Changes in Gill Physiology and Energy Requirements of Darter Species (*Etheostoma* spp.) due to Effluent in the Grand River

by

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Author's declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Abstract

The effluent from municipal wastewater treatment plants is a major point source of contamination in Canadian waterways. Improvement of effluent quality to reduce contaminants like pharmaceuticals and personal care products, natural and synthetic hormones, metals, or pesticides before being released into the environment is necessary to reduce the impacts on organisms that live in the river downstream. This thesis aims to characterize the metabolic and gill physiological responses of rainbow (*Etheostoma caeruleum*), fantail (*Etheostoma flabellare*), and greenside (*Etheostoma blennioides*) darters to the effluent in the Grand River downstream from the recently upgraded Waterloo municipal wastewater treatment plant. In the summer and fall of 2019, metabolism measurements of darters were recorded using a portable field respirometry system to collect data on the energetic costs associated with living in effluent contaminated water. Additionally, body measurements were recorded for overall health assessment. The gills of darters were sampled in fall 2013/2014, fall 2018 and summer of 2019 for histological and molecular analysis to determine changes in morphology and function, which may indicate disruption to the key homeostatic functions of the gill (oxy- and ionoregulation).

Collection of water samples along the Grand River showed measurable amounts of contaminants and compounds downstream from the MWWTP that are of concern such as ammonia, carbamazepine, venlafaxine and diclofenac and their metabolites. Additionally, the physicochemical water properties are altered (i.e. dissolved oxygen, conductivity and pH). Some fishes showed evidence of growing longer and with increased mass than fish from the reference site. The routine metabolism of darters was not affected by effluent exposure, but some species had increased maximum metabolic rates leading to an increased aerobic scope. Gill samples from

effluent exposed fishes showed evidence of more pathologies and variation in morphology; additionally, the gene expression of key ion regulating proteins were altered.

Field research provides information on the direct environmental response of fishes to contaminants in real time. The importance of considering interspecies differences are highlighted, due to the variances in darter responses across the three species. The results suggest that darters can metabolically adjust to effluent contaminated water and may also be adapting to the urban and agricultural inputs. The modification and damage to the gills provides a useful water quality indicator but does not necessarily reflect how well acclimated the species is to the environment due to a lack of evidence of poor fish health.

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AS – aerobic scope
BET – basal epithelium thickness
BOD – biological oxygen demand
CEC – contaminant(s) of emerging concern
EIT – Economical Insurance Trail way; immediately downstream of MWWTP
FMR – field metabolic rate
FTD – Fantail darter (*Etheostoma flabellare*)
FWY – Fairway; further downstream from MWWTP
GSD – greenside darter (*Etheostoma blennoides)*
ID – interlamellar diameter
KIW – Kiwanis; upstream of MWWTP, urban area
MMR – maximum metabolic rate
MRC – mitochondria rich cell
MWWTP – municipal wastewater treatment plant
PPCP – pharmaceutical and personal care product(s)
RBD – rainbow darter (*Etheostoma caerulem*)
RMR – routine metabolic rate
SLL – secondary lamellar length
SLW – secondary lamellar width
TSS – total suspended solids
WMR – West Montrose; upstream reference site

1.0.0 General Introduction

1.1.0 The Grand River

The Grand River, located in Southwestern Ontario, is a 280 km river that drains into Lake Erie. Along the river there are recreational parks and trails, large agriculturally dominated areas and heavily urbanized locations. The river is a resource to nearly one million people that reside in the watershed, which applies pressure on the river to provide and maintain sustainability. There are multiple sources of contamination that affect the Grand River, and each source varies in its volume of contribution seasonally. In summer, municipal wastewater treatment plants (MWWTP) effluent dominates inputs during low flow, while in spring, agricultural run-off spikes phosphorus levels (Anderson and GRWMP Assimilative Capacity Working Group, 2012). Run-off from urban developments and intense agricultural areas along with stormwater drainages are other sources of contamination and water quality degradation (Chambers *et al.*, 1997; Cooke, 2006).

MWWTPs utilize the river flow to assimilate effluent which is the largest point source burden to Canadian waterways (Chambers *et al.*, 1997; Anderson and GRWMP Assimilative Capacity Working Group, 2012). Along the Grand River, there are thirty MWWTP that serve the 11 municipalities and two First Nations territories, ~600,000 residents total, with the remaining using private systems. The development of wastewater treatment plants has mainly reduced the transmission of pathogens and some nutrient inputs into the water systems; focus is now shifting to minimizing the contaminants that affect the aquatic biota, especially contaminants of emerging concern (CECs) which are mostly synthetic, unregulated substances (Prasse *et al.*, 2015). The effluent that is released from the MWWTP is discharged into the river and affects the flow and water quality, physically and chemically where aquatic life is at risk, at distances from the discharge point (Chambers *et al.*, 1997; Carey and Migliaccio, 2009). Due to increasing

populations, urbanization, and agriculture processes, in combination with climate change, water quality downstream from MWWTPs is at risk of being in decline, which impacts the ecology of the river.

1.1.1 Municipal Wastewater Treatment Plants

MWWTPs receive waste that contains solids, inorganic, and organic contaminants mixed, and treatment reduces the concentration of contaminants that are released back into the water. Initially, the waste is put through a primary treatment step to settle solids that will move to digestion and form biosolids. Liquids are moved to secondary treatment which decreases biological oxygen demand (BOD) and nitrogenous compounds. The final step, tertiary treatment, is more filtration or oxidation and is a valuable step to limit contaminants and nutrients that will be released as final effluent back into the river system. The tertiary treatment step is not a required process but many facilities are upgrading to incorporate tertiary treatment when upgrading to the required secondary treatment levels and comply with federal and provincial legislation (CCME and Environment, 2014; Government of Canada, 2016).

There are concentrations and limits set on known contaminants (i.e. pesticides, organics, heavy metals) and physical water qualities by the Ontario Ministry of Environment and Energy in the Provincial Water Quality Objectives to protect aquatic life and recreational uses of surface waters (Environment and Energy Ontario, 1994; Loomer and Cooke, 2011). Limits are set using literature from toxicology studies that focus on aquatic toxicity, bioaccumulation and mutagenicity (Environment and Energy Ontario, 1994). Water quality parameters such as temperature, total suspended solids (TSS), BOD, chlorine, un-ionized ammonia and nitrogen compounds, conductivity, total phosphates and pH extremes are all found to be highest or most variable immediately downstream from MWWTPs and become diluted further downstream (Tsai, 1973;

Chambers *et al.*, 1997; Carey and Migliaccio, 2009). Increased TSS, BOD and turbidity from effluent adds to increased water temperature and hypoxia, due to increased sunlight absorption and metabolism of microorganisms causing eutrophication (Carey and Migliaccio, 2009; Water Quality Working Group, 2013). Un-ionized ammonia and nitrogen compounds can impact the ability of organisms to survive and reproduce in their environment due to direct toxicity. In fish, damage to gill tissue, pH imbalances, osmo- and oxy-regulatory transport disruption, and immunocompromising are all possible effects of exposure to increased levels of un-ionized ammonia and nitrogen compounds with effects amplified when exposed to multiple stressors (Camargo and Martinez, 2007; Gomez Isaza, Cramp and Franklin, 2020). The combination of these factors detrimentally affect fish to varying degrees, depending on species and life stage (Hui-Peng Lin *et al.*, 1992; Paerl and Huisman, 2009; McBryan *et al.*, 2013; Cumming and Herbert, 2016).

Effluent post treatment contains ng/L to µg/L of contaminants of emerging concern (CECs) that are not broken down during the treatment process or are transformed into active metabolites (Carey and Migliaccio, 2009). For example, human and veterinary pharmaceuticals and metabolites, hormones, hygiene and toiletry products are of concern with new products being developed constantly (Santos *et al.*, 2008; Prasse *et al.*, 2015). The hydrophobic CEC compounds are long lasting and can remain in the environment, or can be difficult to break down, depending on their chemical properties (Tran and Gin, 2017). Many of these CECs, which are monitored but not currently regulated, can affect the hydrology, microbiology, nutrient levels, oxygen, conductivity, nitrate/nitrites, ammonia, turbidity, and chloride levels of the water compared to upstream locations, which vary seasonally (Tsai, 1968; Santos *et al.*, 2008; Carey and Migliaccio, 2009; Loomer and Cooke, 2011). Many CECs are measurable and can be detected but there are

also an unknown number of other contaminants and transformation products that can become stressors to organisms, especially in the complex mixtures of effluent. There exist provincial guidelines on the limits of concentrations of known contaminants (Provincial Water Quality Objectives) in the surface waters which were set, in part, to minimize disruption to aquatic life (Environment and Energy Ontario, 1994). The upgrades help to reach these guidelines by causing a reduction in the concentration of chemicals and contaminants in the effluents, but there are still many CECs that remain in the discharged effluent that can affect aquatic life (Hicks, 2017; Srikanthan, 2019). The list includes organics, pesticides, heavy metals but does not include CECs that are hard to degrade or settle into biomass with the possibility of being taken up into the body. Fish are sensitive vertebrate species that can be affected by effluent exposure and are commonly used for toxicology studies on the effects of contaminants found in the effluent (Porter and Janz, 2003). This study aims to measure the effects of the upgrades using darter (*Etheostoma* spp.) in the Grand River that live downstream from the MWWTP to determine if the quality of effluent has been improved enough to minimize sublethal effects of contaminant exposure

1.1.2 The Waterloo Municipal Wastewater Treatment Plants

The Waterloo MWWTP serves 98,000 residents, and recently has undergone upgrades to reduce ammonia, biological oxygen demand, total suspended solids and chloride (Hicks *et al.*, 2017; Srikanthan, 2019). The MWWTP process upgrades included increased solids retention time, added aeration/nitrification process, and UV disinfection which improves effluent quality and odour and improved process efficiency. This upgrade followed guidelines set by the Fisheries Act Canada and the Ministry of Justice in 2012, requiring all MWWTPs to be upgraded to at least secondary treatment. The upgrades took place from 2009 until 2018; these measures were implemented to reduce BOD, TSS, and chlorine and ammonia levels. In Table 1, the upgrade goals

are compared to a second MWWTP, Kitchener, found downstream serving a larger population that was also recently upgraded (Srikanthan, 2019).

Table 1.1 Summary of goals for effluent quality after upgrades to tertiary treatment at the Kitchener and Waterloo MWWTPs. 'Current' column represents values measured pre-upgrades. 'Future' is the final goal once all upgrades are online and optimized. Upgrades were complete in early 2018 but the plant was still working towards optimization later in the year (Modified from Srikanthan, 2019).

Parameter	Kitchener		Waterloo	
Effluent limits	Current	Future	Current	Future
Carbonaceous Biochemical Oxygen Demand	25	15	15	15
Total Suspended Solids (mg/L)	25	15	15	15
Total Phosphorus	1	0.4	0.8	0.6
Total Ammonia Nitrogen (mg/L)	-	4 (May-Nov) 7 (Dec-Apr)	-	1.8
pH	6-9.5	6-9.5	-	6-9.5
E. coli (org/100 mL)	200	200	200	200
Total Residual Chlorine (mg/L)	-	-	0.5	-

Recently, a study by Srikanthan et al. (2019) measured the impacts of the recent upgrades to the Kitchener and Waterloo MWWTPs effluent quality and measured concentrations of CEC's that remain in the treated effluent. There were several CECs released from the MWWTPs, in which pharmaceuticals and personal care products (PPCPs) were a general categorization. These PPCPs included endocrine disrupting compounds (EDCs; synthetic hormones, personal care products), SSRIs (Selective Serotonin Reuptake Inhibitors - antidepressants), analgesics and anti-

inflammatories (NSAIDs; Non Steroidal Anti-Inflammatory Drugs , B-blockers, antiepileptics (Srikanthan, 2019). These CECs are not specifically targeted for removal, but the concentrations of some contaminants decreased with the upgrades (attributed to nitrification reducing ammonia concentrations), while there was no difference in the removal rates of others (Srikanthan, 2019). Fish, as keystone species, can serve as bioindicators to help assess the health of an ecosystem (Adams and Greeley, 2000), and understanding effluent composition can help link effects that are seen in downstream fish populations to contaminant exposure.

1.2.1 Biomonitoring and Field Studies

The complex mixture of effluent and seasonally changing water conditions makes laboratory studies only partially insightful as to how fish populations are being affected because they tend to focus on individual stressors (i.e. hypoxia, temperature or single contaminants (Choi, Alsop and Wilson, 2018). Biomonitoring is meant to measure an environment's quality and identify what is a stressor or pressure to potentially mitigate or remove risks to a given area. Field studies (*in situ)* are more ecologically relevant because samples are collected directly from the environment which will take into account multiple stressor interactions (Hegelund *et al.*, 2004; Coors and De Meester, 2008; Segner, Schmitt-Jansen and Sabater, 2014). Comparing fish from a reference site to an affected site can help determine what effects are occurring due to contamination versus natural variation in river conditions, additionally, long term studies assist in making meaningful conclusions on changes to sites (Munkittrick and Dixon, 1989; Arciszewski and Munkittrick, 2015). Biological monitoring is difficult because it is time consuming and must consider many environmental variables over time to gain a broad understanding of what is happening in the river and can have a limited reach in what conclusions can be drawn from the data (Prasse *et al.*, 2015). Ultimately, a combination of *in situ* and *in vivo* measurements would

present the clearest picture of effluent effects. Monitoring throughout the year, using field studies and natural population sampling, alongside chemical water quality measurements are useful tools to gain a greater understanding of the impacts of effluent in combination with the dynamic river conditions. In this case, biomonitoring and sub-lethal indicators are used to measure the effects of the MWWTP effluent and determine if the upgrades were enough to mitigate stressors in that area of the Grand River.

Quantitative or qualitative sub-lethal monitoring at the physiological level of the different fish species in the Grand River is crucial to understanding overall river health, and such monitoring is currently limited (Water Quality Working Group, 2013; Diamond *et al.*, 2016; Servos, 2016). The concentrations of contaminants found in effluent and subsequently in the environment are too low to affect humans but can have effects on aquatic organisms. The extent of these effects is largely unknown because of the complexity of the effluent mixtures (Brooks, Riley and Taylor, 2006). Concentration, bioavailability, exposure time (duration and life stage), and environmental conditions play a role in how toxic contaminants may be to organisms (Prasse *et al.*, 2015). Additionally, temperature and hypoxic conditions that are out of the optimal limits of a given species are known to be physiological harmful for organisms. Being exposed to many factors that are physiologically exerting to an organism means the organism is exposed to 'multiple stressors'(Segner, Schmitt-Jansen and Sabater, 2014). When fish are living downstream from MWWTPs they may be exposed to multiple stressors, such as when eutrophication events occur creating hypoxic conditions (Santos *et al.*, 2008; Ansari, Gill and Khan, 2010; Water Quality Working Group, 2013). By measuring the uptake of these contaminants through biomarkers in fihs, a whole organism response is documented, taking into account all stressors faced by the fish (Segner, Schmitt-Jansen and Sabater, 2014).

1.2.2 Impacts of MWWTP Effluent on Fishes

Studies to date have examined the impact of effluents on various physiological endpoints of fish populations living downstream from MWWTPs. At the molecular level, mRNA levels for different genes (e.g. immunological, apoptotic, metabolic) are altered at downstream locations (Arstikaitis, Gagné and Cyr, 2014; Marjan *et al.*, 2017). Decreases in diversity or a species shift of dominant fish populations are recorded (Tsai, 1968, 1973; Porter and Janz, 2003). Other recorded effects include impacts on condition factor/growth rates, feeding rates, behaviour, osmoregulation, reproduction, metabolic rate and reduced gill integrity (Hui-Peng Lin *et al.*, 1992; Rowe and Dean, 1998; Porter and Janz, 2003; Lowe, Morrison and Taylor, 2015).

More specifically, CECs can affect non-target organisms, through the same mode of action as in humans/pets or have a different, toxic effect. For example, fluoxetine, an SSRI, was found to alter stress hormones in the brains in Japanese Medaka (Brooks, Riley and Taylor, 2006). The contaminants of major concern in the Grand River are largely the EDCs (Tetreault *et al.*, 2011; Arlos *et al.*, 2015, 2018; Bahamonde *et al.*, 2015). Some of these pharmaceuticals are known, from separate studies, to individually have effects on the fish populations downstream. EDCs in low levels over a chronic exposure period, can cause feminization of male fish, changes in vitellogenin (egg yolk mRNA and protein) levels, and disruption to gonad and egg formation (Liney *et al.*, 2006; Kidd *et al.*, 2007). It was demonstrated in spottail shiners that EDCs act over time, bioaccumulating and then disrupting the function of gap junctions during spermatogenesis, largely altering the reproductive ability of the fish (Marcogliese *et al.*, 2015). Rainbow darters downstream from the MWWTP in the Grand River have experienced changes in gene expression, skewed sex ratios, an altered gonad size and intersex or feminized fish populations are found, largely due to the presence of EDCs (Hicks, 2017; Arlos *et al.*, 2018). The overall implications for being exposed

to these contaminant cocktails on behaviour (reproduction, predation, competition) are difficult to predict. The effects of effluent on fish populations living downstream is detrimental and there is a need for physiological monitoring throughout the year to gain a better picture of overall fish health and how it relates to effluent composition and discharge. With increasing temperatures, turbidity and eutrophication events due to climate change, the ability of the fish to survive in effluent contaminated waters will become increasingly difficult if no action is taken by MWWTPs (Whitehead *et al.*, 2009; Paerl, Hall and Calandrino, 2011).

1.2.3 Darters and the Rainbow Darter as a Sentinel Species

The Grand River is habitat to 43 different fish species, all with differing physiology and tolerance to changing water conditions (Mandrak *et al.*, 2010). In previous studies, the rainbow darter (RBD; *Etheostoma caeruleum)* has been used as the sentinel species in the Grand River due to its abundance, small movement patterns and a sensitivity to pollution (Tetreault *et al.*, 2011; Mehdi *et al.*, 2018). Rainbow darters were found to have a high degree of intersex in males along with skewed sex ratios where females were more prominent downstream from MWWTPs, evidently being affected by effluent discharge in the Grand River (Bahamonde *et al.*, 2015). Fish are attracted to and choose to live in these downstream locations because there is an increase in nutrients, which allows for increased energy towards growth and reproduction, as demonstrated by increased body condition downstream from WWTPs (Tetreault *et al.*, 2011; Fuzzen *et al.*, 2016; McCallum *et al.*, 2019). In a study done prior to the upgrades, darters were less abundant downstream from the MWWTP compared to non-urbanized areas, likely due to being sensitive to pollution making them an ideal candidate to study effects of effluent exposure (Tetreault *et al.*, 2011).

In this study, two additional darter species were selected (fantail darters, FTD, *Etheostoma flabellare* and greenside darters, GSD, *Etheostoma blennioides*), to gain a broader understanding of how the species differ in ability to cope with environmental stressors. The three darter species chosen for this study are found in the Grand River, each of which have slightly differing life histories, occupying slightly different ecological niches. Using similar species to measure effluent effects will give insight on how fish vary in their tolerances, even when closely related. Darters are perch-like fish in the family Percidae (Mayden, Page and Burr, 1992). The genus is largely restricted to bodies of water that have a high oxygen level and low temperatures and are found to have relatively low metabolic rates compared to cyprinids and salmonids (Ultsch, Boschung and Ross, 1978). Darters are short lived species, 1+ year darters breed from April to June, putting a lot of resources towards reproduction efforts annually with efforts towards reproduction reduced over winter (Miller, 1968; Paine, 1990). They are small bodied fishes that prey on small invertebrates (midges, caddisfly, mayfly) and small fish eggs during the day, and are prey themselves to larger fishes as well as hosts to many parasites, positioning themselves as key species in the food web (Crane *et al.*, 2011). The FTD and RBD occupy silty/rocky areas, the FTD able to fit into smaller locations than the RBD; GSD prefer larger rocks with algal growth. The low metabolic rate of darters gives them an advantage in that they have a lower energy requirement and therefore need less food than larger or more active fishes (Ultsch, Boschung and Ross, 1978). The major differences between these species, that allow them to live in the same area are differences in body size and head, jaw, and fin shape as well as reproductive behaviour; these characteristics vary the animals feeding ecology and habitat use which allows the darter species to coexist (Paine, 1990; Carlson, Wainwright and Near, 2009). Darters also have slightly varying thermal preferences and will take up different locations in the stream bed; temperature preferences restrict where the darters

can physiologically survive with CTmax of FTD, RBD and then GSD being highest to lowest. CTmax is the temperature at which equilibrium is lost and is indicative of their ability to tolerate high temperature and variable waters (Hlohowskyj and Wissing, 1985).

