type VI isomer concentrations being 3-10x greater than Type III isomers.

Liver concentrations of *ent*-15-isoP were time-dependent within the control and stressed treatments, increasing by 58% in control fish from 4h to 48h (see Figure 3.3). In stressed fish, an opposite trend was observed: liver concentrations decreased 2-fold to 1.7ng g^{-1} protein from 4h to 48h. A potential treatment effect was also observed: compared to their respective control groups, levels of *ent*-15-isoP were 2-fold higher in stressed fish at 4h, and significantly lower at 48h. However, only the E-4h group differed significantly from the baseline fish sampled at t = 0h.

These trends were apparent for the other three isomers, however differences were not always significant. Levels of 5-isoP, 8-isoP, and 8,12-isoP decreased 2-fold respectively in stressed fish from 4h to 48h, but temporal effects were not significant in control fish. Concentrations of these isomers were 2-fold and 1.3 fold lower in E-48h relative to the C-48h, but changes were not significant for 8-isoP. Concentrations of 5-isoP and 8,12-isoP were greater than baseline levels in the E-4h and C-48h groups respectively.

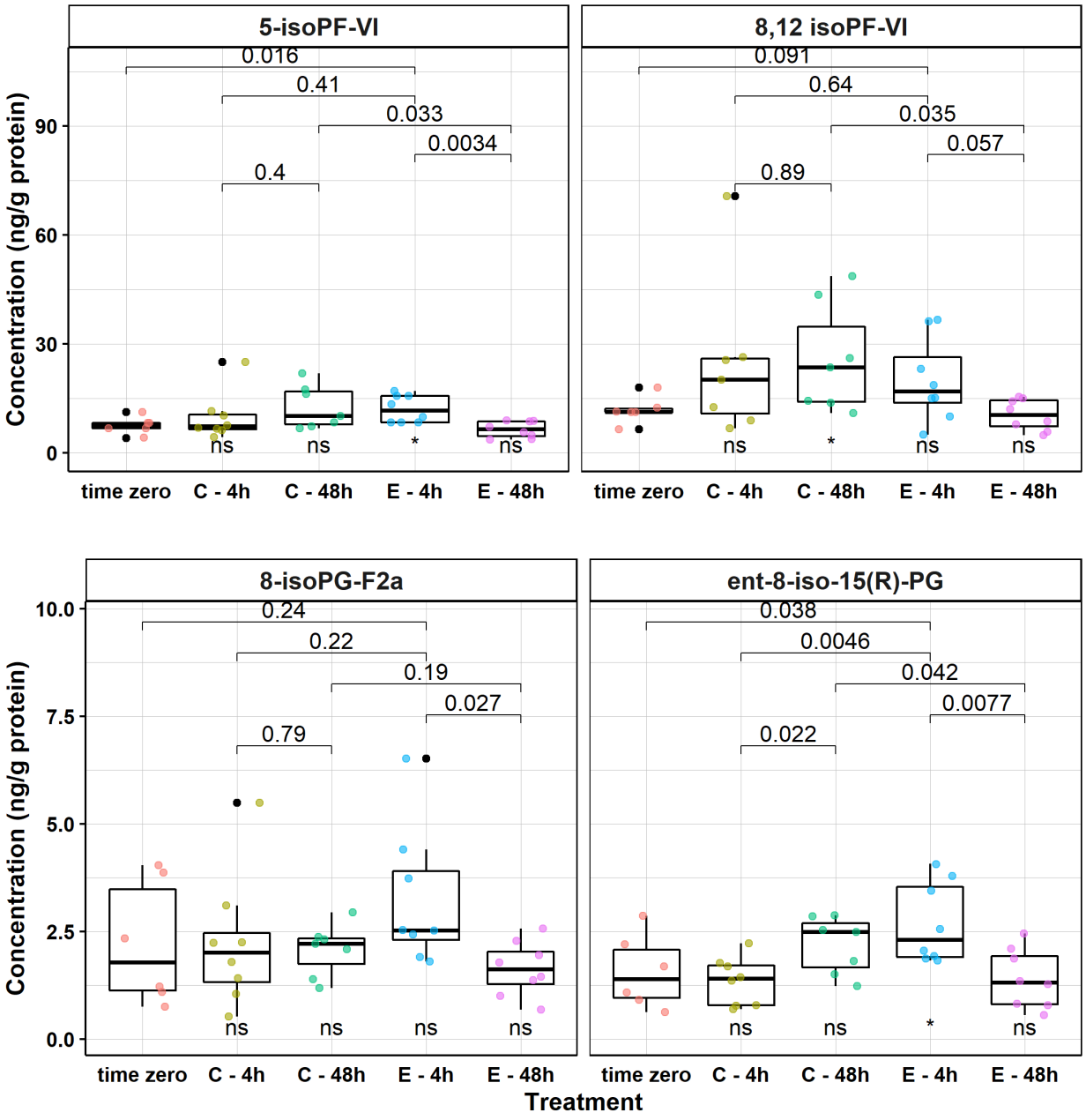

Figure 1.3 F_2-isoprostane concentrations in rainbow trout liver, with pair-wise comparisons shown. C = control, E = stressed. Pair-wise comparison significance level: * = P < 0.05, ** = P < 0.01, ns = not significant.

3.3.6 F_2-Isoprostanes in gill tissue

Several samples returned Type III isoprostane concentrations below the detection limits, therefore treatment groups were compared statistically using survival analysis methods for these compounds to better account for censored (non-detect, or ND) data. A contingency table test was performed to compare the occurrence of censored data between groups. As seen in Figure 3.6, ND occurrence increased from 0% in the baseline group to a maximum of 43% in the E-4h group. Results from a Fisher's exact test used to assess differences in ND occurrence showed a near-significant increasing trend ($P < 0.07$), indicating a likely correlation between treatment and ND frequency. ND occurrence increased from 18.8 to 43% in groups C-4h and E-4h. Non-detect occurrence was nearly identical in fish sampled at 48h, differing by only 2.4%. These data suggest that F_2-IsoPs decreased in the gills, possibly in response to a combination of physical stress and enclosure effects.

F_2-isoprostane concentrations followed a decreasing trend over time: in general, levels were lower in the fish sampled at 48h than in the 4h groups (see Figures 3.4, 3.5). For example, 8,12-isoP decreased by 43% and 46% in control and stressed fish from 4h to 48h ($P < 0.05$). Concentrations of 8-isoP also decreased from 4h to 48h, but the change was not statistically significant.

Possible treatment effects were also observed: 8-isoP was approximately 3-fold lower in relation to the baseline group in the C-48h, E-4h, and E-48h treatment groups ($P < 0.05$) and 8,12-isoP was 2.5-fold lower in group E-48h ($P < 0.05$). Concentrations of 8,12-isoP in stressed fish were 33% lower than in controls at both time points, however these differences were only near-significant ($P = 0.07$ and 0.08). Similarly, 5-isoP levels were depleted in E-4h fish relative to C-4h fish ($P < 0.05$).

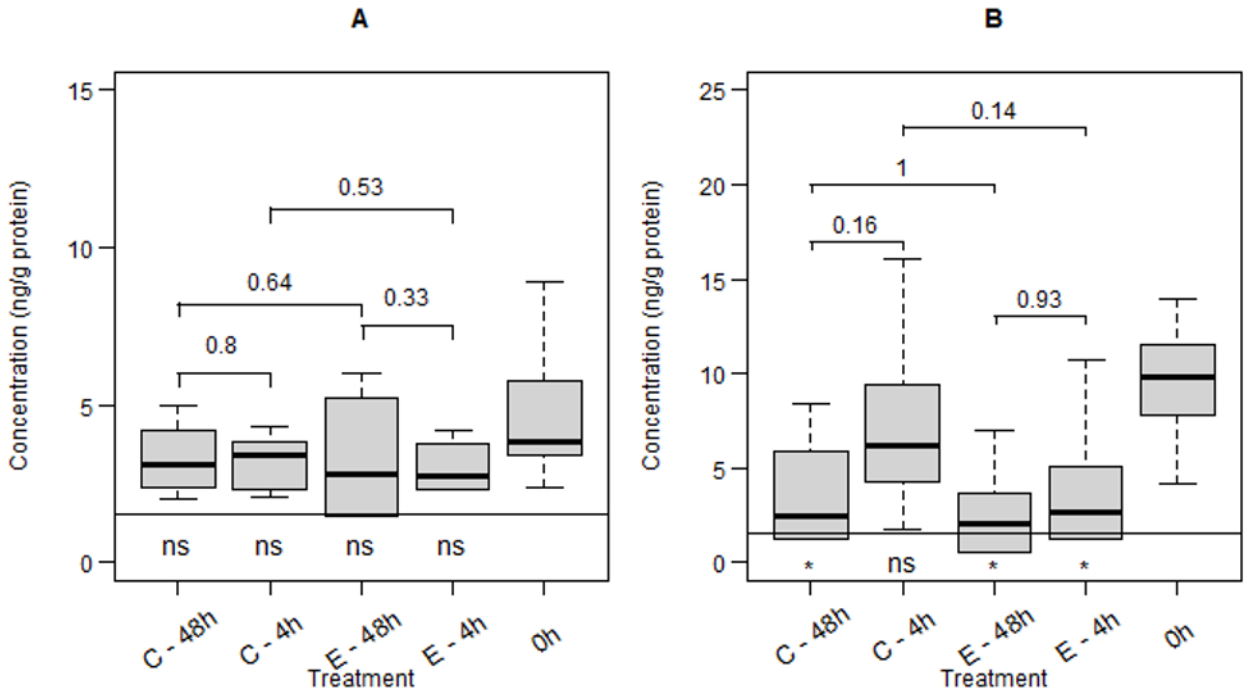

Figure 1.4 Censored boxplots of Type III F_2-isoprostanes in gill tissue. A: *ent*-15-isoP, B: 8-isoP. Pairwise comparisons are made using a generalized Wilcoxon rank-based test to account for censored (non-detected) data. C = control, E = stressed. Pair-wise comparison significance level: * = $P < 0.05$, ns = not significant. Solid line represents the limits of detection.

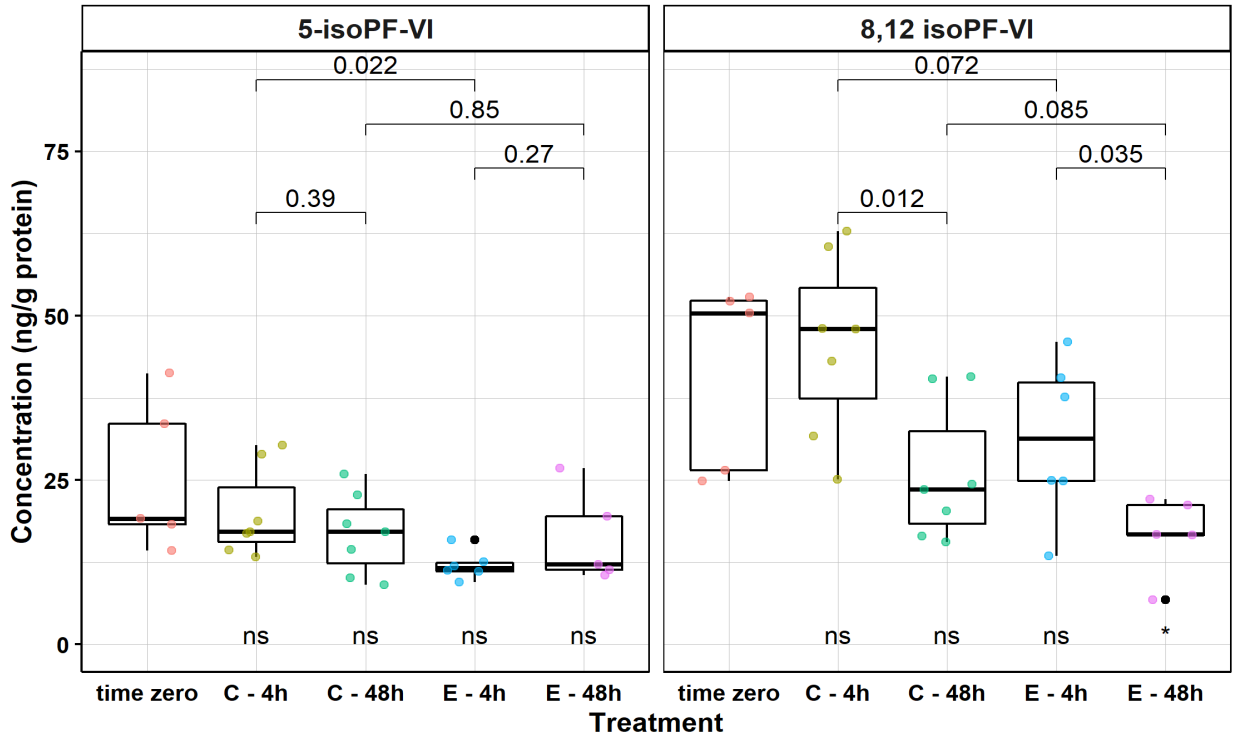

Figure 1.5 Type VI F_2-isoprostane concentration in gill tissue. C = control, E = stressed. Pair-wise comparison significance level relative to baseline group: * = P < 0.05, ns = not significant.

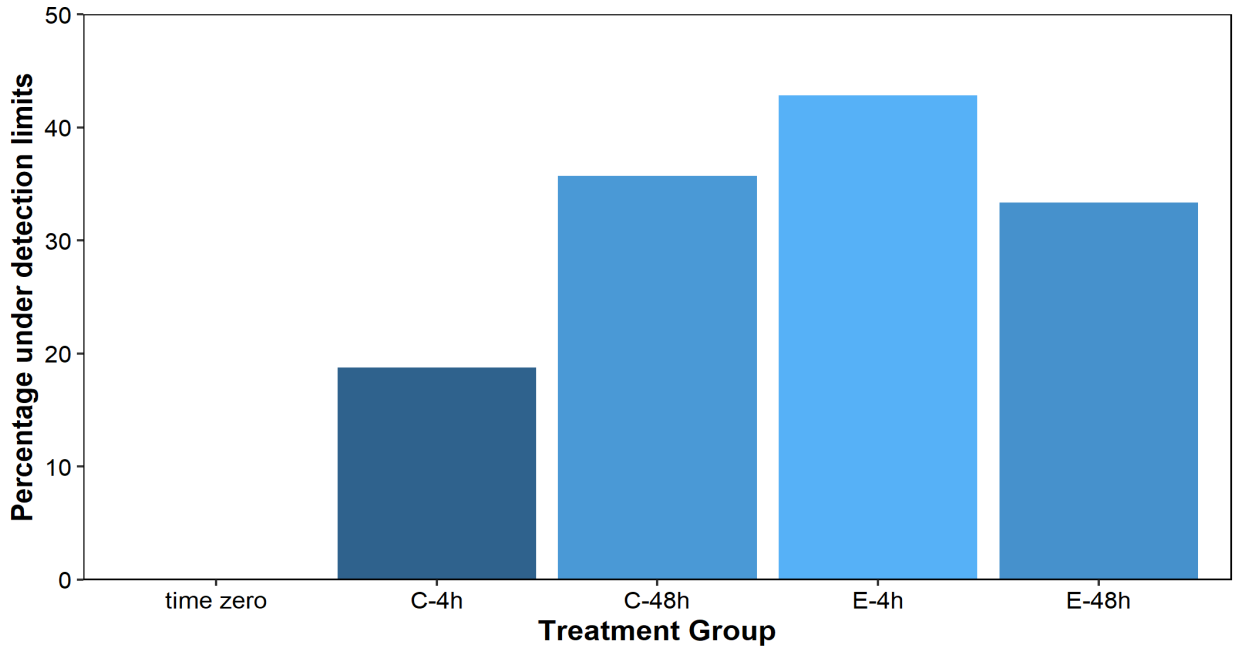

Figure 1.6 Occurrence of non-detects for Type III F_2-isoprostanes in rainbow trout gill tissue.

3.3.7 Glutathione and GSSG:GSH Ratio

Mean GSH and GSSG:GSH ratios for liver and gill tissue are shown in Table 3.2. Mean liver tGSH was approximately double gill tGSH in nmol/g wet tissue, however once protein normalized, concentrations were actually greater in the gills. In both tissues, the glutathione redox ratio was highest in the C-4h fish but was only statistically greater from the baseline in the liver.

Temporal effects within treatment types were not observed. Rather, small but significant treatment effects were seen, with GSSG:GSH ratios and tGSH being depressed in groups E-4h and E-48h relative to their respective controls. Gill GSSG:GSH ratios were 42% lower in both stressed treatment groups than baseline fish, however only the E-48h group was statistically different ($P < 0.05$). Similarly, tGSH was depleted in the E-4h and E-48h groups ($P < 0.05$). These data are shown in Figure 3.6.

GSH redox ratios were overall more constant in the liver with the exception of group C-4h (described previously). tGSH decreased by 15% in stressed fish from 4h to 48h, and was 21% lower in E-48h relative to C-48h.

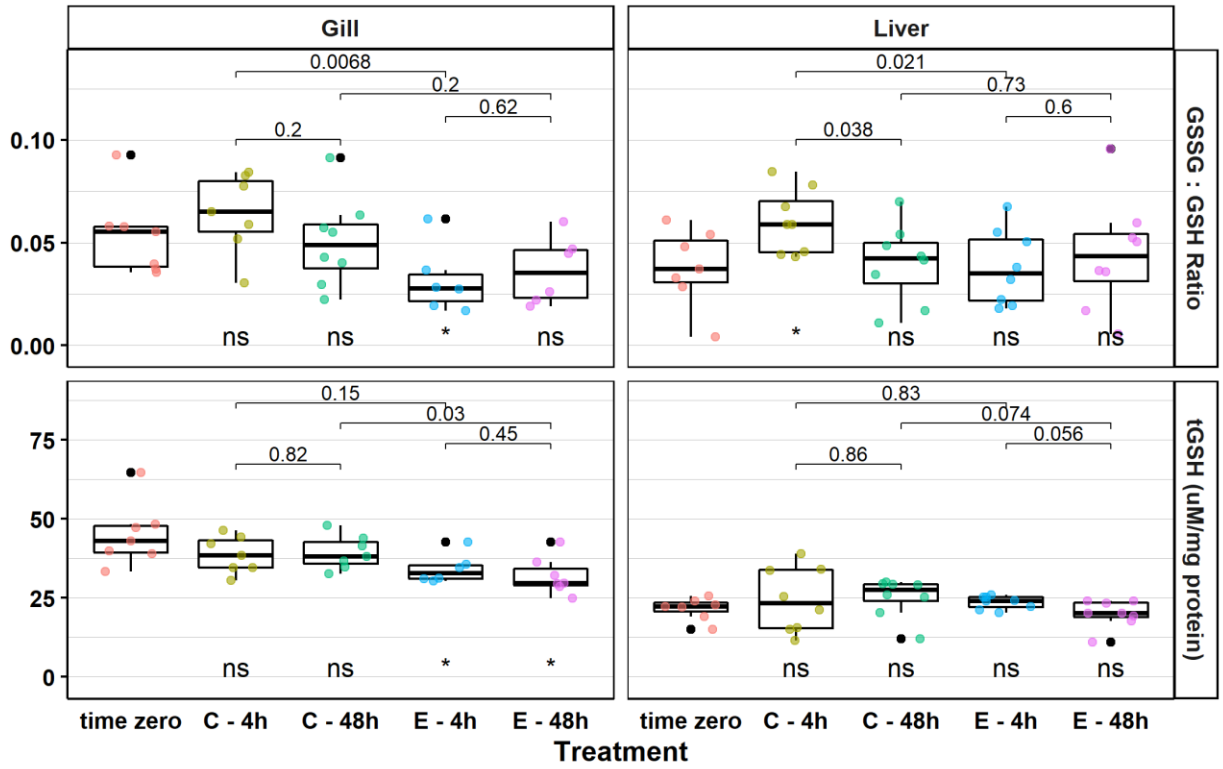

Figure 1.7 Total glutathione and GSSG:GSH ratio in rainbow trout liver and gill tissue. C = control, E = stressed. Pair-wise comparison significance level: * = P < 0.05, ns = not significant.

Table 1.2 Glutathione and glutathione redox ratios in rainbow trout

Treatment	TGSH[a]	SD[b]	GSSG : GSH[c]	SD[b]
Liver				
time zero	21.534	3.493	0.038	0.019
C - 4h	24.358	10.258	0.060	0.016
C - 48h	25.125	6.225	0.040	0.019
E - 4h	23.554	2.052	0.038	0.018
E - 48h	19.921	4.288	0.044	0.028
Gill				
time zero	45.030	10.036	0.054	0.020
C - 4h	38.657	5.822	0.064	0.019
C - 48h	43.972	13.968	0.050	0.022
E - 4h	34.185	4.624	0.032	0.016
E - 48h	31.913	5.905	0.031	0.020

[a] Total glutathione in uM/mg protein

[b] Standard deviation

[c] Ratio of oxidized glutathione to reduced glutathione

3.3.8 Biomarker correlations between tissues

Concentrations of F_2-IsoPs in the liver were assessed for correlation between liver and plasma in each fish, and between liver and gill tissue using the Pearson method. Gill and plasma were not compared, as limited tissue sample masses resulted in too few pairings for a statistical analysis. No apparent correlation was observed between liver F_2-IsoPs and gill/plasma F_2-IsoPs (see Table S8).

The GSSG:GSH ratio was positively correlated between liver and gill tissue, with the exception of fish in the C-4h group. The correlation was statistically significant in the E-4h group (R = 0.86, p = 0.026) and approached significance in two other treatments (time zero, R = 0.62, p = 0.14; C-48h, R = 0.57, p = 0.14).

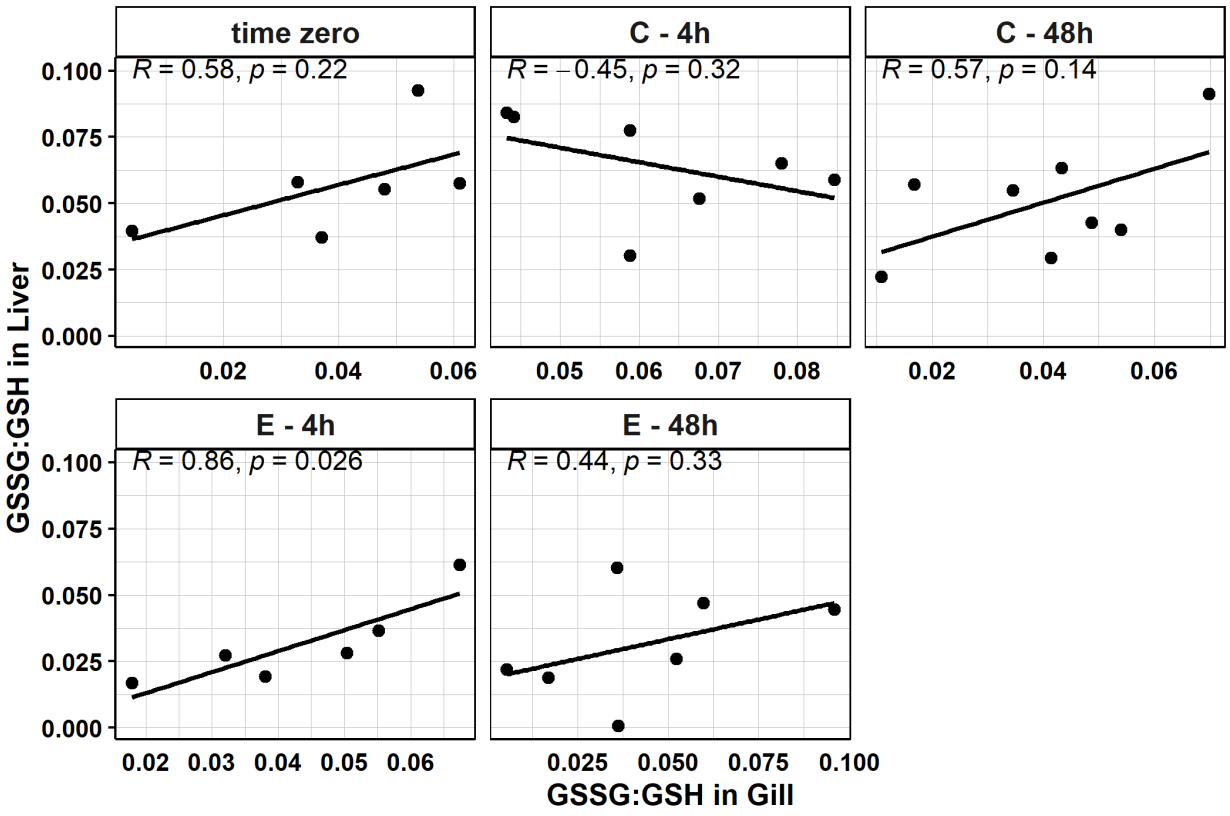

Figure 1.8 Pearson correlations of GSSG : GSH ratios in rainbow trout liver and gill tissue with 95% confidence interval. Statistical significance is P < 0.05.

Correlations of GSSG:GSH and F_2-IsoPs in liver and gill were strongest in the C-4h group, for 5-isoP, 8-isoP, and 8,12-isoP. In the liver, these isomers were negatively correlated to GSSG:GSH (R = -0.46 to -0.58, p = 0.13-0.3), while they were positively correlated in the gills (R = 0.53-0.8, p = 0.03-0.22). In group E-4h, gill 5-isoP was also positively correlated to GSSG:GSH (R = 0.9, p = 0.014). Correlations were otherwise weak and inconsistent in direction.

3.4 Discussion

3.4.1 Plasma cortisol

Cortisol is one of the most abundant corticosteroids produced in teleosts, and several studies have linked physical stress to large increases of plasma cortisol in rainbow trout. Basal levels in unstressed fish can be as low as < 5ng ml^{-1} but are variable: acute stressors were shown to result in concentrations as high as 200ng ml^{-1} [18,19].

In this experiment, baseline fish sampled at t = 0h showed the lowest intra-group variability (19 ± 3 ng ml^{-1}) with the exclusion of an outlier. This outlying data point was excluded on the basis of confounding stressors, as the individual was noted to have a lesion on its tail and signs of deterioration on its caudal fin – a possible indication of bacterial infection or aggression by dominant individuals: rainbow trout are an aggressive, territorial species that rapidly establish social hierarchies in confined environments: cortisol concentrations have been shown to rapidly increase in subordinate fish and remain elevated for at least one week (Sloman et al, 2001). After the exclusion of this outlier, the mean basal concentration was elevated compared to several studies but was well within literature ranges established by Barton et al (1991) [20].

