IMPACTS OF WASTEWATER ON AQUATIC BIOTA IN SUMMER AND WINTER

IMPACTS OF WASTEWATER ON AQUATIC BIOTA IN SUMMER AND WINTER

BY HOSSEIN MEHDI, M. Sc

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TITLE: Impacts of wastewater on aquatic biota in summer and winter

AUTHOR: Hossein Mehdi, M.Sc. (University of Waterloo)

SUPERVISOR: Dr. Sigal Balshine, B.Sc. (University of Toronto), Ph.D. (Cambridge University)

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ABSTRACT

Municipal wastewater treatment plant (WWTP) effluents are a substantial source of aquatic pollution, continuously introducing complex mixtures of contaminants into aquatic environments. While the effects of wastewater effluents have been observed across all levels of biological organization, the impacts on populations and communities remain relatively understudied. Additionally, despite the ubiquity of wastewater effluents and their continuous year-long release, their impacts have rarely been studied during winter. In my thesis, I examined the effects of municipal wastewaters on fish communities (**Chapter 2**), benthic macroinvertebrate communities (**Chapter 3**), and zooplankton communities (**Chapter 4**) along the effluent gradients of the Dundas and Woodward WWTPs in summer and winter. Furthermore, I examined the effects of wastewater exposure on the physiology and behaviour of fathead minnow (*Pimephales promelas*) under simulated summer (20°C) and winter (4°C) conditions (**Chapter 5**). In **Chapter 2**, I showed that fish abundance, species richness, and species diversity were generally highest at sites closest to the WWTP outfalls, but only during winter. I also showed that fish community compositions differed greatly along the effluent gradients of both WWTPs and in both seasons, with community divergence being highest in sites closets and farthest from the WWTP outfalls. In **Chapter 3**, I demonstrated that the effects of wastewater are not generalizable across different WWTPs and are likely dependent on the effluent-receiving environments. I found that at the larger WWTP (Woodward) with its more industrial/urban receiving environment, benthic macroinvertebrate abundance was higher and diversity was lower at sites downstream of the outflow compared to upstream sites in both seasons; whereas the opposite was true for the smaller WWTP (Dundas) with its wetland receiving environment. Community composition differed significantly along the effluent gradients of both WWTPs and in both seasons, with sites closest and farthest from the outfalls being the most dissimilar. In **Chapter 4**, I showed that zooplankton communities were numerically dominated by rotifers in both summer and winter. Furthermore, rotifer abundance was highest near the outfalls of both WWTPs, especially during winter. At the Woodward WWTP, macrozooplankton abundance, richness, and diversity all increased with distance from the outfall in the summer; while in the winter, the opposite trends were observed for abundance and richness. At the Dundas WWTP, only macrozooplankton richness increased with distance from the outfall, and this was observed only during winter. Similar to fish and benthic macroinvertebrate communities, zooplankton community composition differed along the effluent gradients of both WWTPs, with communities closest and farthest from the outfalls being the most distinct. **In Chapter 5**, I demonstrated that temperature plays a key role in modulating the effects of wastewater exposure on fathead minnow physiology and behaviour. Wastewater exposure at 20°C was associated with increased standard metabolic rate, increased haematocrit, and reduced boldness. Whereas exposure to wastewater at 4°C was only associated with reduced sociability. Altogether, my findings suggest that wastewater plumes are a significant source of nutrient enrichment and thermal pollution, particularly in winter. These factors likely make wastewater outfalls an ecological trap for fishes and other aquatic organisms in winter, where the deceptively favourable and attractive conditions are quickly outweighed by the costs of exposure to the various contaminants found in wastewater. I believe this thesis highlights the importance of conducting ecotoxicological research in winter, as it can reveal ecological surprises that would have otherwise been left uncovered.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

DECLARATION OF ACADEMIC ACHIEVMENT

This dissertation is organized in a sandwich format as approved by McMaster University. It consists of six chapters and six appendices. **Chapter 1** introduces the concerns of wastewater contamination around the world and its potential impacts on wild aquatic animals, especially across seasons; it also introduces the idea of an "*ecological trap*" and how wastewater outfalls may act as one. **Chapters 2** and **3** are published manuscripts. **Chapter 4** is a manuscript in preparation for submission. **Chapter 5** is a published manuscript. **Chapter 6** provides a discussion and synthesis of **Chapters 2 – 5**. Appendix A is a published manuscript that provides details on the fish sampling methodology used in **Chapter 2**. Appendix B provides preliminary data for a manuscript in preparation. Appendices C – F provide supplementary material for **Chapters 2 – 5**.

Chapter 1: General introduction

Author: Hossein Mehdi

Chapter 2: Municipal wastewater as an ecological trap: Effects on fish communities across seasons

Authors: Hossein Mehdi, Samantha C. Lau, Caitlyn Synyshyn, Matthew G. Salena, Erin S. McCallum, Melissa N. Muzzatti, Jennifer E. Bowman, Kyle Mataya, Leslie M. Bragg, Mark R. Servos, Karen A. Kidd, Graham R. Scott, Sigal Balshine

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Comments: HM, MRS, KAK, GRS, and SB conceived of this study. HM collected the data, and SCL, CS, MGS, MNM, JEB, KM, LMB, and SB assisted in data collection. HM analyzed the data and ESM assisted with data analysis. HM wrote the manuscript with input from SB.

Chapter 3: Impacts of wastewater treatment plants on benthic macroinvertebrate communities in summer and winter

Authors: Chelsea Aristone, Hossein Mehdi, Jonathan Hamilton, Kelly L. Bowen, Warren J.S. Currie, Karen A. Kidd, Sigal Balshine

Publication: *Science of the Total Environment*, *820*, 153224

Comments: CA and HM contributed equally to this manuscript and are co-first authors. HM, KLB, WJSC, KAK, and SB conceived of this study. CA, HM, and JH collected the data. CA and HM analyzed the data. CA and HM wrote the manuscript with input from SB.

Chapter 4: The impacts of wastewater effluent on zooplankton communities in summer and winter

Authors: Hossein Mehdi, Markelle E. Morphet, Hailey Schultz, Chelsea Aristone, Anittha Thayaparan, Kelly L. Bowen, Warren J.S. Currie, Karen A. Kidd, and Sigal Balshine

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Comments: HM, KLB, WJSC, KAK, and SB conceived of this study. HM, MEM, HS, CA, and AT collected the data. HM analyzed the data. HM wrote the manuscript with input from SB.

Chapter 5: Temperature modulates the impacts of wastewater exposure on the physiology and behaviour of fathead minnow

Authors: Hossein Mehdi, Markelle E. Morphet, Samantha C. Lau, Leslie M. Bragg, Mark R. Servos, Joanne L. Parrott, Graham R. Scott, Sigal Balshine

Publication: *Chemosphere*, *294*, 133738

Comments: HM, LMB, MRS, JLP, GRS, and SB conceived of this study. HM, MEM, SCL, and LMB collected the data. HM analyzed the data. HM and MEM wrote manuscript with input from SB.

Chapter 6: General discussion

Author: Hossein Mehdi

Appendix A: A comparison of passive and active gear in fish community assessments in summer versus winter

Authors: Hossein Mehdi, Samantha C. Lau, Caitlyn Synyshyn, Matthew G. Salena, Markelle E. Morphet, Jonathan Hamilton, Melissa N. Muzzatti, Erin S. McCallum, Jonathan D. Midwood, Sigal Balshine

Publication: *Fisheries Research*, *242*, 106016

Comments: HM and SB conceived of this study. HM, SCL, CS, MGS, MEM, JH, MNM, and SB collected the data. HM analyzed data. HM wrote the manuscript with input from ESM, JDM, and SB. This appendix chapter also includes the supplementary information.

Appendix B: The impacts of wastewater effluent on fish, benthic macroinvertebrates, and zooplankton biomass in summer and winter

Author: Hossein Mehdi

Appendix C: Supplementary materials for Chapter 2

Authors: Hossein Mehdi, Samantha C. Lau, Caitlyn Synyshyn, Matthew G. Salena, Erin S. McCallum, Melissa N. Muzzatti, Jennifer E. Bowman, Kyle Mataya, Leslie M. Bragg, Mark R. Servos, Karen A. Kidd, Graham R. Scott, Sigal Balshine

Appendix D: Supplementary materials for Chapter 3

Authors: Chelsea Aristone, Hossein Mehdi, Jonathan Hamilton, Kelly L. Bowen, Warren J.S. Currie, Karen A. Kidd, Sigal Balshine

Appendix E: Supplementary materials for Chapter 4

Authors: Hossein Mehdi, Markelle E. Morphet, Hailey Schultz, Chelsea Aristone, Anittha Thayaparan, Kelly L. Bowen, Warren J.S. Currie, Karen A. Kidd, and Sigal Balshine

Appendix F: Supplementary materials for Chapter 5

Authors: Hossein Mehdi, Markelle E. Morphet, Samantha C. Lau, Leslie M. Bragg, Mark R. Servos, Joanne L. Parrott, Graham R. Scott, Sigal Balshine

Chapter 1: General Introduction

... وَجَعَلْنَا مِنَ الْمَاءِ كُلَّ شَيْءٍ حَيٍّ - (القرآن الكريم ٢١:٣٠)

...by means of water we give life to everything - (Quran 21:30)

1.1 Motivation for thesis

Water being the source of all living things on earth is by no means an exaggerated statement; therefore, understanding how this valuable resource is impacted by anthropogenic disturbance is of vital importance. That is why I dedicated my PhD research to exploring the impacts of one of the largest sources of pollution on aquatic ecosystems – municipal wastewater treatment plant (WWTP) effluents (Environment Canada, 2001; Holeton et al., 2011; Hamdhani et al., 2020). Municipal wastewater refers to any used water coming from residential, commercial, and some industrial activities. In many developed countries, this used water often flows into a municipal WWTP where it undergoes a series of treatment processes before being discharged in a treated form back into watersheds. Ideally, treatment facilities reduce the amount of pollutants and contaminants found in incoming influents. Under suboptimal conditions, such as when treatment facilities malfunction, sewage overflows, or even when no treatment facility exists (as in many parts of the world including numerous communities in Northern and Atlantic Canada), untreated wastewater is discharged into inland and coastal waters (Environment Canada, 2001). However, even in communities where there is access to treatment facilities, effluents released can impact aquatic biota across multiple levels of organization (Holeton et al., 2011; Hamdhani et al., 2020).

Municipal wastewater effluents are of special concern to aquatic environments for a variety of reasons. First, despite treatment, conventional WWTPs are not capable of removing or fully degrading many contaminants from incoming influents, and as a result, effluents released subject aquatic environments to persistent levels of pollution in the form of excess nutrients, pesticides, metals, pharmaceuticals and personal care products (PPCPs), detergents, and plastic by-products (Daughton and Ternes, 1999; Kolpin et al., 2002; Ternes et al., 2004; Holeton et al., 2011; McCormick et al., 2016). Exposure to such contaminants, even at the low concentrations typically detected in receiving waters, can impact aquatic organisms across all levels of biological organization, spanning from molecular initiating events to population- and community-wide effects (Holeton et al., 2011; Hamdhani et al., 2020). A second cause of concern stems from the sheer volumes and the continuous release of wastewater effluents into receiving environments. In Canada for example, ~14.4 million cubic metres of treated effluent is discharged daily, making it the largest source of aquatic pollution by volume (Environment Canada 1999, 2001). This trend is only expected to increase; as urban population growth increases, so too will the reliance on WWTPs (Luthy et al., 2015). The continuous release of effluents at such high volumes subjects aquatic ecosystems to chronic persistent exposures, as contaminants are continuously introduced into aquatic systems (Jones et al., 2005; Jelić et al., 2012). The third reason for concern is that despite the continuous release of wastewater effluents all-year long, wastewater impacts have rarely been investigated across different seasons. Much of our understanding on the impacts of wastewater effluents on aquatic ecosystems comes solely from research conducted during warmer periods of the year, when most field work occurs. This knowledge gap is further exacerbated by the fact that winter can be a dominant season in many parts of the world (e.g., Canada), with its effects lasting between 4-8 months of the year. Furthermore, the quality of wastewater effluents can vary significantly across seasons; effluents released during winter contain higher concentrations of PPCPs, higher nitrogenous waste products, and higher oxygen demand due to poorer degradation at colder temperatures (Vieno et al., 2005; Sui et al., 2011; Yu et al., 2013; Kot-Wasik et al., 2016). Taken together, I believe these concerns call for more ecotoxicological research to be conducted across seasons, particularly in winter.

During my PhD research, I studied the impacts of wastewater effluents on aquatic communities, including fishes (**Chapter 2**), benthic macroinvertebrates (**Chapter 3**), and zooplankton (**Chapter 4**) in both summer and winter. A comprehensive methodological approach was also explored in my thesis regarding how fish communities ought to be sampled in shallow wetland and creek receiving environments in summer and winter (**Appendix A**). Finally, I was interested in exploring some of the costs that fish may face when exposed to wastewater under two controlled acclimation temperatures, 20°C and 4°C, simulating summer and winter conditions, respectively (**Chapter 5**). In the remainder of this introductory chapter, I briefly review the theoretical background underlying my PhD research projects, which are described in greater detail in **Chapters 2 – 5**. I also describe where and how my studies were conducted as well as how my study area can serve as a useful case study for other research conducted in anthropogenically disturbed environments.

1.2 Theoretical background

1.2.1 What is wastewater?

In Canada, especially in larger municipalities, wastewater undergoes multiple levels of treatment before getting discharged back into receiving environments. Wastewater treatment plants (WWTPs) can be roughly categorized into one of three levels of treatment – primary, secondary, or tertiary (Environment Canada 2001). Primary treatment refers to the removal of large debris as well as smaller organic and inorganic material. Wastewater is first passed through grated screens to remove large debris. Later, heavier organic solids (sludge) that settle by gravity are removed and pumped out for further treatment. Effluent from primary treatment is then transferred to a secondary treatment stage, where wastewater is mixed with known populations of bacteria and aerated heavily, thereby allowing bacterial digestion to occur. In some cases, an additional level of treatment is used to remove additional dissolved and/or suspended solids through physical, chemical, or biological treatment processes. This level of treatment (i.e., tertiary or advanced treatment) varies in its form across plants and depends greatly on what pollutants are being targeted (e.g., phosphorus or ammonia). Lastly, before the final effluent is released into receiving waterbodies, it often gets disinfected (e.g., using chlorine, ultraviolet radiation, ozone) to reduce its pathogenicity. Despite significant investments and substantial improvements in wastewater treatment technologies, effluents from municipal WWTPs remain a major stressor in aquatic environments as they still contain a complex mixture of nutrients, pesticides, metals, micro- and macroplastics, pharmaceuticals and personal care products (PPCPs), as well as natural and synthetic hormones; Daughton and Ternes, 1999; Kolpin et al., 2002; Ternes et al., 2004; Holeton et al., 2011; McCormick et al., 2016).

1.2.2 Biological responses to wastewater

Wastewater effluent and its constituents can impact fish and other aquatic organisms across multiple levels of biological organization (Holeton et al., 2011; Hamdhani et al., 2020). Fish collected from effluent-dominated environments or exposed to wastewater in laboratory settings often show signs of molecular disruption represented by altered gene expression, male feminization and poor fertilization success, impaired stress responsiveness, elevated metabolic rates and altered energetic substrate levels, irregular courtship and aggressive behaviours, as well as morphological abnormalities (Saaristo et al., 2014; Bahamonde et al., 2015; Fuzzen et al., 2015; Du et al., 2018, 2019; McCallum et al., 2017; McLean et al., 2019; Mehdi et al., 2018; Nikel et al., 2021). While the impacts of wastewater effluent have been well established on the organismal level, comparatively, few studies have addressed how wastewater may impact aquatic populations and communities despite the importance of these endpoints when evaluating risk and assessing the health of impacted watersheds (Fausch et al., 1990; Cvetkovic et al., 2010). The status of aquatic populations and communities is a reflection of the environmental conditions that they reside in; as such higher-level endpoints provide a holistic assessment of environmental health (Munkittrick and Dixon, 1989; Clements and Newman, 2002). The use of community ecology to assess risk in impacted environments has numerous benefits that may not be attainable through conventional direct measures taken at the individual level. Aquatic organisms (e.g., fishes and benthic macroinvertebrates) vary in their sensitivity, tolerance, and resilience to environmental stressors; thereby, the presence and absence of certain taxa allows us to infer if certain environmental conditions are met or not. Further, populations and communities of fish and other aquatic organisms can provide us with a more integrative assessment of ecosystem health as communities are reliant on many components of an ecosystem (e.g., inter- and intraspecific interactions, abiotic conditions, habitat characteristics). Additionally, by examining entire aquatic communities, researchers can gauge the effects of environmental disturbances through a cumulative and long-term lens, rather than a snapshot approach provided by other toxicological measures (e.g., laboratory exposures, survival analysis). Furthermore, ecotoxicological effects at the population, community, and ecosystem levels are often the targets of interest of environmental protection agencies. Consequently, the ecological and societal relevance and value of such endpoints is higher than endpoints more commonly employed at the individual scale. However, despite the value and strength of these ecologically-relevant endpoints, obtaining them can be challenging, as the nature of acquiring the necessary data can be exceptionally complex, time consuming, costly, and often has low reproducibility (Kraak and Roessink, 2021). Moreover, the complexity involved in resolving the cause of patterns observed at the population or community level can make it increasingly difficult to link observable ecological effects to a specific stressor/driver (e.g., a specific contaminant or a specific abiotic factor; Clements and Newman, 2002). As such, it is rather unsurprising that ecotoxicological effects at the population, community, and ecosystem levels are understudied, but definitely not undervalued.

1.2.3 Importance of seasonality in ecotoxicological research

As stated above, most of our knowledge regarding the impacts of wastewater effluents comes from research conducted during warmer months of the year. Prior research has shown that the chemical profile of wastewater effluents can vary significantly between seasons. For example, Yu et al., (2013) showed that occurrence and concentration of 14 endocrine disrupting compounds (EDCs) and PPCPs in the influent and effluent of five WWTPs in southern California, USA were considerably higher in winter than in summer. Similarly, Sui et al., (2011) demonstrated that the occurrence and concentration of various

PPCPs were also higher in winter compared to other seasons in both the influent and effluent of two WWTPs in Beijing, China. The observed seasonal differences of chemical concentrations in wastewater influents and effluents are often attributed to the increased human consumption of PPCPs during winter and to the poorer degradation of wastewater contaminants in winter (Yu et al. 2013). Yet, despite these known significant seasonal differences in effluent quality of municipal WWTPs, seasonality is rarely considered in ecotoxicological research.

1.2.4 Can wastewater plumes act as ecological traps?

In its broadest sense, an ecological trap occurs when organisms cannot rely on environmental cues to accurately assess the true quality of an environment and instead make maladaptive decisions based on these cues (Dwernychuk and Boag 1972; Schlaepfer et al., 2002). Ecological traps arise when there is a sudden mismatch between the true quality of a habitat and the cues that are emitted by that habitat. These sudden mismatches often happen due to an anthropogenic disturbance, thereby rendering cues that were once reliable and honest, useless, or false (Tinbergen 1958; Levins 1968; Williams and Nichols, 1984; Schlaepfer et al., 2002). In such cases, an organism can be “trapped” by their poor choices resulting in reduced fitness (Schlaepfer et al., 2002). An illustrative example comes from the West Indian Manatee (*Trichechus manatus*), where during winter, these animals follow thermal cues in search of warm habitats ($\geq$20°C). However, their search of warmer habitats can lead them to warm effluents discharged from power plants – such effluents create deceptively enticing habitats that can leave manatees stranded in inhospitable waters during maintenance periods of these power plants (Shane 1984; Packard et al., 1989).

Wastewater effluents can alter receiving water temperatures by as much ~10°C in winter, potentially creating enticing environments for fishes and other aquatic organisms to seek refuge in when environments elsewhere may seem suboptimal (Environment Canada, 2001; Kinouchi et al., 2007). Temperature, however, is not the only factor that creates this deceptively enticing environment. The continuous influx of nutrients from municipal WWTPs can create environments that are extremely productive, potentially leading to higher food availability near WWTPs. High nutrient concentrations downstream of WWTPs may support productivity and growth of phytoplankton and invertebrate populations – making these areas attractive for fishes and other aquatic organisms, especially during winter, when food is scarce and difficult to encounter elsewhere (Sommer et al., 1986; Byström et al., 2006; Hurst, 2007; Holeton et al., 2011). The higher productivity found in effluent-dominated environments has previously been associated to higher abundance of fishes as well as increased growth, body mass, and body condition of fishes caught downstream (Chambers et al., 1997; McMaster et al., 2005; Brown et al., 2011; Tetreault et al., 2011; Lazaro-Côté et al., 2021; Nikel et al. 2021). The combination of a steady supply of nutrients and thermal enhancement in effluent-receiving environments likely creates an ecological trap for aquatic organisms, as these environments may be perceived by individuals as beneficial or favourable, but are also a major source of contamination, potentially posing detrimental costs to reproduction and survival (Schlaepfer et al., 2002; Battin, 2004; Holeton et al., 2011).

1.3 Study sites

For my PhD research, I examined the effects of wastewater effluent on the community structure of fishes, benthic macroinvertebrates, and zooplankton. I focused on two distinct municipal WWTPs (Dundas and Woodward WWTPs, both described in detail in **Chapters 2 – 4**). The Dundas and Woodward WWTPs discharge their effluents into Hamilton Harbour, a designated Area of Concern by the International Joint Commission (Great Lakes Water Quality Agreement, 2012). Historically, Hamilton Harbour has been subjected to pollution from the once booming steel and iron industries. Today, while the harbour still suffers from industrial contamination of the past as well as the present, it also is at the receiving end of effluents from three municipal WWTPs (Dundas, Woodward, and Burlington Skyway WWTPs; Fig 1.1) as well as urban runoff from the cities of Hamilton and Burlington.

The Dundas WWTP serves a population of ~41,000 and is characterized as a conventional activated sludge facility with tertiary sand filtration with chlorine disinfection. The Woodward WWTP is a secondary conventional activated sludge plant that serves ~480,000 people in Hamilton, Stoney Creek, and Ancaster, Canada; it has a daily capacity of 409 million litres, making it the largest WWTP in

Hamilton (City of Hamilton, 2019). At the time of writing this thesis, the Woodward WWTP was undergoing several upgrades to improve its treatment capabilities and daily treatment capacity including elevating its treatment capabilities from secondary to tertiary level, as well as reducing the levels of phosphorus, ammonia, and suspended solids in its effluent. The upgrades to the Woodward WWTP come at a cost of $340 million, representing the largest investment for the City of Hamilton's Clean Harbour Program, while also signifying the importance and magnitude of wastewater treatment in urban populations (City of Hamilton, 2022). Despite ongoing remediation efforts in Hamilton Harbour, effluents from municipal WWTPs continue to be one of its largest stressors, where by today's estimates, approximately 50% of the harbour's non-lake inflows are of wastewater origin (Lawrence et al., 2004; Government of Canada, 2017). The research highlighted in my thesis will provide a pre-upgrade baseline and be especially relevant to environments impacted by wastewater pollution, like those in the Hamilton Harbour watershed.

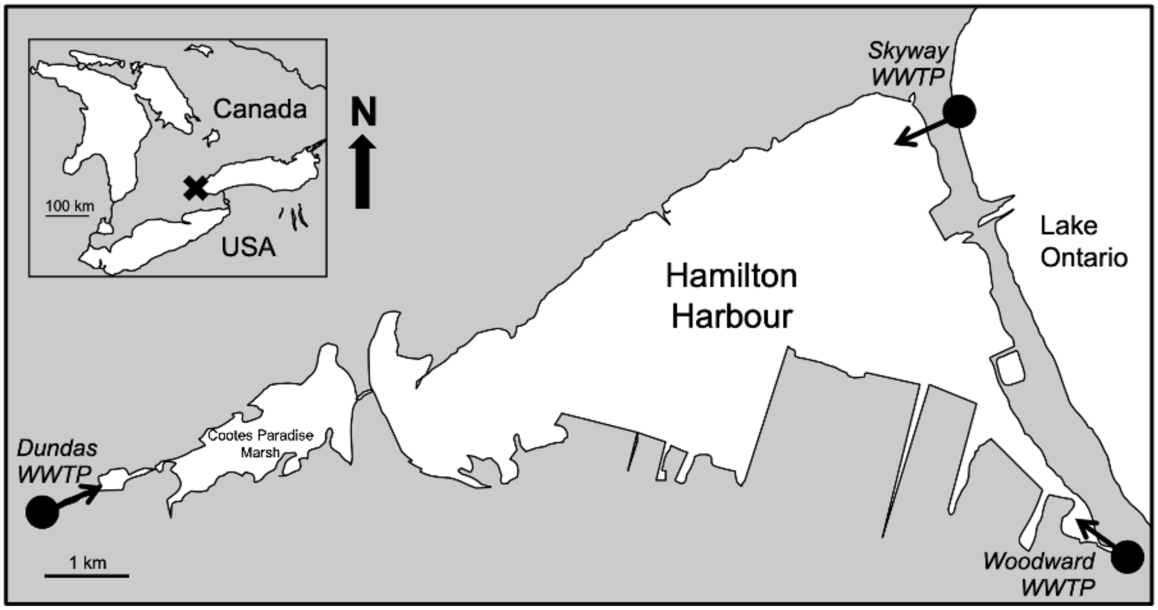

Figure 1.1: Map of Hamilton Harbour, ON, Canada (marked with X) to show its relevant geographical location to the Laurentian Great Lakes. The black circles identify the locations of the three WWTPs that discharge their effluents into Hamilton Harbour: Dundas, Woodward, and Skyway WWTPs. Map was adapted from J. R. Marentette (2011) and E.S. McCallum (2016).

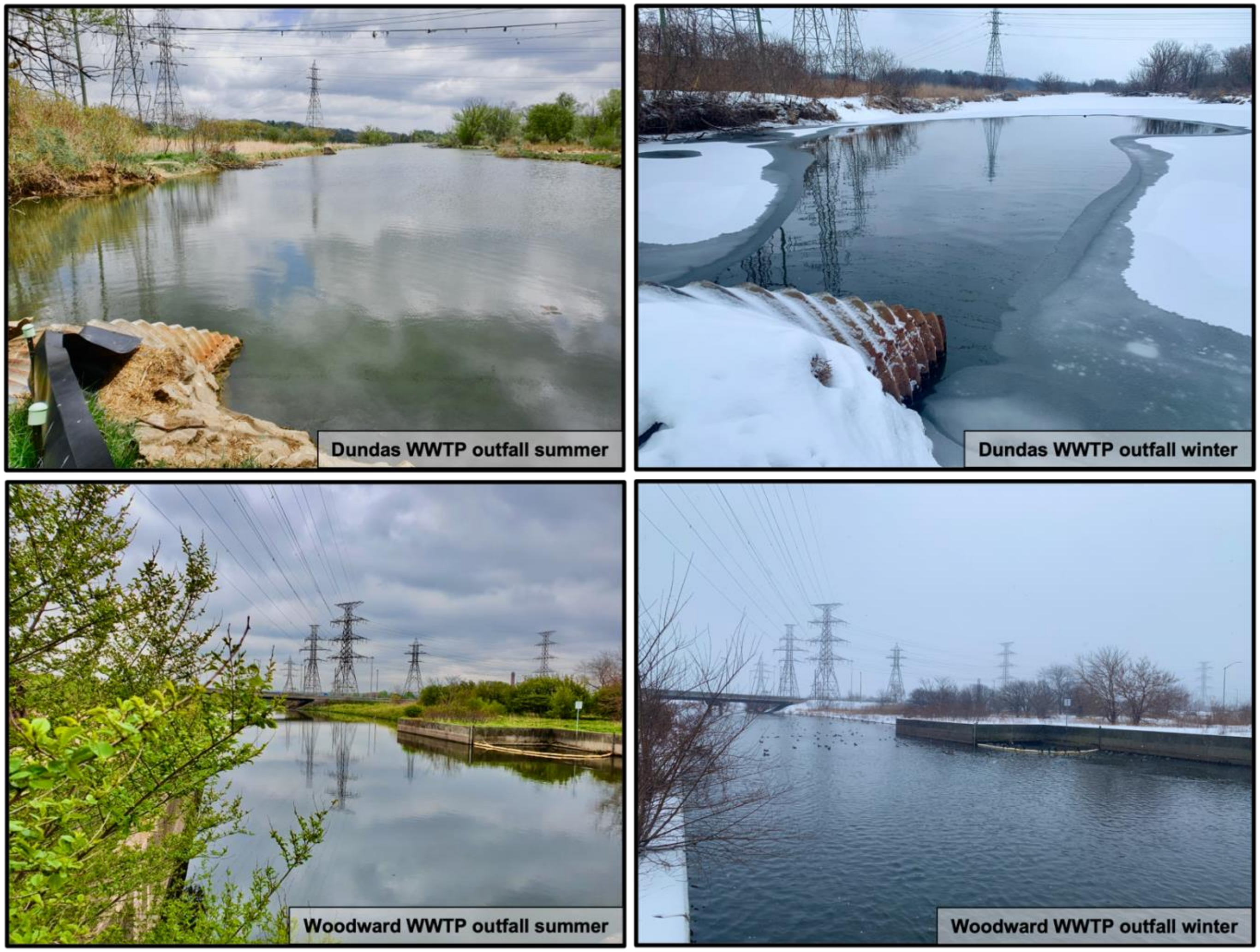

Figure 1.2. Photographs of the outfall sites at the Dundas and Woodward WWTP in summer and winter 2022. Photo credit: H. Mehdi (January 24, 2022) and S. Zarini (May 19, 2022).

1.4 Aims of the thesis

The specific aims of my thesis are as follows:

1) To identify the potential impacts of wastewater effluents on fish communities and fish prey communities in summer and winter.
2) To explore how fish, benthic macroinvertebrate, and zooplankton abundance, family richness, family diversity, and community composition vary along the effluent gradients stemming from the Dundas and Woodward WWTP in summer and winter.
3) To assess the effect of wastewater effluents on various water quality parameters in summer and winter. This is supplemented by a characterization of various contaminants of concern in the effluents of the Dundas and Woodward WWTP in both seasons.
4) To assess the effects of wastewater effluent exposure on fathead minnow physiology and behaviour under lab-controlled simulated summer (20°C) and winter (4°C) conditions.

1.5 Thesis structure

Here, I provide a brief overview of the structure of my thesis, briefly outlining the focus of each chapter. In **Chapter 2**, I assessed the impacts of wastewater effluent on fish communities in summer and winter. I also explored key differences in site water quality and effluent quality between seasons. In **Chapter 3**, I built on the previous chapter by exploring how wastewater effluents affect benthic macroinvertebrate communities in summer and winter. In **Chapter 4**, I further investigated how another prey community, zooplankton, are affected by wastewater contamination in summer and winter. In **Chapter 5**, I investigated the impacts of whole wastewater effluent exposure on fathead minnow physiology and behaviour under two lab-controlled temperatures, simulating summer and winter conditions. Finally in **Chapter 6**, I present a conclusion of my thesis where I reviewed and synthesized the major findings of my data chapters. I also proposed potential avenues for future research.

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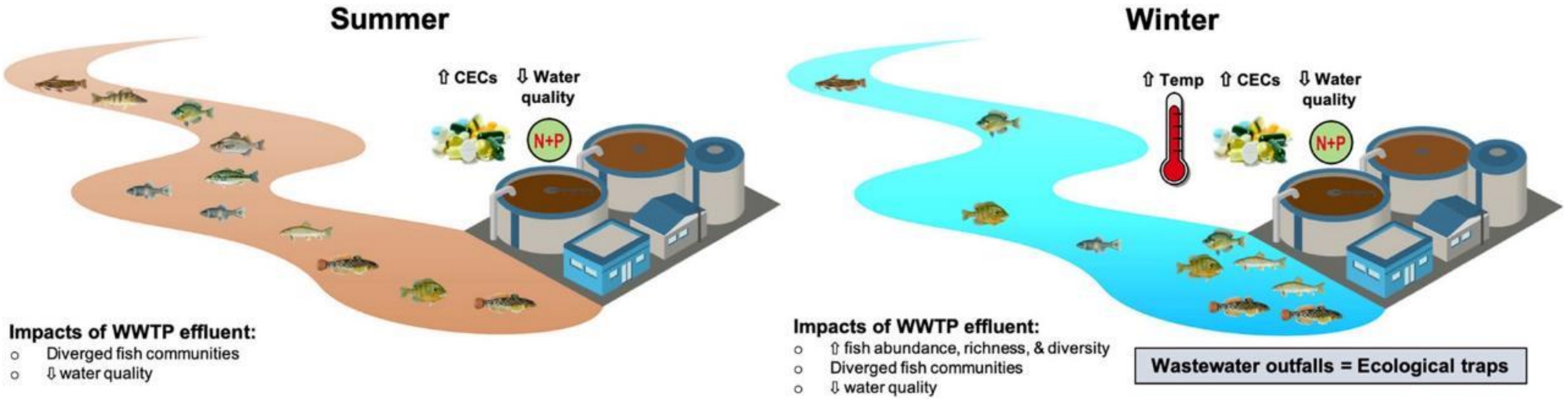
Chapter 2: Municipal wastewater as an ecological trap: Effects on fish communities across seasons



2.1 Abstract

Municipal wastewater treatment plant (WWTP) effluents are a ubiquitous source of contamination whose impacts on fish and other aquatic organisms span across multiple levels of biological organization. Despite this, few studies have addressed the impacts of WWTP effluents on fish communities, especially during the winter—a season seldom studied. Here, we assessed the impacts of wastewater on fish community compositions and various water quality parameters during the summer and winter along two effluent gradients in Hamilton Harbour, an International Joint Commission Area of Concern in Hamilton, Canada. We found that fish abundance, species richness, and species diversity were generally highest in sites closest to the WWTP outfalls, but only significantly so in the winter. Fish community compositions differed greatly along the effluent gradients, with sites closest and farthest from the outfalls being the most dissimilar. Furthermore, the concentrations of numerous contaminants of emerging concern (CECs) in the final treated effluent were highest during the winter. Water quality of sites closer to the outfalls was poorer than at sites farther away, especially during the winter. We also demonstrated that WWTPs can significantly alter the thermal profile of effluent-receiving environments, increasing temperature by as much as ~9 °C during the winter. Our results suggest that wastewater plumes may act as ecological traps in winter, whereby fish are attracted to the favourable temperatures near WWTPs and are thus exposed to higher concentrations of CECs. This study highlights the importance of winter research as a key predictor

in further understanding the impacts of wastewater contamination in aquatic ecosystems.

2.2 Introduction

Municipal wastewater treatment plant (WWTP) effluents, although usually treated, still contain a complex mixture of chemicals, including but not limited to excess nutrients, pesticides, metals, micro- and macroplastics, pharmaceuticals and personal care products (PPCPs), as well as natural and synthetic hormones (Daughton and Ternes, 1999; Kolpin et al., 2002; Ternes et al., 2004; Holeton et al., 2011; McCormick et al., 2016). As a result, effluents from WWTPs are a major source of environmental stressors in aquatic environments, accounting for the largest point source of contamination in Canada, by volume (Environment Canada, 2001; Holeton et al., 2011). Their continuous release into watersheds can significantly impair aquatic environments and ecosystems via chronic exposure of biota to contaminants of emerging concern (CECs), oxygen depletion, eutrophication, and even physical changes to habitats (e.g., alterations in flow, turbidity, and thermal properties; Holeton et al., 2011; Tetreault et al., 2011; Hamdhani et al., 2020).

The impacts of wastewater effluent exposure on aquatic organisms are observed across multiple levels of biological organization. Such impacts include endocrine disruption, represented by severe incidences of intersex, reduced androgen levels, and reduced fertilization success (Bahamonde et al., 2015;

Fuzzen et al., 2015). Exposure to wastewater effluents also has metabolic and behavioural effects, demonstrated by an increase in metabolic rate (Du et al., 2018, Du et al., 2019; Mehdi et al., 2018) and irregular courtship and aggressive behaviours (Saaristo et al., 2014; McCallum et al., 2017a; McLean et al., 2019). Comparatively, few studies have examined the impacts of wastewater effluent exposure at higher levels of biological organization, such as population- and community-level responses. This is surprising, given how relevant such impacts are for evaluating risks. For example, one common wastewater constituent, 17α-ethynylestradiol, has been linked to the collapse of a fathead minnow (Pimephales promelas) population and several ecosystem shifts in a whole-lake experiment (Kidd et al., 2007; Kidd et al., 2014). Other studies have also linked wastewater exposure to higher rates of male feminization and reduced breeding success in fishes, implying potential consequences to population sustainability (Jobling et al., 1998; Harris et al., 2011; Bahamonde et al., 2015; Fuzzen et al., 2015). Wastewater effluent can also reduce species richness and promote the dominance of tolerant and/or invasive species (Ra et al., 2007; Yeom et al., 2007; Brown et al., 2011; Tetreault et al., 2012; McCallum et al., 2019). Therefore, population and community disruptions from wastewater effluent may be extensive, but few other studies have examined fish communities in effluent-dominated environments.

In addition to the paucity of studies on fish communities, our knowledge about population- and community-level effects of WWTP effluents is derived solely from research conducted during warmer months of the year. It is unknown if and how the impacts of wastewater are exacerbated or diminished by colder temperatures. This is of vital concern for a number of reasons. First, in many temperate and polar regions of the globe, winter is a dominant season, lasting 4–8 months. Understanding the effects of WWTP effluents during such a prolonged season is of critical importance. Second, WWTPs often produce effluent of poorer quality in the winter; this is partly due to the higher incidence of human ailments that increase usage of PPCPs during the winter, and the reduced effectiveness of biological degradation of contaminants in WWTPs in colder temperatures (Vieno et al., 2005; Sui et al., 2011; Yu et al., 2013; Kot-Wasik et al., 2016). Third, wastewater effluent can be a source of thermal pollution—altering the temperature of receiving environments by as much as 5–10 °C in the winter (Environment Canada, 2001; Kinouchi et al., 2007; Mehdi et al., 2019). These changes in the thermal profile of aquatic environments may significantly alter aquatic communities, as ectothermic organisms (e.g., fish) are attracted to such thermally-enhanced environments, especially during the winter, when temperatures elsewhere may be suboptimal (Coutant, 1987; Cooke et al., 2004). The thermal plumes created by WWTPs may act as ecological traps (Schlaepfer et al., 2002; Battin, 2004) because fishes may use such environments as thermal refugia during the winter, accentuating exposure to contaminants in wastewater effluent.

