Using Hepatic Gene Expression Assays in English sole (*Parophrys vetulus*) to Investigate the Effects of Metro Vancouver Wastewater Effluents in Fish

by

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B.Sc., Simon Fraser University, 2019

Project Submitted in Partial Fulfillment of the
Requirements for the Degree of
Master of Environmental Toxicology

in the
Department of Biological Sciences
Faculty of Science

SIMON FRASER UNIVERSITY
Spring 2022

Declaration of Committee

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Abstract

This study investigated the effects of Metro Vancouver's wastewater treatment plant (WWTP) effluents on English sole (*Parophrys vetulus*) hepatic gene expression using novel targeted gene expression assays to compliment the 2017 Burrard Inlet Ambient Monitoring Program. Seven locations of varying distance to the WWTPs were included. Twelve genes involved in xenobiotic defense (CYP1A, HSP70), thyroid function (DIO1), lipid and glucose metabolism (FABP1, FASN, GLUT2, PPARδ, PPARγ), protein synthesis (18S rRNA, RPS4X), and reproduction (ERα, VTG) revealed several differences between these impacted sites. Strong evidence of exposure to estrogenic contaminants was observed based on male VTG transcript levels with the highest induction nearest Lions Gate primary WWTP, where females exhibited advanced ovarian development. Future studies that incorporate reference sites and controlled lab studies are critical to better understand the natural variation in all measures in this species, and to further develop these potential biomarkers for more thorough risk assessments of WWTP.

Keywords: Biomarker; Biomonitoring; Burrard Inlet; English Sole; Gene Expression; Metro Vancouver

Dedication

It is with genuine gratitude and warm regard that I dedicate this work to my parents, Dhirubhai and Minaxiben Parekh, whose words of encouragement and drive for tenacity have motivated me daily. I am forever grateful for your hard work and constant sacrifice to pave the path that I walk on today – without you I'd be nowhere near the person I am today.

Acknowledgements

First and foremost, I would like to express my sincere gratitude and thanks to my senior supervisor, mentor, and guide Dr. Vicki Marlatt. Her dedication and keen interest above all her inspiring attitude to help her students succeed in all endeavours has been the sole reason behind my success. Her timely advice, positive criticism, scientific approach, and most importantly genuine support throughout my graduate degree has helped me in every extent. I would also like to thank Dr. Chris Kennedy for his advice, encouragement, and guidance as a member of my graduate committee, director of the MET program, and fellow professor of several of my graduate courses.

I would also like to thank all my fellow Marlatt and Kennedy lab mates for their unwavering encouragement and support. Particularly, I would like to thank Geoffrey Su, Blake Danis, and Mason King for consistently providing welcoming support, guidance, and feedback during my time in the lab. Furthermore, a special thank you to Daniel King for mentoring me with endless opportunities and support throughout my undergraduate and graduate degree, as those opportunities played significant roles in peaking my interests in the field of molecular toxicology.

I also want to take a moment to thank my fellow MET Squad: Manpreet Jhutty, Isabella Borea, Stephanie Renkers, Anup Hura, Chloe Reves, Hannah Jones, Courtney Bogstie, Nathan Young, Nasim Abrishami, and Andrew Mylly for the stimulating discussions, sleepless nights working towards deadlines, and the endless laughter's and jokes during the last three years.

Lastly, I would like to express my upmost gratitude towards my family - without them, all of this would have not been possible. Thank you for your encouragement, support, guidance, and most importantly unconditional love throughout my entire educational journey.

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List of Acronyms

^{o}C	Degrees in Celsius
^{o}F	Degrees in Fahrenheit
µg	Microgram
µL	Microlitre
µg/L	Micrograms per Litre
18S rRNA	18S Ribosomal Ribonucleic Acid
ACTB	Beta Actin
AHH	Aryl Hydrocarbon Hydroxylase
AhR	Aryl Hydrocarbon Receptor
ANOVA	Analysis of Variance
AOP	Adverse Outcome Pathway
ARNT	Aryl Hydrocarbon Receptor Nuclear Translocator
BC	British Columbia
BC SQG	British Columbia Sediment Quality Guideline
BC WQG	British Columbia Water Quality Guideline
BIAMP	Burrard Inlet Ambient Monitoring Program
BOD	Biochemical Oxygen Demand
CCME	Canadian Council of Ministers of the Environment
cDNA	Complimentary Deoxyribonucleic Acid
CEPA	Canadian Environmental Protection Act, 1999
CF	Condition Factor
COC	Contaminant of Concern
Cq	Quantification Cycle
CSOs	Combined Sewer Overflows
CV	Coefficient of Variation
CYP1A	Cytochrome P450 1A
DDT	Dichlorodiphenyltrichloroethane
DIO	Iodothyronine Deiodinase
DNA	Deoxyribonucleic Acid

eEF1α1	Eukaryotic Elongation Factor 1 Alpha 1
E1	Estrone
E2	Estradiol
EE2	17α-Ethinylestradiol
ERα	Estrogen Receptor Alpha
EROD	Ethoxyresorufin-O-Deethylase
EU	European Union
EU WFD	European Union Water Framework Directive
FABP1	Fatty Acid Binding Protein 1
FASN	Fatty Acid Synthase
FET	Fish Embryo Test
GC%	Guanine-Cytosine Content
GLUT2	Glucose Transporter 2
GOI	Gene of Interest
GSI	Gonadosomatic index
GST	Glutathione s-transferase
HSI	Hepatosomatic Index
HSP70	Heat-Shock Protein 70
Hz	Hertz
IDT	Integrated DNA Technologies
IL-1β	Interleukin 1 Beta
IL-6	Interleukin 6
ILWRMP	Integrated Liquid Waste and Resource Management Plan
IRD	Inner Ring Deiodination
Km	Kilometer
Km^2	Square Kilometer
LN	Natural Logarithm
m^3	Cubic meter
MDL	Maximum Daily Load
mg/L	Milligrams per Litre
MIE	Molecular Initializing Event

min	Minute
MIQE	Minimum information for publication of quantitative PCR experiments
ML	Maximum load
mL	Millilitre
mM	Millimolar
mm	Millimeter
MMP23B	Matrix Metallopeptidase 23B
mRNA	Messenger Ribonucleic Acid
N	Sample Size
NCBI	National Center for Biotechnology Information
ng	Nanogram
ng/L	Nanograms per Litre
nM	Nanomolar
NP	Nonylphenol
NTC	No Template Control
OCPs	Organochlorine Pesticides
OD	Optical Density
OECD	Organisation for Economic Co-operation and Development
OP	Organophosphate
OPFR	Organophosphorus Flame Retardants
ORD	Outer Ring Deiodination
PAH	Polycyclic Aromatic Hydrocarbon
PBBs	Polybrominated Biphenyls
PBDEs	Polybrominated Diphenyl Ethers
PCBs	Polychlorinated Biphenyls
PCR	Polymerase Chain Reaction
pmol	Picomole
PPARs	Peroxisome Proliferator Activated Receptors
PPARα	Peroxisome Proliferator Activated Receptor Alpha
PPARδ	Peroxisome Proliferator Activated Receptor Delta

PPARγ	Peroxisome Proliferator Activated Receptor Gamma
PPCPs	Pharmaceuticals and Personal Care Products
PYGL	Glycogen Phosphorylase L
Qc	Quebec
R^2	Coefficient of Determination
RIN	Ribonucleic Acid Integrity Number
RNA	Ribonucleic Acid
RPS4X	Ribosomal Protein S4 X-Linked
RQI	Ribonucleic Acid Quality Indicator
RT-qPCR	Real-Time Quantitative Polymerase Chain Reaction
SFU	Simon Fraser University
SOCS2	Suppressor of Cytokine Signalling 2
SOD1	Superoxide Dismutase Type 1
SOD2	Superoxide Dismutase Type 2
SOD3	Superoxide Dismutase Type 3
SSOs	Sanitary Sewer Overflows
T_2	3,3'-Diiodothyronine
T_3	3,3',5-Triiodothyronine
T_4	Thyroxine
TCDF	2,3,7,8-Tetrachlorodibenzofuran
THRα	Thyroid Hormone Receptor Alpha
THRβ	Thyroid Hormone Receptor Beta
TNFα	Tumor Necrosis Factor Alpha
tOP	Tert-Octylphenol
TSS	Total Suspended Solids
UBC	University of British Columbia
UBQ	Ubiquitin
UDPGT	Uridine Diphosphate Glucuronosyltransferase
US EPA	United States Environmental Protection Agency
UTM	Universal Transverse Mercator
U/µL	Enzymatic Unit per Microlitre

V	Volts
VTG	Vitellogenin
WAP65	Warm Temperature Acclimation Protein 65
WSER	Wastewater Systems Effluent Regulations
WWTP	Wastewater Treatment Plant

Glossary

Adverse Outcome Pathway	Defines a sequence of key events commencing with the interaction of a stressor (e.g., toxicant) on a target cell or tissue and resulting in an adverse outcome for an organism
Biomarker	A measurement of the modification of body fluids, tissues, cells, and/or components of cells in an organism which can indicate exposure to and/or adverse effects associated with a toxin, toxicant, and/or stressor
Downregulation	The process by which a cell decreases the quantity of a cellular component such as RNA or protein in response to an internal or external stimulus
Effluent	Sewage that has been treated and is released into a natural body of water, from a structure such as a sewage treatment plant, sewer pipe, industrial wastewater treatment plant, or industrial outfall
Gene	A distinct sequence of nucleotides forming part of a chromosome, the order of which determines the order of monomers in a polypeptide or nucleic acid molecule which a cell may synthesize
Gene Expression	The process by which the genetic code – the nucleotide sequence – of a gene is used to direct protein synthesis and produce a structure of the cell
Influent	Raw, untreated wastewater that flows into a reservoir, basin, or treatment plant for various treatment processes
In Vitro	Performed or taking place in a test tube, culture dish, or elsewhere outside of the living organism
In Vivo	Performed or taking place in a living organism
Thesis	An extended research paper that is part of the final exam process for a graduate degree. The document may also be classified as a project or collection of extended essays.
Upregulation	The process by which a cell increases the quantity of a cellular component such as RNA or protein in response to an internal or external stimulus
Wastewater	Commonly referred to as sewage, is defined as water that is used in the transport of a number of different pollutants and waste products from domestic and industrial sources

1. Introduction

1.1. Wastewater Treatment and Management in Canada

Wastewater treatment is a critically important service performed by various sewage treatment facilities that are regulated by federal, provincial, and municipal governments to collect, treat, and dispose of sewage from homes, businesses, industrial clients, as well as stormwater run-off. As a result, municipal wastewater treatment plants (WWTPs) are the largest point sources of water pollution in Canada discharging approximately 5.68 billion m^3 of sewage waste annually (Environment and Climate Change Canada, 2017). Wastewater, commonly referred to as sewage, is defined as water that is used in the transport of a number of different pollutants and waste products from domestic and industrial sources, including soap, food scraps, human waste, oils, and other chemicals (Sonune and Ghate, 2004).

Currently WWTPs in Canada are required to treat conventional organic and bacteriological contaminants, including suspended solids, metals, and nutrients (mainly phosphorus and nitrogen; Sonune and Ghate, 2004; Crini and Lichtfouse, 2018). At a minimum, all treatments plants are designed to address two crucial components of wastewater: (1) total suspended solids (TSS); and (2) the biochemical oxygen demand (BOD; Environment and Climate Change Canada, 2017). Wastewater influent is comprised up of small-to-large particulates of matter referred to as TSS that are known to carry bacteria and other pathogenic organisms capable of settling on substratum or floating on the epipelagic zone; ultimately, elevation in TSS lead to the destruction of aquatic habitats and wildlife due to increased sedimentation and/or introduction of pathogenic organisms (Gerardi and Lytle, 2015). Furthermore, the degradation of all organic matter requires an oxidative expenditure measured via the BOD; a measure of the amount of oxygen consumed by microorganisms during the decomposition process of organic matter (Gerardi and Lytle, 2015). As a result, WWTPs are equipped to reduce the BOD by reducing organic matter pollutants discharged in wastewater to minimize environmental degradation. Therefore, currently, TSS and the BOD are ultimately used to gauge the levels of wastewater treatment based on their effectiveness and ability to comply with federal, provincial, and municipal standards (Environment and Climate Change Canada, 2017). However, approximately 30 000 synthetic chemicals registered

for use in Canadian commerce as well as hormones and other biologically active human excretory products (e.g., hormones) are largely unregulated and continuously discharged from conventional WWTPs into receiving waters (Food and Drugs Act F-27, 1985). Exposure to these cocktails of chemicals, pharmaceuticals, and human excretory biological products poses a risk to aquatic wildlife downstream of WWTP facilities, and several studies since the early 1990's have documented the adverse effects of WWTP effluents in fish and invertebrates (Ings et al., 2011; Matthiessen et al., 2018; Purdom et al., 1994).

Wastewater treatment systems are categorized into three main stages, primary, secondary, and tertiary, with progressive reduction of various contaminants at each stage. Primary treatment involves the use of screens to remove large debris and suspended solids which can be of concern to pipes and delicate equipment (Crini and Lichtfouse, 2018). The completion of screening processes generally results in the reduction of BOD and TSS by 30% and 60%, respectively, from raw primary biosolids which contain organic and inorganic matter which are further removed through a sedimentation process (Crini and Lichtfouse, 2019). The secondary stage of wastewater treatment, generally proceeding primary treatment, removes about 90% of both TSS and BOD from organic debris in sewage through the use of aerobic bacteria and aeration processes (Crini and Lichtfouse, 2019). Secondary treatment accelerates the environmental biodegradation process of organic matter by combining air and raw primary biosolids (heavily laden with bacteria) into a close proximity within wastewater; chlorine and other disinfectants are added to the final product to kill pathogenic bacteria and reduce unwanted odor (Crini and Lichtfouse, 2019). However, results of a Swedish study of 15 WWTPs which analyzed for the residues of 164 selected contaminants of concern (COCs) revealed that primary wastewater treatment had minimal reduction of COCs, whereas secondary wastewater treatment noted several cases of negative removal (i.e., bioactivation of parent compounds) (Golovko et al., 2021). Furthermore, even removal efficiencies by secondary wastewater treatment of several pharmaceuticals, antibiotics, and pesticides surveyed for remained quite low (Golovko et al., 2021). Although when nitrification processes were added to the conventional secondary wastewater treatment processes, removal efficiencies of greater than 90% for estrogenic compounds in WWTPs were achieved (Golovko et al., 2021). Ultimately, it is widely accepted that the major source of pharmaceuticals and personal care products

(PPCPs) in receiving aquatic environments is due to the incomplete and inconsistent removal processes in primary and secondary WWTPs (Ebele et al., 2017). As a result, redesigning and optimizing existing wastewater treatment processes to include advanced tertiary treatment systems to remove additional COCs (i.e., PPCPs, human hormones, etc.) could avoid their release into receiving aquatic environments is needed (Golovko et al., 2021; Crini and Lichtfouse, 2019). Tertiary wastewater treatment systems include primary and secondary treatment followed by various physical, chemical, and biological processes to remove organic, bacteriological, and COCs (Crini and Lichtfouse, 2019). For instance, ozonation, activated carbon adsorption, advanced oxidation processes, and photolytic reactions are all forms of tertiary treatment known to inactivate pathogens and remove COCs in urban wastewater (Crini and Lichtfouse, 2019).

Currently, most WWTPs in Canada are primary and secondary treatment systems and effluents discharged are subject to national standards. Indeed, in 2012 the federal government of Canada published the Wastewater Systems Effluent Regulations (WSER; SOR/2012-139) under the Fisheries Act to provide national standards for effluent quality, record-keeping, reporting, and acute rainbow trout toxicity testing that can be achieved by secondary wastewater treatment (Environment and Climate Change Canada, 2017). These national standards solely apply to facilities across Canada which receive an average minimum influent influx of 100 m^3 with the intent to be released into natural environments (Environment and Climate Change Canada, 2017). As of 2015, these minimum effluent quality standards that are intended to be achieved through secondary treatment processes and include BOD and TSS standards of $\leq$ 25 mg/L, as well as maximum residual chlorine and un-ionized ammonia levels of 0.02 mg/L and 1.25 mg/L (at $15^oC \pm 1^oC$), respectively (Environment and Climate Change Canada, 2017). The most recent data shows that between 2013 to 2017 the proportion of Canada's population served by each treatment category remained stable: ~28% primary; 43% secondary; 14% tertiary; 2% no treatment; and 14% not served by municipal wastewater systems (Environment and Climate Change Canada, 2020). Interestingly, as of June 30, 2014, a transitional authorization was provided to wastewater management facilities not meeting the secondary treatment effluent quality limits to upgrade their systems by the end of 2020, 2030, or 2040 based on risk posed on receiving waters set by the WSER (Environment and Climate Change Canada, 2017).

1.2. Wastewater Treatment in Metro Vancouver

The federation of Metro Vancouver spans a land area of 2,883 km^2 in south-western British Columbia and is comprised of 21 municipalities (Statistics Canada, 2017). As a whole, Metro Vancouver collaboratively strategizes and distributes regional scale services to its 2.4 million inhabitants through accessible drinking water, recreational services, and operates the treatment of 450 billion litres of wastewater every year (Environment and Climate Change Canada, 2017; Statistics Canada, 2017). In Metro Vancouver, the region's five major WWTPs and various combined sewer overflows (CSOs) and sanitary sewer overflows (SSOs) are designed and categorized relative to where wastewater effluents are discharged (Metro Vancouver, 2019). The areas of Annacis Island, Lulu Island, and Northwest Langley operate and manage three secondary WWTPs that continuously treat and discharge wastewater effluent into the Fraser River (Metro Vancouver, 2019). Similarly, Iona Island and Lions Gate manage and operate Metro Vancouver's two primary WWTPs that treat and discharge wastewater effluent into the Strait of Georgia and Burrard Inlet, respectively (Metro Vancouver, 2019). In Metro Vancouver, the discharge of wastewater effluents into their respective receiving waterbodies is guided by the Metro Vancouver's Integrated Liquid Waste and Resource Management Plan (ILWRMP) and regulated by the national WSER (Metro Vancouver, 2019). For instance, in 2017, all secondary WWTPs met WSER BOD and TSS standards of $\leq$ 25 mg/L; whereas primary plants were issued transitional authorizations on September 5, 2014, as minimum guidelines were not met (Metro Vancouver, 2019). A summary of Metro Vancouver's WWTPs with respect to municipal populations served, average annual effluent flow volumes, and BOD and TSS removed for the 2017 year are detailed in Table 1.1. Over the last several decades, due to population growth and industrial developments, conventional WWTP facilities in Metro Vancouver have undergone expansions to accommodate for population growth and wastewater treatment demands (Metro Vancouver, 2019). For instance, a new tertiary WWTP, located 2 km east of the Lions Gate primary WWTP, is planned to become operational in the year 2024 to replace the current primary treatment plant (Metro Vancouver, 2019). Overall, given the mass volumes of wastewater discharge that eventually enter our rivers and oceans, Metro Vancouver (2019) recognizes the large volume of inputs of wastewater discharge into BC waters and as part of its ILWRMP is

committed to monitoring, assessing, and forecasting the impacts of liquid waste discharge on receiving waterbodies (Metro Vancouver, 2019).

Table 1.1. Metro Vancouver, which serves a federation of 21 municipalities consisting of over 2.4 million inhabitants, treats approximately 450 billion litres of wastewater across its five major wastewater treatment plant (WWTP) facilities. Of this total, over 235 billion litres received primary treatment (Iona Island and Lions Gate) with the remaining 214 billion litres treated at the three secondary wastewater plants (Annacis Island, Lulu Island, Northwest Langley). For each WWTP, Individual treated effluent flows (ML), quanities of BOD and TSS removed, and the average reduction in BOD and TSS loadings for all plants in 2017 are provided.

	Annacis Island	Iona Island	Lions Gate	Lulu Island	Northwest Langley
Population Size	1'000'000	600'000	180'000	172'000	27'000
Effluent Flow (ML)	183'589	205'085	30'419	25'768	4681
BOD (Tonnes Removed)	31'524	10'939	1663	6777	1331
%BOD Reduction	94	40	44	98	95
TSS (Tonnes Removed)	29'757	14'986	3080	5328	1275
%TSS Reduction	90	56	65	97	95

1.3. Metro Vancouver's Wastewater Treatment Plant Environmental Monitoring Programs

Part of Metro Vancouver's ILWRMP aimed to protect human and environmental health includes monitoring water and sediment quality and conducting biological monitoring studies to assess the habitat and health of various aquatic biota near and far from WWTP discharge sites (Metro Vancouver, 2017). As such, Metro Vancouver's Environmental Monitoring Programs in the Fraser River, Strait of Georgia, and Burrard Inlet operate on a five-year cycle to assess for water and sediment quality and sentinel aquatic wildlife health impacts of the region's five major WWTPs (Metro Vancouver, 2019). The effects of treated wastewater that is discharged from Annacis Island, Lulu Island, and Northwest Langley secondary WWTPs is assessed through the Fraser River Environmental Monitoring Program (Metro Vancouver, 2019). Whereas the Strait of Georgia Environmental Monitoring Program aims to understand the influence of primary

treated wastewater discharged from the Iona Island WWTP (Metro Vancouver, 2019). In addition, the Burrard Inlet Ambient Monitoring Program (BIAMP), which is the focus of this study, was initiated in 2007 and was designed to assess the potential effects on biota, ecological risks, as well as human health risks of liquid waste discharge from the Lions Gate primary WWTP, eight CSOs, three SSOs, and urban runoff (Metro Vancouver, 2017; 2019).

Originally, the BIAMP involved conducting water column and sediment core analyses across seven designated sites of interests at two depths on an annual and biennial basis, respectively, whereas fish/invertebrate health surveys were conducted once every five years across the same seven designated sites (ENKON Environmental, 2015). In 2014, water column and sediment analyses revealed that 88% and 56% of all site-specific objectives were met, respectively (ENKON Environmental, 2015). All sediment sampling sites revealed at least three or more parameters to have exceeded maximum Canadian Environmental Quality guideline concentrations including, but not limited to, chromium, copper, lead, mercury, nickel, zinc, 2-methylnaphthalene, dioxins, and furans, polybrominated diphenyl ether-99 (PBDE-99), aldrin, chlordane, and hexachlorobenzene (ENKON Environmental, 2015). Lastly, water column dissolved oxygen was measured below minimum BC Water Quality Guidelines (BC WQG) for aquatic life throughout the Burrard Inlet with environmental concerns directed towards deeper water sites (ENKON Environmental, 2015). In 2017, the BIAMP was reduced to five sites (BIA-1; BIA-2; BIA-3; BIA-5; BIA-6; Figure 1.1) with the omission of a reference site at the North end of the Indian Arm with no known municipal or industrial inputs (Site BIA-7) and one site at the mouth of the Indian Arm (Site BIA-4; Metro Vancouver, 2017). However, two additional biota sampling sites associated with Iona Island's WWTP (II-NF; II-FF; Figure 1.1) were added to the 2017 round of sampling events to assess for potential effects from the Iona Island WWTP and as a reference site for the BIAMP (Bailey et al., 2003; Metro Vancouver, 2017). II-NF was selected with the intent to be directly influenced by the Iona Island primary WWTP discharge site, whereas II-FF was intended to have minimal or non-existent influence by WWTP effluent exposure (Bailey et al., 2003). Furthermore, II-FF was selected as a reference station from the Iona Island Deep-Sea Outfall Monitoring Program since it resided well outside the influence of the Lions Gate primary WWTP outfall discharge site (Bailey et al., 2003). The 2017 sampling

sites used in the BIAMP, and Iona Receiving Environment are shown illustrated in Figure 1.1 as well as further detailed in Table 1.2.