Intra-group variability was generally much larger in the other groups, with SEM ranging from 20-50%. This may be an artefact of our sampling methodology: for logistical reasons, the remaining 8 fish in each tank were removed in two cohorts of four individuals, anesthetized in MS-222 and rapidly processed. This was done to minimize the number of netting events that may stress the fish (Laidley et al, 1998), while minimizing time between blood sampling of each fish by anesthetizing them simultaneously [21]. In contrast, fish from the baseline group were taken from each of the 4 tanks two at a time (only one cohort). As evidenced in Figure 3.1, mean cortisol

concentrations were 10-213% greater in the second cohort, with the exception of E-48h. Laidley et al (1988) demonstrated that cortisol significantly increased after 12-14min during serial dip net sampling of rainbow trout stocked in aquaria. Therefore, it is possible that the increased intra-group variability was partially caused by stress from two netting events at each time point after zero hours, but does not explain why cortisol was lower in the second cohort of group E-48h, opposite to the other treatments.

According to Barton et al (1991), plasma cortisol tends to peak 0.5-1h after the exposure to acute stress, before declining and returning to basal levels by 24-48h [20]. In my experiment, cortisol levels were not significantly increased relative to baseline in stressed fish after 4h. The selection of 4h as a time point could explain the absence of a plasma cortisol spike in E-4h fish in response to acute physical stress: cortisol levels may have already been declining substantially by the time fish were sampled. Findings from a study by Pickering et al (1991) support this hypothesis, as plasma cortisol in acutely stressed rainbow trout peaked at 0.5h and declined by nearly 80% by four hours [22].

In regards to the apparent increase in plasma cortisol in C-4h group, increased variability introduced by cohort sampling (discussed above) may have affected the results of statistical pairwise comparisons. Referring back to Figure 3.1, it can be seen that the second sampling cohort accounts for 75% of the group C1's highest concentrations, which could upwardly bias the mean concentration and create the illusion of a significant increase relative to the baseline fish. However, the ratio of GSSG:GSH was also significantly elevated in the liver of this group and non-significantly in gill tissue, suggesting that this group somehow differed from other treatments or was affected by an uncontrolled variable. This is surprising considering fish condition factors were

not significantly different between treatment groups by the start of the experiment, and the rigorously controlled experimental conditions.

3.4.2 Liver F_2-IsoPs

Concentrations of 8-isoP ranged from 2-5 ng g^{-1} protein in the liver. Few studies with fish models are currently available for comparison, and results are not always comparable as EIA kits tend to overestimate 8-isoP by factors of 10-20 [23]. For example, Knight et al (2016) detected F_2-IsoP at concentrations of 100-200 ng g^{-1} protein in rainbow trout liver using EIA [24].

Acute physical stress appears to have elicited a small, short-lived oxidative stress response in the liver: concentrations of two isomers, *ent*-15-isoP and 5-isoP, were elevated 1.7-fold relative to baseline levels in stressed fish at 4h. Levels of 8-isoP were also elevated at this time point, however the change was not significant. Overall, concentrations of the four isomers selected in this study followed approximately the same trends, however *ent*-15-isoP was the most sensitive to changes, detecting more statistical differences between groups. Increases in *ent*-15-isoP were similar in scale to those observed in medaka treated with 200-1000μM hydrogen peroxide [25], which suggests that acute physical stress can cause a short-lived oxidative stress response in rainbow trout.

However, the lack of significant change in 8-isoP levels does warrant caution when interpreting these results, as this isomer is the primary model for assessing oxidative stress. Preliminarily, our findings suggest that *ent*-15-isoP and 5-isoP respond similarly, however due to the small scale of the responses, further studies comparing the sensitivities of the isomers as biomarkers of acute oxidative stress should be conducted to determine the accuracy of *ent*-15-isoP and 5-isoP as indicator compounds. This would ensure that the results of this experiment are not a

result of chance: though significance thresholds were selected according to standard practices in toxicology (i.e $p < 0.05$), the relatively small number of fish in each experimental group should be considered as an increased risk of random error in the data. Future studies should use increased replication to strengthen statistical analyses.

3.4.3 Plasma F_2-IsoPs

Levels of F_2-IsoPs in circulation increase dramatically in response to oxidant injury [26]. These compounds are often quantified in plasma for mammalian studies, as collection is relatively non-invasive, however literature sources for fishes are lacking. Plasma concentrations in the current study were consistent with levels found both in humans and in rats, with concentrations of 8-isoP – the most studied isoprostane model – between 20-37 pg ml^{-1} [14,23,26]. Fewer comparisons are available for fish species: basal levels in Atlantic salmon were found to be in the range of 20-50 pg ml^{-1} by Bell et al (2000) using the EIA method. A study by Secci et al (2016) was the only other found with published plasma F_2-IsoPs levels for rainbow trout, and these were far lower than concentrations measured in this experiment (0.0011-0.0016 pg ml^{-1}) [5]. This difference is likely due to a difference in extraction methods: base hydrolysis was not used to free phospholipid-bound F_2-IsoPs in that study.

In the present study, mean concentrations of 5-isoP, 8-isoP, and *ent*-15-isoP were elevated by 1.4- to 1.9-fold in both control and stressed groups at 48h relative to 4h, however these changes were not significant. Though not significant, the increase in plasma concentrations coincides with decreased liver concentrations at 48h, which may indicate F_2-IsoP release from the liver into the bloodstream. This is a known pathway for F_2-IsoPs exiting the liver, which results in elevated plasma levels for an extended period of time: Morrow et al (1992) observed plasma concentrations in CCl_4 exposed rats were still elevated 48h post-exposure [26].

Treatment effects (stressed vs control at same time points) were not observed: mean concentrations increased by only 20-30%, which suggests no detectable oxidative stress response was elicited by the acute physical stress treatment, a conclusion that is at odds with what was observed in the liver tissue. The work of Morrow et al (1992) with CCl_4-administered rats suggests that the sampling times in this experiment may have been inappropriate to accurately capture the maximal response of F_2-IsoPs in the plasma [26]. In their experiment, the highest recorded F_2-IsoP plasma concentrations occurred 8h post-treatment – approximately 200-fold relative to baseline – in tandem with declining liver concentrations (which peaked at 2h post-administration). It is therefore possible that a detectable oxidative stress response brought on by acute physical stress may have been observed at 8h due to the slower response in plasma. Still, it should be noted that in Morrow's work, a 55-fold increase in F_2-isoprostane levels relative to baseline was still observed after 4h, indicating that the response in the plasma is still rapid, however F_2-isoprostane levels 4h post-CCl_4 administration were 77x greater in the liver. This could explain why the modest 1.7-fold increase observed in liver F_2-IsoPs in my experiment resulted in statistically insignificant changes in plasma after 4h.

3.4.4 Gill F_2-IsoPs

As the site of gas exchange between water and the blood stream, fish gills are a highly oxic environment and are therefore more susceptible to oxidative injury. In response, fish have developed defence mechanisms to protect these vital tissues, such as increased antioxidant enzyme activity: basal levels of SOD, CAT, and GST ranging from 7-97x higher than in other tissues have been reported in Rock goby [27]. Incidence of spawning failure in Sockeye salmon was linked to lower antioxidant enzyme expression in gill tissue, illustrating the importance of antioxidant resource allocation to this vital organ [28]. To our knowledge, only a seminal paper about *in vitro*

F_2-IsoP release in gill pavement cells has been published [29], so there is currently no other data available for comparison.

In this study, mean gill tissue concentrations of 5-isoP, 8-isoP, and *ent*-15-isoP were lower in groups C-48h, E-4h, and E-48h, though changes were only significantly different from baseline for 8-isoP. The number of non-detects followed a similar trend, accounting for 43% of measurements in acutely stressed fish sampled at 4h. These findings are unexpected, as an oxidative stress response typically manifests itself via an increase of lipid peroxides such as F_2-IsoPs and protein carbonyls. This could indicate that antioxidant enzyme activity in the gills behaves differently than in the other tissues, as was observed by Vinagre et al (2014) in the Rock Goby. Furthermore, the longer duration of the response (depletion continuing into 48h post-stress) suggests that gills are more susceptible to oxidative injury than the liver.

3.4.5 Glutathione

Total GSH levels were in agreement with other studies performed with rainbow trout, ranging from 0.82 – 2.68 μmol g^{-1} wet weight [30,31]. Ratios of oxidized GSSG were also within expected norms, and levels were not indicative of oxidative stress.[1] Redox ratios were highest in the C1 group in both gill and liver. There were no evident trends in the liver: the relatively stable TGSH concentrations and GSSG:GSH ratios in this tissue is possibly due to high GSH abundance in the liver.

[1] Though GSSG ratios above 1% are generally thought to indicate a pro-oxidant state, our method using 2-VP has been shown to overestimate GSSG concentrations. Therefore, relative ratios were focused on in this study.

Total GSH and GSSG : GSH ratios tended to be lower in the gills, suggesting a potential link between the apparent depletion of F_2-isoPs in the gills and this important antioxidant metabolic pathway. Regarding the decrease in GSSG : GSH ratios, it was discovered that both oxidized and reduced glutathione were significantly depressed relative to baseline fish in stressed fish at 4h and 48h, though the depletion of reduced GSH was proportionally greater, which may indicate a reduction of the total GSH pool as a result of the acute stress. This phenomenon is consistent with the findings of Bayir et al (2011), who observed that brown trout (*Salmo trutta*) exposed to starvation for long periods of time had elevated activities of both glutathione-S-transferase (GST) and glutathione reductase (GR) in their gills [32]. Increased GST activity contributes to a depletion of total GSH available, as the pathway involves the conjugation of GSH with lipid peroxides and its excretion. GR and glutathione peroxidase (GPx) are responsible for detoxifying reactive oxygen species such as hydrogen peroxide by cycling GSH and GSSG:GSSG. Bayir et al (2011) observed a greater increase in GR activity versus GPx (3.5-fold vs 2-fold increase), which would account for the increase in reduced GSH observed in our study. Moreover, this effect was opposite to what was observed in the liver, confirming that differential response indeed occurs in gill tissue, as was seen in our study [32]. Therefore, our GSH findings in gill tissue are consistent with an oxidative stress response in the stressed treatment groups.

This apparent link between F_2-isoPs and glutathione may indicate that GSH plays a role in regulating isoprostane concentrations in the gills, and suggests that antioxidant imbalance may have resulted from acute physical stress, resulting in increased utilization of glutathione. Sinha et al (2014) found that when exposed to high environmental ammonia, rainbow trout were more dependent on the glutathione pathways than goldfish and carp, demonstrating the highest glutathione reductase and glutathione peroxidase activities, and the lowest CAT and SOD activities

of the three teleost species in both liver and gill tissue [33]. In the short term (3-12h), apparent decreases in total GSH were observed prior to rebounding over the next few days. Despite these parallels, Sinha et al (2014) still observed evidence of oxidative stress in the form of increased MDA and xanthine oxidase, contrarily to what was observed here with F_2-IsoPs.

The concurrent decrease in F_2-isoPs and total GSH could therefore be a result of accelerated use of glutathione metabolism pathways by the trout in response to acute physical stress. This hypothesis is supported by the aforementioned study by Morrow et al (1992) in which GSH-depleting agents demonstrably led to much larger increases in F_2-isoPs in CCl_4 treated rats compared to rats only treated with CCl_4, indicating glutathione plays a role in controlling levels of F_2-isoPs in biological tissues [34]. This may occur via the GST metabolism pathway, which is known to facilitate the excretion of lipid hydroperoxides [35], though a mechanism for F_2-IsoP excretion using this pathway has not been studied. Therefore, the absence of elevated F_2-IsoP concentrations in the gill tissue is perhaps a symptom of milder oxidative injury whose effects are adequately mitigated by the trout's antioxidant defences, which would otherwise be overwhelmed by a potent hepatotoxin like CCl_4. However, further studies would need to be conducted to verify this hypothesis.

3.4.6 Biomarker correlations between tissues

In general, a positive correlation would be expected for tissues and organs of an individual during an oxidative stress response in the short-term: in CCl_4 exposed rats and humans subjected to acute exercise, F_2-IsoPs in the plasma and other organs have been shown to rapidly increase [26,36]. In our study, no correlations were observed between F_2-IsoP concentrations in the three tissues at the individual level.

The lack of observable correlation between tissues may be a factor of our sampling times, as was previously discussed, which did not properly capture the peak of an already mild oxidative stress response. Given the limited response that was observed in this study, the variability in correlational strength and direction may be attributed to natural variability between tissues, which could be remedied in the future by the use of greater replication to strengthen statistical power.

As previously discussed, observable trends were seen between F_2-IsoPs and GSH metrics in the gills. This was not seen at the individual level using the Pearson correlation, with the exception of group C-4h between GSSG:GSH ratio and three F_2-IsoP isomers. Given the common trends seen at the group level, a relationship likely exists between these two biomarkers but more studies are required, and should consider different statistical analysis methods to establish how the two biomarkers correlate at the individual level.

3.5 Conclusion

In summary, F_2-isoprostane were successfully measured in gill, liver, and plasma of 1+ year old rainbow trout. Plasma and liver concentrations were similar to levels in mammalian models: to our knowledge, this is the first time that *in vivo* F_2-isoprostane concentrations have been reported for gill tissue in the literature. Evidence of a short lived oxidative stress response was observed after fish were subjected to acute physical stress for a 5 minute period. In the liver, concentrations of two F_2-IsoP isomers were elevated in stressed trout after 4h and returned to baseline by 48h. In the gills, decreases in 8-isoP and 5-isoP occurred after 48h and in stressed fish at 4h and occurred in conjunction with a depletion of total GSH, suggesting that oxidative injury was mitigated through glutathione conjugation of F_2-IsoPs.

The current findings are a promising step in the development of methods to measure F_2-isoprostanes in large-bodied fish models, however several important questions need to be more explicitly answered by future studies. Determining biological levels of F_2-isoPs should be performed using an extraction method appropriate for the analysis of total phospholipid-bound isoprostanes, to ensure limits of detections are exceeded. It is recommended that in future work a more potent stressor be used to guarantee a strong oxidative stress response, such that differential response in tissues and particularly mucus can be determined with the greatest level of certainty: this would also simplify the selection of sampling time points and permit the use of fewer research animals since additional sampling times would not be as crucial. Time resolution should be an important consideration, to ensure that measurements fully capture the response of F_2-IsoPs over the short-term. In exploring the potential link between F_2-isoprostanes and GSH metabolism, measurements of GR, GPx, and GST should be considered essential during experimental design. Finally, the analysis of other important biomarkers of oxidative stress like antioxidant enzymes and protein carbonyls should be incorporated into a future study. Tissue samples are currently being held by our collaborators in the hopes of assaying for TBARS and SOD in all tissues from this experiment, however their analysis has unfortunately been delayed indefinitely due to COVID-19.

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Chapter 4: Quantification of F_2-Isoprostanes in liver and epidermal mucus of juvenile fathead minnows (*Pimephales promelas*) after a 7 day exposure to diluted bitumen.

4.1 Introduction

The Alberta oil sands region (AOSR) of Canada contains some of the world's largest oil reserves, expecting to produce 6 million barrels per day by the year 2030 [1]. The primary export from the AOSR is bitumen, an unconventional crude that is primarily transported to refineries via pipeline. To reduce its viscosity in the pipe, lighter fractions of oil such as natural gas condensates are added, creating a product known as diluted bitumen, or dilbit. Despite the relative safety of pipelines [2], several ruptures have over the years resulted in large volumes of dilbit being spilled into the environment, most notably including the Enbridge spill in the Kalamazoo River in 2011, prompting reviews into the toxicological effects of unconventional crudes in freshwater aquatic habitats [3–5].

The aquatic toxicity of dilbit is in large part mediated by a class of chemicals known as the polycyclic aromatic compounds (PAC), which are composed of multiple fused benzene rings. There is ample evidence that PACs and their alkylated homologues – which are present in high proportions in dilbit – induce ecotoxicological responses in fish through a variety of pathways particularly during developmental life stages, having been implicated in cardiac malformations, DNA damage, hatching success and survival rates, and oxidative stress [6–8]. Oxidative stress can occur as a result of metabolism by CYP450 enzymes, during which a large amount of reactive oxygen species (ROS) are generated. When ROS are present in very high concentrations, an aquatic organism's antioxidant defences can be overwhelmed, leading to ROS reacting with and damaging biomolecules [9].

This has prompted extensive research to identify and quantify oxidative damage using biomarkers of exposure, which now include antioxidant enzyme activity, gene expression, and lipid peroxidation products [10,11]. In mammalian models, the F_2-isoprostanes (F_2-IsoPs) are largely considered to be the gold-standard for quantifying oxidative stress *in vivo* [12,13]. These prostaglandin-like molecules are produced via a non-cyclooxygenase free-radical mediated peroxidation of arachidonic acid, as a result of ROS attack [14]. Elevated F_2-IsoPs have been correlated to exposure to airborne PACs in humans [15,16], but are rarely considered in fish toxicology. Importantly, their production in rats exposed to hepatotoxic carbon tetrachloride was shown to be influenced by CYP450 activity, which is the major metabolic pathway for PACs [17], suggesting PAC exposure could induce a response.

Recently, we published a study in which we successfully resolved and quantified F_2-IsoPs in the epidermal mucus of lake trout (*Salvelinus namaycush*) to explore the potential of non-invasively quantifying oxidative stress in fish [18]. In the present study, juvenile fathead minnows (*Pimephales promelas*) were exposed to a range of diluted bitumen concentrations for seven days in borosilicate beakers under controlled conditions. The objectives were to determine if PACs in the dilbit elicited a dose-dependent oxidative stress response in the fish measurable as increased F_2-isoprostane concentrations in the epidermal mucus and liver, and to compare the response between the two sampled matrices.

4.2 Experimental

4.2.1 Materials and reagents

Hydrochloric acid (HCl), water (HPLC grade), phosphate buffered solution (PBS), butylated hydroxytoluene (BHT), chloroform, ethyl acetate, and KOH were purchased from Fisher

Scientific (Fair Lawn, NJ). (±) 5-iso-prostaglandin-$F_{2\alpha}$-VI, (±) 5-iso-prostaglandin-$F_{2\alpha}$-VI-d_{11} (± indicates a racemic mixture of two enantiomers), 8,12-isoPF-VI, 8,12-isoPF-VI-d_{11}, 8-iso-prostaglandin-$F_{2\alpha}$, and 8-iso-prostaglandin-$F_{2\alpha}$-d_4, *ent*-8-iso-15(S)-prostaglandin- $F_{2\alpha}$ and *ent*-8-iso-15(S)-prostaglandin- $F_{2\alpha}$-d_9 (all >98%) were procured from Cayman Chemicals (Ann Arbor, MI).

4.2.2 Test organisms

Juvenile fathead minnows (*Pimephales promelas*) aged < 3 months were procured from Aquatic Research Organisms (Hampton, NH, USA). Fish were acclimated for 30 days in a circular holding tank equipped with a flow-through system at the Aquatic Toxicology Research Facility at the University of Saskatchewan (Saskatoon, Canada) under the following conditions: holding water was dechlorinated municipal tap water maintained at 25 ± 1°C, with a photoperiod of 12h:12h light/dark cycling. Minnows were fed 2% total body weight twice daily using a combination diet consisting of 70% thawed Hikari frozen bloodworms and 30% Nutrifin Max Golfish flakes. At the end of the acclimation period, sexually immature (sub-adult) fish were randomly selected for each exposure. Sexually mature fish – with visible tubercles or mating coloration in males, or ovipositors in females – were excluded.

4.2.3 Exposure water preparation

A water accommodated fraction (WAF) stock solution of diluted bitumen was prepared by adapting the methods described in Singer et al (2001). Briefly, dilbit was added in a 1:9 ratio to dechlorinated municipal water in a 1L separatory funnels and mixed for 18h at 60 revolutions per minute with a magnetic stir bar. Afterwards, the solution was allowed to settle for 1h and was then decanted into an amber glass bottle as 100% stock WAF solution. Stock solutions were prepared fresh daily, and appropriately diluted in fish holding water to reach the desired nominal

concentrations for dilbit exposures (discussed below). Experimental concentrations of PACs were estimated from concentrations measured by GC-MS/MS in matrix matched standards with nominal concentrations of 0, 3, 6, 12.5, 25, and 50% WAF.

4.2.4 Dilbit exposure design

Juvenile fathead minnows were randomly assigned to four experimental groups and exposed to the following nominal WAF concentrations: 0% (control), 3.0%, 10.0%, or 30.0%. Fish were exposed for 7 days in 1L borosilicate glass beakers (2 fish per beaker). Water temperature was controlled by placing beakers in a temperature-controlled water bath set to 25 ± 1°C, and beakers were aerated continuously with aeration stones throughout the exposure period. Static water changes were performed every 48h: 50% of the holding water was siphoned from each beaker and replaced with freshly prepared WAF solution (warmed to 25 ± 1°C prior to addition). Minnows were fed approximately 4% of body weight once daily for the first 6 days, then were fasted for 48h prior to commencement of ultrasonography measurements described below.

4.2.5 Ultrasonography, dissection, and mucus sampling

At the end of the 7 day exposure period, beakers were transferred to an insulated cooler for ultrasonography. Ultrasononography results are not discussed in this paper but methodology is included to give context on the handling of the fish prior to mucus sampling. Beakers were continuously aerated during this time. Fish were anesthetized using 10 mg L^{-1} Aquacalm (metomidate hydrochloride), and upon reaching stage 3 light anesthesia (Zahl et al, 2011) fish were placed in a foam fish-holder submerged in aerated holding water also containing 10 mg L^{-1} Metacalm. Cardiovascular structure and function was measured *in vivo* using cardiac ultrasound. Immediately afterwards, an electrocardiograph (ECG) was recorded by inserting two needle electrodes into the ventral cavity. Post-ECG, fish were euthanized with an overdose of MS-222,

and tissues were harvested for analysis. Mucus was collected using sterile FloqSwab®, by swabbing the entire body excluding the area near the anus. Swabs were placed into a 15ml Falcon tube and snap frozen. Livers and gallbladders were collected and transferred to sterile microfuge tubes and snap-frozen with liquid nitrogen. All tissue samples were stored at -80°C until analysis.

4.2.6 Total F_2-isoprostanes in liver

Liver isoprostanes were extracted as per Chapter 3. Briefly, liver tissue was homogenized and transferred to glass centrifuge tubes. The lipid fraction was subsequently extracted using chloroform, methanol, and water, and centrifuged for 10 minutes at 800 x g at 4°C. The organic layer was blown down to dryness under a nitrogen stream and reconstituted in 2ml 1M NaOH. The tubes were then incubated for 45min at 45°C to allow base hydrolysis to complete. Then samples were acidified to pH 3 with 1M HCl and diluted to 20ml with 1mM HCl. Solid phase extraction (SPE) was performed using Oasis HLB 20cc cartridges pre-conditioned with methanol and 1mM HCl. After loading samples, the cartridges were washed with 1mM HCl and hexanes, then eluted with 8ml ethyl acetate. Extracts were blown down to dryness and reconstituted in 100µl mobile phase.

4.2.7 Total F_2-isoprostanes in mucus

Swabs were thawed and supplemented with BHT (final concentration 0.05%) to prevent autooxidation of isoprostanes. The swabs were then extracted using 2 x 0.5ml phosphate buffer (0.1M, pH 7.4): swabs were vortexed 2 x 1 minute in 0.5 ml buffer, then rinsed with an additional 0.5ml and blotted dry along the sides of the Falcon tube. The tubes were then centrifuged at 10,000 x g for 20min at 4°C. A 50µl aliquot was removed at this point for protein content determination. To the remaining supernatant, 2ml of 1M methanolic NaOH was added to hydrolize phospholipid-bound isoprostanes. Samples were then acidified as described for liver, spiked with internal

standards (2.5 ng ml^{-1}), and extracted with 3cc Oasis HLB SPE cartridges using the same solvent systems as above, however volumes were 3ml for each. Extracts were prepared for HPLC-MS as described for the liver samples.

4.2.8 Bile PAC metabolites

Extraction and analytic methods for the determination of PAC metabolites in bile are described in Dearnley et al (submitted for review, 2022). Some results relevant to this thesis are discussed below to examine if PAC uptake and metabolism is linked to F_2-IsoP concentrations in mucus and liver samples. Briefly, bile was extracted and analyzed for 1-pyrenol, 2-chrysenol, and several co-eluting phenanthrols using HPLC-fluorescence.

4.2.9 Protein assays

Mucus extract was assayed for protein content using a modified Bradford method. Absorbances at 450 and 590 nm were measured, and the ratio of A_{590} / A_{450} was plotted against concentrations of the standard solutions. Protein content in mucus samples was determined by interpolation.

4.2.10 HPLC-MS/MS

Extracts were analysed with using a Varian 1100 HPLC system coupled to a SCIEX API 365 triple quadrupole mass spectrometer with a custom orthogonal ionization source. The HPLC column was an XSelect® HSS T3 C_{18} (3µm x 2.1mm x 50mm) maintained at 42 ± 0.1°C. Analytes were eluted using the following gradient: 30% B for 1min, increased to 60% B from 1-7min, 80% B by 18min, 100% B from 18.5-20min. Injection volume was 50µl, and source temperature was 500°C. Analyses were performed in negative ion mode: ion fragmentation transitions monitored for the respective F_2-isoP isomers and their internal standards can be found in Table S9.

4.2.11 Statistical Analysis

Statistical comparisons were made using RStudio software. Differences between experimental groups were assessed using student t-tests. Statistical significance was assumed to be $P < 0.05$. To compare concentrations between tissues, data were first tested for normality using the Shapiro test, then correlations were measured using the Pearson method.