The three species were chosen for two main reasons, the RBD is a sentinel species that has been studied in the past in this river due to its high site fidelity and abundance (Hicks and Servos, 2017). The GSD and FTD are closely related, abundant species and have not been extensively studied with respect to WWTP effluent. Previous studies comparing body size to home range in rivers indicates that the small bodied FTD and GSD likely also have a small home range in which they move (<200 m), with the GSD potentially having a larger range due to its larger body size (Minns, 2011). High site fidelity means that fish that are found at a site have likely been in that area their whole life and are chronically exposed to the conditions (Munkittrick and Dixon, 1989; Minns, 2011). The aim is to compare between three species that are living in the same river but may be coping and surviving adverse conditions using different adaptations or strategies. Looking at more than one species is important in determining if one species is outcompeting or has a broader phenotypic response to the conditions than the others, and if one species is focused on, there may be missing information on what is occurring to other fish living in the river.

1.3.1 Metabolic Rate

Metabolism is the integration and exchange of energy between an organism and the environment, using a multitude of biochemical reactions and catalysts. Organisms allocate energy and resources towards survival and excess energy to growth and reproduction processes to increase fitness (see Figure 1.1). When an organism detects a stressor (e.g. contaminant) the general adaptation response occurs where alarm, resistance and exhaustion phases may progress. If an organism cannot move, adapt or does not have the plasticity within its physiology, behavior,

morphology or biochemistry to adjust it may become exhausted and have no fitness or die. As an organism progresses through this cycle, the aerobic scope is consumed for upregulating processes to allow for survival. If an organism's aerobic demand surpasses its maximum output than it will not maintain life in this environment. There are five main environmental factors that contribute to changes in metabolic rate, described by Fry (1947): controlling, limiting, masking, lethal and directive (Claireaux and Chabot, 2016). Temperature and mass are controlling factors; oxygen and ammonia, limiting factors; salinity, a masking factor; pollutants, lethal factors and photoperiod, a directive factor. Each factor contributes to metabolic rate and needs to be considered or controlled to understand environmental effects and measure an organism's metabolic or aerobic scope (Claireaux and Lefrançois, 2007). In fish, aerobic scope (AS) is the difference between maximum metabolic rate (MMR- maximum oxygen uptake) and routine metabolic rate (RMR - minimum oxygen required for maintenance); it is a measure of external, environmental effects on fish and internal compensation and adjustment (Claireaux and Lefrançois, 2007). Using energy as a common currency to the environment allows measurement of how well an organism is adapting and evolving to live in its environment, even when suboptimal (Beyers *et al.*, 1999)

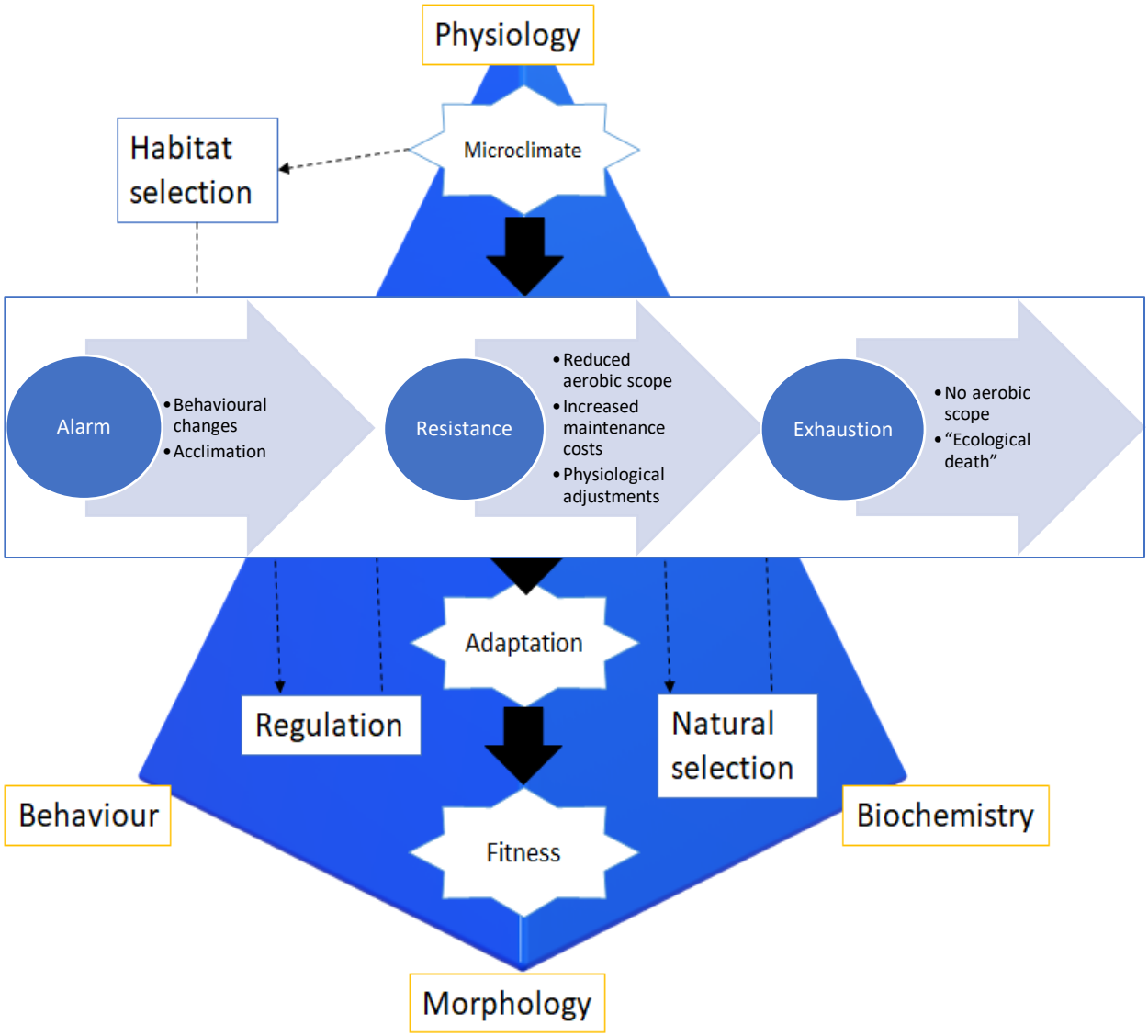


Figure 1.1 Diagram of adaptation to a stressor that an organism uses. With the combination of physiology, behaviour, morphology and biochemistry, an organism can respond and adjust to its environment and increase its fitness. If an organism cannot acclimate, it will become metabolically exhausted and have no fitness. Modified and combined from Beyers et al., (1999) and Gillooly, Brown, West, Savage, & Charnov (2001).

Metabolic rate follows a ¾ power rule, scaling allometrically to body mass, due to fractal branching patterns that limit uptake of resources (West, Brown and Enquist, 1997). Therefore, the larger an organism, the smaller metabolic rate by mass. Metabolism is also tied to temperature. Enzymatic activity increases with increasing temperatures and is expressed as a Q_{10} value, which is a measurement of the rate of change in enzymatic activity for every $10^{o}C$ increase in temperature. This relationship is described by Van't Hoff-Arrhenius equation and the Boltzmann factor, $e^{(-E/kT)}$, specifying how temperature affects rate of reaction (E is energy, k is Boltzmann's constant, T is temperature; (Brown *et al.*, 2004). Lastly, metabolism is tied to stoichiometry, where an organism is limited by the amount of material they can take in and by the required homeostatic functions, to maintain internal chemical composition such as for gradients, coenzymes, excreting waste (Brown *et al.*, 2004)

1.3.2 Respirometry

Respiration rate, in heterotrophs, is equal to metabolic rate because of the carbon oxidizing, energy producing reactions utilized during the citric acid cycle and oxidative phosphorylation. For example, glucose oxidation, the consumption of oxygen to make ATP:

$$C_6H_{12}O_6 + 6\ O_2 + 32\ ADP + 32\ P_i + \rightarrow 32\ ATP + 6\ CO_2 + 6\ H_2O$$

Measuring the oxygen consumption rate (MO_2) of an organism is a proxy measure of the metabolic rate of an organism, when accounting for body mass and temperature, because of oxygen's place as the final electron acceptor in oxidative phosphorylation (Nelson, 2016). Ideally, calorimetry would be used, to give a better measure of metabolic rate because it measures both anaerobic and aerobic metabolism but requires knowledge of a fish's diet and capturing heat loss. Respirometry is used as indirect calorimetry because the bulk metabolism of fish is aerobic and respirometry is much simpler and largely used in the literature (Chabot, McKenzie and Craig, 2016). RMR is the

minimum amount of energy required for physiological life and maintenance; major movement (swimming, food gathering, predator avoidance), growth and reproduction are additional up to the maximum amount of oxygen uptake, maximum metabolic rate (MMR). The difference between RMR and MMR is termed aerobic scope (AS). AS is intimately tied to the environment and can be impacted by perturbations to factors e.g. contaminants. There is evidence that pollutants can modulate aerobic scope of fish, many studies in the past have focused on the effects of hydrocarbons and pesticides (Beyers *et al.*, 1999; Davoodi and Claireaux, 2007; Goodchild, Frederich and Zeeman, 2015).

Measuring differences in AS to measure impacts or factors of living in a certain environment, such as MWWTP effluent, may give insight of how species are making trade-offs or acclimating to an environment that is non-ideal. For example, putting more energy towards detoxification and maintenance processes for survival and compensation, and therefore not having energy to put towards extra life activities (see Figure 1.2; Claireaux and Lefrançois, 2007). Alternatively, AS may decrease through a reduced ability to take up oxygen from the environment, potentially from damage to oxygen uptake organs like the gills (Lannig, Flores and Sokolova, 2006). Knowledge of how fish are living in these environments may be useful for conservation biology efforts.

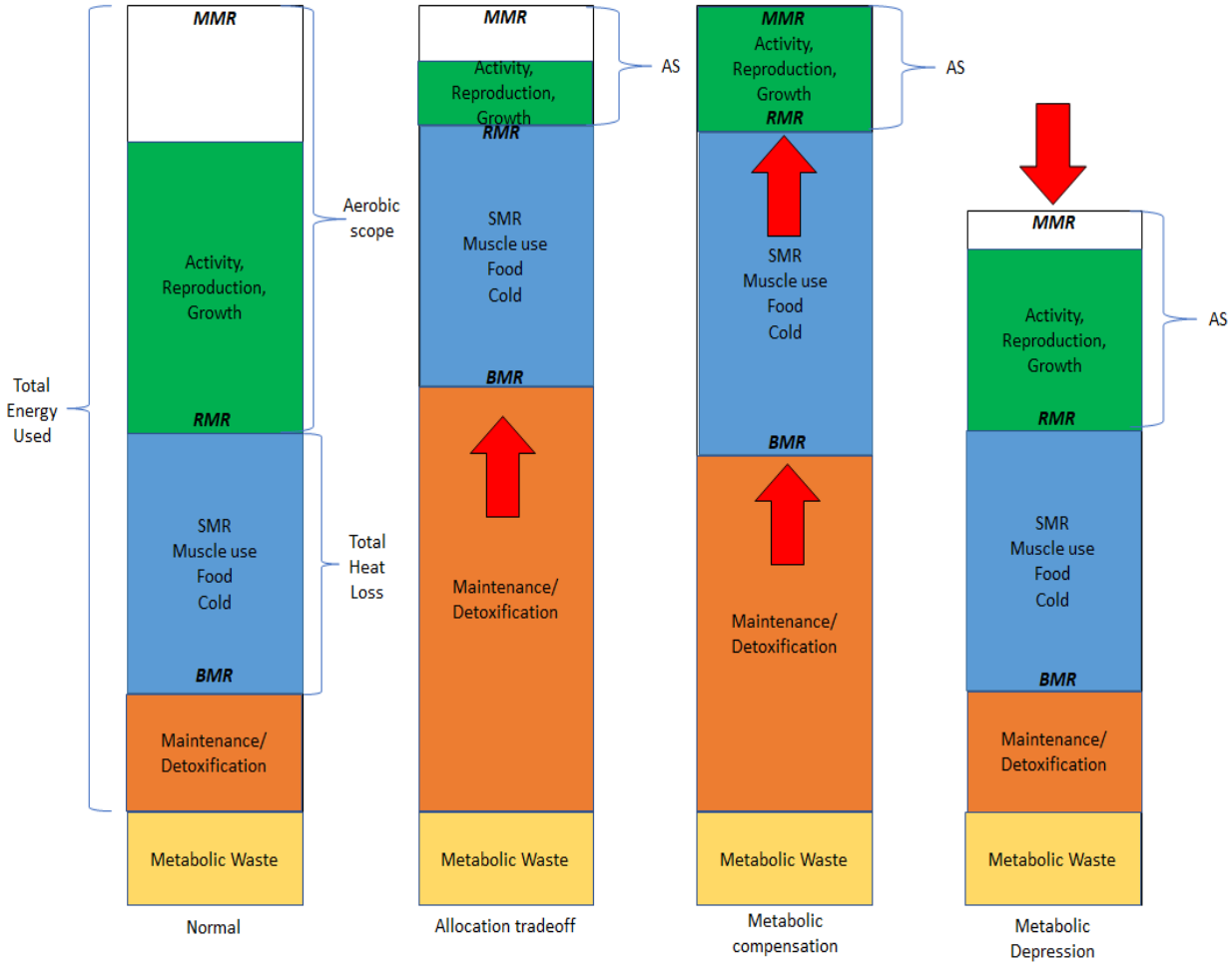


Figure 1.2 Examples of changes to aerobic scope due to modification of metabolism. The left bar indicates a default profile of AS (MMR-RMR). There is some basal level of waste production (yellow), detoxification & maintenance (orange) and environmental adaptation (blue). Extra energy can be allocated to extra-life activities (green). There is some head space for increased oxygen uptake for bouts of intense exercise (white). The second bar shows orange costs increasing and therefore decreasing AS. The third bar shows an increase in both orange and blue costs, AS is reduced and there is a loss of MMR. The fourth bar shows a decrease in the ability to utilize oxygen, also decreasing AS.

1.4.1 Gill Physiology

The gills are an ideal organ to study effluent effects as they control several homeostatic functions at the whole animal level and are at the interface between the internal and external environment of the organism. Major gill functions, focused on in this study, include respiration and osmoregulation. Respiration occurs across the functional surface area of the gill where water passes over gill filaments. Countercurrent exchange maximizes gas exchange on the secondary lamellae mainly over pavement cells (Evans, 1987). Osmoregulation in the gills occurs mostly by specialized, mitochondria rich cells via membrane bound pumps that utilize energy or gradients to maintain salt and water balance along with uptake from food sources (Evans, Piermarini and Choe, 2005). There is a significant metabolic cost to respiration (10-70%) and less so for osmoregulation (2-4%) that can increase when a fish needs to divert energy towards the essential processes (Evans, Piermarini and Choe, 2005).

The gill surface can be changed either by being restructured when undergoing osmorespiratory compromise or in poor water quality conditions to avoid becoming damaged, which can change the energetics of osmoregulation (Wendelaar bonga and Lock, 1991). Osmorespiratory compromise occurs when oxygen levels are low in the water, and the gill structure responds by increasing the surface area of the gill, allowing for increased oxygen uptake, but also comes with an increase in passive ion loss to the environment (Sardella and Brauner, 2007).

1.4.2 The Gills as a Model Organ

Freshwater fish are hypertonic compared to their environment, meaning they are passively losing ions and must use energy to osmoregulate ion stores. Osmoregulatory function of the gill is controlled by enzymatic membrane pumps, co-transporters, and exchangers on filament

epithelium, mostly in mitochondrial rich cells and pavement cells (Evans, 1987; Perry, 1997). The gills are a plastic organ that can be restructured to cope with osmoregulatory stress. Increasing the surface area of the gill available for gas exchange allows for faster oxygen uptake but also increases the concentration of ions being lost passively and therefore more energy must go towards regulating osmolarity. Studies on gill physiology have looked at the impacts of different environmental stressors by looking at the changes in enzyme activity and expression across the membrane (Evans, 1987; Santos *et al.*, 2008).

The gills are in direct contact with the water, so they are prone to becoming damaged due to contaminants or substances in the water - detecting damage to the gills serves as a sublethal indicator of contaminant effects (de la Torre, Salibián and Ferrari, 2007; Flores-Lopes and Thomaz, 2011). In the gills, effluent from MWWTP can cause damage to DNA, increase gill surface area and effect the ability to regulate waste (Bernet *et al.*, 2000; Liney *et al.*, 2006; Du *et al.*, 2018) Gill damage can cause changes in gill structure, often found in the form of epithelial lifting, hyperplasia/hypertrophy of the gill surface, fusion of secondary lamellae or gill aneurysms, all of which can be visualized using histological techniques (Farrell, Kennedy and Kolok, 2004; Camargo and Martinez, 2007). Damage to the gills is important to note because it can have effects on the key functions of the organ (respiration, osmoregulation, acid-base regulation and nitrogen excretion). Aforementioned gill damage and pathologies have been reported downstream from MWWTP outfalls and are a useful biomarker of primary damage due to contaminants (Corbett *et al.*, 2014). In this study, the gills are used to look for markers of sublethal damage due to MWWTP effluent and determine if upgrades have decreased the amount of damage and restructuring the gills undergo in this environment.

1.5.0 Objectives and Hypotheses

In summary, it is understood that there are contaminants released from the MWWTP and changes to the physicochemical properties of the water that can have impacts on the downstream fish populations. Studies tend to focus on the effects of few contaminants in a controlled laboratory environment to determine the effects on low levels of biological organization such as gene expression. However, information regarding the impact of effluent in the environment on higher levels of biological organization, such as metabolic and energetic capacities and gill function in these three darter species (*Etheostoma* spp.) is limited. Therefore, this thesis aims to:

1. Determine changes in metabolic demands of three darter species living in effluent contaminated waters of the Grand River by measuring respiration and metabolic endpoints and compare them to the same species from an upstream reference site. It is hypothesized that fish living downstream from the MWWTP will have increased energetic demand due to costs of detoxification and therefore will have a reduced aerobic scope compared to fish living at upstream control sites.
2. Measure the impacts of diluted MWWTP effluent on gill physiology (structure and function) across different species of darters. It is hypothesized that fish living in effluent will have impaired gill function due to damage and restructuring, meaning more energy is required to maintain respiratory and osmoregulatory physiology.

2.0.0 Field Metabolic Rates of Darters (*Etheostoma* spp.)

2.1.0 Introduction

Individuals vary in how tolerant they are to stress, depending upon their ability to endure and acclimate to conditions which are out of their optimal range (e.g. temperature extremes; Pörtner *et al.*, 2005). Some species can maintain homeostatic balance in unideal environments better than others, but not without consequence (Barton and Iwama, 1991). An organism will undergo a stress response to maintain physiological functions for survival, which includes altering metabolism via energy substrate mobilization due to the release of cortisol, for example, due to an increased energy demand of the gills for detoxification (Vijayan, Aluru and Leatherland, 2010). A valuable endpoint to measure effects of chronic exposure to contaminants is metabolic rate, partly due to this late role in the stress response and its indicator of overall animal health (Beyers *et al.*, 1999).

In the Grand River, contaminant inputs from municipal wastewater treatment plants (MWWTP) pollute the water and have effects on the fish populations living downstream from the effluent outfall. The effluent post-treatment contains pharmaceuticals and other contaminants of emerging concern that can cause sub-lethal effects (Overturf *et al.*, 2015; Park and Park, 2015). In past studies, effects on reproduction, growth, sex-ratios, metabolism, molecular signatures have all been identified as maladaptive phenotypes in downstream locations from MWWTP (Bahamonde *et al.*, 2015; Fuzzen *et al.*, 2016; Mehdi *et al.*, 2018). Fishes exposed to MWWTP effluent have shown evidence of undergoing stress responses. For example, rainbow trout (*Oncorhynchus mykiss)* exposed to diluted effluent had increased plasma cortisol levels and glucocorticoid receptor protein levels, and they struggle to mount a stress response when faced with additional challenges (Ings, Servos and Vijayan, 2011; Ings, Vijayan and Servos, 2012). In

the Grand River, female rainbow darters (RBD; *Etheostoma caeruleum*) were recorded to have an increased level of cortisol when exposed to MWWTP effluent (Mehdi *et al.*, 2018). The objective of this study is to measure metabolism and energetic endpoints of darters living downstream from the MWWTP after upgrades occurred to determine if they are displaying increased metabolic demand due to living in the effluent contaminated environment.