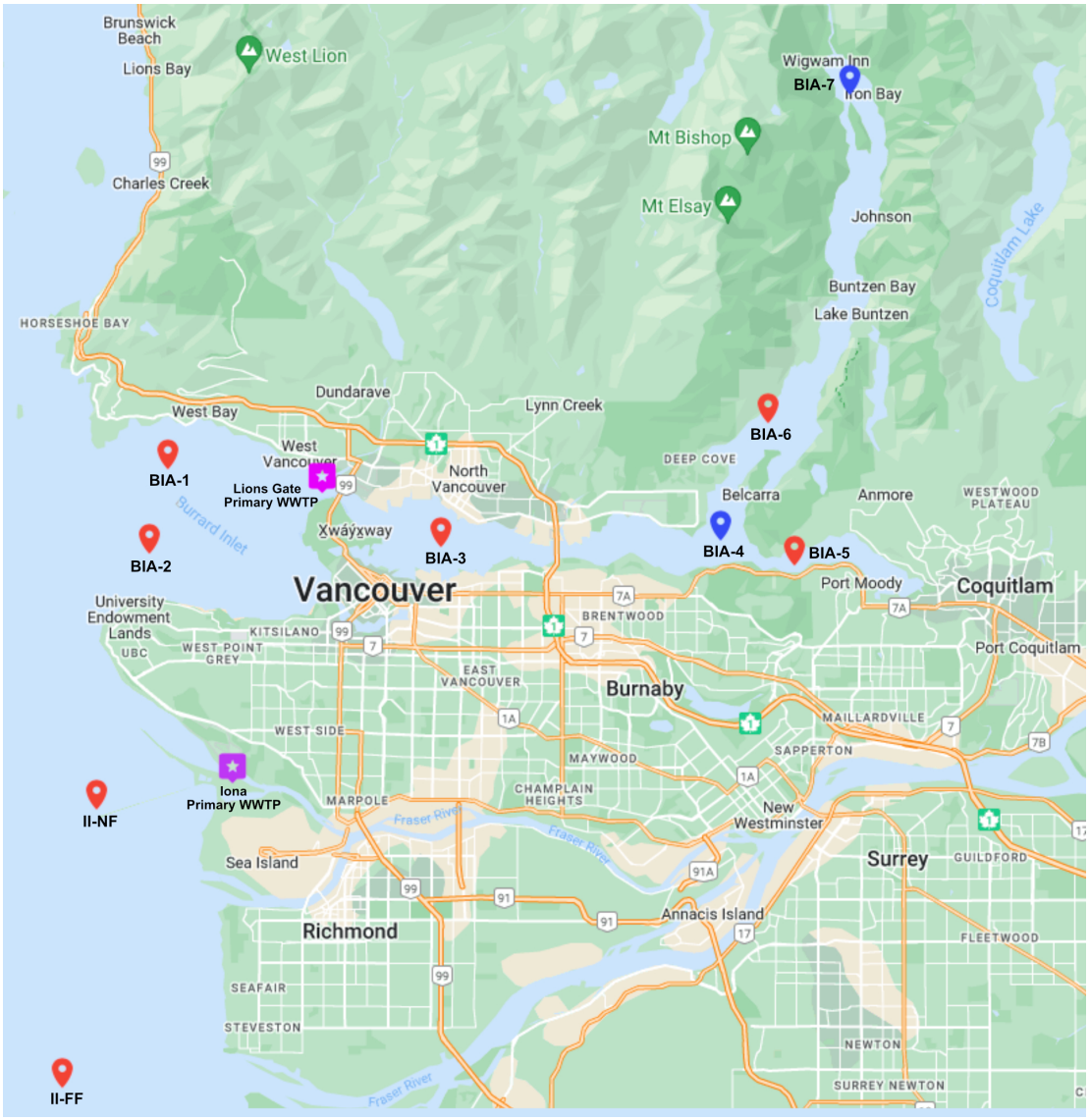

Figure 1.1. Location of seven designated sampling sites along the Burrard Inlet and Iona involved in water, sediment, and English sole (*Parophrys vetulus*) collections for the Burrard Inlet Ambient Monitoring Program (BIAMP). In 2017, the BIAMP included five sites (BIA-1, BIA-2, BIA-3, BIA-5, BIA6) along the Burrard Inlet with the omission of one site at the mouth of the Indian Arm (BIA-4) and a reference site at the North end of the Indian Arm (BIA-7). In addition, two sampling sites associated with Iona Island's primary WWTP were added to assess for potential effects from the Iona Island primary WWTP (II-NF) and act as a reference site (II-FF) for the 2017 BIAMP. Current sampling sites along the Burrard Inlet and Iona are depicted in red with historical sites represented in blue. Metro Vancouver's primary WWTPs, Lions Gate and Iona, are further detailed in purple.

As of 2007, the fish health and tissue chemistry surveys of the BIAMP involved aquatic biota monitoring using the English sole (*Parophrys vetulus*) as a sentinel fish species (Metro Vancouver, 2017). The English sole, commonly referred to as the lemon sole, is known to inhabit the shallow nearshore waters of western North America from the Aleutian Islands, Alaska to Baja California, Mexico (Levings and Ong, 2004). In Canada, the densest population of English sole reside off the coast of British Columbia where adults are known to reside in deep offshore waters and migrate annually to shallow oceans and estuaries to spawn over soft, muddy ocean floors at depths of 60 m to 110 m between the months of January to March (Levings and Ong, 2004). Female English sole reach sexual maturation within 2 to 4 years and can release up to 2 million eggs over the course of their lifetime (Lassuy, 1989). Early larvae begin their first few months in the intertidal zone and shallow waters (10 – 40 m) prior to being transported to estuarine nurseries by tidal currents; a phenomenon that is quite uncommon among flatfish species (Lassuy, 1989; Levings and Ong, 2004). Juveniles can remain in these areas for up to 2 years prior to migrating into deeper ocean waters (40 – 150 m) and maintain diets that consists primarily of crustaceans, worms, small bivalves, and clam siphons (Lassuy, 1989; Levings and Ong, 2004). In the Vancouver Harbour region of Burrard Inlet, English sole are commonly involved in commercial trade, thus are important economically as well as an important oceanic, carnivorous flatfish species closely associated with both the sediment and water column.

Several studies have reported that English sole are sensitive to industrial contaminant exposure due to their ability to accumulate lipophilic hydrocarbons (Levings et al., 2004). Consequently, English sole have been the sentinel species for the BIAMP for adverse health effects to fish due to contaminant exposure via WWTP effluent discharge. This program has been conducted every five years since 2007 and has entailed analyzing composite muscle tissue, blood plasma, and liver samples for the presence of metals, dioxins and furans, polychlorinated biphenyls (PCBs), polybrominated diphenyl ethers (PBDEs), organochlorine pesticides (OCPs), and perfluorinated compounds (PFCs; ENKON Environmental, 2020). The two most recent rounds under the BIAMP in 2012 and 2017 demonstrate several contaminants in WWTP effluents are present in English sole and its habitat. For example, in the 2017 BIAMP, whole body tissue analyses revealed that mercury, methylmercury, PBDE-99, PBDE-100, PCBs, total dichlorodiphenyltrichloroethane (DDT), and 2,3,7,8-

tetrachlorodibenzofuran (2,3,7,8-TCDF) all exceeded the minimum guidelines for the protection of environmental wildlife across a majority of sites (ENKON Environmental, 2020). Furthermore, biochemical liver and plasma analyses were conducted to quantify cytochrome P450 1A (CYP1A) enzyme and vitellogenin (VTG) measurements, respectively, in fish downstream of WWTP effluents (ENKON Environmental, 2015). In 2017, the general findings found higher CYP1A activity in fish from the Port Moody and Indian Arm (BIA-5, BIA-6; respectively) than in fish from the Outer Harbour (BIA- 1 and 2) which correlated with both 2007 and 2012 surveys (Nautilus, 2009; ENKON Environmental, 2015). Furthermore, correlation results suggested that PCBs, 2,3,7,8-TCDF, DDT, and/or a combination of these substances could be contributing to CYP1A induction in English sole in Burrard Inlet (ENKON Environmental, 2015). In 2012, protein plasma VTG concentrations in five male fish per site was quantifiable at all sites in the Port Moody Arm and in Indian Arm South (BIA-3, 4, 5, 6, and 7), whereas only one male fish from the Outer Harbour (BIA-1 and 2) displayed quantifiable plasma VTG (ENKON Environmental, 2015). Such results differed from the 2007 survey which found higher protein plasma VTG concentrations in male fish from the Outer Harbour (BIA-1 and 2) and Port Moody Arm (BIA-5) than in males from the other sites (Nautilus, 2009). Interestingly, correlation analyses suggested that estrogenic substances in 2,3,7,8-TCDF and DDT may have been contributors to VTG production in male English sole in Burrard Inlet (ENKON Environmental, 2015). However, PPCPs and hormones, many of which known to be strong estrogen mimics (i.e., ethynyl estradiol), were not measured in Burrard Inlet in these studies and are likely present and contributing to VTG induction in male English sole.

Table 1.2. List of English sole sampling sites for the Lions Gate and Iona Island primary WWTPs. In the Burrard Inlet Ambient Monitoring Program (BIAMP), English sole samples were collected from five designated sampling sites along the Burrard Inlet in increasing distance from the Lions Gate effluent outfall site: BIA-1; BIA-2; BIA-3; BIA-5; BIA-6. Similar samplings were conducted along Iona Island in increasing distance from the the Iona Island WWTP effluent outfall site: II-NF; II-FF. For each site of interest, location coordinates (Universal Transverse Mercator, UTM) and a brief description of the physical sites are provided.

Site	Location	Coordinates (UTM)	Description
BIA-1	Outer Harbour North	E 483752; N 5464073	1.5 km offshore to the south of Pacific Institute
BIA-2	Outer Harbour South	E 482935; N 5460679	1.5 km north of Spanish Bank
BIA-3	Inner Harbour	E 493841; N 5460963	West of Loch Katrine Bank
BIA-5	Port Moody Arm	E 506788; N 5459875	East of Port Moody Narrows
BIA-6	Indian Arm	E 505549; N 5464824	Representative southern site
II-NF	Iona Island	E 478523; N 5453266	2-4 km north of Iona diffuser
II-FF	Iona Island	E 477283; N 5442000	8-10 km of south Iona diffuser

1.4. Wastewater Effluent Contaminants Impact Aquatic Wildlife

Contaminants have been identified as one of Earth's greatest threats to natural ecosystems (United States Environmental Protection Agency, 2021). As of the late 1800s, industrialization, agriculture, and municipal sewage treatment has resulted in urban waters worldwide, including in Metro Vancouver, to become a central hub for discharge of numerous anthropogenic pollutants from various point and non-point sources (Rehman et al., 2015; Stehr et al., 2004). However, of the several contaminants detected (e.g., metals; PAHs; polychlorinated dibenzo-p-dioxins and polychlorinated dibenzofurans; pesticides; surfactants; PBDEs; PCBs; pharmaceuticals and personal care products (PPCPs); and hormones and hormone-mimicking or antagonizing substances) in the sediments and waters of both near and far-field effluent discharge sites, only few have been assessed for individual or combined risk to aquatic wildlife (United States Environmental Protection Agency, 2021). For example, a field study

conducted in 2015 throughout the coastal regions of British Columbia revealed significantly higher concentrations of PAHs in blue mussels at developed coastal sites, which were also associated with significantly elevated hepatic EROD activity in a seabird (Barrow's goldeneyes) that preys on blue mussels (Willie et al., 2017). Elevated hepatic CYP1A activity has been shown to be induced by several WWTP contaminants such as PCBs, TCDF, and DDT, which adversely affect vertebrates via long-term changes involving gene mutations, loss of survival function, and premature death due to hypothermia (Santana et al., 2018). More recently, as of the 1990s, endogenous human hormones, pharmaceuticals, and personal care products in wastewater effluents have been categorized as contaminants of emerging toxicological concern for aquatic life (Khan et al., 2017). Pharmaceuticals are defined as prescription, over the counter, and veterinary therapeutic drugs used to prevent or treat human and animal diseases, while personal care products such as soaps, deodorants, and cosmetics are used mainly to improve the quality of daily life but are comprised of a variety of human-made chemicals (Khan et al., 2017). For pharmaceuticals that are designed to target various processes in vertebrates, many are inherently toxic to aquatic life at low doses (ng/L to µg/L) alone or as mixtures (i.e., additive, or synergistic; Wang et al., 2021). For instance, chronic exposure of adult zebrafish (*Danio rerio*) to wastewater effluent diluted pharmaceutical mixtures consisting of acetaminophen, carbamazepine, gemfibrozil, and venlafaxine, resulted in significant reductions in ovarian histology, oocyte development, and embryo production within six weeks of exposure (Galus et al., 2013). These datasets suggest that fish populations exposed to pharmaceuticals discharged in wastewater are at risk of negative impacts to reproductive capacity and health (Galus et al., 2013). As a result, in 2001, an initial first list of 33 priority substances was identified under the European Union Water Framework Directive (EU WFD) 2455/2001/EC to identify PPCPs as future emerging priority candidates based on a ranking system for these chemicals according to their perceived relative risk, persistence, bioaccumulation, and toxicity to aquatic wildlife in receiving water bodies (European Union Water Framework Directive, 2001). Based on these procedures, PPCPs such as paracetamol, clotrimazole, procyclidine, bisphenol A (BPA), and tamoxifen were identified as hazardous substances with hopes of ongoing research to decrease current knowledge gaps and provide recommendations for future mitigation strategies (European Union Water Framework Directive, 2001).

Globally, and in Canada, a limited number of ambient quality guidelines exist to assess the aquatic wildlife implications of the thousands of chemicals currently registered under commerce (European Union Water Framework Directive, 2001; Food and Drugs Act F-27, 1985). For example, in Canada nonylphenol, nonylphenol ethoxylates, and 17α-ethynylestradiol (EE2) currently have working water and sediment quality guidelines for the protection of aquatic life (British Columbia Ministry of Environment and Climate Change Strategy, 2021; CCME, 2002). Whereas in Europe, priority pollutant lists under the EU WFD include ambient quality guidelines for estrogenic compounds of biological (e.g., 17β-estradiol [E2], estrone [E1], estriol [E3]) and synthetic nature (e.g., BPA, octylphenol [OP], nonyohenol [NP], and EE2; European Union Water Framework Directive, 2001). Such ambient quality guidelines for estrogenic compounds have only arisen due to the irrefutable data on their ability to disrupt the endocrine system and in particular mimic estrogen (Bergman et al., 2012; Matthiessen et al., 2018). However, close to an additional 800 chemicals are known to be capable of interfering with endocrine systems and are termed as endocrine disrupting compounds (EDCs; Bergman et al., 2012). EDCs are defined as chemicals that interfere directly or indirectly with the endocrine system, either by interacting with hormone receptors or by altering hormone synthesis and/or metabolism and thereby causing adverse effects which are observable in wildlife (Matthiessen et al., 2018). For example, current levels of PCBs, OCPs, and methylmercury in some fish-preying birds and marine mammals such as killer whales (*Orcinus orca*) and harbour seals (*Phoca vitulina*) in British Columbia have been associated with effects on breeding, the immune system, and in some cases resulted in endangerment to the population (Krahn et al., 2007; Rayne et al., 2004; Ross et al., 2000; 2012). Furthermore, estrogenic chemical field measurements in bile of English sole at Puget Sound indicated that BPA and tert-OP (tOP) levels were significantly higher in highly developed sites compared to those classified with either medium or low development (Da Silva et al., 2017). Elevated concentrations of BPA and tOP in wastewater effluents has been shown to be associated with feminisation of male fish, amphibians, and reptiles (Bergman et al., 2012; Matthiessen et al., 2018). Nevertheless, synthetic estrogenic compounds such as EE2, which is a major component of oral contraceptives, have shown to have greater impacts on the production, release, transport, and metabolism of hormones relative to natural estrogens (Bergman et al., 2012; Matthiessen et al., 2018). For instance, *in vivo* assays have shown that the potency of EE2 is 11 to 27 times greater than E2 at inducing VTG in

juvenile male rainbow trout (Thorpe et al., 2003). Interestingly, despite such large bodies of research with feminisation of male aquatic vertebrates, much of it being considered robust and reliable, up until 2007 the question still remained about whether the irrefutable data on estrogenic effects on reproductive variables at the organismal level was a biomarker of population-level effects in fish (Bergman et al., 2012; Matthiessen et al., 2018). This was resolved by Kidd et al. (2007) who conducted a 7-year whole lake experiment at the Experimental Lakes Area (Lake 260) in Ontario, Canada which showed that low-level (5 ng/L) chronic waterborne exposure of fathead minnow (*Pimephales promelas*) to EE2 led to induction of VTG in males and females, gonadal abnormalities, and ultimately caused the near extinction of this species from the lake (Kidd et al., 2007). Collectively, the state of science is clear that contaminants in WWTP effluents, including the growing number of COCs shown to interfere with vertebrate endocrine systems, are causing adverse effects in downstream fish populations (OECD, 2012; Robaire et al., 2022).

1.5. Molecular Changes as Early Onset Indicators of Toxic Effects of Environmental Contaminants

With the advancements in molecular biomarkers, particularly, VTG and EROD activity, great strides have been made in documenting the uptake and subsequent bioavailability of estrogen mimics, planar halogenated and polycyclic aromatic hydrocarbon (PHHs and PAHs) contaminants downstream of WWTPs and other industrial activities. Indeed, developing additional biomarkers indicating impaired biological processes (i.e., metabolism, liver detoxification, etc.) in aquatic wildlife tissues and other biological matrices (e.g., bile, plasma, albumin) show great promise in assessing the uptake, bioavailability, and adverse effects of contaminant exposure (Feist et al., 2004; Richardson et al., 2010; Brusle and I Anadon, 2017). For example, caged fathead minnow studies in North Saskatchewan River (Saskatchewan, Canada) located downstream of a WWTP discharge site presented elevated concentrations of PPCPs including estrones, and androgens, which were significantly associated with elevated levels of CYP3A metabolism and antioxidant enzymes (i.e., glutathione reductase, glutathione-s-transferase) relative to upstream reference sites (Jasinska et al., 2015). Nonetheless, there are few validated, reliable, and robust biomarkers available for fish

and other aquatic biota despite intensive research in this area, and ongoing lab and field-based studies are warranted (Hook et al., 2014; Miller et al., 2018).

Recent developments in the field of molecular biology allowing large scale analysis of DNA, mRNA, proteins, and metabolites through genomics, transcriptomics, proteomics, and metabolomics, respectively, is revolutionizing our understanding of the relationships between molecular level changes and adverse outcomes at the organ and organismal level after toxicant exposure (Ings et al., 2011; Knight et al., 2016). However, despite the wide application of employing VTG and EROD and numerous studies measuring molecular level changes to study toxicant adverse effects in organisms, identifying key underlying molecular level changes indicative of higher biological, physiological, and morphological effects definitively is challenging, slow, and under intensive investigation globally. The term adverse outcome pathways (AOPs) has been coined to describe this area of research (Allen et al., 2014; Ankley et al., 2010). Essentially, an AOP is defined as a biological model that identifies a sequence of molecular and cellular events from the exposure of an organism to a chemical through to an understanding of the toxicological effects at the individual or population level (Allen et al., 2014). For example, higher plasma or liver levels of VTG in male fathead minnows have been used to assess exposures of fish to estrogens and estrogen mimics which can alter reproductive behaviours, impair development of reproductive organs, alter larval developments, and in some cases decrease population trajectories (Bergman et al., 2012; Matthiessen et al., 2018). Through such studies, male teleosts have been shown to be sensitive to estrogen receptor agonists leading to a suite of adverse outcomes. Thus, ongoing research and developments of AOPs will undoubtedly be valuable in predicting toxicological effects of several of the environmental contaminants present in anthropogenic wastes, such as WWTP effluents (Bergman et al., 2012; Dang et al., 2011; Hutchinson, 2002; Länge et al., 2001; Matthiessen et al., 2018).

1.6. Research Objectives

In recent years, reports have indicated the presence of numerous COCs measured in wastewater effluents and the receiving aquatic environment worldwide (Da Silva et al., 2017; Matthiessen et al., 2018). Furthermore, several studies have demonstrated adverse effects of WWTP effluent in several fish species on a vast array of biological processes, such as reproduction, metabolism, and immune functions

(Chambers et al., 1997; Liney et al., 2006). In Metro Vancouver there are several WWTPs, and ongoing, intensive field monitoring programs are conducted every five years to assess the potential adverse effects on aquatic wildlife. One specific program, the BIAMP, focusses on Burrard Inlet and Iona Island to examine various contaminants and their effects on fish and their habitat proximal to two primary treatment WWTPs, Lions Gate and Iona (Metro Vancouver, 2017). The objective of this study was to compliment the most recent BIAMP conducted by Metro Vancouver in 2017 to evaluate the effects of WWTP effluents on English sole liver gene expression profiles for several target genes and their correlation to whole body contaminant concentrations and various health indices. To accomplish this objective, several hepatic genes of interest in female and male English sole collected from various locations in Burrard Inlet and near and far-field from the Iona Deep-Sea Outfall were evaluated for differential expression between sites. The genes of interest investigated were designed to evaluate adverse effects on various biological processes and/or systems, including xenobiotic detoxification, thyroid hormone metabolism, lipid and glucose metabolism, and reproduction. We predicted that xenoestrogen loadings in Metro Vancouver waters would be greatest nearest the Lion's Gate primary WWTP and as a result would induce the greatest transcript abundance of vitellogenin and estrogen receptor alpha in both male and female English sole. However, we also hypothesize that sex-specific differences in reproductive genes (ERα, VTG) are expected due to the natural differential expression that exists between genetic males and females; a result that we do not expect to observe in genes of interests from other biological processes and/or systems. Furthermore, we hypothesized that higher concentrations of 2,3,7,8-TCDF, OCPs (e.g., chlordane and DDT), PAHs, and PCBs in English sole whole-body, liver, and muscle tissue samples will be associated with elevated levels of CYP1A transcript abundance. Finally, we predicted that loadings of endocrine disrupting substances would be most persistent nearest the Lion's Gate primary WWTP and as a result be involved in the disruption of the hypothalamus-pituitary-thyroid axis and genes involved in thyroid homeostasis and energy metabolism.