4.3 Results

4.3.1 Water chemistry

Concentrations of PACs in the exposure water were estimated from a matrix matched standard curve analysed by GC-MS/MS. Standard solutions were analyzed by GC-MS/MS to estimate total PAC (TPAC) in exposure solutions. A strong linear response ($R^2 = 0.9984$) was observed between dilbit WAF % and (TPAC) concentrations. Typical of dilbit, alkyl-PACs accounted for the majority of PACs in the exposure solutions, ranging from 89-94% of the total. Concentrations are listed in Table 4.1 below.

Table 2.1 Total, parent-, and alkyl-PAC concentrations of dilbit WAF exposure solutions estimated from standard solutions.

Treatment (WAF %)	Estimated TPAC(μg/L)	Total Parent (μg/L)	Total Alkyl (μg/L)	Standard used to estimate
L – 3%	5.74	0.305	5.430	B
M – 10%	15.16	1.121	14.042	C
H – 30%	48.21	5.111	43.097	D

A detailed list of concentrations for each compound analyzed can be found in the appendix. Notably, the WAF was low in benzo[a]pyrene, benz[a]anthracene, and other benzylated parent PAC compounds – concentrations of these did not differ from the 0% WAF standard. C1 and C3 chrysenes likewise did not exceed concentrations in the blank. Of the parent PACs, naphthalene,

phenanthrene, fluorene, and acenapthene were the most prevalent. Among the alkyl-PACs, only methylated benzo[a]pyrene and C1/C3 chrysenes were below blank levels in the 3% WAF.

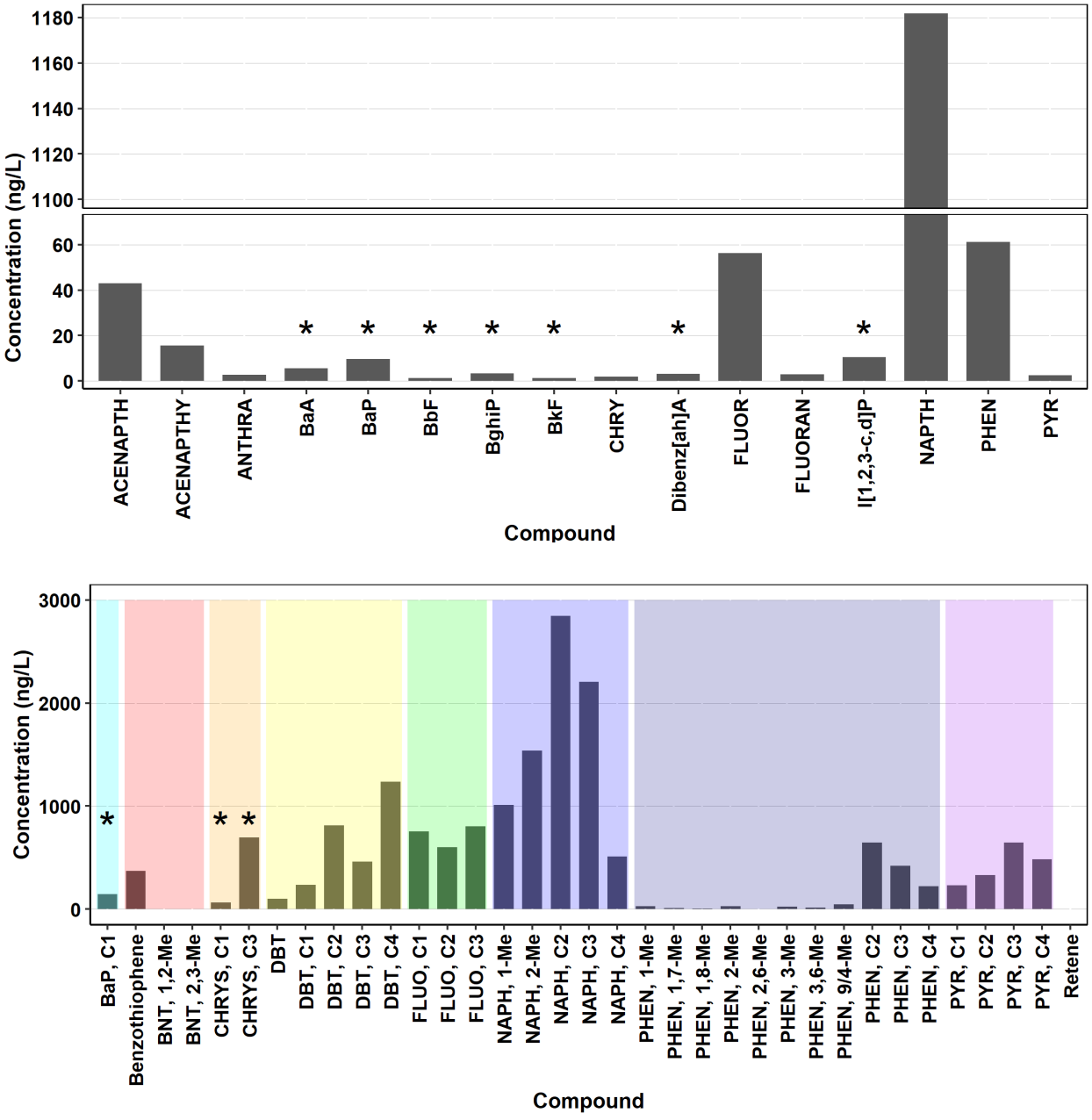

Figure 2.1 Concentrations of parent (top) and alkylated (bottom) PACs in a 12.5% WAF standard of dilbit used for fathead minnow exposure. Compounds that did not exceed levels in the blank are denoted with an asterisk. Alkyl-PACs are grouped by parent compounds (coloured shading).

4.3.2 F_2-isoprostanes

Liver concentrations of Type III F_2-isoprostanes ranged from 0.4-1.3 ng per gram tissue, and were between 5 to 25 times higher for Type VI isomers. Due to the small size of the liver samples, aliquots were not taken for protein content determination. Furthermore, my previous experiments on rainbow trout liver demonstrated that F_2-IsoP concentrations normalized for weight were highly correlated to protein normalized concentrations (R^2 = 0.8 - 0.9), therefore mass normalized concentrations are presented for comparison between the two sample matrices.

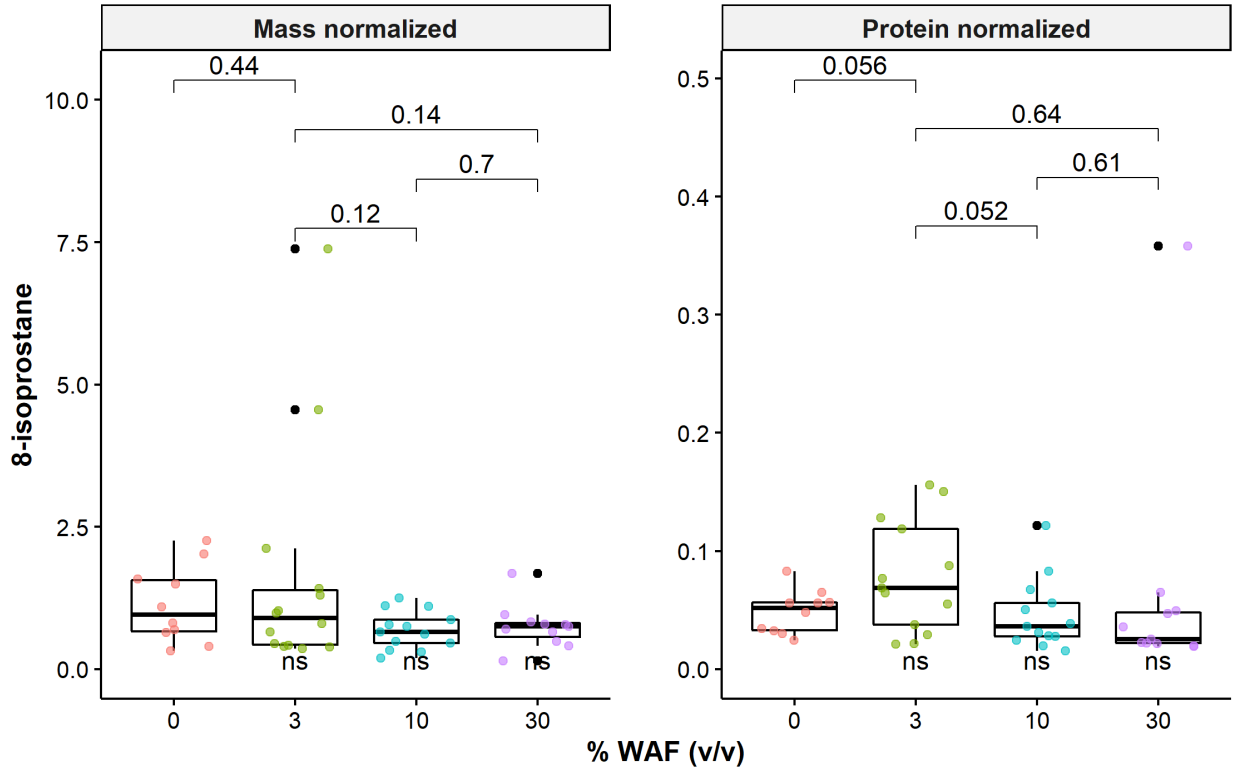

Figure 2.2 Comparison of mucus 8-isoprostane concentrations normalized in units of ng/g tissue and ng/g protein. Protein contents were determined using a modified Bradford assay. P-values are shown for pairwise comparisons; statistical difference from control group ($P < 0.05$) is denoted by *, ns = not significant.

Mean mucus F_2-IsoP concentrations were similar to those measured in liver, however in individual fish, liver F_2-IsoP concentration was a poor predictor of levels in mucus ($R^2 < 0.1$). Additionally, in contrast to the strong correlation seen between mass and protein normalization in liver samples, mucus F_2-IsoPs concentrations normalized for protein content appeared independent of wet sample mass, with an $R^2 < 0.1$. This lack of relationship was observed in all experimental groups. Despite this, mean isoprostane concentrations in the liver and mucus (measured as ng g^{-1} tissue and ng g^{-1} protein) exhibited similar patterns across the treatments groups.

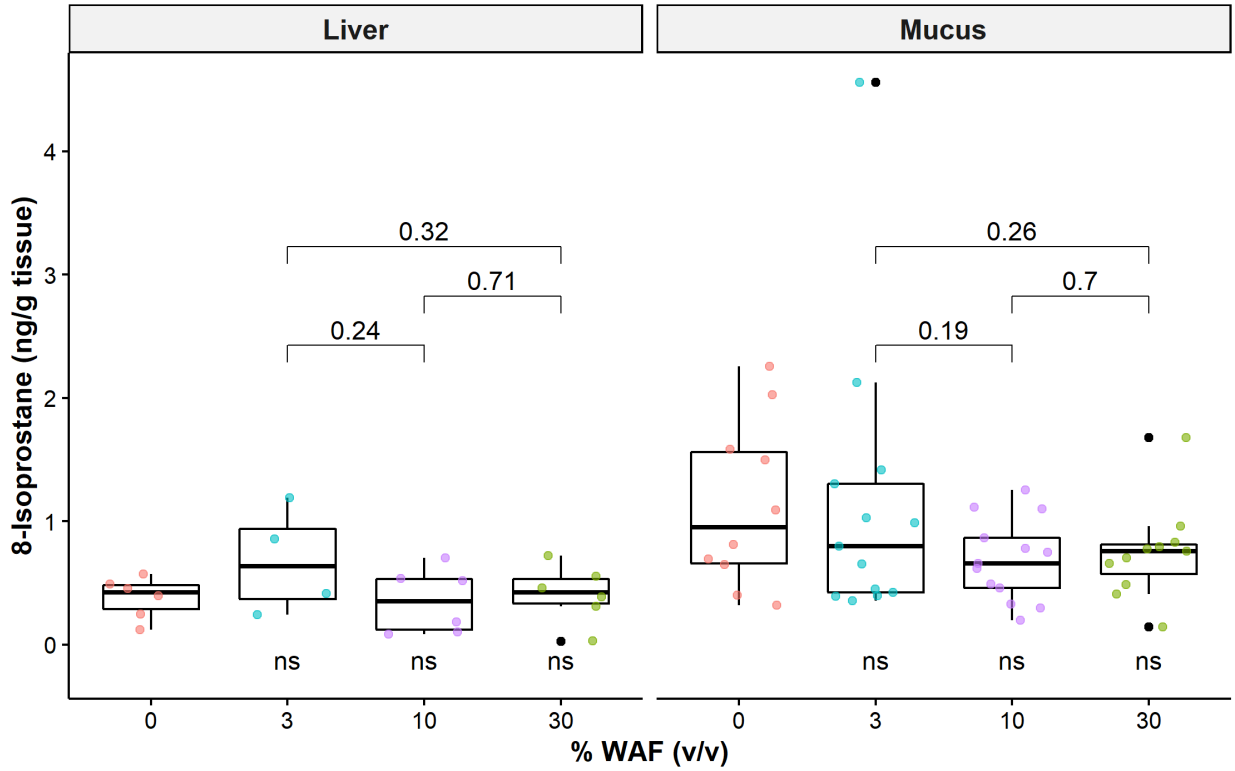

Figure 2.3 8-isoprostane concentration in liver and mucus of juvenile fathead minnows exposed to dilbit WAF. P values are shown for pairwise comparisons; statistical difference from control group ($P < 0.05$) is denoted by *, ns = not significant.

Isoprostane levels did not change significantly in mucus nor liver tissue in response to dilbit WAF exposure. In both biological matrices, mean concentrations were greatest in fish from the "Low" dose group, exposed to 3.0% WAF: mean levels were 30-50% higher in this group than in control or other exposed fish. A linear dose-response relationship was therefore not observed – in fact, no group differed significantly from the control fish.

Concentrations of F_2-IsoP in liver and epidermal mucus were assessed for possible correlations, using only fish from which both tissues were harvested. Shapiro normality tests indicated that data did not differ statistically from a normal distribution, so Pearson correlation tests were used. Positive correlations were observed between the two matrices in the control group. 8,12-isoP was significantly correlated ($R = 0.96$, $p = 0.0028$), and *ent*-iso-15-P was near significant ($R = 0.78$, $p = 0.066$), while the other two isomers were more weakly correlated. A general decreasing trend was observed between dose and magnitude and direction of Pearson R values in all isomers with the exception of 8-isoP, where there appeared to be no discernable correlation.

Table 2.2 F_2-isoprostane concentrations in fathead minnow livers and mucus (ng g^{-1} tissue).

Treatment	Isomer			
	5-isoP	8-isoP	8,12-isoP	*ent*-15-isoP
Liver				
C – 0%	3.379(1.04)	0.76(0.33)	6.463(3.09)	0.645(0.24)
L – 3%	3.973(1.68)	1.251(0.78)	10.855(5.92)	0.976(0.65)
M – 10%	2.673(2.14)	0.71(0.53)	10.691(3.76)	0.462(0.33)
H – 30%	2.033(0.69)	0.821(0.47)	10.471(7.61)	0.408(0.19)
Mucus				
C – 0%	3.672(1.68)	1.232(0.62)	6.094(2.97)	0.967(0.5)
L – 3%	3.788(3.99)	1.749(1.99)	5.893(4.71)	1.363(1.75)
M – 10%	2.258(1.48)	0.829(0.6)	5.157(4.66)	0.52(0.35)
H – 30%	3.41(2.07)	0.827(0.44)	8.343(8.79)	0.708(0.46)

4.3.3 Bile metabolites: quantifying exposure

To quantify uptake of PACs by the fathead minnows, bile was analyzed for 2-chrysenol, several co-eluting phenanthrol isomers, and 1-pyrenol. Phenanthrols and 2-chrysenol were present in the order of 500-1600 ng ml^{-1}, while 1-pyrenol was much less concentrated in the bile, ranging from 1.4-60 ng ml^{-1}. Phenanthrol concentrations were significantly elevated in the High dose relative to the control group ($P < 0.01$). Moreover, despite a lack of significant differences between oiled treatments, phenanthrol concentrations increased linearly ($R^2 = 0.89$) with dose. Increases in henanthrol concentrations were near-significant between the 3% and 30% WAF groups ($P < 0.07$). However, phenanthrol increased only 2.4-fold relative to the Control group, and only 1.6-fold relative to the 3% dose, which indicates uptake of PACs was not proportional to treatment dose. No significant changes nor linear relationships were observed for either 2-chrysenol or 1-pyrenol ($R^2 < 0.1$ for both). Bile metabolites were not correlated to F_2-IsoP concentration in either tissue.

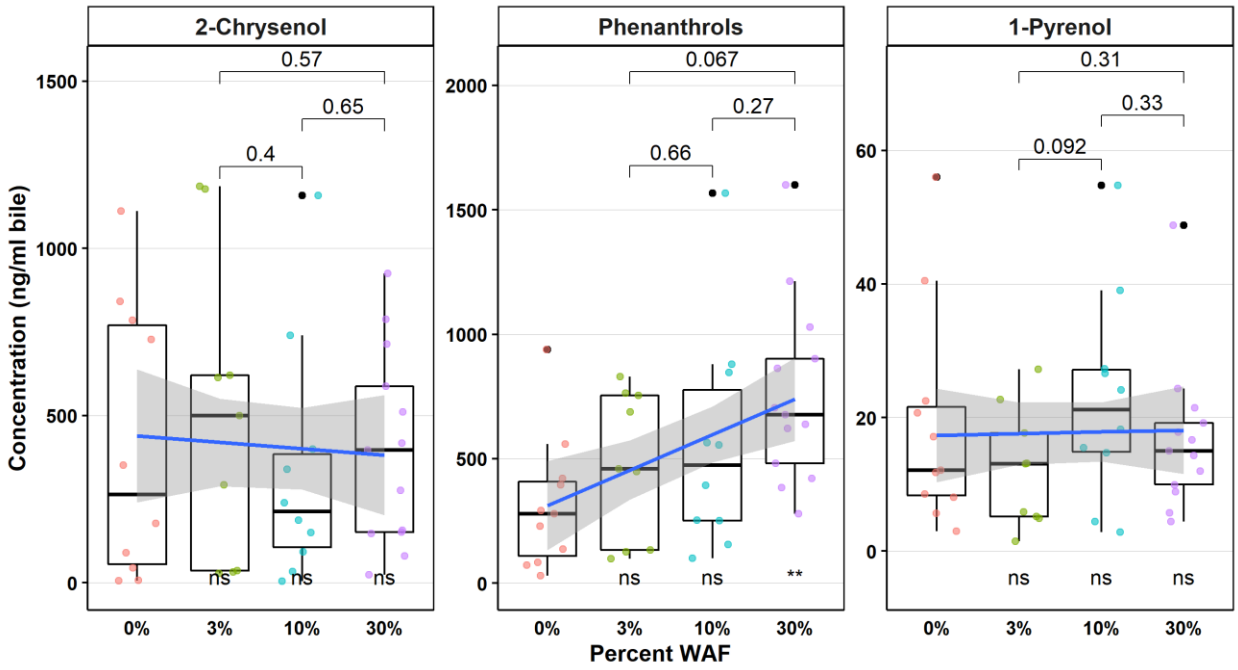

Figure 2.4 Biliary PAC metabolites measured in juvenile fathead minnows after a 7 day exposure to dilbit WAF. Pair-wise statistical comparisons are shown, significance is $P < 0.05$.

4.4 Discussion

Concentrations of TPAC measured in the exposure solutions were comparable to those in studies by Rodriguez et al (2019) and Robidoux et al (2017) [19,20], and are similar to those observed to induce a phase I detoxification response (observable as a significantly increase the expression of cyp1a mRNA in Japanese medaka embryos and (in the 10% and 30% WAF) exceeded the TPAC range where Benetti et al (2014) observed toxic effects in embryonic stages of Pacific herring [7,8]. The dominant compound classes in our study were the alkyl-naphthalenes, followed by several 3- and 4-ring alkylated PACs: alkyl- pyrenes, fluorenes, dibenzothiophenes, and phenanthrenes. PACs with 3 to 5 rings, particularly alkyl-phenanthrenes, have been strongly linked to cardiac malformations in developing herring, and altered metabolic demands in juvenile mackerel chub [21,22].

Accordingly, deleterious effects reported in the literature for this TPAC concentration range were anticipated, but F_2-isoprostane concentrations did not differ significantly from control levels in any of the exposure groups for either liver tissue or mucus. In principle, the induction of CYP450 enzymes leads to greater production of reactive oxygen species (ROS) and in turn enhanced peroxidation of lipids. The role of CYP450 in the formation of F_2-IsoPs was demonstrated by Morrow et al (1992), where inhibiting CYP450 activity in rats exposed to carbon tetrachloride with SKS-525A decreased F_2-IsoP production by 55% relative to uninhibited rats, which saw 55-fold increases in plasma [17]. Though *cyp1a* expression was not measured in this experiment, several literature sources indicate that in early life stages of fish, the TPAC concentrations used in this experiment were sufficiently elevated to induce expression of *cyp1a1* genes, increased CYP1A enzyme activity as measurable by EROD, and other biomarkers of oxidative stress such as GST and MDA [8,11]. Notably, Andrzejczyk et al (2022) discovered that lake trout exposed to low concentrations of dilbit induced upregulation of *cyp1a3* mRNA in epidermal mucus [23], which also suggests that low level dilbit exposures can induce oxidative stress responses in our selected tissues.

However, the PAC exposure apparently induced no F_2-isoprostane response in the exposed fish. This is contradictory to other published results, as studies of fish and humans have both found that chronic exposure to PACs results in elevated 8-isoprostane concentrations: concentrations in rainbow trout (*Onchorhyncus mykiss*) head kidney exposed to a dietary dose of 7.82 μg g fish^{-1} day^{-1} high molecular weight PACs increased 1.5-fold after 50 days of exposure [24]. In humans exposed to airborne PACs, urinary 3-phenanthrol has been positively correlated to 8-isoprostane levels in urine [16]. It is possible that F_2-IsoP responses are not as pronounced in liver and epidermal mucus than in head kidney – differential responses in tissues is common for oxidative

stress biomarkers – however Morrow et al (1992) observed a rapid increase in this organ and concluded that esterified F_2-IsoPs in the plasma were largely produced in the liver, which is consistent with the liver being the primary site of detoxification in the body. In that experiment, liver F_2-IsoP concentrations declined concomitantly with an increase in plasma F_2-IsoPs. A possible explanation for the stable levels in the liver could be that a steady-state release of F_2-IsoPs to the plasma from the liver was achieved by the end of the exposure period, though future study would be required to examine this possibility.

Another possibility is that the PACs in the exposure medium were not bioavailable to the extent implied by the nominal concentrations, which could accounted for a lack of apparent oxidative stress response. Solubility limits and adsorption of PACs to surfaces and particles are two issues that could result in experimental concentration being significantly lower than nominal ones [25]. In this study, the exposure vessels did not contain other substrates to which PACs could adsorb, and GC-MS analysis of the WAF standards showed that concentrations increased linearly to the maximum tested standard concentration of 50% WAF, which was beyond the exposure doses. It is therefore unlikely that PACs were not bioavailable to the fish during the exposure. To confirm the uptake and metabolism of PACs by the exposed fathead minnows, biliary metabolites were quantified. The biliary concentration of the phenanthrols was strongly correlated to dilbit dose, and was significantly greater than in the control fish after 7 days of exposure (in the 30% WAF exposure group). Several studies have demonstrated that biliary metabolite concentrations show dose-dependent increases as a result of chronic exposures to PACs [26,27]. GC-MS analysis of the WAF (Figure 4.1) shows that chrysene isomers and pyrene were present in negligible quantities, which explains their lack of a dose-response relationship. The apparent uptake and metabolism of phenanthrols suggests that the other PAC constituents in the exposure WAF were

metabolized as well as they are detoxified via the same pathways. A caveat of the results is that the concentrations of biliary PAC metabolites, while apparently correlated to TPAC concentration, did not increase proportionally to the dosage: phenanthrols increased less than 50% from the low dose to the high dose despite a nominal 10-fold increase. By contrast, PAC metabolites increased proportionally to nominal concentrations in Atlantic cod (*Gadus morhua*) after one week, and Sanni et al (2017) observed a 4- to 5-fold difference in biliary metabolite concentration between fish exposed to 0.5-1.14μg L^{-1} and those exposed to 5.0-5.2 μg L^{-1} [26,28]. A low uptake rate of nominally available PACs could explain the lack of measurable oxidative stress response in this study. Forthcoming respirometry results collected in parallel from this exposure study will shed more light as to if the treatments caused deleterious effects in the fish.

Based on the lack of an observable oxidative stress response, here we assume that the measured concentrations of F_2-IsoPs in liver and mucus are representative of near-basal levels in the fish. Liver concentrations in the rainbow trout from Chapter 3 were comparable to those measured in this study. 8-isoP concentrations in all treatments were within 0.5ng g^{-1} of head kidney concentrations measured by Bravo et al (2011). Mucus F_2-IsoP concentrations were at the lower end of the range measured in wild lake trout (*Salvelinus namaycush*) by Bulloch et al (2020), though differences in sampling techniques (ie the implementation of swabs for mucus collection) and environmental factors such as spawning stresses in the wild trout make comparisons more difficult [18].

Though present in low concentrations in all mammalian tissues and biological fluids, F_2-IsoPs were found to be most concentrated in rat's livers, where they are produced and subsequently exported throughout the body via plasma [17]. We found concentrations of F_2-IsoPs were generally greater in epidermal mucus than in the liver. In the control group, positive correlations were

observed between concentrations in the two tissues, and negative correlations were observed in the dilbit-exposed groups. Though these were statistically insignificant except for 8,12-isoP in the control ($R = 0.98$, $p = 0.0028$) and high exposure ($R = -0.87$, $p = 0.02$), these data suggest that a relation may exist between basal F_2-IsoP levels of liver and epidermal mucus. A reversal of the correlation direction could be indicative of F_2-IsoPs being released from the liver and accumulating in the mucus, however this needs to be explicitly studied, in conjunction with research into the production and transport of F_2-IsoPs in fish.