2.1.1 Metabolism and Respirometry

Metabolism is the sum of catabolic and anabolic reactions occurring in the body and is a measure of how much energy storage and demand an organism has. It is a sensitive measure that interacts intimately with the environment and can be directly impacted by toxicant uptake and nutrient availability (Jørgensen, Enberg and Mangel, 2016). For example, when metabolism increases due to higher temperatures, the toxicity of a contaminant may increase due to increased enzymatic and chemical reactions (Fischer, Pomati and Eggen, 2013). Metabolism is a good measure of the whole organism response to stressors and can give indication of population level responses (Farrell, 2016). However, it is understudied compared to earlier 'primary' levels of the stress response such as measurements of corticosteroids and hormones (Barton and Iwama, 1991).

Routine metabolic rate (RMR) is the amount of energy needed for basal life processes and is measured by determining the oxygen uptake of fish in a fasted, basal metabolic state. The RMR of Rainbow Darters (*Etheostoma caeruleum*) and Brown Bullheads (*Ameiurus nebulosus*) downstream of MWWTPs in the Grand River was found to be increased compared to fish upstream of the MWWTPs, indicating a higher energy demand in these effluent contaminated locations (Leadley *et al.*, 2016; Mehdi *et al.*, 2018). Just one study has looked at the metabolic rate of *Etheostoma* spp. in the Grand River in the past, and this was before MWWTP upgrades (Mehdi et al., 2018). The RMR of the different species can be compared to their upstream counterparts to

determine to what extent respiration, and therefore metabolic demand, is affected by wastewater effluent. Field metabolic rate (FMR) is higher than routine metabolic rate due to unknown factors of field caught animals, such as specific dynamic action (feeding), however it does provide a useful estimate of metabolic rate of the animal in day to day life (Rolfe and Brown, 1997). The difference between FMR and maximum metabolic rate (MMR; maximum oxygen uptake of an animal) allows for calculation the amount of energy available for extra-life activities, referred to as aerobic scope (AS). Many studies fail to measure MMR (measure only RMR or FMR), when considering impacts of contaminants on metabolism, which could be missing some of the sub-lethal effects on fitness (Auer *et al.*, 2015). Creating models on the aerobic scope of the species can be useful for monitoring fish populations (Clark, Sandblom and Jutfelt, 2013; Rogers *et al.*, 2016). Respirometry testing can be useful for biomonitoring as a sub-lethal indicator of individual organisms and extrapolate to ecosystem health, which can give watershed managers a better understanding of the effects of contaminant inputs (Vaquer-Sunyer and Duarte, 2008).

2.1.2 Darter Ecology and Metabolism

The Grand River has a high population of *Etheostoma* spp., and the focus of many studies in the past has been the RBD, due to its high site fidelity and abundance (Hicks and Servos, 2017). The greenside darter (GSD; *Etheostoma blennioides*) and the fantail darter (FTD; *Etheostoma flabellare*) are also found in the Grand River, living in slightly different habitats than RBD. Although there are 43 recorded different species of fish in the Grand River, darters make up 75-90% of the fish in the Grand River (Loomer and Cooke, 2011; Hicks, 2017). Each of the darters in this study have different life histories and sensitivities to changes in water dynamics (i.e. hydrology, nutrient content, turbidity; Harding et al., 1998). GSD are the most mobile, switching between exposed and non-contaminated sites, whereas FTD move in response to habitat change,

but RBD tend to stay in the same location and are constantly exposed to the effluent; these differences in movement controls how much effluent they are exposed to (Roberts and Angermeier, 2007; Brown *et al.*, 2011; Hicks and Servos, 2017). Carbon and nitrogen stable isotope analyses has shown that RBD and GSD utilize different energy resources downstream from a treatment plant compared to upstream and RBD may have a more competitive advantage at downstream sites (Brown *et al.*, 2011). Additionally, stable isotope analyses has provided evidence that GSD may move in and out of the plume for feeding (Robinson *et al.*, 2016). The three different darter species have differing levels of tolerance to changing oxygen levels and thermal tolerance meaning not all species will be impacted the same way by the changes in water conditions (Hlohowskyj and Wissing, 1985, 1987). The RBD has been found to have a high degree of intersex downstream from MWWTP in the Grand River and is described as a pollution sensitive species, making it a useful sentinel species in the Grand River (Tetreault *et al.*, 2011). GSD has been described as a sensitive species compared to RBD and has also been recorded to display intersex males downstream from the MWWTP with recovery further downstream (Tetreault *et al.*, 2011). The FTD has been grouped with the RBD in terms of habitat preference and is more closely related to RBD than GSD (Hlohowskyj and Wissing, 1987)

2.1.3 Objectives and Hypothesis

The objective of this study is to measure the water quality downstream from the MWWTP in summer and fall of 2019 in the Grand River and determine effects on metabolism and fish health. Metabolic rate and body measurements of three darters (*Etheostoma* spp.) found in the Grand River are used to determine 1) the effects of MWWTP effluent on length, weight and body condition and 2) the effects of the MWWTP on FMR, MMR and AS of each species. Body measurements at sites downstream from the MWWTP are predicted to be increased due to increased nutrient levels. It is

hypothesized that fish exposed to MWWTP effluent in the river will have increased FMR and/or decreased MMR compared to reference sites and therefore have a decreased AS, due to contaminant exposure. More generally, it is predicted that the least resilient species, will have the largest change in AS.

2.2.0 Materials and Methods

2.2.1 Site Descriptions and Fish Collection

In July 2019 (summer 2019) and October 2019 (fall 2019), samples of the three darter species were collected from sites surrounding the Waterloo MWWTP (WMR, KIW, EIT and FWY) to capture seasonal differences in darter responses (Figure 2.1). WMR and KIW are upstream from the Waterloo MWWTP. WMR (43°35′07.54″N 80°28′54.08″W) is located upstream from urban development and is an agriculturally dominated area, KIW (43°30′17.41″N 80°28′28.61″W) is in an urban area. EIT (43°28′24.69″N 80°28′23.99″W) is immediately downstream from the MWWTP. FWY (43°26'40.2"N 80°24'02.7"W) is approximately 10.5 km downstream from EIT and is also in an urban area. At least 14 random samples of each fish species (RBD, GSD, FTD; Figure 2.1) were collected in the morning (starting at 9 A.M.) using a backpack electro-fisher and dip nets then placed into buckets that were aerated and kept at river temperature. Animal collections were approved by the Animal Care Committee at the University of Waterloo under AUPP#40318. Fish smaller than 4.0 cm were not included, as they were considered immature.

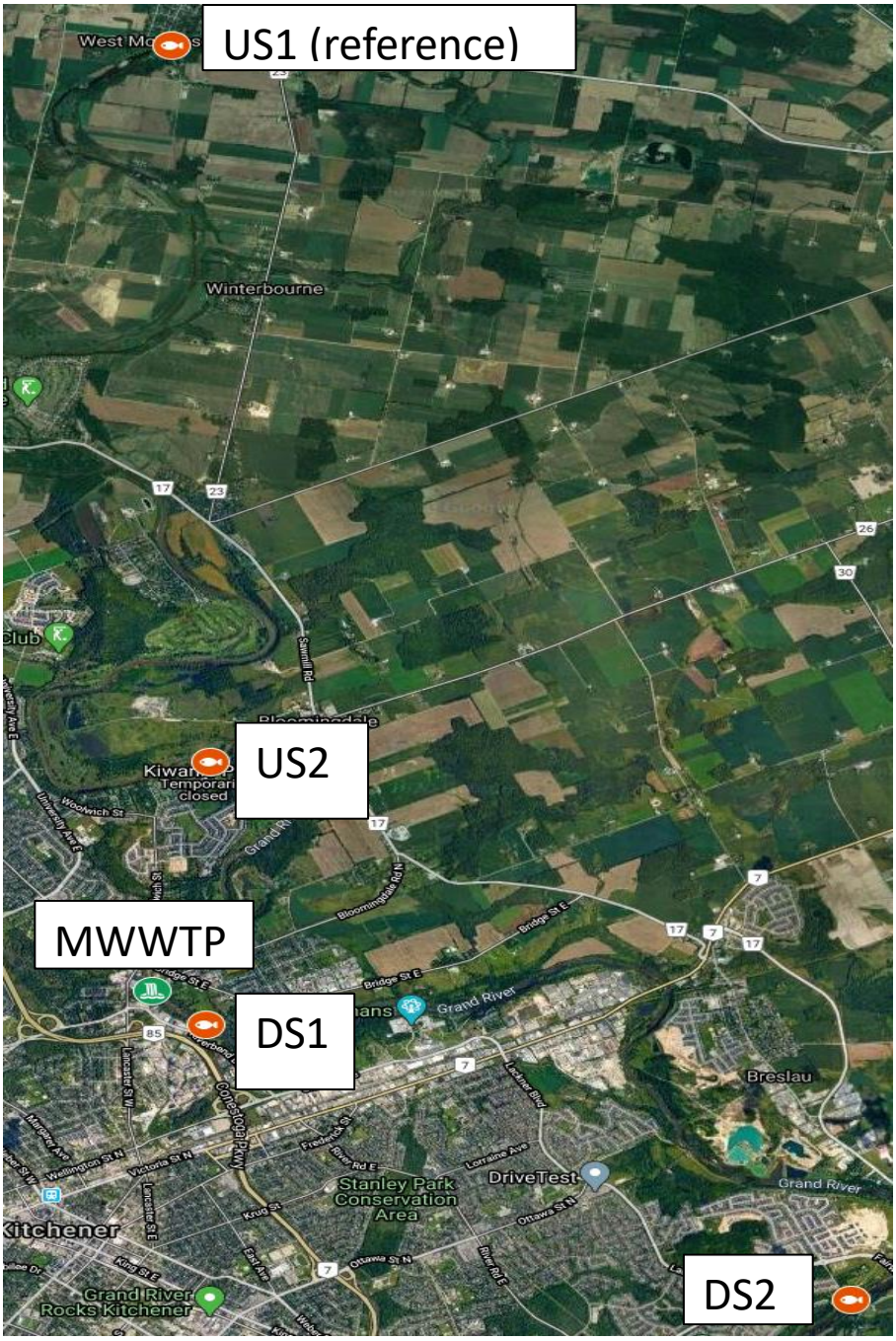


Figure 2.1 Map of sampling locations used for collection of darters for this study. Orange markers indicate general location of capture, green indicates the general location of the effluent outfall (Google MyMaps; Map data ©2020, CNES/Airbus, First Base Solutions, Landsat/Copernicus, Maxar Technologies)

Fish were first used for respirometry trials (detailed below). Following respirometry, fish were measured for total length (±0.1 cm) and weight (±0.01 g) and sacrificed by spinal severance. Body condition (k) was calculated using $k=m/(l^3)$ x100, where m is the mass of fish in grams and l is the length in cm. Fish were then dissected immediately on site for gill samples. The second and third gill arch were extracted from both sides, half going into Davidson's solution (formaldehyde 20%, ethanol 30%, glycerol 10%, acetic acid 10%, water 30%) for histological processing and half snap frozen in liquid nitrogen for enzymatic and molecular studies.

Figure 2.2 The three species of darters (*Etheostoma* spp.) collected. Greenside darters (GSD), rainbow darters (RBD), and fantail darters (FTD) are all common perch-like (Percidae) fish found in the Grand River. The colours displayed by darters are sex-specific and change seasonally (photos by R. Hodgson).

2.2.2 Water Quality

At each site, water quality measurements were taken with a YSI proplus multimeter (YSI Incorporated, OH, USA). In August 2019, grab samples of river water were collected in 125 mL amber glass bottles for analysis of nutrients and pharmaceuticals and general contaminants, sent to the Servos Lab (Waterloo, ON); using LC-MS techniques previously described by Fuzzen *et al.* (2016). Samples of river water (250mL) were preserved using 1mL 49% sulfuric acid and measured for concentrations of total ammonia, nitrite, and nitrate by Maxxam Analytics (Mississauga, ON). In October 2019, samples for pharmaceutical, general contaminants and nutrient analyses were taken but issues with instrumentation delayed results of pharmaceutical/contaminant concentrations. Nutrient concentrations were not taken in summer 2019. The collection of water sample data (pharmaceuticals, physicochemical, nitrogenous compounds) are used to show that effluent and river conditions change seasonally and are not directly linked to biological changes.

2.2.3 Metabolic Rate and Respirometry

Intermittent flow respirometry was used to quantify FMR and MMR in the field following capture. Fish were placed in a system each within a chamber with a volume of 65 mL, that was initially open to allow circulation of water from the reservoir to prevent hypoxia throughout acclimation. Eight chambers, one reserved for background respiration, were used to measure seven randomized fish at a time equally over the day, to minimize diurnal effects during measurements (i.e. 2 FTD:2 GSD:3 RBD, then changing proportions). Three phases of flow occur, the flush, wait and measurement phase (Rosewarne, Wilson and Svendsen, 2016). River water was used for recirculation using a pump to flow water directly from the river to the reservoir and an open loop pump to move water through the chambers and back into the reservoir, a modified set up similar

to that found in Mochnacz *et al.* (2017). When the open loop pump was turned off, each chamber maintained its flow by a closed loop pump to mix water and measure oxygen via a fibre optic oxygen probe for continuous measurements (Mochnacz *et al.*, 2017).

Temperature and oxygen levels were measured throughout the experiment and MO_2 measurements adjusted based on temperature and calibration of the probes. Average temperature of each run was used to correct measurements of FMR and MMR to 20°C for comparison using: $B/M\ 20°C = B/Mte\ (1/20\text{-}1/t)$, where t is body (water) temperature and e (-5.02) is the slope of the line for fish for the equation comparing the effects of temperature to mass normalized metabolic rate (Gillooly *et al.*, 2001). Trials occurred starting at 9:30 A.M until approximately 3 P.M. each day, spreading out the use of each species through the day to minimize effects of diurnal functions. The fish were acclimated in chambers for 60 minutes with fresh, oxygenated river water, because no change in FMR was observed after acclimation after 60 minutes. FMR was then measured for 10 minutes after the open loop pump was turned off, with measurements every second (modified from Mehdi *et al.*, 2018).

As the species we will be collecting are benthic, maximum metabolic rate (MMR) is measured differently than for aerobically active fish (McBryan *et al.*, 2013). The MMR is measured by creating an oxygen debt by holding fish out of water for 2 minutes and immediately placing fish into the respirometer to measure oxygen consumption where the initial decline in O_2 is MMR. This is modified from the chase protocol due to the fish not reacting to being chased (Roche *et al.*, 2013).

2.2.4 Statistics

Data was analyzed using the statistical software GraphPad Prism 8 (San Diego, California, USA). Sex differences were calculated for body measurements but not for metabolic rate due to low n numbers. Figures present the data as means ± standard error of the mean (SEM). Each species-site group was compared using two-way analysis of variance (two-way ANOVA). The data were tested for equal variance and normality with Levine's test and the Kolmogorov–Smirnov test, respectively, to determine if they met the assumptions of an ANOVA. Dunnett's multiple comparisons test was used to compare each species-site group to the reference site (WMR) of the respective species to limit comparisons to a reference site largely unaffected by urbanization. Alpha was set to 0.05 for all tests, where significant differences were any p-values less than this.

2.3.0 Results

2.3.1 Water Quality

Water quality measurements in the Grand River are recorded in Table 2. Means ± SEM were calculated from three measurements at each site (Table 2.1, 2.2; Figure 2.2). Missing values are due to probe calibration issues on that day. In summer 2019, there was usually an increase in the values at EIT, when compared to WMR which sometimes continued downstream. In the fall, KIW had more changes in conditions compared to WMR (Table 2.1). Temperature measurements usually varied based on daily temperature fluctuations. In fall 2019, water samples were analyzed for ammonia, nitrite, nitrate, conductivity and chloride concentrations (see Table 2.2). Values increase downstream from the MWWTP. Most importantly, there are still detectable levels of ammonia ,0.10 mg/, at EIT and FWY.

In summer 2019, water samples were analysed for key contaminants at the four sites surrounding the Waterloo MWWTP (Figure 2.2). There was a significant increase in diclofenac,

atrazine, carbamazepine, venlafaxine, sulfamethoxazole, caffeine, 11,12-epoxide carbamazepine, p-hydroxy atorvastatin, o-hydroxy atorvastatin and desmethyl venlafaxine at EIT when compared to WMR (Dunnett's multiple comparison's, $p<0.05$). Diclofenac, atrazine, carbamazepine, venlafaxine, desmethyl venlafaxine persisted downstream at FWY and were significantly increased compared to WMR (Dunnett's multiple comparison's, $p<0.05$). Atrazine was significantly increased at KIW compared to WMR (Dunnett's multiple comparison's, $p<0.05$).

Table 2.1 A summary of the water conditions during sampling of fishes. Data are presented as mean ± SEM (n=3 per site). Water quality measurements were taken on the same day as sampling at that site where fish were collected from. TDS and pH in summer 2019 and DO (mg/L) measurements from fall 2019 are unavailable due to instrument calibration issues on day of sampling. *Bolded text indicates a significant difference in concentration compared to WMR (Dunnett's multiple comparison's, $p<0.05$).

Season	Site	Temp (ºC)	DO (mg/L)	Avg. Cond (uS/cm)	TDS (ppm)	pH	Turbidity (NTUs)
Summer 2019	WMR	23.47 ± 0.03	7.39 ± 0.03	384.70 ± 0.26	275.60 ± 0.00	8.37 ± 0.03	5.07 ± 0.28
	KIW	23.035 ± 0.03	***8.43 ± 0.09**	422.83 ± 0.41	-	-	4.75 ± 0.66
	EIT	24.52 ± 0.03	***12.86 ± 0.20**	***627.33 ± 27.88**	***416.00 ±18.39**	8.39 ± 0.00	4.68 ± 0.56
	FWY	25.27 ± 0.07	***10.10 ± 0.18**	***512.33 ± 4.84**	***346.67 ± 3.54**	**8.56 ± 0.04**	4.35 ± 0.21
Fall 2019	WMR	9.10 ± 0.19	-	268.10 ± 1.08	251.02 ± 0.49	8.5 ± 0.0	3.34 ± 0.48
	KIW	***11.53 ± 0.07**	-	***373.07 ± 0.40**	***326.52 ± 0.47**	8.46 ± 0.0	6.90 ± 0.76
	EIT	9.20 ± 0.05	-	***479.57 ± 7.17**	***446.12 ± 6.80**	***7.48 ± 0.1**	5.43 ± 0.23
	FWY	9.07 ± 0.05	-	***351.77 ± 2.03**	295.45 ± 27.23	8.33 ± 0.0	3.50 ± 0.14

Table 2.2 Summary of total ammonia, nitrite, nitrate, and chloride concentrations at sites where fish were collected during this study in fall 2019. Data are presented as mean ± SEM (n=3 per site). Concentrations were only available from the fall 2019 sampling period. The values show that there are increased, measurable amounts of nitrogenous compounds and chloride downstream from the MWWTP. *Bolded text indicates a significant difference in concentration compared to WMR (Dunnett's multiple comparison's, $p<0.05$).