2. Materials & Methods

2.1. Sampling of English Sole (Parophrys vetulus)

Wild English sole (*Parophrys vetulus*) collected during the 2017 Burrard Inlet Fish and Sediment Ambient Monitoring Program by Enkon Environmental, Dr. Vicki Marlatt and Metro Vancouver were analyzed in the present study to examine changes in hepatic gene expression associated with Metro Vancouver's WWTP effluent outfalls. A total of seven sites were sampled for fish communities: BIA-1; BIA-2; BIA-3; BIA-5; BIA-6; II-NF; and II-FF. The number of fish collected at each site ranged between 17 to 22. At each site, a minimum of three x ten-minute bottom trawls were conducted using a scientific otter trawl net to collect English sole at a consistent depth and no more than 50 m from the sampling site. The trawl net was 12 m wide (10 cm mesh) at the entrance connected to steel doors tapering to 1 m width at the cod end (2.5 cm mesh). The vessel was equipped with an aeration chiller system to maintain constant dissolved oxygen concentrations and seawater temperatures in the holding tank. Mature English soles were retained and later sacrificed via blunt force trauma prior to extraction of organs, sex identification and measurement of additional health indices and morphometrics. The female English sole were identified by viewing their larger gonad structure through the ventral surface, whereas male English sole were selected by squeezing the gonads and observing the excretion of sperm. For the fish health and tissue survey, a minimum of 10 male and female English sole from each sampling site were sacrificed and processed to determine total body length (mm), whole body weight (g), liver weight (g), and gonad weight (g). Liver tissues were collected and immediately snap frozen and stored on dry ice, followed by transfer to -80^{o}C for long-term storage until further analyses. During liver tissue collection, all instruments were cleaned and sterilized using 10% peroxide and double rinsed with ultrapure water to ensure that all equipment was nuclease-free in between each animal. All work with English soles was performed in compliance with the guidelines of the Canadian Council for Animal Care and with a permit issued by Metro Vancouver (Vancouver, BC, Canada). Although the plasma vitellogenin level results of the 2017 Burrard Inlet Fish and Sediment Ambient Monitoring Program are not currently available, significant effects on vitellogenin levels in males and females representing a 2.2 and 997-fold increase relative to reference sites, respectively, have been observed (Su and Marlatt, unpublished data). As a result, the present study focuses on evaluating

hepatic genes involved in several biological processes to investigate the additional potential effects of WWTP effluents on wild English sole.

2.2. RNA Isolation and cDNA Synthesis

Total RNA isolation and cDNA synthesis for English sole liver samples used for subsequent real-time quantitative PCR (RT-qPCR) experiments adhered to the guidelines demonstrated by Bustin et al. (2010) for appropriate sample acquisition, assay design and validation, and data analysis.

Total RNA was isolated from the English sole livers using TRIzol® Reagent as described by the manufacturer's instructions (Invitrogen, Burlington, ON, Canada). Individual liver samples were homogenized in safe-lock nuclease-free 1.5 mL Eppendorf microcentrifuge tubes containing 1 mL TRIzol® (per 100 mg of liver tissue) and three to four 1 mm tungsten-carbide beads. Microcentrifuge tubes were homogenized at 30 Hz for a total of 4 minutes in a Retsch Mixer Mill MM400 (Fisher Scientific, Ottawa, ON, Canada); chambers were stopped at 2 minutes and rotated 180^{o} to be homogenized for the final 2 minutes. Total RNA obtained from the TRIzol® RNA isolation procedure was eluted in 50 μL of nuclease-free water and DNase-treated using TURBO DNA-free kits™ (Ambion, Austin, TX) to eliminate any co-extracted DNA. DNase-treated RNA samples were stored at -80^{o}C for long-term storage until further downstream analyses.

Downstream quality assurance checks were conducted to determine RNA concentrations (ng/μL), purity and integrity. An Epoch 2 Microplate Spectrophotometer (BioTek, Winooski, VT, USA) was used to quantify RNA concentrations based on the optical density unit (OD260) and RNA purity was assessed by measuring the OD260/280 and OD260/230 ratios. The integrity of the DNase-treated RNA samples was evaluated by assessing all samples using a Bio-Rad Experion™ Automated Electrophoresis System and Experion software (Version 3.20; Bio-Rad, Mississauga, ON, Canada). RNA concentration, purity and integrity were all assessed to adhere within the MIQE guidelines outlined by Bustin et al. (2010). RNA quality indicatory (RQI) scores for representative samples of at least 50% of the total sample size ≥ 8.0 and OD260/280 and OD260/230 ratios ranging between 1.8-2.1 and 2.0-2.2, respectively, were considered stable and free of significant contamination and were included in subsequent steps of the RT-qPCR experiments.

Reverse transcription cDNA synthesis was performed following instructions provided by the Applied Biosystems High-Capacity cDNA Synthesis Kit (Waltham, MA, USA). Samples of DNase-treated total RNA were briefly thawed on ice and aliquoted to obtain appropriate volumes for 2 μg samples. Respective amounts of nuclease-free water and 5.8 μL of the MultiScribe Reverse Transcriptase master mix were added to the 2 μg of DNase-treated RNA samples to establish a 20 μL reaction mix. Reverse transcription reactions resulted in cDNA samples with a final concentration of 100 ng/μL which were stored at -20 °C until subsequent RT-qPCR experiments were performed.

2.3. Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR)

The RT-qPCR experiments were performed on individual English sole liver cDNA samples to relatively quantify the fold change in mRNA levels of each target gene of interest using the comparative Delta-Delta Cq ($2^{-\Delta\Delta Cq}$) method. In this study, WWTP effluent exposure was assessed on target genes of various biological processes including xenobiotic defense, oxidative stress, protein synthesis, growth and metabolism, immune function, and reproduction. A complete list of target genes of interest is further described in Tables 3.2 and 3.3. Due to the lack of sequencing data available for the English sole, all primer sets were designed based on highly conserved regions found between the European plaice (*Pleuronectes platessa*), European flounder (*Platichthys flesus*), Olive flounder (*Paralichthys olivaceus*) and the Pacific halibut (*Hippoglossus stenolepis*); all which demonstrated to have highly similar phylogenetic ancestries based on 16s rDNA and mitochondrial 12S and 16S sequences (Pardo et al., 2005; Azevedo et al., 2008). Highly conserved regions between English sole and the aforementioned flatfish species were identified through the Clustal Omega Multiple Sequence Alignment tool which aligned and presented nucleotide sequences showing perfect or strong similarities among these species for each target gene of interest (Madeira et al., 2019). A target gene primer set was then designed based on the areas of most conserved nucleotides, optimal melting temperatures (55-60°C) and guanine-cytosine content (GC%, 40-60%) using the Integrated DNA Technologies OligoAnalyzer™ Tool (IDT, www.idtdna.com/calc/analyzer), and sequences obtained from the GenBank National Center for Biotechnology Information (NCBI, https://www.ncbi.nlm.nih.gov/) database for European plaice (*Pleuronectes platessa*), when available, or European flounder

(*Platichthys flesus*), Olive flounder (*Paralichthys olivaceus*) and/or Pacific halibut (*Hippoglossus stenolepis*).

Prior to RT-qPCR experiments, primer sets used to measure gene expression levels were optimized by modifying the annealing temperature, template concentration and primer concentrations through a matrix of reactions. The inclusion of a 7-point standard curve, generated through a 4-fold dilution of 100 ng/µL of cDNA, was used to determine the efficiencies of primers as well as provide valuable information regarding the performance of the reaction such as efficiency, linear range, reproducibility, and acceptable criteria for novel primers (Bustin et al., 2009). To generate a standard curve, a dilution series of 100 ng/µL of template cDNA was prepared by aliquoting 1 µL of cDNA from all reference (17 individual liver cDNA samples) and exposed site (119 individual liver cDNA samples) groups into a 1.5 mL microcentrifuge tube resulting in 136 µL of 100 ng/µL cDNA. Four-fold serial dilutions of the 100 ng/µL cDNA were performed to create a 7-point standard curve in each RT-qPCR assay. Therefore, the 7-point standard curve included the following concentrations: 25, 6.25, 1.56, 0.39, 0.098, 0.024, and 0.006 ng/µL cDNA. For each gene standard curve, 2.5 µL of each diluted template cDNA were added to 10 µL of RT-qPCR master mix in triplicates. Analysis of the standard curve was conducted using the Bio-Rad CFX384™ Real-Time PCR Detection System and CFX Manager™ Software (Mississauga, ON, Canada) which is used to plot the logarithmic function of each diluted concentration against its quantification cycle (Cq). The Cq is referred to as the cycle number at which the fluorescent signal of a cDNA sample surpasses its baseline threshold and can be detected; lower Cq values represent a higher initial copy number of the target gene (Bustin et al., 2009). Based on the logarithmic concentration versus Cq plot, a standard curve can be generated with appropriate values for slope, Y intercepts, reaction efficiencies, and the coefficient of determination (R^2). The acceptable criteria for a standard curve were required to include a single peak denaturation melt curve, reaction efficiencies ranging between 90-110%, acceptable amplification of a minimum of 4 concentration points, and an $R^2 > 0.900$ (Bustin et al., 2009). All gene-specific primer sets meeting these criteria were evaluated for specificity using PCR and gel electrophoresis and were sequenced at the University of British Columbia Sequencing and Bioinformatics Consortium (Vancouver, BC, Canada) to verify the target amplicon sequence identity. English sole liver cDNA samples were PCR amplified using the Bio-

Rad T100 Thermal Cycler (Mississauga, ON, Canada) and ABM Taq DNA Polymerase (Applied Biological Materials, Richmond, BC, Canada) as described by the manufacturer's instructions. PCR reactions were prepared in 0.2 mL individual PCR tubes containing 1 µL of cDNA template (100 ng/µL), 1.5 µL each of the forward and reverse gene-specific primers (10 µM), 5 µL of 10X PCR Reaction Buffer, 3 µL of $MgSO_4$ (25 mM), 1 µL of dNTPs (10 mM), 0.5 µL of Taq DNA Polymerase (5 U/µL), and 36.5 µL of nuclease-free water. PCR tubes were incubated at 94°C for 3 minutes to completely denature the cDNA template prior to performing 35 cycles of amplification as follows: (1) denature at 94°C for 30 seconds, (2) anneal at 55°C for 30 seconds, and (3) extend at 72°C for 15 seconds. Samples were then incubated for an additional 5 minutes at 72°C prior to being maintained at 4°C until subsequent gel electrophoresis. PCR amplified products underwent gel electrophoresis on a 1.5% agarose gel containing SYBR® Safe DNA gel stain (Invitrogen, Burlington, ON, Canada) for 75 minutes at 80 V. Bands were visualized using the Bio-Rad Molecular Imager® ChemiDoc™ XRS+ System (Mississauga, ON, Canada) and gene-specific amplicons were excised on the FisherBiotech Transilluminator (ThermoFisher, Waltham, MA, USA). Gel extraction and purification of excised bands was performed using the GeneJET Gel Extraction Kit (ThermoFisher, Waltham, MA, USA) as described by the manufacturer's instructions. Purified amplicon products were verified through Sanger Sequencing at the University of British Columbia Sequencing and Bioinformatics Consortium (Vancouver, BC, Canada). Sequencing results were analyzed using FinchTV, Version 1.5.0 (Geospiza Inc., Seattle, WA, USA) and aligned with the European plaice (*Pleuronectes platessa*) sequence, when available, or European flounder (*Platichthys flesus*), Olive flounder (*Paralichthys olivaceus*) and/or Pacific halibut (*Hippoglossus stenolepis*) from GenBank National Center for Biotechnology Information (NCBI, https://www.ncbi.nlm.nih.gov/) using Nucleotide Blast (BlastN).

All RT-qPCR assays were performed on the Bio-Rad CFX384™ Real-Time PCR Detection System using Hard-Shell 384 well PCR plates as described by the manufacturer's instructions (Bio-Rad, Mississauga, ON, Canada). Each RT-qPCR reaction well contained the following components in a 10 µL volume of master mix: 0.25 – 0.375 µL each of the forward and reverse gene-specific primers (10 µM); 6.25 µL of the SsoFast™ EvaGreen® Supermix (Bio-Rad, Mississauga, ON, Canada); and 3 µL of nuclease-free water. In addition, 2.5 µL of template cDNA (1:80 dilution, 100 ng/µL) was

added to each reaction well to constitute a final volume of 12.5 µL. Each plate contained two technical replicates per biological sample, which were distributed as follows: II-FF (*n* = 17), II-NF (*n* = 20), BIA-1 (*n* = 17), BIA-2 (*n* = 18), BIA-3 (*n* = 21), BIA-5 (*n* = 21), and BIA-6 (*n* = 22). In addition to gene-specific reaction wells, each RT-qPCR assay contained a standard curve and a no template control (NTC) which were both performed in triplicates to assess precision and accuracy, and confirm the absence of cDNA contamination, respectively. The RT-qPCR assays were performed using the following conditions: activation at 95ºC for 30 seconds followed by 45 cycles of (1) denaturation at 95ºC for 5 seconds and (2) primer annealing at 55ºC for 5 seconds. Upon completion, a melt curve analysis was conducted at an initial temperature of 55ºC and was increased by increments of 0.5ºC every 5 seconds until a maximum temperature of 95ºC was obtained.

2.4. Data Analysis of Gene Expression

In this study, the Livak ΔΔCq method for relative quantitation of target genes between exposure sites was performed using the Bio-Rad CFX Manager™ Software (Mississauga, ON, Canada). To determine the relative expression of each gene of interest, Cq values for all technical replicates per sample were geometrically averaged to provide a biological sample mean per sample per site using the following equation:

$$Geometric\ Mean = \sqrt[n]{x_1 \cdot x_2 \cdots x_n} \tag{2.1}$$

Geometrically averaged Cq values in all experimental samples (i.e., exposure sites) were compared with a calibrator sample (i.e., control site) to calculate a relative gene expression value according to the following equation:

$$\Delta Cq = Calibrator\ Cq - Experimental\ Cq \tag{2.2}$$

With the Livak ΔΔCq method, the ΔCq values for the gene of interest in both the experimental and calibrator samples can be normalized to the quantities of the two reference genes for each sample. As a result, the Livak ΔΔCq method compares the results from experimental samples with both a calibrator, such as the control site in this study, and a normalizer gene (i.e., reference gene) using the following equation:

$$\Delta\Delta Cq = Gene\ of\ Interest\ \Delta Cq - Normalizer\ Gene\ \Delta Cq \tag{2.3}$$

This normalized expression value can also be expressed as a ratio to obtain the relative fold increase or decrease of the target gene in the gene of interest sample relative to the calibrator and normalizer gene. This was calculated using the following equation:

$$Normalized\ Target\ Gene\ Expression = 2^{-\Delta\Delta Cq} \quad (2.4)$$

Reference gene stability was determined by calculating a gene stability measure value, also known as an M-value, using the CFX Manager™ Software Gene Expression Analysis (Bio-Rad, Mississauga, ON, Canada). The M-value, which is based on a pairwise comparison between the analyzed reference gene candidates, was performed to measure the reference gene expression stability relative to all other genes in a particular study. The most stable combination of reference genes will provide the lowest M-value and hence providing a stable baseline to normalize the genes of interest. The criteria for an acceptable M value for two reference genes was defined as < 1, and if the M value exceeded this threshold, it was not considered for this study (Bustin et al., 2009). As a result, normalizing the expression of the target gene to that of the reference gene compensates for any differences in the amount of sample tissue. To illustrate the effects of WWTP effluent discharge on gene expression, normalized expression data (ΔΔCq) was depicted in boxplots for each site and sex. Boxplots were designed in RStudio, Version 1.3.1073 (R Package "ggplot2", Boston, MA, USA) and revised in Affinity Designer, Version 1.9.3 (Serif Europe, West Bridgford, UK).

2.5. Statistical Analyses

All statistical analyses were performed using JMP®, Version 16 (SAS Institute Inc., Cary, NC). Normalized gene expression data values of 17 to 22 biological replicates per site (i.e., individual English sole livers per site; actual n is represented in each figure caption for each gene of interest) were plotted as frequency distribution and normal quantile-quantile plots to visually inspect for evidence of outliers and normal distribution. Normality and homogeneity of variance for each normalized gene expression dataset was also assessed by performing a Shapiro-Wilk's and Levene's test, respectively. Based on a Box Cox transformation ($\lambda = 0$), normalized gene expression data was logarithmically transformed (LN) if it failed normality tests; newly transformed datasets were retested for normality and equal variance. Potential outliers were assessed by

plotting the studentized residuals and if individual observations were scored greater than $\pm$ 3 then they were classified as an outlier and omitted from the dataset (Blatná, 2006). A Two-Way Analysis of Variance (ANOVA) followed by Tukey's post-hoc analysis was used to determine statistical significance of the least square means within and between sites, sexes, and interactions between these two variables. A p-value < 0.05 was used to determine statistical significance for all tests.

3. Results

3.1. RNA Concentrations, Integrity, and Purity

English sole liver RNA samples complied with quality assurance checks for concentration (ng/μL), purity and integrity based on the MIQE guidelines (Bustin et al., 2010) using the Experion™ Automated Electrophoresis System and Experion software, and spectrophotometer readings. The average spectrophotometric OD260/280 ratio ± standard error of the mean for a total of 136 biological samples was 2.11 ± 0.08 indicating relatively pure RNA with minimal non-nucleic acid contamination (i.e., residual TRIzol or other reagents used in the extraction protocol, proteins, etc.). The average RQI score ± standard error of the mean for 50% of a total of 136 biological samples was 8.35 ± 0.18 with the observation of two sharp bands, one each for the large (28S) and small (18S) subunit ribosomal RNAs, with the intensity of the larger band being about twice that of the smaller band; an indicator of intact RNA. RNA concentrations (ng/μL), OD260/280, and OD260/230 ratios for all 136 biological samples are further detailed in Table 3.1.

Table 3.1. List of RNA concentration, purity, and integrity values for all biological liver samples of English sole included in RT-qPCR experiments. Sites refer to the various sampling locations along the Lions Gate and Iona WWTP in increasing distance from the outfall site: BIA-1; BIA-2; BIA-3; BIA-5; BIA-6; II-NF; II-FF. Biological replicates per site are listed by Sample ID with respective RNA concentrations indicated as ng/μL, and total yields of RNA for each sample that was diluted in 50 μL of nuclease free water. RNA purity and integrity are represented by both $OD_{260/280}$ and $OD_{260/230}$ values.

Site	Sample ID	Concentration (ng/μL)	Total Yield (μg)	OD 260/280	OD 260/230
BIA-1	1-1	333.94	16.70	2.121	1.552
	1-5	142.18	7.11	2.068	2.042
	1-6	347.95	17.40	2.106	2.218
	1-7	363.23	18.16	2.105	2.02
	1-8	419.28	20.96	2.11	2.083
	1-9	570.25	28.51	2.131	2.258
	1-10	771.43	38.57	2.13	1.995
	1-11	288.32	14.42	2.132	1.063
	1-12	347.10	17.35	2.136	2.177
	1-13	133.13	6.66	2.078	1.606

Site	Sample ID	Concentration (ng/μL)	Total Yield (μg)	OD 260/280	OD 260/230
	1-14	709.53	35.48	2.127	1.336
	1-15	288.42	14.42	2.078	1.606
	1-16	1271.38	63.57	2.1	2.111
	1-17	503.12	25.16	2.138	1.737
	1-18	781.01	39.05	2.043	1.27
	1-19	695.99	34.80	2.086	1.339
	1-20	814.60	40.73	2.127	1.293
BIA-2	2-1	498.66	24.93	2.138	2.298
	2-3	443.87	22.19	2.117	1.956
	2-4	499.33	24.97	2.138	2.066
	2-5	259.85	12.99	2.127	2.058
	2-6	286.86	14.34	2.127	2.126
	2-7	525.83	26.29	2.134	1.953
	2-9	256.93	12.8465	2.102	1.958
	2-10	708.77	35.44	2.12	1.948
	2-11	417.56	20.88	2.099	2.163
	2-12	190.76	9.54	2.123	2.173
	2-13	280.64	14.03	2.145	2.089
	2-14	442.38	22.12	2.144	2.266
	2-15	368.00	18.40	2.141	2.334
	2-16	257.00	12.85	2.145	2.3
	2-17	565.12	28.26	2.101	2.207
	2-18	504.91	25.25	2.104	1.979
	2-19	435.36	21.77	2.108	2.194
	2-20	666.39	33.32	2.109	1.982
BIA-3	3-1	359.51	17.98	2.122	1.002
	3-2	801.62	40.08	2.124	1.481
	3-3	388.98	19.45	2.107	2.341
	3-4	720.06	36.00	2.124	1.972
	3-05	424.75	21.24	2.122	1.616
	3-06	661.11	33.06	2.123	2.292
	3-7	603.42	30.17	2.109	1.923
	3-8	114.74	5.74	2.141	1.994
	3-09	503.52	25.18	2.153	1.924
	3-10	595.81	29.79	2.148	2.278
	3-11	707.59	35.38	2.133	1.599
	3-12	587.05	29.35	2.104	1.973
	3-13	320.22	16.01	2.092	2.134
	3-14	605.60	30.28	2.118	1.915
	3-15	743.82	37.19	2.128	2.126
	3-16	694.94	34.75	2.104	1.936
	3-17	519.08	25.95	2.123	2.14
	3-18	646.04	32.30	2.099	2.243
	3-19	318.14	15.91	2.117	2.252