4.5 Conclusion

This study successfully extracted and quantified F_2-isoprostanes from the epidermal mucus of juvenile fathead minnows, confirming that measuring these potential biomarkers of exposure using swab-based non-invasive sampling methods is feasible even for small-bodied fish. In this study, F_2-IsoPs did not increase in either liver or mucus in response to a 7 day exposure to dilbit, which is unexpected given the central role of CYP450 enzymes in PAC detoxification and its importance in F_2-IsoP production in rats [17,29], perhaps a result of low uptake of PACs by the fish as suggested by the low concentration of bile metabolites in the higher exposure doses, which did not match dose-response relationships established in other studies [26,28]. Additional studies should be conducted to verify if dilbit elicits a change in F_2-IsoP concentrations which include regular analysis of dissolved PACs during the exposure period to confirm the concentration and bioavailability of the compounds in the WAF. Given that our findings contradict others in regards to CYP450 mediated toxicity, future work should also include a more comprehensive set of biomarker analyses including measures of CYP450 activity (EROD) and *cyp1a* gene expression. Furthermore, the relationship between F_2-IsoPs in epidermal mucus and visceral tissues during dilbit exposure requires further attention, to compare the rapidity and magnitude of their responses

to PAC exposure. The positive correlations observed in the control group suggest that mucus and liver F_2-IsoPs are linked. To the best of our knowledge, no other study has compared the relative concentration of these compounds in visceral tissue and epidermal mucus: with further study, the viability of mucus as a surrogate for liver could be determined. Through the quantification of an important biomarker of oxidative stress in the fathead minnow, this study adds to the growing body of literature supporting epidermal mucus as a viable sampling matrix to measure early effects of contaminants in fish [23,30,31], and warrants further investigation into the response of F_2-IsoPs in fish exposed to crude oil.

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Chapter 5: F_2-Isoprostanes in epidermal mucus of fathead minnows (*Pimephales promelas*) exposed to dilbit in a freshwater ecosystem at the IISD-Experimental Lakes Area

5.1 Introduction

Canadian crude oil production increased to more than four million barrels per day in 2017, 80.7% of which was produced in the Alberta oil sands region (AOSR) [1]. Pipelines are recognized as the safest method of transportation, with fewer spill incidents than rail and truck transport [2], resulting in several new pipeline construction projects being proposed in the last decade. However, pipeline malfunctions and spills still occur. These incidents have prompted public concern over our ability to properly respond to spills [3]. In 2015, a report by the Royal Society of Canada identified several key knowledge gaps in this field, including the efficacy of spill response, the environmental impacts of different remediation methods, and the persistence of unconventional crudes such as diluted bitumen in freshwater ecosystems [4].

In the wake of environmental incidents such as the Deepwater Horizon and Exxon Valdez spills, the toxicity of crude oils has been well established in several marine fish species, particularly in early life stages. Much attention has been devoted to the polycyclic aromatic compounds (PAC), a class of planar compounds containing two to six carbon rings that have been linked to disrupted cardiac function, blue sac disease, and oxidative stress [5–7]. However, relatively few studies have focused on the effects of unconventional crudes in freshwater environments, despite the relevance of AOSR products in the Canadian petroleum industry. In contrast to conventional crudes, the toxic PAC fraction of dilbits contains a much greater proportion of alkylated PACs, raising its potential for chronic toxicity. Additionally, the greater density of dilbit allows it to distribute itself

through the water column and into sediments more readily than lighter crudes [8,9], increasing its persistence in aquatic environments and potentially prolonging its chronic toxicity.

To address this, the Freshwater Oil spill Remediation Study (FOReSt) was developed and undertaken at the International Institute for Sustainable Development – Experimental Lakes Area (IISD-ELA) research station in Northwestern Ontario. The objective of the FOReSt project is to assess the impacts of dilbit and secondary minimally invasive remediation methods on freshwater shoreline environments. In this study, an environmentally relevant volume of diluted bitumen was introduced into shoreline enclosures stocked with wild fathead minnows at Lake 260, in two regions of the lake encompassing rocky or organic shoreline substrates. The targeted volumes were representative of medium to heavy shoreline oiling and a slick thickness of 0.1cm. Three secondary remediation methods were then applied to treat the residual oil: nutrient addition, engineered floating wetlands, or COREXIT® EC9580A shoreline cleaning agent. To determine the efficacy of these remediation methods for reducing impacts to resident fish, we measured F_2-isoprostanes in fathead minnows exposed for 75d in the enclosures. Isoprostanes, markers of oxidative stress, were measured in dermal mucus using a recently developed non-invasive sampling method and quantification via high performance liquid chromatography mass-spectrometry.

5.2 Materials & Methods

5.2.1 Study area

The FOReSt project was carried out in lake 260 (49.694108°, -93.767919°), at the IISD-Experimental Lakes Area in northwestern Ontario, Canada. L260 is a small oligotrophic lake with a surface area of 32.76 ha and a maximum depth of 14.4 metres. The food web and water chemistry

are characteristic of boreal shield lakes, and has been extensively described during previous studies [10,11]. The lake sustains a small native population of fathead minnows which has recovered from a whole lake ethinylestradiol addition experiment in the early 2000s [12]. Its shoreline substrate is variable, ranging from highly organic to rock and cobble.

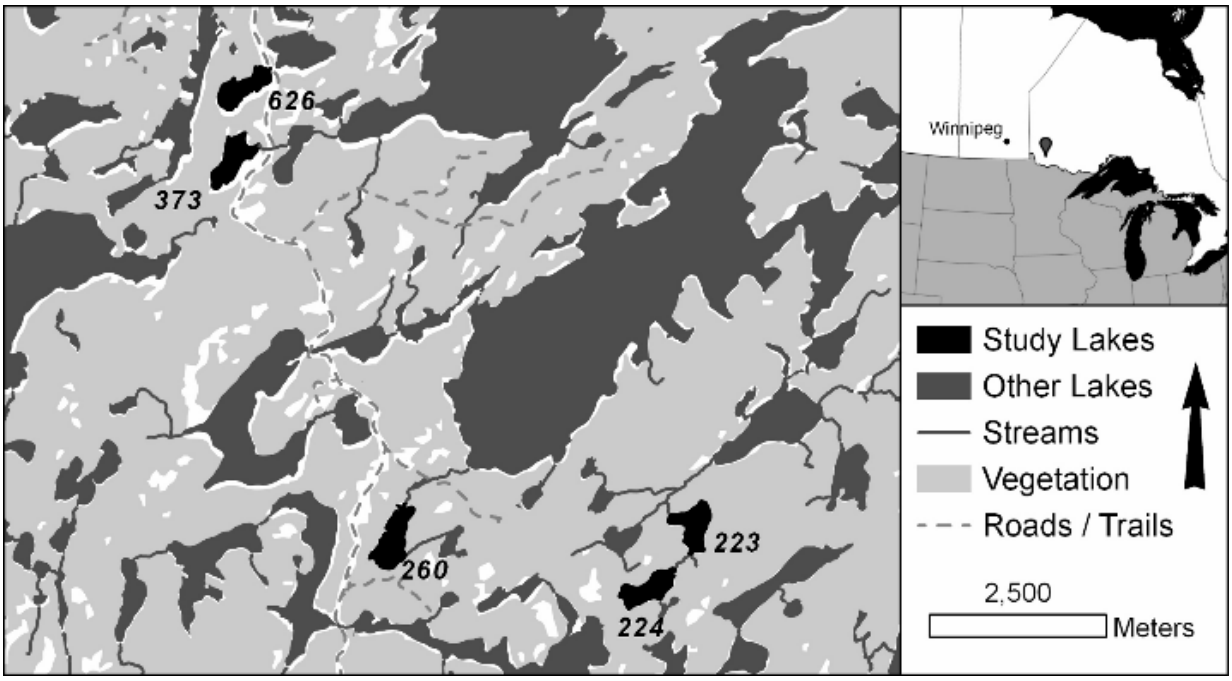

Figure 3.1 Location of IISD-Experimental Lakes Area research station in Northwestern Ontario, Canada (top right) and research lakes sampled for this study (main body). Credit: G. Gunn, IISD-ELA. Data sources: NRCAN and IISD-ELA.

5.2.2 Study species: Fathead Minnow

Fathead minnows (*Pimephales promelas*) were selected as the fish model species in this study. FHM belong to the Cyprinidae family, and besides being native to the IISD-ELA watersheds, are widely distributed across North America. They are opportunistic feeders that consume algae, small invertebrates and crustaceans, insects, and detritus. This varied omnivorous diet, paired with their tolerance to highly varied water chemistry parameters such as pH, turbidity, and temperature, likely contributes to the species' success at proliferating throughout the continent

[13]. Their colouring is olive green, with a white underbelly, and they possess an incomplete lateral line. During spawning periods, males further exhibit secondary sex characteristics including nuptial tubercles on their foreheads, dark banding, and a dark coloured dorsal pad, making them extremely easy to differentiate from females. For their part, females develop ovipositors, a pink fleshy lobe near the anus. Sexual maturity is temperature dependent, but can be reached within a few months post-hatch. Spawning is triggered by water temperature (usually 18°C or higher), after which continuous spawning can occur if favourable conditions persist [14]. Males prepare and aggressively guard nests – such as the undersides of submerged rocks and branches, cleaned to facilitate egg adhesion [13]. Mating involves the inversion of the female by repeated nudging from the male's nuptial tubercles, after which the eggs are lain on the clean underside of the nest. Though the intensive pre-parental care tends to lead to greater mortality in male specimens, females are capable of continuous reproduction during the spawning season.

The fathead minnow has become an essential model species in fish toxicology assays, owing to its short lifespan, wide distribution, and the facility with which their breeding can be controlled by altering water temperature [14,15]. Due to the wealth of toxicity data available on this species, this species was selected for the FOREST project to model the effectiveness of shoreline cleanup methods applied during a diluted bitumen spill.

5.2.3 Experimental design

Nine shoreline enclosures measuring 5 x 15m were deployed in two areas representing the predominant shoreline substrate compositions: peat/organic and rock/cobble (18 enclosures total). Enclosures (Currie Industries, Winnipeg, Canada) consisted of floatation collars with impermeable polypropylene curtains which were sealed to the sediment with sandbags. The enclosures extended 5m onto shore and enclosed 5 x 10m areas of lake water, with a maximum depth of approximately

2m. Enclosure volumes were estimated by spiking tritium: this data is shown in Figure S9, and methodologies are described in Rodriguez et al (2019) [16].

Gee-style minnow traps were used to remove fish trapped inside the enclosures for one week prior to stocking. For enclosure stocking, fathead minnows were trapped from L260 with minnow traps and seine nets, weighed and measured, and tagged with a fluorescent tagging dye for identification. Ten fish were then released to swim freely in each enclosure (5 males and 5 females) for the duration of the study, 75 days.

Dilbit was weathered in 1.2m diameter aluminium pans for 36h by pouring approximately 19kg of fresh dilbit over 220L of lake water. Samples of water accommodated fractions (WAF) of PACs were collected from under the slick after 36h for PAC analysis. Weathered oil was then collected from the surface of the pans using stainless steel utensils, transferred to glass bottles, weighed and transported to the lake. Approximately 1.4kg dilbit was applied to each of 7 enclosures in the two different shoreline environments. Oil was applied to the surface of the water in each enclosure within 50cm of the shoreline using a telescopic bottle holder to ensure even spatial distribution.

No cleanup action was taken for 72h to approximate conservative oil spill response times. After 72h of weathering and interaction with the shoreline, free floating dilbit was removed from the surface of the water using pre-weighed adsorbent oleophilic pads (Spill Ninja, MEP Brothers, Winnipeg, Canada) and low pressure washing of the shorelines with freshwater (1200L delivered through a manifold that traversed the entire width of the enclosures). No attempt was made to remove oil adhered to shoreline substrates or vegetation in the enclosures. Following this primary cleanup, secondary remediation methods were applied to six enclosures at each site: three

enclosures were treated with COREXIT EC9580A shoreline cleaner, which was applied to the remaining oil on the shoreline according to manufacturer recommendations. After allowing the cleaner to interact with the oil for 30 min, oil was again rinsed into the aquatic environment using low pressure freshwater flushing and free floating product was captured with pre-weighed oleophilic pads as described above. Enhanced monitored natural recovery (eMNR) was implemented in three other enclosures. eMNR involves the application of nutrients to stimulate resident bacterial assemblages to enhance microbial degradation of oil constituents. The three enclosures treated with eMNR of the two shoreline environments received ~65g of Scotts Osmocote® Controlled Release Fertiliser (Marysville, Ohio). This rate of addition was chosen to approximate nutrient additions that approximated the Redfield ratio (100:10:1 C:N:P), with the assumption that 87% of the unrecovered oil was added carbon. The fertilizer was added as a one-time broadcast application within 50cm of the shoreline. Finally, secondary remediation of a single enclosure in each shoreline environment was initiated by installing an Engineered Floating Wetland (EFWs). EFWs are intended to facilitate proliferation of a rich bacterial colony on their exposed roots that can help to facilitate oil degradation (reference). Two reference enclosures, untreated with oil, were included in each of the two shoreline environments. The treatment scheme is shown in Figure 5.2.

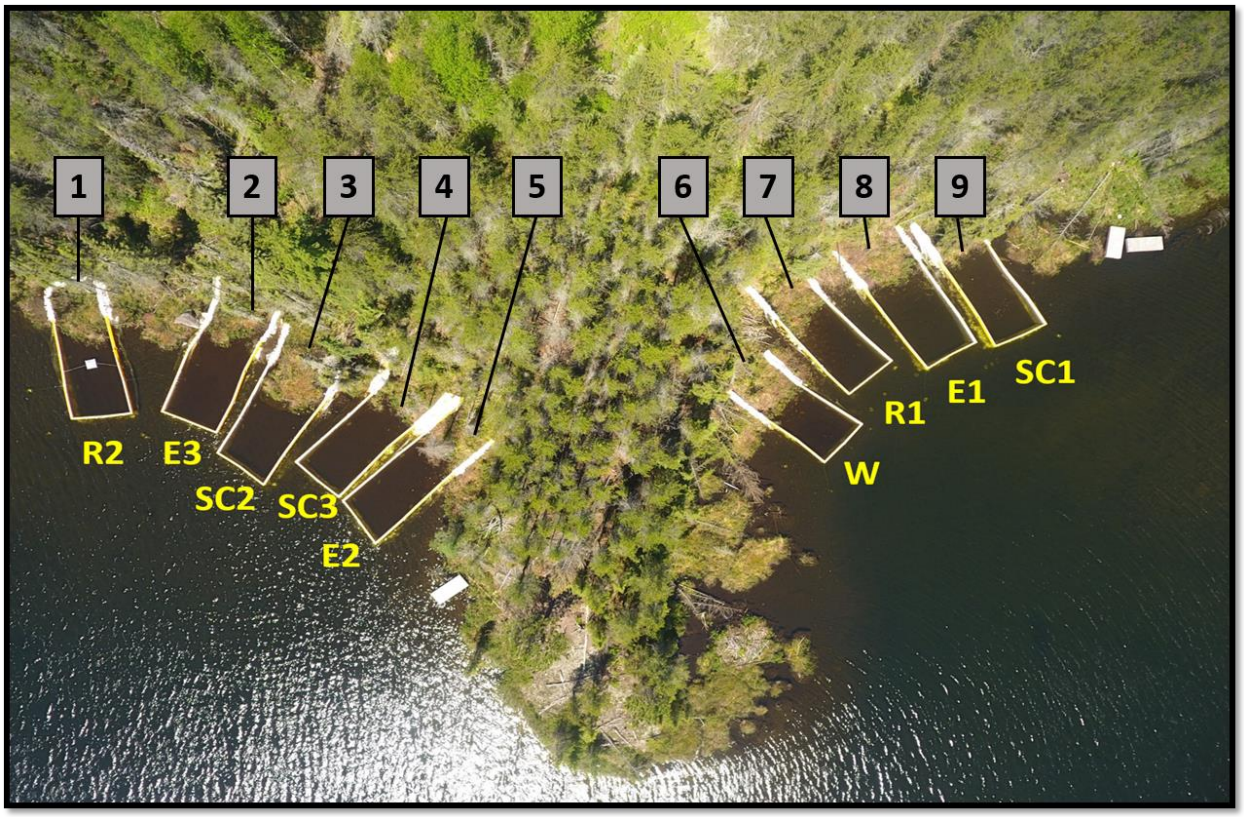

Figure 3.2 FOReSt dilbit exposure study treatment scheme for enclosures in the wetland shoreline environment. Codes refer to secondary remediation method applied after low pressure shoreline washing and adsorbent pads were used to remove free floating dilbit . R = reference (no dilbit), E = enhanced monitored recovery (nutrient addition), SC = COREXIT EC9580A shoreline cleaner, W = engineered floating wetland.

5.2.4 Fish sampling and dissection

At the end of the experimental period, fathead minnows were removed from the enclosures using minnow traps and poll seines. Collected fish were placed in plastic bags full of lake water, oxygenated with pure O_2 and transported (<1hr) to the field station in coolers. Upon arrival, fish were individually euthanized with an overdose of pH neutralized (7.0) MS-222 (0.4g/L) and mucus was collected from one entire side of the fish using Floq-Swabs (COPAN Diagnostics Inc., Murrieta CA). Fish were then weighed and measured (total and fork length). The presence of a

dye tag was verified using a fluorescent lamp to determine that collected fish were the originally stocked fish and not incidental bycatch. Liver, intestine, fin, gill, and gallbladder were dissected from each fish. Fish carcasses were frozen at -20°C for PAC body burden determination. All tissues were placed on dry ice immediately and stored at -80°C until analysis.

5.2.5 Materials and reagents

For the analysis of F_2-IsoPs, the following chemicals were purchased from Fisher Scientific (Fair Lawn, NJ): hydrochloric acid, HPLC-grade water, phosphate buffered solution (PBS), butylated hydroxytoluene (BHT), chloroform, ethyl acetate, and KOH. Analytical standards were purchased from Cayman Chemicals (Ann Arbor, MI): (±) 5-iso-prostaglandin-$F_{2\alpha}$-VI, (±) 5-iso-prostaglandin-$F_{2\alpha}$-VI-d_{11}, 8,12-isoPF-VI, 8,12-isoPF-VI-d_{11}, 8-iso-prostaglandin-$F_{2\alpha}$, 8-iso-prostaglandin-$F_{2\alpha}$-d_4, ent-8-iso-15(S)-prostaglandin- $F_{2\alpha}$, and ent-8-iso-15(S)-prostaglandin- $F_{2\alpha}$-d_9 (all >98%). These compounds are referred to herein as 5-isoP, 8,12-isoP, 8-isoP, and *ent*-15-isoP

For PAC analysis, sodium chloride and sodium sulphate were from Fisher Scientific. A detailed listed of all PAC analytes and deuterated analogues can be found in Tables S11 and S12.

5.2.6 Protein content

Total protein content was determined with a modified Bradford-Lowry method described in Chapter 4. Briefly, absorbance at 450nm and 590nm was measured, and the ratio of A_{590}/A_{450} was used to interpolate sample concentrations.

5.2.7 Water PAC chemistry

Enclosure water was collected from nearshore and far-shore sampling ports using peristaltic pumps and PTFE tubing dedicated to each enclosure. Samples were stored in 1L amber glass bottles and shipped on ice to Winnipeg for extraction within 48h of collection. Water was

samples were stored in a temperature controlled room set to 4°C upon arrival at the University until extraction. In the laboratory, 250ml aliquots were first filtered through 0.45µm polycarbonate filter membranes. Samples were then transferred to 1L separatory funnels. Sodium chloride (10g) was added and PAC internal standards were spiked (final concentration 100 ng ml^{-1}). Water was extracted with 2x50ml dicholoromethane. Shaking was performed for 1 minute, twice, for each volume of DCM. The DCM layer was collected in 125ml round bottomed flasks, concentrated to approximately 2ml via rotary evaporation and dried with sodium sulphate (pre-baked for 6h at 250°C). The remaining volume was transferred to a glass test tube, and the round bottom flask was further rinsed thrice with 2ml hexanes. Samples were then blown down under a nitrogen stream to a final volume of 1ml, transferred to GC vials and spiked with instrument performance internal standard (IPIS).

5.2.8 Mucus: F_2-Isoprostanes

Samples were thawed and kept on ice during extraction. Swabs were first spiked with butylated hydroxytoluene (BHT), to reduce autooxidation of isoprostanes. To assess analyte loss during the extraction process, 10ng of the isoprostane metabolite 2,3-dinor-isoPG was spiked into each sample (this compound was not detected in mucus in any previous experiments) as a recovery internal standard (RIS). The swabs were extracted by vortexing twice in 0.5ml volumes of PBS buffer (0.1M, pH 7.4). The swab was additionally rinsed with 0.5ml PBS and blotted dry on the tube walls. Aliquots were centrifuged for 20min at 4°C, after which 50µl supernatant was removed for total protein determination. Phospholipid hydrolysis was performed by adding 2ml of 1M KOH and incubating samples at 45°C for 45min. Samples were then acidified to pH 3 with 1M HCl and spiked with deuterated isoprostane internal standards (final concentration 2.5 ng ml^{-1}). Samples were purified using SPE: 3cc Oasis HLB cartridges were first preconditioned with 3ml methanol

and 3ml 1mM HCl, washed after sample loading with 1mM HCl and hexanes. Then F_2-IsoPs were eluted with 3ml ethyl acetate, blown down to dryness under nitrogen and reconstituted in 100µl mobile phase.

5.2.9 Liquid chromatography – mass spectrometry

Samples were analysed with a Varian 1100 HPLC system coupled to a SCIEX API 365 triple quadrupole mass spectrometer with a custom orthogonal ionization source, in negative ion mode. Source temperature was 500°C, and injection volume was 50µl. Analytes were quantified using MRM, with quantitative and confirmation ion transitions listed in Table S9. Separation of analytes was achieved using an XSelect® HSS T3 C_{18} (3µm x 2.1mm x 50mm) column. The HPLC solvent gradient was: 30% B for 1min, increased to 60% B from 1-7min, 80% B by 18min, 100% B from 18.5-20min. The column heater was maintained at 42 ±0.1°C.

5.2.10 Normalization of concentrations

Concentrations in ng ml^{-1} measured by the mass spectrometer were normalized by weight and by protein content. The two methods were then compared to each other to determine if one produced a clear advantage in terms of intra-group variability. Mass normalization is common for analyses of tissues. Protein normalization has been used for urinary analysis of F_2-IsoPs, using creatinine concentration [17,18], and was selected under the assumption that it might mitigate variability caused by water collected by the swabs along with the mucus. Since the protein composition of mucus is not well known, total protein was used in this study, as measured by the modified Bradford-Lowry method.

5.3 Results

5.3.1 Tritium – enclosure volumes

Tritium concentrations over time revealed that significant leakage occurred in the rock/cobble enclosures, decreasing to nearly zero by day one post-exposure. By contrast, concentrations declined much more slowly in the wetland enclosures. For this reason, the results and discussion sections below will focus only on enclosures from the wetland shoreline area.

5.3.2 Water chemistry

Polycyclic aromatic compounds were monitored daily for the first week of exposure to capture the equilibration of PACs in the enclosures, after which intervals between sampling session were increased incrementally. Pre-exposure TPAC ranged was 27-66 ng/L in seven samples but enclosures 5,6, and 9 had much higher baseline levels, averaging 228 ng/L. Total PAC concentrations peaked 6 days after oil application, with levels exceeding 12,000 ng/L in enclosure 6 (Figure 5.3). A spike was also observed in control enclosures and lake reference samples on this day, despite having no added dilbit: these changes less pronounced, reaching only approximately 2000 ng/L, but still represented a 50-fold increase relative to pre-spill levels. TPAC concentrations generally remained elevated until sampling day 20, where a record rainfall event caused an increase in concentration. PACs were likely released from leftover dilbit disturbed by the precipitation and distributed throughout the enclosures. By day 66 post-exposure, TPACs in dosed enclosures decreased significantly and remained low for the remainder of the monitoring period. When fish were sampled for isoprostane analysis in September, TPAC concentrations in oil treated enclosures ranged from 157-221 ng/L with the exception of enclosure 6 at 377 ng/L, while control enclosures and lake reference samples ranged from 33-66 ng/L. Relative to pre-exposure levels, concentrations in dosed enclosures were therefore still elevated: four enclosures had TPAC

concentrations 2.6- to 9.2-fold greater than baseline. Concentrations at reference sites returned to within 33-66 ng/L.

Table 3.1 Wetland enclosure TPAC concentrations pre-exposure and during fish sampling

Enclosure	Treatment [a]	TPAC: day -3[b]	TPAC: day 87[b]	% difference
lake reference	lake ref.	57.91	65.93	14
1	ref.	65.45	33.34	-49
2	emnr	48.22	220.78	358
3	sc	54.68	201.28	268
4	sc	19.66	200.41	919
5	emnr	290.16	201.87	-30
6	efw	290.24	377.82	30
7	ref. (efw)	27.39	40.97	50
8	emnr	34.43	182.33	430
9	sc	204.70	157.33	-23

[b] emnr = enhanced monitored natural recovery; sc = COREXIT EC9580A shoreline cleaner; efw = engineered floating wetland

[a]Concentration in ng/L

As expected for weathered dilbit, PAC composition was dominated by alkylated compounds in enclosures after oil application, accounting for > 90% of TPAC: parent compounds followed the same temporal trend however concentrations only exceeded 200 ng/L in enclosures 4 and 9. Even in control enclosures and at the lake reference site, parent PAC relative abundance of TPAC decreased from 25-50% to < 2% by day 6, before gradually returning to pre-exposure concentrations. Four-ringed PACs accounted for approximately 33% of the total, followed by 2-

and 3-ringed PACs respectively. Compounds with five or more rings were at far lower concentrations, generally never exceeding 50 ng/L, or less than 0.5% of total PACs.