In this study, we examined the influence of seasonality by assessing the impacts of wastewater effluent on fish communities near two WWTPs during the summer and winter. The two WWTPs chosen are located on the eastern- and western-most ends of Hamilton Harbour, one of 43 Areas of Concern under the Great Lakes Water Quality Agreement (Great Lakes Water Quality Agreement, 2012). Wastewater contamination is a stressor that is of particular concern in Hamilton Harbour, as it is estimated that ~50% of its inflow comes from wastewater (Government of Canada, 2017). We examined various fish community indices along a distance- and therefore contamination-gradient from the two plants to assess the impacts of wastewater exposure on the integrity of these respective ecosystems. In addition, water quality parameters and the concentrations of various PPCPs and other anthropogenic compounds in the final treated effluent were characterized to assess the abiotic impacts of wastewater contamination. We predicted that wastewater inputs would have significant effects on the physical habitat of effluent-receiving environments, thus affecting fish communities in these impacted sites. We expected this to be more apparent during the winter, when the quality of the effluent is expected to decline. If fish seek thermal refuge near WWTP outflows, then we predicted these sites would be highest in fish abundance, species richness, and species diversity, and be most compositionally distinct from sites farther away, particularly during the winter.

2.2 Methods

2.2.1 Sampling Sites

2.2.1.1 Dundas WWTP

The Dundas WWTP is located on the western tip of Cootes Paradise Marsh, the largest wetland west of Lake Ontario. The treatment facility is rated as a conventional activated sludge plant with tertiary filtration. The facility treats the majority of wastewater from the Dundas population (~30,000 people) and has a capacity of 18.2 million litres per day (City of Hamilton, 2019). The plant's effluent is discharged into the Desjardins Canal, an old shipping corridor located on the western-most end of Cootes Paradise Marsh (Theysmeyer and Bowman, 2017). Five sites, varying in distance from the effluent outflow, were sampled in 2018 and 2019. Site selection was based on a previous study (McCallum et al., 2019) and accessibility in both summer and winter. Of the sites sampled, three were in the direct flow path of the effluent discharged from the WWTP: D1 was immediately adjacent to the outflow, and D2 and D3 were 550 and 1000 m downstream, respectively (Fig. 2.1A). In addition, two reference sites were also sampled: D4, located at the mouth of Spencer Creek which was 2800 m downstream, and D5, located at the southwestern edge of Cootes Paradise Marsh which was 3750 m downstream; neither reference site was in the direct flow path of the wastewater effluent (Fig. 2.1A). All sites were sampled four times in the summer (July to August) and three times in the winter (November to March).

2.2.1.2 Woodward WWTP

The Woodward WWTP is located on the southeastern corner of Hamilton Harbour, ON, Canada. The Woodward WWTP is the largest WWTP in Hamilton, serving ~480,000 people and has an average daily capacity of 409 million litres (City of Hamilton, 2019). The facility is a secondary conventional activated sludge plant with sludge dewatering and digestion (City of Hamilton, 2019). Effluent from the plant is released into Red Hill Creek which flows into Hamilton Harbour. Similar to Dundas, five sites were sampled in 2018 and 2019 (Fig. 2.1B). Three sites were in the direct flow path of the effluent: W1 which was immediately adjacent to the outflow, and W2 and W3, which were 350 and 850 m downstream, respectively. Two upstream reference sites were also sampled: W4 and W5 which were 1000 and 1400 m upstream, respectively. Similar to the Dundas WWTP, sites were selected based on accessibility during both seasons. All sites were sampled five times in the summer (July to August) and three times in the winter (December to March).

2.2.2 Fish sampling

Fish communities were surveyed both in the summer and winter on weather permitting days (i.e., days free of storms and ice cover), using passive (minnow and Windermere traps) and active (electrofishing from a boat) sampling gear. At each site, 10 minnow traps, baited with ~20 g of corn, were set ~10 m apart from each other and deployed from land. Two Windermere traps, each baited with ~100 g of corn, flanking the first and last minnow traps, were also deployed. Minnow and Windermere traps were retrieved 24 h post-deployment. In addition, two 50 m-transects were electrofished at each site (1.5-KVA Electrofisher, Smith-Root Inc.). All fish caught were transported in dark-coloured bins with aerators to a field site where they were identified to species level, measured (total and standard lengths), and weighed. To speed up processing and reduce handling stress, the first 15 individuals of a given species, sampling technique, and site were individually measured, while the remaining individuals caught of that species, site, and sampling technique were identified to species level, counted, and then batch-weighed. Native fish were immediately released back to their site of collection, while invasive fish were euthanized with an overdose of benzocaine (small fishes) or by a lethal cephalic blow (large fishes), as required by the Ontario Ministry of Natural Resources (2015). All fish were handled in accordance with approved animal use protocols from McMaster University's Animal Research Ethics Board (AUP 17-12-45).

2.2.3 Water quality, chemistry, and habitat characterization

At each site, the following water quality parameters were measured simultaneously to fish sampling: temperature and dissolved oxygen (YSI ProODO), pH, conductivity, total dissolved solids, and salinity (Oakton multiparameter Testr). Furthermore, 1 L water samples were collected from the middle of the water column at each site using a 2.2 L Van Dorn sampler. Water samples were analyzed for ammonia + ammonium, nitrate, nitrite, o-phosphate, total phosphorus, and total Kjeldahl nitrogen by the City of Hamilton Environmental Laboratory using methods previously described in McCallum et al. (2019). Long-term water temperature was also measured for 14 days by deploying temperature data loggers (HOBO Pendant MX Temp) at the outfall sites and one of the reference sites (D4 and W4) for both WWTPs during the summer (September 2018) and winter (December 2019).

Additionally, 24 h composite samples of final effluent were collected from both the Dundas and Woodward WWTPs twice a week during the months of August 2019 (summer; nDundas = 7, nWoodward = 8) and December 2019 (winter; nDundas = 8, nWoodward = 8). The following water quality parameters were measured in the composite effluent samples: total suspended solids, biochemical oxygen demand, total phosphorus, total Kjeldahl nitrogen, ammonia, nitrate, nitrite, and Escherichia coli (measurements provided by City of Hamilton Environment Laboratory, see Supplementary Table 1 for these values). Composite effluent samples were further analyzed for a wide range of PPCPs and other CECs using already established methods by Arlos et al. (2015). Briefly, solid phase extraction was used to concentrate the compounds found in the composite effluent samples into 500 µL extracts. Extracts were then analyzed using an Agilent 1260 HPLC with 6460 triple quad mass spectrometer (LC-MS/MS) with Agilent Jet Stream (AJS) electrospray ionization in both positive and negative modes. Twenty-one different compounds of nine different classes were analyzed: lipid regulators, antiepileptics, analgesics, stimulants, antibacterials, antibiotics, antidepressants, non-steroidal anti-inflammatory agents (NSAIDs), and herbicides. Detailed analysis parameters are described in Supplementary Tables 2–5.

The habitat characteristics of each sampling site were assessed following protocols developed in a previous study (McCallum et al., 2019) and were based on a subset of metrics used in the Qualitative Habitat Evaluation Index (Taft and Koncelik, 2006; Strickland et al., 2010). The following parameters were assessed: total water depth, water clarity (Secchi disk), substrate type, sediment particle size, shoreline slope, degree of sinuosity, degree of anthropogenic modifications, riparian zone width, degree of estimated bank erosion, and the presence of any aquatic plants (see Supplementary Tables 6 and 7).

2.2.4 Statistical analysis

All statistical analyses were conducted using R (version 3.6.2, R Core Team, 2019). Prior to any analysis of fish communities, counts of species were mean-standardized (multi-gear mean standardization) following methods outlined in Gibson-Reinemer et al. (2017) and McCallum et al. (2019), as this allowed for data from different gear types to be analyzed together. Catch per unit effort (CPUE) was calculated on a per-trap deployed basis for minnow and Windermere traps and per-second shocked for electrofishing. All species, rare and common, were included in the final analyses. Fish abundance, species richness, and effective species diversity (Shannon-Wiener Index) were analyzed using permutation linear mixed effects models (PLMM) with 5000 iterations using the lme4 and predictmeans packages (Bates et al., 2015; Luo et al., 2020). These response variables were analyzed using sampling site (as a numeric factor representing both distance from outfall and contamination load) and season as main effects, and sampling period within each season as a random effect. Principal coordinate analysis (PCoA) was then performed using a Bray-Curtis dissimilarity matrix to assess fish community composition differences between sites and seasons. Beta diversity was then visually explored using PCoA biplots, with site-specific 80% confidence ellipses overlaid to delineate community composition differences (Oksanen et al., 2019). Fish community composition differences between sites and seasons were further analyzed with a permutation ANOVA with 5000 permutations using adonis2 (Vegan package; Oksanen et al., 2019). Furthermore, similarity percentages (SIMPER) analysis was used to identify which key species were driving the differences observed in the community assemblages between the outfall sites and all other sampling sites. While all species were included in the analysis, only those that contributed ≥5% of the total abundance were further interpreted. Permutation tests (n = 5000) were used to identify between-site significant differences in CPUE of key species. Indicator species analysis (De Cáceres and Legendre, 2009) was then used to identify which species were representative or indicative of sites closest (D1-D2 and W1-W2) and farthest (D4-D5 and W4-W5) from the outfall in both seasons. Permutation tests (n = 5000) were carried out to determine the significance of these particular species as indicators.

To reduce the number of analyses needed and for ease of visualization, water quality parameters were plotted and analyzed collectively using principal component analysis (PCA). Overall sampling site differences in water quality parameters, as expressed by the first two principal components, were analyzed using a permutation ANOVA with 5000 permutations using adonis2 (Vegan package; Oksanen et al., 2019). Differences in individual water quality parameters were tested as a function of site and season using a permutation MANOVA with 5000 iterations. Long-term water temperature data were analyzed using linear models (LM) to test for site differences within each season. Finally, a permutation MANOVA was used to analyze the chemical makeup of the composite effluent samples. All 21 compounds that were measured to characterize the effluent were analyzed simultaneously to test for seasonal differences (lmPerm, Wheeler and Torchiano, 2016). For statistical purposes, compounds that were below the detection limit were analyzed as zeros. All analyses were conducted separately between each WWTP. Data are reported as means ± standard error (SE) unless otherwise stated, and in all analyses, a difference was deemed significant when $p < 0.05$.

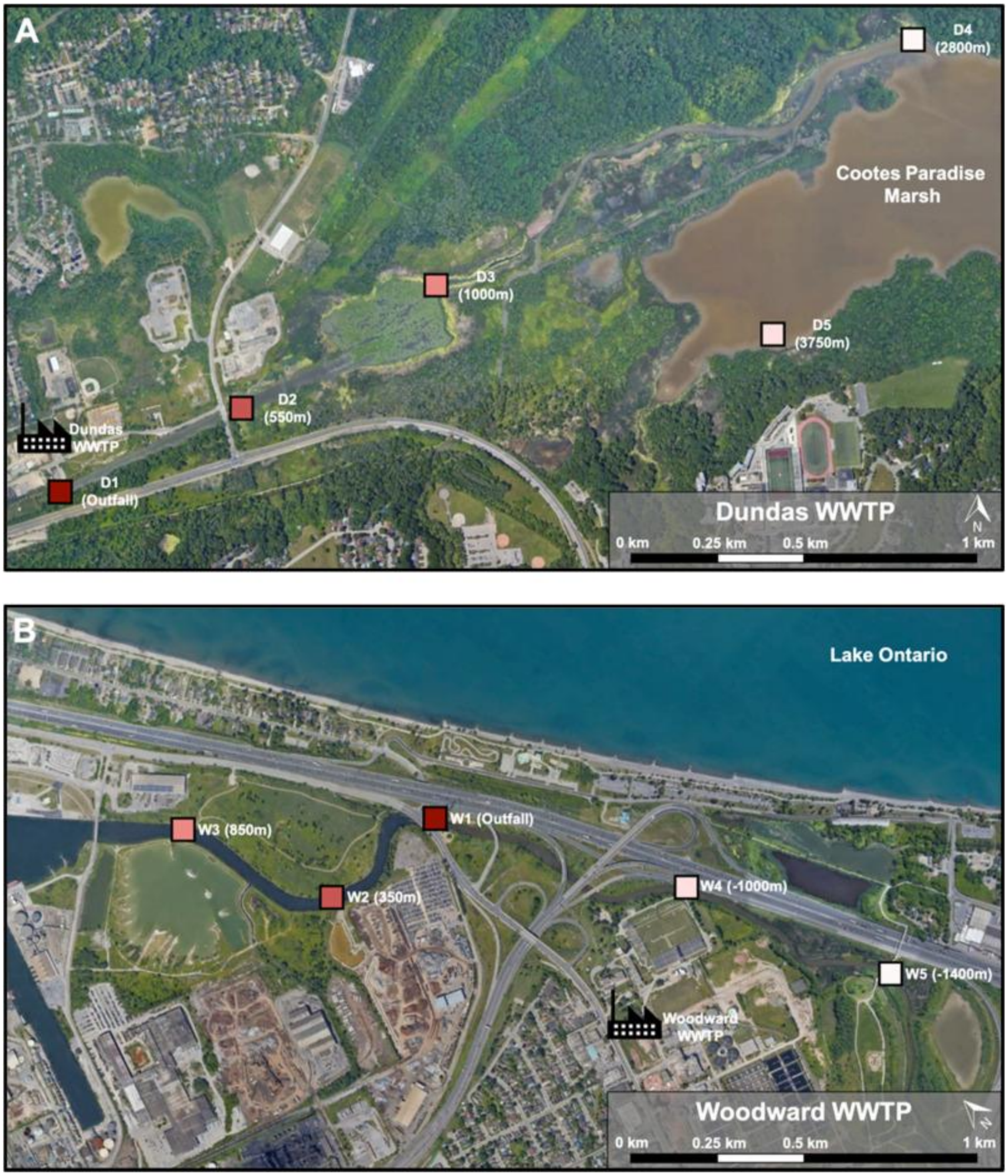


Figure 2.1. Map of our sampling sites along a distance gradient from the (A) Dundas and (B) Woodward WWTPs. The location of each WWTP is also displayed. Maps generated in Google Earth Pro 7.3.2.5776, imagery date 06/30/2018 and accessed on 24/02/2020.

2.3 Results

2.3.1 Fish community metrics

2.3.1.1 Dundas WWTP

At the Dundas WWTP sampling sites, 2388 fish were collected (2112 in the summer and 276 in the winter) consisting of 23 unique species (Supplementary Table 8). Overall, fish were less abundant (based on CPUE) in the winter than in the summer (PLMM, $t = -4.63$, $p < 0.01$; Fig. 2.2A). In the winter, abundance declined with distance from the outfall ($t(\text{Winter}) = -3.13$, $p < 0.01$; Fig. 2.2A); however, no such decline was observed in the summer ($t(\text{Summer}) = 0.83$, $p = 0.39$; Fig. 2.2A). Similarly, species richness was greatly reduced in the winter ($t = -4.08$, $p < 0.001$; Fig. 2.2B). As with abundance in the winter, sites closer to the outfall were more species-rich than those farther away ($t(\text{Winter}) = 3.95$, $p < 0.001$; Fig. 2.2B); however, in the summer, species richness was not linked to proximity to the outfall ($t(\text{Summer}) = 0.52$, $p = 0.58$; Fig. 2.2B). Effective species diversity (Shannon-Wiener index) was lower in the winter than in the summer ($t = -4.39$, $p < 0.01$), and declined with distance from the outfall during the winter ($t(\text{Winter}) = -3.24$, $p < 0.01$) but not in the summer ($t(\text{Summer}) = -1.31$, $p = 0.65$). The tolerance, resilience, trophic level, and the proportion of non-native to native species of fishes caught across sites and seasons are described in the Supplementary Materials.

Fish community composition differed significantly between seasons (Permutation ANOVA, $F(\text{Season}) = 3.33$, $p < 0.001$; Fig. 2.3A and B). In the summer, fish community compositions differed significantly across sites (F(Summer) = 3.13, $p < 0.001$; Fig. 2.3A). Communities at sites closest to the outfall were most different from those farthest away, while there was considerable overlap in the intermediate sites (PCoA; Fig. 2.3A). In the winter, fish communities were separated in a manner similar to in the summer, where sites closer to the outfall were most different than those farther away (F(Winter) = 2.18, $p < 0.001$; Fig. 2.3B). Similarity analysis further highlighted which species were driving the community changes across sites (Table 2.1). In the summer, fish community differences were largely driven by round goby being consistently more abundant in D1 than in all other sites. Similarly, white sucker were more abundant in D1 than all other sites except D5. Green sunfish were more abundant in D1 than D3. Relative to D1, largemouth bass, yellow perch, and goldfish were more abundant in D2, D4, and D5, respectively. During the winter, fewer species appeared to be driving across-site community differences compared to during the summer. Round goby were again more abundant in D1 than in D2 and D3. Bluegill sunfish were more abundant in D1 compared to D2, while common logperch were more abundant in D4 compared to D1. See Supplementary Table 9 for further details on all site and season comparisons. Several indicator species were identified using indicator species analysis. During the summer, sites closer to the outfall were mostly identifiable by the presence of white sucker ($p < 0.01$) and round goby ($p < 0.001$), while sites farther away were more identifiable by the presence of bluegill sunfish ($p = 0.02$) and white perch ($p < 0.001$). During the winter, sites closer the outfall tended to be more identifiable by round goby ($p = 0.05$), green sunfish ($p = 0.05$) and largemouth bass ($p = 0.06$), while sites farther away were more identifiable by yellow perch ($p < 0.01$) and common logperch ($p < 0.001$).

2.3.1.2 Woodward WWTP

At the Woodward WWTP, across all sampling events, 1844 fish were caught (1546 in the summer and 298 in the winter) composed of 26 unique species (Supplementary Table 8). Overall, fish abundance was greater in summer than in winter ($t = 2.51$, $p = 0.049$; Fig. 2.2C). In the winter, fish abundance was highest at the outfall site and decreased with distance ($t(\text{Winter}) = -3.08$, $p = 0.01$; Fig. 2.2C). However, in the summer, abundance was not related to distance from the outfall ($t(\text{Summer}) = 0.001$, $p = 0.91$; Fig. 2.2C). Species richness was greater in summer compared to in winter ($t = 2.72$, $p = 0.03$; Fig. 2.2D) but was not correlated to proximity of the effluent outfall in either season ($t(\text{Summer}) = 1.05$, $p = 0.29$; $t(\text{Winter}) = 1.27$, $p = 0.22$; Fig. 2.2D). Effective species diversity did not show a seasonal pattern ($t = 1.52$, $p = 0.18$), nor was it related to proximity of the outfall in either season ($t(\text{Summer}) = 0.41$, $p = 0.69$; $t(\text{Winter}) = -0.58$, $p = 0.55$). The tolerance, resilience, trophic level, and the proportion of non-native to native species of fish caught across sites and seasons are described in the Supplementary Materials.

Fish community composition also varied significantly between seasons at the Woodward WWTP sampling sites (Permutation ANOVA, $F = 1.79$, $p < 0.001$;

Fig. 2.3C and D). Fish communities differed significantly across sites in the summer (F(Summer) = 1.97, $p < 0.001$ Fig. 2.3C), but not in the winter (F(Winter) = 1.04, $p = 0.40$; Fig. 2.3D). While there was significant overlap in the PCoA output in both seasons, separation of communities was more apparent in the summer, with sites closest and farthest from the outfall being the most dissimilar (Fig. 2.3C and D). Community assemblages were further examined using SIMPER analysis to investigate which species were driving the changes in composition (Table 2.1). During the summer, brook stickleback were more abundant in W1 than in W2 and W3. Spottail shiner were more abundant in W1 than in W3, while W3 had more white sucker than W1. During the winter, cross-site community differences were absent, apart from round goby being more abundant in W1 than in W3. See Supplementary Table 10 for further details on all site and season comparisons. During the summer, indicator species analysis revealed that sites closer to the outfall were only significantly identifiable by brook stickleback ($p < 0.001$), while sites farther away were more identifiable by common carp ($p = 0.02$) and pumpkinseed sunfish ($p < 0.01$). During the winter, only gizzard shad ($p = 0.01$) were identifiable of sites closer to the outfall, while no indicator species were detected elsewhere.

2.3.2 Water quality parameters and habitat characteristics

2.3.2.1 Dundas WWTP

Water quality parameters varied along the effluent gradient in both seasons (Permutation ANOVA, F(Summer) = 3.58, $p < 0.001$, F(Winter) = 5.05, $p < 0.001$; Table 2.2; Fig. 2.4A and B; see Supplementary Table 11 for PCA loadings). Water quality differences were most apparent between sites closer to the outfall and those farthest away in both seasons (PCA; Fig. 2.4A and B). Overall, water temperature, soluble reactive phosphorus, and total phosphorus were higher in the summer; in contrast, pH, conductivity, salinity, total dissolved solids, total ammonia nitrogen, and total nitrate nitrogen were higher in the winter. In the summer, conductivity, total dissolved solids, salinity, total nitrate nitrogen, and total nitrogen decreased with distance from the outfall, while water temperature, pH, and soluble reactive phosphorous increased with distance from the outfall. In the winter, water temperature, conductivity, salinity, total dissolved solids, total ammonia nitrogen ($p = 0.06$), total nitrate nitrogen, total nitrogen, and total phosphorus decreased with distance from the outfall, while dissolved oxygen, and pH increased with distance from the outfall (all contrasts were $p < 0.05$, unless otherwise stated). During the summer, longer-term temperature data were not different between the outfall (D1) and reference (D4) sites (Linear Model, LM, t(Summer) = 1.49; $p = 0.15$; Fig. 2.5A), while in the winter, there was a striking difference between the two sites (t(Winter) = 25.25; $p < 0.001$; Fig. 2.5B), with the outfall site being on average ~ 8 °C warmer than the reference site. See Table 2.2 for further details on all water quality parameters.

The two sites closest to the outfall (D1 and D2) were the most anthropogenically-disturbed. These sites have been heavily impacted with clear modifications to the shoreline and are in close proximity to urban structures. In comparison, sites D3 – D5 appeared to be less disturbed with little to no human modifications. These sites are surrounded with wetland habitats and natural forests, and are farther away from urban structures. Further habitat characteristics are presented in Supplementary Table 6.

2.3.2.2 Woodward WWTP

Water quality parameters varied greatly between the upstream and downstream sites of the Woodward WWTP outflow during both seasons (Permutation ANOVA, F(Summer) = 2.51, $p < 0.01$; F(Winter) = 3.17, $p = 0.01$; Table 2.2; Fig. 2.4C and D; see Supplementary Table 11 for PCA loadings). Reduction of water quality parameters into the first two principal components revealed that sites closer to the outfall clustered together and were more distinct than sites upstream (Fig. 2.4C and D). Of all the water quality parameters, only water temperature was higher in the summer than in winter; whereas pH, conductivity, total dissolved solids, salinity, total ammonia nitrogen ($p = 0.09$) were all higher in the winter. In the summer, total nitrate nitrogen, total nitrogen, soluble reactive phosphorus, and total phosphorus decreased significantly with distance from the outfall, while the reverse trend was true for water temperature and pH. In the winter, water temperature, total ammonia nitrogen ($p = 0.08$), total nitrate nitrogen, total nitrogen, soluble reactive phosphorus, and total phosphorus decreased with distance from the outfall, whereas dissolved oxygen showed the opposite pattern (all contrasts were $p < 0.05$, unless otherwise stated). Similar to the Dundas WWTP, longer-term surface water temperatures were not significantly different between the outfall (W1) and reference (W4) sites during the summer (LM, t(Summer) = 0.42; p = 0.68; Fig. 2.5C), while in the winter, the outfall site was on average ~ 9 °C warmer than the reference site (t(Winter) = 22.30; $p < 0.001$; Fig. 2.5D). See Table 2.2 for further details on all water quality parameters.

All sites near the Woodward WWTP were anthropogenically disturbed with clear modifications to the shoreline, close proximity to urban structures, and relatively narrow riparian zones. Sites upstream (W4 and W5) however, appeared to be marginally less disturbed. While vegetation surrounding the water was different between seasons, all other habitat characteristics remained relatively unchanged between seasons. Further habitat characteristics are presented in Supplementary Table 7.

2.3.3 Effluent CECs characterization

2.3.3.1 Dundas WWTP

In the summer effluents, 16 out of the 21 compounds analyzed were detected at least once, while in the winter, all compounds were detected (Table 2.3). Overall, gemfibrozil, atorvastatin, p-hydroxy atorvastatin, o-hydroxy atorvastatin (lipid regulators and metabolites), carbamazepine (antiepileptic), and caffeine (stimulant) were detected at higher concentrations in the winter. Acetaminophen (analgesic) was only detected once during the winter and never in the summer. Antibacterials showed mixed patterns, where concentrations of triclosan were higher in the winter, while the opposite was true for sulfamethazine. Two of the four antibiotics analyzed (trimethoprim and sulfamethoxazole) were detected at higher concentrations in the winter. Monensin was detected only twice in the winter and not at all in the summer, whereas lincomycin was not detected in the winter and was only detected once in the summer. Fluoxetine, norfluoxetine, venlafaxine, and desvenlafaxine (antidepressants and metabolites) were all detected at higher concentrations in the winter. Two of the three nonsteroidal anti-inflammatory drugs analyzed (naproxen and diclofenac) were detected at higher concentrations in the winter, whereas ibuprofen concentrations were too variable to detect any significant differences between seasons. Finally, atrazine (herbicide) was detected at higher concentrations in the summer.

2.3.3.2 Woodward WWTP

The effluent characteristics from the Woodward WWTP displayed distinct seasonal patterns. While the same 20 out of the 21 compounds analyzed were detected in both seasons, the concentrations varied considerably between the summer and winter (Table 2.3). Three of the four lipid regulators and metabolites analyzed were detected at higher concentrations in the winter (atorvastatin, p-hydroxy atorvastatin, and o-hydroxy atorvastatin); however, gemfibrozil was not significantly different between seasons. Neither carbamazepine (antiepileptic) nor acetaminophen (analgesic) varied significantly between seasons, while caffeine (stimulant) was detected at higher concentrations in the winter. Similar to the effluent from the Dundas WWTP, concentrations of antibacterials showed mixed patterns. Triclosan was detected at higher concentrations in the winter, while sulfamethazine was higher in summer. Of the four antibiotics analyzed, only lincomycin was detected at higher concentrations in the summer, while concentrations of sulfamethoxazole and trimethoprim did not significantly differ between seasons. Monensin was not detected in either season. Both antidepressants, fluoxetine and venlafaxine, were detected at higher concentrations in the summer, however, the concentrations of both metabolites (norfluoxetine and desvenlafaxine) were higher in the winter. Two of the three non-steroidal anti-inflammatory drugs analyzed (ibuprofen and naproxen) were detected at higher concentrations in the winter, while diclofenac concentrations were not significantly different between seasons. Finally, atrazine (herbicide) was detected at higher concentrations in the summer.

Table 2.1. Similarity percentages (SIMPER) analysis showing the contribution of key species to the overall dissimilarity of the outfall site relative to all other sampling sites. Average A and Average B represent the gear-standardized catch per unit effort (abundance) for each species at the pair of sites being compared. Only species that contributed $\geq$ 5% to the overall abundance are shown. Bolded averages indicate significant differences ($p < 0.05$). See Supplementary Tables 9 and 10 for further details on all site and season comparisons.

	Dundas WWTP									
	Summer					Winter				
Comparison	Total dissimilarity	Species	Average A	Average B	Contribution	Total dissimilarity	Species	Average A	Average B	Contribution
A. D1 (Outfall) B. D2 (550 m)	93.01%	*Neogobius melanostomus* (Round goby)	**1.39**	**0.09**	**27.0**	95.51%	*Neogobius melanostomus* (Round goby)	**0.46**	**0.01**	**20.0**
		Ameiurus nebulosus (Brown bullhead)	0.0004	0.86	13.8		*Ameiurus nebulosus* (Brown bullhead)	0.04	0.13	17.2
		Micropterus salmoides (Largemouth bass)	**0.32**	**0.46**	**10.2**		*Lepomis cyanellus* (Green sunfish)	0.14	0.05	12.9
		Carassius auratus (Goldfish)	0.001	0.3	9.67		*Lepomis macrochirus* (Bluegill sunfish)	**0.15**	**0.11**	**11.1**
		Scardinius erythrophthalmus (Rudd)	0.07	0.3	8.82		*Dorosoma cepedianum* (Gizzard shad)	0	0.89	6.86
		Lepomis cyanellus (Green sunfish)	0.16	0.03	7.26		*Scardinius erythrophthalmus* (Rudd)	0	0.05	6.69
		Catostomus commersonii (White sucker)	**0.59**	**0.001**	**5.43**					
A. D1 (Outfall) B. D3 (1000 m)	95.65%	*Neogobius melanostomus* (Round goby)	**1.39**	**0**	**25.7**	97.79%	*Neogobius melanostomus* (Round goby)	**0.46**	**0**	**21.2**
		Carassius auratus (Goldfish)	0.001	0.68	13.1		*Ameiurus nebulosus* (Brown bullhead)	0.04	0.03	10.8
		Ameiurus nebulosus (Brown bullhead)	0.0004	0.63	9.28		*Lepomis macrochirus* (Bluegill sunfish)	0.15	0.05	10.6
		Lepomis cyanellus (Green sunfish)	**0.16**	**0.01**	**8.41**		*Scardinius erythrophthalmus* (Rudd)	0	0.16	10.2
		Catostomus commersonii (White sucker)	**0.59**	**0.04**	**7.10**		*Lepomis cyanellus* (Green sunfish)	0.14	0	9.07
		Dorosoma cepedianum (Gizzard shad)	0	0.05	6.40		*Pimephales promelas* (Fathead minnow)	0.01	0.05	7.41
		Micropterus salmoides (Largemouth bass)	0.32	0.18	5.96					
		Perca flavescens (Yellow perch)	0.06	0.09	5.16					
A. D1 (Outfall) B. D4 (2800 m)	94.53%	*Neogobius melanostomus* (Round goby)	**1.39**	**0.04**	**25.7**	97.50%	*Percina caprodes* (Common logperch)	**0.003**	**0.15**	**27.6**
		Perca flavescens (Yellow perch)	**0.06**	**0.12**	**10.7**		*Neogobius melanostomus* (Round goby)	0.46	0	22.4
		Lepomis macrochirus (Bluegill sunfish)	0.02	0.33	10.6		*Lepomis macrochirus* (Bluegill sunfish)	0.15	0	9.82
		Ameiurus nebulosus (Brown bullhead)	0.0003	0.17	7.98		*Lepomis cyanellus* (Green sunfish)	0.14	0	9.11
		Lepomis cyanellus (Green sunfish)	0.16	0.03	6.71		*Amia calva* (Bowfin)	0	0.02	6.82
		Catostomus commersonii (White sucker)	**0.59**	**0.01**	**6.31**					
		Scardinius erythrophthalmus (Rudd)	0.07	0.15	6.21					
A. D1 (Outfall) B. D5 (3750 m)	97.93%	*Neogobius melanostomus* (Round goby)	**1.39**	**0.01**	**20.0**	98.97%	*Perca flavescens* (Yellow perch)	**0**	**0.32**	**24.4**
		Ameiurus nebulosus (Brown bullhead)	0.0003	0.96	16.5		*Ameiurus nebulosus* (Brown bullhead)	0.04	0.34	19.6
		Lepomis macrochirus (Bluegill sunfish)	0.002	3.24	16.2		*Neogobius melanostomus* (Round goby)	0.46	0	19.4
		Carassius auratus (Goldfish)	0.001	0.36	9.37		*Lepomis macrochirus* (Bluegill sunfish)	0.15	0.001	9.47
		Morone americana (White perch)	**0**	**0.93**	**8.44**		*Lepomis cyanellus* (Green sunfish)	0.14	0	8.08
		Scardinius erythrophthalmus (Rudd)	0.07	0.22	7.59					
		Lepomis cyanellus (Green sunfish)	0.16	0.12	5.45					

	Woodward WWTP									
	Summer					Winter				
Comparison	Total dissimilarity	Species	Average A	Average B	Contribution	Total dissimilarity	Species	Average A	Average B	Contribution
A. W1 (Outfall) B. W2 (350 m)	89.24%	*Culaea inconstans* (Brook stickleback)	**0.70**	**0.36**	**20.1**	83.50%	*Neogobius melanostomus* (Round goby)	0.60	0.31	26.2
		Neogobius melanostomus (Round goby)	0.68	0.13	18.5		*Lepomis cyanellus* (Green sunfish)	0.55	0.14	21.4
		Lepomis cyanellus (Green sunfish)	0.05	0.18	10.9		*Lepomis macrochirus* (Bluegill sunfish)	0.32	0.23	11.6
		Pimephales promelas (Fathead minnow)	1.62	0.08	8.31		*Lepomis gibbosus* (Pumpkinseed)	0.06	0.20	7.31
		Catostomus commersonii (White sucker)	0.03	0.02	6.57		*Ameiurus nebulosus* (Brown bullhead)	0.06	0.23	5.49
		Lepomis macrochirus (Bluegill sunfish)	0.06	0.02	5.36					
		Notropis hudsonius (Spottail shiner)	3.55	0	5.22					
A. W1 (Outfall) B. W3 (850 m)	93.07%	*Culaea inconstans* (Brook stickleback)	**0.70**	**0.18**	**17.5**	89.73%	*Neogobius melanostomus* (Round goby)	**0.60**	**0.20**	**29.9**
		Neogobius melanostomus (Round goby)	0.68	0.01	17.4		*Lepomis cyanellus* (Green sunfish)	0.55	0.10	24.8
		Lepomis cyanellus (Green sunfish)	0.05	0.72	15.3		*Lepomis macrochirus* (Bluegill sunfish)	0.32	0.0004	10.2
		Catostomus commersonii (White sucker)	**0.03**	**0.08**	**8.63**		*Culaea inconstans* (Brook stickleback)	0	0.06	7.11
		Pimephales promelas (Fathead minnow)	1.62	0.24	8.04					
		Notropis hudsonius (Spottail shiner)	**3.55**	**0.15**	**6.6**					
		Carassius auratus (Goldfish)	0.0003	0.23	5.12					
A. W1 (Outfall) B. W4 (-1000 m)	90.40%	*Neogobius melanostomus* (Round goby)	0.68	0.87	21.6	90.23%	*Lepomis cyanellus* (Green sunfish)	0.55	0.20	26.3
		Lepomis macrochirus (Bluegill sunfish)	0.06	0.47	13.9		*Neogobius melanostomus* (Round goby)	0.60	0.08	23.0
		Culaea inconstans (Brook stickleback)	0.70	0	11.7		*Lepomis macrochirus* (Bluegill sunfish)	0.32	0.17	12.9
		Pimephales promelas (Fathead minnow)	1.62	0.17	10.3		*Notropis atherinoides* (Emerald shiner)	0.10	0.02	6.25
		Lepomis cyanellus (Green sunfish)	0.05	0.23	6.7					
		Lepomis gibbosus (Pumpkinseed)	0	0.57	6.21					
		Notropis hudsonius (Spottail shiner)	3.55	0.06	5.26					
A. W1 (Outfall) B. W5 (-1400 m)	89.03%	*Neogobius melanostomus* (Round goby)	0.68	0.41	23.8	90.10%	*Neogobius melanostomus* (Round goby)	0.60	0.12	23.9
		Culaea inconstans (Brook stickleback)	0.70	0	11.8		*Lepomis cyanellus* (Green sunfish)	0.55	0.05	20.3
		Lepomis macrochirus (Bluegill sunfish)	0.06	0.30	11.2		*Lepomis macrochirus* (Bluegill sunfish)	0.32	0.08	15.8
		Lepomis cyanellus (Green sunfish)	0.05	0.37	10.2		*Pimephales promelas* (Fathead minnow)	0.15	0.17	10.6
		Pimephales promelas (Fathead minnow)	1.62	0.13	8.2		*Lepomis gibbosus* (Pumpkinseed)	0.06	0.08	5.72
		Notropis hudsonius (Spottail shiner)	3.55	0.08	5.51					

Table 2.2. Mean (± SE) of water quality parameters taken at each sampling site across both seasons (summer | winter). The following water quality parameters were measured: water temperature, dissolved oxygen saturation (DO), pH, conductivity, total dissolved solids (TDS), salinity, total ammonia nitrogen (TAN), total nitrate nitrogen (TNN), total nitrogen (TN), soluble reactive phosphorus (SRP), and total phosphorus (TP).