Site Fall 2019	Ammonia (mg/L)	Nitrite (mg/L)	Nitrate (mg/L)	Chloride (mg/L)
WMR	0.00 ± 0.00	0.01 ± 0.00	1.56 ± 0.10	27.66 ± 1.30
KIW	0.00 ± 0.00	0.03 ± 0.00	***3.94 ± 0.00**	54.01 ± 0.30
EIT	***0.10 ± 0.00**	***0.08 ± 0.00**	***6.50 ± 1.10**	***77.66 ± 3.60**
FWY	***0.10 ± 0.00**	***0.07 ± 0.00**	***3.69 ± 1.00**	46.29 ± 3.60

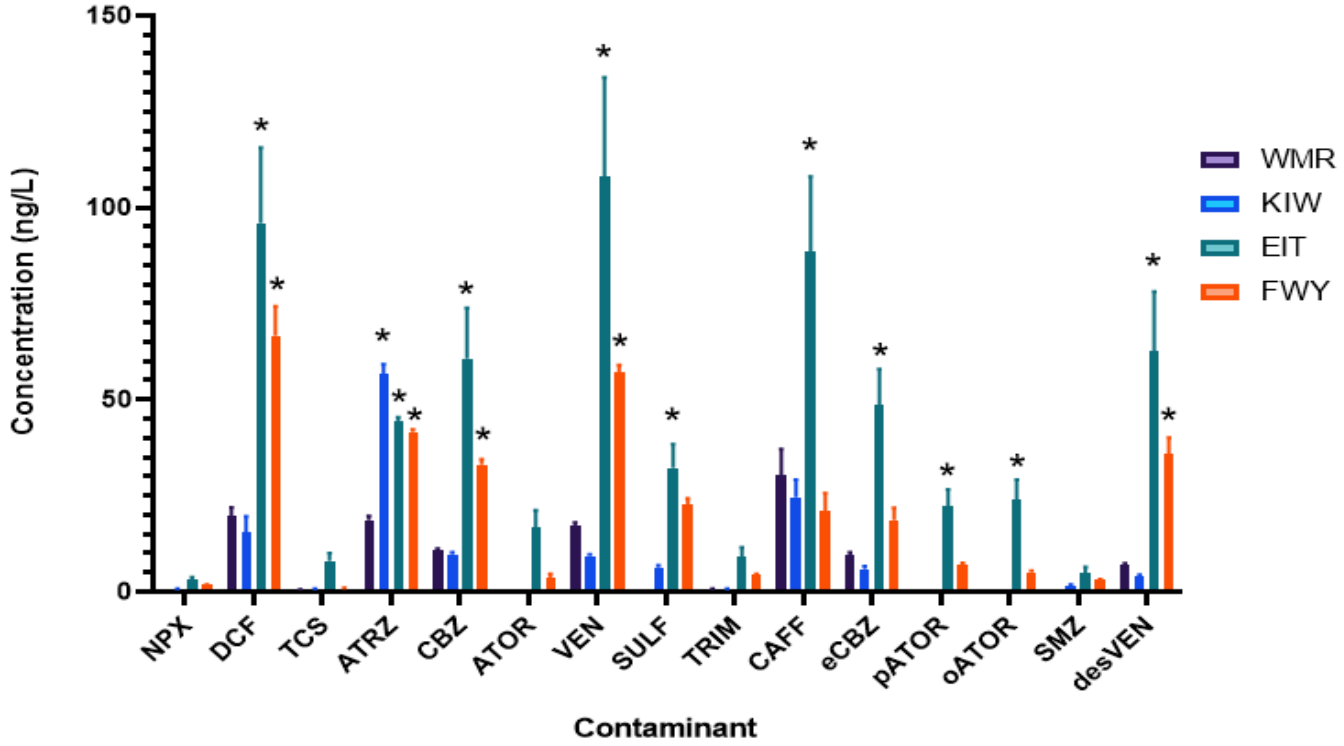


Figure 2.3 Pharmaceutical and contaminants measured in the Grand River in summer 2019. Data are presented as mean ± SEM (n=3 per site). Concentrations (ng/L) of many pharmaceuticals and personal care products are detectable throughout the watershed but usually increase downstream from the MWWTP. Short hands on x axis from left to right: naproxen, diclofenac, triclosan, atrazine, carbamazepine, atorvastatin, venlafaxine, sulfamethoxazole, trimethoprim, caffeine, 11,12-epoxide carbamazepine, p-hydroxy atorvastatin, o-hydroxy atorvastatin, sulfamethazine, des-methyl venlafaxine. An asterisk indicates a significant difference in concentration compared to WMR (Dunnett's multiple comparison's, $p<0.05$)

2.3.2 Length, Mass and Body Condition

Length, mass and body condition are measurements of energy storage and fish health (for summary, see Table 2.3). In fall 2019, no female GSD were collected in random sampling and the mean and SEM were assumed to be the same as the reference site for performing the post-hoc statistical tests. All data passed Levine's test and Kolmogorov–Smirnov test ($p>0.05$).

In summer 2019, there was a significant effect of site on variation (15.17%) in RBD length (Two-way ANOVA, $F(3,52) = 3.329$, $p=0.0265$). There were no significant differences in RBD length in any sex at any site, compared to WMR. FTD length was not significantly affected by sex or site and displayed no significant changes in length in any season at any site. GSD variation in length was explained 8.328% by sex and 17.12% by site (Two-way ANOVA, $F_{sex}(1, 57) = 8.204$, $p=0.0058$; Two-way ANOVA, $F_{site}(3, 57) = 5.623$, $p=0.0019$). There was a significant increase in length of female GSD at EIT in summer 2019 (Dunnett's multiple comparisons, $p<0.0001$).

There was a significant effect of site on the variation seen in RBD mass (8.203%; Two-way ANOVA, $F_{site}(1,52)=5.387$, $p=0.0242$). There were no differences between WMR and any site in RBD. There were no significant changes observed in FTD mass in summer 2019. There was a significant effect of sex (8.159%) and site (16.08%) on variation of mass in summer 2019, in GSD (Two-way ANOVA, $F_{sex}(1, 55) = 7.999$, $p=0.0065$; Two-way ANOVA, $F_{site}(3, 55) = 5.254$, $p=0.0029$). The mass of female GSD at EIT in summer 2019 was significantly larger than WMR (Dunnett's multiple comparisons, $p=0.0001$). There were minimal differences between body condition at sites, in each season. In summer 2019, there was a significant effect of sex on body condition in RBD which accounted for 7.28% of the variation observed (Two-way ANOVA, $F(1, 52) = 4.438$, $p=0.04$). There were no other significant differences in body condition in summer 2019 for any species.

Variation in length of RBD in fall 2019 was 11.39% explained by sex and 11.28% by site (Two-way ANOVA, F_{sex} (1, 48) = 8.625, p=0.0051; Two-way ANOVA, F_{site} (3, 48) = 2.848, p=0.0472). In fall 2019, there was a significant increase in length of male RBD (Dunnett's multiple comparisons, p=0.0062). FTD length was significantly affected by sex (20.44%; Two-way ANOVA, F(1,48)=12.79, p=0.0008). There were no differences in FTD or GSD length between any site compared to WMR, in either sex. RBD mass in fall 2019 was affected by sex (13.07%) and site (16.65%) (Two-way ANOVA, F_{sex} (1, 48) = 10.77, p=0.0019; Two-way ANOVA, F_{site} (3, 48) = 4.572, p=0.0068). Male RBD were heavier at both EIT and FWY, compared to WMR (Dunnett's multiple comparisons, p=0.0020; Dunnett's multiple comparisons, p=0.0270). FTD variation in mass was 17.23% explained by sex (Two-way ANOVA, F_{sex}(1,48)=10.23, p=0.0024). There were no differences in mass of FTD when compared to WMR. Lastly, GSD variation in mass was 13.01% explained due to site (Two-way ANOVA, F_{site}(1,56)=3.361, p=0.0250). Female GSD were smaller at KIW in fall 2019 (Dunnett's multiple comparisons, p=0.0310). RBD body condition had significant variation due to sex (12.6%) and site (17.3%) (Two-way ANOVA, F_{sex} (1, 48) = 9.543, p=0.0033; Two-way ANOVA, F_{site} (3, 48) = 4.367, p=0.0085). Female RBD had significantly increased k at EIT compared to WMR (Dunnett's multiple comparisons, p=0.0132). GSD and FTD did not show any differences in body condition in fall 2019.

Table 2.3 Summary table of length (cm), mass (g) and body condition (k = 100x(m/L^3), m= mass in grams, L = length in cm) of three darters species collected throughout the study. F is female, M is male. Data are presented as mean ± SEM. N numbers are presented with sex, in order from WMR to FWY. *Average values that are significantly different (Dunnett's multiple comparisons, $p<0.05$) than found at the reference site, WMR, are bolded.

Season	Species	Sex (n)	Length (cm)				Mass (g)				Body condition (*k*)			
			WMR	KIW	EIT	FWY	WMR	KIW	EIT	FWY	WMR	KIW	EIT	FWY
Summer 2019	RBD	F (7,7,8,8)	5.9 ±2.2	5.6 ±2.1	5.4 ±1.7	5.1 ±2.1	2.52 ±0.89	2.47 ±0.87	2.04 ±0.64	1.77 ±0.72	1.21 ±0.46	1.32 ±0.49	1.26 ±0.40	1.23 ±0.50
		M (7,7,10,6)	5.96 ±2.3	5.99 ±2.2	5.79 ±2.05	5.44 ±1.9	3.03 ±1.07	2.97 ±1.05	2.75 ±0.97	2.14 ±0.76	1.40 ±0.53	1.33 ±0.50	1.40 ± 0.50	1.27 ±0.45
	FTD	F (11,9,8,4)	5.7 ±2.8	5.7 ±2.5	5.7 ±1.5	5.3 ±1.6	1.80 ±0.80	2.02 ±0.82	2.20 ±0.59	1.61 ±0.51	0.98 ±0.49	1.08 ±0.48	1.22 ±0.33	1.09 ±0.33
		M (4,5,14,11)	5.9 ±1.8	5.5 ±1.8	5.8 ±2.1	6.1 ±3.1	1.92 ±0.58	1.82 ±0.57	2.04 ±0.72	2.45 ±1.23	0.95 ±0.29	1.07 ±0.36	1.01 ±0.36	1.07 ±0.54
	GSD	F (6,6,12,9)	6.9 ±2.4	7.0 ±2.9	***8.0 ±2.2**	7.3 ±3.3	4.16 ±1.38	4.00 ±1.41	***5.89 ±1.70**	4.68 ±2.09	1.14 ±0.40	1.12 ±0.46	1.14 ±0.33	1.19 ±0.53
		M (8,6,13,5)	7.7 ±3.1	7.3 ±3.0	7.6 ±2.2	8.1 ±2.69	5.47 ±2.07	4.57 ±1.62	5.05 ±1.52	4.68 ±2.12	1.20 ±0.5	1.1 ±0.46	1.13 ±0.33	1.21 ±0.40
Fall 2019	RBD	F (6,9,6,7)	5.2 ±2.1	5.1 ±1.7	5.2 ±2.1	5.08 ± 1.9	1.54 ±0.63	1.67 ±0.56	1.93 ±0.79	1.73 ±0.65	1.08 ±0.44	1.17 ±0.39	***1.27 ±0.52**	1.17 ±0.44
		M (8,5,8,7)	5.25 ±1.9	5.12 ±2.29	***6.26 ±2.2**	6.01 ±2.3	1.83 ±0.65	1.69 ±0.75	3.32 ±1.17	2.98 ±1.12	1.25 ±0.44	1.17 ±0.53	1.32 ±0.47	1.33 ±0.50
	FTD	F (5,4,7,6)	5.02 ±2.2	4.63 ±2.3	4.96 ±1.9	4.95 ±2.0	1.34 ±0.60	0.96 ±0.48	1.23 ±0.47	1.21 ±0.50	1.04 ±0.47	0.92 ±0.46	0.95 ±0.36	0.97 ±0.39
		M (9,10,7,8)	5.64 ±1.9	5.84 ± 1.9	5.70 ±2.2	5.46 ± 1.9	1.69 ±0.56	1.79 ±0.57	2.06 ±0.78	1.81 ±0.64	0.93 ±0.31	0.87 ±0.27	0.98 ±0.37	1.05 ±0.37
	GSD	F (8,3,N/A,2)	6.75 ± 2.4	5.43 ±3.14	N/A	5.65 ± 4.0	3.57 ±1.26	***1.58 ±0.91**	N/A	1.73 ±1.23	1.12 ±0.40	0.98 ±0.56	N/A	0.94 ±0.67
		M (6,11,14,12)	6.05 ±2.5	5.98 ±1.8	5.81 ±1.6	6.16 ±1.8	2.57 ±1.05	1.95 ±0.59	2.18 ±0.58	2.56 ±0.74	1.03 ±0.42	0.93 ±0.28	1.06 ±0.28	1.03 ±0.30

2.3.3 Respirometry

FMR and MMR of RBD, GSD and FTD were measured in summer and fall of 2019. AS is the difference between FMR and MMR. All data passed Levine's test and Kolmogorov–Smirnov test ($p>0.05$). There was no significant effect of sex on metabolic rate when data were separated, additionally, there were low sample numbers, so samples were pooled together for greater power (<4% of variance in all species). In summer 2019, RBD and FTD showed significant increases in AS downstream from the MWWTP (Figure 2.3). There were no significant changes in FMR, the increase observed was from increased MMR. Values are presented as means ± SEM.

There was a significant source of variation due to species and site (Two-Way ANOVA, $F_{species}$ (2, 137) = 6.62, p=0.0018, 7.87% variation; F_{site} (3, 134) = 3.819, p=0.0115, 6.804% variation). In RBD, there was a significant 2.2-fold increase of AS (mg O_2/kg/hr) at FWY compared to WMR (Dunnett's multiple comparisons test, p=0.0287, n = 14, 10, 10, 14). There were no other significant differences, but a trend of increased AS at EIT (2.1-fold; n.s.). RBD AS at KIW was 0.9-fold lower than at WMR. There were no significant differences in AS of GSD (n = 13,12,13,14). AS of GSDs was 1.2-fold higher at KIW, 1.0-fold at EIT and 1.6-fold at FWY. FTD (n = 11,11,11,13) at EIT had a significant 2.7-fold increase in AS compared to WMR (Dunnett's multiple comparisons test, p =0.0130). FTD AS increased 1.8-fold at KIW and 1.5 at FWY but were not significantly significant.

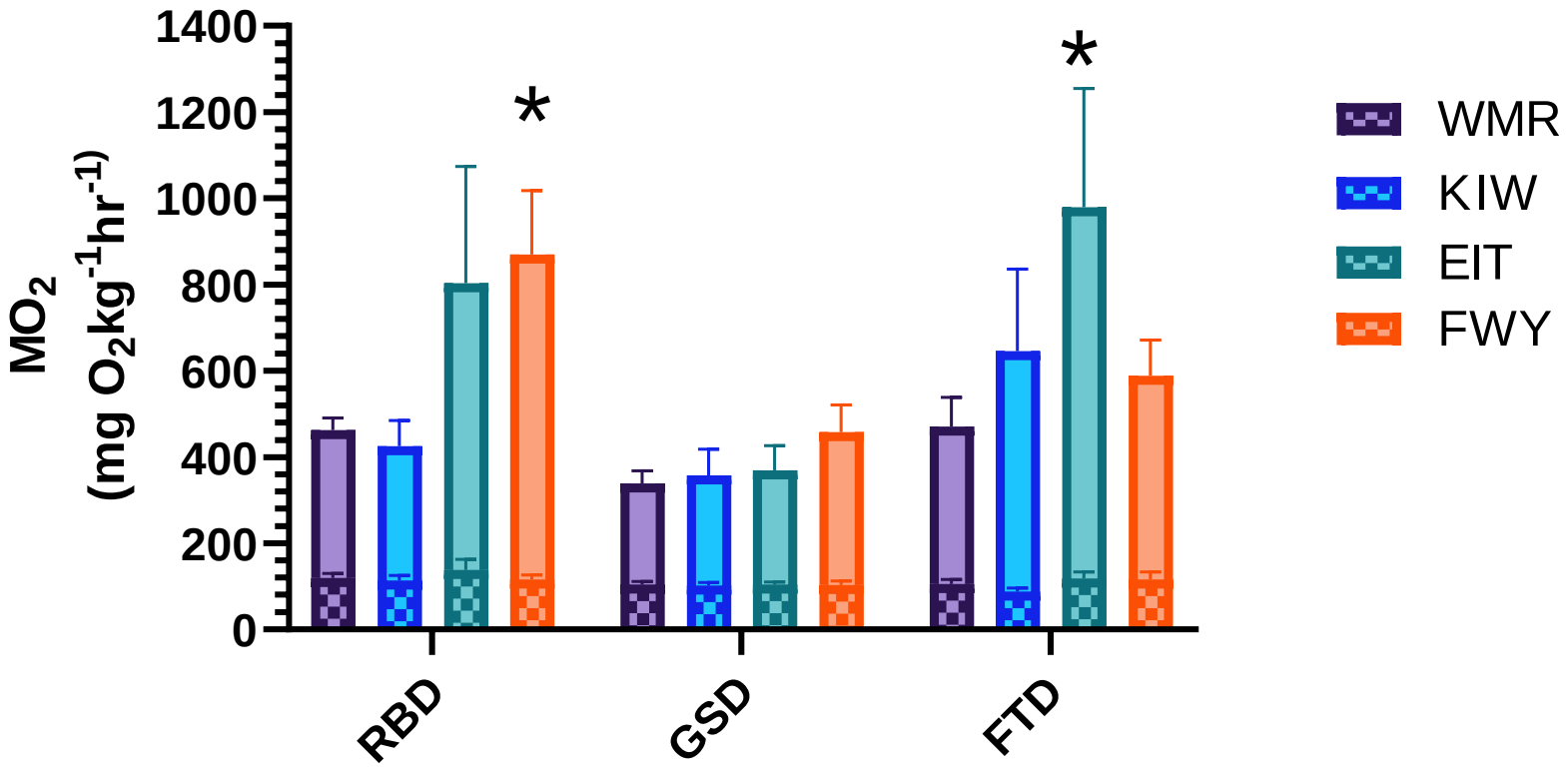


Figure 2.5 FMR and MMR of RBD, GSD and FTD in summer 2019. Data are represented as means ± SEM (MO2 - mg O2/kg/hr). AS is represented as the smooth area [MMR (entire bar) - FMR (checkered bar)]. Measurements are corrected to 20°C. The Waterloo MWWTP is between KIW and EIT. An asterisk indicates a significant difference ($p<0.05$) between WMR (reference site) and that location using a Dunnett's post hoc test.

In fall 2019 (Figure 2.4), there was no significant impact on variance from species or site (Two-Way ANOVA, $F_{species}(2, 144) = 0.2304$, p=0.7945, 0.305% variation; F_{site} (3, 144) = 0.7386, p=0.5307 1.468% variation. Non statistically significant fold changes in AS for RBD compared to WMR (n = 12,12,12,14) was 0.86-fold at KIW, 0.6-fold at EIT and 1.0-fold at FWY. GSD (n = 13,11,13,14) AS fold change compared to WMR were 1.4 at KIW, 1.1 at EIT and 0.92 at FWY, none of which were significantly different. FTD (n=14,14,14,13) AS (mg O_2/kg/hr) compared to WMR was 0.8-fold at KIW, 0.75-fold at EIT and 0.8-fold at FWY.

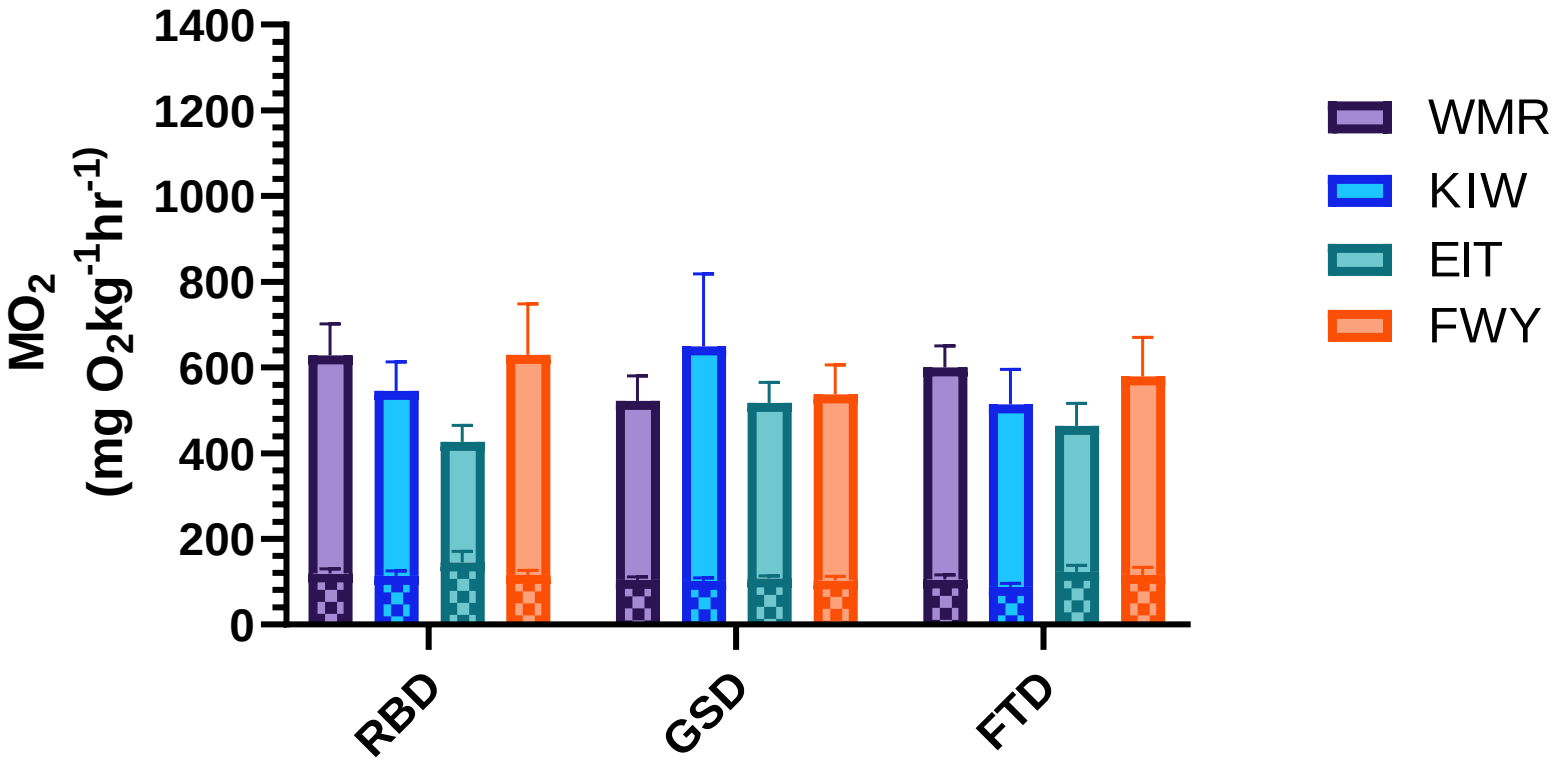


Figure 2.6 FMR and MMR of RBD, GSD and FTD in fall 2019. Data are represented as means ± SEM (MO2 - mg O2/kg/hr). AS is represented as the smooth area [MMR (entire bar) - FMR (checkered bar)]. Measurements are corrected to 20oC. The Waterloo MWWTP is between KIW and EIT.