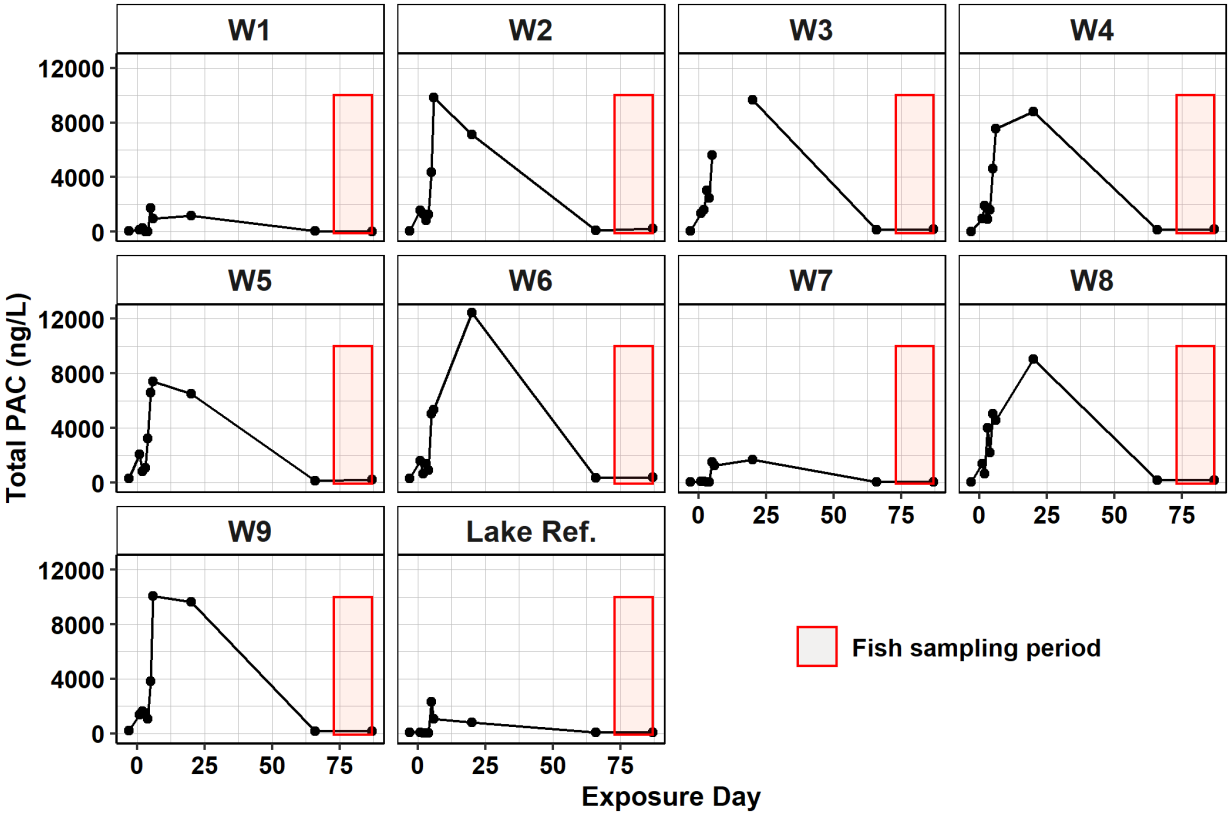

Figure 3.3 Total PAC concentrations in water from the wetland enclosures over time.

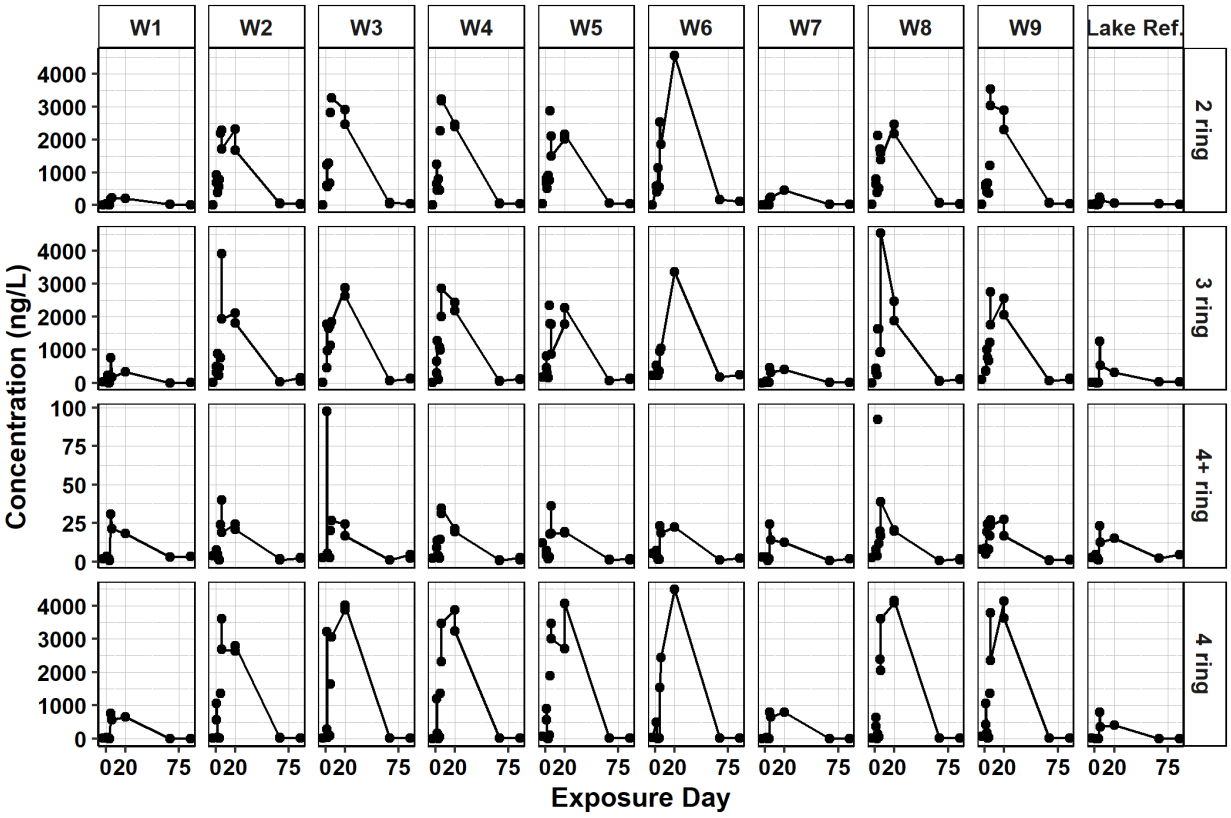

Figure 3.4 PAC concentration time lapse in the enclosures according to number of aromatic rings.

5.3.3 Fathead minnows

Tagged fathead minnows were only recovered from two enclosures at the wetland site, W3 (SC2) and W4 (SC3), for a total of eleven fish or a retrieval rate of 6%. Mortalities were not observed, and so it is unclear if tagged fish perished over the course of the exposures. However, tritium concentrations in the enclosure declined, indicating that some leakage may have occurred and suggesting that escape may have contributed to the low recovery rates of tagged fish. A total of 148 were fish were recovered from the wetland enclosures, with the majority being untagged fathead minnows and finescale dace (*Phoxinus neogaeus*). A small number of pearl dace (*Margariscus margarita*) and lake chub (*Couesius plumbeus*) were also recovered, however due

to their small numbers were not analyzed. Apart from one enclosure (enclosure 8), fathead minnows and finescale dace presence was mutually exclusive in relation to enclosures. Finescale dace and fathead minnows were selected for further analysis, as the former was the selected species for the FOREST study and the latter was the species of interest in a previous dilbit study conducted at the IISD-ELA in 2018.

5.3.4 LC-MS method efficacy

Internal standard recoveries for 5-isoP, 8-isoP, and *ent*-15-isoP ranged between 70 and 95%, and 8,12-isoP recovery was 60%. The RIS spiked at the very start of the extraction process, 2,3-dinor-8-isoPG, had an average recovery of 73 ± 10%. These results indicate that isoprostanes were efficiently extracted from mucus using this protocol.

The two normalizing methods using mucus mass and protein showed weak correlation between paired data points with a correlation $R^2 < 0.1$. Despite this, mean concentrations followed similar trends for both methods. Protein normalized concentrations (units of ng mg^{-1} protein) are presented in the following sections.

5.3.5 F_2-isoprostanes in fathead minnows

Concentrations of F_2-IsoPs did not differ significantly between tagged and untagged fathead minnows in either enclosure from which they were recovered, nor did they differ between enclosures in tagged fish. Treatment effects were considered constant, as both enclosures were treated with the same secondary remediation method (COREXIT EC9580A shoreline cleaner) and had TPAC concentrations in September within 0.5% of each other. Interestingly, a small but significant difference in levels of *ent*-15-isoP was observed between fish of these enclosures as a whole, with concentrations being 40% greater in enclosure 4, but not in any other isomer.

Type III isomers 8-isoP and *ent*-15-isoP concentrations showed some differences between enclosures, as shown in Figure 5.6: mean 8-isoP concentrations were 1.8- to 2.8-fold greater in enclosures 8 and 9 relative to the other three, though sample sizes were considerably smaller in enclosures 5, 8, and 9. Type VI isomers showed no change between enclosures, however for both regioisomer classes, all enclosures differed significantly from lake reference fish, ranging from 2-4 times lower in the enclosures. Within the enclosures, there was no significant trend between mean isoprostanes and TPAC concentrations, though 8-isoP showed a near-significant decreasing trend ($p = 0.058$, $R^2 = 0.67$). Other variables such as sex, condition factor, HSI, and GSI were not found to significant effects on the observed concentrations.

5.3.6 F_2-isoprostanes in fine scale dace

Though fine scale dace were not stocked in the enclosures, they were recovered in sufficient numbers to warrant analysis. Mean concentrations of mucus isoprostanes were similar to those of fathead minnows, ranging from 0.05-0.15 ng g^{-1} protein for Type III isomers. No significant differences were observed for any isomer between dosed enclosures, however concentrations were significantly elevated in relation to the reference enclosure 1 and lake references fish (see Figure 5.6). Notably, 8-isoP and *ent*-15-isoP concentrations in fish from enclosure 7 (reference with engineered floating wetland) were also elevated compared to the other reference fish and were statistically indistinguishable from dosed enclosures. In contrast with the fathead minnows, mucus F_2-IsoPs in lake reference fish were much lower: while only significant differences were observed between lake reference and enclosures 6-8, concentrations were 2.5- to 8-fold greater in enclosures, with the exception of enclosure 1. Due to the lack of overlap in species in the enclosures, no further comparisons can be made between the two species.

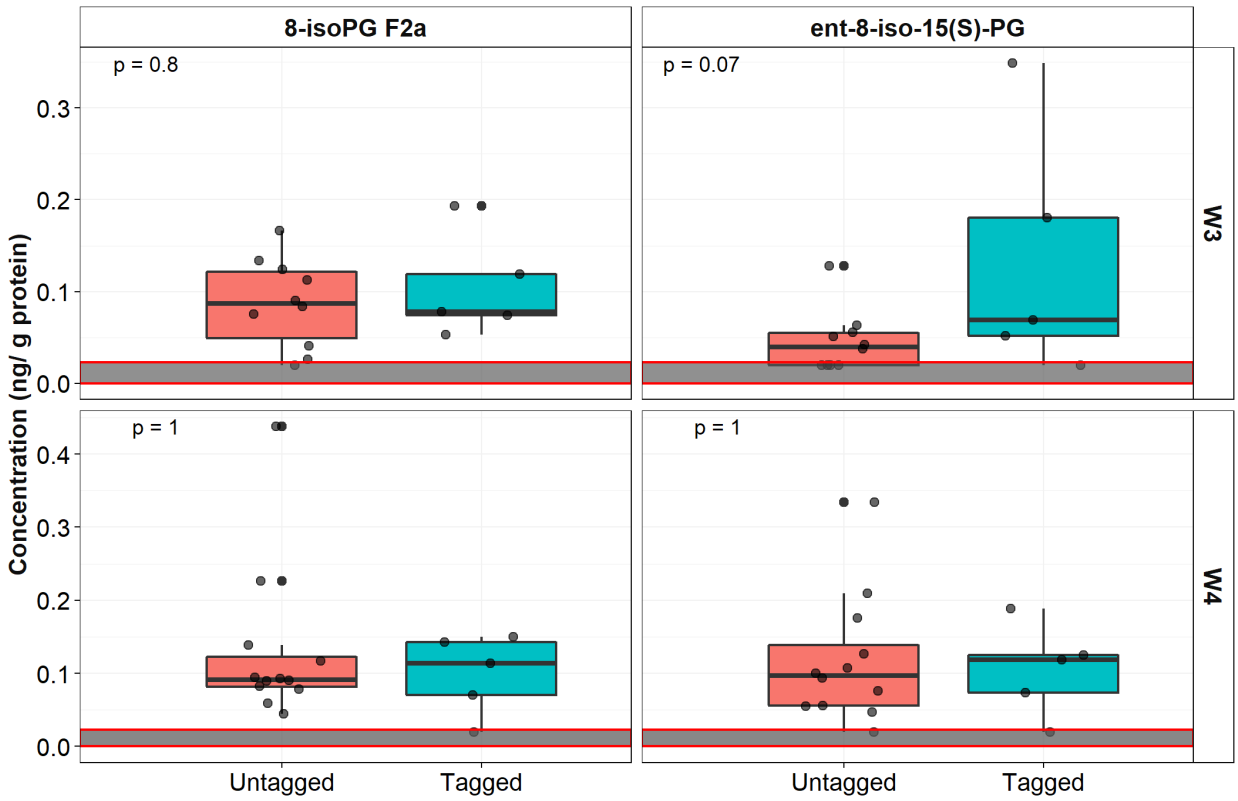

Figure 3.5 Comparison of two F_2-isoprostane isomers in mucus from tagged and untagged fathead minnows. Data points in the grey shaded area are below the method's detection limits. Tagged fathead minnows were only recovered from enclosures 3 and 4. Statistical significance is shown in the top left of each panel ($p < 0.05$ is significant).

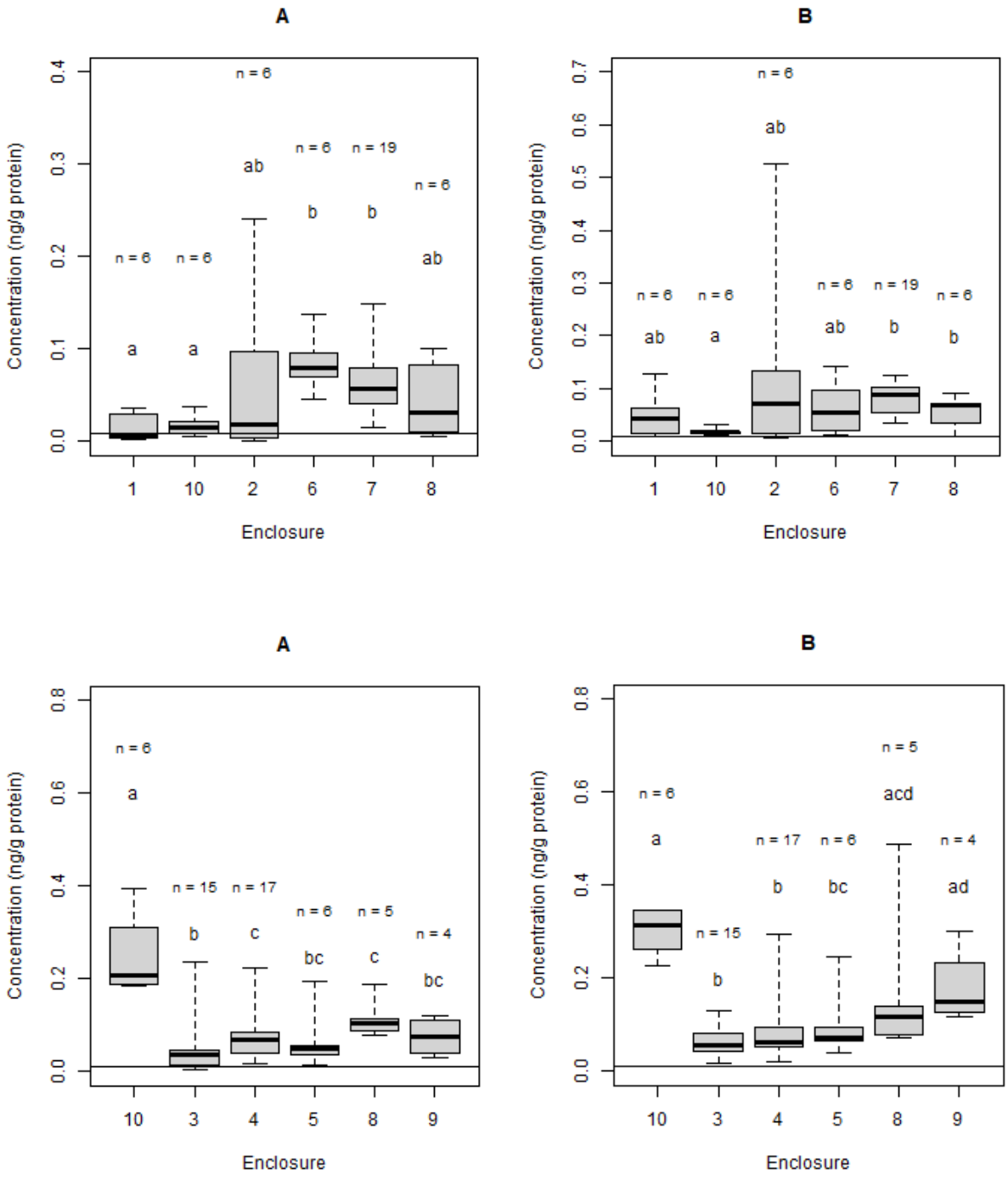

Figure 3.6 Concentrations of Type III F_2-isoprostanes (**A**: *ent*-15-isoP, **B**: 8-isoP) in mucus of finescale dace (top) and fathead minnows (bottom). Box plots labelled with different letters are significantly different from each other. Statistical significance was determined using survival analysis to account for censored data. Significance level is $P < 0.05$.

5.4 Discussion

5.4.1 Method efficacy

Acceptable recoveries of all internal standards indicated that SPE successfully extracted isoprostanes from mucus. The recovery standard also showed that losses during sample preparation were small, as a combined analyte loss and matrix effect of only 23% was achieved. Overall, the developed extraction protocol was effective for mucus samples collected with swabs. Normalization to protein content was used to correct for variable water content in the mucus samples and for any impurities that may contribute to mass. However it was not a good predictor of isoprostane concentration in samples, showing no correlation whatsoever, and had no effect on variance within enclosures. Lipid normalization has been used in other studies of isoprostanes, but may require larger sample sizes than what can be practically collected from small bodied fish.

5.4.2 Fish recovery: implications for data interpretation

The recovery of only 6% of tagged fish from our enclosures has serious implications when interpreting the presented data. The steady decline of tritium in the enclosures suggests that small openings were present in the enclosure perimeters, which could have allowed fish to enter and escape from the enclosures during the exposure period. It is therefore possible that not all fish sampled were exposed to the same extent to dissolved PACs, particularly if fish entered the enclosure after day 66 post-exposure when concentrations had returned to baseline levels. Likewise, we cannot assume mortality for unrecovered fish due to the possibility of escape. It is unlikely that the untagged fish are young of the year who spawned in the enclosure: mean total length was 57mm for both male and female untagged fatheads recovered. According to Smith (1978), fatheads typically reach 45-50mm by 90 days post-hatch and begin spawning when water temperatures reach 18°C. Mean enclosure water temperature at day zero was 18.4 °C, and fish

were retrieved starting on day 71 post-exposure, therefore the minnows are unlikely to have had sufficient time to grow to the observed mean size. This eliminates potential confounding effects associated with exposure to PACs during peak development period.

5.4.3 Water chemistry

Baseline [TPAC] (i.e. prior to oil additions) were relatively constant, with the exception of three enclosures: in enclosures 5, 6, and 9 [TPAC] was approximately five times greater with an average of 223 ng L^{-1}. The occurrence of these higher baseline levels was not limited to one of the two small bays in which the enclosures were deployed, nor were they in directly adjacent enclosures (see Figure 5.2). Therefore it is unlikely that contaminants were introduced unintentionally during enclosure setup. It is possible that greater disturbance of highly organic sediments occurred in these enclosures causing enhanced release of PACs from the sediment, as each enclosure required different levels of physical disruption to ensure proper sealing to the bottom sediment and shorelines with variable morphology. Pending analyses on soil and sediment PAC should provide further insights on these discrepancies between baseline levels in different enclosures. However, it should be noted that [TPAC] of 223 ng L^{-1} is still considered very low: US EPA drinking water guidelines for benzo[a]pyrene alone are 200 ng L^{-1} [19].

The predominance of alkylated PACs in the enclosures is consistent with expected distributions of PACs in dilbit, where alkyl-PACs can account for 85-95% of the total [20]. The size distribution of alkyl-PACs in water was dominated by the 2-4 ring compounds, which ranged between 2-4μg L^{-1}, with 5-ring compounds occurring at much lower concentrations. As observed by Conmy et al (2017), major alkylated dilbit constituents were naphthalenes (2-ring), and phenanthrenes and dibenzothiophenes (3-ring) [21]. However, [PAC] in the enclosure water was

actually dominated by C2-pyrenes, which accounted for ~33% of [TPAC], which is greater than has been reported in other studies [21–23].

Total PAC concentrations reached a maximum of 12.4 μg L^{-1} on day 5 post-addition. The increase over the 0-5d period is likely a factor of natural dispersion processes and wind speed and direction: from June 21 to June 26, (days 0-5), the wind direction gradually changed from the southeast to a westerly wind: the latter direction would help disperse dilbit adsorbed to the shoreline into the enclosure, releasing PACs. Concentrations also spiked by a factor of 40-50 in control enclosures and the lake reference samples, which may be a result of incomplete sealing of enclosures, or atmospheric deposition. Maximal gust speed measured in Kenora, ON on day 5 was 61 km/h, the highest recorded for June 2019 – a windier day may have led to the observed spike in concentration (Kenora RCS Weather Station, Environment and Climate Change Canada). Several studies have demonstrated chronic toxicity at this concentration range in early life stages of fish: malformation occurrence EC50 values of 20.3μg L^{-1} and 0.6-0.9 μg L^{-1} were determined experimentally in medaka and fathead minnow embryos, respectively [7,24], while Alderman et al (2015) demonstrated that Pacific salmon parr exposed to dilbit WAF at concentration of 3.5μg L^{-1} had elevated EROD activity after four weeks and upregulated cardiac *cyp1a* at 16.45 μg L^{-1} after the same period [25]. However, it should be noted that embryonic stages are more susceptible to toxic effects due to the high rate of cellular division and biological systems development, and that data on adult life stages is lacking. Furthermore, as seen in Figure 5.3, [TPAC] in the enclosure returned to < 0.36μg L^{-1} by day 66 post-exposure, which may have implications on the F_2-IsoP levels observed in the fish retrieved from the enclosures and is further discussed below.

5.4.4 F_2-isoprostanes in mucus

It is widely accepted that PAC toxicity is partially related to induction of CYP450 enzymes, which gives rise to reactive intermediate species including semiquinones that can undergo redox cycling and produce large amounts of ROS [26,27], or cause dysregulation of other genes associated with oxidative stress [28]. Several studies have linked F_2-isoprostane levels in humans with PAC-induced toxicity, but these have focused primarily on air pollution [29,30]. As discussed in the previous section, embryotoxicity of dilbit has already been associated with [TPAC], but F_2-IsoPs have not been studied in this field.

In our experiment, there was no clear association between mucus F_2-IsoPs and [TPAC] nor with treatment. 8-isoP was elevated by 1.8- and 2.8-fold in fathead minnows from enclosures 8 and 9 (shoreline cleaner and eMNR, respectively) relative to fatheads in other oiled enclosures, however the differences were not significant for *ent*-15-isoP. Additionally, these two enclosures actually had lower [TPAC], though all oiled enclosures were < 0.36μg L^{-1}. The return of [TPAC] to baseline levels by the time fish were collected possibly contributed to the lack of effect observed in our experiment, as this is below experimentally determined threshold concentrations determined for embryotoxicity in other studies. Ideally, fish should have been collected between days 6 to 30 post-exposure, as [TPAC] were in a range shown to cause chronic effects (see Figure 5.3). Unfortunately, this was not possible due to the disruptive effects of fishing out the enclosures, which could have compromised other components of the FOReSt project.

Though only 6% of tagged fathead minnows were recovered, some inferences can still be made regarding the effects of dilbit. As Figure 5.5 demonstrated, there was no statistical difference in mucus F_2-IsoPs between tagged fish and untagged fish in enclosures 3 and 4. Regardless whether

untagged fish were not exposed as long, the lack of change suggests that neither dilbit components nor secondary remediation caused chronic changes in F_2-IsoP levels.

Surprisingly, lake reference fatheads had elevated concentrations of all four measured F_2-isoprostane isomers relative to enclosure fish: the mean concentration was 2 to 4 times greater than any other enclosure. The total lack of fatheads retrieved from reference enclosures is problematic in trying to explain this observation, as enclosure and treatment effects cannot be differentiated. The later collection date of the lake reference fish – on September 23-25 – due to logistical reasons likely did not account for this discrepancy in mucus F_2-IsoPs. Environmental conditions such as temperature and changes in dissolved oxygen (DO) are known to impact oxidative status [31–33], however until fish collection from the enclosures, lake temperature was within 1°C of enclosure water, a deviation that is unlikely to account for such a difference in oxidative status based on the available literature [34–36]. Likewise, DO levels in the enclosures differed from the general lake environment by 0.1-1.6 mg L^{-1}, and exceeded 5.6 mg L^{-1} five days prior to fish collection from the enclosures, which is well above the threshold for even moderate hypoxia. It could be argued that the two week difference may have been sufficient to alter the oxidative status of the lake reference fish in a measurable way, however this trend was not observed – and in fact opposite – in the reference fine scale dace that were collected in the same time period.

A more likely experimental explanation for the elevated F_2-IsoPs in lake reference fatheads is that these fish were collected prior to a belated delivery of dry ice, and were consequently stored at only 0°C for a few hours. The effects of storage temperature on autooxidation of F_2-IsoPs has been well documented [37], therefore it is not unlikely that concentrations in these samples are artificially elevated as a result of this extenuating circumstance. This is supported by the findings with the finescale dace, who were only collected September 25 and had very low F_2-IsoP.

Mean F_2-IsoP concentrations in finescale dace were slightly lower than in fathead minnows but the two species were only concomitantly retrieved from enclosure 8. Given the apparent lack of treatment effects in both species, it is possible that baseline levels of F_2-IsoPs are lower in finescale dace. Differences in reproductive strategies are a possible contributor to the observed difference, as finescales breeding typically ends in June while fathead minnows are fractional spawners whose mating season overlapped with the exposure period. Reproductive behaviours have been shown to alter oxidative status of other fish species such as smallmouth bass (*Micropterus dolomieu*) [38], though further studies would be required to determine if and how these species might differ.