	Dundas WWTP				
	D1 (Outfall)	D2 (550 m)	D3 (1000 m)	D4 (2800 m)	D5 (3750 m)
Water temperature (°C)	21.1±0.679 | 9.97±0.578	23.3±0.578 | 7.67±0.689	24.2±0.702 | 4.87±1.24	23.7±1.12 | 3.23±0.667	26.7±1.22 | 3.60±1.56
DO (%)	124±6.73 | 91.1±4.99	134±12.0 | 98.2±5.54	90.7±17.3 | 110.±1.63	87.0±7.64 | 101±1.54	132±24.3 | 108±2.41
pH	7.57±0.114 | 7.69±0.223	7.88±0.271 | 7.84±0.194	7.49±0.229 | 8.40±0.240	8.22±0.147 | 8.78±0.211	8.34±0.272 | 8.80±0.163
Conductivity (µS)	1190±45.9 | 1450±75.1	1150±46.6 | 1530±92.1	1130±74.7 | 1490±82.2	944±101 | 942±90.7	886±64.8 | 967±98.0
TDS (ppm)	845±34.1 | 1010±47.4	819±32.0 | 1090±64.7	807±82.9 | 1070±58.7	670.±70.8 | 670.±65.3	626±45.7 | 690.±70.1
Salinity (ppm)	563±29.3 | 719±34.6	544±20.7 | 756±38.1	537±30.8 | 732±28.7	441±44.6 | 462±59.3	414±27.0 | 476±60.4
TAN (mg/L)	0.030±0.006 | 0.507±0.437	0.046±0.011 | 0.223±0.159	0.060±0.020 | 0.137±0.058	0.060±0.039 | 0.043±0.015	0±0 | 0.020±0.010
TNN (mg/L)	15.4±0.766 | 17.1±1.17	11.3±1.82 | 16.1±1.02	4.84±1.57 | 11.6±1.29	0.493±0.314 | 1.07±0.195	0±0 | 0.690±0.205
TN (mg/L)	16.3±0.515 | 18.5±0.695	12.5±1.68 | 17.0±1.03	6.43±1.52 | 13.1±0.973	1.53±0.398 | 1.64±0.229	1.38±0.222 | 1.21±0.252
SRP (mg/L)	0±0 | 0±0	0±0 | 0±0	0.013±0.013 | 0±0	0.030±0.018 | 0±0	0.04±0.002 | 0±0
TP (mg/L)	0.117±0.020 | 0.079±0.010	0.117±0.020 | 0.079±0.015	0.183±0.034 | 0.071±0.003	0.118±0.007 | 0.065±0.019	0.194±0.054 | 0.052±0.003
	Woodward WWTP				
	W1 (Outfall)	W2 (350 m)	W3 (850 m)	W4 (-1000 m)	W5 (-1400 m)
Water temperature (°C)	21.7±0.371 | 11.3±0.954	22.2±0.250 | 10.7±0.832	22.7±0.425 | 9.77±0.291	23.3±0.473 | 4.13±1.07	23.3±1.28 | 4.43±1.08
DO (%)	74.2±2.60 | 69.0±1.26	69.4±4.47 | 68.9±0.666	63.3±4.10 | 67.3±2.87	75.4±24.5 | 92.9±2.53	93.6±13.0 | 95.6±0.088
pH	7.07±0.106 | 7.77±0.231	7.08±0.128 | 7.40±0.091	7.06±0.105 | 7.55±0.091	8.05±0.231 | 8.66±0.427	8.13±0.189 | 8.71±0.317
Conductivity (µS)	1170±43.5 | 2020±361	1150±53.2 | 2030±299	1150±56.9 | 1820±289	1150±208 | 2350±289	1110±221 | 2020±349
TDS (ppm)	828±30.9 | 1460±238	817±36.8 | 1440±219	815±40.7 | 1100±90.1	812±148 | 1670±206	780.±156 | 1600±274
Salinity (ppm)	540.±21.0 | 1010±175	534±25.2 | 1000±159	534±28.1 | 948±122	535±101 | 1150±142	550.±99.7 | 1110±180.
TAN (mg/L)	0.463±0.183 | 2.32±1.65	0.893±0.433 | 2.23±1.53	1.20±0.481 | 2.26±1.42	0.120±0.032 | 0.043±0.019	0.040±0.017 | 0.027±0.017
TNN (mg/L)	11.3±0.734 | 10.9±0.508	9.54±1.16 | 10.4±0.717	9.14±1.04 | 9.52±1.16	2.10±0.804 | 1.09±0.135	1.66±0.667 | 1.15±0.063
TN (mg/L)	13.1±1.03 | 16.1±3.21	12.2±1.41 | 15.1±3.66	12.2±1.76 | 14.6±4.01	2.81±0.868 | 1.50±0.094	2.62±0.959 | 1.54±0.093
SRP (mg/L)	0.228±0.048 | 0.187±0.052	0.228±0.055 | 0.187±0.058	0.228±0.058 | 0.193±0.058	0.028±0.016 | 0±0	0±0 | 0.023±0.023
TP (mg/L)	0.452±0.048 | 0.443±0.038	0.406±0.057 | 0.374±0.052	0.361±0.065 | 0.364±0.065	0.13±0.02 | 0.080±0.011	0.157±0.067 | 0.081±0.023

Table 2.3. Mean (± SE) concentrations in [ng/L] of various classes of chemicals measured in the final effluent of the Dundas and Woodward WWTPs in summer ($n_{Dundas} = 7$, $n_{Woodward} = 8$) and winter ($n_{Dundas} = 8$, $n_{Woodward} = 8$). Zeros indicate concentrations measured below detection limit. Bolded averages indicate a significant difference ($p < 0.05$) between seasons within each WWTP.

		Dundas WWTP		Woodward WWTP	
		Summer	Winter	Summer	Winter
Lipid regulator	Gemfibrozil	0 ± 0	**9.65 ± 2.80**	13.2 ± 3.42	9.76 ± 1.18
	Atorvastatin	28.2 ± 0.672	**305 ± 20.9**	23.3 ± 0.867	**292 ± 17.1**
	p-hydroxy Atorvastatin	160. ± 37.8	**484 ± 45.2**	126 ± 13.1	**425 ± 20.5**
	o-hydroxy Atorvastatin	18.3 ± 5.41	**480. ± 38.4**	46.7 ± 5.84	**431 ± 19.3**
Antiepileptic	Carbamazepine	293 ± 7.59	**406 ± 23.4**	310. ± 14.1	268 ± 20.0
Analgesic	Acetaminophen	0 ± 0	2.56 ± 2.56	30.1 ± 30.1	16.4 ± 6.33
Stimulant	Caffeine	15.8 ± 0.889	**212 ± 122**	218 ± 43.4	**494 ± 70.1**
Antibacterial	Triclosan	0 ± 0	**22.4 ± 1.75**	36.9 ± 3.88	**62.2 ± 2.99**
	Sulfamethazine	**3.61 ± 2.63**	0 ± 0	**64.0 ± 17.8**	7.84 ± 2.71
Antibiotic	Monensin	0 ± 0	0.333 ± 0.221	0 ± 0	0 ± 0
	Trimethoprim	7.96 ± 4.46	**223 ± 14.0**	156 ± 17.0	136 ± 8.17
	Lincomycin	0.313 ± 0.313	0 ± 0	**10.6 ± 3.75**	1.14 ± 1.08
	Sulfamethoxazole	12.9 ± 2.63	**651 ± 51.8**	325 ± 32.8	398 ± 44.6
Antidepressant	Fluoxetine	14.1 ± 11.7	**45.5 ± 0.938**	**88.2 ± 11.6**	26.6 ± 0.502
	Norfluoxetine	5.30 ± 1.05	**9.67 ± 0.505**	3.31 ± 0.492	**9.48 ± 0.340**
	Venlafaxine	195 ± 65.8	**939 ± 68.0**	**865 ± 52.9**	578 ± 46.1
	Desvenlafaxine	6.13 ± 2.73	**1830 ± 110.**	696 ± 70.6	**1060 ± 78.5**
NSAID	Ibuprofen	6.99 ± 1.82	231 ± 162	90.0 ± 14.9	**530. ± 184**
	Naproxen	0 ± 0	**252 ± 115**	141 ± 16.2	**583 ± 32.0**
	Diclofenac	176 ± 18.3	**1090 ± 41.8**	720. ± 48.0	802 ± 42.9
Herbicide	Atrazine	**32.9 ± 1.02**	25.2 ± 1.17	**36.2 ± 1.70**	25.6 ± 1.89

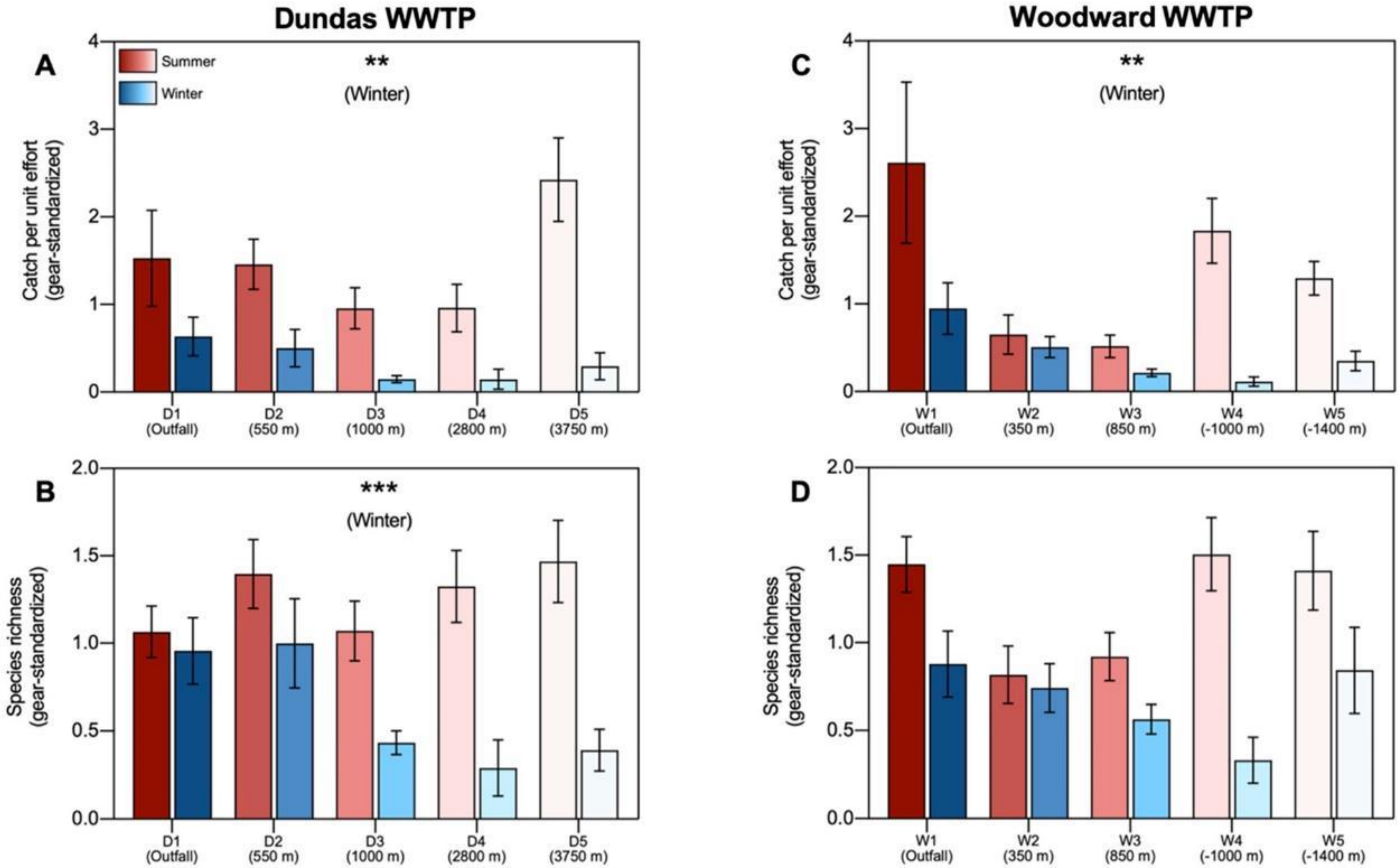


Figure 2.2. Mean (± SE) gear-standardized **(A, C)** abundance (catch per unit effort) and **(B, D)** species richness, of fish caught along the effluent gradient from the Dundas and Woodward WWTP. In the winter, a significant effect of proximity to the outfall is indicated by (** $p < 0.01$) and (*** $p < 0.001$). No significant trends were observed in the summer.

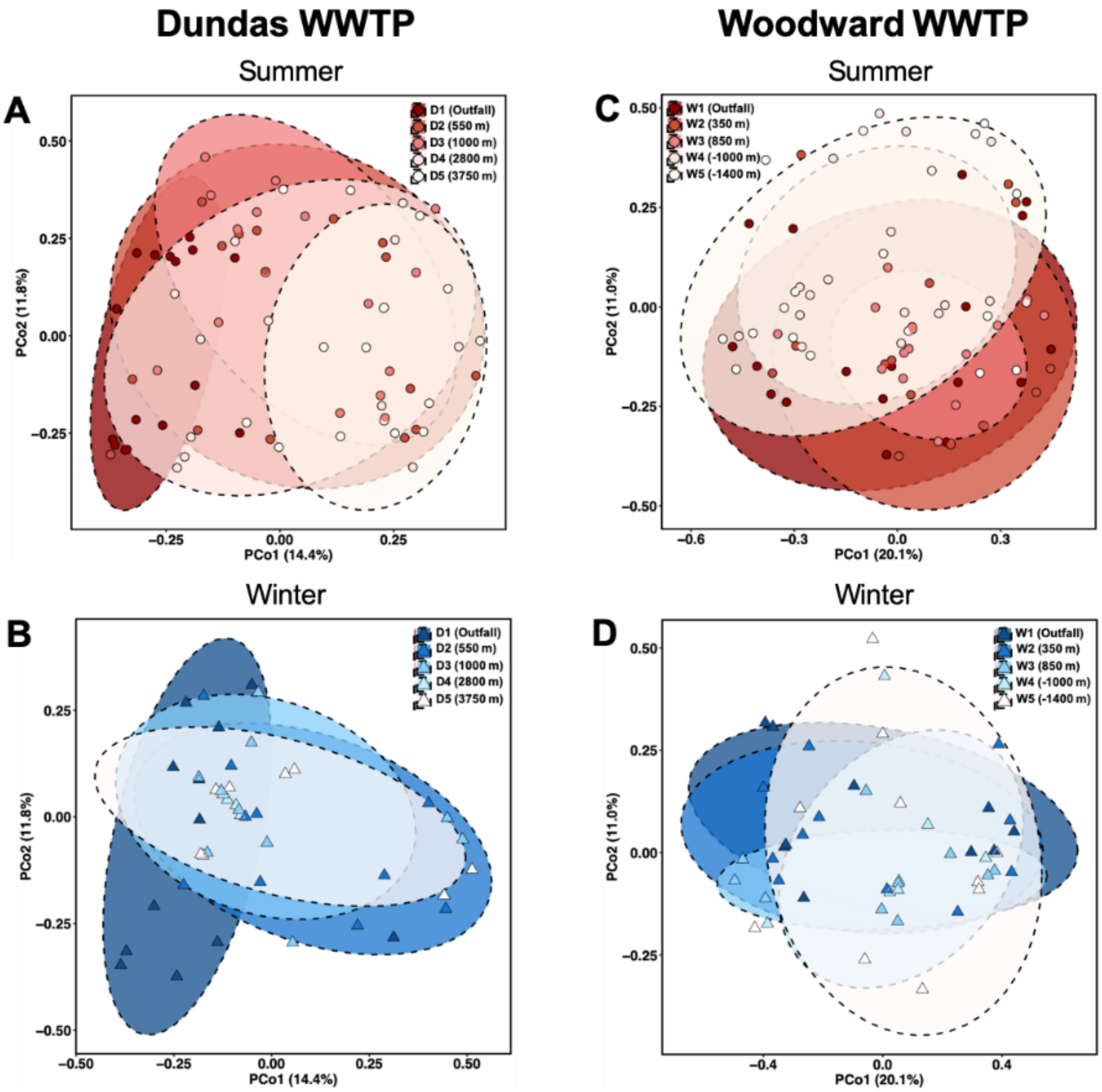


Figure 2.3. Principal coordinate analysis (PCoA) ordination output of fish community compositions with 80% confidence ellipses overlaid on each site. (**A, B**) PCoA biplots from the Dundas WWTP in summer and winter, respectively. (**C, D**) PCoA biplots from the Woodward WWTP in summer and winter, respectively.

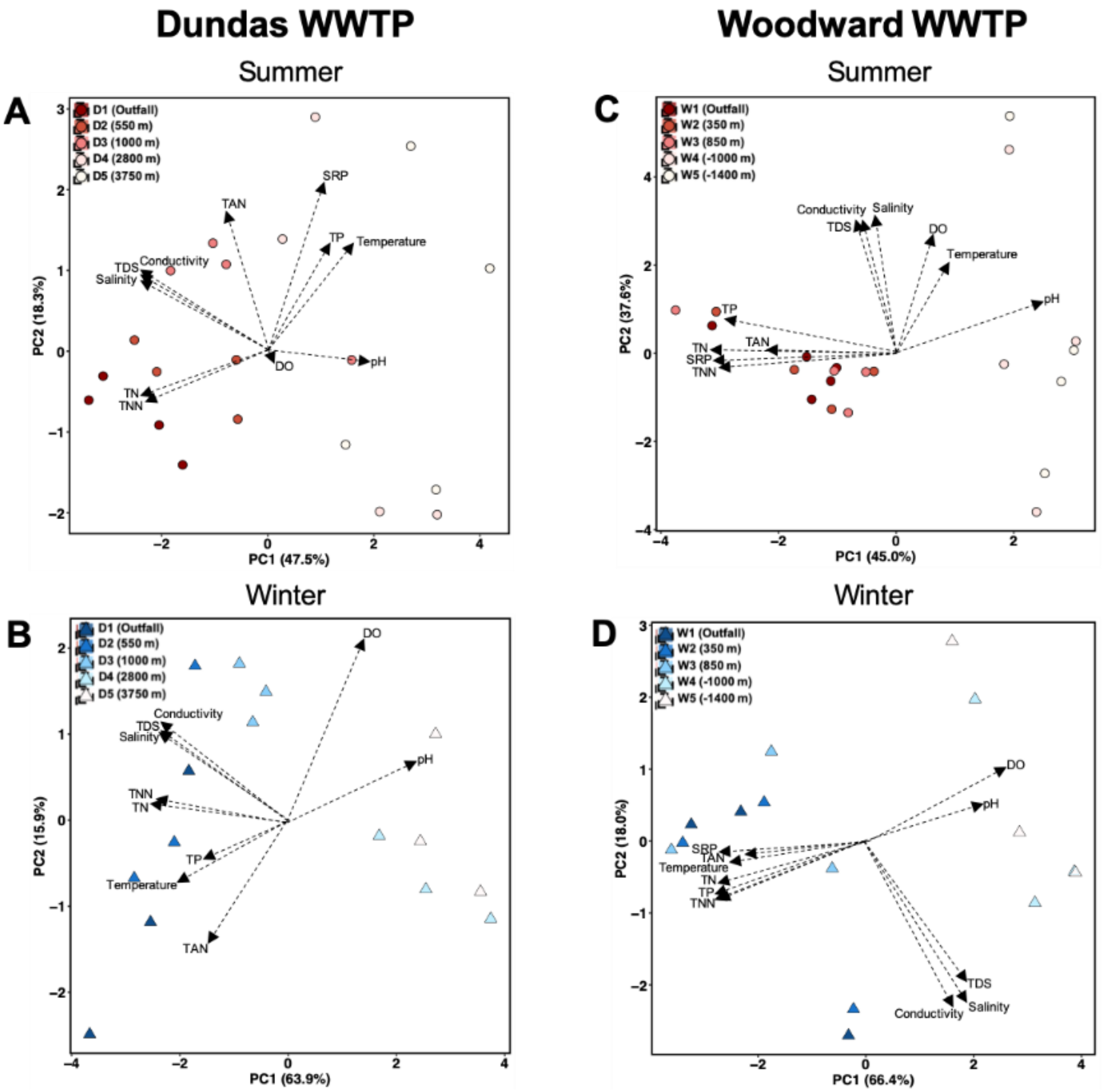


Figure 2.4. Principal component analysis (PCA) biplots on water quality parameters measured along the effluent gradients of both WWTPs. (**A, B**) PCA biplots from the Dundas WWTP in summer and winter, respectively. (**C, D**) PCA biplots from the Woodward WWTP in summer and winter, respectively. Each data point represents a field sampling event. The dashed lines represent the strength of the loadings and direction of the respective water quality parameters in two-dimensional space. See Supplementary Table 11 for PCA loadings.

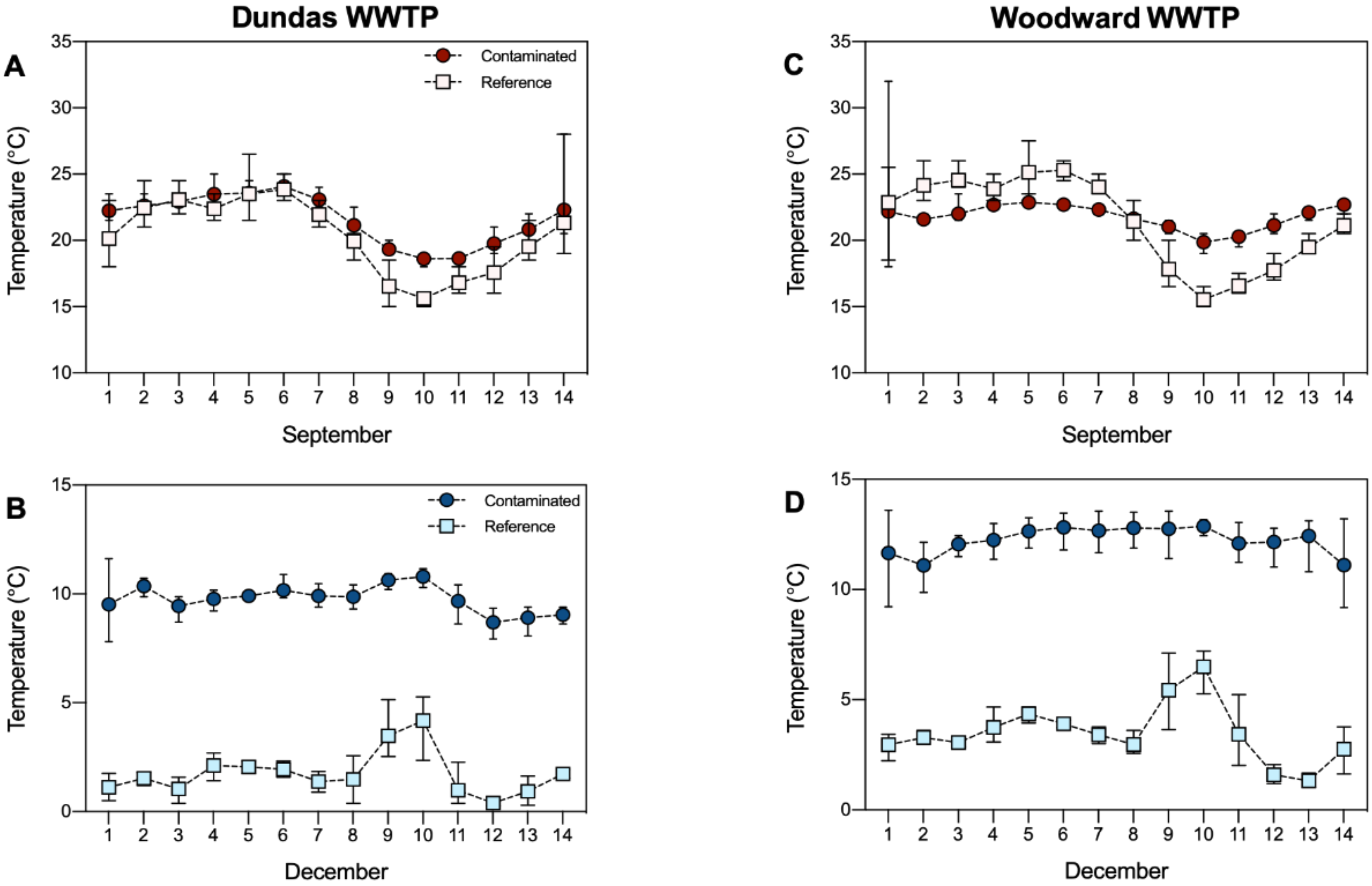


Figure 2.5. Mean, minimum and maximum daily temperatures of the outfall site (D1, W1) and one reference site (D4, W4) measured along a 14-day period in the summer and winter. (**A, B**) Temperature data from the Dundas WWTP in summer and winter, respectively. **(C, D**) Temperature data from the Woodward WWTP in summer and winter, respectively. Temperature data was recorded once every 30 min in the summer and once every 15 min in winter.

2.4 Discussion

Our study explored the impacts of wastewater contamination on fish communities between the summer and winter. Fish communities were sampled along contamination gradients generated by two WWTPs in Hamilton, ON, Canada during both seasons. In the winter, sites closer to the effluent outfall generally had higher fish abundance, higher species richness, and higher species diversity compared to sites farther away. This trend, however, was not as apparent in the summer. Wastewater plumes are a significant source of nutrients in aquatic environments, so fish may seek such highly productive environments, especially during the winter, when food is scarce and difficult to encounter (Sommer et al., 1986; Byström et al., 2006; Hurst, 2007; Holeton et al., 2011). The increase in productivity in effluent-receiving environments likely leads to higher food availability and is potentially the cause of the increase in growth and body condition observed in fishes caught in such environments (Chambers et al., 1997; McMaster et al., 2005; Brown et al., 2011; Tetreault et al., 2011). Additionally, effluent released from WWTPs can significantly alter the thermal conditions of receiving environments (Environment Canada, 2001; Kinouchi et al., 2007; Mehdi et al., 2019). In the summer, we observed that temperature increased with distance from the outfall, while during the winter, sites closer to the outfall were significantly warmer than sites farther away. Sites closer to the outfall also appeared to be more thermally stable than sites farther away. Thermal enhancement of effluent-receiving environments may create perceived thermal refugia for aquatic organisms, particularly during the winter, when survival elsewhere may be challenging (Brodersen et al., 2011). Fish may select sites with temperatures closer to their optima (i.e., sites closer to the outfall), rather than sites with cooler temperatures (i.e., sites farther away from the outfall; Cooke et al., 2000, Cooke et al., 2004). The combination of a steady supply of nutrients and thermal enhancement in effluent-receiving environments may create an enticing ecological trap for aquatic organisms, as these environments may be perceived by individuals as beneficial or favourable, but are also a major source of contamination, and may pose detrimental costs to reproduction and survival (Schlaepfer et al., 2002; Battin, 2004; Holeton et al., 2011). Empirical assessment of fish thermal preferences and food availability across seasons in wastewater-impacted environments would be a natural next step for understanding why fish might be attracted to such environments.

During the summer, fish community composition (dissimilarity, tolerance, proportion of native to non-native species, and the average trophic level) differed along the effluent gradients at one or both WWTPs, with the outfall sites being most dissimilar from the reference sites. Previous studies conducted in the summer have also demonstrated that wastewater promotes the presence of tolerant, non-native, and omnivorous fishes (Tetreault et al., 2012; McCallum et al., 2019). A study conducted along the same effluent gradients as our own found that both fish abundance and species richness was highest near the outfalls during the summer (McCallum et al., 2019). Our study demonstrated a similar pattern in the sites closest to the outfalls, however, sites farther away were relatively more variable. Such differences may be attributable to our study using 1) more gear types, 2) different reference sites, and 3) sampling over a shorter period (July and August only). In our study, fish abundance, species richness, and species diversity, and overall composition varied between the two WWTPs. Such varying patterns between the two WWTPs indicate that it is difficult to generalize the impacts of wastewater contamination on fish communities. The different results may be due, in part, to WWTPs varying in almost every aspect (e.g., population served, daily capacity, treatment technologies; see Methods section for differences between the two WWTPs). Furthermore, the habitats that wastewater effluent is discharged into can also influence fish community responses (Midwood et al., 2015; McCallum et al., 2019; see Supplementary Tables 6 and 7). Despite such differences, key similarities were identified. Specifically, more fish were found near the outflows and certain key species were present in sites closer to the outfall (e.g., round goby, white sucker, green sunfish, and spottail shiner). Previous studies have demonstrated that these species are often found in polluted environments and tend to be tolerant and/or resilient to a wide range of environmental conditions (e.g., dissolved oxygen, temperature, salinity, turbidity, and pollution; Reash and Berra, 1987; Froese and Pauly, 2020; Eakins, 2018; Anseeuw et al., 2012; Kornis et al., 2012; Hernandez, 2014). The overrepresentation of these species in impacted sites may indicate that only certain species are able to tolerate wastewater exposure while reaping the benefits associated with polluted environments. It is important to note that during the winter, species richness and species diversity were both significantly higher in sites closer to the outfall, especially at the Dundas WWTP, suggesting fishes are either staying and/or moving into wastewater plumes. Telemetry studies tracking movement of fishes in and out wastewater plumes across seasons would be critical in further understanding how wastewater outfalls may act as ecological traps.

Water quality parameters varied along the effluent gradients generated by both WWTPs and between seasons, further establishing the impacts that municipal wastewater can have on the chemical and physical characteristics of effluent-impacted environments. Additionally, of the chemical compounds analyzed, the majority were more frequently detected and detected at higher concentrations in the winter compared to summer, implying that contaminant loading worsens during the winter. Similar trends have previously been demonstrated in other countries that experience comparable climates to Canada, e.g., China (Sui et al., 2011), Finland (Vieno et al., 2005), Poland (Kot-Wasik et al., 2016), and the USA (Yu et al., 2013). Such seasonal differences in the concentrations of PPCPs have previously been attributed to increased pharmaceutical consumption and to poorer biological degradation of contaminants in WWTPs during colder months of the year. Indeed, antibiotics, analgesics, and antidepressants are more likely to be prescribed during the winter (Vieno et al., 2005; Gardarsdottir et al., 2010; ter Laak et al., 2010; Sui et al., 2011; Yu et al., 2013; Suda et al., 2014); however, we could not find data on seasonal pharmaceutical prescription and consumption rates of the specific compounds we measured in Canadian populations. Seasonal differences in the sewershed flows and inputs as well as in treatment efficiencies may have influenced the CECs distribution in the final effluent. In the Dundas WWTP effluent, but not in the Woodward WWTP, there was a distinct increase in treatment-resistant compounds such as carbamazepine and venlafaxine during the winter. Further studies are needed to better understand the seasonal distribution of these chemicals entering the environment. Several of the compounds analyzed in our study have been linked to sublethal effects in fishes (e.g., venlafaxine and fluoxetine), including impacts on metabolism (Best et al., 2014; Mennigen et al., 2010; Mehdi et al., 2019), stress response (Ings et al., 2011a, Ings et al., 2011b; Melnyk-Lamont et al., 2014), reproductive capacity (Lister et al., 2009; Weinberger and Klaper, 2014), and routine behaviours (Martin et al., 2017; McCallum et al., 2017b; Martin et al., 2019). However, these impacts may be more severe in the winter, as the metabolic scope of ectotherms is greatly reduced in colder temperatures, potentially limiting the capacity to detoxify contaminants found in wastewater effluent (Lemly, 1993; Lemly, 1996). Moreover, fish may also suffer from endogenous exposure to contaminants during the winter as reliance on lipid stores expectedly increases, which may mobilise tissue stores of some contaminants (Paterson et al., 2007; Treberg et al., 2016). Also, lower water temperatures in the winter may reduce metabolism and gill ventilation, thereby decreasing uptake, elimination, and remobilization of contaminants (Capkin et al., 2006; Buckman et al., 2007; Noyes et al., 2009). The physiological impacts of winter temperatures on fish contaminant exposures downstream of WWTP outfalls warrant further exploration.

Few compounds were present in effluents at higher concentrations in the summer. Atrazine, lincomycin, monensin, and sulfamethazine are predominantly used in agriculture, making their seasonal concentrations less surprising (Couperus et al., 2016). While most of the compounds assessed in our study were measured at concentrations lower than what would be considered lethal (Brausch and Rand, 2011; Brausch et al., 2012), it should be noted that there are no water quality guidelines for the majority of PPCPs found in wastewater effluents. However, ammonia is a pollutant that is actively monitored in wastewater treatment facilities, as it is toxic in freshwater ecosystems (Canadian Council of Ministers of the Environment, 2010). Mean LC50 values reported for freshwater fishes typically range from 0.56 to 2.37 mg/L, where toxicity is often higher at lower temperatures and pH values (Environment Canada, 1999). These concentrations are within the range of what we observed in our study, especially downstream of the Woodward WWTP during the winter (up to 5.62 mg/L ammonia/ammonium). Ammonia exposure has been linked to numerous adverse effects in fishes, including reproductive and developmental impairments as well as morphological abnormalities (Randall and Tsui, 2002; Yuen and Chew, 2010). High concentrations of toxic nitrogenous products, like those found in wastewater effluent, may significantly disturb fish populations and communities in effluent-receiving environments, especially in the presence of other contaminants

(Environment Canada, 1999; Canadian Council of Ministers of the Environment, 2010; EPA, 2013). Hence, our study further emphasizes the importance of winter research in ecotoxicology, as research conducted only in warmer seasons may not reveal the full scope of the impacts of wastewater effluent in aquatic ecosystems.

This study is unique in its approach to studying the impacts of wastewater on fish communities and water quality during the winter—a season seldom studied in ecotoxicology. We demonstrated that effluent-receiving environments may act as ecological traps for fishes, especially during the winter, where the effluent provides enhancement and stability to the temperature profile of receiving water bodies, as well as potentially increasing the availability of food when it is scarce elsewhere. Effluent quality was also predictably worse during the winter compared to the summer. To better understand why fish might choose sites with greater contaminant exposure, future research should further investigate the relative costs and benefits of living in effluent-dominated environments using lab and field manipulation studies in fish and other aquatic organisms, especially during the winter. Such research would aid conservation and management efforts of aquatic ecosystems that are heavily impacted by wastewater pollution and urbanization.

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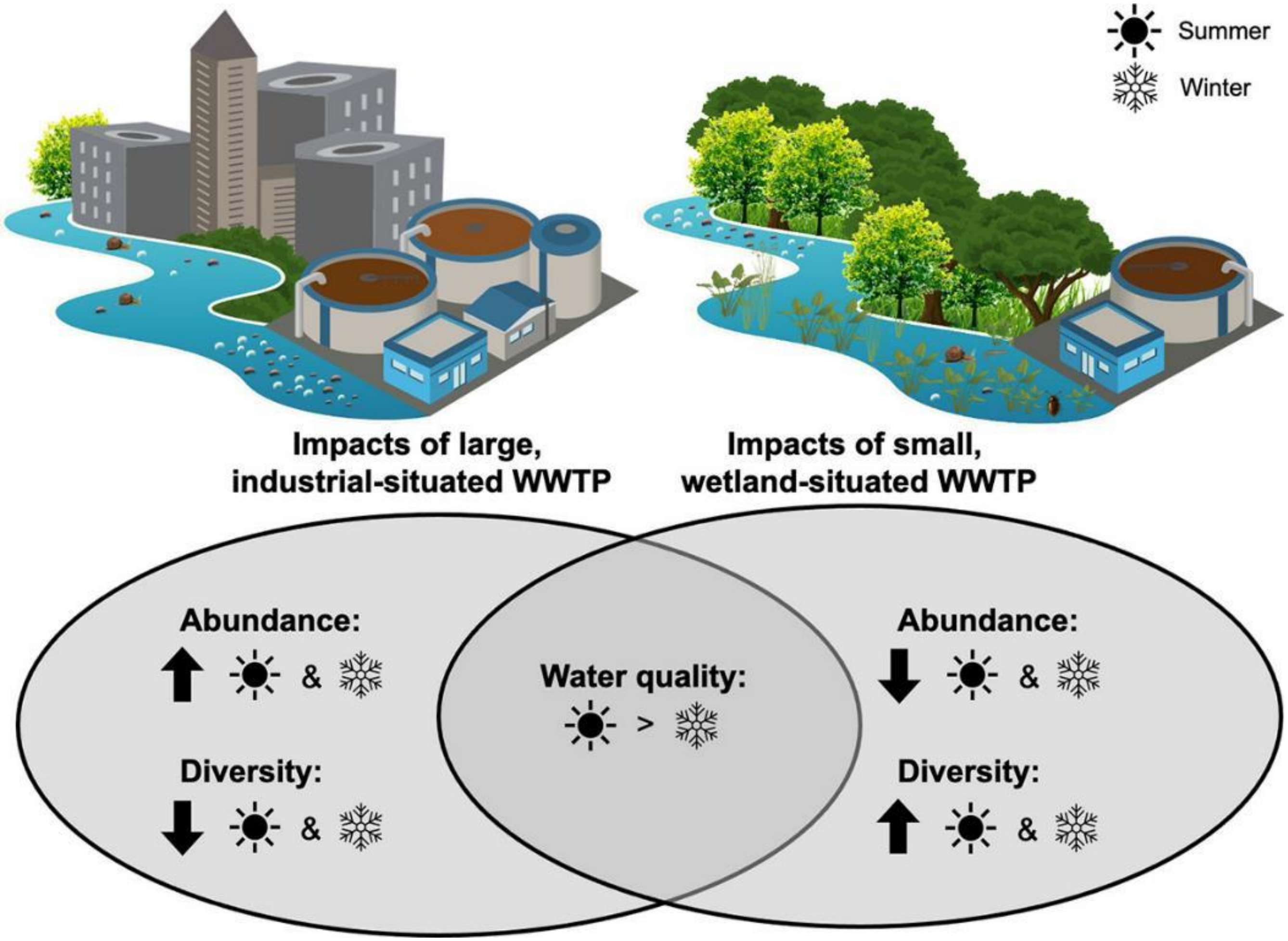
Chapter 3: Impacts of wastewater treatment plants on benthic macroinvertebrate communities in summer and winter



3.1 Abstract

Treated effluent from municipal wastewater treatment plants (WWTPs) is a major source of contamination that can impact population size, community structure, and biodiversity of aquatic organisms. However, because the majority of field research occurs during warmer periods of the year, the impacts of wastewater effluent on aquatic communities during winter has largely been neglected. In this study, we assessed the impacts of wastewater effluent on aquatic benthic macroinvertebrate (benthos) communities along the effluent gradients of two WWTPs discharging into Hamilton Harbour, Canada, during summer and winter using artificial substrates incubated for 8 weeks. At the larger of the two plants, benthic macroinvertebrate abundance was higher and diversity was lower at sites downstream of the outfall compared to upstream sites in both seasons. Whereas at the smaller plant, the opposite was observed, abundance increased and diversity decreased with distance from the outfall in both seasons. While the impacts of wastewater on benthic communities were largely similar between seasons, we did detect several general seasonal trends – family diversity of macroinvertebrates was lower during winter at both WWTPs and total abundance was also lower during winter, but only significantly so at the smaller WWTP. Further, benthic macroinvertebrate community composition differed significantly along the effluent gradients, with sites closest and farthest from the outfall being the most dissimilar. Our contrasting results between the WWTPs demonstrate that plants, with different treatment capabilities and effluent-receiving environments

(industrial/urban versus wetland), can dictate how wastewater effluent impacts benthic macroinvertebrate communities.