2.4.0 Discussion

2.4.1 Water Quality

Collection of water samples downstream from the MWWTP in summer 2019 revealed diclofenac, atrazine, carbamazepine, venlafaxine, sulfamethoxazole, caffeine, 11,12-epoxide carbamazepine, p-hydroxy atorvastatin, o-hydroxy atorvastatin and desmethyl venlafaxine were present in higher concentrations than upstream (Fig 2.2). Of the highest contaminant concentrations detected, carbamazepine (antiepileptic), diclofenac (anti-inflammatory) and venlafaxine (antidepressant) are persistent pharmaceuticals that are not easily broken down and remain at high concentrations after release into the river even after upgrades to the MWWTP (Vieno and Sillanpää, 2014; Tran and Gin, 2017; Srikanthan, 2019). In the summer, there is a decrease in flow in the river and this may be exposing the fishes downstream to a higher concentration of contaminants than in other season due to less dilution (Anderson and GRWMP Assimilative Capacity Working Group, 2012). Although concentrations have significantly decreased compared to pre-upgrade concentrations, they are not completely removed and have the potential to cause sub-lethal effects on fish populations (Marjan *et al.*, 2017; Srikanthan, 2019). The complex mixtures of contaminants make it difficult to predict concentration limits for effects due to potential synergistic, antagonistic or additive effects of contaminant combinations (Coors and De Meester, 2008; Metcalfe *et al.*, 2010). The pharmaceuticals detected may directly alter the fishes general adaptation syndrome by altering behaviour, ability to mount a stress response, or alter energetic cost of adaptation (Mehdi *et al.*, 2018; Du *et al.*, 2019; McCallum *et al.*, 2019). As mentioned previously, the Grand River is a heavily urbanized area, and effects due to the MWWTP specifically can be hard to pull apart from effects due to this. Looking at results while considering seasonal changes in river flow and other physical river properties is important. Table 2.1 and 2.2 show that seasonal variance causes

changes throughout the river but the MWWTP has some effect on these variables (e.g. conductivity). As shown, although the upgrades occurred before this study, there are still measurable amounts of pharmaceuticals and personal care products detectable throughout the Grand River and increased amounts downstream from the MWWTP (Fig 2.2). It was expected that a gradient response of metabolic rate and energetic endpoints would be seen, but there were no obvious trends between sites and seasons that followed similarly to contaminants. More subtle trends were seen, including at the site upstream from the MWWTP (KIW) which may indicate that fish are responding to agricultural and urban inputs, additionally there are upstream tributaries that could be contributing dilute MWWTP effluent. These non-point sources should not be ignored, and effects can be hard to differentiate from the MWWTP inputs (Park and Park, 2015).

2.4.2 Body Measurements and Metabolic Rate

Body measurements can be used to give information on the overall health of fish and their ability to assimilate nutrients into growth and condition. In summer, GSD had a modified body measurement and in fall, RBD had altered body measurements downstream from the MWWTP. Differences in body sizes may vary the impacts effluent has on the fish. For example, Tetreault (2011), suggested that larger RBD may be more affected by effluent outputs due to their age and their territorial behaviour, which increase their exposure time. Alternatively, here GSD are larger but they are thought to be more mobile than the RBD and may be benefiting from the increased nutrients but limiting exposure to effluent (COSEWIC, 2006). A combination of life history, behaviour and physiology play a role in how fish respond to stressors such as contaminant exposure.

There were no significant changes in FMR in any species, site or season. RBD modulate their FMR in response to living in effluent, but perhaps the improvement in treatment has reduced

their need for detoxification (lower ammonia and contaminant levels; Mehdi et al., 2018). Previous to the upgrades, exposed RBD from EIT (which used a different method to collect MO_2, so direct comparisons are avoided) were recorded to have RMRs up to 600 mgO_2/kg/hr, with reference sites around 200 mgO_2/kg/hr, these results indicate that the RMR levels are now closer to reference site than exposed (Mehdi *et al.*, 2018). The field metabolic rates measured in this study were in the range of those of other fishes that are closely related (Gonzalez and Mcdonald, 1994). Fish species vary in their tolerance to effluent exposure. Round Goby showed no increase in routine metabolic rate when exposed to effluent and is thought to be quite tolerant to MWWTP effluent (McCallum *et al.*, 2017). In contrast, bluegill sunfish had an increased FMR when exposed to effluent (Du *et al.*, 2019). Therefore, because the typical responses to contaminant exposure were not seen, the effects of the MWWTP may not be due to contaminants but physical changes to the water i.e. nutrients, oxygen concentrations and temperature changes. If sub-lethal effects of contaminants are still occurring but undetectable by these endpoints, perhaps behavioural studies would be more ideal. Recently, Martin *et al.* (2019) demonstrated that field-realistic exposure to an antidepressant (fluoxetine) disrupted foraging and aggression in mosquitofish. While there may be no change in routine metabolism, a change in behaviour could free up enough energy for detoxification and maintenance functions (Handy *et al.*, 1999; Campbell, Handy and Sims, 2002), however this remains to be studied for darter species.

Aerobic scope was increased in RBD and FTD in sites downstream from the MWWTP in summer 2019. Increased temperature and oxygen availability are a likely explanation for the increase in MMR (that caused the change in AS) at EIT and FWY in RBD and FTD. There is some evidence of increased body measurements downstream from the MWWTP. Increased energy availability seen in studies looking at fish exposed to effluent could help explain the increase in

AS (Tetreault et al., 2011; Melvin, 2015). The DO may vary in the habitats the darters choose to live in, giving them an increase in oxygen availability (Hlohowskyj and Wissing, 1987; Claireaux *et al.*, 2000). In another study, AS had increased in fish that were exposed environmental perturbations, contrary to the expected decrease when faced with an environmental challenge (Rummer *et al.*, 2013). Wild fish can vary their hematocrit levels to alter their ability to transport oxygen to the tissues, which may explain how fish downstream from the MWWTP are compensating to increase MMR (Borowiec *et al.*, 2016). Fishes can also increase perfusion and recruitment of the gills to increase MMR (Evans, Piermarini and Choe, 2005).

Darters preferentially select water temperatures just above 20℃, which may explain why there is an increase in their aerobic capacity in the summer over the fall (Ingersoll and Claussen, 1984). Additionally, the food and habitat resources of darters overlap, causing intraspecific competition, specifically between RBD and FTD (Hlohowskyj and Wissing, 1986). These two species are more closely related than GSD and have similar prey; in the summer they may be similarly affected by ecological variability (Schlosser and Toth, 1984). Since GSD do not face the same degree of competition, the metabolic adjustments may not be necessary. Alternatively, GSD are more sensitive to changes in oxygen availability and temperature preference, and therefore have less ability to modify aerobic capacity (Hlohowskyj and Wissing, 1985, 1987). The increase in response further downstream from the MWWTP could be caused by an increase of exposure to urban run-off in combination with MWWTP input and agricultural inputs from upstream. The contaminant profile only measures for targeted CECs and therefore could be missing key inputs that are having an impact on the fish's metabolism. However, evidence presented here suggests there are differential species-specific responses to living in MWWTP effluent contaminated sites.

2.4.4 Limitations and Conclusion

For future studies measuring MMR, a combination of techniques to elicit MMR may be more beneficial, such as a chase protocol in combination with the air exposure. It is hard to determine when the fish has reached complete exhaustion using a manual chase protocol which may underestimate MMR (Svendsen et al., 2010). Darters specifically could have a strong reliance on anaerobic tissues that may underestimate the metabolic costs of the organism, due to their benthic life history and sporadic movements. The behaviour of darters has been described as shy and high alert (Reeves, 1907). The ability to conduct metabolic measurements in the field may be affected by this behaviour that would likely take several days of acclimation to overcome. This behaviour, in addition to varied levels of feeding (specific dynamic action), and differences in individual behaviour while measuring metabolic rates, can result in intraspecific variation that may make distinguishing differences in energy requirements challenging (Clark, Sandblom and Jutfelt, 2013). Therefore, the resting metabolic rate is instead referred to as field metabolic rate, due to factors that are not controllable in this study design.

Darters were collected to measure the differences in metabolism and energetic endpoints to give a perspective of whole animal metabolic response to MWWTP effluent after upgrades improved effluent quality. It was hypothesized that fish living downstream from the MWWTP would have increased FMR and overall a decreased AS, to cope with the increased energy cost associated with contaminant exposure from the effluent. However, RBD and FTD showed increased MMR and therefore an increased AS. GSD was unaffected. RBD and FTD sizes were unaltered when metabolic adjustments were seen, GSD were significantly larger and did not alter their aerobic scope, indicating that species specific differences in metabolic adjustments may be occurring. Overall, there is no clear pattern of the impacts of the MWWTP effluent or detrimental

effects of contaminants, due to the variation in the fish's physiological responses at the various sites. Increased AS and body measurements indicate that fish living in the urbanized areas of the Grand River may utilize the greater nutrient and oxygen availability.

3.0.0 The Impacts of Municipal Wastewater Effluent on Gill Physiology of Darters (*Etheostoma* spp.)

3.1.0 Introduction

Over the past 100 years, increasing population, urban sprawl and agricultural activities have influenced the characteristics of the Grand River (Loomer and Cooke, 2011; Water Quality Working Group, 2013). With a large and growing population in the basin area, greater than 98,000 residents in Waterloo, respectively, significant infrastructure is imperative to mitigate wastewater contamination. Two MWWTPs support the Waterloo region, which has an impact on the river itself. MWWTP effluent is released into the river post-treatment but still contains small amounts (ng/L to µg/L) of pharmaceuticals, heavy metals, pesticides, natural and synthetic hormones and hygiene and toiletry products (Arlos *et al.*, 2015; Overturf *et al.*, 2015; Srikanthan, 2019). Impacts of effluent on the organisms living in the river are from population level, where skewed sex ratios are occurring, down to the molecular level, for example the change in mRNA levels of various genes (immune, apoptosis, metabolism, etc.; Arstikaitis et al., 2014; Bahamonde et al., 2015; Fuzzen et al., 2016; Hicks, 2017; Marjan et al., 2017). The water located downstream from MWWTPs therefore presents a significant stressor on the fish populations. Recently, upgrades to the Waterloo MWWTP occurred, increasing load holding time and nitrification which has significantly decreased ammonia levels detected downstream from the MWWTP (Srikanthan, 2019). Upgrades to the Kitchener plant were successful in reducing some of the impacts seen on fish living downstream (Marjan *et al.*, 2017). This study aims to measure fish health downstream from the Waterloo MWWTP post-upgrade, at organismal and molecular levels of organization, and compare this to the health of fish in the Grand River pre-upgrade.

3.1.1 The Gills as a Bioindicator Organ

Biomarkers are indicators of how well a biological system is working and are useful tools for investigating the impacts of perceived stressors on an environment (McCarthy and Shugart, 1990). Biomarkers of pollution are important because they provide sensitive information on the health of a species and, more broadly, an ecosystem (Van der Oost, Beyer and Vermeulen, 2003). The gills are an excellent model organ to use because of their sensitivity to environmental changes and their key roles in maintaining homeostatic functions in the body i.e. gas exchange, ion exchange, acid-base balance, waste excretion, and immune function (Evans, 1987; Laurent and Perry, 1990, 1991). Many studies have used the gills as an indicator of pollution and effluent exposure effects by studying molecular, structural, and functional changes that occur (Schwaiger *et al.*, 1997; Pawert, Müller and Triebskorn, 1998; Triebskorn *et al.*, 2004; Lujić, Marinović and Miljanović, 2013; Corbett *et al.*, 2014; Choi, Alsop and Wilson, 2018). Few studies have examined *in situ* gill biomarkers; many compare a stress free control set-up rather than a reference site, often neglecting the variable conditions fishes face in a natural environment (Li *et al.*, 2009; Saravanan, Ramesh and Petkam, 2013; Rodrigues *et al.*, 2017; Choi, Alsop and Wilson, 2018)

The gills of teleost fishes include four holobranches, each composed of two hemibranches, that create a sieve that is exposed to water for uptake and release of gasses and solutes (reviewed by Wilson & Laurent, 2002). The gill filaments have folded lamellae that increase the surface area of the epithelium which contains important ionoregulatory cells while also supporting respiratory requirements of the fish. Below the filament and lamellar epithelium are cardiovascular structures, responsible for perfusion of the gills for oxygen exchange (Claiborne, Edwards and Morrison-Shetlar, 2002). The lamellar epithelium is thinner than the filament epithelium and more suited for efficient gas exchange. The epithelium is largely composed of pavement cells connected to each

other with tight junctions; much fewer ionoregulatory cells called mitochondria rich cells (MRCs) are found mostly in between lamellae on one side of filaments (Evans, Piermarini and Choe, 2005).

3.1.2 Ionoregulatory function of the gill

With freshwater fishes, ions are passively lost from the blood via gill tissue to the surrounding hypoosmotic water. To maintain the needed level of salt in the blood, MRCs of the gill contain ion pumps that actively transport solutes from the water to the blood (McCormick, 1995). The ion pumps focussed on in this study are Na^+K^+ATPase and the H^+ATPase. In freshwater fish, the H^+ATPase is located on the apical side of MRCs and the Na^+K^+ATPase is basally located (see Figure 3.1). Although, even within freshwater teleosts, ion pump presence varies in terms of cellular location and presence/absence, usually due to either rearing conditions or genetic diversity (Wilson, 2000).

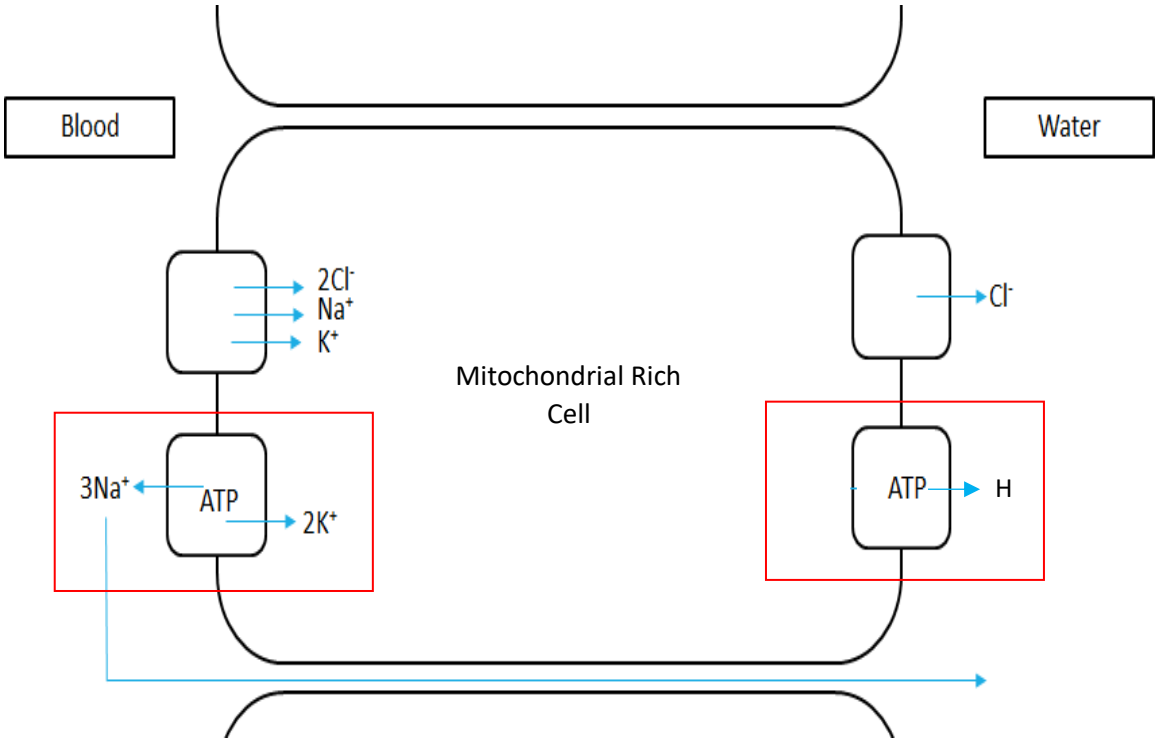


Figure 3.1 A basic diagram of the freshwater teleost mitochondrial rich cell (MRC), with the location of the ion transporters focused on in this study highlighted. Na^+K^+ATPase is located on the basolateral side of the MRC, with contact to the blood. The H^+ATPase is on the apical side, with contact with water. The NKCC and CFTR transporters are other membrane bound enzymes that assist with ion regulation in the gill.

The Na^+K^+ATPase is the most useful ionoregulatory bioindicator of fish health to study because it is sensitive to contaminants and changes in water quality (Twitchen and Eddy, 1994). Basolaterally located in MRCs, the Na^+K^+ATPase hydrolyzes adenosine triphosphate (ATP) and pumps 3 Na^+ and 2 K^+ ions opposite directions across the basolateral side of the cell, against their electrochemical gradient for salt uptake (McCormick, 1995). This pump is important because it assists with acid base balance, osmoregulation and establishes the electrochemical gradient in which other pumps rely on (Evans, 2008; Fernandes, Moron and Sakuragui, 2016). The role of the apical H^+ATPase is not entirely clear, but it does assist with creating the electrochemical gradient for movement of sodium into the cell and may play a role in acid and ammonia secretion (Lin Hong *et al.*, 1994; Wright and Wood, 2012). The activity and expression of Na^+K^+ATPase (and other ion transporters) can decrease due to effluent effects i.e. metals, pharmaceuticals (Parvez, Sayeed and Raisuddin, 2006; Atli and Canli, 2007; Gravel *et al.*, 2009); activity may increase due to stress, brackish water conditions, and some hormones (Tipsmark *et al.*, 2002; Atli and Canli, 2007; Wright and Wood, 2012; Fernandes, Moron and Sakuragui, 2016). The importance of these transporters is their role in acclimation, each has been shown to change in expression, location or activity when freshwater fish are facing osmoregulatory challenge (Laurent and Perry, 1991; Evans, 2008). Therefore, gill ion transporters may play a role in how fish can adapt to other stressors, such as contaminants found in MWWTP effluent.

3.1.3 Techniques to Study Gill Physiology

Histological imaging has been used to understand fish health in many studies; it is a visual technique that targets the organ level of biological organization (McKenzie *et al.*, 2007). It is useful for studying gills, as they are plastic structures that can change morphology to handle changes in environmental conditions For example, in areas of low available oxygen, fish will undergo

reversible restructuring of the gills to maintain the required level of oxygen consumption (Sollid and Nilsson, 2006). This increases the functional surface area of the gill for oxygen uptake but also increases passive ion loss and is a visible increase in interlamellar cell mass (Fernandes et al., 2016; Sinha, Matey, Giblen, Blust, & De Boeck, 2014). Termed osmo-respiratory compromise, this remodelling allows fish to survive in areas of lower oxygen levels, or as a response to high temperature or salinity, but causes a change in the effort required to maintain homeostasis of ions concentration in the blood via the gills (Sollid and Nilsson, 2006). The remodelling of gills and maintenance of gill integrity is largely controlled by the stress hormone cortisol associated with sensing a change in environmental conditions (Laurent and Perry, 1990; Sollid and Nilsson, 2006). Fish downstream from a MWWTP show evidence of a stress response (increase in plasma cortisol levels and cortisol released from gills; Ings et al., 2012; Pottinger, Williams, & Matthiessen, 2016) and the increase in cortisol may therefore play a role in restructuring of the gills.