Site	Sample ID	Concentration (ng/μL)	Total Yield (μg)	OD 260/280	OD 260/230
	3-20	423.85	21.19	2.103	2.229
	3-21	789.93	39.50	2.078	1.115
	5-1	865.13	43.26	2.101	1.598
	5-02	268.59	13.43	2.129	2.388
	5-3	961.79	48.09	2.116	1.589
	5-4	1288.84	64.44	2.079	1.877
	5-5	1188.53	59.43	2.115	2.014
	5-6	454.65	22.73	2.117	1.458
	5-7	477.10	23.86	2.077	0.676
	5-8	640.32	32.02	2.115	1.988
	5-9a	597.65	29.88	2.115	1.96
	5-9b	349.06	17.45	2.129	1.691
BIA-5	5-10	780.38	39.02	2.119	2.194
	5-11	656.91	32.85	2.124	1.385
	5-12	477.54	23.88	2.131	1.718
	5-13	421.18	21.06	2.09	2.322
	5-14	1035.47	51.77	2.125	1.946
	5-15	353.81	17.69	2.141	1.54
	5-16	1122.90	56.14	2.117	2.173
	5-17	356.92	17.85	2.211	1.997
	5-18	2678.30	133.91	1.116	0.969
	5-19	735.98	36.80	2.109	1.993
	5-20	633.69	31.68	2.106	1.993
	6-1	480.36	24.02	2.086	1.94
	6-02	405.35	20.27	2.177	2.213
	6-3	685.57	34.28	2.109	2.057
	6-4	658.45	32.92	2.089	1.592
	6-5	578.19	28.91	2.141	1.999
	6-6	742.60	37.13	2.135	2.072
	6-07	583.41	29.17	2.152	2.135
	6-8	483.78	24.19	2.111	2.097
	6-09	486.29	24.31	2.12	1.465
	6-9	677.62	33.88	2.104	2.036
BIA-6	6-10	606.60	30.33	2.09	2.082
	6-11	480.36	24.02	2.086	1.94
	6-12	684.47	34.22	2.111	2.189
	6-13	382.15	19.11	2.127	2.186
	6-14	1534.47	76.72	2.092	2.181
	6-15	462.00	23.10	2.122	2.134
	6-16	1051.34	52.57	2.104	2.222
	6-17	977.08	48.85	2.11	2.246
	6-18	662.08	33.10	2.1	1.635
	6-19A	452.34	22.62	2.149	1.886
	6-19B	462.00	23.10	2.122	2.134

Site	Sample ID	Concentration (ng/μL)	Total Yield (μg)	OD 260/280	OD 260/230
	6-20	425.28	21.26	2.076	0.574
	NF-01	389.44	19.47	2.1	0.895
	NF-2	595.31	29.77	2.079	0.674
	NF-3	626.87	31.34	2.143	1.792
	NF-4	523.98	26.20	2.138	0.985
	NF-5	842.52	42.13	2.119	1.304
	NF-06	717.94	35.90	2.122	1.832
	NF-7	1217.59	60.88	2.143	2.258
	NF-8	497.44	24.87	2.138	0.985
	NF-9	845.58	42.28	2.089	1.087
II-NF	NF-10	675.69	33.78	2.105	2.024
	NF-11	768.73	38.44	2.129	1.945
	NF-12	1017.59	50.88	2.135	1.445
	NF-13	1013.92	50.70	2.09	1.874
	NF-14	497.44	24.87	2.149	1.538
	NF-15	792.27	39.61	2.11	1.838
	NF-16	716.95	35.85	2.107	1.841
	NF-17	730.14	36.51	2.103	1.888
	NF-18	689.81	34.49	2.159	2.092
	NF-19	842.52	42.13	2.149	1.538
	NF-20	758.32	37.92	2.108	1.968
	FF-1	849.24	42.46	2.13	2.165
	FF-2	1055.99	52.80	2.138	2.162
	FF-03	444.24	22.21	2.099	0.915
	FF-04	470.78	23.54	2.128	2.049
	FF-05	357.08	17.85	2.097	0.985
	FF-7	860.43	43.02	2.119	1.217
	FF-8	688.05	34.40	2.111	1.711
	FF-9	1079.07	53.95	2.156	1.94
II-FF	FF-10	470.78	23.54	2.099	0.915
	FF-11	585.72	29.29	2.097	1.492
	FF-12	569.15	28.46	2.093	1.198
	FF-13	300.68	15.03	2.054	1.123
	FF-16	644.06	32.20	2.11	2.272
	FF-17	1112.87	55.64	2.099	2.009
	FF-18	585.72	29.29	2.097	1.492
	FF-19	1048.72	52.44	2.124	2.225
	FF-20	1046.91	52.35	2.108	1.843

3.2. Gene Primer Set Design and Evaluation

A total of 27 gene primer sets were designed and tested in this study based on their biological functions relating to either xenobiotic detoxification, oxidative stress, lipid

metabolism and transport, glucose metabolism, growth and development, immune response, and or reproduction (Table 3.2 and 3.3). All primers were evaluated based on a minimum 4-point standard curve with acceptable efficiency values (90-110%), a single sharp melt peak curve and a goodness of fit of linear regression ($R^2 > 0.900$); a total of 12 gene primer sets met this criterion and were used in subsequent RT-qPCR experiments (Table 3.2). One minor exception to these criteria was the case of HSP70 which exhibited an efficiency of 112% but was deemed acceptable due to the presence of a single sharp melt peak and $R^2 = 0.971$ (Table 3.2). Reference gene stability was assessed using the CFX Manager™ Software Gene Expression Analysis tool and calculated a mean M-value for the two reference genes, ACTβ and eEF1α1, of 0.58 with an average coefficient variance (CV) of 0.20 (Table 3.2).

Some primer sets were excluded from the RT-qPCR experiments due to displaying very low or no amplification of cDNA and not meeting the MIQE criteria. Specifically, these included AhR, IL-1β, IL-6, MMP23B, SOD2, SOCS2, THRα, TNFα and WAP65 (Table 3.3). These conclusions were based on the following failure to meet MIQE criteria: (1) less than 4 points in standard curve showing amplification; (2) gene amplification appears at Cq values greater than 35 for the three greatest concentration points (25, 6.25, 1.56 ng/μL); and (3) efficiency values appeared outside of the acceptable 90-110% range. In addition, GST, SOD1, SOD3, THRβ and UBQ showed multiple peaks in the melt curve analysis which indicated the presence of numerous amplicons, likely due to non-specific binding of primer sets to multiple gene isoforms or non-specific gene products rather than primer dimers since NTCs displayed no amplification or sources of DNA contamination. Generally, the presence of primer dimers is exhibited at a lower melting temperature relative to that of the amplicon and most often will also appear in the NTCs due to the excess abundance of primers (Real-Time qPCR Handbook, Life Technologies, 2012). Lastly, PYGL displayed good efficiency and a single melt curve peak, however, failed sequence validation based on the Sanger sequencing reads not matching the published sequence for this target gene. For PYGL, the Sanger sequencing results provided 58 and 61 base pair sequences for the forward and reverse pair primers, respectively, and exhibited a 94% and 78% nucleotide sequence similarity compared to 18S rRNA when aligned against Atlantic halibut (*Hippoglossus hippoglossus*). As a result, PYGL was excluded from further study.

Primers displaying multiple peaks in the melt curve analysis were further analyzed by testing amplification at different concentrations, annealing temperatures or redesigning the primers. Multiple attempts to redesign unique primers for SOD1 and SOD3 were unsuccessful due to the high sequence similarity within the exonic regions of these target genes. Predicted amino acid sequence comparisons revealed that SOD1 and SOD3 demonstrated areas of high amino acid homology in the exonic regions encoding for the [Cu-Zn] catalytic site (Zelko et al., 2002). As a result, in this study both SOD1 and SOD3 exhibited multiple peaks in all melt curve analyses and therefore were excluded from subsequent RT-qPCR experiments. A full list of all unsuccessful genes of interest including data related to primer design as well as detailed reasons for exclusion are provided listed in Table 3.3.

Table 3.2. List of successful primer sequences designed to amplify genes of interest to examine the effects of wastewater effluents in English sole through RT-qPCR experiments. For each target gene of interest, the National Center for Biotechnology and Information (https://www.ncbi.nlm.nih.gov/) accession identifiers, forward and reverse primer sequences (5' → 3'), annealing temperatures (Tm), amplicon sizes, efficiency of primer pairs (%E) and goodness of fit of linear regression for the relative standard curves (R^2) are provided for control genes ACTβ and eEF1α1, and target genes 18S rRNA, CYP1A, ERα, FABP1, FASN, GLUT2, HSP70, DI01, PPARδ, PPARγ, RPS4X and VTG.

Target Gene	Accession ID	Primer Sequence	Tm (^{0}C)	Amplicon Size	Efficiency (%)	R^2
18S rRNA	XR_004613416.1	F: GGTCTGTGATGCCCTTAGATG R: GCTTATGACCCGCGCTTAC	55.8	210	104.5	0.972
ACTβ	XM_035181811.1	F: GACCAACTGGGATGACATGG R: GCGTACAGGGACAGCACAGC	61	204	103	0.972
CYP1A	AJ310693.1	F: TGTGAGGACAGGAAGCTGGA R: GCTCCAAACAGGTCGTTGACA	58	86	96.9	0.992
DI01	AB362421.1	F: ACAGATGGTTGGGCCTTCAC R: TGACTTTCCCAGCCTGAAGC	57.7	196	108.1	0.992
eEF1α1	XM_035620145.1	F: AAGATCCACATCAACATCGTG R: CAAACTTCCACAGAGCGATG	56.7	229	98.8	0.998
ERα	XM_035143352.1	F: GCTGAGGGATTTGAGATGGCT R: ATGTAGTCATTGTGACCCTGGATG	56.9	143	106.1	0.96
FABP1	XM_020099114.1	F: GAAGGTCAAGGCGGTGGTTC R: ACATGCGTTTGCTCGTCCTC	58.4	155	108.4	0.989

Target Gene	Accession ID	Primer Sequence	Tm (°C)	Amplicon Size	Efficiency (%)	R^2
FASN	XM_035162863.1	F: GCAACGGCAATGACAAAGAGC R: TTTGTCTGGTTTCCGTGCCA	57.6	143	107.8	0.952
GLUT2	AY521663.1	F: CCGCGCTACCTCTACATCGT R: TGCTGCCTGTAGACGGAAGA	58.6	182	102.6	0.969
HSP70	AF187726.1	F: CAGTGCCCGCCTACTTCAAT R: TTCTGACCCAACCTTCTTGTCC	57.3	143	111.7	0.971
PPARδ	XM_035158970.1	F: GACCTCGCTCCACCCTTTAC R: TCCAAGCCCGAATGTGGAAC	57.7	157	98.5	0.988
PPARγ	AJ243956.2	F: TGTCAGTCACGCTCTGCTGAA R: TAGGAGATCAGGGTCCCGTCT	58.8	176	102.4	0.988
RPS4X	XM_034569090.1	F: GTTTGATACTGCCAACCTGTGC R: TTGGAGAGCCTGGTAGCGAA	57.5	150	99.7	0.976
VTG	XM_035177204.1	F: CAAGAGCCAGAGTTCACACA R: TGCAACAGCATAAGTCTCAAC	57.3	143	103.6	0.971

Table 3.3. List of primer sequences designed to amplify genes of interest in English Sole that were unsuccessful for RT-qPCR experiments, and the reasons for exclusion. For each target gene of interest, the National Center for Biotechnology and Information (https://www.ncbi.nlm.nih.gov/) accession identifiers, forward and reverse primer sequences (5' → 3') and amplicon sizes are provided.

Target Gene	Accession ID	Primer Sequence	Amplicon Size	Reason For Exclusion
AhR	XM_020106318.1	F: TTCCTCCACGGTCAGAGCAG R: CCAGCTTGTGCTTGGTCCTG	151	>1 Peak Melt Curve Efficiency > 110% Failed Standard Curve
GST	X63761.1	F: AAGTCAGCCGAGGTGATGAGC R: CAGGCGGCGTAGGATTCATTC	92	>1 Peak Melt Curve
IL-1β	AJ010640.1	F: CGAGTGGAGGACAAGACCATG R: GCTCGGATGTGCTGATGTACC	145	>1 Peak Melt Curve Efficiency > 110% Failed Standard Curve
IL-6	GU985454.1	F: GGCTTGTCTCCACAGGTTGG R: CAGACAAGTTCAGGGCTCACTC	197	>1 Peak Melt Curve Efficiency > 110% Failed Standard Curve
MMP23B	XM_020089187.1	F: TCATAAACGCCAGTGACACACG R: CCGTGATGCCGTCGAAACA	186	Efficiency > 110% Failed Standard Curve
PYGL	XM_035167960.1	F: AGCTGGAGGAGATGGAGGAG R: CAAGAAACACGCTGCCAGTC	71	Failed Sanger Sequencing

Target Gene	Accession ID	Primer Sequence	Amplicon Size	Reason For Exclusion
SOD1	AJ291980.1	F: AGATGCTCACCCTCAATGGC R: TTCCAAGATCGTCAGCTTTCTCA	78	>1 Peak Melt Curve
SOD2	XM_020106318.1	F: TTCCTCCACGGTCAGAGCAG R: CCAGCTTGTGCTTGGTCCTG	151	>1 Peak Melt Curve Efficiency > 110% Failed Standard Curve
SOD3	XM_020100342.1	F: CCAGAGGTCATGCTGCA R: GGTGGCAATACATTCTTTGCTC	85	>1 Peak Melt Curve Efficiency > 110% Failed Standard Curve
SOCS2	XM_034578888.1	F: ATCAGACGAGAGCCGCATCG R: GGCGGAGATGGTGAACAGGT	169	>1 Peak Melt Curve Efficiency > 110% Failed Standard Curve
THRα	D16461.1	F: CCGCCTCATTGTCCTGTGATT R: CCCTGGAACAGAGACGCTAAG	187	>1 Peak Melt Curve Efficiency > 110% Failed Standard Curve
THRβ	D45245.1	F: GCCAAGCGGAAGCTAATCGA R: ATGCGGATGAGGTCCCACTC	113	>1 Peak Melt Curve
TNFα	AB040448.1	F: ATTCACTCAGGGCGGCTTC R: TCCGTGAAGAGCCATACCCT	183	>1 Peak Melt Curve Efficiency > 110% Failed Standard Curve
UBQ	XM_034582323.1	F: CTGGAGGATGGACGCACACT R: CGCAGACGCAGAACCAGATG	74	>1 Peak Melt Curve
WAP65	KC521544.1	F: TCATCACATCGCTCACATCGTT R: AAGAGGGAAATCGTCTGTTCGTTT	99	Poor Efficiency (> 110%) / Low Expression (< 4 Points in Standard Curve)

3.3. RT-qPCR Gene Expression

Mean normalized expression ($\pm$ standard error of the mean) values of 7-11 biological replicates per site per sex for a total of 12 genes revealed significant differences in several target genes between sites and sexes, and in some instances the interactive effects between these two main factors. Target genes involved in protein synthesis which included 18S rRNA (Figure 3.1) and RPS4X (Figure 3.2) both exhibited significant changes in normalized gene expression. Normalized expression in 18S rRNA was found to have a main effect of site ($p < .0001$), sex ($p < .0001$), and the interaction ($F(6,119) = 3.7874$, $p = .0017$). Specifically, normalized expression was significantly elevated in females at BIA-1 (3.75 ± 0.58) in comparison to some sites further from the Lion's Gate WWTP (BIA-3 (1.23 ± 0.18), BIA-6 (0.66 ± 0.22)) and compared to both Iona WWTP sites investigated (II-NF = 0.76 ± 0.12, II-FF = 1.06 ± 0.52; Figure 3.1). However, changes in 18S rRNA appeared to be sex specific since no significant changes were observed for males between sites ($p > 0.05$; Figure 3.1). In addition,

differences in the expression of 18S rRNA was significantly different between sexes at one site, whereby, BIA-6 females (0.66 $\pm$ 0.22) exhibited significantly lower normalized expression of 18S rRNA compared to males (1.39 $\pm$ 0.20) at this site (Figure 3.1). Normalized expression in RPS4X was found to have a main effect of site ($p < .0001$) and sex ($p < .0008$) but no significant interaction was observed ($F(6,120) = 1.5506$, $p = .1676$). Specifically, in males normalized expression was significantly elevated at BIA-3 (1.90 $\pm$ 0.66) in comparison to BIA-6 (0.18 $\pm$ 0.05) and to the near-field Iona WWTP site (II-NF = 0.34 $\pm$ 0.10); whereas in females at BIA-6 revealed significantly lower normalized expression of RPS4X in comparison to BIA-5 (0.83 $\pm$ 0.23) and BIA-3 (1.10 $\pm$ 0.44; Figure 3.2). Interestingly, at both of the Iona WWTP sites, males and females exhibited similar levels of RPS4X that were not significantly different from any of the Lion's Gate WWTP sites, except for females at II-FF (0.08 $\pm$ 0.02) which differed from several sites (BIA-3 = 1.10 $\pm$ 0.44, BIA-5 = 0.83 $\pm$ 0.23; Figure 3.2).

Target genes involved in xenobiotic detoxification and transcriptional regulation which included CYP1A (Figure 3.3) and HSP70 (Figure 3.4) both exhibited significant changes in normalized gene expression. For CYP1A there was no significant effect of sex ($p = .0623$) or interactive effects between sex and site ($F(6,113) = 1.9735$, $p = .0752$), but site as a main effect was significant ($p < .0001$). Within females, normalized expression was significantly lower nearest to the Lion's Gate WWTP at BIA-1 (0.08 $\pm$ 0.01) in comparison to sites further from the Lion's Gate WWTP (BIA-2 = 0.38 $\pm$ 0.05, BIA-3 = 0.43 $\pm$ 0.09, BIA-5 = 1.03 $\pm$ 0.20, BIA-6 = 1.10 $\pm$ 0.39), and compared to the near-field Iona WWTP site (II-NF = 0.44 $\pm$ 0.13; Figure 3.3). However, within males, no significant differences were observed across Lion's Gate and Iona WWTP sites, but BIA-6 (1.09 $\pm$ 0.20) exhibited higher CYP1A expression relative to II-NF (0.32 $\pm$ 0.07; Figure 3.3). Normalized expression in HSP70 was found a main effect of site ($p < .0001$) and sex ($p = .0258$) but no significant interaction was observed ($F(6,121) = 1.1356$, $p = 0.3459$; Figure 3.4).

Target genes involved in homeostasis and metabolic function like DI01 exhibited significant changes in normalized gene expression (Figure 3.5). Normalized expression in D101 was found to have a main effect of site ($p < .0002$), sex ($p < .0001$), and the interaction ($F(6,120) = 2.2008$, $p = .0475$). Specifically, normalized expression was significantly elevated in females at BIA-1 (1.29 $\pm$ 0.26) in comparison to some sites

further from the Lion's Gate WWTP (BIA-3 (0.41 $\pm$ 0.08), BIA-5 (0.26 $\pm$ 0.05), BIA-6 (0.33 $\pm$ 0.05)) and compared to one of the Iona WWTP sites, II-NF (0.26 $\pm$ 0.05; Figure 3.5). However, changes in DI01 appeared to be sex specific since no significant changes were observed for males between sites ($p > 0.05$). In addition, differences in the expression of DI01 was significantly different between sexes at two sites, whereby, BIA-5 (0.26 $\pm$ 0.05) and II-NF (0.26 $\pm$ 0.05) females exhibited significantly lower normalized expression of DI01 compared to males (BIA-5 = 0.96 $\pm$ 0.28, II-NF = 0.82 $\pm$ 0.14; Figure 3.5) at these sites.

Target genes involved in lipid and glucose metabolism included FABP1 (Figure 3.6), FASN (Figure 3.7), PPARδ (Figure 3.8), and PPARγ (Figure 3.9), and all exhibited significant changes in normalized gene expression. For FABP1 there was no significant main effect of sex ($p = .1509$) or interactive effects between sex and site ($F(6,121) = 1.9191$, $p = .083$), but site as a main effect was significant ($p < .0034$). Within females, normalized expression was significantly higher nearest to Lion's Gate WWTP at BIA-1 (2.33 $\pm$ 0.69) in comparison to sites further from the Lion's Gate WWTP (BIA-2 = 0.64 $\pm$ 0.96, BIA-3 = 0.66 $\pm$ 0.29, BIA-6 = 0.67 $\pm$ 0.22) and both of the Iona WWTP sites (II-NF = 0.78 $\pm$ 0.19, II-FF = 0.67 $\pm$ 0.39, Figure 3.6). However, changes in FABP1 appeared to be sex specific since no significant changes were observed for males between sites ($p > 0.05$). For FASN there was no significant main effect of sex ($p = .8472$) or interactive effects between sex and site ($F(6,121) = 1.957$, $p = .0771$), but site as a main effect was significant ($p < .0001$). Within males, normalized expression was significantly higher nearest to Lion's Gate WWTP at BIA-1 in comparison to BIA-5 (0.8 $\pm$ 0.14) and compared to both Iona WWTP sites investigated (II-NF = 0.57 $\pm$ 0.04, II-FF = 0.64 $\pm$ 0.06, Figure 3.7). Interestingly, females exhibited similar levels of FASN that were not significantly different from any of the Lion's Gate WWTP sites, except BIA-1 (1.22 $\pm$ 0.09) which exhibited significantly higher normalized expression of FASN compared to Iona WWTP sites (II-NF = 0.60 $\pm$ 0.06, II-FF = 0.73 $\pm$ 0.11, Figure 3.7). Normalized expression in PPARδ was found to have a main effect of site ($p < .0001$) and the interaction ($F(6,114) = 0.4520$, $p = .8423$) but no significant main effect of sex was observed ($p = .1868$). Within females, normalized expression was significantly higher nearest to Lion's Gate WWTP at BIA-1 (2.78 $\pm$ 0.52) in comparison to some sites further from the primary WWTP (BIA-3 = 0.63 $\pm$ 0.14, BIA-5 = 0.68 $\pm$ 0.08, BIA-6 = 0.60 $\pm$ 0.16), and compared to both Iona WWTP sites investigated (II-NF = 0.34 $\pm$ 0.07, II-FF =

0.35 $\pm$ 0.06; Figure 3.8). However, within males the far-field Iona WWTP site (II-FF = 0.30 $\pm$ 0.03) exhibited lower PPARδ expression relative to several sites at Lion's Gate WWTP (BIA-1 = 1.07 $\pm$ 0.20, BIA-2 = 1.34 $\pm$ 0.22, BIA-3 = 1.00 $\pm$ 0.27) whereas the near-field Iona WWTP site (II-NF = 0.39 $\pm$ 0.08) only exhibited lower PPARδ expression relative to BIA-2 (1.34 $\pm$ 0.22; Figure 3.8). For PPARγ there was no significant main effect of sex ($p = .8708$), but site as a main effect ($p < .0093$) and the interactive effects between these two main factors ($F(6,121) = 3.9343$, $p < .0013$) was significant. Specifically, normalized expression was significantly elevated in females at BIA-5 (0.72 $\pm$ 0.15) in comparison to the nearest site to the Lion's Gate WWTP (BIA-1 = 0.21 $\pm$ 0.07; Figure 3.9). Similarly, normalized expression was significantly elevated in males at BIA-6 (0.53 $\pm$ 0.13) in comparison to the BIA-2 (0.20 $\pm$ 0.06). Lastly, normalized expression in GLUT2 indicated no main effect of site ($p = .0751$), sex ($p = .4418$), nor an interactive effect between these two main factors ($F(6,114) = 0.4520$, $p = .8423$; Figure 3.10).