3.2 Introduction

Effluents discharged from municipal wastewater treatment plants (WWTPs) are one of the largest sources of aquatic pollution (by volume) in many parts of the world (Holeton et al., 2011; Hamdhani et al., 2020). Wastewater effluents, although treated in many jurisdictions, still contain a wide variety of contaminants beyond just phosphates and nitrogenous waste products, such as pharmaceuticals and personal care products (PPCPs), natural and synthetic hormones, micro- and macroplastics, agricultural and industrial chemicals, and metals (Daughton and Ternes, 1999; Kolpin et al., 2002; Ternes et al., 2004; Holeton et al., 2011; McCormick et al., 2016; Hamdhani et al., 2020). The continuous release of wastewater effluents into waterbodies subjects aquatic biota to chronic exposure of complex mixtures of contaminants, eutrophication, oxygen depletion, thermal pollution, and overall habitat degradation (Brown et al., 2011; Holeton et al., 2011; Tetreault et al., 2013; Hamdhani et al., 2020). As a result, aquatic organisms residing in effluent-receiving habitats are affected across all levels of biological organization, from molecular initiating events all the way up to population and community responses (Saaristo et al., 2014; Bahamonde et al., 2015; Fuzzen et al., 2015; McCallum et al., 2017, McCallum et al., 2019; Du et al., 2018, Du et al., 2019; McLean et al., 2019; Mehdi et al., 2018, Mehdi et al., 2021; Lau et al., 2021).

As urban populations continue to grow, so too will the reliance on WWTPs, and by extension, the concerns regarding the impacts of their effluents on aquatic ecosystems (Sumpter, 2009; Bernhardt et al., 2017).

To date, most studies on the impacts of WWTP effluents have been conducted during warmer months of the year, and as a result, it is unclear whether similar impacts occur at colder temperatures, and if so, to what degree. In many parts of the world, winter is a dominant season, with its effects lasting 4–8 months of the year; therefore, understanding the impacts of such a ubiquitous contaminant as wastewater effluent in a season as dominant as winter is of crucial importance. Additionally, the effectiveness of WWTPs and therefore the quality of their effluents is poorer at colder temperatures (i.e., winter) than at warmer temperatures (i.e., summer; Vieno et al., 2005; Sui et al., 2011; Yu et al., 2013; Kot-Wasik et al., 2016). This is mainly due to poorer influent degradation at colder temperatures as well as elevated usage of PPCPs, caffeine, and health products during winter (Vieno et al., 2005; Sui et al., 2011; Yu et al., 2013; Kot-Wasik et al., 2016). The higher concentrations of PPCPs, nitrogenous waste products, and nutrients, and the overall poorer quality of wastewater effluent during winter suggests that the impacts on aquatic organisms may also be greater during winter. Further, effluents discharged during winter can increase water temperature by as much as 5–10 °C in effluent-receiving environments, potentially providing thermal refuge for aquatic organisms (Environment Canada, 2001; Kinouchi et al., 2007). Taken together, the effects of thermal pollution and nutrient enrichment may increase food availability, potentially causing wastewater outfalls to act as ecological traps, particularly during winter, when the impacts of wastewater effluent exposure may be magnified (McCallum et al., 2019; Mehdi et al., 2021).

While impacts of wastewater effluent have been well established on the individuals, comparatively, few studies have addressed how wastewater may impact aquatic populations and communities. This is surprising given how relevant such ecological endpoints are in determining habitat quality and evaluating risks, especially in environments impacted by anthropogenic disturbances (Fausch et al., 1990; Cvetkovic et al., 2010). One strategy for examining the impacts of wastewater effluent on ecosystem health is the use of aquatic benthic macroinvertebrates as bioindicators. Benthic macroinvertebrates are commonly used as bioindicators of water quality in rivers and lakes as they (i) are highly diverse and range widely in sensitivity to disturbances, (ii) typically have small home ranges, and (iii) are easily collected and identified (Krumhansl et al., 2015; Resh and Unzicker, 1975; Jones et al., 2007). Benthic macroinvertebrates also play an important role in the transformation of nutrients (Krumhansl et al., 2015; Gleason and Rooney, 2017) and are an important food source for fish, amphibians, and birds (Covich et al., 1999). Although little is known about the impacts of wastewater effluent on benthic macroinvertebrate communities, a few notable studies have demonstrated that wastewater effluent can indeed affect food web structure and function through shifts in community composition and leaf litter composition, changes in trophic status of receiving systems, and changes in biodiversity and in biological integrity indices (Ortiz et al., 2005; Englert et al., 2013; Huong et al., 2017; Burdon et al., 2019; Peschke et al., 2019; dos Reis Oliveira et al., 2020; Jesus et al., 2020). However, as mentioned above, the seasonal impacts posed by wastewater effluent on ecosystems have rarely been investigated, particularly during the winter – a season seldom studied in ecotoxicology.

The aim of the present study was therefore to compare the impact of wastewater treatment plant effluent on benthic macroinvertebrate communities between summer and winter. To do this, we examined the impacts of wastewater effluent on benthic macroinvertebrate communities near a large and small WWTP during summer and winter. The two WWTPs are both located within the Hamilton Harbour watershed in Ontario, Canada, one of 43 areas of concern under the Great Lakes Water Quality Agreement (2012). Wastewater pollution is a stressor of special concern in Hamilton Harbour, as it is estimated that ~50% of its non-lake inflow is of WWTP origin (Lawrence et al., 2004; Government of Canada, 2017). Benthic macroinvertebrate samples were collected along a distance and contamination gradient from each WWTP, thereby allowing us to assess the longitudinal effects of wastewater effluent in both summer and winter. Additionally, we measured a suite of water quality parameters and habitat quality characteristics to assess the abiotic impacts of wastewater contamination. We predicted that wastewater would significantly impair the physical and chemical quality of effluent-receiving environments, thereby affecting benthic macroinvertebrate communities at these impacted sites (Hamdhani et al., 2020; van der Meer et al., 2021).

Because the continuous discharge of wastewater effluents leads to the degradation of benthic habitats in effluent-receiving environments, we expected sites closer to the effluent outfalls to have reduced richness and diversity of benthic macroinvertebrates, and be most compositionally distinct from sites farther away (Walsh et al., 2005). Since effluent quality and therefore, water quality of receiving environments would likely be worse during the winter, we expected the associated impacts on benthic macroinvertebrate communities to be more apparent during the winter.

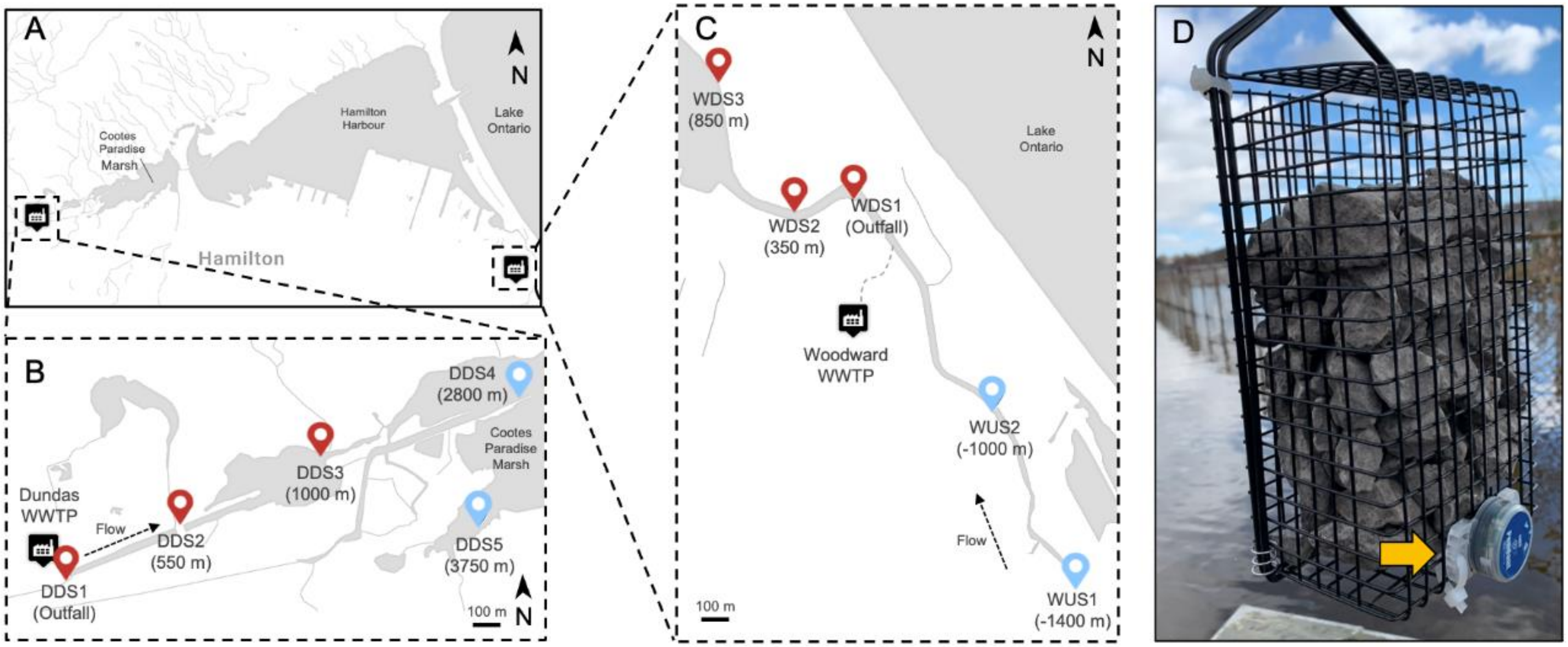


Figure 3.1. Maps showing **A)** the location of the Dundas and Woodward WWTPs in Hamilton Harbour as well as a close-up view of the sampling sites: **B**) the Dundas WWTP (with an arrow indicating the direction flow in Desjardins Canal) and **C**) the Woodward WWTP (with the grey dotted line indicating the wastewater outflow from the plant and an arrow indicating the direction of flow in Red Hill Creek). **D**) Photograph of a representative rock basket filled with substrate and equipped with a temperature logger (see arrow) before deployment. Sampling sites in red are in the direct flow of the effluent outfall, whereas sites in blue are not in the direct flow of the effluent outfall. Sites were named as follows: Dundas downstream (DDS); WUS (Woodward upstream); WDS (Woodward downstream).

3.3 Methods

3.3.1 Sampling regime

This study was conducted during the summer of 2018 and the winter of 2019–20. Benthic macroinvertebrates were collected using artificial substrates deployed at 10 sites located along the effluent gradients of the Dundas and Woodward WWTPs discharging into Hamilton Harbour (further described in 2.1.1 Large Woodward WWTP, 2.1.2 Small Dundas WWTP; Fig. 3.1). Site selection was based on accessibility in both summer and winter and because these sites were part of a long-term research program (McCallum et al., 2019; Mehdi et al., 2021; Nikel et al., 2021).

3.3.1.1 Large Woodward WWTP

The Woodward WWTP is a secondary conventional activated sludge plant that serves ~480,000 people in Hamilton, Stoney Creek, and Ancaster, Canada; it has a daily capacity of 409 million litres, making it the largest WWTP in Hamilton (City of Hamilton, 2019). This plant releases its effluent into the Red Hill Creek which connects to the southeastern corner of Hamilton Harbour (Fig. 3.1A). Five sites were sampled along the effluent gradient of the Woodward WWTP, three of which were downstream: WDS1 (outfall), WDS2 (350 m), and WDS3 (850 m), and two of which were upstream located in Red Hill Creek: WUS1 (1400 m upstream) and WUS2 (1000 m upstream). Two reference upstream sites were selected because in 2022, one of the reference sites (WUS2) will become the new outfall site of the Woodward WWTP as part of ongoing upgrades to the plant (City of Hamilton, 2019). Therefore, our study may serve as a baseline of the conditions of benthic macroinvertebrate communities and water quality prior to the upgrades.

The Woodward WWTP is situated in a heavily industrialized part of Hamilton (East Hamilton Harbour), and all sampling sites were anthropogenically modified. All sites were in close proximity to urban structures, showed clear modifications to the shoreline, had relatively narrow riparian zones, and substrate was predominantly comprised of cobble and boulder in the downstream sites and cobble and silt in the upstream reference sites. See Supplementary Material Table S1 for further details on habitat characteristics of our sampling sites.

3.3.1.2 Small Woodward WWTP

The smaller Dundas WWTP is a conventional activated sludge plant with tertiary filtration that serves the majority of the Dundas population (~30,000 people) and has a daily capacity of 18.2 million litres. It is located on the west end of Cootes Paradise Marsh, the largest wetland of western Lake Ontario (City of Hamilton, 2019). Effluent from the plant is discharged along an old shipping corridor, the Desjardins Canal, located on the westernmost end of Cootes Paradise Marsh (Theysmeyer and Bowman, 2017). Three of the sites sampled were in the direct flow of the effluent: DDS1 (outfall), DDS2 (550 m downstream), and DDS3 (1000 m downstream). Additionally, because the WWTP outfall is located at the head of the stream, there were no upstream sites of the Dundas WWTP, therefore two distant, but downstream reference sites were sampled: DDS4 (2800 m downstream) and DDS5 (3750 m downstream). Neither of these sites were in the direct flow of the effluent, therefore effluent exposure was less than in sites that were in the direct flow of the effluent (DDS1, DDS2, and DDS3).

The Dundas WWTP is situated in a less industrialized part of Hamilton (West Hamilton Harbour) and has a much larger surrounding riparian zone than the Woodward WWTP. Sites closest to the outfall of the Dundas WWTP (DDS1 and DDS2) were the most anthropogenically disturbed with clear modifications to the shoreline and were in close proximity to urban structures. Sites farther away from the outfall (DDS3–DDS5) were more natural, surrounded by wetland and natural forest habitats, and were less disturbed than sites near the outfall. See Supplementary Material Table S1 for further details on habitat characteristics of our sampling sites.

3.3.2 Benthic macroinvertebrate collection, enumeration, and identification

Benthic macroinvertebrates were sampled using wire baskets filled with 2 kg of prewashed crushed granite rocks (mean ± SE surface area of all rocks within a basket = 24.6 ± 1.21 cm2; Fig. 3.1D). The prewashed and premeasured rocks in the wire baskets were used to standardize the type and amount of available substrate across the sampling sites (actual site substrate included: boulder, cobble, gravel, sand, and silt; see Supplementary Table S1 for details on substrate and habitat characteristics). Rock baskets were placed at the sampling sites by lowering them onto the substrates, at a depth of 0.5–1.0 m and within 2 m from the shoreline. Baskets were left in contact with the existing sediment and suspended by rope to prevent them from sinking into the substrate if the substrate was too soft. The baskets were left to be colonized by benthic macroinvertebrates for 8 weeks in both seasons. In the summer of 2018, 60 rock baskets (n = 6/site) were deployed from July 10th until September 4th at the Dundas WWTP sampling sites and from July 15th until September 10th at the Woodward WWTP sampling sites; of these, 51 were retrieved. Winter baskets were initially deployed in December 2018, however, high water levels led to difficulties with their retrieval causing the winter sample collection to be delayed until winter 2019–20. Forty-five rock baskets (n = 5/site) were deployed from November 16th, 2019 to January 10th, 2020 at the Dundas WWTP sampling sites and from November 17th, 2019 to January 11th, 2020 at the Woodward WWTP sampling sites; of these, 38 were retrieved. Rock baskets could not be deployed in the winter at one of the reference sites (DDS5; Dundas WWTP) due to ice cover and all rock baskets deployed at WDS3 (Woodward WWTP) in the winter were lost due to vandalism. See Supplementary Table S2 for additional details on rock basket deployment. Each rock basket was retrieved by lifting it out of the water inside a D-net (500 μm mesh size) to prevent sample loss, and then the rocks were washed into a 500 μm-sized sieve to collect the invertebrates. Samples were immediately preserved in 10% sugar-buffered formalin before being transferred into 70% ethanol. For the majority of samples, all invertebrates were identified and counted. However, if the samples were too dense

(>400 individuals in the first quadrant), then they were subsampled in halves or in quarters (see Supplementary Table S2 for details on subsampling). For enumeration, samples were emptied into a large dishpan and benthic macroinvertebrates were identified to the lowest practical taxonomic level (family) following West Virginia Department of Environmental Protection (n.d.), St. Lawrence River Institute Environmental Sciences (2005), and Witty and Sarrazin-Delay (2014).

3.3.3 Habitat characterization and water quality

Habitat characteristics were assessed based on the protocols of McCallum et al. (2019) and a subset of metrics of the Qualitative Habitat Evaluation Index (Taft and Koncelik, 2006; Strickland et al., 2010). At each sampling site, the following parameters were assessed: water depth, water clarity (Secchi disk), substrate type, sediment particle size, shoreline slope, degree of sinuosity, degree of anthropogenic modifications, riparian zone width, degree of estimated bank erosion, and the presence of any aquatic plants (see Supplementary Table S1).

At each site, four times in summer and three times in winter, we measured the following water quality parameters: water temperature and dissolved oxygen (YSI ProODO), pH, conductivity, total dissolved solids (TDS), and salinity (Oakton multiparameter Testr); (Table 3.1). Also, long-term temperature data were collected for 14 days using HOBO Pendant MX temperature loggers (Onset Computer Corp) deployed at the outfall site and a reference site of each WWTP in both seasons at a depth between 0.5 and 1.0 m (Fig. 3.1D). At each site, 1 L water samples were collected at mid-water depth using a 2.2 L Van Dorn sampler (Wildco Alpha) and later analyzed for total ammonia + ammonium, nitrate, nitrite, ortho-phosphate, total phosphorus, and total Kjeldahl nitrogen by the City of Hamilton Environmental Laboratory (methods as in McCallum et al., 2019). Additionally, 24-h composite samples of the effluent were collected twice a week directly from each WWTP just before discharge during the summer and winter of 2019 (summer: nDundas = 7, nWoodward = 8; winter: nDundas = 8, nWoodward = 8). The following water quality parameters were measured in the composite effluent samples: total suspended solids, biochemical oxygen demand, total phosphorus, total Kjeldahl nitrogen, ammonia, nitrate, nitrite, and Escherichia coli (measurements provided by the City of Hamilton and can be found in Supplementary Table 3).

3.3.4 Statistical analysis

Statistical analyses were performed using R (version 4.0.4; R Core Team, 2021) and graphics were made with Prism (version 9) and R. Water quality parameters were analyzed collectively using a permutation MANOVA with 5000 permutations to assess the effects of proximity to the outfall and season. For all biodiversity metrics calculated, we used each rock basket to represent a replicate and an individual observation. Total abundance, family richness, and family diversity (Shannon's Index) were analyzed (after being log-transformed due to heterogeneity of variance) using permutation linear models (PLMs) with 5000 iterations from the lme4 and predictmeans packages (Bates et al., 2015; Luo et al., 2020). We analyzed the response variables for each WWTP separately because the sampling site order differed between the two plants. At the Woodward WWTP, sampling site type (i.e., upstream or downstream; categorical), season (summer or winter; categorical), and their interaction were included in the model. Whereas at the Dundas WWTP, sampling site order (numeric), season (categorical), and their interaction were included in the model. This type of analysis allowed for response variables to be interpreted along a 'gradient of contamination' at the Dundas WWTP and as 'upstream versus downstream' at the Woodward WWTP. Principal Coordinate Analysis (PCoA) with a Bray-Curtis dissimilarity matrix were used to analyze benthic macroinvertebrate community composition differences between sites and seasons for each WWTP (Oksanen et al., 2019). PCoA biplots with 80% confidence ellipses overlaid on each site were used to visualize beta diversity differences across sites within each WWTP and season (Oksanen et al., 2019). Community composition differences across sites and between seasons were further analyzed using a permutation ANOVA with 5000 permutations using adonis2 (Vegan package; Oksanen et al., 2019). Similarity percentages analysis (SIMPER; Oksanen et al., 2019) assisted with identifying which family groups were driving the between-site differences in community composition in each season. While all benthic macroinvertebrate families sampled were included in the analysis, only those that contributed ≥5% to the total abundance were further interpreted using permutation tests (n = 5000). In all analyses, a difference was deemed significant when $p < 0.05$.

3.4 Results

3.4.1 Water quality

3.4.1.1 Large Woodward WWTP

Water quality differed significantly between downstream (WDS1, WDS2, and WDS3) and upstream sites (WUS1 and WUS2) in both seasons at the Woodward WWTP (Table 3.1). Across all sites, pH, conductivity, total dissolved solids, salinity, and total ammonia nitrogen ($p = 0.08$) were higher in the winter, whereas only water temperature was higher in the summer. At the downstream sites in summer, total nitrogen, total nitrate nitrogen, total phosphorus, and soluble reactive phosphorus were all significantly higher relative to sites upstream, while the opposite was true for dissolved oxygen and pH. Whereas at the downstream sites in winter, water temperature, total nitrogen, total ammonia nitrogen, total nitrate nitrogen, total phosphorus, and soluble reactive phosphorus were all higher relative to the upstream sites, the opposite was true for dissolved oxygen and pH (all contrasts were $p < 0.05$ unless otherwise stated). Data from the temperature loggers revealed no difference in water temperature between the outfall site (WDS1) and the reference site (WUS2) in the summer (Linear Model, $t_{Summer} = 0.42$, $p = 0.68$); however, during the winter, the outfall site was on average ~9 °C warmer than the upstream site (Linear Model, $t_{Winter} = 22.30$, $p < 0.001$).

3.4.1.1 Small Dundas WWTP

Similar to the Woodward WWTP, water quality varied significantly with distance from the effluent outfall in both seasons (Table 3.1). Across all sites, water temperature, total phosphorus, and soluble reactive phosphorus were all higher in summer, whereas pH, total nitrogen, total ammonia nitrogen, total nitrate nitrogen, conductivity, total dissolved solids, and salinity were higher in winter. In summer, total nitrogen, total nitrate nitrogen, conductivity, total dissolved solids, and salinity were all highest near the outfall site, and decreased with distance from the WWTP, while pH, temperature, and soluble reactive phosphorus increased with distance from the outfall. In winter, water temperature, total nitrogen, total ammonia nitrogen, total nitrate nitrogen, conductivity, total dissolved solids, and salinity were all highest near the outfall and decreased with distance from the treatment plant, while only pH increased with distance from the outfall. Temperature data loggers revealed that the outfall (DDS1) and reference (DDS4) sites had similar water temperatures during summer (Linear Model, tSummer = 1.49, $p = 0.15$), but the outfall site was on average ~8 °C warmer than the reference site in winter (Linear Model, tWinter = 25.25, $p < 0.001$).

3.4.2 Benthic macroinvertebrate community metrics

3.4.2.1 Large Woodward WWTP

At the Woodward WWTP sampling sites, we collected 36,854 benthic macroinvertebrates (mean ± SE = ~929 ± 189/basket in summer and ~681 ±

229/basket in winter; Table 3.2). Overall, benthic macroinvertebrate abundance and family richness did not significantly differ between summer and winter (PLM; tAbundance(1,41) = 1.04 p = 0.30; tRichness(1,41) = 1.48, p = 0.09; Fig. 3.2A and B; S1A and S1B). However, family diversity was significantly lower in winter than in summer (PLM; tDiversity (1,41) = −3.98, p < 0.001; Fig. 3.2E and F). Benthic macroinvertebrate abundance was higher in sites downstream of the effluent outfall compared to upstream sites in both summer (PLM; t(1,41) = 7.35, p < 0.001; ~21 times; Fig. 3.2A) and winter (t(1,41) = 4.64, p < 0.001; ~149 times; Fig. 3.2B). In contrast, family diversity was significantly lower at sites downstream of the outfall relative to those upstream in both seasons (PLM, tSummer(1,41) = −5.57, p < 0.001; Fig. 3.2E; tWinter(1,41) = −5.26, p < 0.001; Fig. 3.2F). Family richness on the other hand did not differ between downstream and upstream sites in either season (PLM, tSummer(1,41) = 1.50, p = 0.12; Fig. S1A; tWinter(1,41) = 0.63, p = 0.23; Fig. S1B).

3.4.2.2 Small Woodward WWTP

At the Dundas WWTP sampling sites, we collected a total of 15,082 benthic macroinvertebrates (mean ± SE = ~499 ± 149/basket in the summer and ~117 ± 23/basket in the winter; Table 3.2). The abundance of macroinvertebrates was significantly higher in summer than in winter (PLM; t(1,40) = 2.55, p = 0.02; Fig. 3.2C and D). Similarly, family richness was higher in summer than in winter (PLM; t(1,40) = 1.42, p = 0.02; Supplementary Fig. S1C and D), whereas family diversity did not differ between seasons (PLM; t(1,40) = 0.83, p = 0.13; Fig. 3.2G and H). In contrast to the large Woodward WWTP, total abundance increased with distance from the outfall during both summer (PLM; t(1,40) = 4.92, p < 0.001; Fig. 3.2C) and winter (t(1,40) = 5.47, p < 0.001; Fig. 3.2D). Family diversity, however, decreased with distance from the outfall in both seasons (PLM; t(Summer 1,40) = −1.97, p = 0.04; t(Winter 1,40) = −3.26, p < 0.001; Fig. 3.2G and H). Conversely, family richness was not influenced by proximity to the outfall in either season (PLM; t(Summer 1,40) = 1.45, p = 0.24; t(Winter 1,40) = 1.49, p = 0.14; Supplementary Fig. S1C and D).

3.4.3 Benthic macroinvertebrate community composition

3.4.3.1 Large Woodward WWTP

During summer, we identified 24 different families of benthic macroinvertebrates in all sampling sites, and the samples were largely comprised of isopods, snails, and leeches. During winter, we identified only 20 families, and the samples were mostly comprised of isopods and amphipods. See Fig. 3.3A and B and Table 3.2 for the community makeup of benthic macroinvertebrates at each sampling site in each season. Benthic macroinvertebrate community composition differed significantly between seasons (Permutation ANOVA; F(1,44) = 7.62, p < 0.001; Fig. 3.4A and B). In summer, communities downstream of the outfall were the most distinct from those upstream (PCoA; Permutation ANOVA; FSummer(4,24) = 7.35, p < 0.001; Fig. 3.4A). In the winter, upstream communities displayed a degree of overlap with each other but were distinct from those at sites downstream; although the two downstream sites were distinct from one another (PCoA; Permutation ANOVA; FWinter(3,19) = 16.7, p < 0.001; Fig. 3.4B). Similarity analysis indicated that differences between WDS1 (outfall) and all other sites were mainly driven by a high abundance of Asellidae (freshwater isopods) at WDS1 in both seasons (Table 3.3 and Supplementary Table S4). Furthermore, dissimilarity scores were highest between the outfall site and the two reference sites upstream, and this was observed in both summer and winter (Table 3.3).

3.4.3.2 Small Woodward WWTP

During summer, we identified 30 families of benthic macroinvertebrates across all sampling sites, and the samples were mostly comprised of isopods, amphipods, and dipterans. In winter, we identified 21 families and like in summer, the samples were mainly comprised of isopods, amphipods, and dipterans. The community makeup of benthic macroinvertebrates at each sampling site in both seasons can be found in Fig. 3.3C and D and Table 3.2. Like the Woodward WWTP, benthic macroinvertebrate community composition at the Dundas WWTP differed significantly between seasons (Permutation ANOVA; F(1,43) = 13.7, p < 0.001; Fig. 3.4C and D). In summer, the communities closest to the outfall (DDS1 and DDS2) were the most distinct from those farther away (DDS4 and DDS5), while DDS3 was an intermediate between those sites (PCoA; Permutation ANOVA; FSummer(4,25) = 10.5, p < 0.001; Fig. 3.4C). During winter, the benthic macroinvertebrate community at the outfall site (DDS1) was most different from the site farthest away (DDS4); while sites in between (DDS2 and DDS3) shared a considerable amount of overlap (PCoA; Permutation ANOVA; FWinter(3,17) = 9.76, $p < 0.001$; Fig. 3.4D). Similarity analysis further indicated which families were driving the majority of community compositional differences across sites. In summer, community differences between DDS1 and DDS2 were largely driven by the higher abundance of Chironomidae (midges) and Caenidae (square gill mayflies) at DDS2, and a higher abundance of Coenagrionidae (damselflies) at DDS1. Differences between DDS1 and DDS3 were driven by the higher abundance of Coenagrionidae at DDS1, but also by the higher abundance of Asellidae (isopods) at DDS3. The difference between DDS1 and DDS4 was mostly driven by Gammaridae (amphipods) which were considerably more abundant at DDS4. Differences between DDS1 and DDS5 were driven by Naididae (clitellate oligochaete worms) and Erpobdellidae (proboscisless leeches) being more abundant at DDS1 and Asellidae being more abundant at DDS5. In winter, higher abundances of both Caenidae and Asellidae were found at DDS2 compared to DDS1. Between DDS1 and DDS3, the differences were driven by a higher abundance of Coenagrionidae at DDS3. Differences between DDS1 and DDS4 were attributed mostly to the higher abundance of Gammaridae at DDS4 (Table 3.3 and Supplementary Table S5). Furthermore, dissimilarity scores were highest between the outfall site and the reference site(s) in both summer and winter (Table 3.3).

Table 3.1. Mean (± SE) of water quality parameters measured at the sampling sites of the Woodward and Dundas WWTPs in summer | winter. Water quality parameters shown are water temperature, dissolved oxygen saturation (DO), pH, conductivity, total dissolved solids (TDS), salinity, total ammonia nitrogen (TAN), total nitrate nitrogen (TNN), total nitrogen (TN), soluble reactive phosphorus (SRP), and total phosphorus (TP). Data based on measurements in the summer (n = 4/site) and winter (n = 3/site). Water quality data previously reported in Mehdi et al. (2021).

	Woodward WWTP				
	WUS1 (-1400 m)	WUS2 (-1000 m)	WDS1 (Outfall)	WDS2 (350 m)	WDS3 (850 m)
Water temperature (°C)	23.25±1.28 | 4.43±1.08	23.25±0.47 | 4.13±1.07	21.72±0.37 | 11.3±0.95	22.17±0.24 | 10.7±0.83	22.65±0.42 | 9.76±0.29
DO (%)	93.55±12.98 | 95.63±0.08	75.42±24.53 | 92.86±2.53	74.2±2.6 | 69.03±1.26	69.35±4.47 | 68.9±0.66	63.32±4.1 | 67.26±2.87
pH	8.13±0.18 | 8.7±0.31	8.05±0.23 | 8.65±0.42	7.07±0.1 | 7.77±0.23	7.08±0.12 | 7.4±0.09	7.05±0.1 | 7.55±0.09
Conductivity (µS)	1113±220.58 | 2021.66±349.31	1147.25±207.64 | 2351.33±288.89	1166.25±43.47 | 2021.33±360.99	1148±53.24 | 2033.66±298.55	1147.25±56.91 | 1824±288.92
TDS (ppm)	779.75±156.15 | 1606.66±273.57	812±147.65 | 1670±205.5	828±30.88 | 1463.33±238.35	816.75±36.83 | 1443.33±218.8	815±40.7 | 1100.66±90.11
Salinity (ppm)	550.25±99.67 | 1110±180.36	534.75±100.49 | 1153.33±142.4	540.25±21.01 | 1011.33±174.49	534±25.2 | 1001.66±159.17	534±28.08 | 947.66±121.54
TAN (mg/L)	0.04±0.01 | 0.02±0.01	0.12±0.03 | 0.04±0.01	0.46±0.18 | 2.32±1.65	0.89±0.43 | 2.23±1.53	1.2±0.48 | 2.26±1.42
TNN (mg/L)	1.66±0.66 | 1.15±0.06	2.09±0.8 | 1.09±0.13	11.25±0.73 | 10.88±0.5	9.54±1.16 | 10.36±0.71	9.14±1.04 | 9.52±1.16
TN (mg/L)	2.62±0.95 | 1.54±0.09	2.81±0.86 | 1.5±0.09	13.14±1.03 | 16.14±3.21	12.18±1.41 | 15.13±3.66	12.19±1.76 | 14.62±4.01
SRP (mg/L)	0±0 | 0.02±0.02	0.02±0.01 | 0±0	0.22±0.04 | 0.18±0.05	0.22±0.05 | 0.18±0.05	0.22±0.05 | 0.19±0.05
TP (mg/L)	0.15±0.06 | 0.08±0.02	0.13±0.02 | 0.08±0.01	0.45±0.04 | 0.44±0.03	0.4±0.05 | 0.37±0.05	0.36±0.06 | 0.36±0.06

	Dundas WWTP				
	DDS1 (Outfall)	DDS2 (550 m)	DDS3 (1000 m)	DDS4 (2800 m)	DDS5 (3750 m)
Water temperature (°C)	21.05±1.35 | 9.96±1	23.32±1.15 | 7.66±1.19	24.22±1.4 | 4.86±2.15	23.65±2.24 | 3.23±1.15	26.7±2.43 | 3.6±2.7
DO (%)	124.02±13.46 | 91.06±8.65	134.32±23.9 | 98.23±9.6	90.7±34.57 | 109.86±2.81	86.97±15.28 | 100.83±2.67	132.42±48.53 | 107.93±4.17
pH	7.57±0.22 | 7.69±0.38	7.88±0.54 | 7.84±0.33	7.49±0.45 | 8.4±0.41	8.21±0.29 | 8.78±0.36	8.33±0.54 | 8.8±0.28
Conductivity (µS)	1188.25±91.89 | 1452±130.03	1149.25±93.2 | 1530±159.48	1134.25±149.38 | 1495.33±142.33	943.5±201.07 | 942±157.08	885.75±129.63 | 966.66±169.78
TDS (ppm)	844.5±68.14 | 1005.33±82.12	818.75±63.94 | 1086.33±112.07	806.75±105.7 | 1065.33±101.71	670.25±141.5 | 670.33±113.03	626.25±91.45 | 690±121.5
Salinity (ppm)	562.5±58.59 | 718.66±59.87	544.25±41.39 | 756±65.93	536.75±61.68 | 732.33±49.66	441±89.22 | 461.66±102.75	414±54.06 | 476.33±104.64
TAN (mg/L)	0.03±0.01 | 0.5±0.75	0.05±0.02 | 0.22±0.27	0.06±0.04 | 0.13±0.1	0.06±0.07 | 0.04±0.02	0±0 | 0.02±0.01
TNN (mg/L)	15.35±1.53 | 17.09±2.03	11.26±3.64 | 16.1±1.76	4.84±3.14 | 11.62±2.24	0.49±0.62 | 1.06±0.33	0±0 | 0.69±0.35
TN (mg/L)	16.25±1.03 | 18.53±1.2	12.49±3.36 | 17.03±1.77	6.43±3.04 | 13.12±1.68	1.52±0.79 | 1.64±0.39	1.38±0.44 | 1.21±0.43
SRP (mg/L)	0±0 | 0±0	0±0 | 0±0	0.01±0.02 | 0±0	0.03±0.03 | 0±0	0.03±0.04 | 0±0
TP (mg/L)	0.11±0.04 | 0.07±0.01	0.11±0.04 | 0.07±0.02	0.18±0.06 | 0.07±0	0.11±0.01 | 0.06±0.03	0.19±0.1 | 0.05±0

Table 3.2. Benthic macroinvertebrate counts at each sampling site (abundances are denoted as Summer **|** Winter, “-” indicates no information for that specific site). Count data are shown as the total number of invertebrates collected in all rock baskets per site (see Supplementary Table S3 for sample sizes by site and season).