Restructuring of the gills and the general stress response due to environmental perturbations can be quantified using histological and cellular physiology techniques. Alternative changes to the gill epithelium, such as thickening of the epithelium or pathological changes to the epithelial surface can be visualized. Pathological effects of xenobiotics are evidence of a fish undergoing a general stress response, which can show if fish are being exposed to a significant level of environmental contaminants (Mallatt, 1985). Changes in gill morphology have previously been shown in a study focused on MWWTP effluent exposure, in bluegill sunfish (*Lepomis macrochirus*) living downstream from a MWWTP, where gill morphology was markedly different from upstream counterparts (Du *et al.*, 2019). Patterns of gene expression are also becoming an area of focus in ecotoxicology that may help reveal mechanisms of responses to stressors at a finer level in combination with information from higher biological levels of organization (Van Aggelen

et al., 2010). Contaminants can compete for transporters and cause changes in their activity and abundance as a compensatory response (Laurent and Perry, 1991). Using molecular and histological techniques to determine the sub-lethal impacts of effluent exposure can give insight into the mechanisms that fishes use to adjust and adapt to unideal conditions. In this study, histological and molecular techniques are used to determine if fish are experiencing an increased physiological stress response in the gills when exposed to MWWTP effluent in the river compared to living in upstream agricultural and urbanized locations. This is investigated by measuring changes in the morphology and ionoregulatory function of the gills. It is hypothesized that gills of fishes living downstream from the MWWTP will have increased changes in morphology, pathologies and an impacted ability to maintain their physiological function compared to those fish from sites unaffected by effluent inputs.

3.2.0 Materials and Methods

3.2.1 Site Descriptions

In October 2018 (fall 18), rainbow darter (RBD; *Etheostoma caeruleum*), greenside darter (GSD; *Etheostoma blennioides*) and fantail darter (FTD; *Etheostoma flabellare*) samples were collected from two sites along the Grand River (WMR, EIT,) using backpack electrofishing (Smith Root, LR-20). After this season, two sites were added - KIW, FWY. In July 2019 (summer 2019) and October 2019 (Fall 2019) samples of the three darter species were collected from four sites surrounding the Waterloo MWWTP (WMR, KIW, EIT and FWY). Previous sampling of RBD in the Grand River at WMR, KIW, EIT and FWY sites in Fall 2013/2014 provided historical gill samples collected the same way (no prior respirometry measurements conducted though), before upgrades to the MWWTP were completed and just UV disinfection had been implemented. Historical gill samples from 2013 and 2014 were pooled as “before upgrade” reference samples

because there were not enough samples from one year. Only RBD samples were available for analysis, which were measured and scored for pathologies. WMR (43°35′07.54″N 80°28′54.08″W) is located upstream from urban development and is an agriculturally dominated area and KIW (43°30′17.41″N 80°28′28.61″W) is in an urban area. EIT (43°28′24.69″N 80°28′23.99″W) is immediately downstream from the MWWTP. FWY (43°26'40.2"N 80°24'02.7"W) is approximately 10.5 km downstream from EIT and is in a more heavily urbanized area.

3.2.2 Fish Collection

Fish were collected using dip nets and placed into buckets that were aerated and kept at river temperature. Animal collections were approved by the Animal Care Committee at the University of Waterloo under AUPP#40318. Fish were first used for respirometry trials for a parallel study, for approximately 1.5 hours. Following respirometry, fish were measured for total length (±1.0 mm) and weight (±0.01 g) and sacrificed by spinal severance. Fish were then dissected immediately on site for gill samples. The second and third gill arch were extracted from both sides, half going into Davidson's (formaldehyde 20%, ethanol 30%, glycerol 10%, acetic acid 10%, water 30%) solution for histological processing and half snap frozen in liquid nitrogen for enzymatic and molecular studies.

3.2.3 Histological Analysis

Gill tissue was examined histologically for changes in morphology and evidence of pathologies at all sites, using WMR as a reference site. Gill tissue was fixed in Davidson's solution for 24hr then moved to 70% ethanol until processing. Samples were dehydrated and embedded in paraffin wax longitudinally. Samples were sliced with a microtome set to 5 µm until whole gill arches, filaments and lamellae were seen in sections that were placed on glass slides and stained

using an automated hematoxylin and eosin stain procedure. Images of gills were recorded using Leica microscope (DM 1000 LED) and camera (MC170 HD) at 100x using Leica Application Suite (LAS) software (Nussloch, Germany). A random image was taken of the gill section that had at least three primary filaments present with interior cartilage visible to standardize location of analysis. Images were labelled blindly so no knowledge of which image belonged to which site was known until after scoring was complete. The morphological assessment used is described by Tetreault et al. (2012). Each image had 9 measurements (see Figure 3.2) of secondary lamellar length (SLL), secondary lamellar width/diameter (SLW), inter-lamellar diameter (ID) and basal epithelium thickness (BET) each, spread along the base, middle and distal edge of the filament. SLL and BET were used to calculate percentage available for gas exchange, PAGE = SLL/(SLL+BET)*100. PAGE gives a percentage of the epithelium exposed for gas exchange with the environment. Measurements were taken using the software Fiji - Fiji Is Just ImageJ (Schindelin et al., 2012) and calibrated using the Leica scale bars.

Gills were scored for pathologies, using a scale from 1-4 on five gill pathologies to assess the degree of damage in the gill tissue (see Figure 3.3 and Table 3.1). Hypertrophy is the increase in an individual cell's volume; hypertrophy is cell proliferation; epithelial lifting is the peeling of the epithelium from the sinus of the gill; fusion is the (complete or partial) connecting of secondary lamellae and edema of the lamellae is enlargement of the sub-epithelium spaces. Gill scoring is modified from Poleksić & Mitrovic-Tutundzic (1994), where a literature study was done on the usefulness of gill scoring and severity of pathologies. Each sample had the scores of all pathologies summed and the average score for samples at that site and species was compared to the reference site for each species.

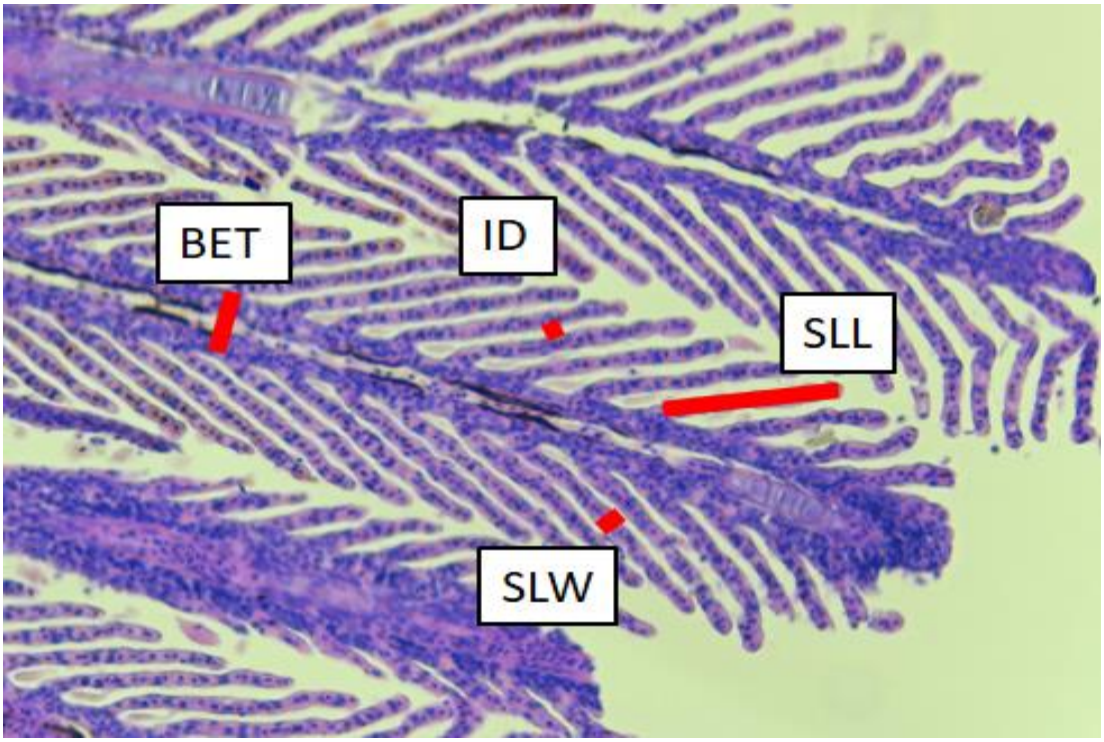


Figure 3.2 A sample of morphometric measurements of the gill. BET = basal epithelium thickness; ID = interlamellar diameter; SLL = secondary lamellar length; SLW= secondary lamellar width. Fiji (Image J) software was used to measure each parameter on 3 filaments 9 times total at the base, middle and distal area.

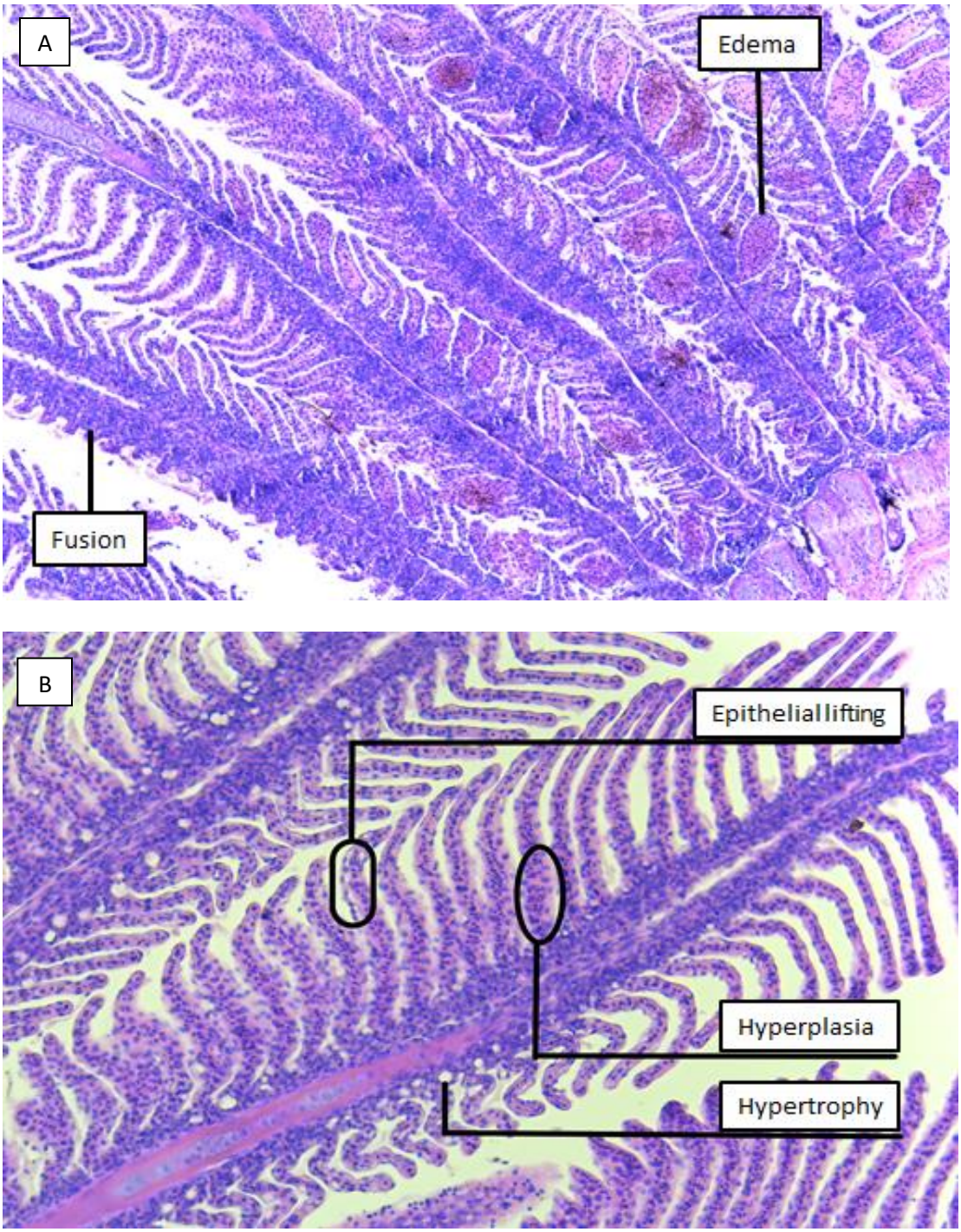


Figure 3.3 Examples of the gill pathologies scored. Five pathologies were scored out of a scale from 1-4 for severity and summed. A) Edema and fusion of the secondary lamellae. B) Epithelial lifting, hyperplasia and hypertrophy. See text for description of pathologies. The average score of the pathologies was compared to the reference site (WMR).

Table 3.1 Gill pathology scoring table. The description of the five common pathologies that were scored in each histological sample. An average score of all pathologies was taken from the sum of each sample at each site and compared to the reference sites average to test for differences.

Pathology	Score	Description
Hypertrophy	1	1-2 cells swollen
	2	Swollen cells on multiple lamellae
	3	Multiple affected cells on many lamellae
	4	Most lamellae have many affected cells
Hyperplasia	1	Some 2 lamellae (1-5) show hyperplasia
	2	Most lamellae have some hyperplasia
	3	All lamellae show hyperplasia; beginning to fuse
	4	Lamellae are in distinguishable and all have severe hyperplasia
Epithelial Lifting	1	Mild lifting at the base of the lamellae, on 1-2
	2	3-5 lamellae showing minor lifting
	3	Many lamellae have lifted epithelium moving to tip
	4	Lamellae is lifted on the entirety of the lamellae and on most to all
Lamellar fusion	1	1-2 of lamellae are fused/overlapping
	2	fusion of the many tips of lamellae
	3	most of the lamellae are completely fused otherwise tips fused
	4	there are no distinguishable parts of lamellae, all fused
Edema	1	One or two lamellae are mildly swollen
	2	1-4 lamellae are more severely swollen, very blown up
	3	4+ lamellae are affected, 1-2 are severe
	4	Many lamellae are completely swollen to the filament base. At least 3+ severe

3.2.4 Molecular Analysis

Total RNA was isolated from summer 2019 gill samples that were snap frozen in the field following respirometry trials. After homogenization, using the OMNI tissue homogenizer (NW Kennesaw, GA, USA), RNA was extracted following RNeasy Mini Kit (Qiagen; Hilden, Germany) protocol. RNA concentrations and quality (280:260μm and 260:230μm) were measured using the SpectraMax 190 from Molecular Devices (San Jose, California, USA). Each reaction had 1 μg of template RNA (volume depending on concentration) in the reaction mixture with 1μL reverse transcriptase (RT), 4μL RT buffer, 1 μL RT primer mix, plus 2 μL gDNA wipeout, and n μL of RNAse free water to bring the reaction up to 20 μL. RNA was converted to cDNA using QuantiTect Reverse Transcription Kit (Qiagen; Hilden, Germany), following manufacturer's instructions. Primers were developed for ion pumps by gathering known sequences of closely related species through NCBI the Nucleotide database for Na^+K^+ATPase (catalytic subunit - *atp1α*) and H^+ATPase (transporting subunit - *atp6v1a*). Additionally, two housekeeping genes (β – actin and Glyceraldehyde-3-phosphate dehydrogenase) that have consistent expression were previously developed and used to normalized measurements of target genes (Table 3.2). A consensus sequence was created by aligning multiple sequences using CLUSTALW (Kyoto University Bioinformatics Center) and Jalview (Waterhouse et al., 2009), then blasted in NCBI's Primer designing tool to find forward and reverse primers (Table 3.2). PCR followed by gel electrophoresis was then conducted to confirm amplicon size to verify the correct product was replicated. Sangar sequencing was used to determine the most probable nucleotide sequences of the products for further verification against assumed sequences. RT-qPCR followed cDNA creation using SSo advanced SYBR green to stain DNA and Biorad CFX 96 machine with CFX Maestro software from Biorad (Hercules, California, USA). The reaction mixture included 2 μL

of cDNA, 1µL of water, 1 µL of each forward and reverse primer and 5 µL of SYBR green for a total of 10 µL. The reaction was carried out in duplicate in Biorad low-profile clear plates with bio-rad optically clear flat caps (Hercules, California, USA). Primers were validated using a dilution series (4, 16, 64, 256x) to test for primer efficiency and dimerization. The PCR conditions were a 30 second activation at 95°C, a 10 second denaturation at 95°C, an annealing and extension phase for 15 seconds and 60°C for 40 cycles. The melt curve was at 65°C to 95°C every 0.5°C to check for amplification of just one product.

Table 3.2 Primer information. There are two housekeeping genes and two targets. NCBI database I.D., amplicon size, and sequences are given.

Target	NCBI I.D	Info	Amplicon Size (b.p.)	Forward (5'-3') Reverse(5'-3')
actb	XM_032537833.1	β – actin Housekeeping gene	200	F:CGACATCCGTAAGGACCTGT R:GCTGGAAGGTGGACAGAGAG
gapdh	XM_034874299.1	Glyceraldehyde-3-phosphate dehydrogenase Housekeeping gene	217	F: GAACATCATTCCCGCCTCTA R:GTATCCCAGAATGCCCTTCA
atp1a	XM_034864776.1	Na^+K^+ ATPase Subunit α-1	196	F:GCTCTGAAGAAGGCCGACAT R:GGGTGATCTCGGGGATGTTAC
atp6v1a	XM_032504460.1	H^+ATPase Transporting V1 subunit A	146	F:CTGCGGACAGGAAAACCTCT R:TTGAGGGCTCCGATGTTCAC

3.2.5 Statistical Analysis

Data were analyzed using the statistical software GraphPad Prism 8 (San Diego, California, USA). Figures present the data as means ± standard error of the mean (SEM). For histological analyses, enzyme activity and mRNA relative abundance, each species-site group was compared using analysis of variance (ANOVA). The data were tested for equal variance and normality with Levine's test and the Kolmogorov–Smirnov test, respectively, to determine if they met the assumptions of an ANOVA. Dunnett's multiple comparisons test was used to compare each species-site group to the reference site of the respective species. Alpha was set to 0.05 for all tests, where significant differences were any p-values less than this.

3.3.0 Results

3.3.1 Histological Analysis

In fall 2013/2014, fall 2018 and summer 2019, gill samples were collected and analyzed for morphological changes and pathologies and compared to the upstream location, WMR (Figure 3.4, 3.5 3.6 and 3.7). In all seasons, only SLW, a measurement of oxygen diffusion distance, showed significant differences (see below). Hyperplasia, fusion, epithelial lifting, edema and hypertrophy were seen in samples from all sites and all years, but severity of pathologies was most prevalent at the immediate downstream location, EIT. The most common and severe pathology seen was hyperplasia of the secondary lamellae (Figure 3.3.B); if severe enough, hyperplasia led to fusion of the secondary lamellae. Fusion could also occur if the tips of secondary lamellae became fused together by bending. Epithelial lifting and edema were third and fourth common. Hypertrophy was the least common pathology seen in all samples. No season or species had significant differences in SLL, BET, PAGE or ID.

In 2013/2014, RBD showed significantly increased SLW (Dunnett's multiple comparisons, $p = 0.0471$) and pathologies (Dunnett's multiple comparisons, $p = 0.0380$) at the downstream site closest to the MWWTP, at EIT, compared to WMR. KIW and FWY showed a trend of increased pathologies but there were no significant differences compared to WMR.

In fall 2018, after upgrades to the Waterloo MWWTP were completed, RBD was the only species to have significant changes to gill structure compared to the control site. RBD had increased secondary lamellar diameter (Dunnett's multiple comparisons, $p=0.0012$) and increased average gill score pathologies (Dunnett's multiple comparisons, $p<0.0001$). GSD and FTD did not display significant increases in lamellar diameter or gill pathology when compared to RBD control site fish. Overall, the most prominent pathologies were hyperplasia and fusion of the secondary lamellae. There were not enough samples to compare FTD fall 2018 samples (due to sample loss) to their own control site species and were compared to RBD but there were no significant differences.

In summer 2019, there was an increase in gill lamellae diameter in RBD at EIT (Dunnett's multiple comparison's, $p=0.0196$). There was a significant effect of site on pathology in RBD (One-way ANOVA, $F=3.619$, $p=0.0443$), but no significant between site differences.

GSD had significantly increased lamellae diameter at EIT (Dunnett's multiple comparisons test, $p=0.0247$) and FWY (Dunnett's multiple comparisons test , $p= 0.0305$) and increased pathology at KIW (Dunnett's multiple comparisons test, $p=0.0221$) and EIT (Dunnett's multiple comparisons test, $p=0.0488$) in summer 2019 compared to WMR.