Interestingly, 8-isoP and *ent*-15-isoP in enclosure 7 – a reference control containing an EFW – were significantly elevated relative to lake reference fish and the other reference enclosure (*ent*-15-isoP only). Mucus samples from the two EFW enclosures (6 and 7) yielded the fewest proportion of non-detects out of all the enclosures and had among the highest concentrations of all F_2-IsoPs. Given that no dilbit was applied to enclosure 7, these findings might point to an enclosure effect associated with the EFW itself, though it bears underlining that the uncertain origin of the finescale dace in the enclosures detracts from the weight of these findings.

To conclude, this study found that F_2-IsoPs in fathead minnow epidermal mucus were not impacted significantly by an environmentally relevant concentration of dilbit after 80 days. It is unknown if F_2-IsoPs spiked prior to [TPAC] returning baseline conditions on day 66 and subsequently recovered, or if no change occurred throughout. More studies in complex environmental settings should place an emphasis on measuring F_2-IsoPs at regular intervals during the exposure period to accurately capture the biomarker response in exposed fish. The low recovery of tagged fish and possible intrusion of fish into the enclosures also confounds the

interpretation of these results, since exposure to dilbit cannot be guaranteed in untagged individuals. Data from study components taken on by other members of the FOReSt project focusing on the expression of *cyp1a* and PAC uptake, when available, will provide additional insights into the toxicity and oxidative impacts of dilbit and secondary remediation.

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Chapter 6: Synthesis and considerations for future studies

6.1 Summary of findings

In recent years, the growing demand for study into the effects of crude oil spills in freshwater ecosystems has highlighted key knowledge gaps in the literature. One of these is the lack of background data, which complicates the quantification of toxicological effects and assessing the recovery of impacted fish populations. Though more sampling is desirable to establish background databases, conventional monitoring practices used in marine settings are not always appropriate in freshwater ecosystems, as lethal sampling practices can threaten the health of smaller fish populations. Epidermal mucus has shown promise as a non-lethal matrix for measuring oxidative stress in fish, however few studies consider the F_2-isoprostanes, which are considered the most reliable indicator of lipid peroxidation *in vivo* in mammals. This thesis addressed these knowledge gaps by developing a method for quantifying F_2-IsoPs in epidermal mucus and testing it in fish exposed to dilbit.

Chapter 2 served as a first step, as we tested the hypothesis that F_2-IsoPs were present in the epidermal mucus of fish. In this study, we developed and validated a method to extract F_2-IsoPs and demonstrated they were quantifiable in lake trout (*Salvelinus namaycush*) mucus collected from pristine boreal lakes. We successfully resolved and quantified three isomers (8-iso-PG-$F_{2\alpha}$, 5-isoPF-VI, and *ent*-8-iso-15(S)-PG-$F_{2\alpha}$) using HPLC-MS/MS. The HPLC-MS/MS method was shown to be robust and repeatable, and only small matrix effects were noted. This study served as a proof of concept that F_2-IsoPs were present in detectable concentrations in freshwater fish species in unpolluted environments and thus should be considered for baseline monitoring of fish populations, which could help address a crucial data gap in how we currently monitor recovery in fish populations after a dilbit spill.

In Chapter 3, the effect of acute physical stressed on oxidative stress biomarker responses was examined in 18-month old rainbow trout (*Onchorynchus mykiss*). F_2-IsoP concentrations were compared between three tissues (plasma, liver, and gills) and to glutathione, another biomarker of oxidative stress. Cortisol was also quantified in the plasma to assess stress in the fish. Liver F_2-IsoPs were elevated 4h after trout were exposed to 5 minutes of netting, chasing, and emersion, then returned to baseline levels by 48h, suggesting a short-lived mild oxidative stress response. In the gills, F_2-IsoP and glutathione concentrations decreased in stressed fish after 4h, and remained depressed even after 48h, which suggests a link between the two biomarkers and a possible susceptibility to oxidative stress in this tissue, given the longer duration of the response. To our knowledge, this was the first time F_2-IsoPs were quantified *in vivo* in gills. These findings demonstrated that acute physical stress can be a confounding variable when analyzing F_2-IsoPs in fish, which is an important consideration for the design of future studies. They also showed that biomarker responses are complex, as they are expressed differently depending on sampling time and tissue type.

In Chapter 4, an in-laboratory exposure of juvenile fathead minnows to a range of dilbit concentrations was performed. F_2-IsoPs were extracted and quantified from liver and epidermal mucus. The methodology used in Chapter 2 was refined: changes included the use of swabs to collect the mucus and an SPE-based extraction protocol instead of LLE, to include phospholipid-bound isoprostanes. F_2-IsoPs were successfully quantified in both matrices, however concentrations did not change significantly from the baseline group in any of the treatments after the 7-day exposure. We noted that biliary PAC metabolite concentrations did not increase proportionally to exposure dose as in other studies, identifying this as a potential reason for the lack of observed response. Despite the lack of dose-dependent response, other findings proved

interesting. In the control fish not exposed to dilbit, positive correlations were observed between F_2-IsoP concentrations in the liver and epidermal mucus, while weak negative correlations occurred in the fish from the high dilbit dose. The association between these two tissues should be studied further to determine how this correlation is affected during the oxidative stress response, and if it can be exploited to utilize non-invasively collected epidermal mucus rather than liver in the future.

In Chapter 5, we explored the effects of dilbit on fathead minnows (*Pimephales promelas*) using concentrations of dilbit typical of pipeline spills and the effects of different secondary remediation methods on potential toxicity. Due to a large amount of bycatch from possible intruding fish, finescale dace (*Phoxinus neogaus*) were also assessed. F_2-IsoPs were successfully quantified in both species, but after 80 days of exposure there was no clear effect on the epidermal mucus concentrations of F_2-IsoPs. Instead, differences between enclosure fish and lake reference fish were seen, indicating an effect of the enclosures themselves. Given that TPAC concentrations had returned to baseline levels by day 66 post-exposure, these findings suggest that dilbit exposure does not cause lasting changes to the F_2-IsoP concentration in mucus and that future studies are needed to properly assess the response of these compounds during the peak TPAC concentrations.

6.2 Recommendations for future studies

6.2.1 Fish models

In this thesis, F_2-IsoPs were successfully quantified in four freshwater fish species. The most pertinent of these to future laboratory studies are the fathead minnow and the rainbow trout: due to their widespread use in toxicology research [1–3], they benefit from extensive databases including genome sequencing, DNA libraries, and established methods, all of which facilitates

comparing results to other studies. Other fish models with such advantages include the zebrafish and the medaka [3,4]. Using one or several of these fish models would be especially valuable during the earlier stages of research, for example to elucidate on F_2-IsoP production and transport in the body, to determine if they are produced *in situ* in epidermal mucus, and how F_2-IsoP response relates to other biomarkers – particularly if toxicity mechanisms are known for the selected biomarkers and the chosen stressor. In the case of dilbit exposure in freshwater environments for example, a consideration when selecting a fish model species might be whether non-native medaka and zebrafish are as relevant as fathead minnows when considering habitats in North America.

Though lacking some of the advantages of the model species listed above, fish species can be selected depending on the desired application and scientific context. Fish models excel at producing generalizable mechanistic information on the effects of contaminants on fish physiology, however results from laboratory studies are often limited in their application to fish populations in real-world environments where complex environmental variables including reproductive strategies, inconsistent diet, and morphological plasticity can alter the response of fish to contaminants [5,6]. Moreover, certain behaviours which may affect F_2-IsoP – and other biomarkers' – response (such as spawning migration for semelparous salmonids) are absent in the described model species and cannot be replicated in a laboratory study. Future F_2-IsoP research should therefore be designed in consideration of environmental conditions in the region and the species of interest.

6.2.2 Experimental design considerations

The experiments performed as part of this thesis highlighted a number of important parameters that should be accounted for in order to design successful future experiments of F_2-IsoPs in fish.

The timing of sampling events were a recurring source of uncertainty in this thesis. In Chapter 3, additional sampling events 2h, 8 or 12h, and 24h hours would have provided a clearer idea of the progression the F_2-IsoP response, particularly in light of the modest scale of the response. In the chronic study from the FOReSt project, the selected sampling window was likely too far removed from peak TPAC concentrations in the enclosure water, meaning fish who had suffered sublethal oxidative stress responses may have already recovered. In light of this, the added logistical complexity – more time, more experimental subjects, and higher analytical budgets – brought on by increasing sampling frequency should also be considered during the design phase.

The dilbit exposure study in Chapter 4 highlighted the importance of using multiple biomarkers to verify the oxidative stress response in these first studies on dilbit's impacts on F_2-IsoPs in epidermal mucus: CYP1A1 activity – an important biomarker of PAC metabolism – and other biomarkers of oxidative stress were not measured in conjunction with F_2-IsoPs. Incorporating these biomarkers would have yielded a greater level of confidence to the results in this study, which suggested PAC exposure did not occur to a significant extent in the fathead minnows. The relationship observed in Chapter 3 between F_2-IsoPs and glutathione suggests that other such relationships with F_2-IsoPs may exist, which could include biomarkers more relevant to dilbit exposure such as CYP1A1.

In this thesis, correlations between F_2-IsoP concentrations in mucus and other visceral tissues were not conclusive. The presence or absence of these correlations should be examined in

detail, as the dynamics of F_2-IsoPs in fish have yet to be elucidated as they have in mammalian models. It is likely that as in mammals, a large portion of F_2-IsoP production occurs in the liver, after which they are released into the bloodstream and transported throughout the body, however F_2-IsoP research would benefit from tissue comparisons and tracer studies examining the fate of F_2-IsoPs in fish, similar to those performed in rats.

These studies will be important for validating epidermal mucus F_2-IsoPs as a biomarker of oxidative stress since it is not known if they are produced *in situ* and/or transported to the mucus from the liver: findings from Morrow et al (1992) suggest that isoprostane-containing lipids in plasma originate in the liver [7], which lends credence to this route being the major source of F_2-IsoPs in other biofluids such as epidermal mucus. However, Morrow also noted that it was possible other organs contributed to bodily concentrations of F_2-IsoPs in rats exposed to CCl_4 [7]. In fish, there is already evidence of *in situ* production in isolated gill pavement cells [8], therefore it is likely the case that other organs are involved in producing F_2-IsoPs. Thus, it is possible that *in situ* production of F_2-IsoPs occurs in the mucus given it contains phospholipids [9]. Interestingly, mucus phospholipid concentrations are linked to viscosity and can vary significantly between fish species [9], and could be explored as a source of interspecies variability in mucus F_2-IsoPs.

Determining how F_2-IsoPs respond to chemical stressors in different organs and tissues of fish will also assist in validating epidermal mucus as a non-lethal sampling matrix by identifying those tissues that can be used to verify results in mucus. This thesis has identified liver and gills as strong potential candidates, but kidney, head kidney, heart, brain, and muscle tissue should also be considered based on their use in the literature for determinations of oxidative stress [7,10–13]. Muscle in particular provides interesting opportunities, as biopsies can be collected non-lethally

and F_2-IsoPs have previously been quantified in medaka muscle exposed to hydrogen peroxide [12].

In these respects and many others, Morrow et al (1992) serves as an excellent model experiment for studying the effects of an acute stressor causing oxidative damage on F_2-IsoP release and production in fish. Using CCl_4 would ensure oxidative damage is the mechanism of toxicity to the fish specimens. Concentrations of F_2-IsoPs and other biomarkers could then be measured in epidermal mucus and several of the aforementioned tissues to establish correlations between biomarkers and how F_2-IsoPs distribute in fish. Furthermore, Morrow's study also describes a method for assessing the relationship between CYP450 activity and F_2-IsoP concentrations. If this relationship was found to be comparable in fish and rats, a follow-up study using dilbit WAF instead of CCl_4 would provide a strong indication of if dilbit induces an F_2-IsoP response in fish.

6.2.3 Experimental design considerations: field-based studies

While laboratory based studies will be essential to validating mucus F_2-IsoPs as viable biomarkers of oxidative stress in dilbit-exposed fish, field-based studies will be also be necessary to validate the method in a complex environment, where a host of confounding variables can mask or modify biomarker response [5,6,14]. Future field experiments of F_2-IsoPs should be carefully designed to account for variables such as temperature, dissolved oxygen, diet, and reproductive strategies (these variables are discussed in Section 1.5).

Mesocosm studies and limnocorral studies such as the FOReSt project present several advantages which could bridge the gap between laboratory and ecological conditions. In Chapter 5, enclosures were constructed within a small area of a lake during a short time window: the physical proximity and timing of setup provided relatively uniform water chemistry and microbial

communities. Enclosures can be stocked with fish selected for species, age cohort, size, and sex, which will be exposed to approximately equivalent habitats. Temperature and dissolved oxygen can be regularly monitored. Onshore mesocosms provide many of these same benefits, and are more appropriate in most cases; environmental regulations prohibit the introduction dilbit to *in situ* enclosures. In these ways and many more, mesocosms and limnocorrals can control environmental variables to help deconvolute an F_2-IsoP response caused by dilbit from environmentally induced changes in oxidative status. While still having limitations in how their results can be generalized to different ecosystems [15], these types of studies would provide valuable insights into the behaviour of F_2-IsoPs in fish mucus under ecological conditions.

These variables are more difficult to control in larger-scale studies where several fish species and waterbodies may be studied, such as opportunistic studies of oil spills. Design and results interpretation should therefore be mindful of their impacts to oxidative stress and to F_2-IsoP response. For example, in Chapter 2, lake trout mucus contained higher concentrations of F_2-IsoPs in the lakes sampled later in October, which could have signified impacts of spawning stress. However, the study was designed to determine presence or absence of F_2-IsoPs and thus did not consider the spawning status of the fish. In this specific case, the role of spawning in lake trout could be assessed by a longitudinal study involving repeated sampling of mucus from fish fitted with acoustic telemetry tags. The effects of spawning, temperature, oxygen availability, diet, and age could be determined individually: this could be performed at a smaller scale using laboratory studies prior to field studies. There are several examples of experiments measuring effects of these variables on the oxidative response of fish [10,16–19]. Even with these relationships determined beforehand, control groups in field studies should be designed in consideration of these variables,

as site-specific conditions can alter how different populations of the same fish species respond under ecological conditions [20].

6.3 Conclusion

Taken together, these studies provide a foundation for the future study of F_2-IsoPs as an oxidative stress biomarker in fish. Future laboratory-based studies are needed to determine how epidermal mucus F_2-IsoPs correlate to visceral tissues in response to dilbit exposure, to assess the viability of mucus use as a surrogate for tissues such as liver. Though the objective is to eventually maximize the use of non-lethal epidermal mucus, this thesis illustrates that a broader understanding of how F_2-IsoPs are produced and transported in fish is also needed. More comparisons to glutathione and other biomarkers are also warranted, particularly in the gills, where we identified a possible link between the two compounds. As important as glutathione, the relationship between the CYP450 pathway and the F_2-IsoPs in fish during exposure to crude oil should be examined, as was underlined by the apparent lack of effects in Chapter 4. It is critical that future studies study CYP1A and F_2-IsoPs in tandem, and that the sampling intervals be carefully selected. Future field-based studies will be equally important once some of these questions have been resolved in the lab. The effects of complex environmental variables and multiple stressors will be important considerations if epidermal mucus F_2-IsoPs are to be successfully implemented as a measure of oxidative stress in wild fish exposed to crude oil. Specifically, the effects of spawning stress, life history, temperature, dissolved oxygen all require attention. Though much research is still needed, this thesis contributes to the growing field of non-lethal analysis and provides direction for future studies that will further our understanding of F_2-IsoPs in fish.

6.4 References

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Appendix

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Table S5. Student t-test outputs for comparisons of plasma cortisol between rainbow trout sampling cohorts 1 and 2.

Table S6. RStudio outputs of Shapiro-Wilkes normality tests for GSSG:GSH ratios in rainbow trout liver and gill.

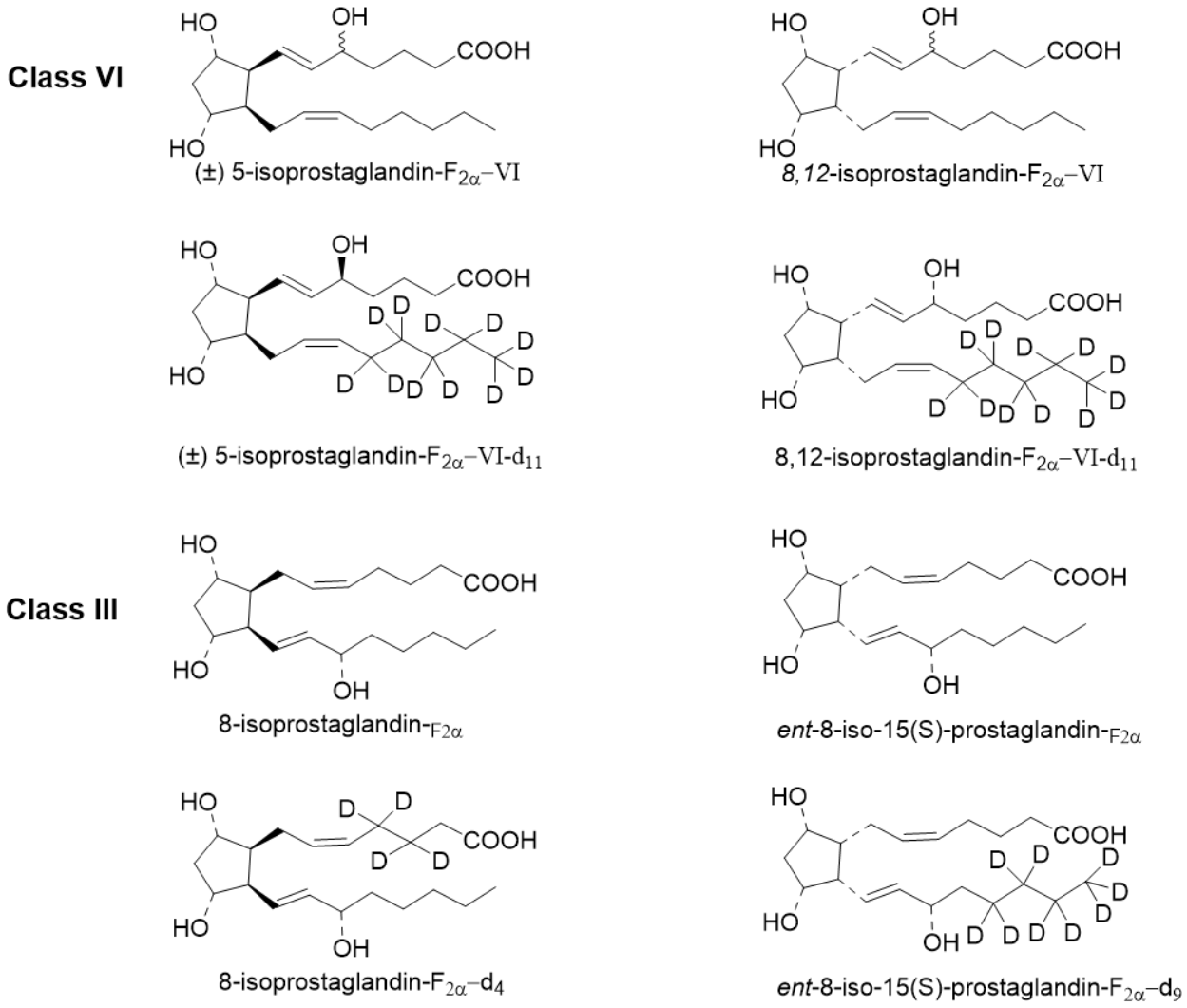


Figure S1. F_2-isoprostane isomers measured in this study. Regioisomers of Type III and VI are shown.

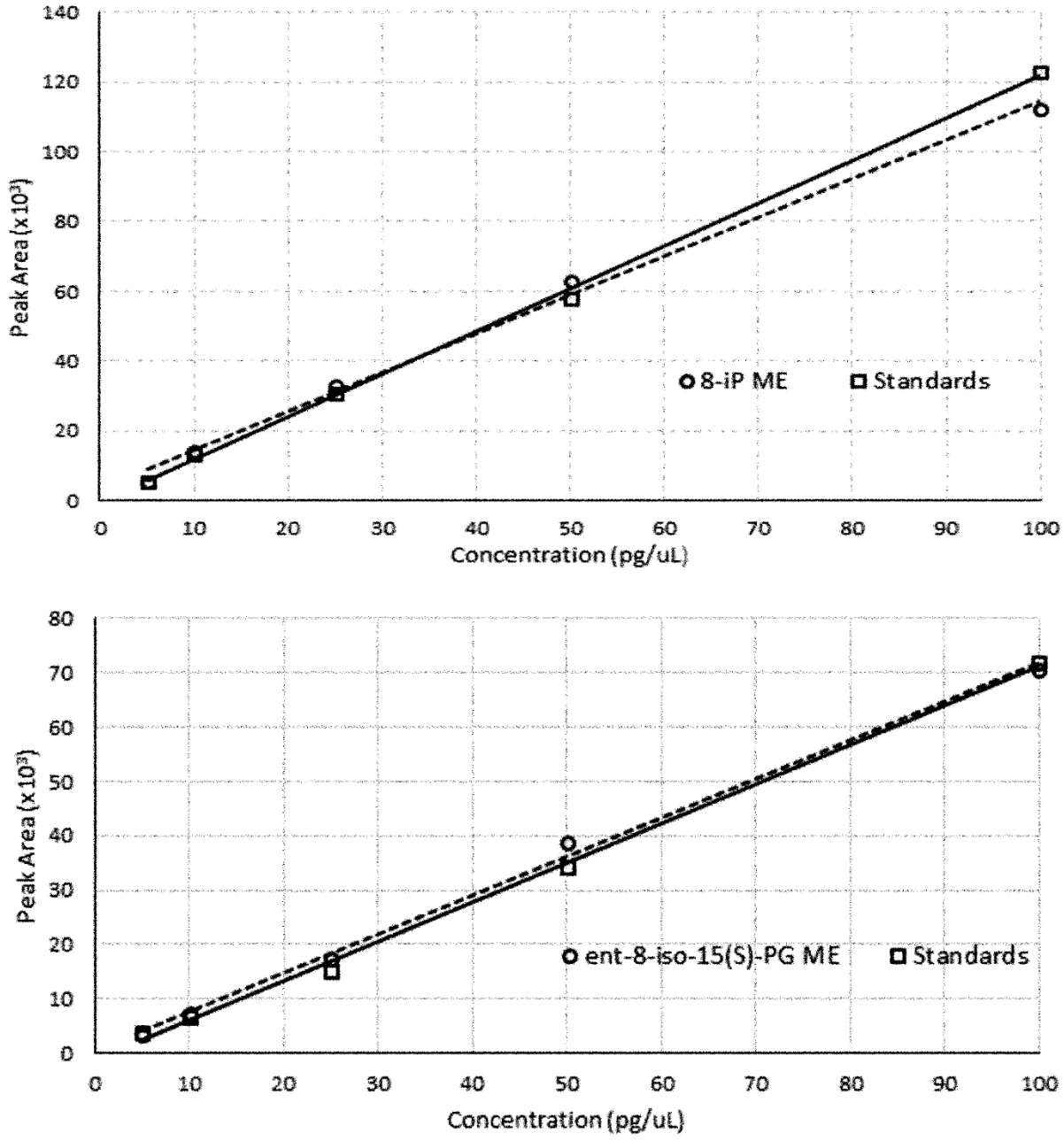
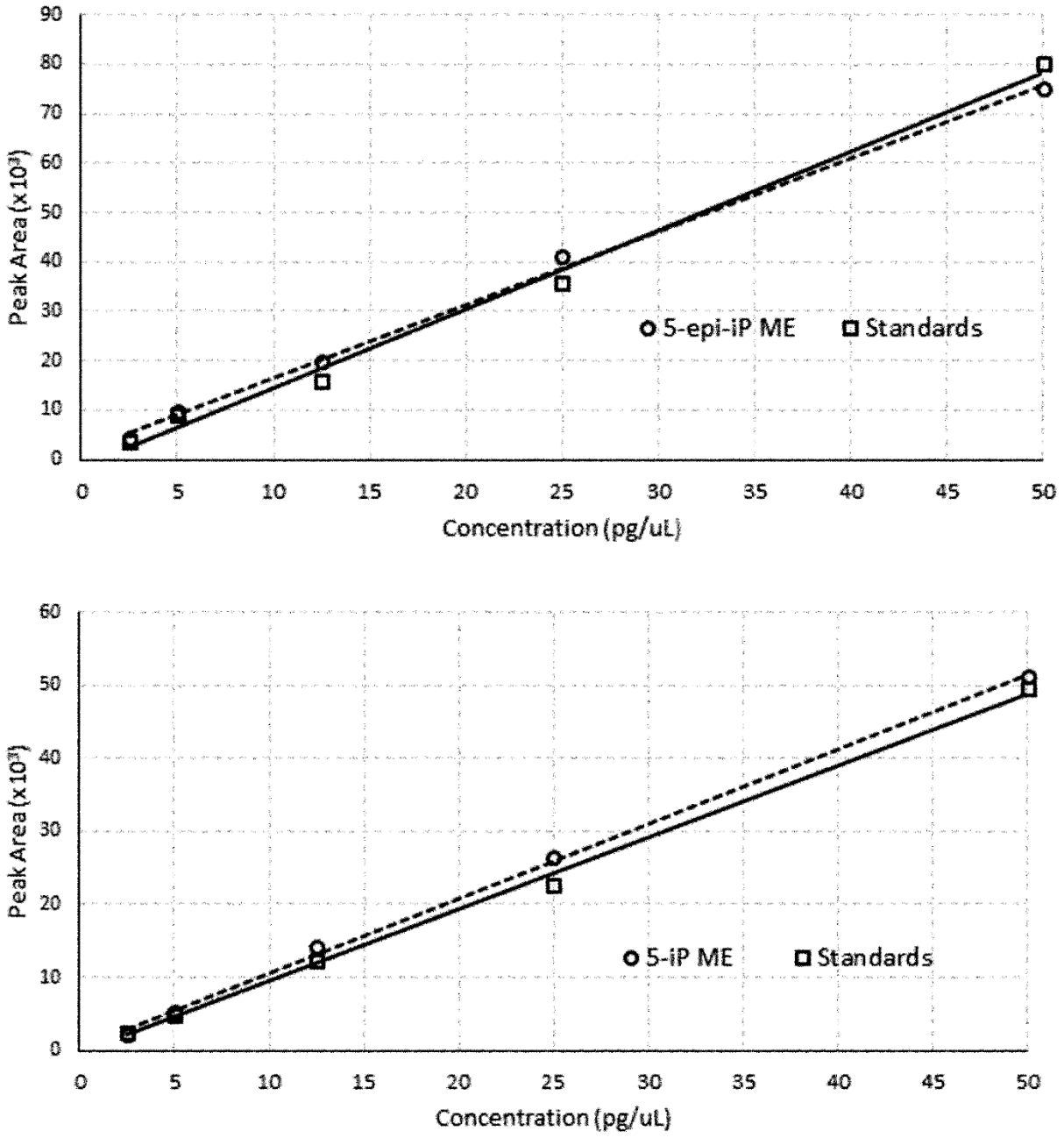


Figure S2. Matrix effect calibration curves. Matrix matched calibration deuterated isoprostane standards are plotted against deuterated isoprostane standards prepared in methanol.