Target genes involved in reproduction included ERα (Figure 3.11) and VTG (Figure 3.12), and both exhibited significant changes in normalized gene expression. Normalized expression in ERα was found to have a main effect of site ($p < .0001$) and sex ($p < .0001$) but no interactive effects between these two main factors was observed ($F(6,121) = 0.3278$, $p = .9213$). Within females, normalized expression was significantly higher nearest to Lion's Gate WWTP at BIA-1 (4.23 $\pm$ 1.10) in comparison to some sites further from the primary WWTP (BIA-5 = 0.36 $\pm$ 0.20, BIA-6 = 0.47 $\pm$ 0.25) and compared to the near-field Iona WWTP site (II-NF = 0.67 $\pm$ 0.37; Figure 3.11). Similarly, within males, normalized expression was also significantly higher nearest to Lion's Gate WWTP at BIA-1 (0.67 $\pm$ 0.26) in comparison to BIA-5 (0.02 $\pm$ 0.004) and compared to the near-field Iona WWTP site (II-NF = 0.03 $\pm$ 0.008; Figure 3.11). Normalized expression in VTG was found to have a main effect of site ($p < .0001$), sex ($p < .0001$), and the interaction ($p < .0014$). Specifically, normalized expression was significantly elevated in females nearest to Lion's Gate WWTP at BIA-1 (7.58 $\pm$ 1.63) in comparison to all sites further from the primary WWTP (BIA-2 = 0.83 $\pm$ 0.75, BIA-3 = 0.13 $\pm$ 0.09, BIA-5 = 0.54 $\pm$ 0.51, BIA-6 = 0.02 $\pm$ 0.01) and compared to both Iona WWTP sites investigated (II-NF = 0.15 $\pm$ 0.10, II-FF = 0.65 $\pm$ 0.52; Figure 3.12). However, changes in VTG appeared to be sex specific since no significant changes were observed for males between sites ($p > 0.05$). In addition, differences in the expression of VTG was

significantly different between sexes at two sites, whereby, BIA-6 (0.88 $\pm$ 0.33) and II-NF (0.30 $\pm$ 0.11) males exhibited significantly higher normalized expression of VTG compared to females (BIA-6 = 0.02 $\pm$ 0.007, II-NF = 0.15 $\pm$ 0.10; Figure 3.12) at these sites.

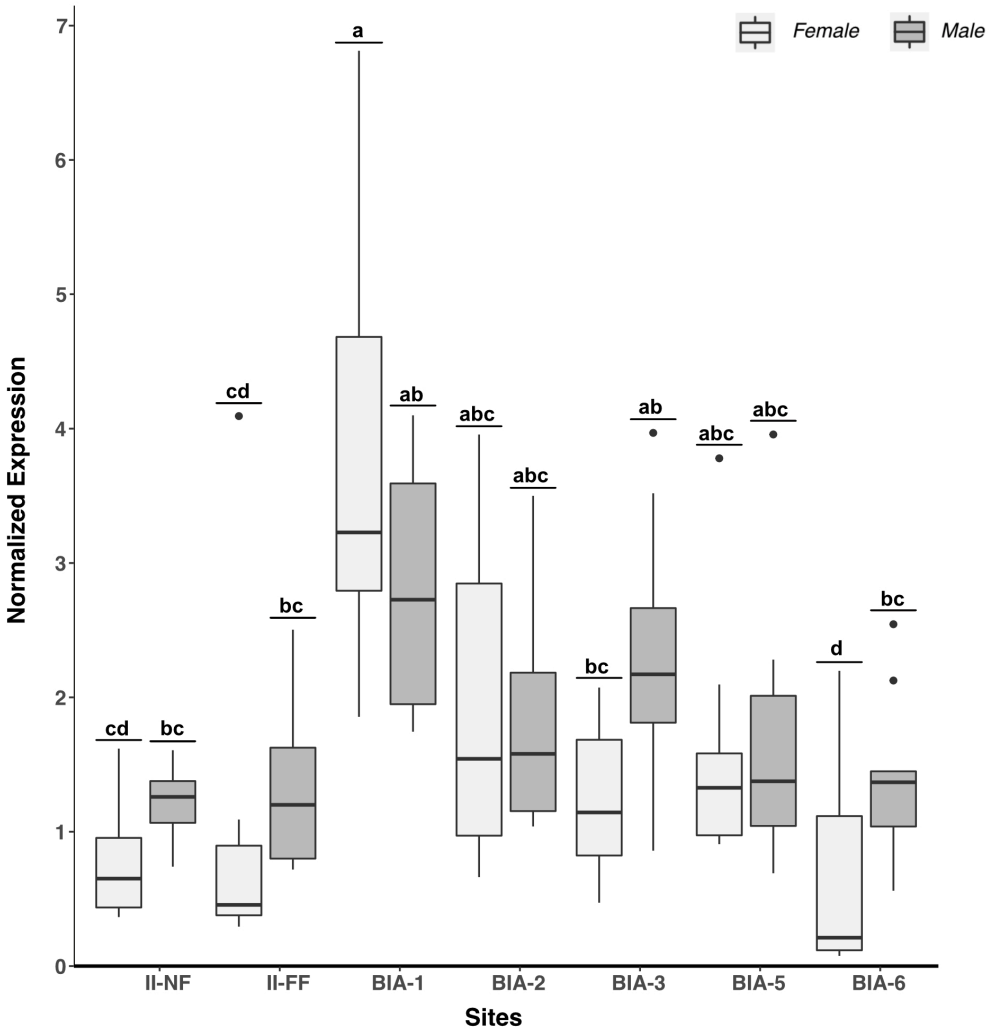

Figure 3.1. **Hepatic gene expression levels of 18S in English sole *(Parophrys vetulus)* at various sites near- and far-field from the Iona and Lion's Gate Wastewater Treatment Plant in Burrard Inlet. Normalized gene expression data was calculated based on the $\Delta\Delta C_q$ method for relative quantitation of a target gene using two reference genes (ACTB and eEF1A1). Boxplots demonstrating the median values (horizontal black lines within box), the lower and upper 25th and 75th percentiles (upper and lower boundaries of the box), the minimum and maximum values (whiskers) and outliers (black points) are presented. Biological replicates for each site for females and males are as follows: II-NF, n = 10 and 10; II-FF, n = 7 and 9; BIA-1, n = 8 and 9; BIA-2, n = 9 and 9; BIA-3, n = 10 and 11; BIA-5, n = 11 and 10; BIA-6, n = 11 and 9. A Two-Way Analysis of Variance (ANOVA) followed by Tukey's post-hoc analysis was used to determine significant differences between the main effects (site and sex) and interaction between the main effects. A significant effect of site ($p < .0001$), sex ($p < .0001$), and an interactive effect ($F(6,119) = 3.7874$, $p = .0017$) was observed. Different superscript letters indicate significant differences ($p < 0.05$).**

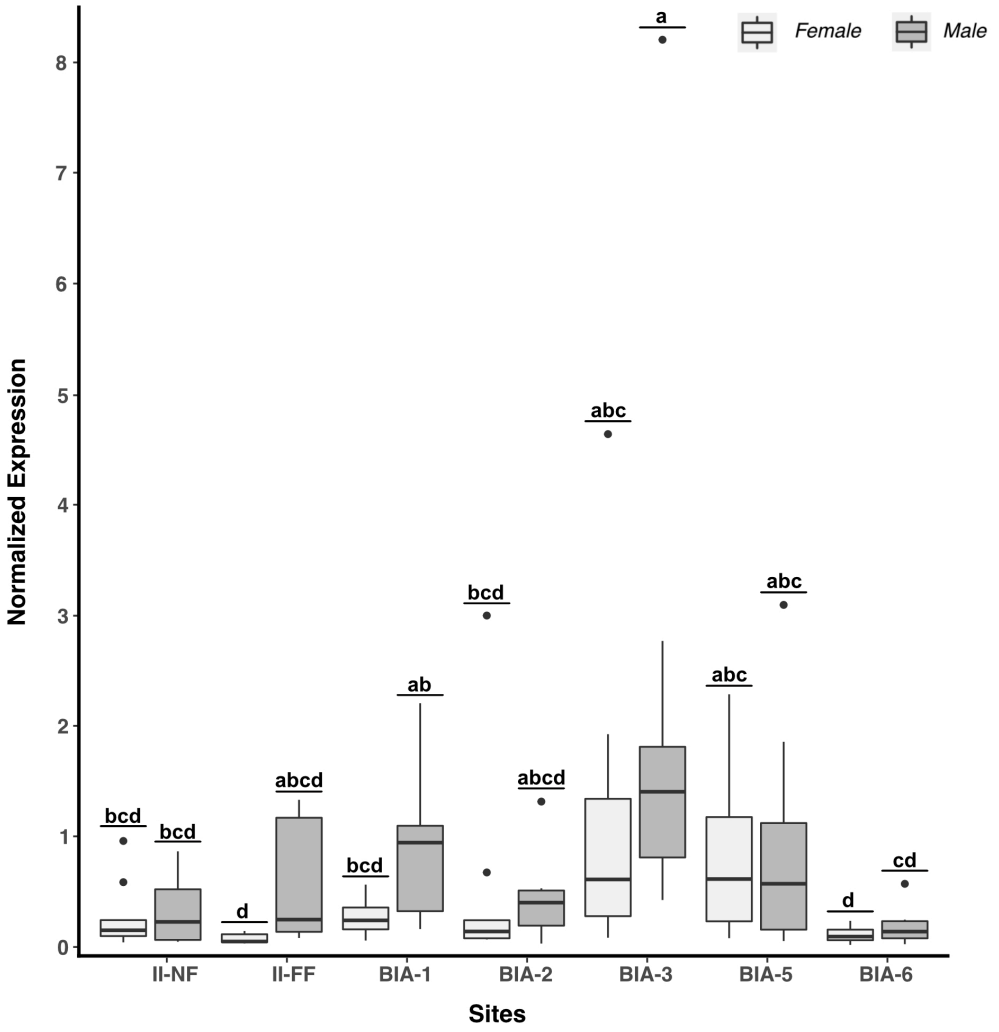

Figure 3.2. **Hepatic gene expression levels of RPS4X in English sole *(Parophrys vetulus)* at various sites near- and far-field from the Iona and Lion's Gate Wastewater Treatment Plant in Burrard Inlet. Normalized gene expression data was calculated based on the $\Delta\Delta C_q$ method for relative quantitation of a target gene using two reference genes (ACTB and eEF1A1). Boxplots demonstrating the median values (horizontal black lines within box), the lower and upper 25th and 75th percentiles (upper and lower boundaries of the box), the minimum and maximum values (whiskers) and outliers (black points) are presented. Biological replicates for each site for females and males are as follows: II-NF, n = 10 and 10; II-FF, n = 7 and 9; BIA-1, n = 8 and 9; BIA-2, n = 9 and 8; BIA-3, n = 10 and 11; BIA-5, n = 11 and 10; BIA-6, n = 12 and 10. A Two-Way Analysis of Variance (ANOVA) followed by Tukey's post-hoc analysis was used to determine significant differences between the main effects (site and sex) and interaction between the main effects. A significant effect of site ($p < .0001$) and sex ($p < .0008$), but no interactive effect ($F(6,120) = 1.5506$, $p = .1676$) was observed. Different superscript letters indicate significant differences ($p < 0.05$).**

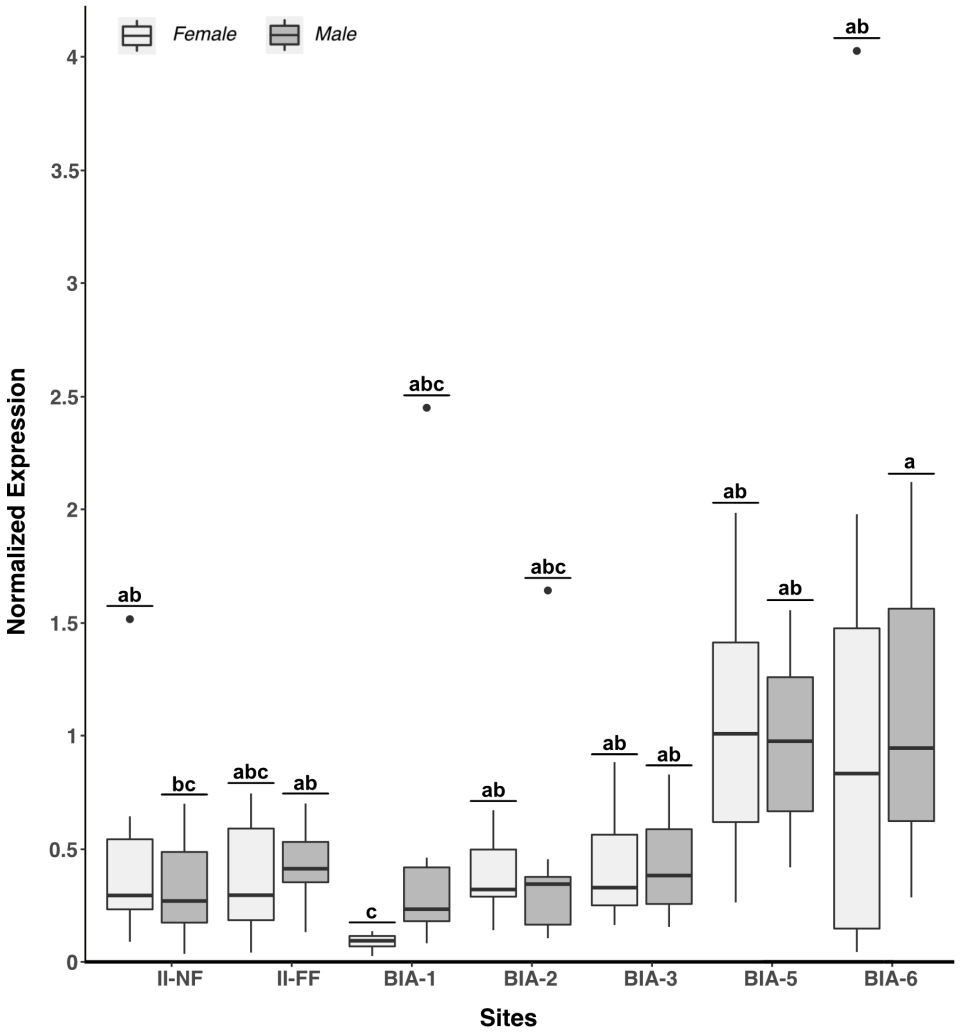

Figure 3.3. **Hepatic gene expression levels of CYP1A in English sole *(Parophrys vetulus)* at various sites near- and far-field from the Iona and Lion's Gate Wastewater Treatment Plant in Burrard Inlet. Normalized gene expression data was calculated based on the $\Delta\Delta C_q$ method for relative quantitation of a target gene using two reference genes (ACTB and eEF1A1). Boxplots demonstrating the median values (horizontal black lines within box), the lower and upper 25th and 75th percentiles (upper and lower boundaries of the box), the minimum and maximum values (whiskers) and outliers (black points) are presented. Biological replicates for each site for females and males are as follows: II-NF, n = 10 and 10; II-FF, n = 7 and 9; BIA-1, n = 7 and 9; BIA-2, n = 9 and 8; BIA-3, n = 10 and 11; BIA-5, n = 9 and 8; BIA-6, n = 10 and 10. A Two-Way Analysis of Variance (ANOVA) followed by Tukey's post-hoc analysis was used to determine significant differences between the main effects (site and sex) and interaction between the main effects. A significant effect of site ($p < .0001$), but no effect of sex ($p = .0623$) or interactive effect ($F(6,113) = 1.9735$, $p = .0752$) was observed. Different superscript letters indicate significant differences ($p < 0.05$).**

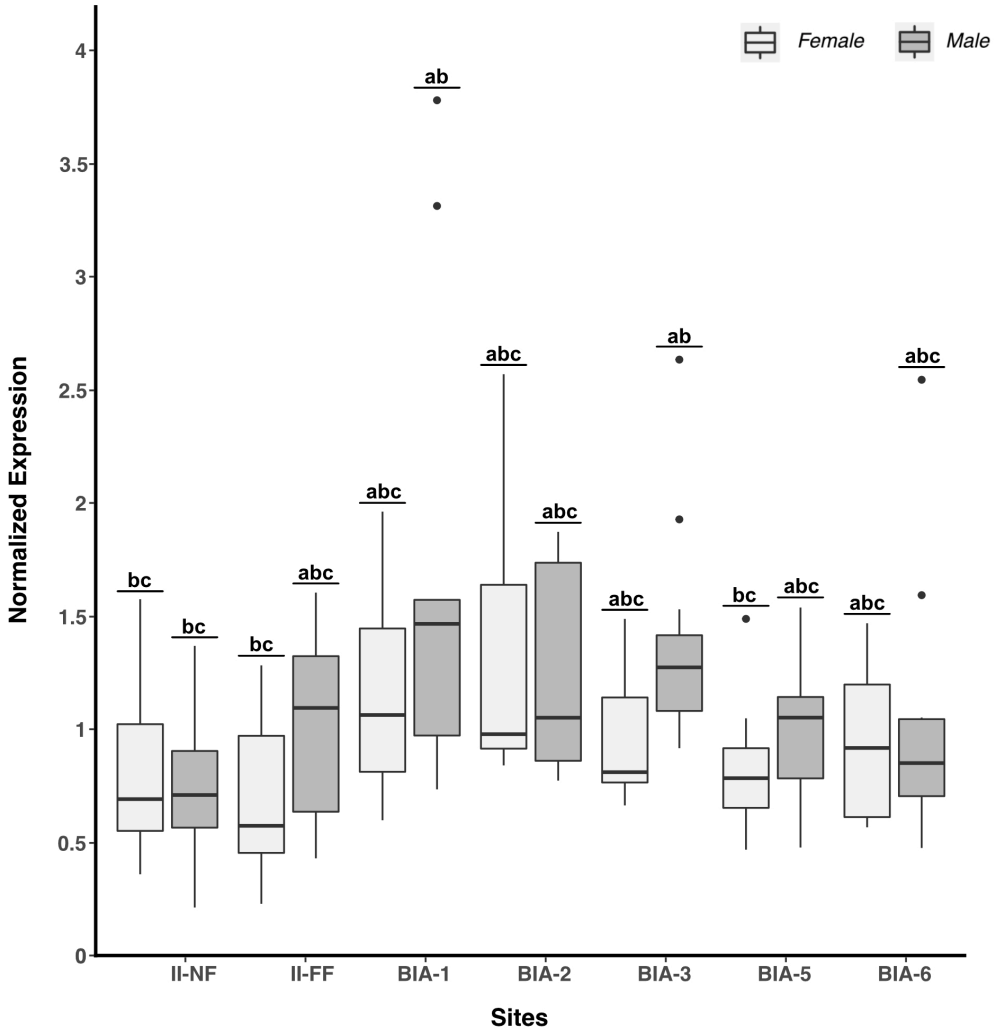

Figure 3.4. **Hepatic gene expression levels of HSP70 in English sole *(Parophrys vetulus)* at various sites near- and far-field from the Iona and Lion's Gate Wastewater Treatment Plant in Burrard Inlet. Normalized gene expression data was calculated based on the $\Delta\Delta C_q$ method for relative quantitation of a target gene using two reference genes (ACTB and eEF1A1). Boxplots demonstrating the median values (horizontal black lines within box), the lower and upper 25th and 75th percentiles (upper and lower boundaries of the box), the minimum and maximum values (whiskers) and outliers (black points) are presented. Biological replicates for each site for females and males are as follows: II-NF, n = 10 and 10; II-FF, n = 7 and 9; BIA-1, n = 8 and 9; BIA-2, n = 9 and 9; BIA-3, n = 10 and 11; BIA-5, n = 11 and 10; BIA-6, n = 12 and 10. A Two-Way Analysis of Variance (ANOVA) followed by Tukey's post-hoc analysis was used to determine significant differences between the main effects (site and sex) and interaction between the main effects. A significant effect of site ($p < .0001$) and sex ($p = .0258$), but no interactive effect ($F(6,121) = 1.1356$, $p = 0.3459$) was observed Different superscript letters indicate significant differences ($p < 0.05$).**

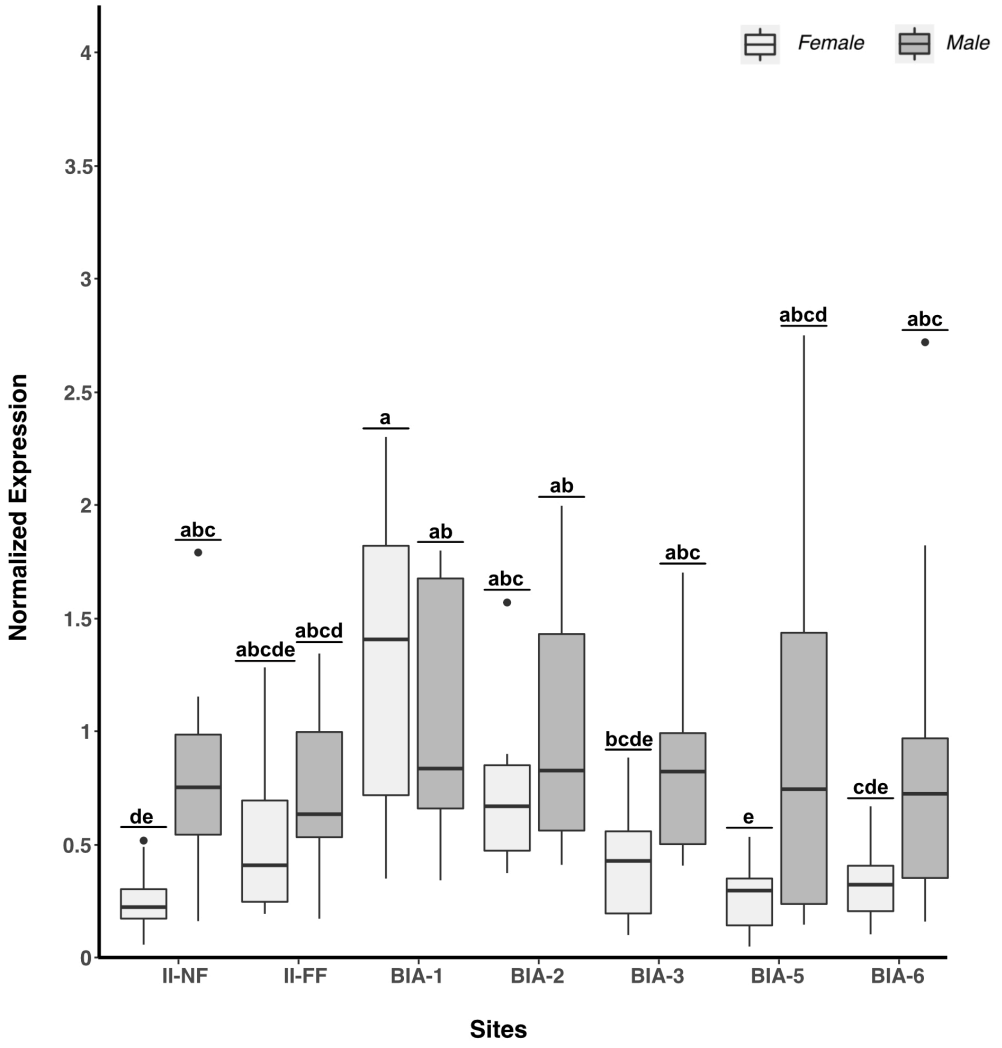

Figure 3.5. **Hepatic gene expression levels of DI01 in English sole *(Parophrys vetulus)* at various sites near- and far-field from the Iona and Lion's Gate Wastewater Treatment Plant in Burrard Inlet. Normalized gene expression data was calculated based on the $\Delta\Delta C_q$ method for relative quantitation of a target gene using two reference genes (ACTB and eEF1A1). Boxplots demonstrating the median values (horizontal black lines within box), the lower and upper 25th and 75th percentiles (upper and lower boundaries of the box), the minimum and maximum values (whiskers) and outliers (black points) are presented. Biological replicates for each site for females and males are as follows: II-NF, n = 10 and 10; II-FF, n = 7 and 9; BIA-1, n = 8 and 9; BIA-2, n = 9 and 9; BIA-3, n = 10 and 11; BIA-5, n = 11 and 9; BIA-6, n = 12 and 10. A Two-Way Analysis of Variance (ANOVA) followed by Tukey's post-hoc analysis was used to determine significant differences between the main effects (site and sex) and interaction between the main effects. A significant effect of site ($p < .0002$), sex ($p < .0001$), and an interactive effect ($F(6,120) = 2.2008$, $p = .0475$) was observed. Different superscript letters indicate significant differences ($p < 0.05$).**