<table>
<tr><th></th><th></th><th></th><th colspan="5">Woodward WWTP</th><th colspan="5">Dundas WWTP</th></tr>
<tr><th>Organism Type</th><th>Order</th><th>Family</th><th>WUS1 (-1400 m)</th><th>WUS2 (-1000 m)</th><th>WDS1 (Outfall)</th><th>WDS2 (350 m)</th><th>WDS3 (850 m)</th><th>DDS1 (Outfall)</th><th>DDS2 (550 m)</th><th>DDS3 (1000 m)</th><th>DDS4 (2800 m)</th><th>DDS5 (3750 m)</th></tr>
<tr><td>Amphipods</td><td>Amphipoda</td><td>Gammaridae</td><td>27 | 66</td><td>23 | 1032</td><td>0 | 0</td><td>6 | 2</td><td>0 | -</td><td>2 | 6</td><td>57 | 221</td><td>56 | 216</td><td>9344 | 1046</td><td>160 | -</td></tr>
<tr><td>Isopods</td><td>Isopoda</td><td>Asellidae</td><td>42 | 33</td><td>41 | 47</td><td>7711 | 10966</td><td>5260 | 912</td><td>4164 | -</td><td>95 | 16</td><td>98 | 70</td><td>719 | 9</td><td>100 | 58</td><td>479 | -</td></tr>
<tr><td>Leeches</td><td>Arhynchobdellida</td><td>Erpobdellidae</td><td>63 | 6</td><td>32 | 6</td><td>56 | 45</td><td>527 | 108</td><td>100 | -</td><td>0 | 2</td><td>31 | 0</td><td>26 | 0</td><td>4 | 1</td><td>112 | -</td></tr>
<tr><td></td><td>Rhynchobdellida</td><td>Glossiphoniidae</td><td>20 | 0</td><td>8 | 1</td><td>0 | 0</td><td>483 | 0</td><td>136 | -</td><td>0 | 0</td><td>1 | 0</td><td>6 | 0</td><td>0 | 0</td><td>45 | -</td></tr>
<tr><td>Seg. Worms</td><td>Haplotaxida</td><td>Naididae</td><td>115 | 14</td><td>8 | 16</td><td>10 | 6</td><td>11| 4</td><td>0 | -</td><td>8 | 2</td><td>1 | 3</td><td>15 | 0</td><td>0 | 0</td><td>149 | -</td></tr>
<tr><td>Flatworms</td><td>Tricladida</td><td>Planariidae</td><td>0 | 0</td><td>1 | 2</td><td>1 | 0</td><td>46 | 4</td><td>4 | -</td><td>0 | 2</td><td>0 | 0</td><td>0 | 0</td><td>0 | 0</td><td>0 | -</td></tr>
<tr><td>Snails</td><td>Basommatophora</td><td>Physidae</td><td>14 | 1</td><td>15 | 3</td><td>29 | 66</td><td>118 | 5</td><td>68 | -</td><td>8 | 1</td><td>5 | 0</td><td>2 | 4</td><td>4 | 14</td><td>9 | -</td></tr>
<tr><td></td><td></td><td>Planorbidae</td><td>1 | 0</td><td>3 | 0</td><td>0 | 0</td><td>10 | 0</td><td>72 | -</td><td>1 | 0</td><td>0 | 0</td><td>1 | 0</td><td>4 | 0</td><td>4 | -</td></tr>
<tr><td></td><td></td><td>Lymnaeidae</td><td>1 | 0</td><td>1 | 1</td><td>0 | 0</td><td>0 | 0</td><td>0 | -</td><td>0 | 0</td><td>0 |0</td><td>0 | 0</td><td>0 | 0</td><td>0 | -</td></tr>
<tr><td></td><td>Heterostropha</td><td>Valvatidae</td><td>1 | 0</td><td>1 | 1</td><td>0 | 0</td><td>0 | 0</td><td>2348 | -</td><td>0 | 0</td><td>0 | 0</td><td>0 | 0</td><td>0 | 0</td><td>5 | -</td></tr>
<tr><td></td><td>NA</td><td>Snails w/ No Shell</td><td>0 | 0</td><td>1 | 0</td><td>4 | 16</td><td>11 | 2</td><td>0 | -</td><td>0 | 0</td><td>0 | 1</td><td>0 | 0</td><td>0 | 0</td><td>0 | -</td></tr>
<tr><td></td><td>Neotaenioglossa</td><td>Hydrobiidae</td><td>28 | 4</td><td>107 | 4</td><td>0 | 0</td><td>125 | 13</td><td>684 | -</td><td>0 | 0</td><td>17 | 0</td><td>9 | 0</td><td>52 | 0</td><td>52 | -</td></tr>
<tr><td></td><td>Venerida</td><td>Sphaeriidae</td><td>46 | 13</td><td>12 | 61</td><td>0 | 0</td><td>0 | 0</td><td>4 | -</td><td>0 | 0</td><td>0 | 1</td><td>1 | 0</td><td>0 | 0</td><td>0 | -</td></tr>
<tr><td>Beetles</td><td>Coleoptera</td><td>Dryopidae</td><td>0 | 0</td><td>1 | 0</td><td>0 | 0</td><td>0 | 0</td><td>0 | -</td><td>0 | 0</td><td>10 | 0</td><td>0 | 0</td><td>40 | 0</td><td>0 | -</td></tr>
<tr><td></td><td></td><td>Haliplidae</td><td>0 | 0</td><td>0 | 0</td><td>0 | 0</td><td>0 | 0</td><td>0 | -</td><td>0 | 0</td><td>2 | 0</td><td>0 | 0</td><td>0 | 0</td><td>0 | -</td></tr>
<tr><td></td><td></td><td>Hydrophilidae</td><td>5 | 0</td><td>42 | 0</td><td>0 | 0</td><td>0 | 0</td><td>0 | -</td><td>0 | 0</td><td>2 | 1</td><td>0 | 0</td><td>0 | 0</td><td>0 | -</td></tr>
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<tr><td>Flies</td><td>Diptera</td><td>Chironomidae</td><td>8 | 16</td><td>0 | 104</td><td>334 | 15</td><td>13 | 5</td><td>0 | -</td><td>42 | 7</td><td>144 | 14</td><td>36 | 10</td><td>68 | 17</td><td>1 | -</td></tr>
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</table>

Table 3.3. Similarity percentages analysis (SIMPER) indicating the relative contribution of family groups to the overall dissimilarity score of the Woodward outfall site (WDS1) to all other Woodward sites and the Dundas outfall site (DDS1) to all other Dundas sites. Average A and B are based on the abundance for each family group at the sites being compared. Sites are ordered according to proximity to outfall and contamination load. Only families that contributed ≥ 5% to overall abundance are shown. Bolded values indicate significant differences between sites ($p <$ 0.05).

	Woodward WWTP									
	Summer					Winter				
Comparison	Dissimilarity	Family	Average A	Average B	Contribution	Dissimilarity	Family	Average A	Average B	Contribution
A. WDS1 (Outfall)	54.00%	*Asellidae*	1927.75	876.67	43.27	82.07%	***Asellidae***	**2193.20**	**182.40**	**80.48**
B. WDS2 (350 m)										
A. WDS1 (Outfall)	46.23%	*Asellidae*	1927.75	1041.00	22.09	NA				
B. WDS3 (850 m)		*Valvatidae*	0.00	587.00	15.14					
A. WDS1 (Outfall)	98.23%	***Asellidae***	**1927.75**	**6.83**	**90.11**	98.67%	***Asellidae***	**2193.20**	**9.40**	**86.56**
B. WUS2 (-1000 m)							*Gammaridae*	0.00	206.40	9.42
A. WDS1 (Outfall)	97.33%	***Asellidae***	**1927.75**	**8.40**	**88.74**	98.97%	***Asellidae***	**2193.20**	**6.60**	**96.65**
B. WUS2 (-1400 m)										

	Dundas WWTP									
	Summer					Winter				
Comparison	Dissimilarity	Family	Average A	Average B	Contribution	Dissimilarity	Family	Average A	Average B	Contribution
A. DDS1 (Outfall)	61.69%	***Chironomidae***	**8.40**	**24.00**	**12.82**	89.02%	*Gammaridae*	1.20	44.20	40.81
B. DDS2 (550 m)		*Asellidae*	19.00	16.33	10.66		*Coenagrionidae*	1.20	18.20	16.43
		Coenagrionidae	**27.80**	**14.50**	**10.40**		***Caenidae***	**0.40**	**15.80**	**13.43**
		Caenidae	**1.00**	**18.33**	**9.77**		***Asellidae***	**3.20**	**14.00**	**11.45**
		Gammaridae	0.40	9.50	5.79					
A. DDS1 (Outfall)	74.62%	***Asellidae***	**19.00**	**119.83**	**40.20**	92.99%	*Gammaridae*	1.20	72.00	44.02
B. DDS3 (1000 m)	-	***Coenagrionidae***	**27.80**	**12.50**	**13.18**		***Coenagrionidae***	**1.20**	**53.33**	**39.28**
A. DDS1 (Outfall)	96.32%	***Gammaridae***	**0.40**	**1868.80**	**90.43**	94.53%	***Gammaridae***	**1.20**	**209.20**	**86.32**
B. DDS4 (2800 m)										
A. DDS1 (Outfall)	86.46%	***Asellidae***	**19.00**	**119.75**	**34.80**	NA				
B. DDS5 (3750 m)		*Gammaridae*	0.40	40.00	11.51					
		Naididae	**1.60**	**37.25**	**10.34**					
		Erpobdellidae	**0.00**	**28.00**	**9.12**					
		Coenagrionidae	27.80	0.50	8.70					

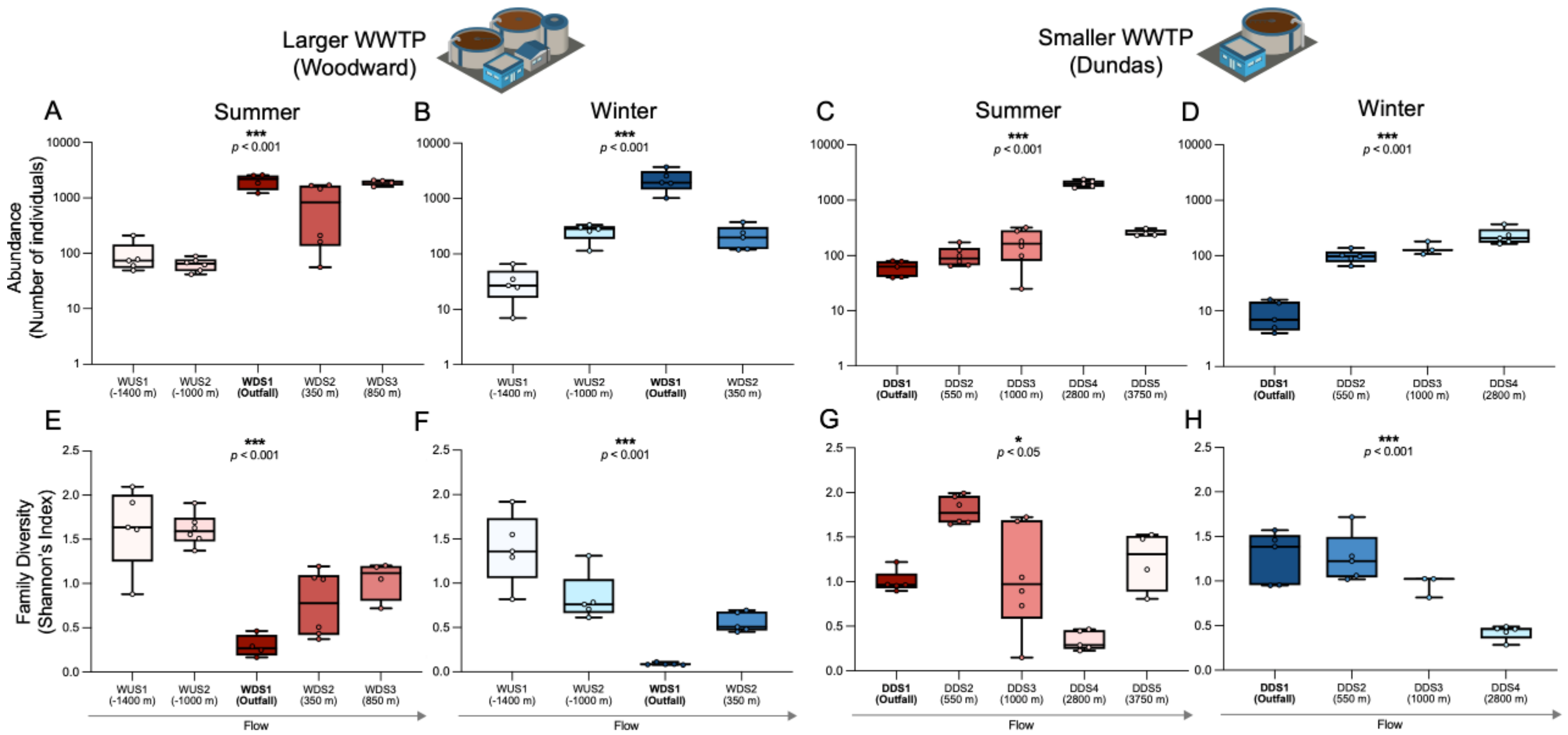

Figure 3.2. Benthic macroinvertebrate abundance (on a log scale) for the Woodward WWTP in the summer **(A)** and winter **(B),** and for the Dundas WWTP in summer **(C)** and winter **(D)**. Family diversity (Shannon's Index) shown for the Woodward WWTP in summer **€** and winter **(F),** and for the Dundas WWTP in summer **(G)** and winter **(H)**. Boxplots show the median and inter-quartile range, whiskers show minimum and maximum values, and individual data points are jittered to improve visualization. In all figures, summer data are represented in red and winter data are represented in blue; the intensity of the colours signify proximity to the outfall (darker colours being closest and light colours being most distant). Direction of flow is shown by arrows under the x-axes (with the outfall site indicated in bold font). All data are presented as per basket (N = 3 – 6 samples/site; see Supplementary Table S3 for sample

sizes by site and season). Significant effects of proximity to the outfall in Dundas and differences between upstream and downstream sites at Woodward are indicated by *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, or no significance (n.s.).

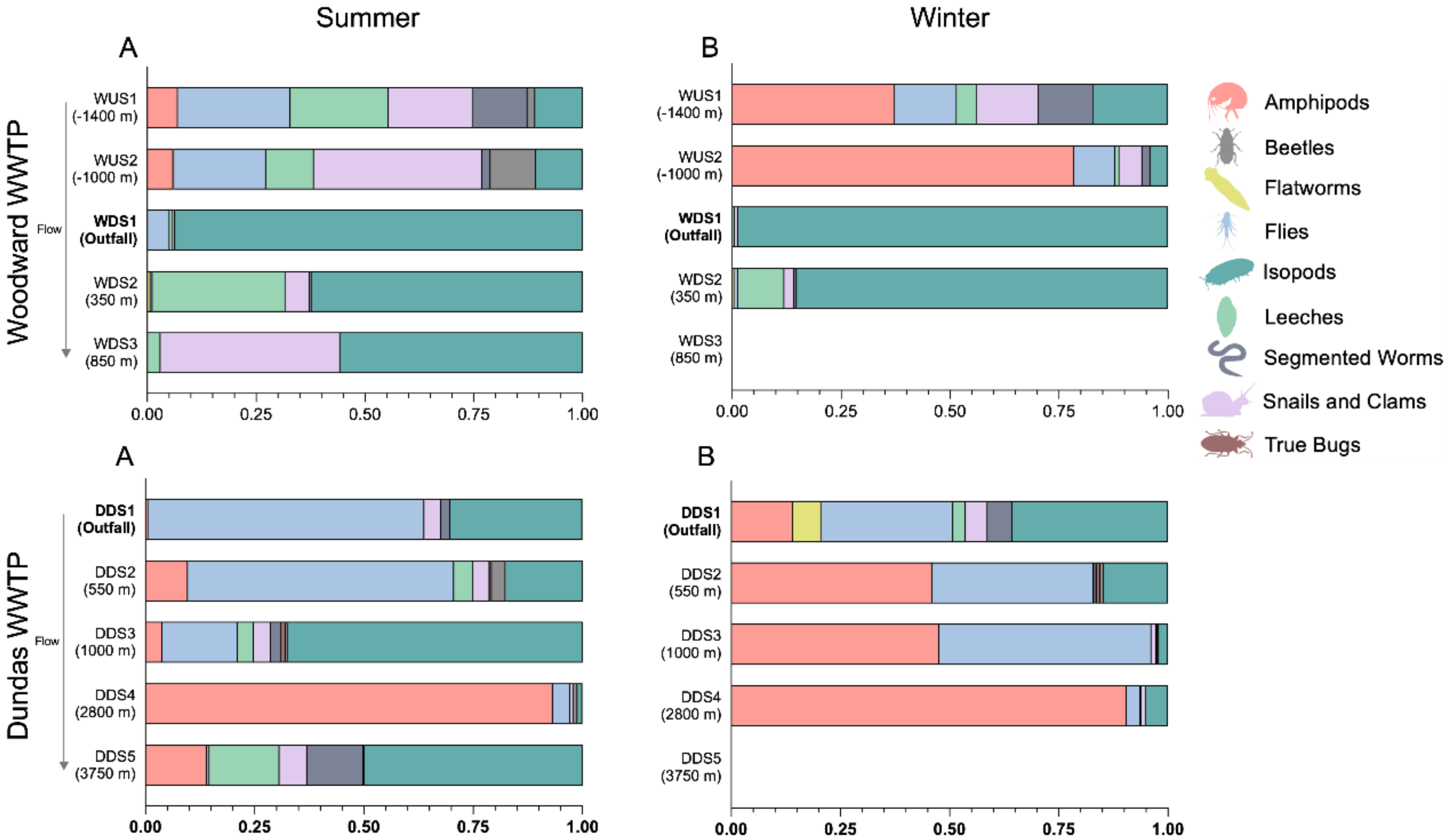

Figure 3.3. Proportions of different types of benthic macroinvertebrates from the Woodward WWTP sampling sites in summer **(A)** and winter **(B)**, and from the Dundas WWTP sampling sites in summer **(C)** and winter **(D)**. Proportions based on total abundance of macroinvertebrates within each site and season (all basket replicates combined per site). Direction of flow is shown by arrows next to the y-axes (with the outfall site shown in bold font).

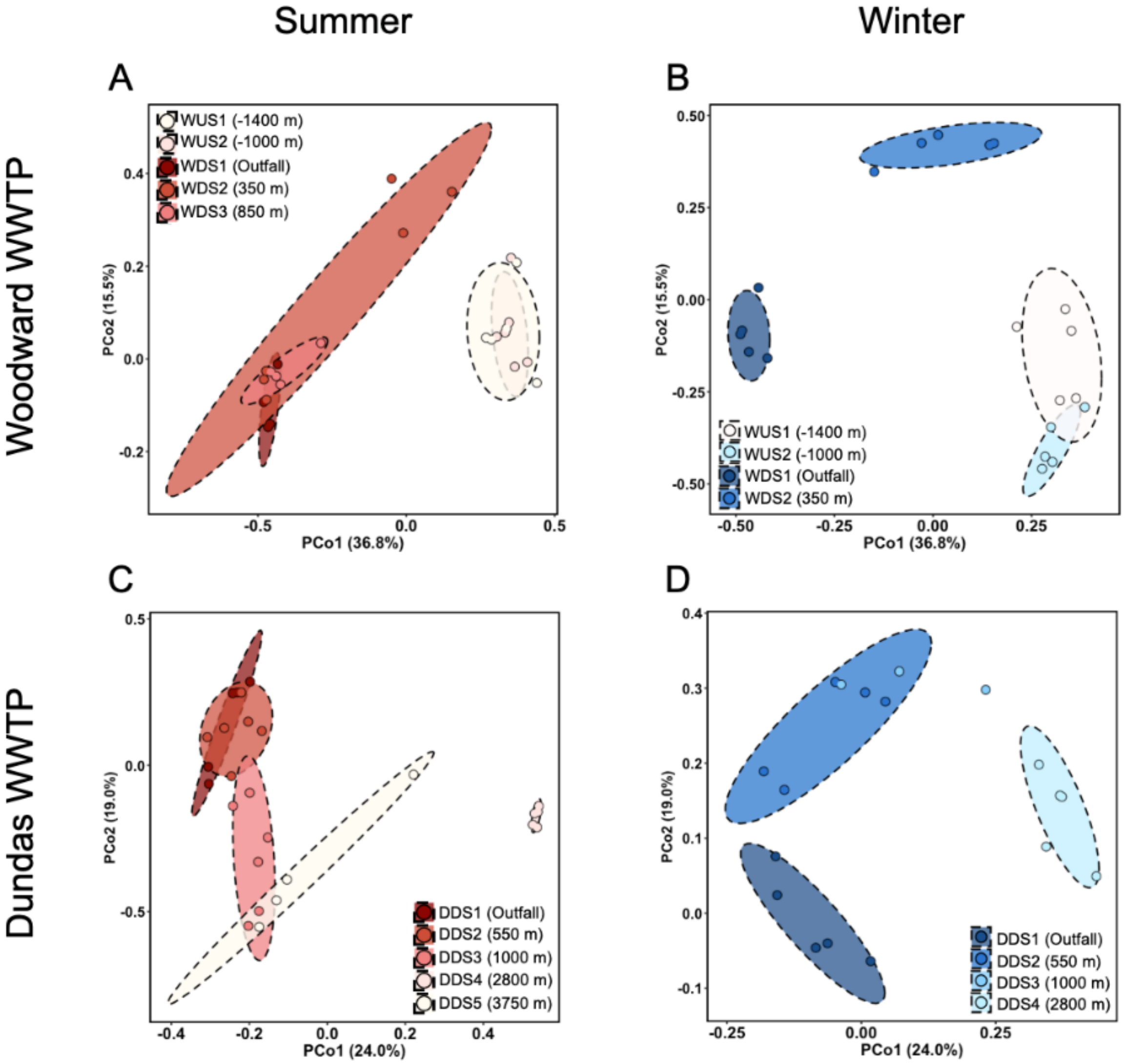

Figure 3.4. Principle coordinate analysis (PCoA) ordination biplots for benthic macroinvertebrate community composition at the Woodward WWTP sites in summer **(A)** and winter **(B)**, and at the Dundas WWTP sites in summer **(C)** and winter **(D)**. Each site is overlaid with 80% confidence ellipses (except for Dundas DDS3 in winter due to small sample size; n < 4).

3.5 Discussion

In this study, we examined the impacts of wastewater effluent in summer and winter on benthic macroinvertebrate communities near two WWTPs that discharge their effluents into very different habitats. We demonstrated that the effluents from the two WWTPs impacted the biodiversity of benthic macroinvertebrates differently. In both seasons, the larger Woodward WWTP, with its less effective secondary treatment and location in a highly industrialized area, had higher abundance but lower diversity of benthic macroinvertebrates at sites downstream of the outfall compared to sites upstream. In contrast, the smaller Dundas WWTP, with its enhanced tertiary treatment and location in a wetland, had lower abundance but higher diversity of benthic macroinvertebrates near the outfall sites compared to sites farther away in both seasons. Additionally, in both seasons and at both WWTPs, community composition of benthic macroinvertebrates differed significantly between sites closer to the outfall and sites farther away. Finally, we detected significant water quality deterioration in sites closer to the outfall, with water quality generally being poorer during the winter. The degraded water quality at the impacted sites manifested in higher nutrient concentrations, conductivity, salinity, and total dissolved solids in both seasons, as well as WWTP-induced thermal pollution, whereby sites closer to the outfall were 8–9 °C warmer during winter. Although the water quality varied among seasons, we did not detect concurrent seasonal differences in benthic macroinvertebrate community responses to wastewater at either plant.

3.5.1 Contrasting patterns between the two WWTPs

Poor water quality and high nutrient concentrations have been associated with higher abundance but lower measures of diversity of aquatic communities (Birge et al., 1989; Hickey and Clements, 1998; Walsh et al., 2005; Brown et al., 2011; Grantham et al., 2012; Tetreault et al., 2013; Zokan and Drake, 2015; Tuncay, 2016; McCallum et al., 2019; Jesus et al., 2020; Mehdi et al., 2021). Our results from sites along the Woodward WWTP effluent gradient are consistent with these previously reported findings. The high nutrient inputs at the Woodward WWTP appear to be supporting more benthic macroinvertebrates, and specifically, those with high tolerance to poor water quality (e.g., Asellidae). Interestingly, our results from along the effluent gradient of the Dundas WWTP revealed an opposite trend to that observed at the Woodward WWTP, with abundance increasing and diversity decreasing with distance from the effluent outfall. The Dundas WWTP is a much smaller plant with a higher level of treatment compared to the Woodward WWTP. Additionally, its daily effluent discharge is significantly lower than that of Woodward's (18.2 versus 409 million litres per day). Those two factors could explain why the water quality downstream of the Woodward WWTP was worse than water quality downstream of the Dundas WWTP. For example, total phosphorus, one of the main water quality indicators of productivity (Schindler, 1978; McQueen et al., 1986; Chapra and Robertson, 1977), was significantly higher in the sites downstream of the Woodward WWTP relative to sites downstream of the Dundas WWTP in both seasons, but this difference was more obvious in the winter. Furthermore, the Dundas WWTP releases its effluent into Cootes Paradise Marsh, the largest wetland west of Lake Ontario and a nature sanctuary that is of vital importance for migratory waterfowl and provides a significant habitat for many reptiles, amphibians, and fish (Leslie and Timmins, 1992; Smith and Chow-Fraser, 2010). Moreover, wetlands are known to buffer the effects of aquatic pollution as many wetland plants are able to absorb nutrients and toxic substances (Brix, 1994; Gopal, 1999; Hamoda et al., 2004). The Woodward WWTP discharges its effluent in an engineered channel, with relatively high flow rates and harder substrate (comprised mostly of boulder and cobble), and is surrounded by a heavily industrialized part of Hamilton with relatively little to no riparian zones. Taken together, the smaller size and smaller effluent footprint of the Dundas WWTP, its superior treatment, and the wetland environment receiving its effluent could help explain the contrasting biodiversity patterns observed between the two plants.

3.5.2 Limited differences between summer and winter patterns

Although water quality was strongly influenced by seasonality, with water quality in winter being significantly more impaired than in summer, the impacts of wastewater on benthic macroinvertebrate communities were similar between seasons. Deterioration of water quality during winter is consistent with prior studies demonstrating reduced contaminant removal/degradation efficiency by WWTPs at colder temperatures and increased usage and therefore concentrations of pharmaceuticals, caffeine, and other products during winter (Vieno et al., 2005; Gardarsdottir et al., 2010; ter Laak et al., 2010; Sui et al., 2011; Yu et al., 2013). The limited seasonal shifts in benthic macroinvertebrate community responses to wastewater is intriguing. During winter, many aquatic invertebrates are inactive, are only present as immobile eggs, migrate to more suitable habitats, and/or burrow into the sediment to withstand the cold/ice cover (Frouz et al., 2003; Hill et al., 2016). Additionally, some adult life stages of aquatic invertebrates, such as trichoptera and coleoptera, are known to take winter refuge in adjacent terrestrial habitats, and both of these taxa were absent in our winter samples (Chadd, 2010; Hill et al., 2016). These explanations should have led to several taxa being missed in our aquatic artificial substrate colonization technique during winter, resulting in large differences between seasons. This was evidently the case for total abundance and richness at Dundas WWTP sampling sites and for diversity at the Woodward WWTP sampling sites, as all were lower during winter. However, we did not find a strong seasonal influence on the impacts of wastewater on benthic macroinvertebrate communities; impacted sampling sites remained relatively high in abundance at Woodward and low in abundance at Dundas in both seasons.

The contrasting patterns in total macroinvertebrate abundance between the two WWTPs types could be due to bottom-up effects. The effluent released from the Woodward WWTP was of poorer quality than effluent released from the Dundas WWTP, particularly during winter. At the larger Woodward WWTP, the higher levels of nutrients, organic matter (TDS and TSS as measured in the effluent), and temperature at sites near the outfall during the winter may have allowed certain taxa to remain high in abundance via bottom-up control despite the expected winter dormancy effects described above. This was further supported by the higher fold-difference in benthic macroinvertebrate abundance between downstream and upstream sites at the Woodward WWTP in winter (~149×) compared to summer (~21×). In contrast, at the smaller Dundas WWTP, the lower abundance of benthic macroinvertebrates during winter may be attributed to the overall lower levels of total phosphorus that characterized all sampling sites. As mentioned before, phosphorus is one of the main water quality indicators of productivity (Schindler, 1978; McQueen et al., 1986; Chapra and Robertson, 1977), therefore, the lower concentrations of phosphorus measured at the Dundas sampling sites could explain the overall lower abundance of benthic macroinvertebrates in winter. However, this was not the case at Woodward, where total phosphorus levels remained relatively stable between seasons, particularly at the downstream sites. Overall, the lack of strong seasonal effects on benthic macroinvertebrate community responses to wastewater contradicted our initial predictions. These predictions were based on our previous work that demonstrated strong seasonal effects, where fish (Mehdi et al., 2021) and zooplankton (Mehdi et al., in prep) abundance were significantly higher near wastewater outfalls, but only during the winter. The different seasonal responses to wastewater effluent demonstrated by different trophic levels remains largely unexplored and warrants further research.

3.5.3 Conclusions

Our study is unique because we compared the impacts of wastewater effluent contamination on benthic macroinvertebrate communities and water quality between summer and winter. Despite finding major seasonal differences in water quality in effluent-receiving environments as well as finding general differences in community metrics between seasons, the effects of wastewater on benthic macroinvertebrate communities were similar in summer and winter. Interestingly, the two WWTPs sampled in our study demonstrated opposite trends in their impacts on benthic macroinvertebrate communities. Further research should investigate why and how WWTPs with different treatment capabilities and effluent-receiving environments might impact benthic communities differently. Differences in WWTPs' effluent footprints, treatment capabilities, source populations, and receiving habitats must be taken into consideration when evaluating their impacts on aquatic environments. Such research would improve the direction and precision of remediation strategies in restoring aquatic communities in effluent-receiving habitats. Additionally, studies of this kind demonstrate the importance of conducting research during winter, a season largely neglected in ecotoxicology (Powers and Hampton, 2016; Salonen et al., 2009; Hampton et al., 2015; McMeans et al., 2020). Our findings will contribute to the recent focus of understanding winter ecology, particularly in temperate and polar regions around the world, where field work is especially challenging during that time of year.

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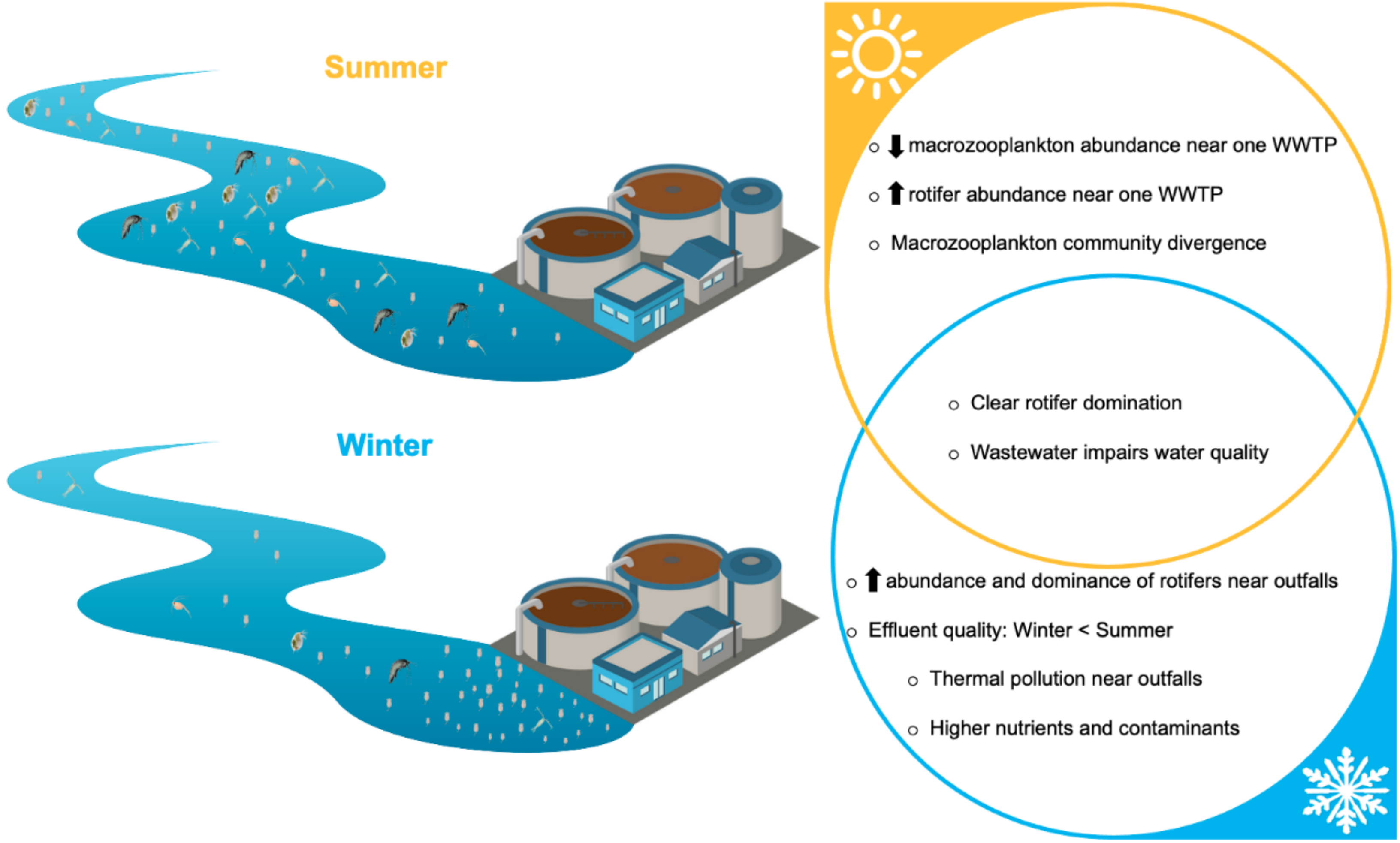
Chapter 4: Impacts of wastewater effluent on zooplankton communities in summer and winter



- ↓ macrozooplankton abundance near one WWTP
- ↑ rotifer abundance near one WWTP
- Macrozooplankton community divergence

- Clear rotifer domination
- Wastewater impairs water quality

- ↑ abundance and dominance of rotifers near outfalls
- Effluent quality: Winter < Summer
- Thermal pollution near outfalls
- Higher nutrients and contaminants

4.1 Abstract

Effluents from municipal wastewater treatment plants (WWTPs) are a large and ubiquitous source of aquatic pollution, with well-demonstrated impacts on aquatic life across multiple levels of biological organization. Despite this, our knowledge on how wastewater affects zooplankton communities is limited, particularly during winter – a season often ignored in ecotoxicology. In this study, we examined the effects of wastewater contamination on zooplankton communities along effluent gradients of two distinct municipal WWTPs discharging into Hamilton Harbour, Canada during both summer and winter. Zooplankton communities were numerically dominated by rotifers both in summer and winter. At the larger of the two WWTPs, macrozooplankton abundance, richness, and diversity all increased with distance from the outfall in summer; while in winter, the opposite trends were observed for abundance and richness. At the smaller WWTP, only macrozooplankton richness appeared to increase with distance from the outfall and this pattern was only observed during winter. One clear trend observed at both WWTPs was the high abundance of rotifers near the outfall, especially during winter. Furthermore, macrozooplankton community composition differed along the effluent gradients of both WWTPs, with the sites closest and farthest from the outfalls being the most dissimilar, particularly during summer. Overall, we show that wastewater outfalls are a significant source of nutrients, contaminants, thermal pollution, and productivity, predominantly so during winter. Our results indicate that effluent-receiving environments support high abundances of prey

populations; thereby, further supporting the notion that such environments may act as an ecological trap for fishes and other aquatic organisms.

4.2. Introduction

Aquatic environments are under continuous pressure from population growth and urbanization, resulting in poor water quality and habitat degradation (Vörösmarty et al. 2010; Reid et al. 2018). Effluents discharged from municipal wastewater treatment plants (WWTPs) are a major and continuous contributor to this degradation, accounting for the largest source of aquatic pollution (by volume) in many regions around the world (Environment Canada, 2011; Holeton et al. 2011). Although treated, wastewater effluents still contain complex mixtures of pesticides, natural and synthetic hormones, nutrients, pharmaceuticals and personal care products (PPCPs), and micro- and macroplastics (Daughton and Ternes, 1999; Kolpin et al. 2002; Holeton et al. 2011; Hamdhani et al. 2020). The continuous discharge of wastewater into surface waters means aquatic biota are chronically exposed to various contaminants of concern, excess nutrients, hypoxic conditions, and thermal alterations (Odjadjare and Okoh, 2010; Tetreault et al. 2011; Holeton et al. 2011; Melvin et al. 2016; Hamdhani et al. 2020; Mehdi et al. 2021). As urban populations continue to grow and expand, pressure on WWTPs will continue to increase, raising concerns over wastewater's growing impacts on aquatic ecosystems (Sumpter, 2009; Bernhardt et al. 2017).

Wastewater effluents are known to affect aquatic organisms across multiple levels of biological organization, from molecular to whole-organism responses (Holeton et al. 2011; Hamdhani et al. 2020). Responses include endocrine, sexual, and reproductive disruptions (Bahamonde et al. 2015; Fuzzen et al. 2015), increased metabolic costs (Du et al. 2018, 2019 Mehdi et al. 2018; Lau et al. 2021), altered behaviours (Saaristo et al. 2014; McCallum et al. 2017; McLean et al. 2019), morphological abnormalities (Nikel et al. 2021), and changes in gut or whole-body microbiomes (Millar et al. 2021; Restivo et al. 2021). In contrast to the ample research on the individual level, few studies have addressed how aquatic populations and communities respond to these wastewaters, despite the importance of such higher-level endpoints to management agencies when evaluating risks (Cvetkovic et al. 2010). The handful of studies that have examined the impacts of wastewater on populations and communities have predominantly focused on fishes, and generally demonstrate an overall increase in fish abundance, with a higher proportion of tolerant or invasive species found closer to effluent outfalls (Brown et al. 2011; Tetreault et al. 2013; McCallum et al. 2019; Mehdi et al. 2021).