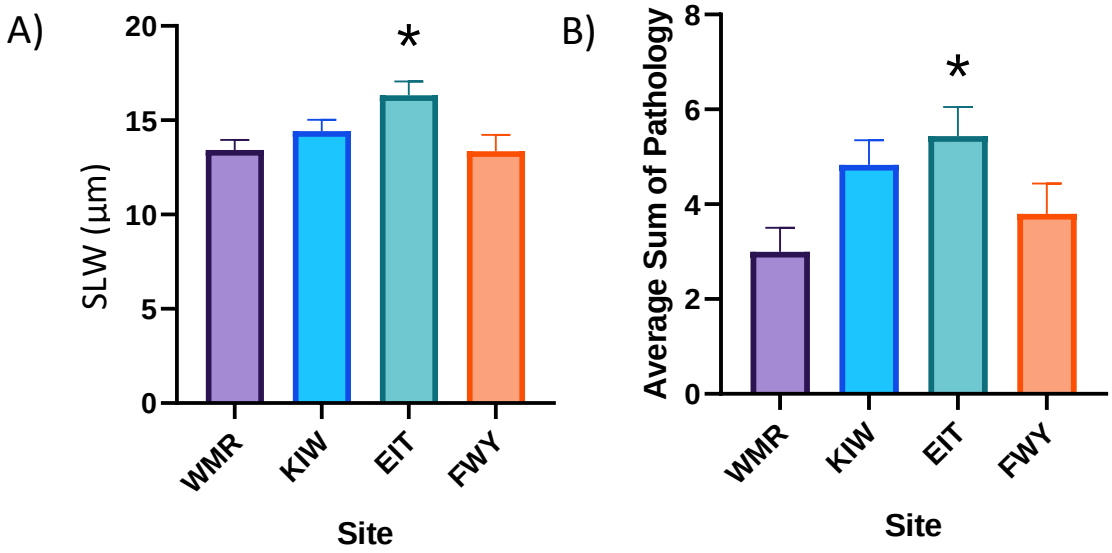


Figure 3.4 **A)** The average SLW of RBD in fall 2013/2014. An asterisk represents a significant difference in diameter compared to WMR, compared using a one-way ANOVA and Dunnett's multiple comparison's test ($p<0.05$, n=8,18,16,15). EIT is downstream from the MWWTP. **B)** The average pathology scores of RBD in fall 2013/2014. An asterisk represents a significant difference in diameter compared to WMR, compared using a one-way ANOVA and Dunnett's multiple comparison's test ($p<0.05$, n=8,18,16,15). EIT is downstream from the MWWTP. The stacked bar represents the added averages of each pathology.

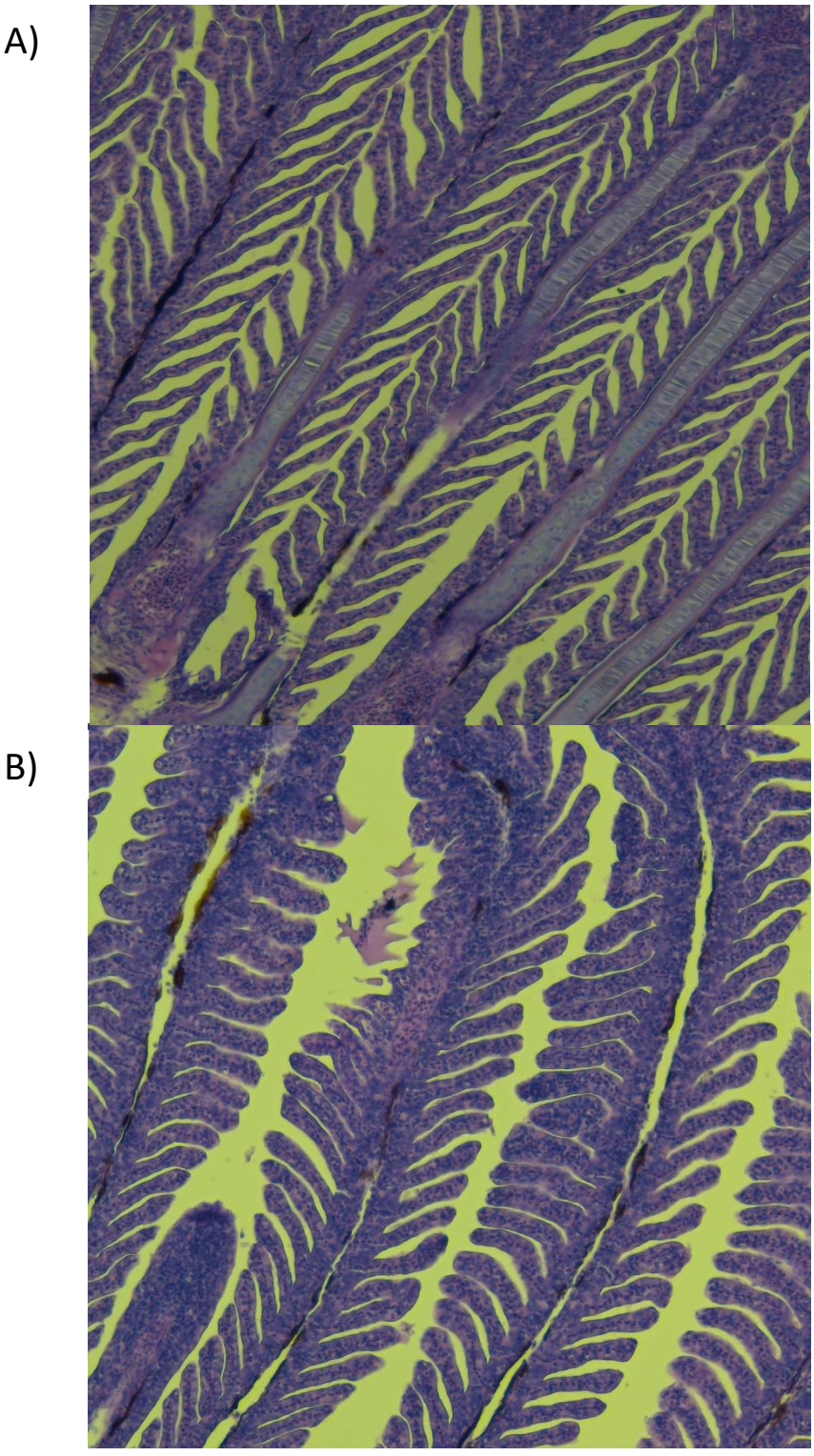


Figure 3.5 **A)** RBD fall 2013/2014 sample from WMR. **B)** RBD fall 2013/2014 sample from EIT.

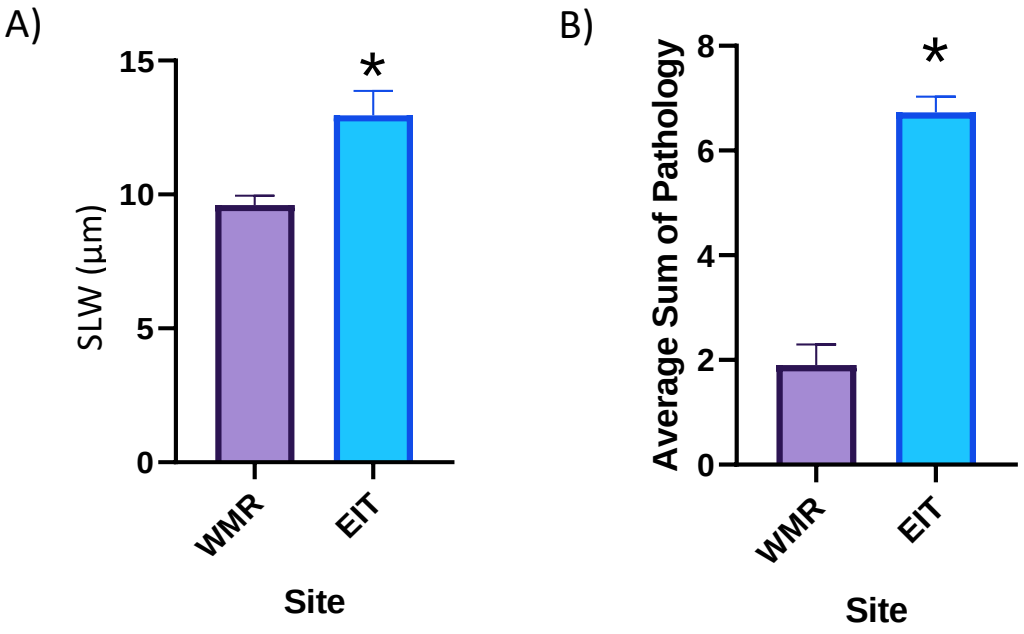
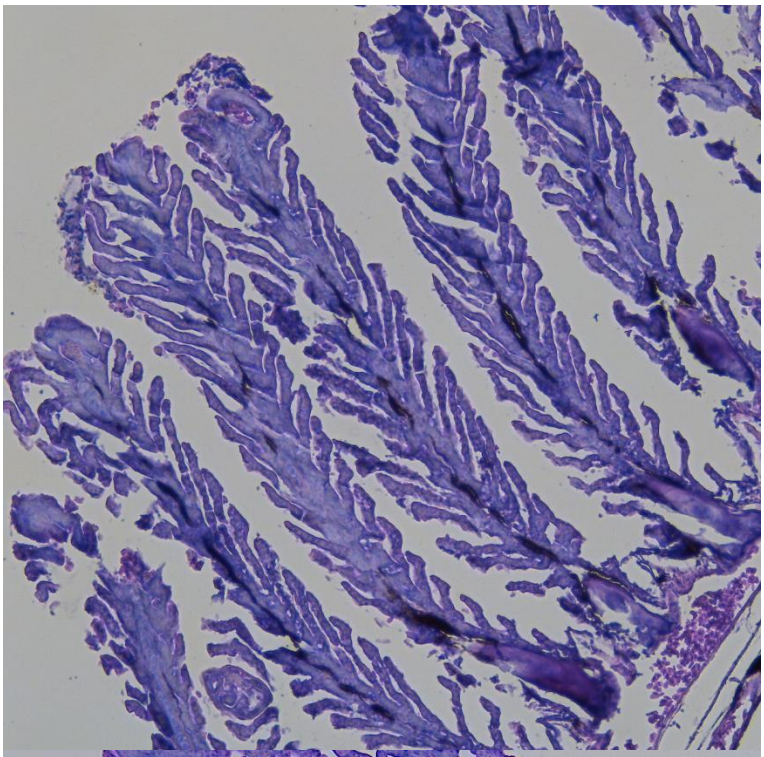


Figure 3.5 **A)** The average SLW of RBD in fall 2018. An asterisk represents a significant difference in diameter compared to WMR, compared using a one-way ANOVA and Dunnett's multiple comparison's test ($p<0.05$, $n=20,11$). EIT is downstream from the MWWTP. **B)** The average pathology scores of RBD in fall 2018. An asterisk represents a significant difference in diameter compared to WMR, compared using a one-way ANOVA and Dunnett's multiple comparison's test ($p<0.05$, $n=20,11$). EIT is downstream from the MWWTP. The stacked bar represents the added averages of each pathology.

A)



B)

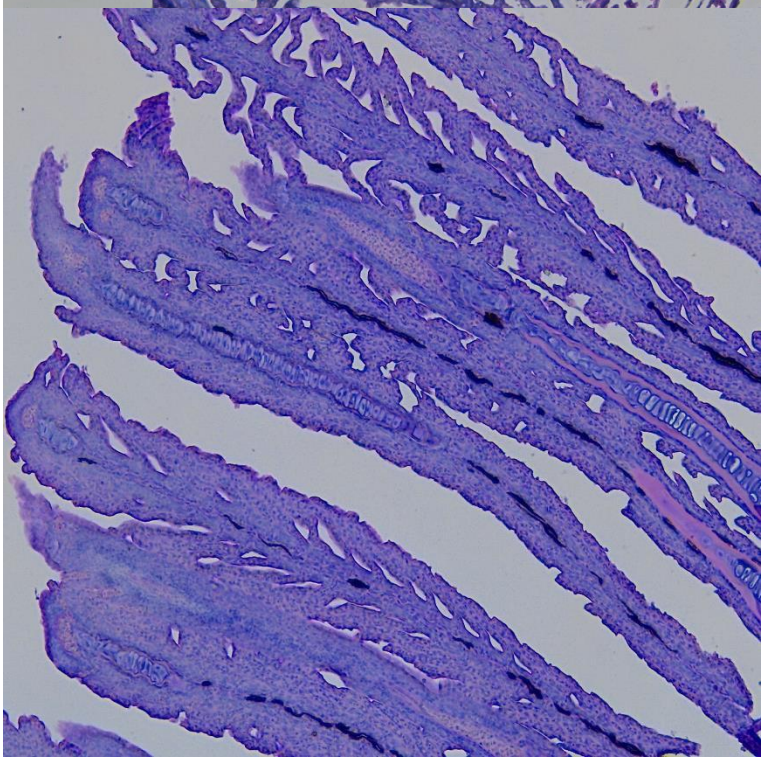


Figure 3.6 **A)** RBD fall 2018 gill sample from WMR. **B)** RBD fall 2019 gill sample from EIT.

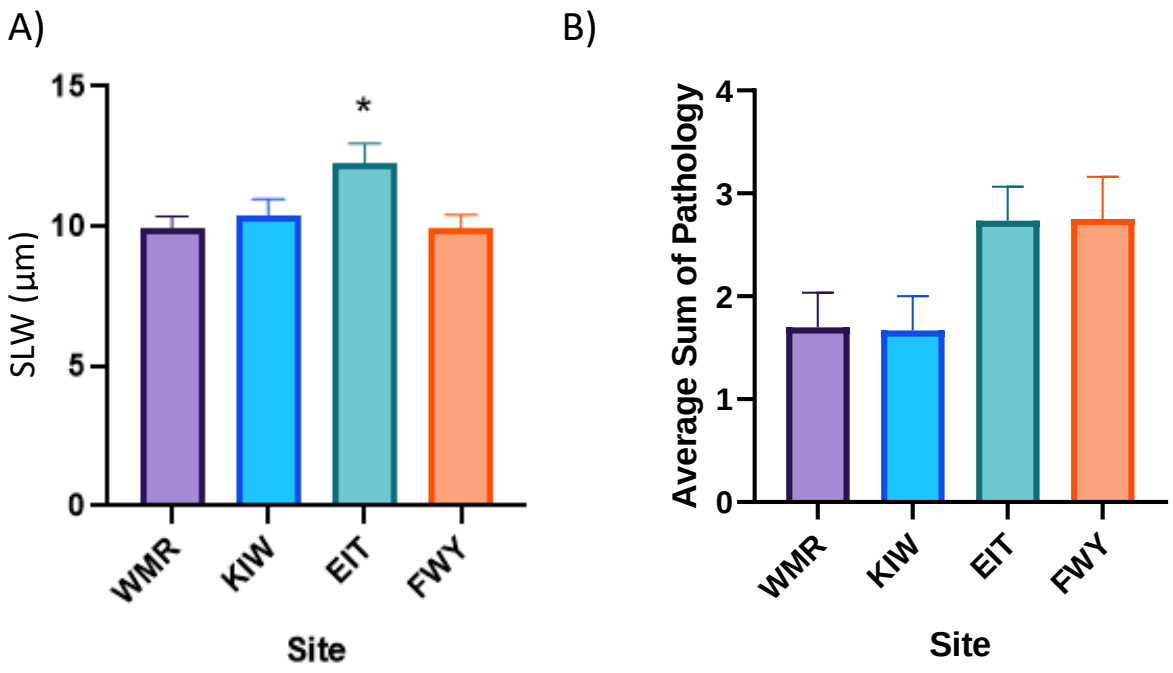


Figure 3.7 **A)** The average SLW of RBD in summer 2019. An asterisk represents a significant difference in diameter compared to WMR, compared using a one-way ANOVA and Dunnett's multiple comparison's test ($p<0.05$, n=9,11,14,11). EIT is downstream from the MWWTP. **B)** The average pathology scores of RBD in summer 2019. An asterisk represents a significant difference in diameter compared to WMR, compared using a one-way ANOVA and Dunnett's multiple comparison's test ($p<0.05$, n=9,11,14,11). EIT is downstream from the MWWTP. The stacked bar represents the added averages of each pathology.

A)

B)

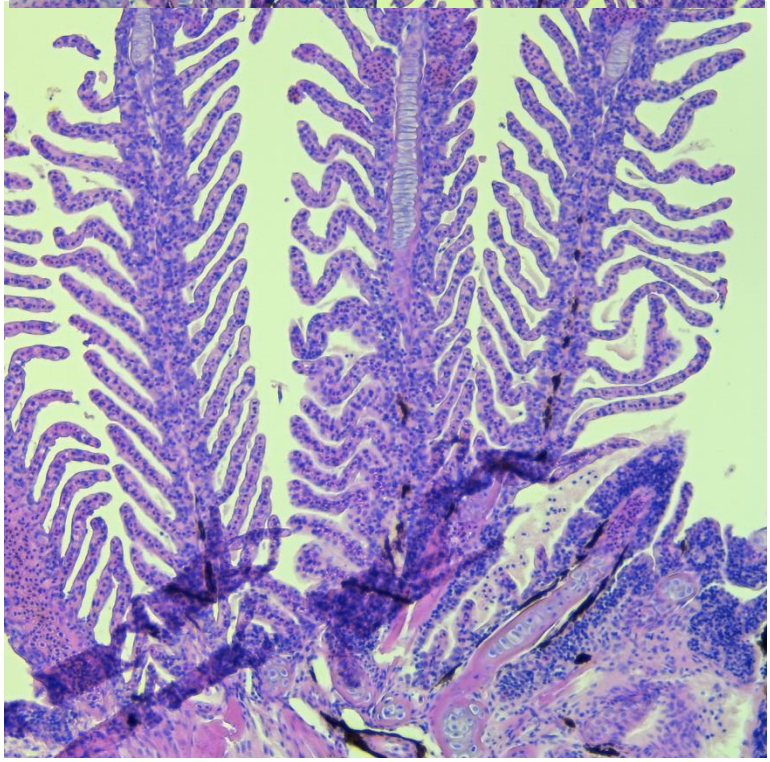


Figure 3.8 **A)** RBD summer 2019 gill sample from WMR. **B)** RBD summer 2019 gill sample from EIT.

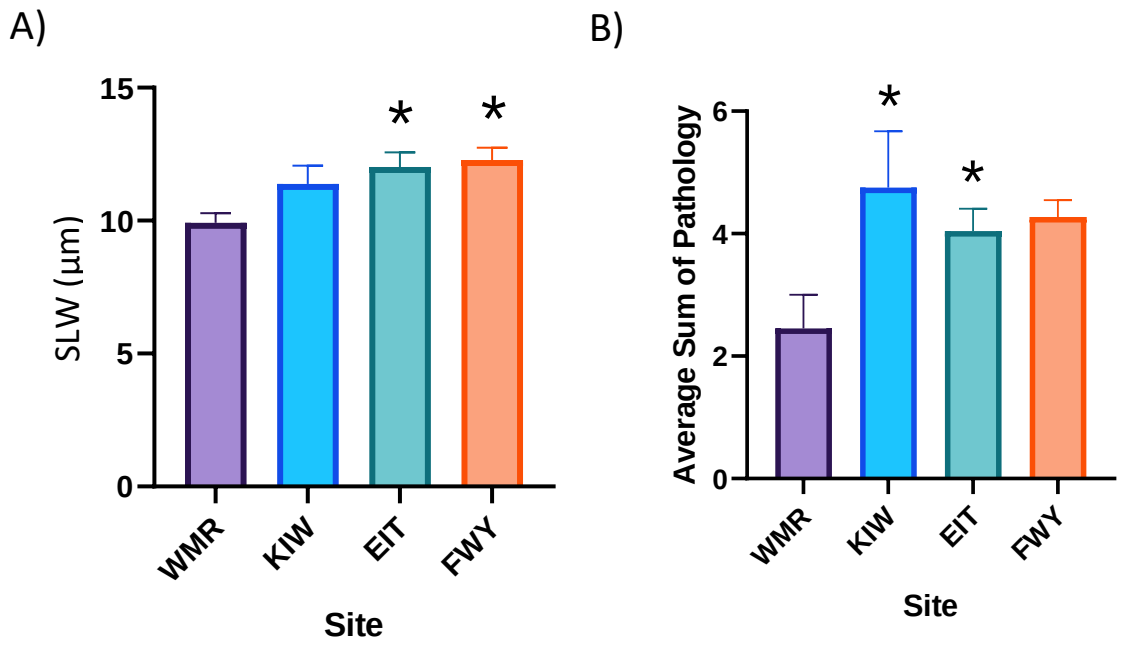
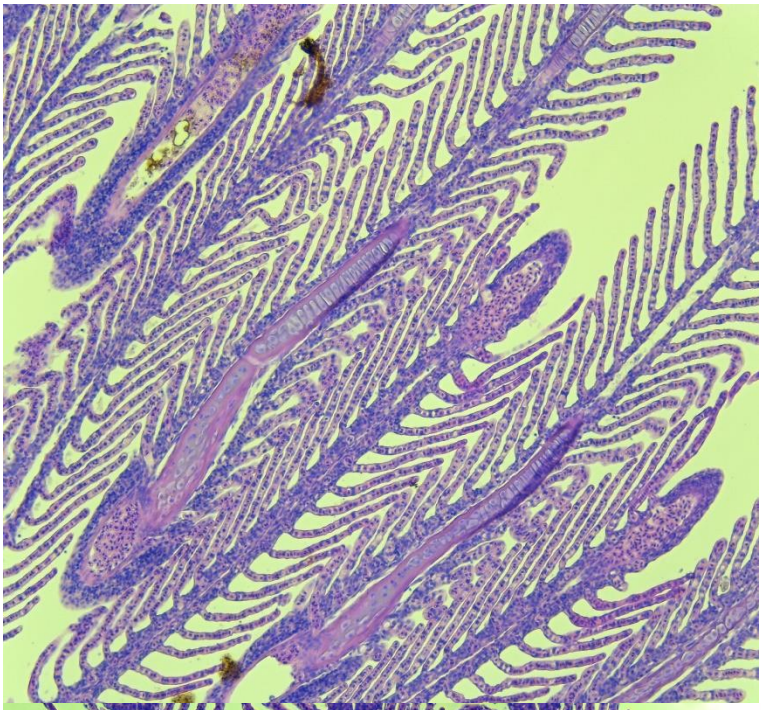


Figure 3.9 **A)** The average SLW of GSD in summer 2019. An asterisk represents a significant difference in diameter compared to WMR, compared using a one-way ANOVA and Dunnett's multiple comparison's test ($p<0.05$, n=11,8,24,11). EIT is downstream from the MWWTP. **B)** The average pathology scores of GSD in summer 2019. An asterisk represents a significant difference in diameter compared to WMR, compared using a one-way ANOVA and Dunnett's multiple comparison's test ($p<0.05$, n=11,8,24,11). EIT is downstream from the MWWTP. The stacked bar represents the added averages of each pathology.