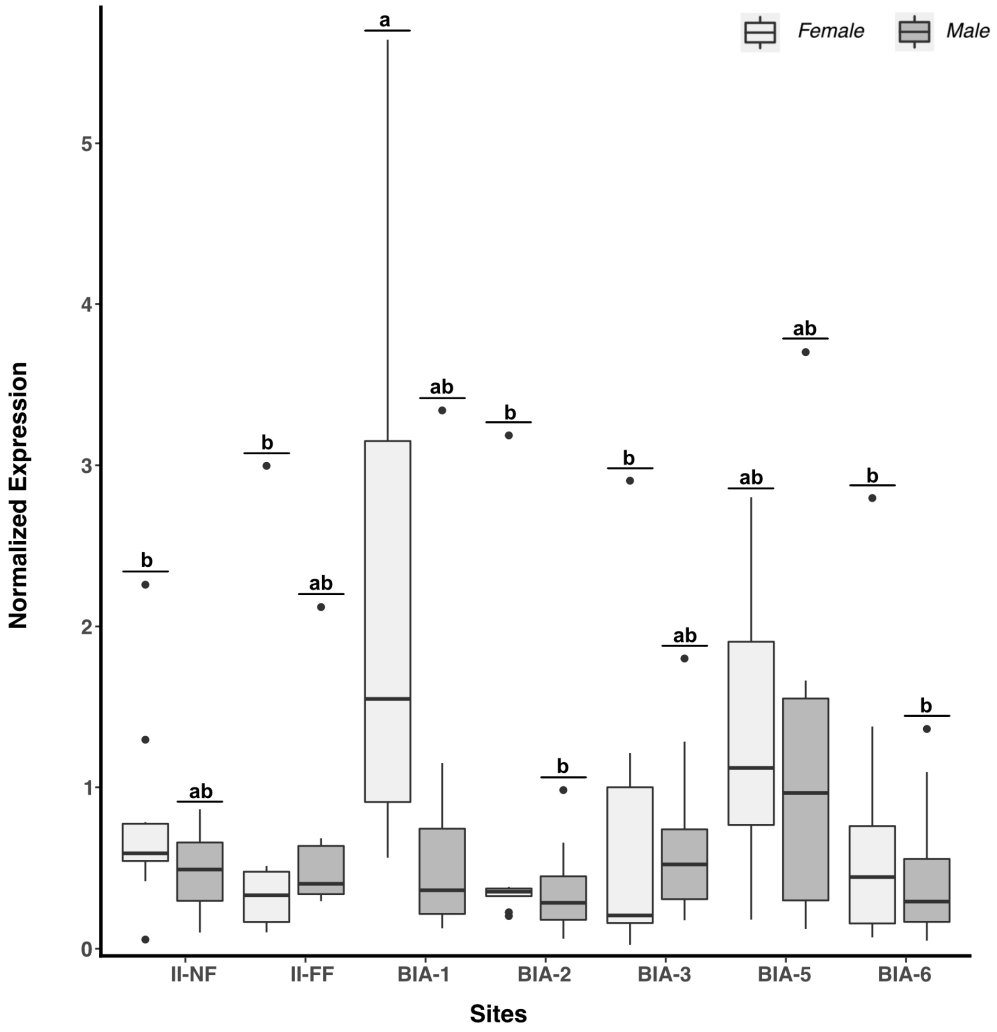

Figure 3.6. **Hepatic gene expression levels of FABP1 in English sole *(Parophrys vetulus)* at various sites near- and far-field from the Iona and Lion's Gate Wastewater Treatment Plant in Burrard Inlet. Normalized gene expression data was calculated based on the $\Delta\Delta C_q$ method for relative quantitation of a target gene using two reference genes (ACTB and eEF1A1). Boxplots demonstrating the median values (horizontal black lines within box), the lower and upper 25th and 75th percentiles (upper and lower boundaries of the box), the minimum and maximum values (whiskers) and outliers (black points) are presented. Biological replicates for each site for females and males are as follows: II-NF, n = 10 and 10; II-FF, n = 7 and 9; BIA-1, n = 8 and 9; BIA-2, n = 9 and 9; BIA-3, n = 10 and 11; BIA-5, n = 11 and 10; BIA-6, n = 12 and 10. A Two-Way Analysis of Variance (ANOVA) followed by Tukey's post-hoc analysis was used to determine significant differences between the main effects (site and sex) and interaction between the main effects. A significant effect of site ($p < .0034$), but no effect of sex ($p = .1509$) nor an interactive effect ($F(6,121) = 1.9191$, $p = .083$) was observed. Different superscript letters indicate significant differences ($p < 0.05$).**

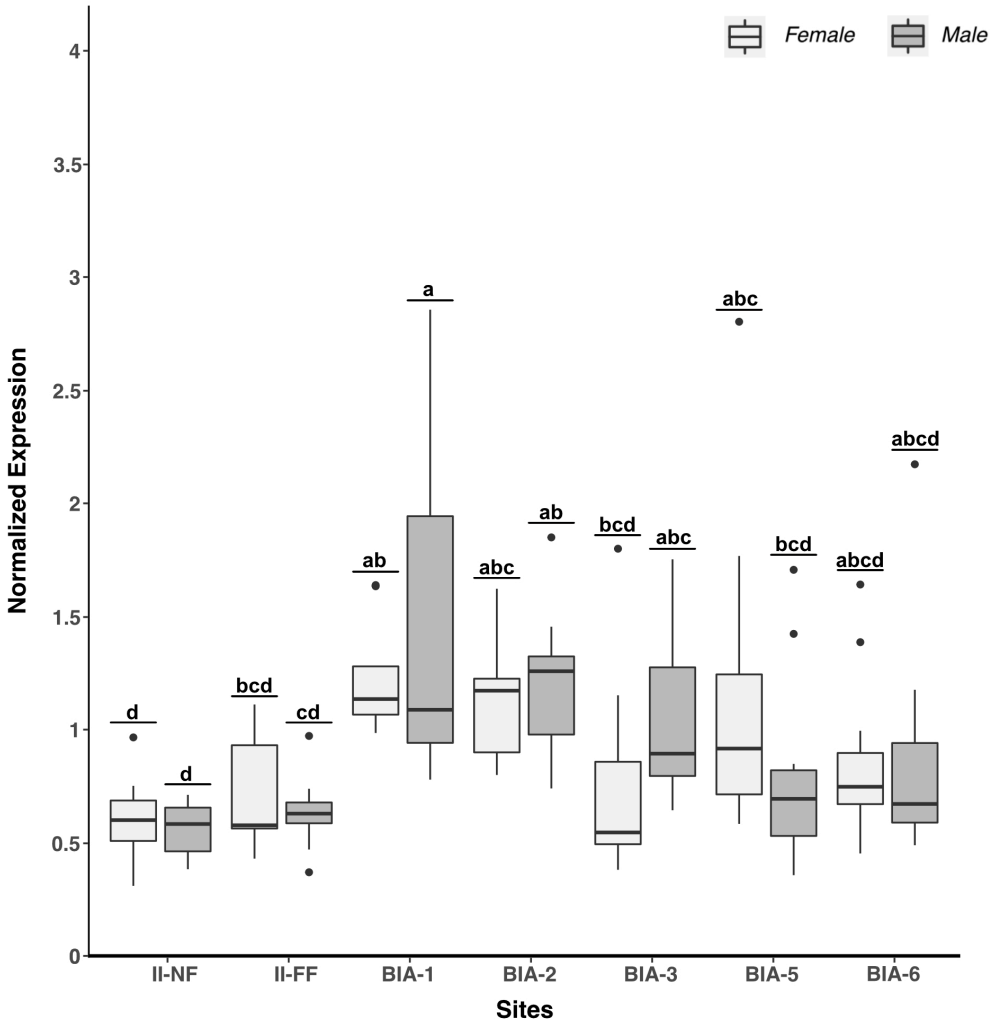

Figure 3.7. **Hepatic gene expression levels of FASN in English sole *(Parophrys vetulus)* at various sites near- and far-field from the Iona and Lion's Gate Wastewater Treatment Plant in Burrard Inlet. Normalized gene expression data was calculated based on the $\Delta\Delta C_q$ method for relative quantitation of a target gene using two reference genes (ACTB and eEF1A1). Boxplots demonstrating the median values (horizontal black lines within box), the lower and upper 25th and 75th percentiles (upper and lower boundaries of the box), the minimum and maximum values (whiskers) and outliers (black points) are presented. Biological replicates for each site for females and males are as follows: II-NF, n = 10 and 10; II-FF, n = 7 and 9; BIA-1, n = 8 and 9; BIA-2, n = 9 and 9; BIA-3, n = 10 and 11; BIA-5, n = 11 and 10; BIA-6, n = 12 and 10. A Two-Way Analysis of Variance (ANOVA) followed by Tukey's post-hoc analysis was used to determine significant differences between the main effects (site and sex) and interaction between the main effects. A significant effect of site ($p < .0001$), but no effect of sex ($p = .8472$) nor an interactive effect ($F(6,121) = 1.957$, $p = .0771$) was observed. Different superscript letters indicate significant differences ($p < 0.05$).**

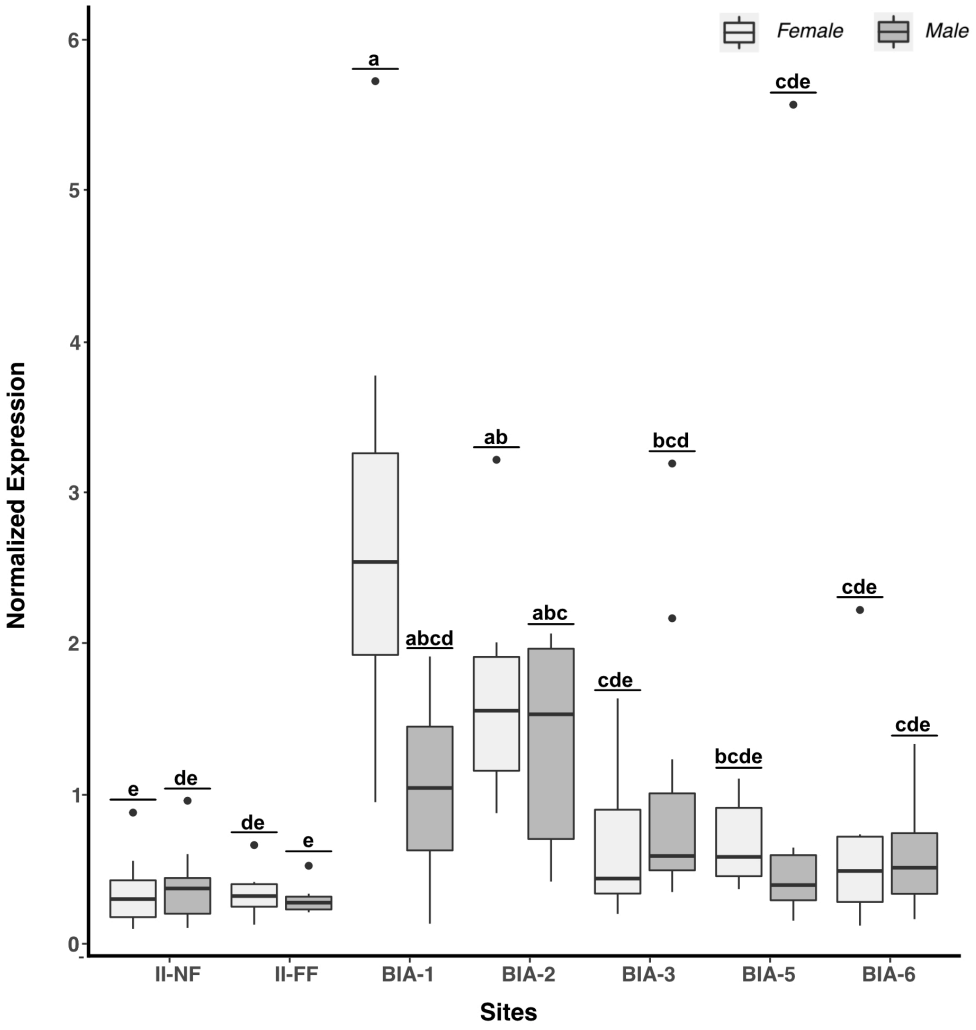

Figure 3.8. **Hepatic gene expression levels of PPARδ in English sole *(Parophrys vetulus)* at various sites near- and far-field from the Iona and Lion's Gate Wastewater Treatment Plant in Burrard Inlet. Normalized gene expression data was calculated based on the $\Delta\Delta C_q$ method for relative quantitation of a target gene using two reference genes (ACTB and eEF1A1). Boxplots demonstrating the median values (horizontal black lines within box), the lower and upper 25th and 75th percentiles (upper and lower boundaries of the box), the minimum and maximum values (whiskers) and outliers (black points) are presented. Biological replicates for each site for females and males are as follows: II-NF, n = 10 and 10; II-FF, n = 7 and 9; BIA-1, n = 8 and 9; BIA-2, n = 9 and 9; BIA-3, n = 10 and 11; BIA-5, n = 11 and 10; BIA-6, n = 12 and 10. A Two-Way Analysis of Variance (ANOVA) followed by Tukey's post-hoc analysis was used to determine significant differences between the main effects (site and sex) and interaction between the main effects. A significant effect of site ($p < .0001$), and interactive effect ($F(6,114) = 0.4520$, $p = .8423$), but no effect of sex ($p = .1868$) was observed. Different superscript letters indicate significant differences ($p < 0.05$).**

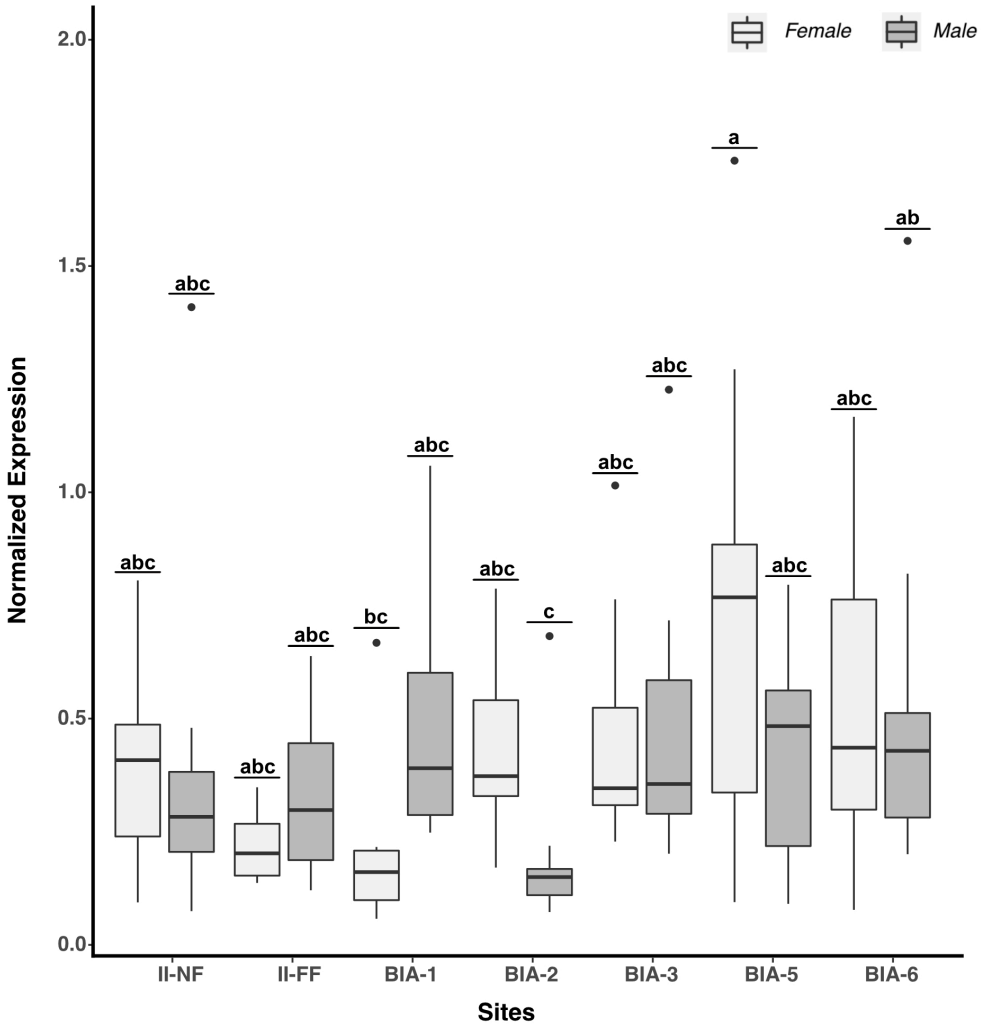

Figure 3.9. **Hepatic gene expression levels of PPARγ in English sole *(Parophrys vetulus)* at various sites near- and far-field from the Iona and Lion's Gate Wastewater Treatment Plant in Burrard Inlet. Normalized gene expression data was calculated based on the $\Delta\Delta C_q$ method for relative quantitation of a target gene using two reference genes (ACTB and eEF1A1). Boxplots demonstrating the median values (horizontal black lines within box), the lower and upper 25th and 75th percentiles (upper and lower boundaries of the box), the minimum and maximum values (whiskers) and outliers (black points) are presented. Biological replicates for each site for females and males are as follows: II-NF, n = 10 and 10; II-FF, n = 7 and 9; BIA-1, n = 8 and 9; BIA-2, n = 9 and 9; BIA-3, n = 10 and 11; BIA-5, n = 11 and 10; BIA-6, n = 12 and 10). A Two-Way Analysis of Variance (ANOVA) followed by Tukey's post-hoc analysis was used to determine significant differences between the main effects (site and sex) and interaction between the main effects. A significant effect of site ($p < .0093$) and interactive effect ($F(6,121) = 3.9343$, $p < .0013$), but no effect of sex ($p = .8708$) was observed. Different superscript letters indicate significant differences ($p < 0.05$).**

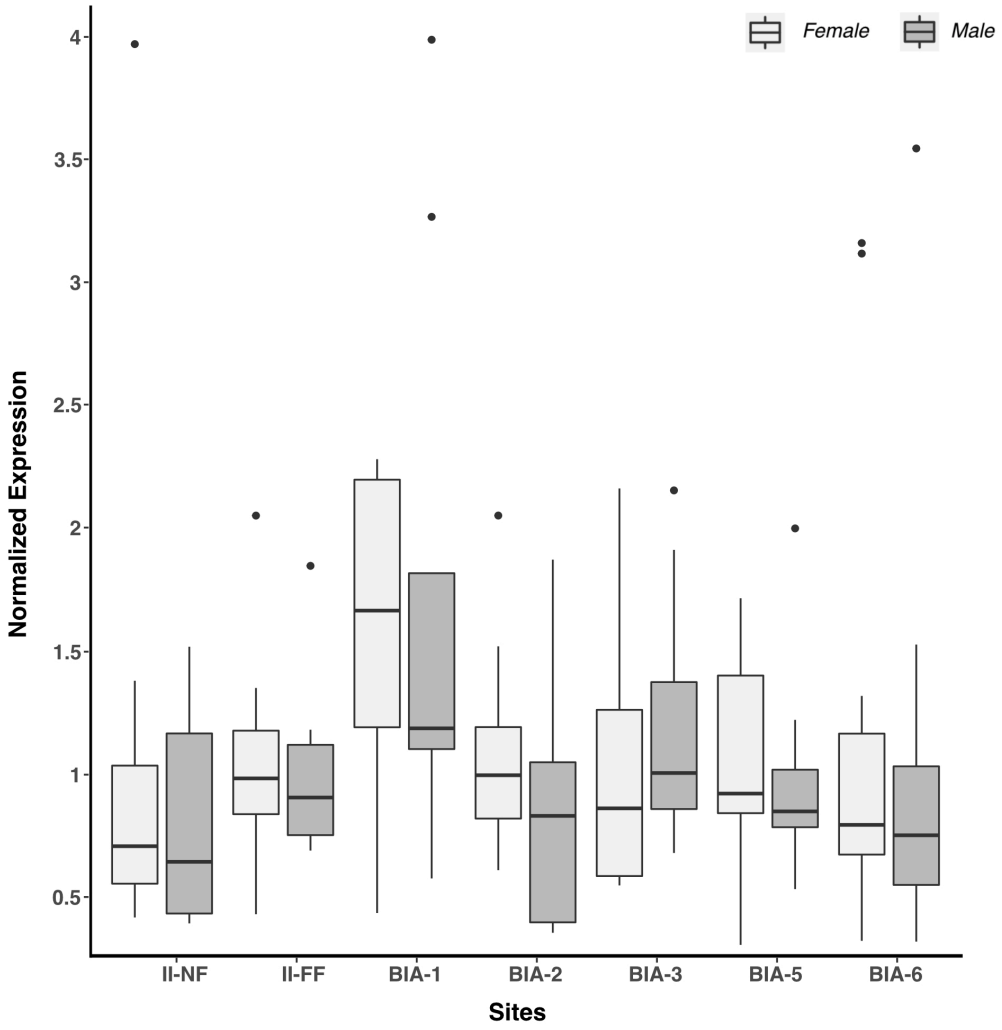

Figure 3.10. Hepatic gene expression levels of GLUT2 in English sole *(Parophrys vetulus)* at various sites near- and far-field from the Iona and Lion's Gate Wastewater Treatment Plant in Burrard Inlet. Normalized gene expression data was calculated based on the $\Delta\Delta C_q$ method for relative quantitation of a target gene using two reference genes (ACTB and eEF1A1). Boxplots demonstrating the median values (horizontal black lines within box), the lower and upper 25th and 75th percentiles (upper and lower boundaries of the box), the minimum and maximum values (whiskers) and outliers (black points) are presented. Biological replicates for each site for females and males are as follows: II-NF, n = 10 and 9; II-FF, n = 7 and 9; BIA-1, n = 7 and 8; BIA-2, n = 9 and 9; BIA-3, n = 10 and 11; BIA-5, n = 10 and 8; BIA-6, n = 11 and 10. A Two-Way Analysis of Variance (ANOVA) followed by Tukey's post-hoc analysis was used to determine significant differences between the main effects (site and sex) and interaction between the main effects. No significant effect of site (p = .0751), sex (p = .4418), nor interactive effect (F(6,114) = 0.4520, p = .8423) was observed.