In addition to knowing little about community responses, it is the case that much of our current understanding of the impacts of WWTP effluents on aquatic ecosystems comes from research conducted during warmer months, despite the year-round release of treated effluent and the prolonged cold conditions in some regions when wastewater treatment is less effective. The poorer quality of wastewater effluents during winter months is due to the reduced biological degradation of many contaminants at colder temperatures, as well as the higher prescription and consumption rates of PPCPs and other health products (Vieno et al. 2005; Sui et al. 2011; Yu et al. 2013; Kot-Wasik et al. 2016; Mehdi et al. 2021). In addition, the relatively warm effluents released from WWTPs in winter can increase temperatures of receiving environments by 5-10ºC, creating thermally enhanced and nutrient rich zones that are attractive for aquatic organisms (Environment Canada, 2001; Kinouchi et al. 2007; Mehdi et al. 2021). Indeed, these wastewater plumes appear to act as an ecological trap for fishes, as their abundance, richness, and diversity has been reported to be highest near wastewater outfalls, especially so in winter, even though overall abundance lower compared to summer (Mehdi et al. 2021). Aggregation of fishes near wastewater outfalls may be linked to higher food availability, suggesting that zooplankton and benthic macroinvertebrates productivity are also likely to high in such nutrient-rich environments. Zooplankton are ubiquitous in most aquatic systems and are often used as bioindicators of water quality and ecosystem health (Zhou et al. 2008; Deksne, 2011; Argawal and Gopal, 2013). Their roles as primary consumers and as prey for higher-trophic-level organisms makes them effective bioindicators of food web dynamics and ecosystem function (Jeppesen et al. 2011; Bowen and Currie, 2017; Garcia-Chicote et al. 2018). Moreover, the presence and absence of certain zooplankton taxa and their overall community composition in an area can be used to make inferences about the quality of habitats through bottom-up and top-down mechanisms (Gannon and Stemberger, 1978).

In this study, we examined the effects of municipal wastewater effluents on zooplankton communities in both summer and winter along effluent gradients of two WWTPs, the Dundas and Woodward WWTPs. Both these WWTPs discharge their effluents into Hamilton Harbour, a large eutrophic embayment on the western end of Lake Ontario, Canada, and a declared Area of Concern under the Great Lakes Water Quality Agreement (2012). Wastewater contamination is of particular concern to Hamilton Harbour, as it is estimated that up to ~50% of the water that enters the harbour (not including periodic lake inflows) is of wastewater origin (Lawrence et al. 2004; Government of Canada, 2017). Because effluents contain nutrients and diverse chemicals and may provide a thermal refuge in winter, we predicted that zooplankton abundance and community composition would differ between outfall-impacted and reference sites across both seasons, and that these differences would be greatest in winter months.

4.3 Materials and methods

4.3.1 Sampling design and study sites

Zooplankton were sampled (in triplicates) at each site using vertically-integrated Van-Dorn hauls conducted four times in summer (July and August) and three times in winter (November, December, and March) of 2018 and 2019 (n=3/site/date) along the effluent gradients created by the Dundas and Woodward WWTPs. Water quality parameters and habitat characteristics were also measured and used to describe the abiotic conditions in each of our sampling sites. All the sites along the gradient of each WWTP were sampled on the same day to minimize temporal variability. Sampling sites were selected for their accessibility and because they were part of a long-term research program investigating the impacts of wastewater on aquatic communities (Aristone et al., 2022; McCallum et al. 2019; Mehdi et al. 2021; Nikel et al. 2021).

4.3.1.1 Dundas WWTP

The Dundas WWTP is a conventional activated sludge plant with tertiary filtration and is located on the western tip of Cootes Paradise Marsh which flows into Hamilton Harbour. The plant treats wastewater from the town of Dundas (~30,000 people) and has a daily capacity of 18.2 million litres (City of Hamilton, 2019). The treated effluent from the plant is discharged into the western-most tip of Cootes Paradise Marsh via an old shipping corridor, the Desjardins Canal (Theysmeyer and Bowman, 2017). Zooplankton samples were collected from five sites at increasing distances from this WWTP's outfall (Fig. 4.1A). Three of the sites sampled were in the direct flowpath of the WWTP effluent outfall: D1 was immediately downstream of the outflow, D2 was 550 m downstream of the outflow, and D3 was 1000 m downstream of the outflow. Two additional reference sites were sampled, D4 (2800 m downstream) is located on the mouth of Spencer Creek and D5 (3750 downstream) is located on the southwestern edge of Cootes Paradise Marsh; neither reference site was in the direct path of the effluent flow, so wastewater impacts were assumed to be dampened there. Sites closest to the outfall at the Dundas WWTP (D1 and D2) had clear modifications to the shoreline and were in close proximity to urban structures. Sites farther away from the outfall (D3 – D5) were more natural, surrounded by wetland and natural forest habitats, and were overall less disturbed than sites near the outfall. See Supplementary Table 4.1 for further details on habitat characteristics at these sites.

4.3.1.2 Woodward WWTP

The Woodward WWTP, a secondary conventional activated sludge plant with sludge dewatering and digestion is located on the southeastern corner of Hamilton Harbour (City of Hamilton, 2019). The plant treats the majority of wastewater generated by the city of Hamilton population (~480,000 people) and has an average daily capacity of 409 million litres (City of Hamilton, 2019). Five sites were sampled for zooplankton and included three sites that were downstream of the effluent outfall: W1 (outfall), W2 (350 m downstream), and W3 (850 m downstream), as well as two upstream reference sites: W4 (1000 m upstream) and W5 (1400 m upstream). The Woodward WWTP is situated in a heavily industrialized part of Hamilton (East Hamilton Harbour), and all sites were significantly more anthropogenically modified than sites near the Dundas WWTP. All Woodward sampling sites had clear modifications to the shoreline, were in close proximity to urban structures, and had relatively narrow riparian zones. See Supplementary Table S1 for further details on habitat characteristics in all our sampling sites.

4.3.2 Habitat characterisation and water quality assessment

The habitat characteristics of the sites were evaluated following McCallum et al. (2019) and were based on a subset of the metrics used in the Qualitative Habitat Evaluation Index (QHEI; Taft and Koncelik, 2006; Strickland et al. 2010). Habitat metrics measured included: total water depth, water clarity (Secchi depth), substrate type, sediment particle size, shoreline slope, degree of sinuosity, degree of anthropogenic modifications (i.e., physical alterations of the shore-water interface), riparian zone width, degree of estimated bank erosion, and the presence of any aquatic plants. Across the two gradients sampled for this study, the physical habitat characteristics remained relatively unchanged between seasons, apart from vegetation being more prominent and abundant during the summer.

At each site and on each sampling day, the following water quality parameters were measured: water temperature and dissolved oxygen (YSI ProODO), pH, conductivity, salinity, and total dissolved solids (Oakton multiparameter Testr). Long-term temperature was also monitored using HOBO Pendant MX temperature loggers (Onset Computer Corp) which recorded water temperature over 14-day periods in the summer and winter at the two WWTP outfall sites (D1 and W1) and two of the corresponding reference sites (D4 and W4) of both WWTPs. The loggers recorded water temperature every 30 minutes in the summer and every 15 minutes in the winter. Additionally, at each site and on each sampling date, a 1 L water sample was collected at the mid-water column level using a 2.2 L Van Dorn sampler (Wildco Alpha) where it was later analysed by the City of Hamilton Environmental Laboratory for the following parameters: ammonia and ammonium, nitrate, nitrite, total Kjeldahl nitrogen ortho-phosphate, and total phosphorus. Additionally, 24-hour composite effluent samples were collected from each WWTP during the summer and winter of 2019 and analyzed for total suspended solids, biochemical oxygen demand, total phosphorus, total Kjeldahl nitrogen, ammonia, nitrate, nitrite, and *Escherichia coli* (measurements provided by the City of Hamilton and can be found in Supplementary Table S2).

4.3.3 Sample acquisition and enumeration

All zooplankton samples were collected between 0800 and 1400 h on weather permitting days based on methods previously described in Lougheed and Chow-Fraser (2002), Deksne (2011), and Bowen and Currie (2017). Briefly, before sample collection at each site, total water depth was measured by lowering a Secchi disk to the bottom. This depth was divided into four equal zones and a 2.2L Van Dorn sampler was then used to collect water from each of the four zones. Water from all four Van Dorn hauls at each site was pooled (8.8 L total) and filtered using 64 μm sieve. Material collected on the sieve was preserved immediately with 4% sugar-buffered formalin solution on site. This process was repeated to yield three replicates for each sampling site during each visit, resulting in 12 samples per site during the summer and 9 samples per site during the winter.

Zooplankton were enumerated based on a protocol adapted from the technical report of Rozon et al. (2016). All macrozooplankton (not including rotifers) in a sample were enumerated using a 6 mL Bogorov counting chamber under a dissecting microscope (Nikon SMZ1500 Zoom Stereomicroscope) equipped with a digital camera (QImaging Retiga 2000R CCD) and video capture software (QCapture Suite Plus version 3.1.3.10). Whole-sample enumeration of macrozooplankton was possible due to their relatively low density in our samples. Macrozooplankton were counted and identified to most practical taxonomic levels: Bosminidae (family), copepods (sub class), Chydoridae (family), Daphniidae (family), and Sididae (family). Rotifers were identified to phylum and because rotifer abundance was high, they were subsampled and enumerated on a 1 mL Sedgewick-Rafter chamber under a compound light microscope (Leica DM2500). Rotifers were counted in random cells on the Sedgewick-Rafter chamber (using a random number generator; random.org). If the first two cells enumerated contained fewer than 25 rotifers, then all remaining cells were counted. However, if the first two cells contained more than 25 rotifers cumulatively then additional randomly selected cells were counted until at least 200 rotifers were enumerated. Abundance (i.e., density per L) was then calculated for each individual family or subclass of macrozooplankton and total rotifers.

4.3.4 Statistical analyses

All statistical analyses were conducted using R (version 4.0.4; R Core Team, 2021). Water quality parameters were plotted and analyzed using principal component analysis, thereby, minimizing the total number of statistical analyses needed and improving data visualization by creating two-dimensional biplots for principal components 1 and 2. Differences in water quality parameters among sites were collectively analyzed using permutation ANOVAs with 5,000 permutations within each season (adonis2; Vegan package; Oksanen et al. 2019). Additionally, differences in individual water quality parameters were analyzed as a function of sampling site order from the outfall (i.e., representing both physical distances and contamination load differences across sites) and season using permutation MANOVAs with p-values adjusted to account for multivariate analysis. Long-term water temperature data were analyzed using linear models (LMs) to assess temperature differences between the outfall and reference sites within each season.

Zooplankton abundance (macrozooplankton and rotifers), ratio of macrozooplankton to rotifers, zooplankton richness (family/subclass level), and diversity (family/subclass level; Shannon-Weiner Index) were all analyzed using permutation linear mixed effects models (PLMM) with 5,000 iterations (lme4 and predictmeans packages; Bates et al. 2015; Luo et al. 2020). These response variables were analyzed in relation to sampling site order from the outfall and season as main effects, as well as replicate number and sampling period within each season as random effects. Zooplankton community composition differences were calculated using a Bray-Curtis dissimilarity matrix and later visualized using Principal Coordinate Analysis (PCoA) biplots with 80% site-specific confidence ellipses overlain to ascertain community compositional differences across sites and between seasons (Oksanen et al. 2019). The differences in zooplankton community composition between seasons and across sites (based on Bray-Curtis dissimilarity matrix) were later analyzed using permutation ANOVA with 5,000 permutations (Vegan package; Oksanen et al. 2019). Furthermore, similarity percentages (SIMPER) analysis was used to identify which key macrozooplankton taxa were responsible for the observed differences in community composition across all site combinations. While all zooplankton taxa were included in the analysis, only those that contributed $\geq$5% to the total abundance were further examined and interpreted. Finally, to assess how various zooplankton metrics varied in relation to water quality parameters, macrozooplankton abundance, rotifer abundance, and richness were analyzed in relation to overall water quality parameters (principal components 1 and 2) within each season using linear multiple response permutation models (lmp and multresp function; lmPerm package; Wheeler and Torchiano, 2016). Because the gradients sampled in our study were generated by WWTPs that varied significantly in size and treatment, all analyses were conducted separately between each WWTP. Data are reported as means ± standard error (SE) unless otherwise stated. In all analyses, a difference was deemed significant when $p < 0.05$.

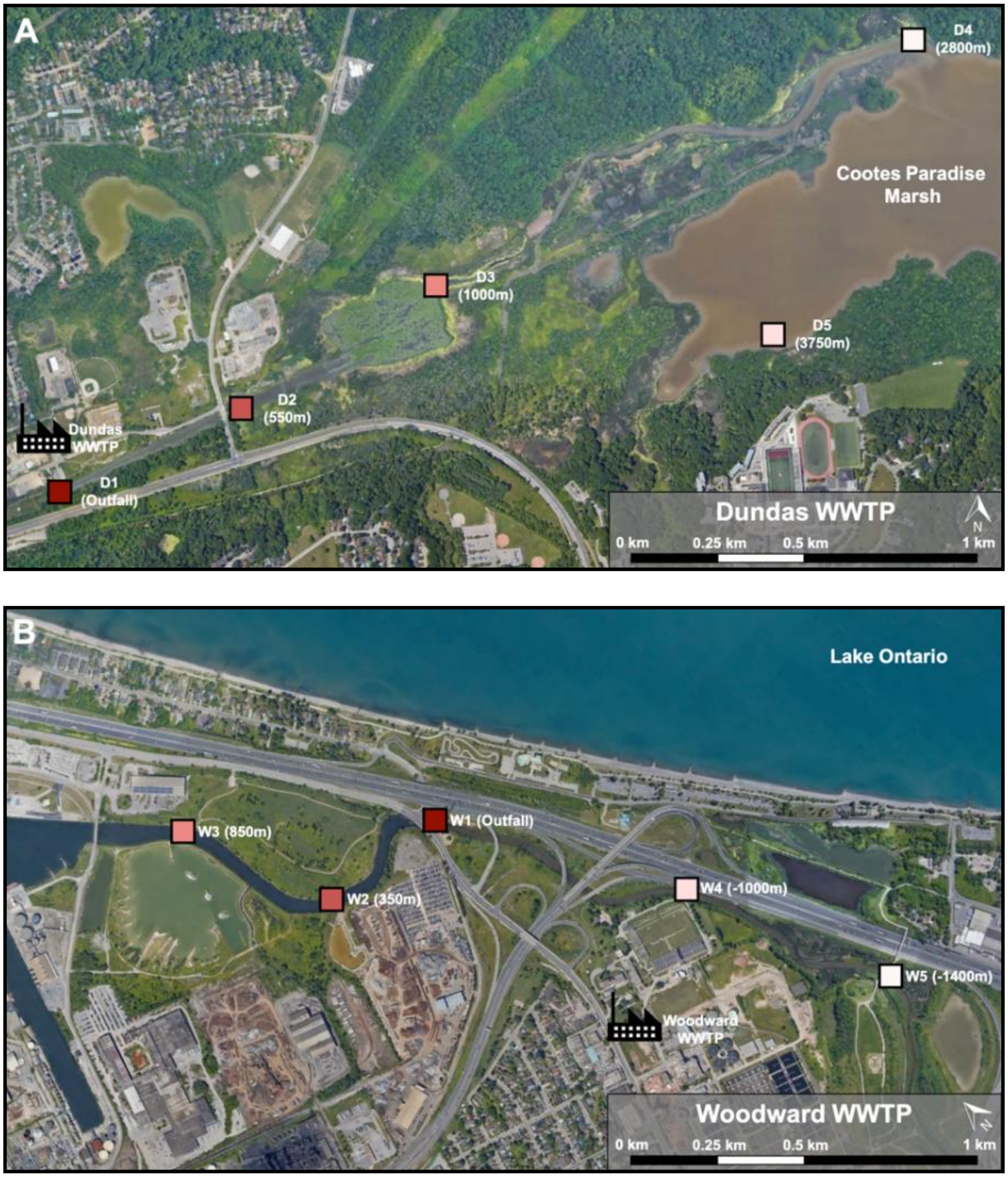


Figure 4.1. Map of our sampling sites along a distance gradient from the **(A)** Dundas and **(B)** Woodward WWTPs. The location of each WWTP is also displayed with a factory icon. The colours of the squares indicate the presumed contamination load of each sampling site (with darker red = more contaminated) Maps were generated in Google Earth Pro 7.3.2.5776, imagery date 06/30/2018 and accessed on 24/02/2020 and also were based on Mehdi et al. (2021).

4.4 Results

4.4.1 Water quality

4.4.1.1 Dundas WWTP

Water quality at sites closer to the outfall was poorer than those at sites farther away in both seasons (Permutation ANOVA, $F_{(Summer)} = 3.58$, $p < 0.001$; $F_{(Winter)} = 5.05$, $p < 0.001$; Figs. 4.2A and 4.2B; Table 4.1). In the summer, total nitrogen, total nitrate nitrogen, conductivity, total dissolved solids, and salinity were highest at the outfall site, and decreased in concentration with distance from the WWTP. In the winter, water temperature, total nitrogen, total ammonia nitrogen ($p = 0.06$), total nitrate nitrogen, total phosphorus, conductivity, total dissolved solids, and salinity and were all highest near the outflow site, and decreased with distance from the treatment plant, while dissolved oxygen and pH increased with distance from the outfall (all contrasts were $p < 0.05$, unless otherwise stated). In the summer, water temperature did not differ between the outfall (D1) and reference (D4) sites (LM, $t_{(Summer)} = 1.49$, $p = 0.15$), but in the winter, temperature was on average ~8ºC higher at the outfall site that it was at the reference site (LM, $t_{(Winter)} = 25.25$, $p < 0.001$).

4.4.1.2 Woodward WWTP

As with the Dundas WWTP, sites downstream of the outfall had poorer water quality when compares to upstream sites, and this was the case in both seasons (Permutation ANOVA, $F_{(Summer)} = 2.51$, $p < 0.01$; $F_{(Winter)} = 3.17$, $p = 0.01$; Figs. 4.2C and 4.2D; Table 4.1). During the summer, total nitrogen, total nitrate nitrogen, total phosphorus, and soluble reactive phosphorous were higher in the downstream sites than in the upstream sites, while the opposite trend was observed for water temperature and pH. During the winter, water temperature, total nitrogen, total ammonia nitrogen (p = 0.08), total nitrate nitrogen, total phosphorus, and soluble reactive phosphorus were all highest near the outfall and lowest in the sites upstream, while the opposite was true for dissolved oxygen (all contrasts were $p < 0.05$, unless otherwise stated). In the summer, water temperature did not vary significantly between the outfall (W1) and reference (W4) sites (LM, $t_{(\mathrm{Summer})} = 0.42$, $p = 0.68$); however in the winter, the outfall site was on average ~9ºC warmer than the reference site (LM, $t_{(\mathrm{Winter})} = 22.30$, $p < 0.001$).

4.4.2 Zooplankton community metrics

4.4.2.1 Dundas WWTP

At the Dundas WWTP sampling sites, the macrozooplankton community was comprised of copepods and the cladoceran families Bosminidae, Chydoridae, Daphnidae, and Sididae. Macrozooplankton abundance was not influenced by season (PLMM, $t = 0.71$, $p = 0.45$; Fig. 4.3A) nor was it influenced by proximity to the effluent outfall (PLMM, $t = 0.65$, $p = 0.74$; Fig. 4.3A). In contrast, rotifer (microzooplankton) abundance differed between seasons (PLMM, $t = 2.76$, $p = 0.03$; Fig. 4.3B), with ~12 times more rotifers found during summer than winter. Additionally, rotifer abundance declined with distance from the outfall during winter but not during summer ($t_{(\mathrm{Winter})} = -16.65$, $p < 0.001$; $t_{(\mathrm{summer})} = 0.01$, $p = 0.80$; Fig. 4.3B). Macrozooplankton richness was significantly lower in winter compared to summer (PLMM, t = 29.64, p < 0.001; Fig. S1A) and increased with distance from the outfall in winter (t = 4.86, p = 0.02; Fig. S1A), but not in summer (t = 1.99, p = 0.17; Fig. S1A). Similarly, macrozooplankton diversity (Shannon-Weiner Index) tended to be lower in the winter relative to the summer (PLMM, t = -2.18, p = 0.08; Fig. S1B). Diversity tended to be highest at sites farther away from the outfall in winter but not in summer ($t_{\text{(Winter)}}$ = 1.78, p = 0.09; $t_{\text{(Summer)}}$ = 0.11, p = 0.78; Fig. S1B).

The community composition of macrozooplankton differed significantly between seasons (Permutation ANOVA, $F_{\text{(Season)}}$ = 11.34, p < 0.001; Fig. 4.4A). In the summer, divergence in community composition across sites was most apparent between the outfall site and reference sites, while intermediate sites had significant overlap in their community compositions ($F_{\text{(Summer)}}$ = 4.89, p < 0.001; Fig. 4.4A). This change in community structure was further confirmed using similarity analysis (Table 4.2 and Supplementary Table S3). The degree of dissimilarity in community composition between the outfall site and the other sites steadily increased the farther away each site was from the outfall: $77.3\%_{\text{(D1 vs. D2)}}$, $88.2\%_{\text{(D1 vs. D3)}}$, $88.2\%_{\text{(D1 vs. D4)}}$, and $92.8\%_{\text{(D1 vs. D5)}}$. In the summer, this community divergence was mainly attributed to the higher proportion Bosminidae at the outfall site (D1) relative to all other sites, as well as to the lower proportion of Sididae at the outfall site (D1) compared to one of the reference sites (D5; Fig. 4.5A; Table 4.2). In the winter, while macrozooplankton community compositions varied across sites ($F_{\text{(Winter)}}$ = 3.55, p < 0.001; Fig. 4.4B), there was considerable overlap between the sites in their community composition (PCoA; Fig. 4.4B). This was further confirmed by similarity analysis (Table 4.2), where community composition differences did not follow a clear pattern of distance from impact as was observed in the summer: 68.3%$_{(\text{D1 vs. D2})}$, 65.3%$_{(\text{D1 vs. D3})}$, 75.0%$_{(\text{D1 vs. D4})}$, and 70.0%$_{(\text{D1 vs. D5})}$. Differences between the outfall site (D1) and the other sites in the winter were mainly attributed to the outfall site (D1) having lower relative abundance of Chydoridae compared to the D3 site, a higher relative abundance of copepods than at the D4 site, and lower relative Bosmindae abundance than at the D5 site (Fig. 4.5B; Table 4.2).

4.4.2.2 Woodward WWTP

At the Woodward WWTP sampling sites, the macrozooplankton community was also comprised of copepods and the cladoceran families Bosminidae, Chydoridae, Daphnidae, and Sididae. Macrozooplankton abundance was ~4.3 times higher in summer than in winter (PLMM, t = 1.70, p = 0.05; Fig 4.3C). A significant interaction was detected, indicating that the influence of proximity to the outfall depended on whether samples were collected in summer or winter (PLMM, t = 3.40, p = 0.001; Fig 4.3C). In summer, sites upstream of the effluent outfall had higher abundance of macrozooplankton compared to the sites downstream (PLMM, t = 3.33, p = 0.002; Fig 4.3C). However, during the winter, the opposite was observed, macrozooplankton abundance was highest near the outfall and lowest in the upstream sites (PLMM, t = -1.71, p = 0.04; Fig. 4.3C). In contrast to the patterns with macrozooplankton, rotifer abundance was significantly higher in the winter than in the summer (PLMM, $t = 5.05$, $p < 0.001$; Fig 4.3D). Additionally, rotifer abundance decreased with increasing distance from the outfall during both seasons. However, a significant interaction was detected (PLMM, t = 5.11, p < 0.001; Fig 4.3D), revealing that the influence of outfall proximity was much greater in the winter than it was in the summer (PLMM, $t_{\text{(Winter)}} = -7.34$, $p < 0.001$; $t_{\text{(Summer)}} = -3.07$, $p = 0.003$ Fig 4.3D). Macrozooplankton richness depended on both season and proximity to the outfall (PLMM, t = 3.92, $p < 0.001$; Fig. S1C); in the summer, richness increased significantly with distance from the outfall (PLMM, $t = 2.59$, $p = 0.01$; Fig. S1C), while the opposite pattern was observed in the winter (PLMM, $t = -2.02$, $p = 0.04$; Fig. S1C). Similarly, macrozooplankton diversity (Shannon-Weiner Index) was dependent on both season and proximity to the outfall (PLMM, $t = 2.77$, $p = 0.009$; Fig. S1D); in the summer, diversity increased with distance from the outfall (PLMM, $t = 2.67$, $p = 0.01$; Fig. S1D), while in the winter, proximity did not influence diversity (PLMM, $t = 1.43$, $p = 0.12$; Fig. S1D).

Macrozooplankton community composition differed significantly between seasons (Permutation ANOVA, $F_{\text{(Season)}} = 6.06$, $p < 0.001$; Fig. 4.4C). In the summer, macrozooplankton community composition differed across the different sites ($F_{\text{(Summer)}} = 4.87$, $p < 0.001$; Fig. 4.4C). This was evident from the PCoA biplots (Fig. 4.4C) where a clear separation was found between sites downstream of the WWTP outfall and those upstream. This divergence in community composition was further confirmed using similarity analysis (Table 4.2 and Supplementary Table S3), where community composition differences increased the farther away the site was from the outfall: 66.5%$_{(\text{W1 vs. W2})}$, 75.9%$_{(\text{W1 vs. W3})}$, 88.7%$_{(\text{W1 vs. W4})}$, and 90.0%$_{(\text{W1 vs. W5})}$. This community divergence was mainly driven by a lower Bosminidae and Daphniidae abundance at the outfall (W1) site compared to the W2 and W3 sites, lower Siddidae abundance at the W1 site compared to the W4 site, and lower copepod abundance at the W1 site than at the W5 site (Fig. 4.5C; Table 4.2). In the winter, macrozooplankton community composition did not appear to differ significantly across sites (Permutation ANOVA, $F_{(\text{Winter})} = 1.52$, $p = 0.10$; Fig. 4.4D). The overlap between sites in the winter was also confirmed via similarity analysis (Table 4.2); the differences in communities between sites did not follow a clear pattern with distance from the outfall as was found in the summer: 71.4%$_{(\text{W1 vs. W2})}$, 73.0%$_{(\text{W1 vs. W3})}$, 68.8%$_{(\text{W1 vs. W4})}$, and 80.02$_{(\text{W1 vs. W5})}$. Although overall zooplankton community composition did not vary significantly between sites in the winter, there were a few macrozooplankton groups that did differ between sites. Most notably, Daphniidae were less abundant at W1 than at W3 and copepods were more abundant at W1 than at W5 (Fig. 4.5D; Table 4.2).

4.4.3 Zooplankton community responses to water quality

4.4.3.1 Dundas WWTP

Water quality parameters were examined using principal component analysis (Figs. 4.2A and B; Supplementary Table S4). In the summer, pH, conductivity, total dissolved solids, salinity, total nitrogen, and total nitrate nitrogen loaded strongly on PC1, which explained 47.5% of the variation in water quality. However, despite the high proportion of variance explained, none of the zooplankton biodiversity metrics correlated significantly with PC1 (Table 4.3). Temperature, total ammonia nitrogen, total phosphorus, and soluble reactive phosphorus were all associated with PC2, which explained only 18.3% of the total variation (Fig. 4.2A; Supplementary Table S4), and only macrozooplankton abundance was correlated with PC2 (Table 4.3). In the winter, temperature, pH, total nitrogen, total nitrate nitrogen, conductivity, total dissolved solids, and salinity all loaded highly on PC1, which explained 63.9% of the total variation (Fig. 4.2B; Supplementary Table S4). Both rotifer abundance and macrozooplankton richness were significantly correlated with PC1 (Table 4.3). PC2 explained only 15.9% of the variation with only dissolved oxygen and total ammonia nitrogen loading highly on it (Fig. 4.2B; Supplementary Table S4). Macrozooplankton abundance was the only significant correlation with PC2 (Table 4.3).

4.4.3.2 Woodward WWTP

In the summer, pH, total nitrate nitrogen, total ammonia nitrogen, total phosphorus, and soluble reactive phosphorus loaded highly on PC1, which explained 45.0% of the total variation in water quality (Figs. 4.2C and D; Supplementary Table S4). Macrozooplankton abundance, rotifer abundance, and macrozooplankton richness were all significantly correlated with PC1 (Table 4.3). PC2 on the other hand explained 37.6% of the variation, with temperature, dissolved oxygen, conductivity, total dissolved solids, and salinity all loading highly on it (Fig. 4.2C; Supplementary Table S4). PC2 was strongly correlated with both macrozooplankton and rotifer abundance (Table 4.3). In the winter, temperature, dissolved oxygen, pH, total nitrogen total nitrate nitrogen, total ammonia nitrogen, total phosphorus, and soluble reactive phosphorus highly loaded on PC1, which explained 66.4% of the variation (Fig. 4.2D; Supplementary Table S4) and correlated significantly with all zooplankton metrics (Table 4.3). PC2 explained 18.0% of the total variation, with only conductivity, total dissolved solids, and salinity loading highly on it (Fig. 4.2D; Supplementary Table S4). PC2 only significantly correlated with rotifer abundance (Table 4.3).

Table 4.1. Mean (± SE) of water quality parameters taken at each sampling site across both seasons (summer | winter). The following water quality parameters were measured: water temperature, dissolved oxygen saturation (DO), pH, conductivity, total dissolved solids (TDS), salinity, total ammonia nitrogen (TAN), total nitrate nitrogen (TNN), total nitrogen (TN), soluble reactive phosphorus (SRP), and total phosphorus (TP). Water quality data previously reported in Mehdi et al. (2021).

	Dundas WWTP				
	D1 (Outfall)	D2 (550 m)	D3 (1000 m)	D4 (2800 m)	D5 (3750 m)
Water temperature (°C)	21.1±0.679 | 9.97±0.578	23.3±0.578 | 7.67±0.689	24.2±0.702 | 4.87±1.24	23.7±1.12 | 3.23±0.667	26.7±1.22 | 3.60±1.56
DO (%)	124±6.73 | 91.1±4.99	134±12.0 | 98.2±5.54	90.7±17.3 | 110.±1.63	87.0±7.64 | 101±1.54	132±24.3 | 108±2.41
pH	7.57±0.114 | 7.69±0.223	7.88±0.271 | 7.84±0.194	7.49±0.229 | 8.40±0.240	8.22±0.147 | 8.78±0.211	8.34±0.272 | 8.80±0.163
Conductivity (µS)	1190±45.9 | 1450±75.1	1150±46.6 | 1530±92.1	1130±74.7 | 1490±82.2	944±101 | 942±90.7	886±64.8 | 967±98.0
TDS (ppm)	845±34.1 | 1010±47.4	819±32.0 | 1090±64.7	807±82.9 | 1070±58.7	670.±70.8 | 670.±65.3	626±45.7 | 690.±70.1
Salinity (ppm)	563±29.3 | 719±34.6	544±20.7 | 756±38.1	537±30.8 | 732±28.7	441±44.6 | 462±59.3	414±27.0 | 476±60.4
TAN (mg/L)	0.030±0.006 | 0.507±0.437	0.046±0.011 | 0.223±0.159	0.060±0.020 | 0.137±0.058	0.060±0.039 | 0.043±0.015	0±0 | 0.020±0.010
TNN (mg/L)	15.4±0.766 | 17.1±1.17	11.3±1.82 | 16.1±1.02	4.84±1.57 | 11.6±1.29	0.493±0.314 | 1.07±0.195	0±0 | 0.690±0.205
TN (mg/L)	16.3±0.515 | 18.5±0.695	12.5±1.68 | 17.0±1.03	6.43±1.52 | 13.1±0.973	1.53±0.398 | 1.64±0.229	1.38±0.222 | 1.21±0.252
SRP (mg/L)	0±0 | 0±0	0±0 | 0±0	0.013±0.013 | 0±0	0.030±0.018 | 0±0	0.04±0.002 | 0±0
TP (mg/L)	0.117±0.020 | 0.079±0.010	0.117±0.020 | 0.079±0.015	0.183±0.034 | 0.071±0.003	0.118±0.007 | 0.065±0.019	0.194±0.054 | 0.052±0.003
	Woodward WWTP				
	W1 (Outfall)	W2 (350 m)	W3 (850 m)	W4 (-1000 m)	W5 (-1400 m)
Water temperature (°C)	21.7±0.371 | 11.3±0.954	22.2±0.250 | 10.7±0.832	22.7±0.425 | 9.77±0.291	23.3±0.473 | 4.13±1.07	23.3±1.28 | 4.43±1.08
DO (%)	74.2±2.60 | 69.0±1.26	69.4±4.47 | 68.9±0.666	63.3±4.10 | 67.3±2.87	75.4±24.5 | 92.9±2.53	93.6±13.0 | 95.6±0.088
pH	7.07±0.106 | 7.77±0.231	7.08±0.128 | 7.40±0.091	7.06±0.105 | 7.55±0.091	8.05±0.231 | 8.66±0.427	8.13±0.189 | 8.71±0.317
Conductivity (µS)	1170±43.5 | 2020±361	1150±53.2 | 2030±299	1150±56.9 | 1820±289	1150±208 | 2350±289	1110±221 | 2020±349
TDS (ppm)	828±30.9 | 1460±238	817±36.8 | 1440±219	815±40.7 | 1100±90.1	812±148 | 1670±206	780.±156 | 1600±274
Salinity (ppm)	540.±21.0 | 1010±175	534±25.2 | 1000±159	534±28.1 | 948±122	535±101 | 1150±142	550.±99.7 | 1110±180.
TAN (mg/L)	0.463±0.183 | 2.32±1.65	0.893±0.433 | 2.23±1.53	1.20±0.481 | 2.26±1.42	0.120±0.032 | 0.043±0.019	0.040±0.017 | 0.027±0.017
TNN (mg/L)	11.3±0.734 | 10.9±0.508	9.54±1.16 | 10.4±0.717	9.14±1.04 | 9.52±1.16	2.10±0.804 | 1.09±0.135	1.66±0.667 | 1.15±0.063
TN (mg/L)	13.1±1.03 | 16.1±3.21	12.2±1.41 | 15.1±3.66	12.2±1.76 | 14.6±4.01	2.81±0.868 | 1.50±0.094	2.62±0.959 | 1.54±0.093
SRP (mg/L)	0.228±0.048 | 0.187±0.052	0.228±0.055 | 0.187±0.058	0.228±0.058 | 0.193±0.058	0.028±0.016 | 0±0	0±0 | 0.023±0.023
TP (mg/L)	0.452±0.048 | 0.443±0.038	0.406±0.057 | 0.374±0.052	0.361±0.065 | 0.364±0.065	0.13±0.02 | 0.080±0.011	0.157±0.067 | 0.081±0.023

Table 4.2. Similarity percentages (SIMPER) analysis showing the contribution of key macrozooplankton taxonomic groups to the overall dissimilarity of the outfall site relative to all other sampling sites. Only groups that contributed >5% to the overall abundance are shown. Bolded averages indicate significant differences ($p < 0.05$). See Supplementary Table S3 for further details on all site and season comparisons.

Dundas WWTP

	Summer					Winter				
Comparison	Total Dissimilarity	Group	Average A	Average B	Contribution	Total Dissimilarity	Group	Average A	Average B	Contribution
A. D1 (Outfall) B. D2 (500 m)	77.3%	Bosminidae	**31.4**	**5.20**	**53.2%**	68.3%	Chydoridae	1.78	4.89	37.8%
		Sididae	12.0	0.923	12.5%		Copepods	1.93	1.19	28.0%
		Copepods	0.393	1.76	6.31%					
A. D1 (Outfall) B. D3 (1000 m)	88.2%	Bosminidae	**31.4**	**1.92**	**57.8%**	65.3%	Chydoridae	**1.95**	**1.78**	**45.1%**
		Copepods	0.393	3.61	14.0%		Copepods	1.93	1.09	19.7%
		Sididae	12.0	0.398	11.7%					
A. D1 (Outfall) B. D4 (2800 m)	88.2%	Bosminidae	**31.4**	**0.530**	**65.2%**	75.0%	Copepods	**1.93**	**1.35**	**42.7%**
		Sididae	12.0	0.237	14.0%		Chydoridae	1.78	0.871	28.2%
		Copepods	0.393	1.04	5.90%					
A. D1 (Outfall) B. D5 (3750 m)	92.8%	Bosminidae	**31.4**	**0.123**	**47.4%**	70.0%	Copepods	1.93	3.28	33.7%
		Sididae	**12.0**	**61.6**	**29.4%**		Chydoridae	1.78	0.492	18.0%
		Copepods	0.393	5.31	12.8%		Bosminidae	**0.013**	**0.568**	**13.9%**

Woodward WWTP

	Summer					Winter				
Comparison	Total Dissimilarity	Group	Average A	Average B	Contribution	Total Dissimilarity	Group	Average A	Average B	Contribution
A. W1 (Outfall) B. W2 (350 m)	66.5%	Bosminidae	**1.09**	**0.165**	**19.4%**	71.4%	Chydoridae	1.16	1.74	50.1%
		Copepods	1.19	0.238	19.2%		Copepods	0.213	0.13	13.7%
		Daphnidae	**0.052**	**0.114**	**11.7%**					
		Chydoridae	0.021	0.085	11.0%					
		Sididae	0.031	0.043	5.15%					
A. W1 (Outfall) B. W3 (850 m)	75.9%	Bosminidae	**0.165**	**5.58**	**28.7%**	73.0%	Chydoridae	1.16	2.77	46.5%
		Copepods	0.238	0.341	17.3%		Daphnidae	0.043	0.215	15.3%
		Chydoridae	0.207	0.298	17.3%		Copepods	0.213	0.126	9.36%
		Daphnidae	**0.052**	**0.426**	**10.6%**					
A. W1 (Outfall) B. W4 (-1000 m)	88.8%	Sididae	**0.031**	**4.43**	**43.9%**	68.8%	Chydoridae	1.16	0.313	44.3%
		Copepods	0.238	1.97	25.6%		Copepods	0.213	0.114	12.8%
		Chydoridae	0.021	0.369	12.8%		Daphnidae	0.043	0.057	5.90%
							Bosminidae	0	0.085	5.88%
A. W1 (Outfall) B. W5 (-1400 m)	90.0%	Copepods	**0.238**	**6.43**	**40.3%**	80.2%	Chydoridae	1.16	0.227	48.7%
		Sididae	0.031	2.96	28.8%		Copepods	0.213	0.028	26.1%
		Chydoridae	0.624	0.021	15.3%		Daphnidae	0.043	0	5.36%

Table 4.3. Association strength and significance of all water quality parameters condensed into Principal Component 1 and 2 against macrozooplankton abundance, rotifer abundance, and macrozooplankton richness. Bolded values are significant ($p < 0.05$). See Supplementary Table S4 for all PCA loadings.