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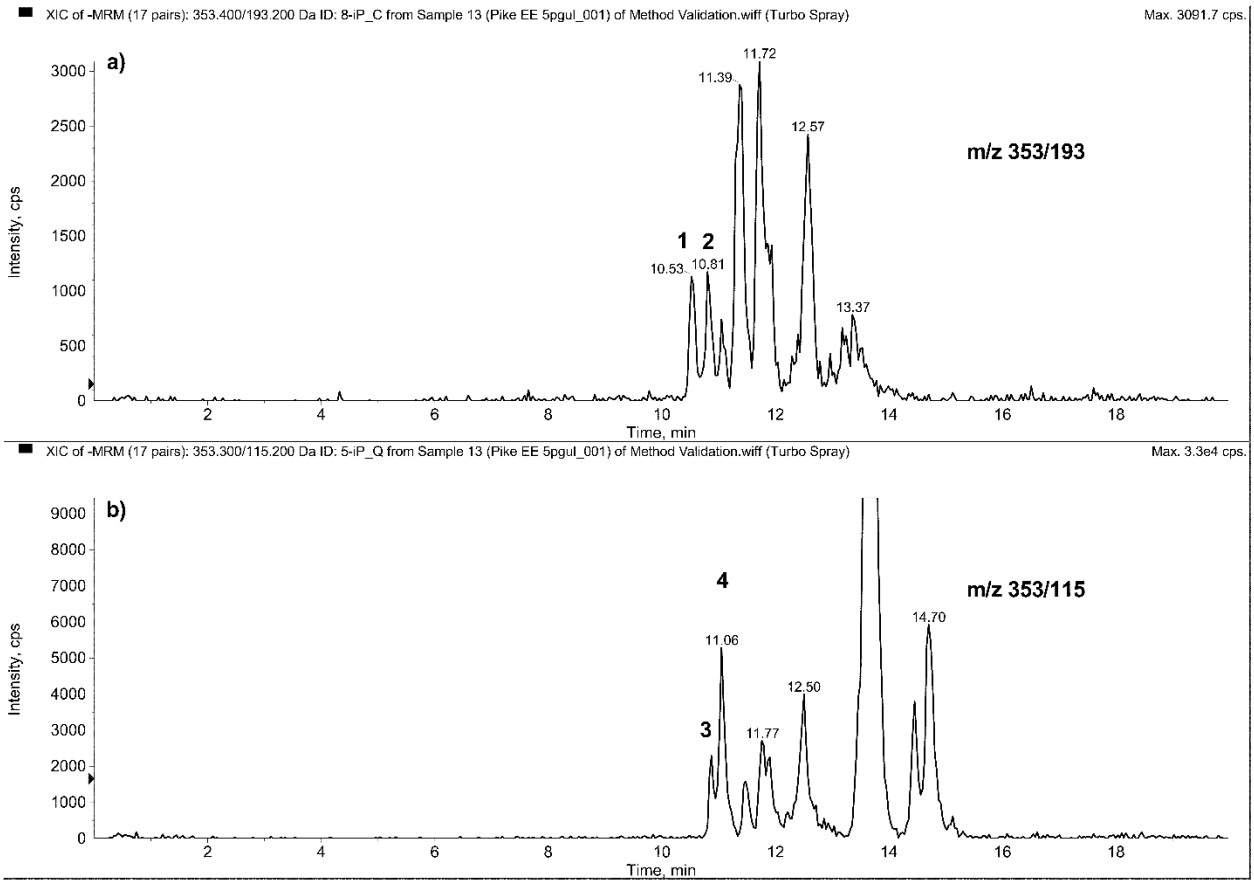

Figure S4. HPLC-MS/MS elution of **a)** endogenous *ent*-8-iso-15(S)- prostanglandin-$F_{2\alpha}$ (**Peak 1**) and 8-iso-prostanglandin-$F_{2\alpha}$ (**Peak 2**) and **b)** 5-iso-prostaglandin-$F_{2\alpha}$-VI (**Peak 3**) and 5-*epi*-iso-prostaglandin-$F_{2\alpha}$-VI in a Northern pike mucus sample. Other isoprostane peaks in the mucus have not yet been posively identified.

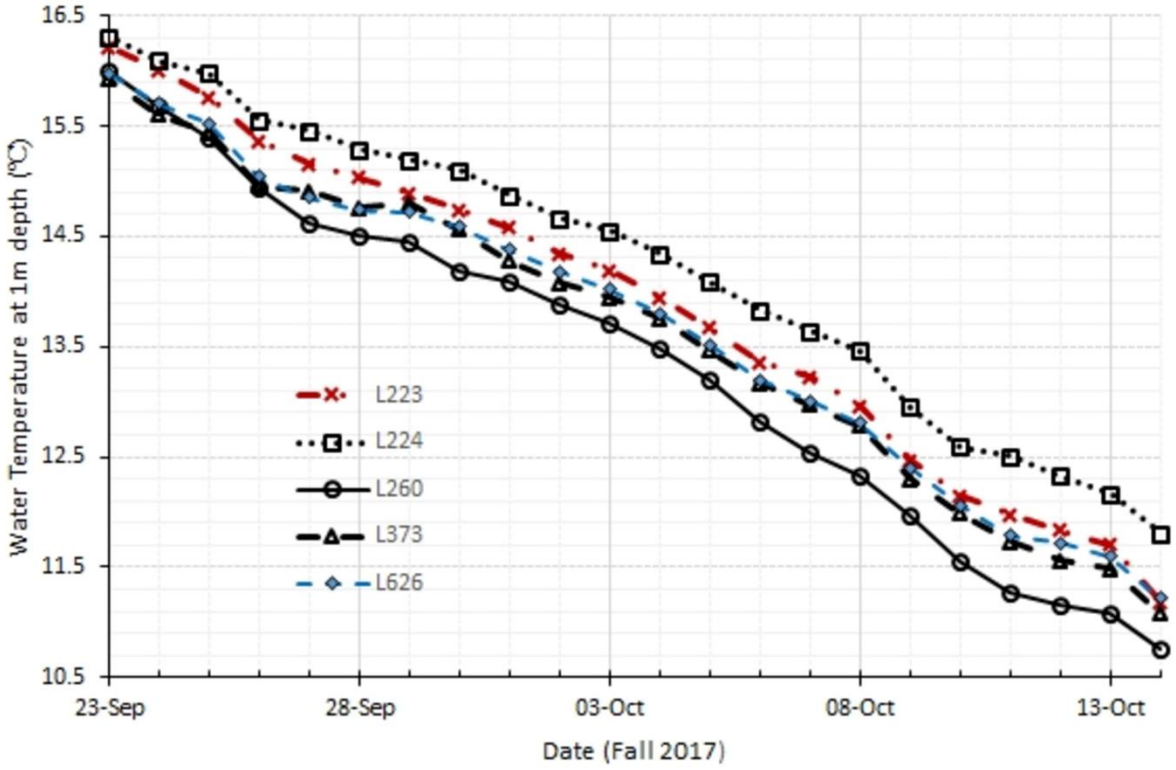

Figure S5. Mean daily water temperatures at 1m depth in the five experimental lakes during the temporal range of our lake trout sampling. Temperatures were logged hourly using Hobo Water Temp Pro v2 loggers. Data source: IISD-ELA.

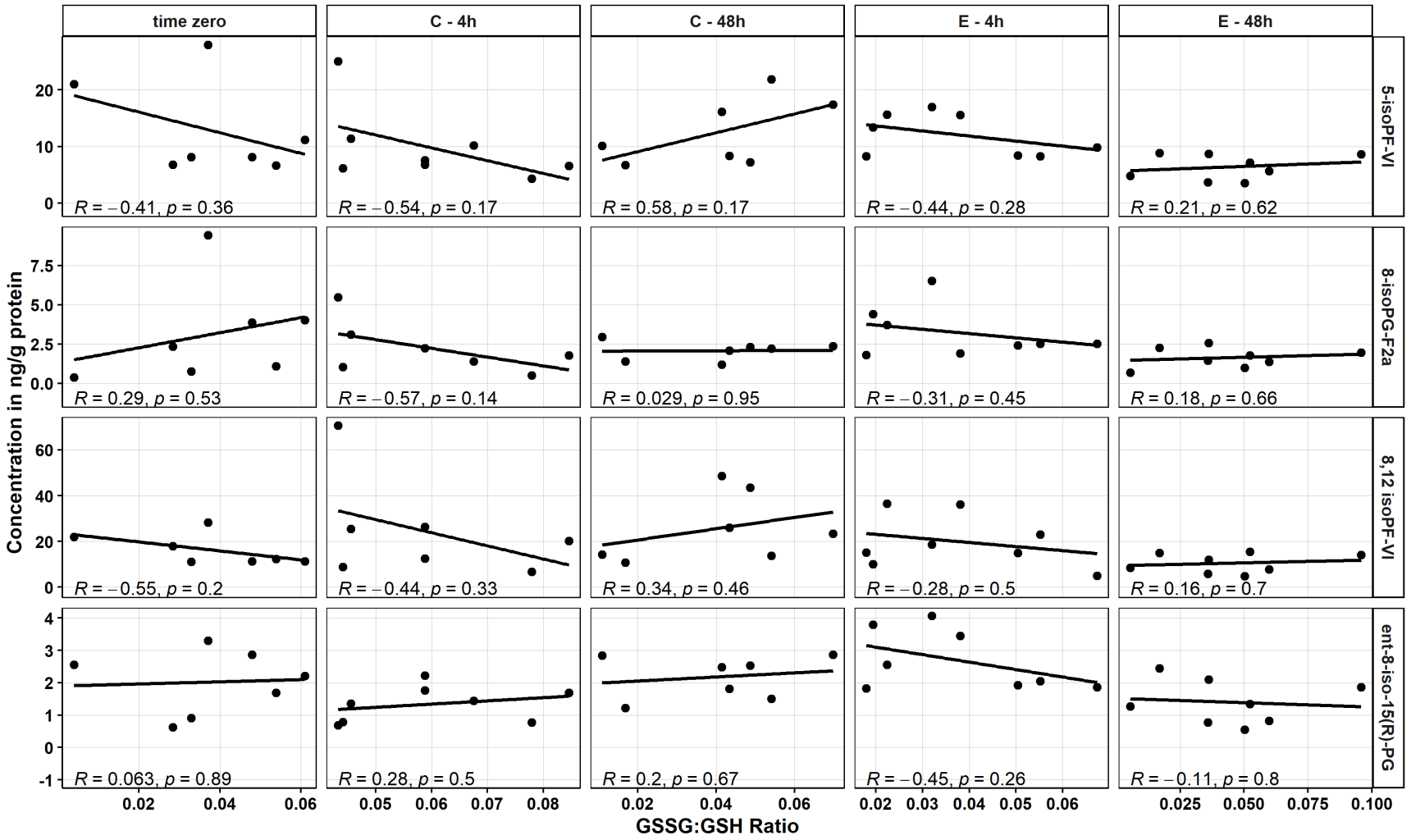

Figure S6. Pearson correlation of GSSG:GSH ratio and F_2-Isoprostanes in rainbow trout liver.

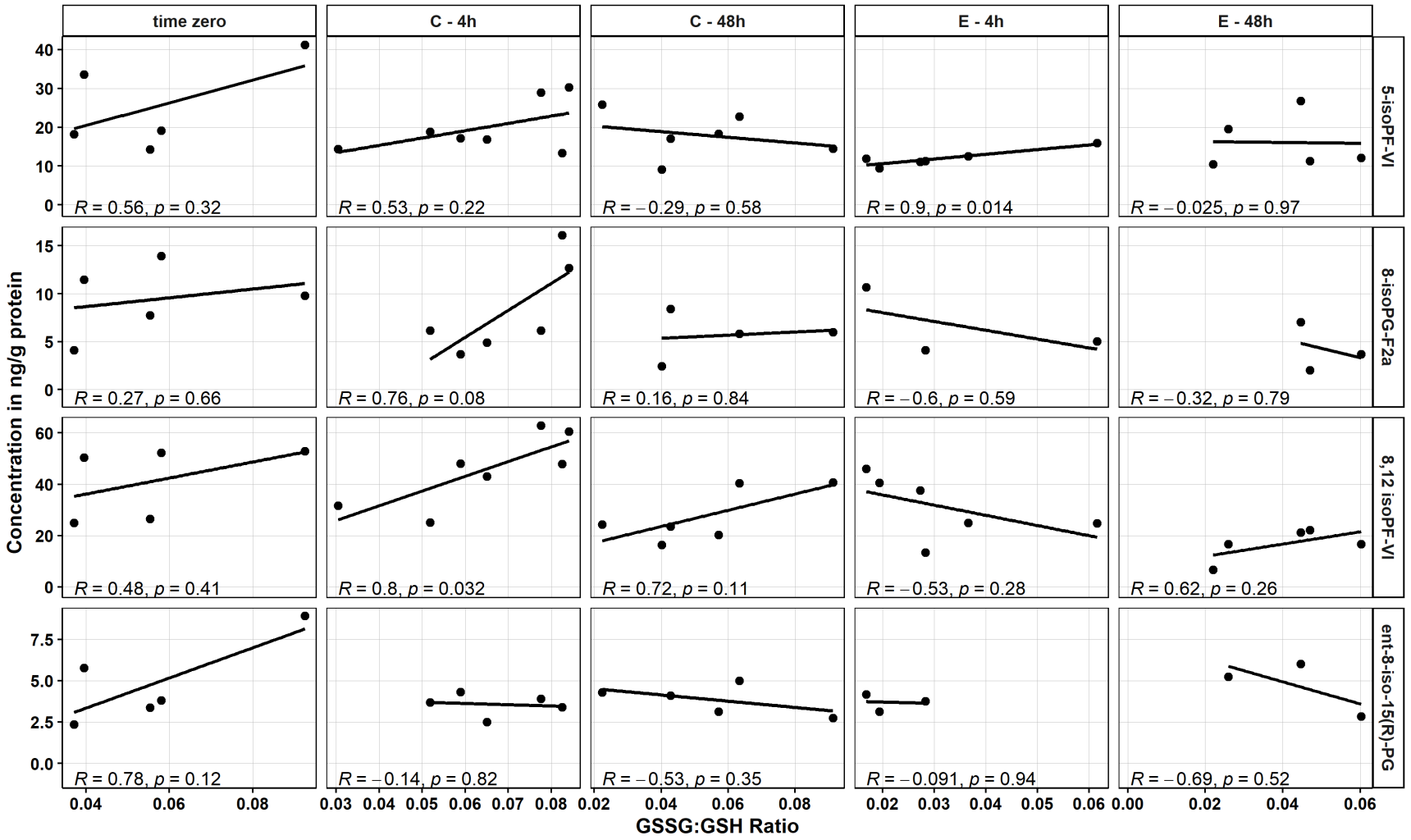

Figure S7. Pearson correlation of GSSG:GSH ratio and F_2-Isoprostanes in rainbow trout gills.

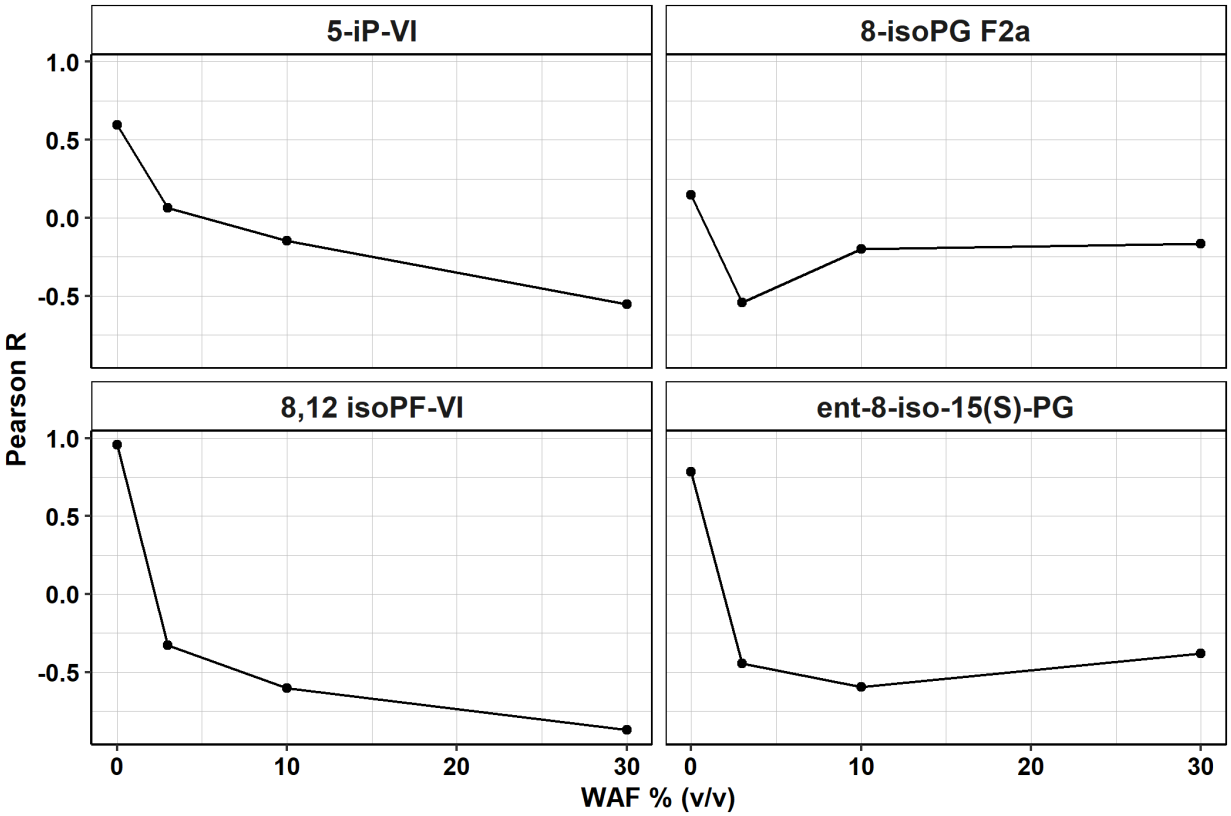

Figure S8. Change in Pearson R correlation amplitude and direction epidermal mucus F_2-Isoprostane concentration in relation to dilbit dose.

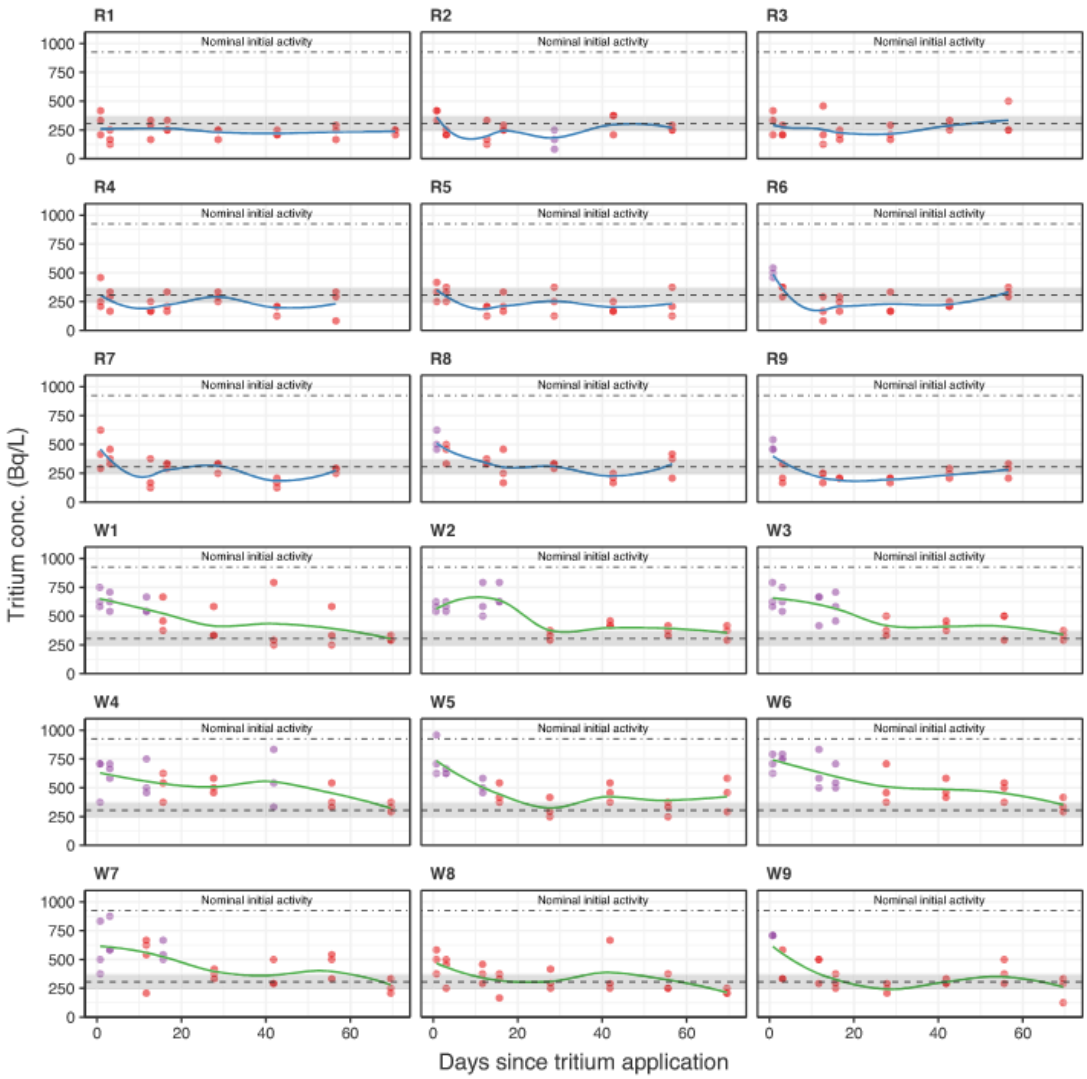

Figure S9. Tritium concentrations in the FOReSt enclosures post-addition of dilbit. Figure created by Dr. Jose-Luis Rodriguez (IISD-ELA) for the FOReSt project.

Table S1. Detection and identification of F_2-isoP isomers in mucus samples of lake trout and pike by use of high-resolution liquid chromatography-quadrupole-time-of-flight mass spectrometry (LC-Q-TOF-MS).

	Peaks	RT	Formula	Exact Mass	Theoretical Mass	Experimental Mass	ΔMass [a] (ppm)
Reference Standard	1	11.6	$C_{20}H_{34}O_5$	$[M-H]^-$	353.2333	353.2332	0.28
	2	12.0	$C_{20}H_{34}O_5$	$[M-H]^-$	353.2333	353.2333	<0.01
	3	12.1	$C_{20}H_{34}O_5$	$[M-H]^-$	353.2333	353.2335	0.57
Lake Trout	1	13.0	$C_{20}H_{34}O_5$	$[M-H]^-$	353.2333	353.2333	<0.01
	2	13.3	$C_{20}H_{34}O_5$	$[M-H]^-$	353.2333	353.2333	0.00
	3	13.6	$C_{20}H_{34}O_5$	$[M-H]^-$	353.2333	353.2331	0.57
	4	13.8	$C_{20}H_{34}O_5$	$[M-H]^-$	353.2333	353.2329	1.13
	5	14.3	$C_{20}H_{34}O_5$	$[M-H]^-$	353.2333	353.2331	0.57
Pike	1	13.0	$C_{20}H_{34}O_5$	$[M-H]^-$	353.2333	353.2334	0.28
	2	13.3	$C_{20}H_{34}O_5$	$[M-H]^-$	353.2333	353.2342	2.55
	3	13.6	$C_{20}H_{34}O_5$	$[M-H]^-$	353.2333	353.2338	1.42
	4	13.8	$C_{20}H_{34}O_5$	$[M-H]^-$	353.2333	353.2335	0.57
	5	14.3	$C_{20}H_{34}O_5$	$[M-H]^-$	353.2333	353.234	1.98

[a] "Δ Mass" means the mass difference between theoretical and experimental *m/z* masses.

Table S2. Parameters of experimental lakes used for trout sampling in autumn 2017.

Lake	Max. Depth (m)	Lake Area (ha)	Date Sampled	Avg. Temp (°C) on sampling date
223	14.4	27.27	September 28	15.0
224	27.4	25.92	September 27	15.5
260	14.2	32.76	September 26	14.9
373	21.2	27.3	October 10	12.0
626	13.2	25.77	October 6	13.2

Data source: IISD-ELA

Table S3. F_2-Isoprostanes and deuterated analogues analyzed in Chapters 3 to 5.[1]

Analyte	Internal Standard
5-iso-prostaglandin-$F_{2\alpha}$-VI (±)	5-iso-prostaglandin-$F_{2\alpha}$-VI-d_{11} (±)
8,12-isoPF-VI, 8,12-isoPF-VI	8,12-isoPF-VI, 8,12-isoPF-VI-d_{11}
8-iso-prostaglandin-$F_{2\alpha}$	8-iso-prostaglandin-$F_{2\alpha}$-d_4
ent-iso-15(S)-prostaglandin- $F_{2\alpha}$	ent-iso-15(S)-prostaglandin- $F_{2\alpha}$-d_9

[1] All were purchased from Cayman Chemical Inc. (Ann Arbour, MI).