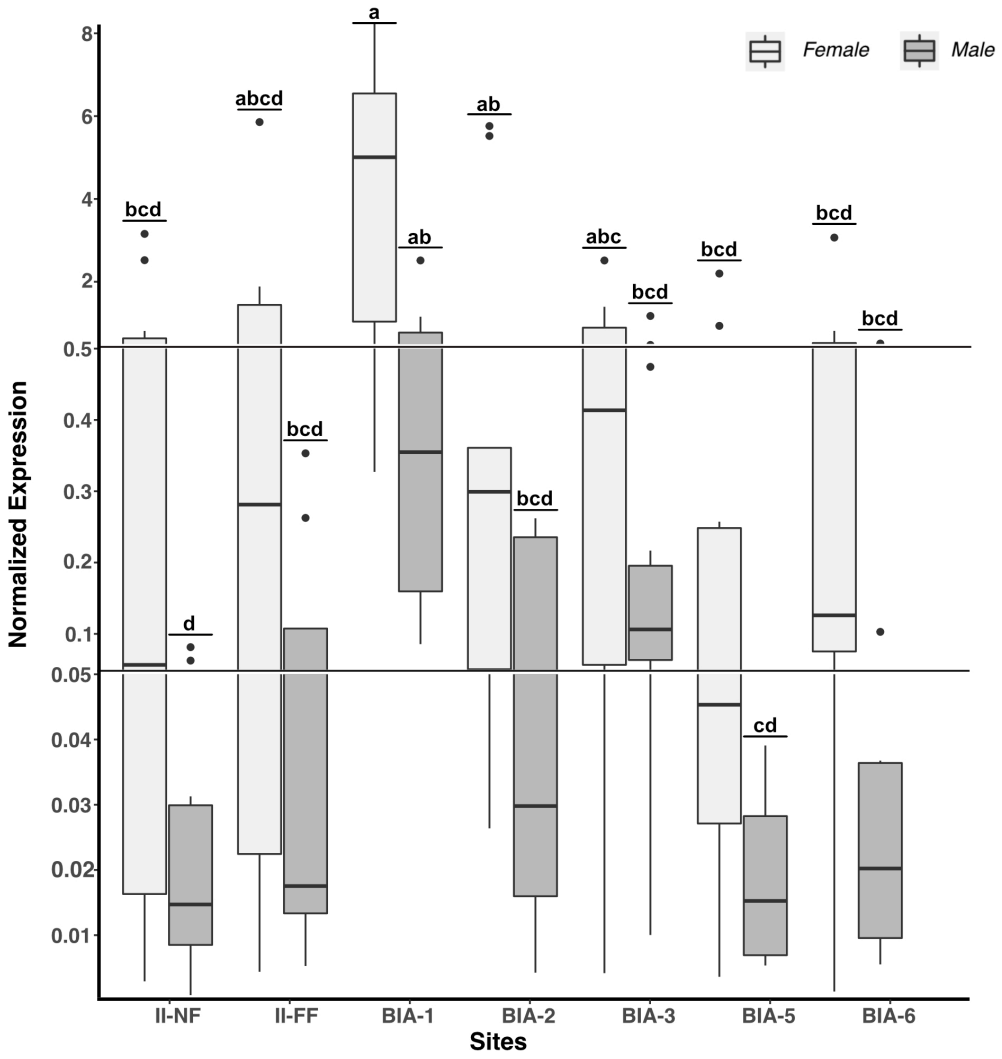

Figure 3.11. **Hepatic gene expression levels of ERα in English sole *(Parophrys vetulus)* at various sites near- and far-field from the Iona and Lion's Gate Wastewater Treatment Plant in Burrard Inlet. Normalized gene expression data was calculated based on the $\Delta\Delta C_q$ method for relative quantitation of a target gene using two reference genes (ACTB and eEF1A1). Boxplots demonstrating the median values (horizontal black lines within box), the lower and upper 25th and 75th percentiles (upper and lower boundaries of the box), the minimum and maximum values (whiskers) and outliers (black points) are presented. Biological replicates for each site for females and males are as follows: II-NF, n = 10 and 10; II-FF, n = 7 and 9; BIA-1, n = 8 and 9; BIA-2, n = 9 and 9; BIA-3, n = 10 and 11; BIA-5, n = 11 and 10; BIA-6, n = 12 and 10. A Two-Way Analysis of Variance (ANOVA) followed by Tukey's post-hoc analysis was used to determine significant differences between the main effects (site and sex) and interaction between the main effects. A significant effect of site ($p < .0001$), sex ($p < .0001$), but no interactive effect ($F(6,121) = 0.3278$, $p = .9213$) was observed. Different superscript letters indicate significant differences ($p < 0.05$).**

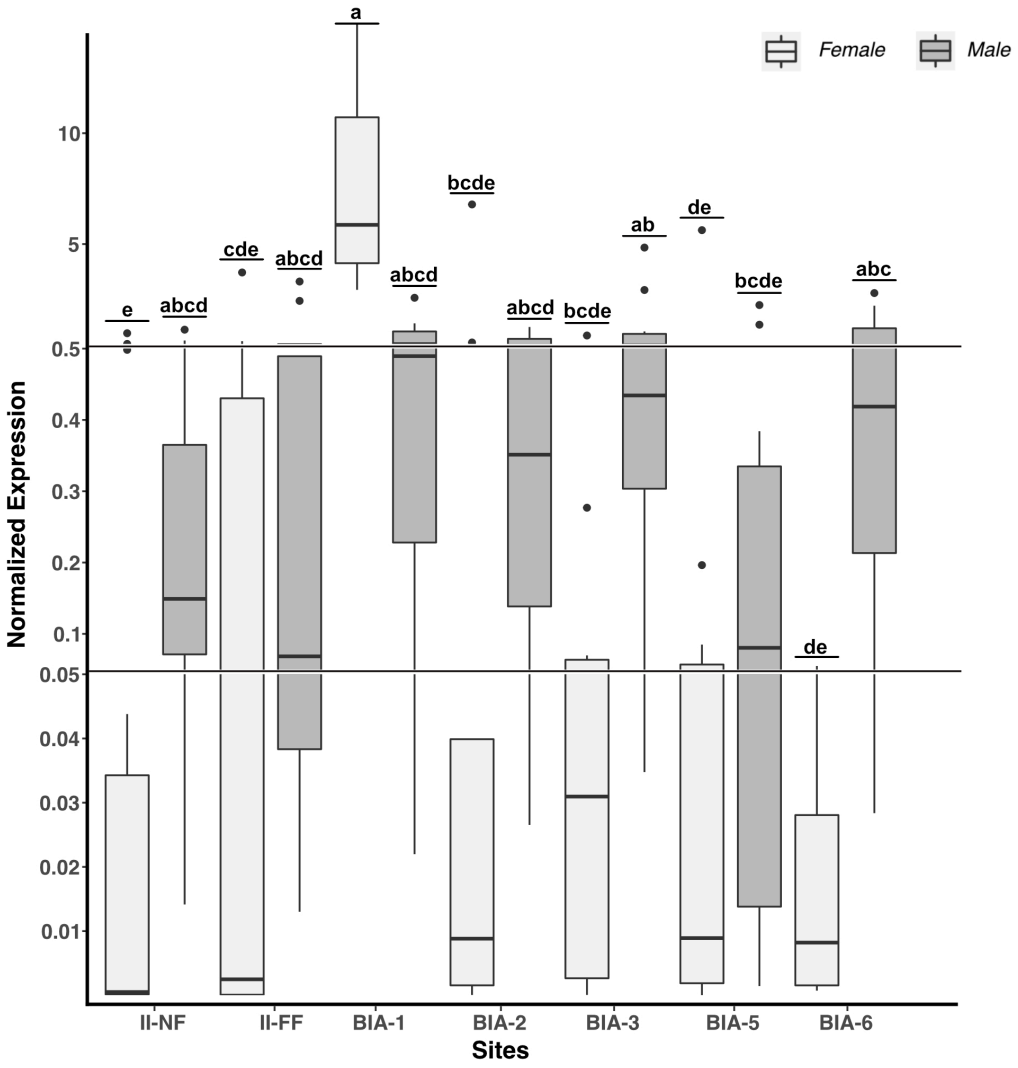

Figure 3.12. **Hepatic gene expression levels of VTG in English sole *(Parophrys vetulus)* at various sites near- and far-field from the Iona and Lion's Gate Wastewater Treatment Plant in Burrard Inlet. Normalized gene expression data was calculated based on the $\Delta\Delta C_q$ method for relative quantitation of a target gene using two reference genes (ACTB and eEF1A1). Boxplots demonstrating the median values (horizontal black lines within box), the lower and upper 25th and 75th percentiles (upper and lower boundaries of the box), the minimum and maximum values (whiskers) and outliers (black points) are presented. Biological replicates for each site for females and males are as follows: II-NF, n = 10 and 10; II-FF, n = 7 and 9; BIA-1, n = 8 and 8; BIA-2, n = 9 and 9; BIA-3, n = 10 and 11; BIA-5, n = 11 and 10; BIA-6, n = 11 and 9. A Two-Way Analysis of Variance (ANOVA) followed by Tukey's post-hoc analysis was used to determine significant differences between the main effects (site and sex) and interaction between the main effects. A significant effect of site ($p < .0001$), sex ($p < .0001$), and interactive effect ($p < .0014$) was observed. Different superscript letters indicate significant differences ($p < 0.05$).**

4. Discussion

This study is the first to use targeted gene expression assays for English sole in a Metro Vancouver wastewater effluent monitoring program. This study revealed significant differences between the sites examined and sexes in the abundance of hepatic transcripts known to be involved in various biological processes and/or systems, including the defense against xenobiotics detoxification, transcriptional regulation and synthesis of proteins, lipid and glucose metabolism, thyroid hormone metabolism, and reproduction. A key finding of the present study is that males exhibited higher VTG transcript levels than females at all sites, indicating widespread exposure to estrogenic contaminants throughout the Burrard Inlet. This study presents 11 other novel English sole primers and RT-qPCR assays that were developed and met MIQE guidelines (Bustin et al., 2009). However, future studies using English sole in controlled lab studies are critical to further develop these additional potential biomarkers and provide linkage to individual contaminants or mixtures thereof and organism and/or population level adverse effects. In addition, to better understand the natural variation in traditional apical measures (i.e., morphometrics, gonad and liver size, etc.) and transcript abundance in English sole, future studies that incorporate reference sites unimpacted by human development alongside contaminated sites are also critical. Collectively, this study demonstrates the potential of molecular biomarkers of urban contaminant exposure in wild caught English sole for use in diagnosing a broader range of adverse health effects when combined with conventional whole organism health indicators with an overview outlined in Table 4.1.

Table 4.1. Overview of molecular biomarker results of urban contaminant exposure in wild English sole (*Parophrys vetulus*). For each site of interest, known point sources of contamination are provided with whole organism health indicators (e.g., EROD activity; external and internal abnormalities; fork length; GSI; plasma VTG; and weight) from 2007, 2012, and 2017 Burrard Inlet Ambient Monitoring Program reports. Gene expression changes from this study (Parekh, 2022) are summarized in reference to each site of interest.

Site	Known Point Sources of Contamination	Burrard Inlet Ambient Monitoring Program Results	Gene Expression Changes[4]
BIA-1 (Outer Harbour North)	Lions Gate Primary WWTP	↓ EROD Activity[1,2,3] ↑ Fork Length & Weight[1,2,3] ↑ GSI[1,3] ↑ Plasma VTG[1] ↓ Plasma VTG[2]	↑ 18S rRNA ↓ CYP1A ↑ DIO1 ↑ ERα ↑ FABP1 ↑ FASN ↑ PPARδ ↑ VTG
BIA-2 (Outer Harbour South)	Lions Gate Primary WWTP	↓ EROD Activity[1,2] ↑ GSI[2] ↑ Plasma VTG[1] ↓ Plasma VTG[2]	↑ FASN ↑ PPARδ
BIA-3 (Inner Harbour)	Shipping Activities & CSOs (e.g., Vancouver Wharves, BC Sugar, Neptune Bulk Terminal, Canada Harbour Place)	↑ External & Internal Abnormalities[2]	↑ RPS4X
BIA-5 (Port Moody Arm)	Industrial Sources (e.g., Imperial Oil Ioco, Flavelle Cedar, Burrard Thermal)	↑ EROD Activity[1,2,3] ↑ External & Internal Abnormalities[2,3] ↑ Liver Lesions[1,2,3] ↑ Plasma VTG[1,2]	↑ CYP1A ↓ DIO1 ↑ FABP1
BIA-6 (Indian Arm)	Hydroelectric Plants (e.g., Buntzen Plants)	↑ EROD Activity[2,3] ↑ External & Internal Abnormalities[1,3] ↑ Liver Lesions[1,2,3]	↓ 18S rRNA ↑ CYP1A ↓ RPS4X
II-NF (Iona Near-Field)	Iona Island Primary WWTP		↓ FASN ↓ PPARδ
II-FF (Iona Far-Field)	Downstream of South Arm of Fraser River		↓ FASN ↓ PPARδ

[1] 2007 Metro Vancouver Ambient Burrard Inlet Monitoring Program Fish Health Survey Report (Nautilus; 2009)
[2] 2012 Burrard Inlet Ambient Monitoring Program (ENKON Environmental; 2015)
[3] 2017 Burrard Inlet Ambient Monitoring Program (ENKON Environmental; 2020)
[4] This Study (Parekh; 2022)

4.1. 2017 Burrard Inlet Ambient Monitoring Program English Sole Health and Body Metrics

Adult English sole are a widely recognized sentinel species for contaminant studies because of their abundance, ability to be easily sampled, and broad distribution throughout northwestern Canada (Moser et al., 2003; Stein et al., 1993). Adult English sole mature typically at 2-3 years and 3-4 years for males and females, respectively (Lassuy, 1989); and 2017 BIAMP reports were indicative of mature English sole since ages ranged from 5 to 16 years for males and 4 to 16 years for females (ENKON Environmental, 2020). On average, the oldest fish were present at BIA-3, where the mean ages for males and females, respectively, were 11.4 years and 9.5 years; whereas the youngest fish were found at BIA-2, where the mean ages for males and females, respectively, were 7.8 years and 6.2 years (ENKON Environmental, 2020). Furthermore, sex-related differences were observed in growth rates where males appeared to grow more slowly than females. Specifically, the average fork lengths of males and females from the seven sampling sites, respectively, ranged from 240 mm to 286 mm and 253 mm to 326 mm which is consistent with the observation of Lassuy (1989) that females grow faster than males after the second year (ENKON Environmental, 2020). However, the overall longest and heaviest female English sole occurred at the Outer Harbour (BIA-1) nearest to the Lion's Gate WWTP discharge site, which was not observed in males (ENKON Environmental, 2020; Lassuy, 1989; Sol et al., 1998). However, condition factor (CF), which reflects the physical and biological circumstances and fluctuations by interaction among feeding conditions, parasitic infections, and physiological factors (Le Cren, 1951), did vary slightly in males at BIA-2 being significantly higher than that of males from BIA-6 and females at II-NF and BIA-1 were significantly higher than those of females at BIA-3 and BIA-6 (ENKON Environmental, 2020). Interestingly, the hepatosomatic index (HSI), which is an indicator of energy reserves, was similar in both males and females with highest levels observed at BIA-5 and lowest at BIA-6 (ENKON Environmental, 2020).

Several studies have successfully demonstrated the application of the gonadosomatic index (GSI) to improve accuracy and precision in determining sexual maturity and fertility in fish (Flores et al., 2015; Lowerre-Barbieri et al., 2011). The heavier body weight of females at BIA-1 was likely partially attributed to the significantly higher GSI in these females, the latter indicating ovaries in an advanced stage of

development and closer to a ripe ovary observed just prior to spawning. With all English sole of adult age, one would expect the reproductive status to be similar across all sites, thus it is possible that WWTP contaminants, particularly those with estrogenic modes of action may have caused advanced ovarian development in females residing nearest the Lions Gate WWTP effluent discharge site (BIA-1; Guiguen et al., 2010; Li et al., 2019). Although no detailed studies of English sole reproductive cycles and gonad size in BC have been conducted, English sole gonad size has been shown to vary seasonally in other Pacific Northwest populations. Specifically, GSI and gonad sizes increase during the months leading up to winter spawning at which point GSI and gonad sizes peak, and this which is then followed by reduced size immediately post-spawning of spent gonads and continued gonadal recrudescence into the summer and/or early fall. For example, during the reproductive seasons in the months of October to February of English sole in adults 5 years or older, GSI values ranged from 3.67 to 4.59 and 2.94 to 4.04 in females collected from a reference site and industrial site in Puget Sound, Washington (Johnsten et al., 1997). Johnsten et al. (1997) reported no developing eggs (i.e., recrudescence) present in the ovary and completion of spawning by April and that fish in the impacted site began to mature at a younger age then the unimpacted site. Another study of Puget Sound English sole reported average GSI for females of 1.4% in immature fish, 20% in pre-spawning females with mature oocytes, and 2.8% in spawned-out females; and for males 0.6% in immature fish, 1.6% in spawning males, and 0.8% in spawned-out males (Sol et al., 1998). The present study where female English sole collected in September 2017 from the Outer Harbour (BIA-1) nearest the Lion's Gate primary WWTP displayed significantly higher GSI levels (average of ~13%) relative to all other collection sites (average ranged from ~1.3% to 3.7%; ENKON Environmental, 2020). This advanced ovarian development at BIA-1 may be due to estrogenic contaminants known to be present in WWTP effluent (i.e., 17β-estradiol, estriol, estrogen 17α-ethynyl estradiol, etc.), and all other sites may be exhibiting underdeveloped ovaries. Indeed, in some cases elevated GSIs have been indicative of nutritional enrichment, whereas lowered GSIs being associated with delayed developments due to environmental exposures to pulp mill effluents (Khan et al., 2006; Leblanc et al., 1997; Sepulveda et al., 2003), municipal wastewater treatment plant effluents (Vajda et al., Undated; Andersson, 2007), landfill leachates (Noaksson et al., 2004), and metals (James et al., 2003). Although clearly differences in female GSI were evident, the inclusion of multiple reference sites in

study design to better understand basic reproductive biology of local female and male English sole populations in Metro Vancouver is warranted.

4.2. Hepatic CYP1A Gene Expression Varies with Urban Pollutants

Although no significant differences in male English sole in the present study were observed, CYP1A levels in females were lowest closest to the Lion's Gate WWTP in the Outer Harbour (BIA-1) compared to sites further along the Inner Harbour (BIA-3), Port Moody Arm (BIA-5), and Indian Arm (BIA-6). The induction of hepatic CYP1A is a widely recognized biomarker of environmental exposure to certain planar halogenated and non-halogenated hydrocarbons (Hook et al., 2014), and the present study showed significant differences in the expression levels of this transcript in female English sole between sites of interest. The cytochrome P450 detoxification system is an extensively studied enzyme system that is involved in the first phase oxidation reactions of the two-phase metabolism of xenobiotics and its activity, transcript, and protein abundance has been shown to be related to sediment, whole-body, and liver tissue concentrations of various planar halogenated and non-halogenated hydrocarbons (Bucheli et al., 1995; Široká and Drastichova, 2004). Indeed, several studies over the past two decades have demonstrated the induction of hepatic CYP1A enzymes or transcripts after exposure to several compounds including PCBs, PBDEs, OCPs, DDT, and PAHs, primarily through the binding and activation of the AhR pathway (Bucheli et al., 1995; Oris and Roberts, 2009). For instance, Roy et al. (2003) conducted a field study in 2000 to investigate the impacts of sediment PCBs and PAHs on CYP1A protein induction in English sole collected from sites surrounding the outfall of the Orange County (CA, USA) municipal wastewater discharge. CYP1A protein concentrations were significantly elevated in English sole at sites nearest the discharge site relative to both upstream and downstream reference sites (Roy et al., 2003). Furthermore, regression analyses indicated a dose-dependent, positive correlation between sediment PCBs and CYP1A protein induction (Roy et al., 2003). Similarly, Stein et al. (1993) reported elevated hepatic monooxygenase EROD activity from PAH- and PCB-contaminated sites in Puget Sound (WA, USA) in English sole, starry flounder (*Platichthys stellatus*), and rock sole (*Lepidopsetta bilineata*). Interestingly, a field-based monitoring study conducted by Johnson et al. (2015) from 2000 – 2004 investigated the impacts of aluminum smelter-

derived PAH metabolites on the health of adult English sole in the marine waters of Kitimat (BC, Canada) via hepatic aryl hydrocarbon hydroxylase (AAH) enzyme activity and DNA adduct levels measured by 32P-postlabeling. Johnson et al. (2015) reported significantly elevated PAHs in sediments, PAH metabolites in bile, AHH activity and DNA adducts closest to the smelter. Interestingly, Stein et al. (1992) studied English sole in urbanized areas of Puget Sound and showed similar trends for these exposure and biological effect metrics, albeit at somewhat higher magnitudes compared to those reported by Johnston et al. (2015). In any case, planar halogenated and non-halogenated hydrocarbons are clearly present in both urban and industrial wastewater discharges, including the present study reporting significant variation in hepatic CYP1A transcript abundance in English sole in urbanized Metro Vancouver waters.