		Dundas WWTP				**Woodward WWTP**			
		Percentage Variance	Macrozooplankton Abundance	Rotifer Abundance	Macrozooplankton Richness	Percentage Variance	Macrozooplankton Abundance	Rotifer Abundance	Macrozooplankton Richness
PC1	Summer	42.7%	$R^2 = 0.012$, $p = 0.86$	$R^2 = 0.090$, $p = 0.08$	$R^2 = -0.009$, $p = 0.40$	45.0%	**$R^2 = 0.129$, $p < 0.001$**	$R^2 = 0.040$, $p = 0.05$	$R^2 = 0.049$, $p = 0.09$
	Winter	63.9%	$R^2 = -0.018$, $p = 0.64$	**$R^2 = 0.170$, $p < 0.001$**	**$R^2 = 0.077$, $p = 0.04$**	66.4%	**$R^2 = 0.101$, $p < 0.01$**	**$R^2 = 0.404$, $p < 0.001$**	**$R^2 = 0.064$, $p = 0.04$**
PC2	Summer	20.5%	$R^2 = 0.026$, $p = 0.23$	**$R^2 = 0.234$, $p < 0.001$**	**$R^2 = 0.097$, $p = 0.03$**	37.6%	**$R^2 = 0.313$, $p < 0.001$**	**$R^2 = 0.642$, $p < 0.001$**	**$R^2 = 0.103$, $p = 0.01$**
	Winter	15.9%	**$R^2 = 0.162$, $p < 0.01$**	$R^2 = -0.022$, $p = 1.00$	$R^2 = 0.020$, $p = 0.24$	18.0%	**$R^2 = 0.234$, $p < 0.001$**	$R^2 = 0.040$, $p = 0.14$	$R^2 = 0.019$, $\mathrm{p} = 0.19$

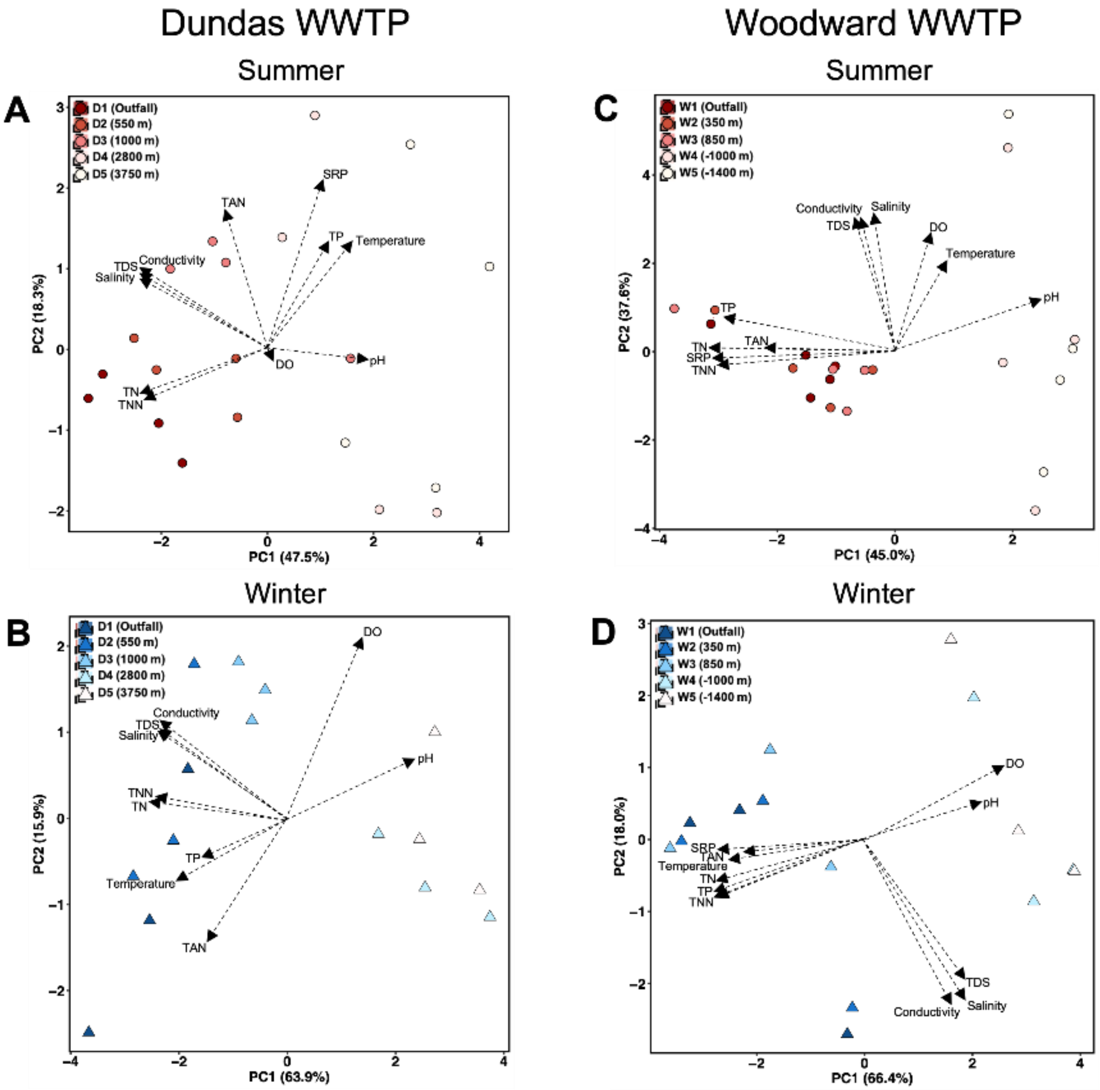


Figure 4.2. Principal component analysis (PCA) biplots of water quality parameters measured along the effluent gradients of the Dundas WWTP in the **(A)** summer and **(B)** winter, and the Woodward WWTP in **(C)** summer and **(D)** winter. Each data point represents a sampling event, and the dashed arrows represent the strength of the loading and its direction in two-dimensional space. See Supplementary Table S4 for all PCA loadings. Water quality data was previously reported in Mehdi et al. (2021).

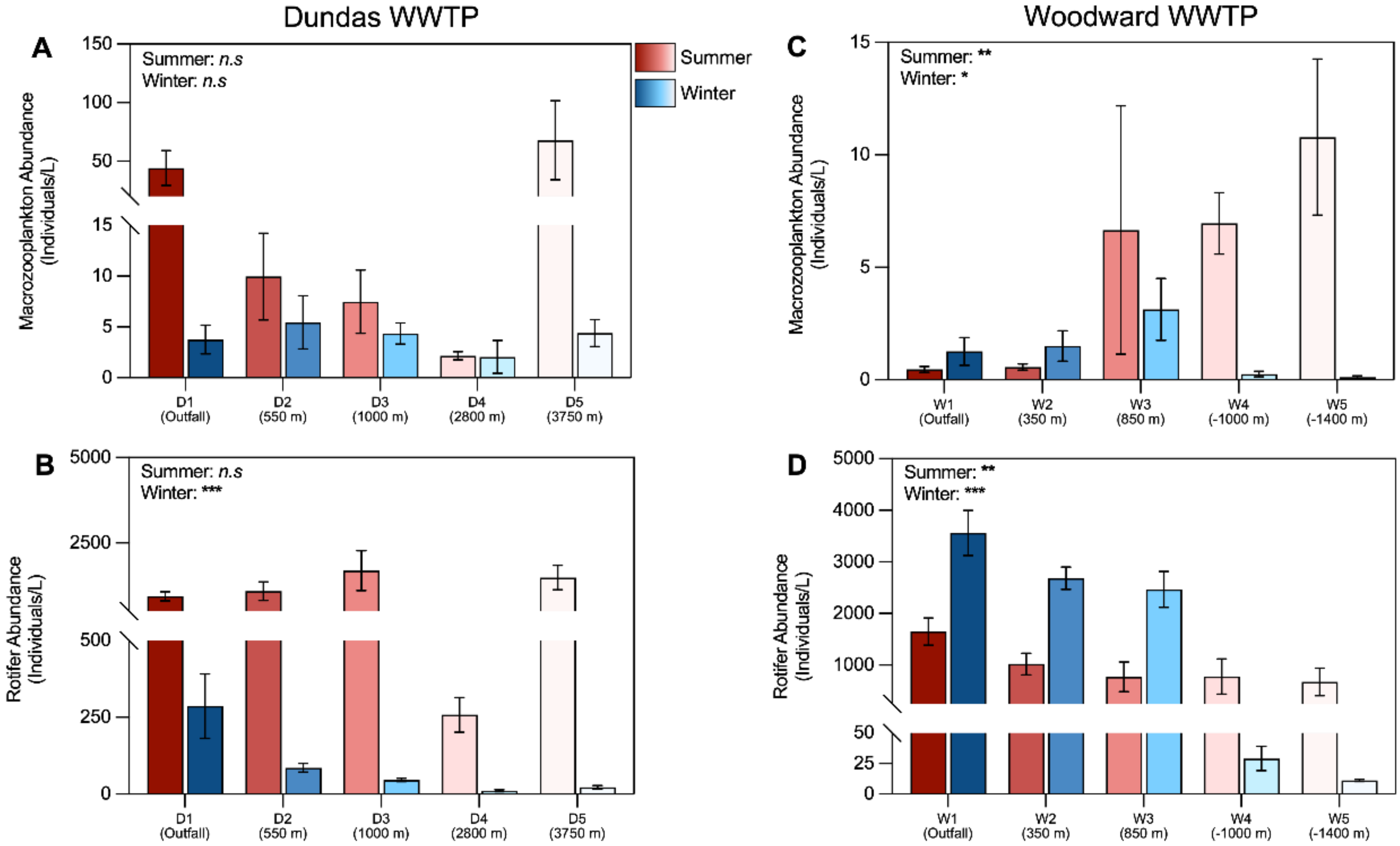


Figure 4.3. Mean (± SE) macrozooplankton abundance and rotifer abundance along the effluent gradients of the **(A, B)** Dundas and **(C, D)** Woodward WWTPs. Seasonal significant effects of proximity to the outfall are indicated by (*$p \leq 0.05$), (**$p < 0.01$), (***$p < 0.001$), and (no significance, *n.s.*).

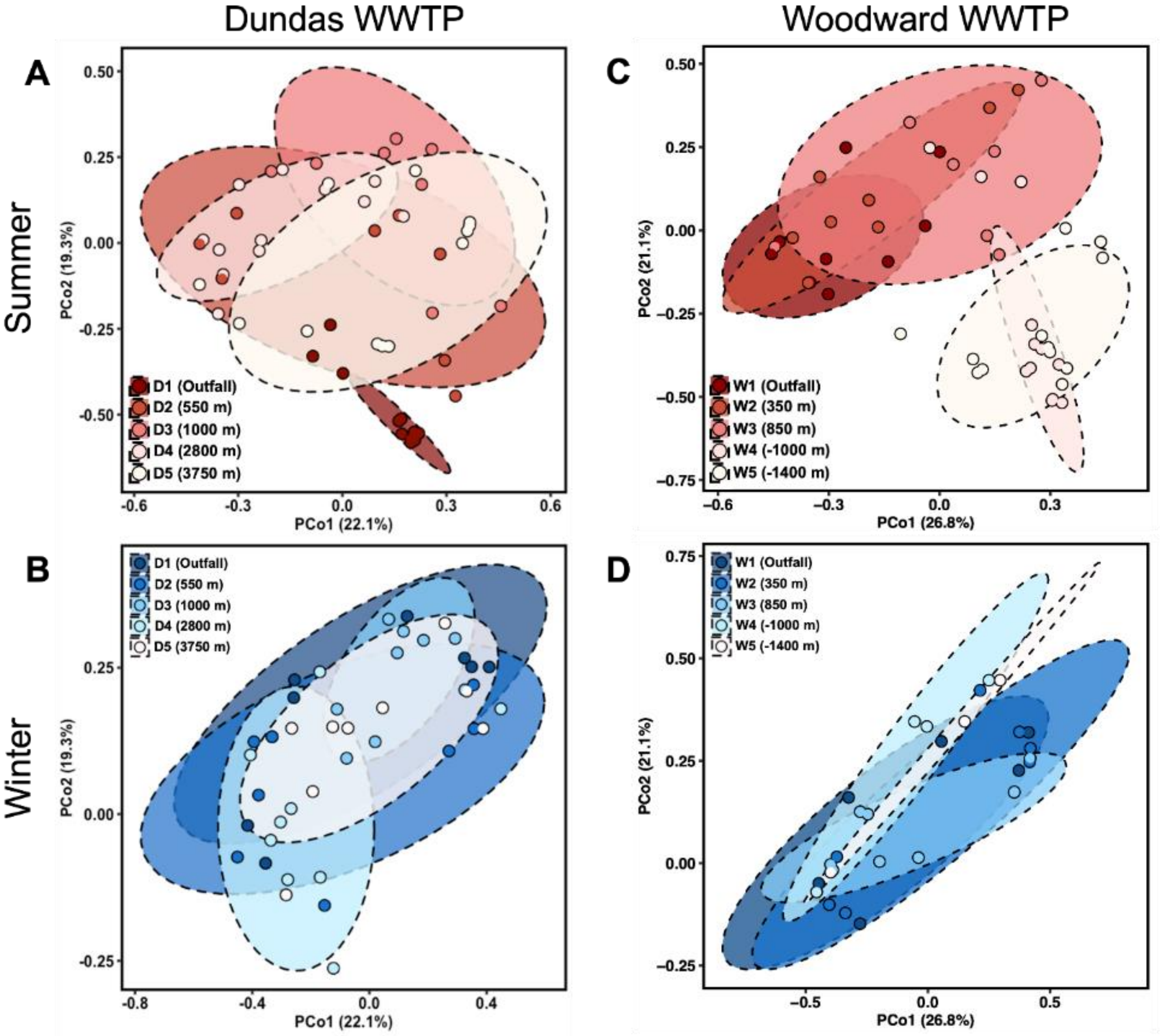


Figure 4.4. Principal coordinate analysis (PCoA) ordination output of macrozooplankton community compositions with 80% confidence ellipses overlain on each site. (**A, B)** PCoA biplots of the Dundas WWTP in summer and winter, respectively. **(C, D)** PCoA biplots of the Woodward WWTP in summer and winter, respectively.

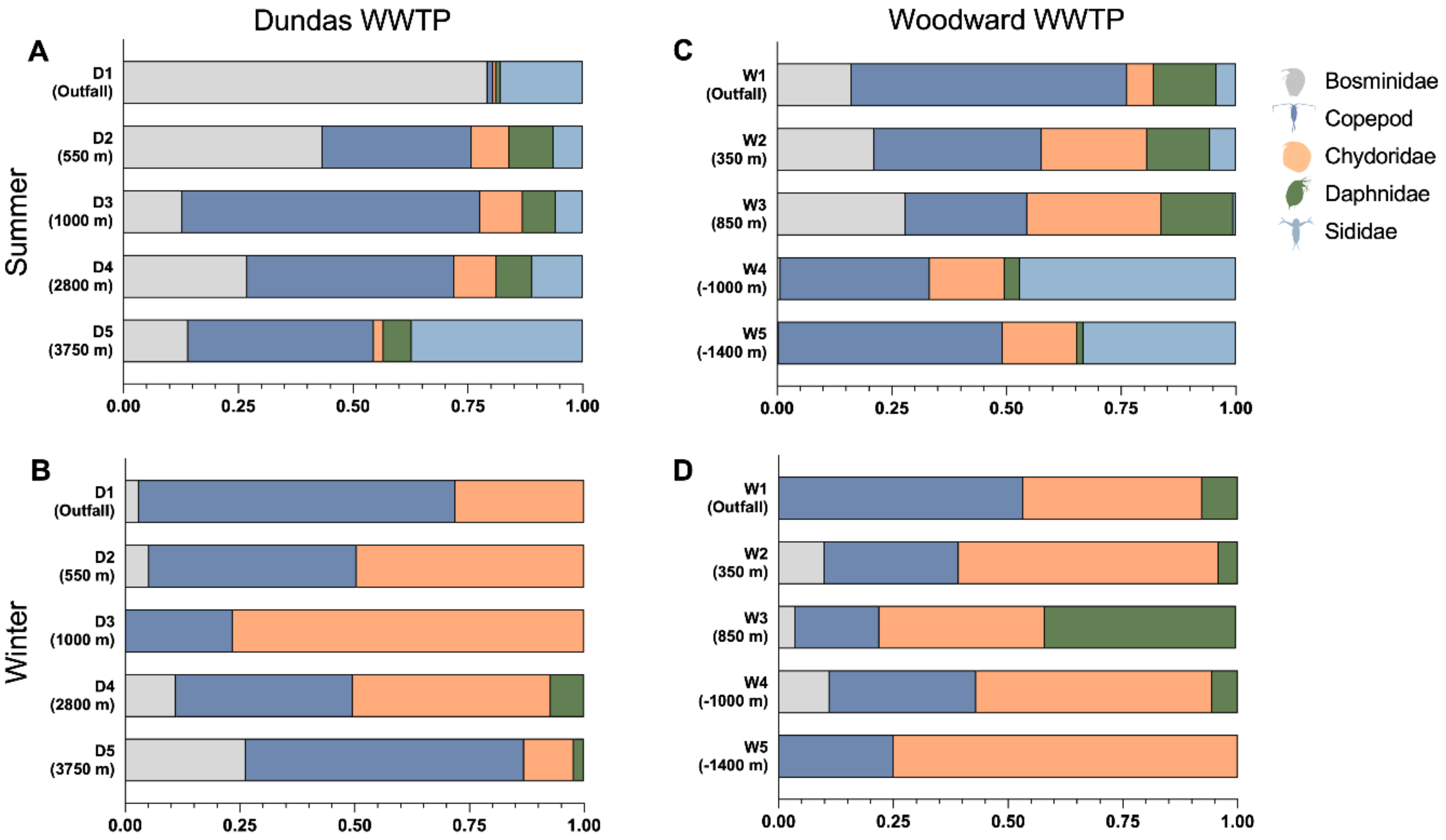


Figure 4.5. Macrozooplankton taxonomic composition for the Dundas WWTP sampling sites in the **(A)** summer and **(B)** winter, and the Woodward WWTP sampling sites in the **(C)** summer and **(D)** winter. Proportions based on total abundance of macrozooplankton across all sampling events within each season.

4.5 Discussion

Despite the ubiquity of wastewater contamination and its wide-reaching impacts on biota across multiple levels of biological organization, its effects on aquatic communities remain poorly understood, especially during the winter. In this study, we sampled zooplankton communities along the effluent gradients of two distinct WWTPs in Hamilton, Ontario, Canada during summer and winter. In both seasons, zooplankton communities were largely numerically dominated by rotifers and contained relatively few macrozooplankton. Rotifers were more abundant at sites closer to the effluent outfalls compared to sites farther away, particularly during winter. Additionally, differences in zooplankton community composition were most apparent between sites closer to the outfall and sites farther away, however, this divergence was most apparent in the summer. Our results suggest that not only are WWTPs a major source of contamination, excess nutrients, and thermal pollution, but are also capable of altering aquatic habitats and their zooplankton communities. This may be the result of abiotic microhabitat changes, bottom-up and/or top-down control – potentially altering food web structures directly or indirectly in impacted environments.

4.5.1 Bottom-up control of zooplankton communities in effluent-receiving environments

In our study, zooplankton communities were largely made up of rotifer species, and the dominance of rotifers was most apparent near both wastewater outfalls, especially during winter. Zooplankton community responses in relation to wastewater inputs have rarely been studied, and are almost never examined in shallow creeks, rivers, and wetlands like our study sites. However, the increase in zooplankton productivity, decrease in zooplankton mean size, and the dominance of smaller zooplankton species in community assemblages has been reported in multiple large bodies of freshwater suffering from eutrophication and aquatic pollution (Jeppesem et al. 2000; Sommer et al. 2012; Moody and Wilkinson, 2019). For example, a study in Iowa, USA found that eutrophic lakes in close proximity to agricultural lands were largely comprised of smaller zooplankton taxa (Moody and Wilkinson, 2019). Similarly, Hamilton Harbour, a eutrophic embayment in Lake Ontario that has long suffered from industrialization, urbanization, and invasive species has been demonstrated to have high zooplankton productivity and that is numerically dominated by small zooplankton taxa (e.g., Bosminidae and rotifers; Bowen and Currie, 2017). These trends are common in eutrophic waterbodies as eutrophication does not promote the success of larger zooplanktons (e.g., Daphniidae) and instead improves the competitive ability of smaller zooplankton (e.g., rotifers and Bosminidae). Therefore, mean size of zooplankton is generally considered an indicator of trophic status in aquatic ecosystems, where smaller zooplankton and rotifer species become more dominant with increasing nutrient levels (Lampert, 1987; Hansson et al. 2007; Sun et al. 2012; Vehmaa et al. 2018). Further, the addition of flow from WWTPs in shallow habitats makes larger zooplankton (e.g., cladocerans and Daphniidae less successful, thereby, reducing the predation pressure on smaller zooplankton (e.g., rotifers; Walks, 2007; Rozon et al. 2018). In our study, this trend was also apparent; sites closer to the effluent outfalls at both WWTPs had higher nutrient concentrations (total phosphorus and total nitrogen), higher TDS, higher bacterial loads, and higher flow rates than sites farther away and reflected by a clear dominance of smaller zooplankton species (rotifers) at these sites, particularly during winter. Rotifers can be important indicators of water quality due to their relatively short life cycles and their ability to reproduce parthenogenically, which allows them to react more quickly to seasonal changes in water quality when compared to some larger macrozooplankton with longer and more complex life histories (e.g., copepods; Gilbert and McPeek, 2013). We found that effluent quality and water quality of impacted sites were worse in winter than in summer, similar to results found by others (Vieno et al. 2005; Sui et al. 2011; Yu et al. 2013). Higher nutrient and contaminant concentrations during winter may limit the growth and reproduction of larger zooplankton, as their populations take longer to recover and are less tolerant to shifts in environmental conditions relative to smaller, r-selected taxa (Elser et al. 1996; Hansen et al. 1997).

4.5.2 Top-down control of zooplankton communities in effluent-receiving environments

Rotifer dominance of zooplankton communities, particularly during winter could also be reinforced by top-down control, where fish predation may reduce the number of larger, more energetically desirable prey items (i.e., larger zooplankton). Selective predation on larger zooplankton by fishes has been shown to shift zooplankton community compositions to be dominated by smaller-sized groups (Brooks and Dodson, 1965; Jenberg et al. 2017). A study in the subtropical lakes along the Yangtze River, China showed that the combination of eutrophication and fish predation led to an increase of small zooplankton species like rotifers and a decrease in larger species like Daphniidae (Li et al. 2017). Wastewater outfalls are a significant source of nutrients and thermal enhancement in aquatic environments, potentially driving fish and other aquatic organisms to select these highly productive environments, especially during winter, when food is scarce and difficult to encounter elsewhere (Sommer et al. 1986; Byström et al. 2006; Hurst, 2007; Holeton et al. 2011). Several previous studies have demonstrated that not only are fish more abundant near effluent outfalls but are also larger and in better body condition compared to fish from reference sites (Chambers et al. 1997; McMaster et al. 2005; Brown et al. 2011; Tetreault et al. 2011; McCallum et al. 2019; Lazaro-Côté et al. 2021; Mehdi et al. 2021; Nikel et al. 2021). The relatively high abundance of fishes (predominantly in the winter) and the dominance of smaller zooplankton species near effluent outfalls suggests that the composition of zooplankton communities is likely driven by top-down control (Brooks and Dodson, 1965; Drenner, 1977; Gilbert, 1985; Gilbert, 1988; Li et al. 2020). While planktivorous fish prefer larger zooplankton over smaller ones, the high rotifer abundance in our study, distinctly near the effluent outfalls, may force fish to incorporate more rotifers in their diet, and perhaps more so during winter, when food availability elsewhere is reduced. Numerical dominance of rotifer species may also influence the makeup of fish communities in these polluted environments. Gizzard shad (*Dorosoma cepedianum*), a detritivores species that incorporates rotifers in its diet (Buynak and Mitchell, 1993), were commonly found near the wastewater outfalls of the Dundas and Woodward WWTPs (McCallum et al., 2019; Mehdi et al. 2021).

4.5.3 Conclusions

Our study is distinctive in its approach to examining the impacts of wastewater effluent contamination on zooplankton communities and water quality during winter, a season seldom studied in ecotoxicology. The high productivity and increased dominance of smaller-sized zooplankton at impacted sites is likely a combination of bottom-up and top-down control and abiotic changes. High food availability and warm effluent may create enticing ecological conditions for aquatic organisms to seek impacted environments during winter, but at the cost of being exposed to a complex mixture of contaminants found in wastewater effluents – often at elevated concentrations during winter. Our study further highlights how wastewater outfalls may act as ecological traps for fish and other aquatic organisms, potentially influencing aquatic populations and communities of lower trophic levels (McCallum et al. 2019; Mehdi et al. 2021). Understanding and assessing the impacts of wastewater effluents on aquatic biota at multiple trophic levels during winter is an important yet challenging task. However, despite its challenges, we believe that winter ecotoxicology is a key component in unraveling the impacts of such a pervasive source of contamination in aquatic environments.

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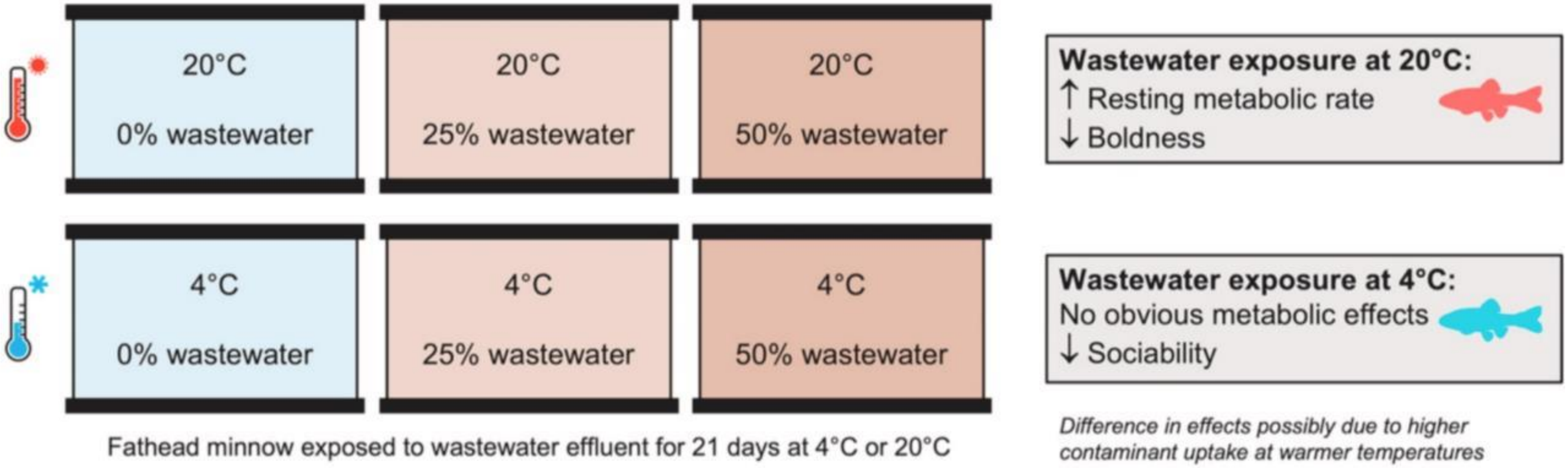
Chapter 5: Temperature modulates the impacts of wastewater exposure on the physiology and behaviour of fathead minnow



5.1 Abstract

Municipal wastewater treatment plant (WWTP) effluent is a substantial source of pollution in aquatic habitats that can impact organisms across multiple levels of biological organization. Even though wastewater effluent is discharged continuously all year long, its impacts across seasons, specifically during winter, have largely been neglected in ecotoxicological research. Seasonal differences are of particular interest, as temperature-driven metabolic changes in aquatic organisms can significantly alter their ability to respond to chemical stressors. In this study, we examined the effects of multiple levels of wastewater effluent exposure (0, 25, or 50% treated effluent) on the physiological and behavioural responses of adult fathead minnow (*Pimephales promelas*) at temperatures simulating either summer (20 °C) or winter (4 °C) conditions. At 20 °C, wastewater exposure posed a metabolic cost to fish, demonstrated by higher standard metabolic rate and was associated with increased haematocrit and a reduction in boldness. In contrast, fish exposed to wastewater at 4 °C experienced no change in metabolic rate but performed fewer social interactions with their conspecifics. Taken together, our results demonstrate that wastewater exposure can lead to metabolic and behavioural disruptions, and such disruptions vary in magnitude and direction depending on temperature. Our findings highlight the importance of studying the interactions between stressors, while also underscoring the importance of research during colder periods of the year to broaden and deepen

our understanding of the impacts of wastewater contamination in aquatic ecosystems.

5.2 Introduction

Municipal wastewater treatment plant (WWTP) effluents are one of the largest and most ubiquitous sources of aquatic contamination around the world (Environment Canada, 2001; Strayer and Dudgeon, 2010). WWTPs are not capable of removing all contaminants from wastewater, and as a result, treated effluent released into watersheds still contains a complex mixture of contaminants of emerging concern (CECs), including pesticides, metals, micro- and macroplastics, ammonia, pharmaceuticals and personal care products (PPCPs), and natural and synthetic hormones (Daughton and Ternes, 1999; Kolpin et al., 2002; Ternes et al., 2004; Holeton et al., 2011; Blair et al., 2013; McCormick et al., 2016; Jorgenson et al., 2018). The concentration of such contaminants is often relatively low (in the ng/L - µg/L range); however, due to the continuous discharge of wastewater effluent into receiving waterbodies, fish and other aquatic organisms residing near effluent outfalls can be subjected to chronic exposure of contaminants (Kolpin et al., 2002; Jelić et al., 2012; Jones et al., 2005; Blair et al., 2013). In addition to contaminant exposure, aquatic organisms residing near effluent outfalls are also subjected to poor habitat conditions as a result of excess nutrient loading, eutrophication, oxygen depletion, increased conductivity, and changes in temperature (Odjadjare and Okoh, 2010; Holeton et al., 2011; Tetreault et al., 2012; Melvin, 2016;

Hamdhani et al., 2020; Mehdi et al., 2021). The impacts of wastewater effluent exposure have been a subject of growing research and concern, especially since our reliance on WWTPs continues to increase as urban populations grow (Grimm et al., 2008; Holeton et al., 2011; Boxall et al., 2012; Rudd et al., 2014; Hamdhani et al., 2020).

Prior ecotoxicological research on the impacts of wastewater effluent exposure has largely been focused on reproductive endpoints (i.e., endocrine disruption represented in reproductive physiology and behaviour). This has mainly been driven by the abundance of endocrine-active compounds found in wastewater effluent (e.g., 17α-ethinylestradiol) motivating a growing area of research focused on the endocrine-related impacts of wastewater effluent exposure (Kidd et al., 2007; Harris et al., 2012; Tetreault et al., 2011; Bahamonde et al., 2015; Fuzzen et al., 2015). Such impacts include disruption of endogenous hormone levels, an increase in male feminization rates, and a reduction in fertilization success (Bahamonde et al., 2015; Fuzzen et al., 2015). The endocrinological and reproductive impacts of wastewater effluent exposure are commonly studied, as these parameters have direct implications on population growth and sustainability (European Chemicals Agency, 2011; Ågerstrand et al., 2020). However, our understanding of how other fitness-linked facets of biological organization (e.g., metabolic physiology and behaviour) may be impacted by wastewater effluent exposure is still in its infancy. Behaviours such as foraging, predator avoidance, sociability, exploration, and aggression are all essential to fitness and survival (Scott and Sloman, 2004; Brodin et al., 2014; Saaristo et al., 2018). Similarly, metabolic physiological endpoints such as standard metabolic rate, maximal metabolic rate, and aerobic scope (the difference between standard metabolic rate and maximal metabolic rate) provide an excellent insight on how contaminants influence energy transfer within an organism and are tightly linked to growth, reproduction, and many behaviours that are important for survival (Brown et al., 2004; Scott and Sloman, 2004; Biro and Stamps, 2010; Clark et al., 2013). Despite the overall scarcity of research on the physiological and behavioural impacts of wastewater exposure, a handful of studies have recently emerged demonstrating that exposure to wastewater effluent can inflict metabolic costs in both wild-caught and lab-reared fishes, manifesting as increases in whole animal metabolic rate (O2 consumption rate; Du et al., 2018; Mehdi et al., 2017; Du et al., 2019). Such metabolic costs can be associated with increases in the activities of various metabolic enzymes, changes in metabolic substrate levels (e.g., glycogen), and even tissue- and whole-body morphological abnormalities (Ings et al., 2012; Du et al. 2018, 2019; Mehdi et al., 2017; Nikel et al., 2021). Furthermore, an even smaller but growing number of studies have shown that exposure to wastewater effluent affects various non-reproductive behaviours in fishes, including altered aggression, dampened anti-predator responses, and reduced swimming performance (Saaristo et al., 2014; Melvin, 2016; McCallum et al., 2017a; McLean et al., 2019).

The paucity of research focusing on non-reproductive physiological and behavioural endpoints of wastewater effluent exposure is further exacerbated by the fact that the majority of our current knowledge comes from research conducted during warmer periods of the year and/or under warm lab-rearing temperatures (Lemly, 1993, 1996; Bennett and Janz, 2007; Driedger et al., 2009; Mehdi et al., 2021). The synergistic effects of temperature on the toxicity of wastewater effluent, particularly at the colder end of the spectrum, have largely been ignored in ecotoxicological research. Knowledge of potential interactions between temperature and contaminant exposure is of vital importance for a number of reasons. First, in many temperate and polar regions of the world, effluent from WWTPs is released into cold, near-freezing environments during colder periods of the year, sometimes lasting between 4 and 8 months. Therefore, understanding how effects of wastewater differ across temperatures is critical, particularly, at the colder end of the spectrum. Second, temperature has an overarching influence on numerous biological functions, especially in ectotherms (e.g., fishes; Hochachka and Somero, 2002). Colder temperatures during the winter could reduce whole-animal metabolism and gill ventilation, thereby reducing contaminant uptake and lessening the impacts of wastewater exposure. However, colder temperatures could also reduce the rates of contaminant elimination and remobilization or limit the aerobic supply of energy needed to fuel detoxification, thereby accentuating the impacts of wastewater exposure (Lemly, 1993, 1996; Capkin et al., 2006; Buckman et al., 2007; Noyes et al., 2009; Mehdi et al., 2019). Furthermore, fish during the winter may suffer from endogenous exposure of lipophilic contaminants stored in adipose tissue, as their reliance on lipid energy stores increases when food is scarce (Paterson et al., 2007; Treberg et al., 2016). Even though research on this topic is currently limited, a study by Lemly (1993) found that selenium exposure under simulated cold winter conditions caused reductions in activity, feeding, and lipid stores, as well as significant increases in mortality in juvenile bluegill sunfish (Lepomis macrochirus). Whereas under warmer conditions, fish continued to actively feed and lipid depletion did not occur, despite an increase in oxygen consumption. Otherwise, the question of whether colder temperatures reduce or accentuate the impacts of wastewater exposure remains largely unanswered. Studies considering realistic seasonal changes in temperature are much needed when evaluating the toxicity of wastewater effluent as well as other contaminants.

In this study, we examined the influence of temperature on the physiological and behavioural effects of wastewater effluent exposure in fathead minnow (*Pimephales promelas*). Adult fish were exposed to one of three wastewater treatments differing in the proportion of treated effluent (Control 0%; Low 25%; High 50%) for 21-days at temperatures typical of summer (20 °C) or winter (4 °C). We measured various facets of metabolism (standard metabolic rate, maximal metabolic rate, and aerobic scope) in addition to various behavioural endpoints (boldness, sociability, foraging, and response to predator). Based on previous studies, we hypothesized that wastewater effluent exposure would pose metabolic costs, represented by an increase in standard metabolic rate that would lead to a reduction in aerobic scope (Du et al. 2018, 2019; Mehdi et al., 2017). Such metabolic costs would further be manifested in dampening behaviour, with reductions in boldness, sociability, foraging, and anti-predator responses. We further hypothesized that these effects would be less pronounced at 4 °C than at 20 °C, as metabolism, respiration rates, and therefore contaminant uptake, are subdued at lower temperatures.

5.3 Methods

5.3.1 Study organism and housing

We used fathead minnow in this study, as they are a well-established laboratory toxicological model organism and are commonly found year-long in effluent-receiving environments across North America (Ankley and Villeneuve, 2006; Mehdi et al., 2021). Adult fathead minnow of both sexes were acquired from lab-reared stocks and maintained in 38 L glass tanks (density of 20 fish/tank; 18 tanks). Each tank was equipped with a sponge filter and an aerator and supplied with Hamilton tap water that underwent reverse osmosis and UV sterilization. Fish were held under 16L:8D light schedule and fed until satiation with Nutrafin Basix Staple Food once daily. The tanks were held within two large environmental chambers at the Canada Centre for Inland Waters, Burlington, ON, Canada. Both environmental chambers were maintained at 20 °C before the start of the experiment, but the temperature in one of the environmental chambers was then incrementally reduced from 20 °C to 4 °C over a period of 12 days. Once the desired temperature was reached, fish were held at that temperature for another two weeks prior to the start of wastewater effluent exposures. All the procedures employed were approved by the animal utilization protocols from the McMaster University Animal Research Ethics Board (# 17-12-45) and the Department of Fisheries and Oceans/Environment Canada Joint Animal Care Committee for the Canada Centre for Inland Waters (# 1956; Burlington, ON, Canada) in accordance with the guidelines of the Canadian Council on Animal Care.