Table S4. Morphometric data for rainbow trout (*Onchorhynchus mykiss*) exposed to acute physical stress.

Fish code	Treatment	Sex	Weight (g)	Fork length (mm)	Total length (mm)	Liver weight (g)	Condition factor
C1-1	time zero	NS	173	236	245	2.573	1.18
C1-2	time zero	NS	182	233	247	3.278	1.21
C1-3	C - 4h	NS	140.5	232	241	1.74	1
C1-4	C - 4h	NS	167.5	228	232	2.308	1.34
C1-5	C - 4h	NS	94.5	202	215	1.482	0.95
C1-6	C - 4h	NS	105	216	225	1.48	0.92
C1-7	C - 4h	NS	154	226	235	1.779	1.19
C1-8	C - 4h	NS	171.5	246	255	2.148	1.03
C1-9	C - 4h	F	143	235	249	1.675	0.93
C1-10	C - 4h	F	191.5	235	245	3.214	1.3
C2-1	time zero	NS	157.5	235	247	1.793	1.05
C2-2	time zero	NS	145.5	210	217	2.126	1.42
C2-3	C - 48h	NS	207.5	252	260	1.983	1.18
C2-4	C - 48h	F	141	215	227	2.153	1.21
C2-5	C - 48h	F	190	236	250	2.947	1.22
C2-6	C - 48h	F	196.5	250	260	4.134	1.12
C2-7	C - 48h	NS	119	211	221	1.976	1.1
C2-8	C - 48h	NS	172.5	235	247	3.488	1.14
C2-9	C - 48h	F	172.5	234	245	2.902	1.17
C2-10	C - 48h	NS	189	259	270	2.749	0.96
E1-1	time zero	F	138	234	255	2.536	0.83
E1-2	time zero	F	140.5	236	245	2.106	0.96
E1-3	E - 4h	F	121.5	212	222	2.578	1.11
E1-4	E - 4h	F	173.5	237	250	1.854	1.11
E1-5	E - 4h	F	189	246	262	2.701	1.05
E1-6	E - 4h	F	133.5	221	233	1.774	1.06
E1-7	E - 4h	F	198	246	261	2.011	1.11
E1-8	E - 4h	NS	173.5	145	259	1.754	1
E1-9	E - 4h	NS	139	223	235	2.323	1.07
E1-10	E - 4h	NS	154	236	248	2.349	1.01
E2-1	time zero	NS	127.5	211	222	2.519	1.17
E2-2	time zero	NS	157.5	240	247	2.678	1.05
E2-3	E - 48h	NS	94	198	213	1.803	0.97
E2-4	E - 48h	F	126	210	222	2.789	1.15
E2-5	E - 48h	NS	159	234	245	2.96	1.08
E2-6	E - 48h	F	167	239	250	1.367	1.07
E2-7	E - 48h	NS	159.5	235	248	2.455	1.05
E2-8	E - 48h	NS	111.5	202	211	1.673	1.19
E2-9	E - 48h	NS	166	245	258	2.245	0.97
E2-10	E - 48h	NS	146.5	240	259	2.315	0.84

Table S5. Student t-test outputs for comparisons of plasma cortisol between rainbow trout sampling cohorts 1 and 2.

treatment	estimate	estimate1	estimate2	statistic	p.value	parameter	conf.low	conf.high
C - 4h	-4.5	60.15	64.65	-0.168	0.873	5.554	-71.402	62.402
C - 48h	-23.1	8.57	31.67	-2.191	0.160	2.005	-68.358	22.158
E - 4h	-23.825	14.85	38.68	-1.545	0.200	3.822	-67.431	19.781
E - 48h	97	140.7	43.7	2.630	0.213	1.099	-283.057	477.057

Table S6. RStudio outputs of Shapiro-Wilkes normality tests for GSSG:GSH ratios in rainbow trout liver and gill.

Tissue	Treatment	P value	Method
liver	time zero	0.104107	Shapiro-Wilke normality test
liver	C - 4h	0.327232	Shapiro-Wilke normality test
liver	C - 48h	0.864875	Shapiro-Wilke normality test
liver	E - 4h	0.456576	Shapiro-Wilke normality test
liver	E - 48h	0.805556	Shapiro-Wilke normality test
gill	time zero	0.096313	Shapiro-Wilke normality test
gill	C - 4h	0.490026	Shapiro-Wilke normality test
gill	C - 48h	0.764251	Shapiro-Wilke normality test
gill	E - 4h	0.188633	Shapiro-Wilke normality test
gill	E - 48h	0.867514	Shapiro-Wilke normality test

Table S7. RStudio outputs of Shapiro-Wilkes normality tests for isoprostane concentrations in rainbow trout liver, gill, and plasma.

		Liver		Gill		Plasma	
Treatment	Isomer	Statistic	P value	Statistic	P value	Statistic	P value
time zero	5-isoPF-VI	0.8057	**0.0329***	0.8782	0.3014	0.7725	**0.0144***
	8-isoPG-F2a	0.8014	**0.0296***	0.9904	0.9811	0.9061	0.3697
	8,12 isoPF-VI	0.8954	0.2627	0.7459	**0.0273***	0.9742	0.9270
	ent-8-iso-15(S)-PG	0.9466	0.6773	0.9080	0.4557	0.8783	0.1814
C - 4h	5-isoPF-VI	0.7928	**0.0239***	0.8277	0.0761	0.8578	0.1143
	8-isoPG-F2a	0.9072	0.3348	0.8462	0.1467	0.9401	0.6120
	8,12 isoPF-VI	0.8055	**0.0464***	0.9436	0.6712	0.7661	**0.0123***
	ent-8-iso-15(S)-PG	0.9611	0.8203	0.9502	0.7387	0.9236	0.5319
C - 48h	5-isoPF-VI	0.8860	0.2544	0.9869	0.9803	0.8871	0.3030
	8-isoPG-F2a	0.9332	0.5786	0.9461	0.6920	0.9633	0.8445
	8,12 isoPF-VI	0.8726	0.1956	0.8418	0.1350	0.6808	**0.0023***
	ent-8-iso-15(S)-PG	0.8895	0.2719	0.9510	0.7441	0.9035	0.3953
E - 4h	5-isoPF-VI	0.8345	0.0661	0.9092	0.4313	0.9583	0.8044
	8-isoPG-F2a	0.8387	0.0731	0.8521	0.2460	0.8742	0.2433
	8,12 isoPF-VI	0.9129	0.3749	0.9427	0.6812	0.8428	0.1054
	ent-8-iso-15(S)-PG	0.8356	0.0679	0.9841	0.7589	0.9710	0.8817
E - 48h	5-isoPF-VI	0.8680	0.1441	0.8381	0.1598	0.8627	0.1599
	8-isoPG-F2a	0.9838	0.9793	0.9659	0.6451	0.7708	**0.0208***
	8,12 isoPF-VI	0.9003	0.2909	0.8584	0.2226	0.8440	0.1082
	ent-8-iso-15(S)-PG	0.9383	0.5944	0.9196	0.4510	0.7255	**0.0113***

Table S8. Pearson correlation statistics of F_2-Isoprostanes in rainbow trout tissues.

treatment	isomer	estimate	statistic	p.value	parameter	conf.low	conf.high
Liver : Gill							
time zero	5-isoPF-VI	-0.130	-0.227	0.835	3	-0.908	0.850
	8-isoPG-F2a	-0.915	-3.919	**0.030***	3	-0.994	-0.168
	8,12 isoPF-VI	-0.385	-0.723	0.522	3	-0.946	0.753
	ent-8-iso-15(R)-PG	-0.416	-0.793	0.486	3	-0.950	0.737
C - 4h	5-isoPF-VI	0.696	2.170	0.082	5	-0.119	0.951
	8-isoPG-F2a	0.343	0.731	0.505	4	-0.649	0.903
	8,12 isoPF-VI	0.646	1.691	0.166	4	-0.349	0.956
	ent-8-iso-15(R)-PG	0.846	2.753	0.071	3	-0.142	0.990
C - 48h	5-isoPF-VI	-0.738	-2.184	0.094	4	-0.969	0.184
	8-isoPG-F2a	0.335	0.503	0.665	2	-0.923	0.980
	8,12 isoPF-VI	0.289	0.604	0.579	4	-0.683	0.891
	ent-8-iso-15(R)-PG	-0.041	-0.072	0.947	3	-0.891	0.873
E - 4h	5-isoPF-VI	-0.486	-1.111	0.329	4	-0.930	0.538
	8-isoPG-F2a	-0.972	-4.144	0.151	1	NA	NA
	8,12 isoPF-VI	0.375	0.810	0.463	4	-0.627	0.910
	ent-8-iso-15(R)-PG	-0.942	-2.796	0.219	1	NA	NA
E - 48h	5-isoPF-VI	0.908	3.755	**0.033***	3	0.130	0.994
	8-isoPG-F2a	0.979	4.825	0.130	1	NA	NA
	8,12 isoPF-VI	0.206	0.365	0.739	3	-0.826	0.921
	ent-8-iso-15(R)-PG	0.964	3.613	0.172	1	NA	NA

Cont. on next page

treatment	isomer	estimate	statistic	p.value	parameter	conf.low	conf.high
Liver : Plasma							
time zero	5-isoPF-VI	-0.425	-1.149	0.294	6	-0.869	0.399
	8-isoPG-F2a	-0.304	-0.714	0.507	5	-0.860	0.582
	8,12 isoPF-VI	-0.853	-3.652	**0.015***	5	-0.978	-0.279
	ent-8-iso-15(R)-PG	-0.524	-1.507	0.183	6	-0.897	0.287
C - 4h	5-isoPF-VI	0.314	0.811	0.448	6	-0.501	0.834
	8-isoPG-F2a	-0.060	-0.147	0.888	6	-0.734	0.673
	8,12 isoPF-VI	0.365	0.876	0.421	5	-0.535	0.877
	ent-8-iso-15(R)-PG	-0.708	-2.005	0.115	4	-0.965	0.244
C - 48h	5-isoPF-VI	-0.315	-0.664	0.543	4	-0.897	0.667
	8-isoPG-F2a	0.640	1.666	0.171	4	-0.357	0.955
	8,12 isoPF-VI	0.019	0.042	0.968	5	-0.745	0.761
	ent-8-iso-15(R)-PG	0.372	0.802	0.467	4	-0.629	0.909
E - 4h	5-isoPF-VI	0.261	0.605	0.572	5	-0.612	0.848
	8-isoPG-F2a	0.349	0.746	0.497	4	-0.645	0.905
	8,12 isoPF-VI	0.507	1.317	0.245	5	-0.398	0.912
	ent-8-iso-15(R)-PG	0.740	1.906	0.153	3	-0.410	0.981
E - 48h	5-isoPF-VI	0.548	1.465	0.203	5	-0.349	0.921
	8-isoPG-F2a	0.273	0.634	0.554	5	-0.605	0.851
	8,12 isoPF-VI	0.545	1.452	0.206	5	-0.353	0.920
	ent-8-iso-15(R)-PG	0.445	0.995	0.376	4	-0.573	0.923

Table S9. Mass spectrometry MRM ion transitions for F_2-Isoprostanes and their internal standards, and retention time on the HPLC column.

Analyte	Average Retention time (min)[1]	Quant. Transition (m/z)	Confirmation (m/z)
5-iso-prostaglandin-$F_{2\alpha}$-VI (±)	16.34	353/115	353/219
8,12-isoPF-VI, 8,12-isoPF-VI	17.65	353/115	353/219
8-iso-prostaglandin-$F_{2\alpha}$	16.03	353/193	353/247
ent-iso-15(S)-prostaglandin- $F_{2\alpha}$	15.66	353/193	353/247
5-iso-prostaglandin-$F_{2\alpha}$-VI-d_{11} (±)	16.18	364/115	364/219
8,12-isoPF-VI, 8,12-isoPF-VI-d_{11}	17.58	364/115	364/219
8-iso-prostaglandin-$F_{2\alpha}$-d_4	15.94	357/197	357/251
ent-iso-15(S)-prostaglandin-$F_{2\alpha}$-d_9	15.34	362/193	362/318

[1] Retention times shown are averages based on elution times in Chapters 4 and 5. The HPLC gradient and run duration were adjusted to accommodate retention time shift over the lifespan of the HPLC column. Retention times are lower for Chapter 3, but the elution order and relative retention times (i.e time resolving) were the same. The method in Chapter 2 utilizes a similar gradient and different column: retention times are shown in Table S1.

Table S10. Morphometric data for juvenile fathead minnows (*Pimephales promelas*) exposed to diluted bitumen.

Group	Fish ID	Weight (mg)	FL[a] (mm)	Liver (mg)	Group	Fish ID	Weight (mg)	FL (mm)	Liver (mg)
Control	1	1257	42	20.42	Medium	1	1209	43	10
Control	2	1503	45	8.15	Medium	2	1210	45	16
Control	3	1319	42	18.8	Medium	3	1100	41	11
Control	4	1281	44	7.4	Medium	4	1439	45	16
Control	5	1090	41	10.2	Medium	5	1236	46	11
Control	6	1471	46	24.2	Medium	6	998	41	16
Control	7	1633	46	29.1	Medium	7	1028	41	11
Control	8	1059	42	1.2	Medium	8	1244	43	11
Control	9	1259	43	10	Medium	9	1228	44	16
Control	10	1294	43	13	Medium	10	1154	43	18
Control	11	1003	42	11	Medium	11	Mortality		
Control	12	1277	43	11	Medium	12	Mortality		
Control	13	1330	43	24	Medium	13	1498	44	17
Control	14	1405	44	18	Medium	14	1476	46	13
Control	15	1206	42	27	Medium	15	1103	40	17
Control	15	1370	44	19	Medium	16	1685	45	29
MEAN		1297.31	43.25	15.78	MEAN		1257.71	43.36	15.14
STD.DEV		163.73	1.48	7.94	STD.DEV		197.45	1.98	4.88
Low	1	1072	41	5	High	1	1317	42	9.1
Low	2	1073	41	5	High	2	1701	46	14.2
Low	3	1345	45	19	High	3	1555	46	31.1
Low	4	1279	42	17	High	4	1407	44	9.2
Low	5	1286	43	19	High	5	1109	43	14
Low	6	1070	40	13	High	6	Mortality		
Low	7	1420	44	15	High	7	1126	40	13.9
Low	8	922	40	7	High	8	1203	43	6.1
Low	9	1137	42	21	High	9	1174	42	20
Low	10	1724	47	31	High	10	1169	42	11
Low	11	1428	45	24	High	11	1054	41	13
Low	12	1436	46	16	High	12	1173	42	22
Low	13	1166	43	12	High	13	936	39	6
Low	14	1169	42	9	High	14	1106	42	12
Low	15	1145	44	7	High	15	1525	46	22
Low	16	1792	47	22	High	16	1202	43	20
MEAN		1279.00	43.25	15.13	MEAN		1250.47	42.73	14.91
STD.DEV		238.29	2.29	7.46	STD.DEV		209.93	2.09	6.91

[a] Fork length

Table S11. Parent PACs and internal standards analyzed by GC-MS/MS.[1]

Analyte	Internal Standard
Acenapthene	Acenaphthene-d_{10}
Acenapthylene	Acenaphthylene-d_8
Anthracene	Anthracene-d_{10}
Benz[a]anthracene	Benz[a]anthracene-d_{12}
Benzo[a]pyrene	Benzo[a]pyrene-d_{12}
Benzo[b]fluoranthene	Benzo[b]fluoranthene-d_{12}
Benzo[g,h,i]perylene	Benzo[g,h,i]perylene-d_{14}
Benzo[k]fluoranthene	Benzo[k]fluoranthene-d_{12}
Chrysene	Chrysene-d_{12}
Dibenzo[a,h]anthracene	Dibenzo[a,h]anthracene-d_{14}
Fluoranthene	externally calibrated
Fluorene	Fluorene-d_{10}
Indeno[1,2,3-c,d]pyrene	Indeno[1,2,3-c,d]pyrene-d_{14}
Naphthalene	Naphthalene-d_8
Phenanthrene	Phenanthrene-d_{10}
Pyrene	Pyrene-d_{10}

[1] Parent compounds and d_{10}-anthracene were purchased from Acccustandard Inc. (New Haven, CT). Remaining internal standards were from Cambridge Isootope Laboratories Inc. (Tewsbury, MA).

Table S12. Alkylated PACs analyzed by GC-MS/MS[1]

Analyte	Analyte (cont.)
Benzo[a]pyrene, C1	Naphthalene, C3
Benzothiophene	Phenanthrene, 1-methyl
1,2-BNT	Phenanthrene, 2-methyl
2,3-BNT	Phenanthrene, 3-methyl
Chrysene, C1	Phenanthrene, 9/4-methyl
Chrysene, C3	Phenanthrene, 1,7-dimethyl
Dibenzothiophene	Phenanthrene, 1,8-dimethyl
Dibenzothiophene, C1	Phenanthrene, 2,6-dimethyl
Dibenzothiophene, C2	Phenanthrene, 3,6-dimethyl
Dibenzothiophene, C3	Phenanthrene, C2
Dibenzothiophene, C4	Phenanthrene, C3
Fluorene, C1	Phenanthrene, C4
Fluorene, C2	Pyrene, C1
Fluorene, C3	Pyrene, C2
Naphthalene, 1-methyl	Pyrene, C3
Naphthalene, 2-methyl	Pyrene, C4
Naphthalene, 1-methyl	Retene
Naphthalene, C2	

[1] Standards were purchased from Accustandard Inc. (New Haven, CT) and Caledon Laboratory Chemicals (Georgetown, ON).

Table S13. Estimated concentrations in ng L^{-1} of PACs in experimental WAF and linear correlation coefficients of calibration curves.[1]

Compound	3% WAF	10% WAF	30% WAF	R^2 cal. curve	P value
Acenapthene	9.4	34.1	112.5	1.0000	0.00000
Acenapthylene	1.1	11.5	21.3	0.9080	0.00786
Anthracene	1.5	2.9	7.1	0.9820	0.00069
Benz[a]anthracene	3.4	5.5	3.0	-0.0720	0.45552
Benzo[a]pyrene	3.3	6.3	5.0	-0.3080	0.82442
Benzo[b]fluoranthene	5.3	2.2	2.3	-0.2300	0.65078
Benzo[g,h,i]perylene	3.0	4.7	19.8	-0.2840	0.75619
Benzo[k]fluoranthene	6.2	1.5	1.3	-0.1350	0.52132
Chrysene	0.8	2.0	4.7	0.5540	0.09237
Dibenzo[a,h]anthracene	2.2	3.2	2.3	-0.2540	0.69346
Fluoranthene	1.8	2.6	5.0	0.9670	0.00166
Fluorene	8.8	47.3	133.4	0.9990	0.00001
Indeno[1,2,3-c,d]pyrene	5.6	8.6	5.0	-0.0580	0.44171
Naphthalene	235.7	1,072.7	4,298.8	0.9780	0.00088
Phenanthrene	16.4	57.2	164.1	0.9780	0.00089
Pyrene	0.9	2.6	3.4	0.8660	0.01395

[1] Estimated from standard solutions of 3, 6, 12.5, 25, and 50% WAF.

Table S14. Estimated concentrations in ng L^{-1} of APACs in experimental WAF and linear correlation coefficients of calibration curves.[1]

Compound	3% WAF	10% WAF	30% WAF	R^2 cal. curve	P value
C1 Benzo[a]pyrene	81.8	160.3	84.7	-0.3300	0.93271
1-Methylnaphthalene	223.3	960.3	3,801.9	0.9790	0.00086
2-Methylnaphthalene	336.7	1,434.3	5,632.0	0.9850	0.00050
C2 Naphthalene	763.3	2,948.4	10,264.4	0.9880	0.00037
C3 Naphthalene	556.5	1,882.1	5,931.7	0.9840	0.00054
C4 Naphthalene	265.9	500.8	1,074.7	0.9880	0.00038
1-Methylphenanthrene	10.3	28.3	88.5	0.9950	0.00009
1,7-Dimethylphenanthrene	0.8	5.7	14.7	0.8130	0.02329
1,8-Dimethylphenanthrene	0.6	5.2	13.6	0.9910	0.00025
2-Methylphenanthrene	9.7	26.0	82.6	0.9930	0.00017
2,6-Dimethylphenanthrene	1.3	3.0	12.2	0.9920	0.00021
3-Methylphenanthrene	7.6	22.2	72.4	0.9960	0.00008
3,6-Dimethylphenanthrene	1.6	14.9	25.0	0.9160	0.00683
9/4-Methylphenanthrene	15.4	42.2	133.9	0.9950	0.00011
C2 Phenanthrene	166.7	637.5	1,316.5	0.9870	0.00043
C3 Phenanthrene	225.3	413.5	608.0	0.9890	0.00030
C4 Phenanthrene	99.5	221.4	316.2	0.7100	0.04625
C1 Pyrene	100.7	234.8	388.1	0.9810	0.00074

Compound	3% WAF	10% WAF	30% WAF	R^2 cal. curve	P value
C2 Pyrene	95.0	255.4	395.8	0.8730	0.01291
C3 Pyrene	315.9	526.5	599.6	0.7710	0.03184
C4 Pyrene	170.3	385.5	373.4	0.5750	0.08519
C1 Chrysene	32.0	55.4	35.2	-0.2630	0.70934
C3 Chrysene	339.0	529.6	343.4	-0.2520	0.68890
C1 Fluorene	214.8	911.2	2,755.9	0.9820	0.00068
C2 Fluorene	224.8	583.0	1,731.9	0.9940	0.00014
C3 Fluorene	240.7	751.2	2,402.0	0.9900	0.00026
C1 Dibenzothiophene	70.8	205.6	720.8	0.9930	0.00015
C2 Dibenzothiophene	324.5	712.9	2,233.4	0.9530	0.00283
C3 Dibenzothiophene	92.4	348.0	776.1	0.9770	0.00097
C4 Dibenzothiophene	334.8	1,064.3	2,399.5	0.9970	0.00005
Dibenzothiophene	25.1	86.4	273.7	0.9860	0.00047
1,2-BNT	0.5	1.3	2.3	0.9260	0.00568
2,3-BNT	1.7	1.2	4.0	0.8780	0.01201
Benzothiophene	79.1	294.1	897.4	0.9960	0.00007
Retene	1.5	3.7	14.6	0.9870	0.00041

[1] Estimated from standard solutions of 3, 6, 12.5, 25, and 50% WAF.

Table S15. Shapiro-Wilkes normality test Rstudio outputs for liver and mucus F_2-IsoP concentrations for Chapter 4 data.

Isomer	Treatment	Statistic	p.value	Method
5-iP-VI	Control	0.900276	0.375562	Shapiro-Wilk normality test
8-isoPG F2a	Control	0.940065	0.659719	Shapiro-Wilk normality test
8,12 isoPF-VI	Control	0.839847	0.129986	Shapiro-Wilk normality test
ent-8-iso-15(S)-PG	Control	0.9614	0.830466	Shapiro-Wilk normality test
5-iP-VI	Low	0.718739	0.018792	Shapiro-Wilk normality test
8-isoPG F2a	Low	0.865342	0.2798	Shapiro-Wilk normality test
8,12 isoPF-VI	Low	0.705805	0.013537	Shapiro-Wilk normality test
ent-8-iso-15(S)-PG	Low	0.863968	0.27467	Shapiro-Wilk normality test
5-iP-VI	Medium	0.891659	0.326972	Shapiro-Wilk normality test
8-isoPG F2a	Medium	0.968123	0.879596	Shapiro-Wilk normality test
8,12 isoPF-VI	Medium	0.912487	0.452957	Shapiro-Wilk normality test
ent-8-iso-15(S)-PG	Medium	0.900603	0.377507	Shapiro-Wilk normality test
5-iP-VI	High	0.929626	0.577216	Shapiro-Wilk normality test
8-isoPG F2a	High	0.882196	0.279292	Shapiro-Wilk normality test
8,12 isoPF-VI	High	0.937472	0.638904	Shapiro-Wilk normality test
ent-8-iso-15(S)-PG	High	0.959659	0.81711	Shapiro-Wilk normality test

Table S16. Pearson correlation RStudio outputs for liver and mucus F_2-IsoP concentrations in Chapter 4.

isomer	treatment	estimate	statistic	p.value	parameter	conf.low	conf.high
5-iP-VI	Control	0.595684	1.483244	0.21216	4	-0.41791	0.94864
8-isoPG F2a	Control	0.147711	0.298698	0.780045	4	-0.75427	0.856587
8,12 isoPF-VI	Control	0.95628	6.539763	0.002825	4	0.646299	0.995361
ent-8-iso-15(S)-PG	Control	0.782286	2.511674	0.06594	4	-0.08018	0.974906
5-iP-VI	Low	0.064929	0.092017	0.935071	2	-0.9558	0.965751
8-isoPG F2a	Low	-0.54398	-0.91682	0.45602	2	-0.98835	0.874094
8,12 isoPF-VI	Low	-0.32661	-0.4887	0.673385	2	-0.98006	0.924759
ent-8-iso-15(S)-PG	Low	-0.44641	-0.70552	0.553591	2	-0.98493	0.901423
5-iP-VI	Medium	-0.14653	-0.29625	0.781785	4	-0.85626	0.754791
8-isoPG F2a	Medium	-0.20111	-0.41061	0.702403	4	-0.87058	0.729518
8,12 isoPF-VI	Medium	-0.60326	-1.5128	0.204878	4	-0.94981	0.408097
ent-8-iso-15(S)-PG	Medium	-0.59469	-1.47941	0.213124	4	-0.94849	0.41918
5-iP-VI	High	-0.55382	-1.33028	0.254204	4	-0.94199	0.468162
8-isoPG F2a	High	-0.16734	-0.33947	0.751332	4	-0.86186	0.745459
8,12 isoPF-VI	High	-0.87165	-3.55685	0.023653	4	-0.98583	-0.20537
ent-8-iso-15(S)-PG	High	-0.37888	-0.81882	0.458869	4	-0.91048	0.624794