High molecular weight PAHs (e.g., naphthalene, fluorene, anthracene, pyrene) are commonly associated with petroleum operations, lumber production, WWTPs, and commercial use (Latimer and Zheng, 2003); and while the BIAMP aims to monitor and assess the impacts of effluents from the primary Lion's Gate WWTP, 2017 tissue chemistry results for arsenic, lead, PCBs, OCPs (represented by total DDT and total chlordane), dioxins and furans, and the PAH metabolite (pyrene 1-glucuronide) in bile was highest in fish from BIA-5 and BIA-6 (ENKON Environmental, 2020). It is possible that this is due to the Port Moody and Indian Arm being subjected to a larger influx of PAHs from multiple contributors such as Imperial Oil Ioco, Flavelle Cedar, Burrard Thermal, and active hydroelectric plants relative to the Outer Harbour and the Lion's Gate WWTP (Nautilus, 2009; Rao et al., 2019). Furthermore, liver English sole CYP1A activity measured using an *in vitro* EROD assay was significantly higher in the eastern portions of Burrard Inlet (BIA-5, BIA-6) compared to the western Outer Harbour region (BIA-1) in 2007, 2012, and 2017 (Nautilus 2009; ENKON Environmental, 2015; 2020). These results suggest exposure of English sole to CYP1A-inducing chemicals (e.g., dioxins and furans, PCBs, PAHs, and 2,3,7,8-TCDF) in Metro Vancouver waters and generally support the hypothesis that sites furthest from the Lion's Gate WWTP had higher levels of these chemicals due to local contaminant sources in the Indian Arm and Port Moody Arm (ENKON Environmental, 2015; 2020; Nautilus, 2009; Rees et al., 2003; Van Der Oost et al., 2003). Nevertheless, PCBs and PBDEs have been measured at concentrations equal to or exceeding Canadian Environmental Quality Guidelines in Metro Vancouver's WWTP but are also prevalent from a variety of other sources in

urban areas relative to both industrial and urban wastewater discharges (ENKON Environmental, 2015).

4.3. Potential Disruption of Thyroid Hormone Metabolism by Sewage Effluent

In the present study transcript abundance of a deiodinase enzyme involved in peripheral thyroid hormone metabolism, DIO1, in female English sole was highest closest to the Lion's Gate WWTP effluent discharge site in the Outer Harbour North (BIA-1) compared to sites further away (i.e., Inner Harbour (BIA-3), Port Moody Arm (BIA-5), Indian Arm (BIA-6), and near-field north of the Iona diffuser (II-NF)). Interestingly, over the past ~three decades there is mounting evidence that contaminants detected in wastewater effluents (e.g., plasticizers, i.e., bisphenol F, and bisphenol S; flame retardants, i.e., PBDEs; surfactants, i.e., perfluorinated chemicals; and pharmaceuticals i.e., methimazole, dexamethasone) affect thyroid hormone homeostasis via peripheral control of triiodothyronine (T_3) and thyroxine (T_4) metabolism, and thus have the potential to disrupt the actions of these hormones known to influence a wide array of biological processes (i.e., metabolism osmoregulation, growth, differentiation, reproduction, metamorphosis, and development; Brown et al., 2009; Dang et al., 2021; Norris and Carr, 2013; Jargue and Pina, 2014). In teleosts, peripheral T_4 can be deiodinated to biologically active T_3 hormone or inactivated to reverse T_3 (rT_3) or 3,3'-diiodothyronine (T_2) via deiodinase enzymatic activity and are known to be influenced by physiological (e.g., nutritional state, stress, and hormones) and environmental stressors (e.g., metals, dioxins, furans, OPs, PAHs, PCBs, PPCPs; Brown et al., 2009; Dang et al., 2021; Noyes and Stapleton, 2014). The DIO1 enzyme in particular is involved in both T_4-outer ring deiodination (ORD) and T_4-inner ring deiodination (IRD) catalytic reactions to produce active T_3 and inactive rT_3 hormone, respectively, whereas DIO2 and DIO3 participate solely in ORD and IRD reactions, respectively (Noyes and Stapleton, 2014). Although currently no specific genes are validated in fish as biomarkers of contaminants that disrupt thyroid hormone metabolism, several studies, including the present study, demonstrate the potential of using enzymes key to the synthesis, degradation, metabolism, or excretion of thyroid hormones as indicators of impaired functioning of the hypothalamic-pituitary-thyroid (HPT) endocrine axis in aquatic biota (Brown et al., 2009; Chen et al., 2012; Noyes and Stapleton, 2014;

Thambiraiah et al., 2022; Wang et al., 2019). For example, controlled laboratory studies by Chen et al. (2012) and Wang et al. (2019) showed significant increased DIO1 transcript levels in zebrafish larvae exposed to some brominated flame retardants (i.e., 2,2',3,3',4,4',5,5',6,6'-decabromodiphenyl ether (BDE-209) and decabromodiphenyl ethane (DBE)) causing increased T_3 levels and significant changes in T_4 levels (BDE-209 caused a decrease while DBE caused an increase). Of particular relevance to the present study however are more recent field-based studies in fish demonstrating the impacts on the abundance of deiodinase enzyme levels in waters downstream of WWTP discharge sites. In Canada, Reinling et al. (2017) demonstrated major urban wastewater effluents in Montreal (QC) were associated with increased hepatic DIO2 transcript levels, free and total plasma T_3 levels, and total lipids in female northern pike (*Esox lucius*). These findings in female pike are consistent with studies of immature walleye (*Sander vitreus*) collected downstream from the Ottawa and Gatineau (Canada) WWTPs, whereby walleye exhibited significantly greater T_3 plasma levels compared to reference sites and elevated gene expression of hepatic DIO2 (Picard-Aitken et al., 2007). In light of several suspected and known EDCs with modes of action that disrupt the HPT endocrine axis of vertebrates (Gore et al., 2015), the findings in the present study and the presence of these contaminants in Metro Vancouver sewage effluents (i.e., alkylphenols, dioxins and furans, metals, PAHs, PBDEs, PCBs, pesticides, and PPCPs; ENKON Environmental, 2015), it is likely that English sole are experiencing some disruption of the HPT axis. However, further investigations including thyroid hormone and metabolic (i.e., lipid levels) measures combined with a suite of genes involved in thyroid hormone metabolism, regulation, and signalling would be beneficial in better establishing the risks these contaminants pose to fish exposed to Metro Vancouver WWTP effluents.

4.4. Fatty Acid Metabolism Transcripts as Potential Biomarkers of Sewage Effluent Exposure

The present study concurs with several other studies reporting that WWTP effluents have the potential to alter metabolism by reducing tissue energy reserves and altering the expression of metabolic genes (Cazenave et al., 2014; Ings et al., 2012; Melvin, 2016; Smolders et al., 2003; Vidal-Dorsch et al., 2013; Sherry et al., 2019). In the present study transcript abundance of a fatty acid binding protein, FABP1, in female

English sole were highest closest to the Lion's Gate WWTP effluent discharge site in the Outer Harbour North (BIA-1) compared to sites further downstream (i.e., Outer Harbour South (BIA-2), Inner Harbour (BIA-3), Indian Arm (BIA-6), and near- and far-field north of the Iona diffuser (II-NF, II-FF)). Similarly, in both females and male's transcript abundance of a fatty acid synthase, FASN, was highest closest to the Lion's Gate WWTP effluent discharge site in the Outer Harbour North (BIA-1) compared to sites north of the Iona diffuser (i.e., II-NF, II-FF) and Port Moody Arm (BIA-5, males only). In teleost, lipids play a major role in the storage and provision of energy through lipogenesis pathways occurring in the cytosol via FASN, a multienzyme complex which catalyzes the conversion of acetyl-CoA and malonyl-CoA to 16- and 18-carbon fatty acids (palmitic and stearic acids, respectively; Olivares-Rubio and Vega-López, 2016; Tocher, 2003); while intracellular transport and metabolism of dietary lipids and exogenous lipophilic compounds are catalyzed by cytoplasmic FABP1 (Schulz, 2008). Although, FABP1 and FASN catalyze the metabolism and transport of fatty acids, nuclear receptors (i.e., peroxisome proliferator-activated receptors, PPARs) are key to these processes and bind a broad range of ligands (e.g., fatty acids and fatty acid derivatives) and serve as major transcriptional regulators of enzymes involved in fatty acid metabolism and transport (e.g., FABP1 and FASN, Poulsen et al., 2012; Schulz, 2002; Tocher, 2003). Interestingly, PPARδ, in female English sole was also highest closest to the Lion's Gate WWTP in the Outer Harbour North (BIA-1) compared to sites further along the Inner Harbour (BIA-3), Indian Arm (BIA-6), and near- and far-field north of the Iona diffuser (II-NF, II-FF). Similarly, male English sole from near- and far-field sites north of the Iona diffuser (II-NF, II-FF) were significantly lower compared to sites along the Burrard Inlet (i.e., Outer Harbour (BIA-1, BIA-2) and Inner Harbour (BIA-3), Olivares-Rubio and Vega-López, 2016). Together, in the present study a trend of elevations in two enzymes and a transcriptional regulator involved in lipid metabolism at the site most proximal to a primary WWTP effluent discharge site combined with the significantly heavier and longer females at this site provides evidence of altered metabolism in fish exposed to WWTP effluent. Whether these larger fish exhibited different levels of lipids or other metabolites or organ level alterations in metabolism was not measured in the study but should be considered in future monitoring programs.

Although several contaminants discharged in primary and secondary WWTP effluents have been detected globally (i.e., hormones, PAHs, PCBs, pesticides, and

PPCPs) and several have been shown to alter metabolism in fish, lipid regulating pharmaceuticals are likely important contributors to such observations (Olivares-Rubio and Vega-López, 2016). Indeed, lipid regulating pharmaceuticals are prescribed to alter blood lipid levels and are known to reduce the risk of cardiovascular disease in humans (Zhang et al., 2020). A recent review of the two main types of lipid-regulating agents, the statins, and fibrates, reported that these and cardiovascular drugs are the most consumed pharmaceuticals worldwide, and routinely detected in the ng/L to µg/L range (Zhang et al., 2020). For example, statins were found in untreated sewage samples at concentrations between 4 and 177 ng/L and in treated sewage samples at 1 and 59 ng/L (Miao and Metcalfe, 2003a; Miao and Metcalfe, 2003b). Additionally, statins were also detected in surface water at concentrations of 15 ng/L (Metcalfe et al., 2004). Although not measured in the Metro Vancouver BIAMP, it is expected that these biologically active pharmaceuticals targeting lipid metabolism in vertebrates are present to some extent proximal to Metro Vancouver's' WWTP. Furthermore, the alterations in transcripts related to lipid metabolism in the present study supports this hypothesis. However, most studies reporting significant effects of fibrates and statins on lipid metabolism and other endpoints are laboratory based and variable, but some WWTP effluent field-based exposures do exist and demonstrate effects on lipids and lipid metabolites. For example, in laboratory-based studies exposing female fathead minnow to gemfibrozil (fibrate that targets PPARα) for two days caused an increase in plasma triglycerides at 15 and 600 µg/L (Skolness et al. 2012). Sex specific changes in several hepatic genes involved in lipid metabolism were observed after these waterborne gemfibrozil exposures, for example, the induction of hepatic PPARα (600 µg/L) and a reduction of FABP1 (15 and 600 µg/L) after 8 days in males (Skolness et al., 2012). Field studies by Simmons et al. (2017) deploying caged goldfish (*Carassius auratus*) in waters affected by WWTP effluents in Lake Ontario, Canada for three weeks provide further support of the wide range of contaminants in these affected waters and showed the highest bioaccumulation factors for gemfibrozil and the antidepressant fluoxetine. Indeed Simmons et al. (2017) observed lower plasma fatty acids, bile acids, and phosphatidylcholines and changes in 13 proteins and metabolites involved in lipid metabolism in wild male goldfish compared to caged goldfish at the reference site, indicating inhibition of lipid synthesis and accumulation in wild male goldfish near WWTP effluent discharge site. Interestingly, recent studies have also shown some phase I enzymes (e.g. CYP1) to be under activation of various PPARs (Shaban et al., 2004). For instance, clofibric acid and

gemfibrozil, which have been detected in several environmental samples at concentrations of 2100 ng/L in WWTP effluent, is a common ligand of PPARα and PPARδ and has been associated with the downregulation of AhR and CYP1A mRNA levels in rat models (Metcalfe et al., 2004; Shaban et al., 2004). Although this is a poorly studied topic in fish exposed to such contaminants, the present study has shown elevated levels of PPARδ, FABP1, and FASN to be associated with significantly downregulated mRNA expression levels of CYP1A nearest to the Lion's Gate WWTP in the Outer Harbour North (BIA-1). Though the induction of PPARs and changes in enzyme transcript levels involved in lipid metabolism by various pharmaceuticals contaminants associated with wastewater effluents have been linked to organismal level effects on energy stores and metabolism, further studies must be conducted to determine their specificity as a proxy for particular contaminants and the sensitivity of these potential biomarkers for environmental contaminant risk assessment.

4.5. Disruption of the Reproductive Endocrine Axis

Strong evidence of exposure to contaminants with an estrogenic mode of action was observed in this study based on male English sole VTG transcript levels either equivalent or exceeding female levels of this gene at all sites investigated. In oviparous vertebrates and some invertebrates (i.e., bivalves), the glycolipophosphoprotein VTG, is the precursor of vitellin which is used as the primary nutritional source for the developing embryo (Martyniuk et al., 2020). It is produced in the liver and regulated by estrogens and ERs (ligand activated transcription factors) that mediate estrogen action, and then exported into the blood and sequestered in oocytes (Martyniuk et al., 2020). Although males possess the genes to naturally produce VTG, this naturally occurs at much lower levels than that of female. As a result, VTG at the transcript and protein level have been validated as reliable and robust biomarkers of exposure to estrogenic substances (Amano et al., 2019; Barber et al., 2019; Denslow et al., 1999; Eidem et al., 2006; Tran et al., 2019; Zhang et al., 2019). In addition, in most teleosts investigated to date exhibit sex specific differences in ERa levels with females exhibiting higher levels than males (Nelson and Habibi, 2020), however, this was not observed in the present study. This is further evidence of the presence of estrogenic contaminants in Metro Vancouver waters. Interestingly, site BIA-1 nearest to the primary treatment Lion's Gate WWTP where females exhibited advanced ovarian development and elevated ERα levels, the males

collected from this site exhibited the largest magnitude VTG transcript induction. Previous Metro Vancouver BIAMPs conducted in 2007 and 2012 measured protein levels of VTG and did report elevated levels in males nearest to the Lion's Gate WWTP discharge site in both years and some induction in the Central Harbour (BIA-3), Port Moody Arm (BIA-5), and Indian Arm (BIA-6; n = 5 males per site). Collectively, multiple years of monitoring programs suggest that females spawning nearest the Lion's Gate WWTP discharge site may occur abnormally early and out of synch with males, but also that male teste structure and/or function may be impaired due to exposure to estrogenic compounds.

A key finding of the present study is that males exhibited higher VTG transcript levels than females at all sites. We hypothesize this indicates widespread exposure to estrogenic contaminants throughout the Burrard Inlet. In addition, it also underscores the need for the inclusion of reference sites to clarify the natural background fluctuations in English sole males to better understand the magnitude of VTG induction and how this translates into impacts on the reproductive endocrine axis and the population dynamics. Nonetheless, in light of numerous contaminants in WWTP effluent known to be estrogen mimics or antagonists (i.e., BPA, phthalates, natural estrogens, synthetic estrogens, alkylphenols, etc.) and several studies reporting alterations in the reproductive endocrine system of feral fish downstream of sewage discharge sites, it is widely accepted that xenoestrogens are adversely affecting fish populations downstream of WWTP effluent discharges in most continents (Bergman et al., 2013; Osachoff et al., 2013; 2016; Robaire et al., 2022). For instance, studies conducted in Metro Vancouver (BC, Canada) by Osachoff et al. (2014) evaluated the treatment of synthetic wastewater containing PPCP cocktails and assessed removal efficiencies via conventional activated sludge primary treatment to effects observed on juvenile rainbow trout (*Oncorhynchus mykiss*). Significant inductions of VTG mRNA transcript and plasma protein levels were observed after exposure to both wastewater influent and effluent, indicating the presence of estrogenic contaminants discharged from primary and secondary WWTPs in Metro Vancouver (Osachoff et al., 2014). With respect to ng/L natural and synthetic estrogens discharged in primary and secondary WWTPs, the 7-year, whole-lake experiment by Kidd et al. (2007) is particularly relevant. Kidd et al. (2007) demonstrated that chronic low-level exposure of fathead minnows to EE2 (5-6 ng/L) led to feminization of males measured via elevated VTG mRNA and protein levels, increased incidence of intersex

gonads in males, and altered oogenesis in females, which ultimately caused a near extinction of this species from the lake (Kidd et al., 2007). Similarly, Da Silva et al. (2013) developed a rapid method to measure BPA, E2, and EE2 in bile of English sole collected from Puget Sound using enzymatic hydrolysis and solid-phase extraction and ultra-performance liquid chromatography. The distribution of BPA and E2 in male English sole bile showed that the most urbanized regions of Puget Sound had high occurrences of BPA and E2 (13 ng/mL and 66 ng/mL, respectively) coupled with abnormal levels of VTG in male and immature female English sole than non-urban sites (5.6 ng/mL and 5.1 ng/mL, respectively; Da Silva et al., 2013). Finally, since the most common endocrine disrupting chemicals known to date bind to and activate or inhibit estrogen and androgen receptors in multiple vertebrate species, routine VTG and steroid receptor assays are recommended for future monitoring in Burrard Inlet (Robaire et al., 2022). However, with clear evidence of organismal and population level impacts due to disruption of the reproductive endocrine axis in fish downstream of sewage effluent discharge sites based on data collections since the 1990s (reviewed in Robaire et al., 2022), mitigation measures such as improved WWTP treatment processes (i.e., tertiary level) are the ideal solution.

4.6. Expression Stability of Ribosomal Genes for RT-qPCR Normalization in English sole Studies

In vertebrate studies, ribosomal proteins such as 18S rRNA and RPS4X have commonly been a popular choice of house-keeping genes based on their basic cellular functions and availability throughout various teleost species (Chapman and Waldenström, 2015). However, the present study concurs with several other studies that the utility of such genes is limited in many cases due to differential expression across species, tissue types, cell lines, developmental stages, and/or in response to a plethora of natural and anthropogenic chemicals discharged into aquatic environments (Brown et al., 2004; Filby and Tyler, 2007; Hoffmann et al., 2006; Li et al., 2020). In the present study transcript abundance of a structural ribosomal RNA, 18S rRNA, in female English sole was highest closest to the Lion's Gate WWTP effluent discharge site in the Outer Harbour North (BIA-1) compared to sites further downstream (i.e., Inner Harbour (BIA-3), Indian Arm (BIA-6), and near- and far-field north of the Iona diffuser (II-NF, II-FF)). Similarly, in both females and male's transcript abundance of a ribosomal protein,

RPS4X, was highest in the Inner Harbour (BIA-3) and Port Moody Arm (BIA-5, females only) compared to sites along the Indian Arm (BIA-6) and near-field north of the Iona diffuser (II-NF, males only). In teleosts, 18S rRNA plays a crucial role in the active center of 40S ribosomal subunits in eukaryotic cytoplasmic ribosomes and its transcriptional activity increases proportionally to the number of available cytoplasmic ribosomes (i.e., increased gene transcription and protein translation) in response to various biological stimuli and environmental stressors (Chapman and Waldenström, 2015). For instance, Filby and Tyler (2007) evaluated the expression stability of 18S rRNA as an internal control for RT-qPCR assays in adult fathead minnow exposed to EE2. Indeed, hepatic 18S rRNA expression was found to be unaffected by estrogen treatment after a 21-day exposure which concurred with findings by Hoffmann et al. (2006) for an exposure to EE2 on adult zebrafish (liver, 48- and 168-hour exposures). However, using the Affymetrix GeneChip® Zebrafish Genome Microarray revealed upregulation of hepatic 18S rRNA in a concentration-dependent manner in adult zebrafish following a 24-hour exposure to EE2, suggesting that regulation of 18S rRNA by estrogen-mimics may occur in a temporal manner similar to that of low-dose chronic exposures present in wastewater effluent discharge (Hoffman et al., 2006). Thus, in the present where females nearest to the Lion's Gate WWTP exhibited the highest levels of 18S rRNA expression and advanced ovarian development, elevated ERα and VTG levels may be further evidence of 18S rRNA as a potential biomarkers of estrogenic contaminant exposure.

4.7. Conclusions

Field studies, such as the BIAMP conducted by Metro Vancouver, provide invaluable information about point-source environmental pollution associated with WWTP as well as other anthropogenic pollutants and stressors in urban waters. One limitation of the present study investigating Metro Vancouver's WWTP effluents was the lack of a true reference sites unimpacted by WWTP or other anthropogenic activities. This is largely due to the fact that there is natural fluctuations in biological variables/measures that will differ to some extent between sites of interest or periods of time in presence or absence of impact. Therefore, future study designs characterizing English sole responses that incorporate reference sites and/or controlled lab studies are critical to better understand the natural variation in traditional apical measures (i.e.,

morphometrics, gonad and liver size, baseline expression, etc.), and to further develop gene expression biomarkers for assessing the impacts of WWTP effluents more thoroughly in this model teleost. Furthermore, the genome sequence is not yet available for English sole, and this restricted the selection of genes for this study, thus sequencing the genome of this flatfish species would greatly enhance the ability to use molecular tools in this important model species. Despite these challenges, the present study developed novel RT-qPCR assays for 12 genes of interest spanning various biological processes and/or systems, including xenobiotic metabolism (CYP1A, HSP70), transcriptional regulation and protein synthesis (18S rRNA, RPS4X), thyroid hormone homeostasis (DIO1), lipid and glucose metabolism (FABP1, FASN, GLUT2, PPARδ, PPARγ), and the reproductive endocrine axis (ERα, VTG). We report the induction of well characterized biomarkers of reproductive endocrine axis abnormalities (i.e., elevated VTG and ERα in males and females, advanced ovarian development) that were most prevalent nearest the primary treatment Lion's Gate WWTP, but ultimately, the gene expression analyses indicate widespread exposure to estrogenic contaminants throughout the Burrard Inlet. Although few other genes in fish are as well characterized as VTG and ERα, several genes involved in lipid and glucose metabolism and one enzyme involved in thyroid hormone metabolism were also associated with exposure to primary WWTP effluent. We hypothesize the former is at least partly attributed to the lipid regulating pharmaceuticals known to be present near sewage discharge sites. While the latter potential thyroid hormone metabolism may be due to several chemicals present in sewage effluents and other industrial effluents (i.e., alkylphenols, dioxins and furans, metals, PAHs, PBDEs, PCBs, pesticides, and PPCPs), several of which were present in English sole tissue in the 2017 BIAMP (ENKON Environmental, 2017). This study demonstrates the high potential and applicability of molecular biomarkers of urban contaminant exposure in wild caught English sole to assess a wider range of adverse health effects when combined with conventional whole organism health indicators.

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