A)



B)

Figure 3.10 **A)** GSD summer 2019 gill sample from WMR. **B)** GSD summer 2019 gill sample from EIT.

3.3.2 Molecular Analysis

In summer 2019 gill samples were used to measure mRNA relative abundance of ion pumps in the gills (Figure 3.8). The GSD relative abundance of Na^+K^+ATPase (Dunnett's multiple comparison's, p=0.0233) and H^+ATPase (Dunnett's multiple comparison's, p=0.0491) at EIT were significantly increased by 3-fold and 3.5-fold, respectively. RBD had increased H^+ATPase (Dunnett's multiple comparison's, p=0.0304) relative abundance by 2.5-fold at FWY. RBD and FTD did not have significant changes in Na^+K^+ATPase activity. FTD had no differences in H^+ATPase relative abundance.

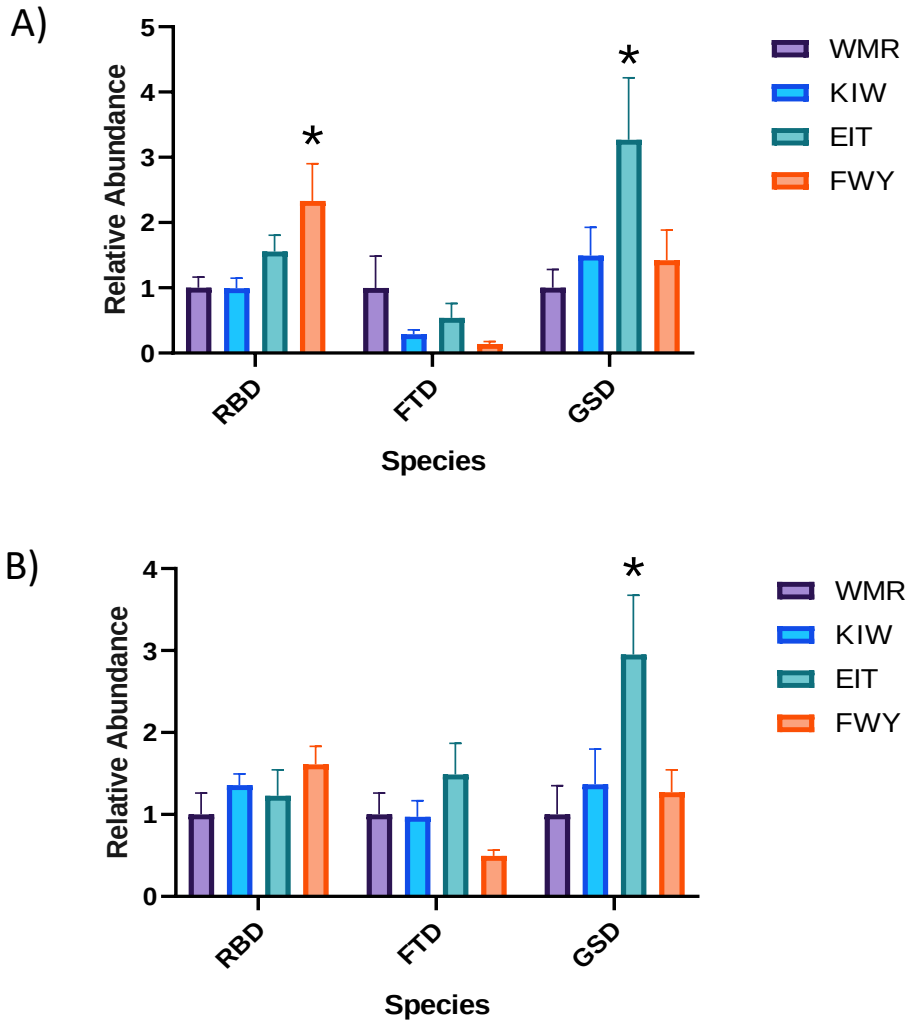


Figure 3.11 **A)** Na^+K^+ATPase relative abundance in RBD, FTD and GSD in summer 2019. Relative abundance is normalized to two housekeeping genes. An asterisk represents a significant difference in relative abundance compared to WMR, compared using a one-way ANOVA and Dunnett's multiple comparison's test ($p<05$, $n=6$). **B)** H^+ATPase relative abundance in RBD, FTD and GSD in summer 2019. Relative abundance is normalized to two housekeeping genes. An asterisk represents a significant difference in relative abundance compared to WMR, where relative abundance at WMR is 1, compared using a one-way ANOVA and Dunnett's multiple comparison's test ($p<05$, $n=4$-6).

3.4.0 Discussion

Effluent from MWWTP are complex mixtures that may cause direct damage to the gills, the major gas and ion and transporting organ in fishes. This study found that downstream from the MWWTP, RBD gills in fall and summer, and GSD gills in summer, were morphologically different and displayed more severe pathologies than at the reference site, regardless of year examined. Interestingly, each darter species may be adjusting and adapting to the urban stream areas differently, with GSD showing more variability in their Na^+K^+ATPase relative abundance than RBD and FTDs, which may indicate greater sensitivity to environmental disruption in GSDs.

3.3.1 Gill Morphology and Pathologies

Damage due to contaminants can directly change the morphology of the gills, for example, epithelial lifting can occur when toxic substances directly interact with the tissue (Nascimento *et al.*, 2012). The most common pathologies associated with chronic, sublethal exposure to various xenobiotics are hyperplasia, clavate lamellae (edemas) and cell proliferation, as a general stress response (Mallatt, 1985). The gills in this study showed evidence of all pathologies at all sites, but a significant increase in the presence and severity as well as thickened lamellar epithelium, at the site downstream from the MWWTP in RBD. Overall, the pathologies are thought to be defense mechanisms of the fish to limit entry of irritants as a general stress response rather than a contaminant specific reaction (Evans, 1987). Thickening of the epithelium, hyperplasia and epithelial lifting all increase oxygen diffusion distance, but may provide a barrier to entry of contaminants (Poleksic and Mitrovic-Tutundzic, 1994). An increase in temperatures during the summer months can explain, in part, the increase in the effects of river conditions on gill morphology, where the increase in water temperature in combination with decrease in water quality could cause an in increase in toxicity of pollutants (Mckim and Erickson, 1991; Schwaiger

et al., 1997). All three species have an epithelium that is "thick" due to their benthic habitat, compared to fish that have a more active life history (Evans, Piermarini and Choe, 2005). An increase in lamellar thickness and a decrease in lamellar surface area, due to severe hyperplasia causing gill fusion, hinders oxygen uptake and decreases carbon dioxide offloading, making this maladaptive (Gilmour, 1997; Wright and Wood, 2012). Over time, fish can resist the effects of contaminants if they are at sublethal concentrations, via functional modifications to the gills different than those mentioned before; for example, varying perfusion of the gills, increasing breathing rate, increasing detoxification processes or stimulating the immune system (Schwaiger *et al.*, 1997; Claiborne, Edwards and Morrison-Shetlar, 2002; Flores-Lopes and Thomaz, 2011). FTDs may be employing different strategies such as these to minimize the effects of contaminants to the gills, however this is currently unknown. Overall, there is evidence of pollution effects on the gill morphology of RBD, and in one season, on GSD, while FTDs do not show any maladaptive response.

3.3.2 Ionoregulatory Adjustments

The increased relative abundance of ion pumps in summer 2019, indicates that GSD and RBD can alter ion pump levels to adapt to their environment. FTD do not show evidence of this trait. The differences seen in ion pump relative abundance in RBD and GSD but not FTD may be related to the restructuring of the gills seen in the previous measurements. Fishes can modify their respiratory epithelium via osmorespiratory compromise, which increases the surface area available for gas exchange and additionally, ion loss (Sardella and Brauner, 2007; Fernandes, Moron and Sakuragui, 2016).

Mechanisms of increased Na^+K^+ATPase and H^+ATPase relative abundance need to be further investigated, however restructuring of the gills could be caused by a change in cortisol,

which plays a role in acclimation by increasing the number of MRCs and ion pumps in the gill (Laurent and Perry, 1990; Dang *et al.*, 2000). In combination with stress, contaminants in the water (such as venlafaxine, diclofenac and carbamazepine, see previous chapter) may also be directly altering the abundance of Na^+K^+ATPase. In a study on juvenile rainbow trout exposed to environmentally relevant concentrations of venlafaxine, the activity and expression of the Na^+K^+ATPase was altered (Best *et al.*, 2014). There is evidence that diclofenac caused an increase in gill Na^+K^+ATPase activity in Indian major carp (*Cirrhinus mrigala*) in a long term exposure (Saravanan, Ramesh and Petkam, 2013). However, carbamazepine has the ability to reduce Na^+K^+ATPase activity in chronic exposures, making predictions of specific causes of change difficult due to the complex mixtures found in effluents (Coors and De Meester, 2008; Li *et al.*, 2009). Alternatively, the water quality could be impacting the ion pumps; increased Na^+K^+ATPase activity has been recorded in marine teleosts that were exposed to high nitrite concentration, although activity of the pump is not always reflected by a change in gene expression (Deane and Woo, 2007; Best *et al.*, 2014). Nitrogenous compounds can competitively bind to ion pumps and compensation for this may be increasing ion pump numbers (Twitchen and Eddy, 1994; Ings, Vijayan and Servos, 2012). There is some evidence that ion pumps, especially H^+ATPase, are tied to ammonia regulation and changes in gene expression could be in response to chronic ammonia exposure (Nawata *et al.*, 2007; Nawata and Wood, 2009; Wright and Wood, 2012). In summary, the ionoregulatory function of the gills in darters was variable. GSD and RBD can modify their ionoregulatory pump expression to adapt to changes in their habitat, but it is not directly due to MWWTP effluent, as effects were seen at various sites along the river.

3.3.3 Conclusion

Using multiple levels of biological organisation is key to understanding the impacts of environmental stressors on an ecosystem. In this study, organ and molecular level experiments were examined to identify specific effects on the gills of *Etheostoma* spp. downstream from the MWWTP. Upgrades to the Waterloo MWWTP reduced ammonia levels and estrogenicity of released effluent (Srikanthan, 2019). A 2016 study in the Grand River demonstrated that the improvement in effluent quality lead to recovered reproductive endpoints (i.e. steroid production) in RBD downstream from the Kitchener MWWTP (Marjan, 2018). Other studies have found that increased dilution factors and longer retention time of water in treatment plants are a key change in minimizing impacts on the downstream environments (Giang *et al.*, 2019). This study showed that there are effects of effluent exposure on the gill morphology of darters found in the Grand River. However, because the results are not consistent across seasons, sites, or species, it appears pulling apart effluent effects from urban and agricultural inputs is difficult using the endpoints selected and may require looking at different levels of biological organization and different physiological functions. However, the evidence presented here suggests GSD and RBD can modify their ionoregulatory function, which may help them adapt to changing river conditions.

4.0 – General Discussion

4.1 Conclusions

The purpose of this thesis was to investigate the response of darters (rainbow darter, RBD, *Etheostoma caeruleum*; greenside darter, GSD, *Etheostoma blennioides* and fantail darter, FTD, *Etheostoma flabellare*) to Waterloo municipal wastewater treatment plant (MWWTP) effluent in the Grand River. The Waterloo MWWTP recently underwent upgrades to improve the quality of the effluent by increasing solids retention time and nitrification. These upgrades were hypothesized to reduce the biological impacts on fishes downstream, as seen after upgrades to the Kitchener MWWTP, also located in the Waterloo region (Marjan *et al.*, 2017; Srikanthan, 2019). Studying the effects of contaminants on the physiology of fishes directly in the field provides comprehensive data on effects of contaminants and incorporates natural environmental variation and the complex interactions of these variables (Munkittrick and Dixon, 1989; Coors and De Meester, 2008; Segner, Schmitt-Jansen and Sabater, 2014; Arciszewski and Munkittrick, 2015). MWWTP effluent has the potential to affect fishes at multiple levels of biological organization, from community to molecular levels (Porter and Janz, 2003; Marjan *et al.*, 2017; McCallum *et al.*, 2019). These studies aimed to characterize the metabolic and gill physiological responses of darter populations exposed to improved Waterloo MWWTP effluent and compare them to fish populations from sites unaffected by effluent. This study highlights potential biomarkers of contaminant exposure and the importance of looking at multiple species when assessing contaminant effects on an ecosystem.

In chapter two, physical and chemical water quality data along with body measurements and metabolic rates of darter samples were collected up and downstream from the MWWTP. The metabolic rate and body measurements of fishes from downstream were compared to those from an upstream site to incorporate changes in water quality due to other sources (i.e. agricultural and

urban non-point sources). Contaminants of emerging concern, such as pharmaceuticals and personal care products, in addition to nitrogenous compounds such as ammonia were found to be increased downstream from the MWWTP. Contrary to what was hypothesized, field metabolic rates of fishes were no different than upstream populations, providing evidence that the MWWTP is not a significant energetic stressor to darter metabolism. Interestingly, the maximum metabolic rate (MMR) and therefore aerobic scope (AS) for two darter species were increased, indicating an increase in energy availability. This may allow fish to maintain or improve reproductive success, anti-predator behaviour and foraging, at a population level (Norin and Clark, 2015). Metabolism measurements (FMR and MMR) are sensitive biomarkers that are useful in detecting stressors to an organism in an environment (Clark, Sandblom and Jutfelt, 2013). The long term exposure to poor quality effluent prior to upgrades may have previously been a metabolic stress for darters (Mehdi *et al.*, 2018); this study provides evidence that the upgrades to the MWWTP do not continue to cause an increase in routine costs of maintaining homeostasis and detoxification. Additionally, their may be some growth (length and weight) advantages due to increased nutrients, but this needs to be further investigated. Overtime, darters can adapt to exposure to contaminants and this, in combination with improved water quality, may allow the darters to benefit from the increased nutrients in effluent while being minimally affected by contaminants (Beyers *et al.*, 1999; Triebskorn *et al.*, 2004; Tetreault *et al.*, 2011). The general adaptation syndrome's primary step is a shift in behaviour via secondary physiological response, a trade-off rather than increase in demand may be sufficient in mitigating stress to the fish (Beyers *et al.*, 1999; Scott and Sloman, 2004).

In chapter three, the morphological and functional effects of chronic exposure of MWWTP effluent on gill tissue in darters was investigated. RBD and GSD downstream from the MWWTP

had significant gill pathologies and increased lamellar thickness. GSD also had changes in gill tissue just upstream from the MWWTP. RBD and GSD also had altered expression of ion pumps in the gill tissue, indicating a change in ionoregulatory function downstream from the MWWTP. Pathologies can alter the surface area of the gill and the ability of a fish to oxy- and ionoregulate; increased lamellar width indicates an increase in oxygen diffusion distance. The fishes may be adjusting their ion pump levels because of this change in gill structure to maintain ionoregulatory function. Additionally, the increase in pumps may be in response to water quality; for example, exposure to ammonia can directly affect ionoregulatory ability (Wright and Wood, 2012). The adjustment of gill tissue is hypothesized to be a generalized response to a stressor (Mallatt, 1985); darters may be altering their structure and functionality of the gill tissue to adapt to the environment. Histological measurements of the gills are a simple, useful tool for measuring a general stress response in fishes. This may prove to be especially insightful for studying the complex effects of MMWTP effluent and natural environmental variation (i.e. multiple stressors) because it highlights the reception and response to stressors by an organism rather than focusing on stressors properties (Segner, Schmitt-Jansen and Sabater, 2014).

Together, the results of this study indicate that fishes can acclimate to effluent and urban contaminated water through respiratory adjustments. This thesis provides evidence of increased metabolic capacity even though there was damage to gill tissue. Cumming & Herbert (2016) tested the ability of damaged gills to uptake oxygen and found there was no impairment on any metabolic endpoint despite the increase in oxygen diffusion distance, which had previously been assumed. Oxygen consumption adjustments such as changes to blood or cardiac physiology may have compensated for effects of contaminate exposure and even improved the oxygen uptake ability, but this needs to be further investigated (Dussault *et al.*, 2001). There is evidence that fishes have

excess lamellae that are not perfused unless required, which is called lamellar recruitment (Nilsson, 2007). Lamellar recruitment is controlled by stress hormones such as cortisol and catecholamines (McDonald, Cavdek and Ellis, 1991). It is possible that the fishes are increasing lamellar perfusion, thereby increasing oxygen uptake ability due to an increase in functional surface area of the gills, in combination with other adjustments (Holbert, Boland and Olson, 1979). This theory also helps to explain the increase in ion pump expression seen, as the perfused surface becomes an area of ion loss and so adjustment may be necessary to maintain salt balance homeostasis. Exposure to ammonia may cause competition for sodium during ion regulation, which may also explain the increase in ion pump expression (Wright and Wood, 2012). Thus, gill physiological endpoints are useful in monitoring water quality but are not necessarily an indicator of how fishes are acclimating to their environment (Flores-Lopes and Thomaz, 2011).

4.2 Recommendations & Future work

Overall, this study provides evidence for species-specific differences in responses to contaminants and stressors in the Grand River. It is important to consider that not all species, even if closely related, will react the same to environmental stressors. It is therefore important to not over interpret data from one species and assume that the same response will occur in other species in the same way. Sentinel species are useful subjects because there are often more studies that can be used to synthesize information about their health, but it cannot be assumed that if a sentinel organism is doing well that the ecosystem is also adjusted (Barrett and Munkittrick, 2010). There is a difficulty in extrapolating effects seen at lower levels of biological organization to higher, ecological level effects because of the plasticity of organisms and the evolution of physiological resistance, which can cause adaptive or maladaptive changes to a population (see Saaristo et al., 2018).

Behavioural studies may give more information on metabolic costs of living in effluent. Although similar routine metabolic rates are seen here, a change in behaviour such as less aggression or movement could be enough to offset the cost of detoxification (Garcia-Reyero *et al.*, 2011; Melvin, 2016). The liver and kidneys, that also process contaminants, present alternative organs to study as they may display different responses to effluent than the gills (Liney *et al.*, 2006; de la Torre, Salibián and Ferrari, 2007; Nawata *et al.*, 2007; Marjan *et al.*, 2017). Additionally, population studies to determine genetic adaptation to chronic contaminant exposure could be useful in learning the ability of fishes to adapt to unideal environments (Medina, Correa and Barata, 2007; Saaristo *et al.*, 2018).

Due to the complexity of interactions between contaminants, along with changes in physical water conditions, predicting the effects of effluent exposure on fish populations is difficult and therefore, comments on the results are limited. This is due to differences in life history, behaviour, physiology, morphology and biochemistry (Brown *et al.*, 2004). Here, I have presented some evidence of urban and agricultural non-point sources affecting populations. Overall, darters displayed some physiological adjustments to living downstream to the MWWTP, but there was minimal evidence of progressive maladaptive traits specifically due to effluent exposure. It is recommended that future studies further incorporate the possible effects of urbanization, agricultural run-off and other point and non-point sources of contamination on the fish populations of the Grand River in the Waterloo Region.

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