5.3.2 Wastewater effluent exposure

Following the initial two-week period of adjustment to the target temperature, fish were exposed to treated wastewater effluent collected from the Woodward WWTP in Hamilton, ON, Canada. The Woodward WWTP is a secondary conventional activated sludge plant that serves the majority of the Hamilton population (~480,000 people) and has an average daily treatment capacity of 409 million litres (City of Hamilton, 2019). Effluent from this facility flows directly into Red Hill Creek, which empties into Hamilton Harbour, one of 43 locations around the Great Lakes designated as an Area of Concern (AOC) by the International Joint Commission (Great Lakes Water Quality Agreement, 2012). Collections of effluent were conducted after the final stage of treatment, immediately prior to being discharged into Red Hill Creek. Fresh effluent was collected in opaque plastic carboys twice every week (between 0900 and 1130 h) from March to May of 2019, and stored for a maximum of 4 days at 4 °C in the dark to slow down degradation.

Fathead minnow were exposed to one of six treatments for 21 days: (i) 0% wastewater effluent at 20 °C (warm control); (ii) 25% wastewater effluent at 20 °C (warm low); (iii) 50% wastewater effluent at 20 °C (warm high); (iv) 0% wastewater effluent at 4 °C (cold control); (v) 25% wastewater effluent at 4 °C (cold low); (vi) 50% wastewater effluent at 4 °C (cold high). The concentrations of wastewater chosen are representative of effluent-receiving environments in Hamilton Harbour (e.g., downstream of the Dundas and Woodward WWTPs). Additionally, the concentrations of wastewater used did not reduce survival (Table S4) and thus any observed effects can be considered sub-lethal effects of exposure. Three tank replicates were used for each exposure treatment, with 20 adult fish per tank. Water changes were made every fourth day of the exposure with 75% of the water being replaced 1 h after feeding. During water changes in the wastewater exposure treatment tanks, the tanks were re-dosed with newly collected wastewater effluent. Wastewater effluent was brought to the appropriate exposure temperatures overnight before water changes were made, while also being continuously aerated to ensure sufficient dissolved oxygen levels. Wastewater effluent was diluted and well-mixed with clean fresh water to match the exposure conditions prior to dosing.

5.3.3 Wastewater effluent exposure

In the exposure tanks, temperature was continuously monitored using temperature loggers (HOBO Pendant Temperature Data Logger) placed in a randomly selected tank of each treatment. Dissolved oxygen (YSI Pro DO), pH, total dissolved solids, conductivity, and salinity (Oakton Multiparameter PCS Testr 35) were measured once a week (Supplementary Table 1). A number of water quality parameters were measured on composite final effluent samples that were collected over a period of 24 h at the Woodward WWTP: total suspended solids, carbonaceous biochemical oxygen demand, total phosphorus, total Kjeldahl nitrogen, ammonia + ammonium, nitrate, nitrite, E. coli, conductivity, and chemical oxygen demand (measurements provided by City of Hamilton, 2019; Supplementary Table 2). Furthermore, during each effluent collection, a 500 mL sample of freshly collected wastewater effluent was preserved and analyzed for a wide range of PPCPs and other CECs using already established methods described by Arlos et al. (2015) and Mehdi et al. (2021). Throughout the exposure, 125 mL samples of tank water were collected from each tank two times — once ~1 h post-dosing and another prior to the next water change. This sampling regimen allowed us to compare the potency of effluent immediately after collection, during dosage, and after a 4-day period in the exposure tanks. Similar to freshly collected wastewater effluent, tank water was also analyzed for PPCPs and other CECs. Briefly, wastewater and tank water samples were concentrated using solid phase extraction. Extracted samples were then analyzed using an Agilent 1260 HPLC with 6460 triple quad mass spectrometer (LC-MS/MS) with Agilent Jet

Stream (AJS) electrospray ionization in both positive and negative modes. Nine different classes of compounds were analyzed: lipid regulators, antiepileptics, analgesics, stimulants, antibacterials, antibiotics, antidepressants, non-steroidal anti-inflammatory agents (NSAIDs), and herbicides.

5.3.4 Respirometry

At the end of the exposure period (day 21), a subset of fish (n = 12) from each treatment was used to measure standard and maximal metabolic rates. Metabolic rate measurements were performed at the appropriate exposure temperature for each treatment in clean water (same source water that was used for exposures) following previously described methods in Borowiec et al. (2015). Fish were placed in 90 mL respirometry chambers situated in a darkened, temperature-controlled, and well-aerated buffer tank. Respirometers were equipped with flush and recirculation pumps. Recirculation pumps were connected in circuit to fibre-optic oxygen sensors (OXROB10 PyroScience) and were continuously turned on to ensure water in the chamber was well mixed and continuously flowing past the oxygen sensors. Oxygen sensors were connected to an optical oxygen meter (FireStingO2 PyroScience) for continuous oxygen concentration measurements. Flush pumps were turned on (7 min) and off (7 min) intermittently to expel the chambers of residual water and supply them with well oxygenated water. Fish were held in respirometry chambers overnight where standard metabolic rate measurements were continuously measured. Standard metabolic rate was determined by calculating the mean of the lowest five metabolic rate measurements. The next day, maximal metabolic rate was determined by transferring fish to a cylindrical tank (diameter = 46.0 cm; height = 20.0 cm) and being chased for 3 min and then air-exposed for 30 s, this method has been demonstrated to elicit maximal metabolic rates in a variety of fish species (Clark et al., 2012; Roche et al., 2013; Norin et al., 2014). Fish were then placed immediately back into their chambers to measure maximal metabolic rate; oxygen consumption rate was continuously measured for another ~60 min. Maximal metabolic rate was determined to be the highest metabolic rate measurement taken during this hour. Absolute aerobic scope was determined by calculating the difference between maximal and standard metabolic rates. Note; fish underwent respirometry experiments after completing the behavioural trials (see below).

5.3.5 Behavioural assays

To assess the effects of wastewater and temperature on fathead minnow behaviour, four key behavioural traits were assessed in a multi-step behavioural assay: (i) boldness, (ii) sociability, (iii) foraging, and (iv) response to predator. These behavioural tests are widely used in behavioural ecology (Bell, 2004; Brodin et al., 2013; Saaristo et al., 2018; McLean et al., 2019; Ågerstrand et al., 2020) and were adapted and validated for fathead minnow using pilot studies. Behavioural trials were also conducted following the 21-day exposure period in a 38L behavioural arena (50.5 × 25.7 × 30 cm) divided into three compartments (all filled with 10 cm of water). Focal fish were placed in the middle compartment (26 × 25.7 × 30 cm) during all assays. Twenty equal-sized grids (5.25 × 5.25 cm) were drawn on the bottom of the focal compartment to facilitate positional scoring of focal fish. The focal compartment was flanked with two equally-divided sides (11.7 × 25.7 × 30 cm), one housing three unexposed conspecific shoal fish, while the other was empty. The two flanked compartments were divided from the focal compartment via permanent transparent barriers that were water-impermeable and two removable black opaque barriers. All behavioural assays were recorded using an overhead camera (GoPro Hero 5); recorded videos were later analyzed using a behavioural annotation software (BORIS v.7.9.4). Behavioural arena setups can be found in Fig. 5.2 – 5.

Before the start of behavioural trials, three size-matched unexposed and unfamiliar conspecific fish were added to one of the side compartments. These stimuli or “shoal” fish were given an hour to adjust to the arena before a focal fish was introduced into a refuge PVC tube (diameter = 5.0 cm; length = 10.0 cm) placed in the middle of the focal arena. For the first behavioural assay, boldness (Fig. 5.2A), focal fish were allowed to adjust to the refuge for 10 min while being closed off from the rest of the arena by a removable door. Following the adjustment period, the refuge door was remotely lifted, and the focal fish were given 10 min to exit the refuge. We recorded the time at which at least half the body of the fish exited the refuge. If the focal fish did not exit during the allotted 10 min (600 s) period, it was assigned a maximum refuge exit time of 600 s. At 10 min, the entire refuge was remotely lifted from the back; this gently forced any remaining focal fish to swim down and exit and prevented the fish from re-entering the refuge during subsequent assays.

Fish were then given 5 additional min to acclimate following the refuge removal and before the start of the second behavioural assay, sociability (Fig. 5.3A). In the sociability assay, the two removable black opaque barriers on either side of the focal central arena were remotely lifted, revealing the two side compartments. One of these side compartments contained a social stimulus (with three shoal fish) and the other side compartment was empty. Using the grids on the bottom of the tank, the focal arena was divided into 5 equally-sized columnar-zones, and the time spent in each zone was recorded for a 10-min period. A sociality index was calculated by multiplying the total time spent in each zone by a zone-specific factor (−2, −1, 0, 1, 2), where the zone closest to the shoal was given a factor of 2 and the zone furthest from the shoal was given a factor of −2. Additionally, the time the focal fish spent interacting with shoal was recorded.

In the third behavioural assay, foraging (Fig. 5.4A), a mesh-lined cassette (2.9 × 4.0 × 0.7 cm) containing ~0.3 g of blood worms (Hikari Bio-Pure), a type of food that our fish were familiar with prior to the exposure period, was remotely dropped into the centre of the focal area. The latency to approach the food cassette by the focal fish as well as the number of interactions with the food cassette were recorded for a 5 min period.

In the fourth and final behavioural assay, response to predator (Fig. 5.5A), a rubber fish model predator (total length = 30 cm) attached to a pole was used to strike the centre of the focal area five times from above. The immediate response to the simulated predator attack as well as the time the focal fish spent being active post-predator attack were recorded for a 5 min period (binned in 1-min increments).

5.3.6 Fish sampling

Following the post exposure physiological and behavioural assays, fish were euthanized by cerebral percussion; the standard length, total length, and body mass were recorded (Ohaus, Scout Pro SP202, accuracy to 0.01 g). Blood was collected in heparinized capillary tubes via caudal severance, centrifuged at 4750 g for 3 min in a Readacrit centrifuge (Clay Adams) for haematocrit measurement (% of packed red blood cells in the sample).

5.3.7 Statistical analysis

All statistical analyses were conducted using R (version 4.0.4, R Core Team, 2021). The impacts of wastewater exposure treatment, exposure temperature, and their interaction were analyzed using (i) linear models, (ii) beta regressions, (iii) negative binomial general linear models (GLMs), and (iv) binomial GLMs; depending on the response variable (see Supplementary Materials for details). Because the conditions of wastewater are dynamic and concentrations of some chemicals can vary between collection dates, we randomly staggered the start date of our tank replicates to help minimize undesired variation between replicates and treatment groups and to ensure sufficient time for all fish to be tested. To account for potential differences in the potency of wastewater effluent across the exposure period, we included the start date of each tank replicate as a covariate. (i) Metabolic rate, haematocrit, boldness, sociability (as measured by zonal scoring), and foraging (as measured by latency to interact with food item) were all analyzed using linear models with sex included as a fixed independent variable and body mass as a covariate. Absolute metabolic rate data was statistically analyzed with body mass as a covariate; however, data is graphically reported as mass-specific metabolic rate (mgO2 gfish−1 h−1) to facilitate comparison with previous literature values. (ii) We fit a beta regression (betareg package, Cribari-Neto and Zeileis, 2010) to analyze the proportion of time the focal fish spent socially interacting with their shoal in the sociability assay. (iii) We used negative binomial GLMs for fitting over-dispersed count data to analyze the number of times the focal fish interacted with the food item. (iv) Binomial GLMs were used to analyze the type of response (i.e., dart or no response) focal fish exhibited when presented with the model predator. Data were log transformed when necessary to meet assumptions of normality and homogeneity of variance. Tukey's HSD post-hoc tests were then used to identify significant pairwise differences between each treatment and control within each exposure temperature (emmeans package; Lenth et al., 2018). Data are reported as means ± standard error (SE) unless otherwise stated, and in all analyses, statistical differences were deemed significant at $\alpha = 0.05$.

5.4 Results

5.4.1 Characterization of the effluent

We detected nine different classes of chemicals in the final effluent: lipid regulators (gemfibrozil, atorvastatin, p-hydroxy atorvastatin, o-hydroxy atorvastatin), anti-epileptic (carbamazepine), analgesic (acetaminophen), stimulant (caffeine), antibacterials (triclosan, sulfamethazine), antibiotics (trimethoprim, lincomycin, sulfamethoxazole), antidepressants (fluoxetine, norfluoxetine, venlafaxine, desvenlafaxine), non-steroid anti-inflammatory agents (ibuprofen, naproxen, diclofenac), and herbicide (atrazine); see Table 1 for concentrations of each chemical. Most of these compounds (19/20) were also detected in the exposure tanks, although at lower concentrations compared to the concentrations measured in freshly collected effluent. Lower concentrations in the exposure tanks suggest that our dilution regimen was effective and that some degradation of the effluent continued during storage. Some compounds sharply declined between the dosing and water changing periods, while others were more stable. See Supplementary Table 3 for all compound concentrations in exposure tanks.

5.4.2 Metabolic rate

Overall, standard metabolic rate (SMR) was greatly influenced by temperature; SMR was on average ~5.3 times higher for fish held at 20 °C compared to fish held at 4 °C (LM, t(1,54) = 211.86, p < 0.001; Fig. 5.1A). At 20 °C, fish exposed to low and high levels of wastewater effluent demonstrated ~33% (t = 3.75, p = 0.001) and ~21% (t = 2.41, p = 0.05) respective increases in SMR relative to control fish. However, fish exposed to wastewater at 4 °C did not exhibit significant changes in SMR relative to control fish (t(Low – Control) = 0.17, p = 0.99); (t(High – Control) = 0.79, p = 0.71). Similarly, maximal metabolic rate (MMR) was significantly influenced by temperature; MMR was on average ~5.1 times higher at 20 °C than at 4 °C (LM, t(1,54) = 101.90, p < 0.001; Fig. 5.1B). Exposure to wastewater had a modest but significant effect on MMR (LM, F(2,54) = 3.43, p = 0.04; Fig. 5.1B), but the direction of the effect was inconsistent between wastewater doses, and we did not detect any significant pairwise differences between fish exposed to wastewater effluent and control fish at either temperature (t(20°C: Low – Control) = 2.09, p = 0.10; t(20°C: High – Control) = 0.98, p = 0.59; t(4°C: Low – Control) = 0.30, p = 0.95; t(4°C: High – Control) = 0.81, p = 0.70).

Similar to SMR and MMR, absolute aerobic scope (AAS), the difference between MMR and SMR, was on average ~5.0 times higher at 20 °C than at 4 °C (LM, t(1,50) = 48.57, p < 0.001; Fig. 5.1C). Exposure to wastewater effluent had a marginal, albeit non-significant effect on AAS (LM, F(2,50) = 2.97, p = 0.06; Fig. 5.1C); no significant pairwise differences between exposed and unexposed fish at either temperature were detected (t(20°C: Low – Control) = 1.28, p = 0.41; t(20°C:

High – Control) = 1.47, p = 0.32; t(4°C: Low – Control) = 0.14, p = 0.99; t(4°C: High – Control) = 0.67, p = 0.78).

5.4.3 Behaviour

5.4.3.1 Boldness

Boldness, measured as the latency to exit refuge in seconds, was not affected by temperature (LM, t(1,134) = 0.01, p = 0.94; Fig. 5.2). At 20 °C, fish exposed to high concentrations of wastewater took on average ~2.4 times longer to emerge from refuge compared to control fish (t = 2.49, p = 0.04), while fish exposed to the low effluent concentrations were similar in their exit times to control fish (t = 0.76, p = 0.73). At 4 °C, exit times were not significantly different between fish from either exposure treatment and control fish (t(Low – Control) = 1.56, p = 0.27; t(High – Control) = 1.55, p = 0.27).

5.4.3.2 Sociability

Overall, fish spent the majority of their time in the social zone of the behavioural arena (68%). However, fish held at 20 °C spent ~12% more time on the social side of the tank compared to fish held at 4 °C (LM, t(1,127) = 5.45, t = 0.02). Wastewater effluent exposure did not affect the amount of time fish spent in the social zone relative to the other zones (LM, F(2,127) = 0.002, p = 0.99). When we examined how fish spent their time in the social zone, we found that those held at 20 °C and exposed to either concentration of wastewater effluent interacted with a shoal to the same extent as fish in the control group (Z(Low – Control) = 0.74, p = 0.74; Z(High – Control) = 0.09, p = 0.99). Whereas at 4 °C, fish exposed to high concentrations of wastewater effluent spent on average ∼70% less time interacting with their shoal compared to fish in the control group (Z(High – Control) = −2.84, p = 0.01), no such differences were observed in fish exposed to the low concentration of effluent (Z(Low – Control) = 1.00, p = 0.58). Furthermore, temperature did not affect the overall time spent by the focal fish interacting with a shoal of conspecifics (Beta Regression: N = 137, χ2 = 0.43, p = 0.51; Fig. 5.3).

5.4.3.3 Foraging

Fish held at 20 °C interacted ∼46 times more frequently with the food (Negative binomial GLM: N = 137, χ2 = 65.98, p < 0.001; Fig. 5.4A) and were also ∼1.7 times quicker to approach the food compared to fish held to 4 °C (LM, t(1,127) = 33.83, p < 0.001; 4B). Wastewater effluent exposure did not affect the fish's latency to first approach the food (LM, F(2,127) = 0.76, p = 0.47) nor did it influence the number of times the fish interacted with the food (Negative binomial GLM: N = 137, χ2 = 2.65, p = 0.27). Although we did detect a significant interaction between temperature and wastewater effluent exposure on the number of times a fish engaged with the food (Negative binomial GLM: N = 137, χ2 = 8.52, p = 0.01; Fig. 5.4A), all pairwise contrasts between treatment groups and control within each temperature were non-significant (Z(20°C: Low – Control) = 0.44, p = 0.90; Z (20°C: High – Control) = 0.61, p = 0.81; Z(4°C: High – Control) = 0.76, p = 0.73;

fish exposed to low concentrations of wastewater at 4 °C did not interact with the food).

5.4.3.4 Response to predator

After the simulated predator attack, 56% of fish darted away while 44% did not respond at all. Fish held at 20 °C were more likely (~71%) to dart away from the predator than fish held at 4 °C (~41%; Binomial GLM, N = 133, $\chi 2 = 4.43$, $p = 0.04$). Wastewater effluent exposure did not have an impact on the type of behavioural response observed (Binomial GLM, N = 133, $\chi 2 = 2.31$, $p = 0.31$). At both exposure temperatures, fish from all treatments responded to the model predator attack with a sharp decline in activity from baseline (Fig. 5.5). This change in activity was not affected by wastewater effluent exposure (LM, $F(2, 117) = 0.45$, $p = 0.64$; Fig. 5.5). At 4 °C, fish appeared to respond less sharply to the model predator attack, but not significantly so (LM, $t(1, 117) = -1.85$, $p = 0.07$; Fig. 5.5). On average, fish returned to their baseline activity between 2 and 5 min post-model predator attack; time to return to baseline activity levels was not affected by wastewater effluent exposure (LM, $F(2,117) = 0.09$, $p = 0.91$) or by temperature ($t(1,117) = 0.43$, $p = 0.67$).

5.4.3.5 Morphology and survival

Total length (TL), standard length (SL), body mass (BM), and body condition (K) were unaltered by wastewater effluent exposure (LM, F(TL; 2,210) = 0.50, p =

0.61; F(SL; 2,210) = 0.55, p = 0.58; F(BM; 2,210) = 0.86, p = 0.42; F(K; 2,210) = 0.63, p = 0.53). However, fish held at 4 °C appeared to be larger and in better body condition than fish held at 20 °C post exposure (LM, t(TL; 1,210) = 3.99, p = 0.047; t(SL; 1,210) = 4.76, p = 0.03; t(BM; 1,210) = 20.28, p < 0.001; t(K; 1,210) = 57.36, p < 0.001). Haematocrit was also significantly higher in fish held at 4 °C than in fish held at 20 °C (LM, t(1,205) = 38.17, p < 0.001). Furthermore, fish exposed to low and high concentrations of wastewater effluent had higher haematocrit than unexposed control fish; however, this was only the case in fish exposed at 20 °C (t(20°C: Low – Control) = 2.37, p = 0.049; t(20°C: High – Control) = 2.67, p = 0.02; t(4°C: Low – Control) = 0.16, p = 0.99; t(4°C: High – Control) = 0.03, p = 0.99; See Supplementary Table 5). Wastewater exposure did not increase mortality relative to clean water control (see Supplementary Table 4 for survival information and sample sizes for each assay performed).

Table 5.1. Mean (±SE) concentration in [ng/L] of chemicals measured in the final effluent upon collection (n = 20). <DL indicates below detection limit. See Mehdi et al. (2021) for detection limits.

Class	**Chemical**	**Concentration (ng/L)**
Lipid regulator	Gemfibrozil	73.8 ± 6.83
	Atorvastatin	197 ± 15.1
	p-hydroxy Atorvastatin	339 ± 28.4
	o-hydroxy Atorvastatin	324 ± 27.1
Anti-epileptic	Carbamazepine	249 ± 21.0
Analgesic	Acetaminophen	775 ± 424
Stimulant	Caffeine	4320 ± 1670
Antibacterial	Triclosan	191 ± 15.0
	Sulfamethazine	90.7 ± 10.2
Antibiotic	Monensin	<DL
	Trimethoprim	178 ± 15.5
	Lincomycin	28.0 ± 6.62
	Sulfamethoxazole	341 ± 26.0
Antidepressant	Fluoxetine	24.6 ± 1.30
	Norfluoxetine	18.8 ± 1.42
	Venlafaxine	611 ± 45.0
	Desvenlafaxine	847 ± 65.1
NSAID	Ibuprofen	2290 ± 301
	Naproxen	2130 ± 190.
	Diclofenac	727 ± 32.5
Herbicide	Atrazine	29.3 ± 1.70

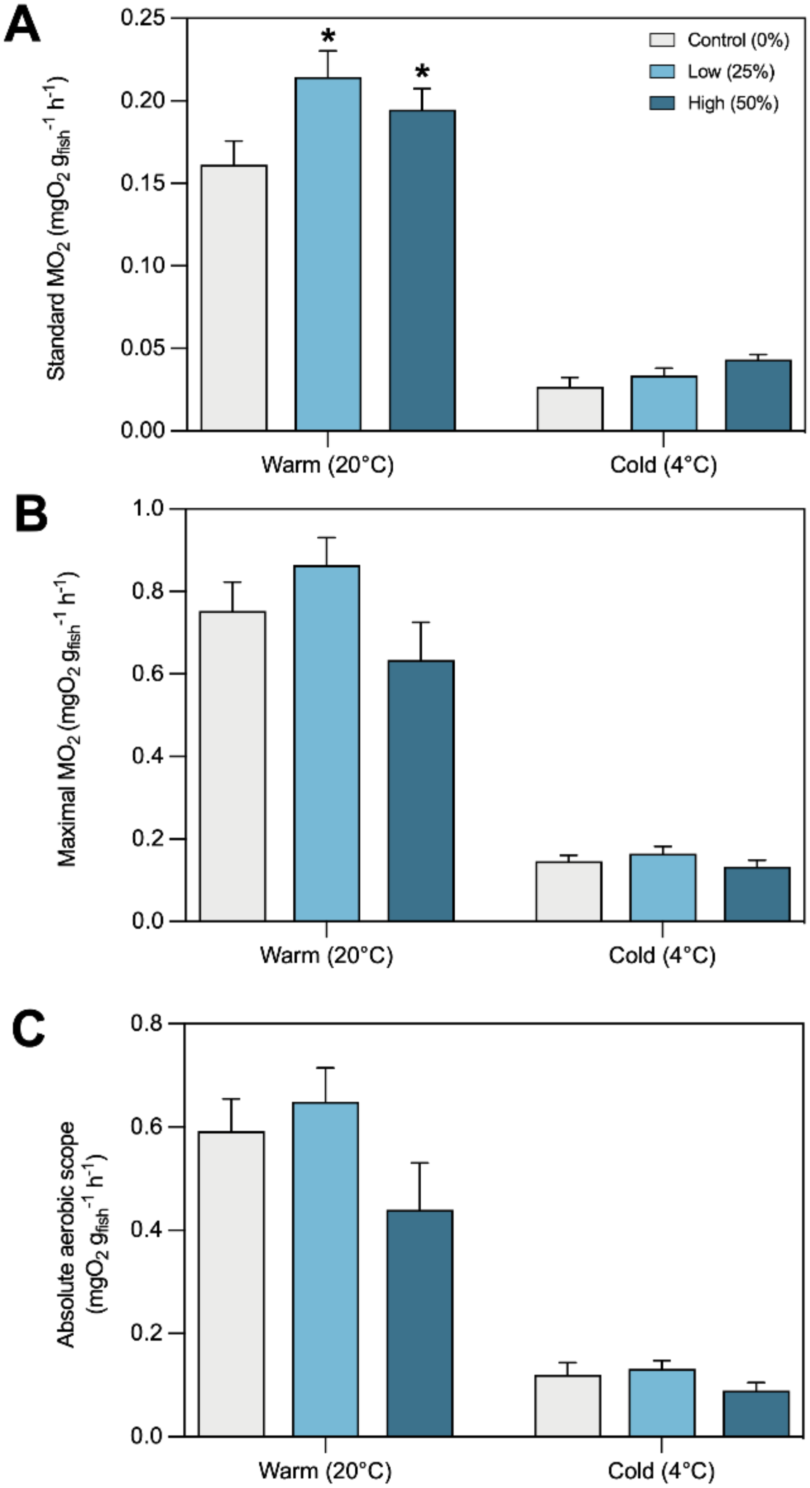


Figure 5.1. Mean ± SEM (**A**) Standard metabolic rate, (**B**) maximal metabolic rate, and (**C**) absolute aerobic scope at 20°C and 4°C. *represents significant pairwise differences between exposed fish and control within each temperature.

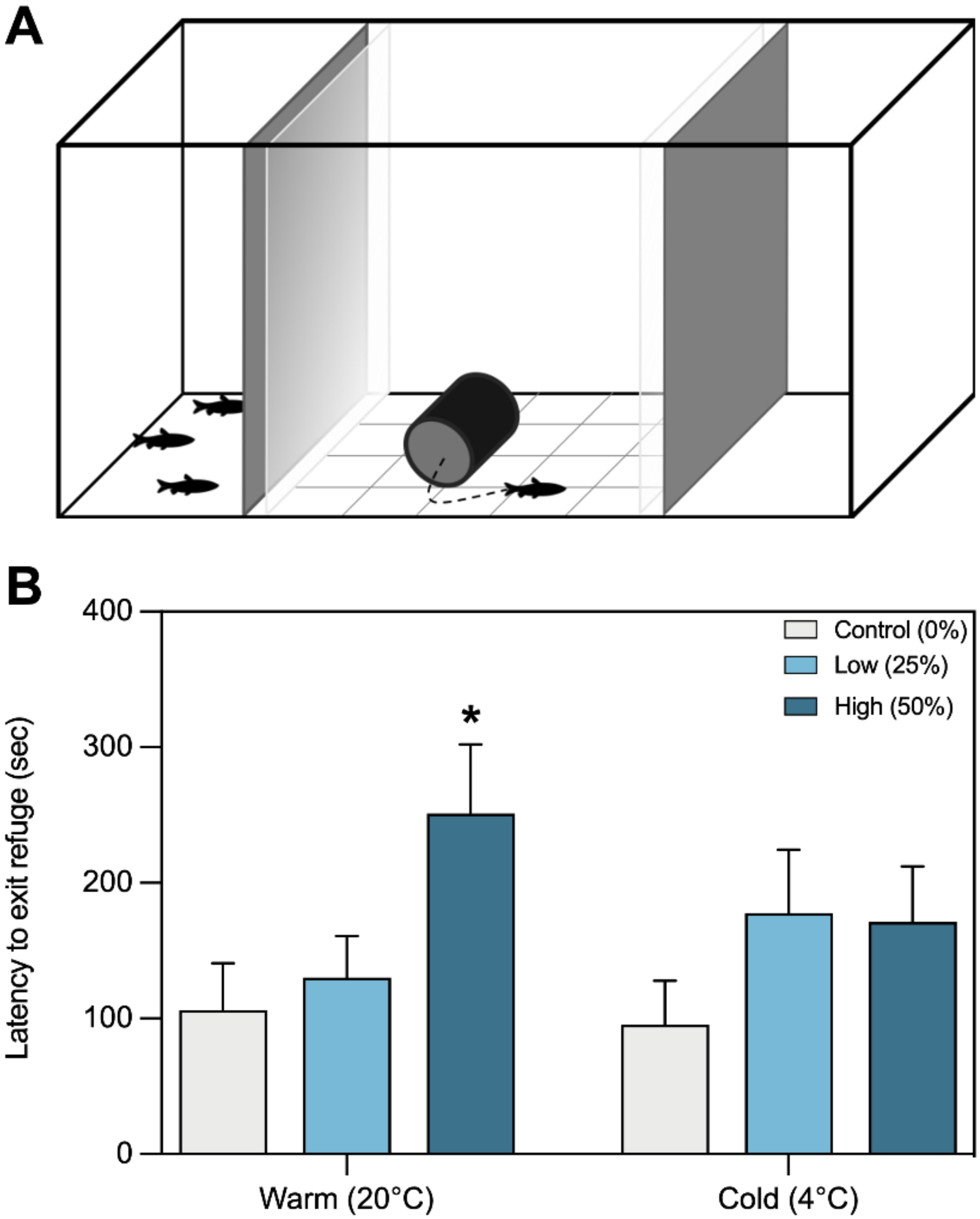


Figure 5.2. (**A**) Boldness assay showing fish exiting refuge and (**B**) mean ± SEM boldness, measured by latency to exit refuge (in seconds) at 20°C and 4°C.*represents significant pairwise differences between exposed fish and control within each temperature.

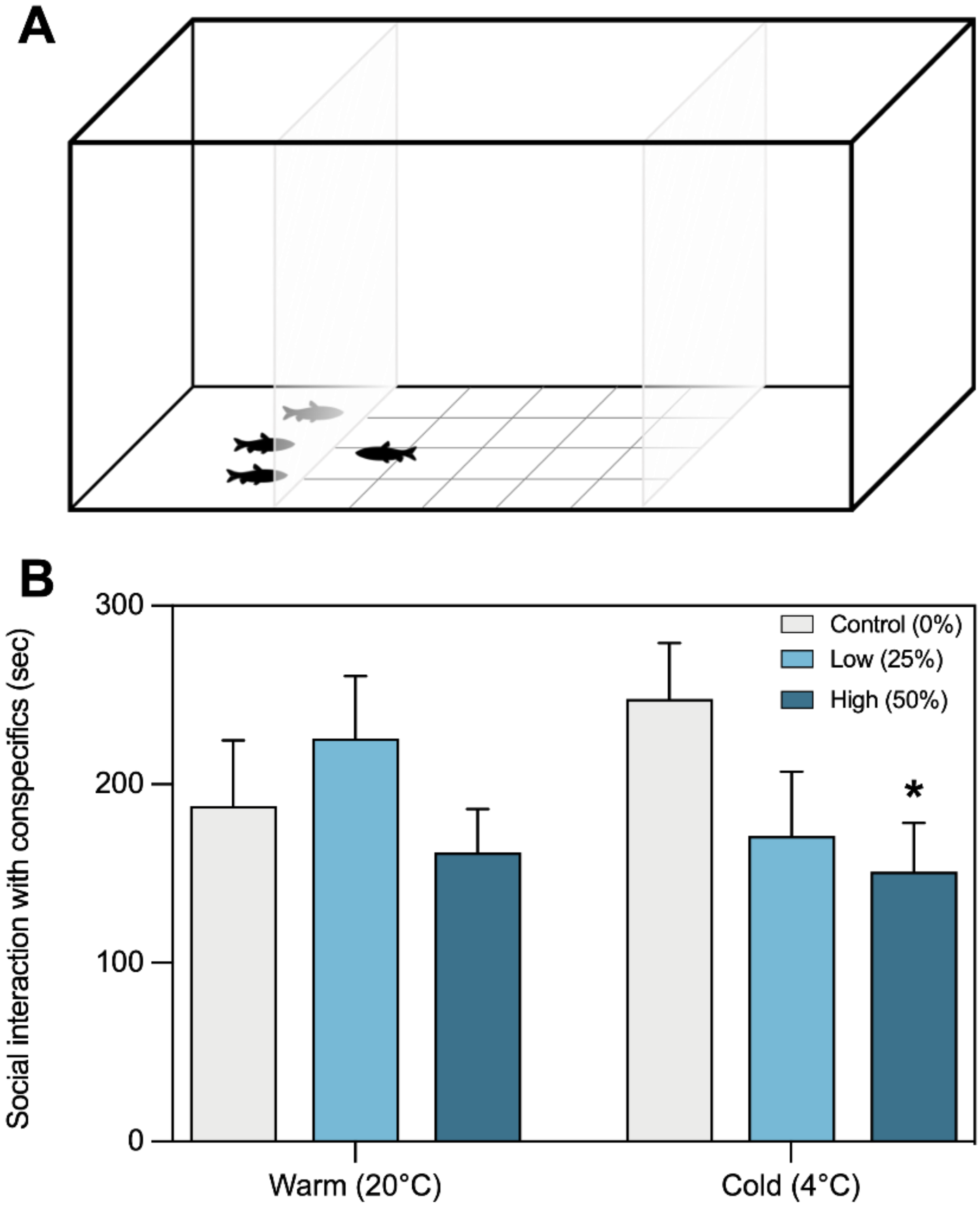


Figure 5.3. (**A**) Sociability assay showing fish interacting with shoal and (**B**) mean ± SEM time focal fish spent interacting with shoal at 20°C and 4°C. *represents significant pairwise differences between exposed fish and control within each temperature.

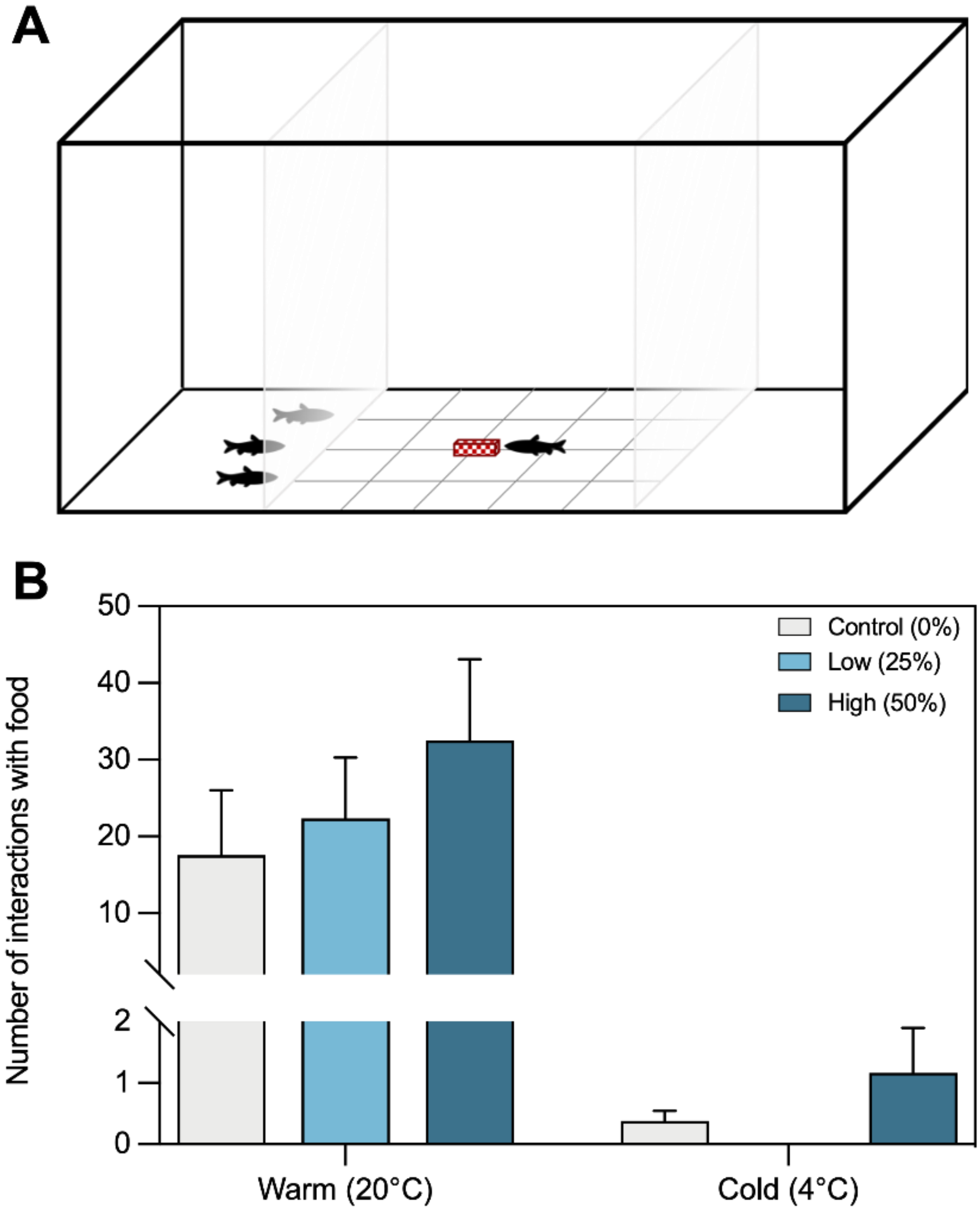
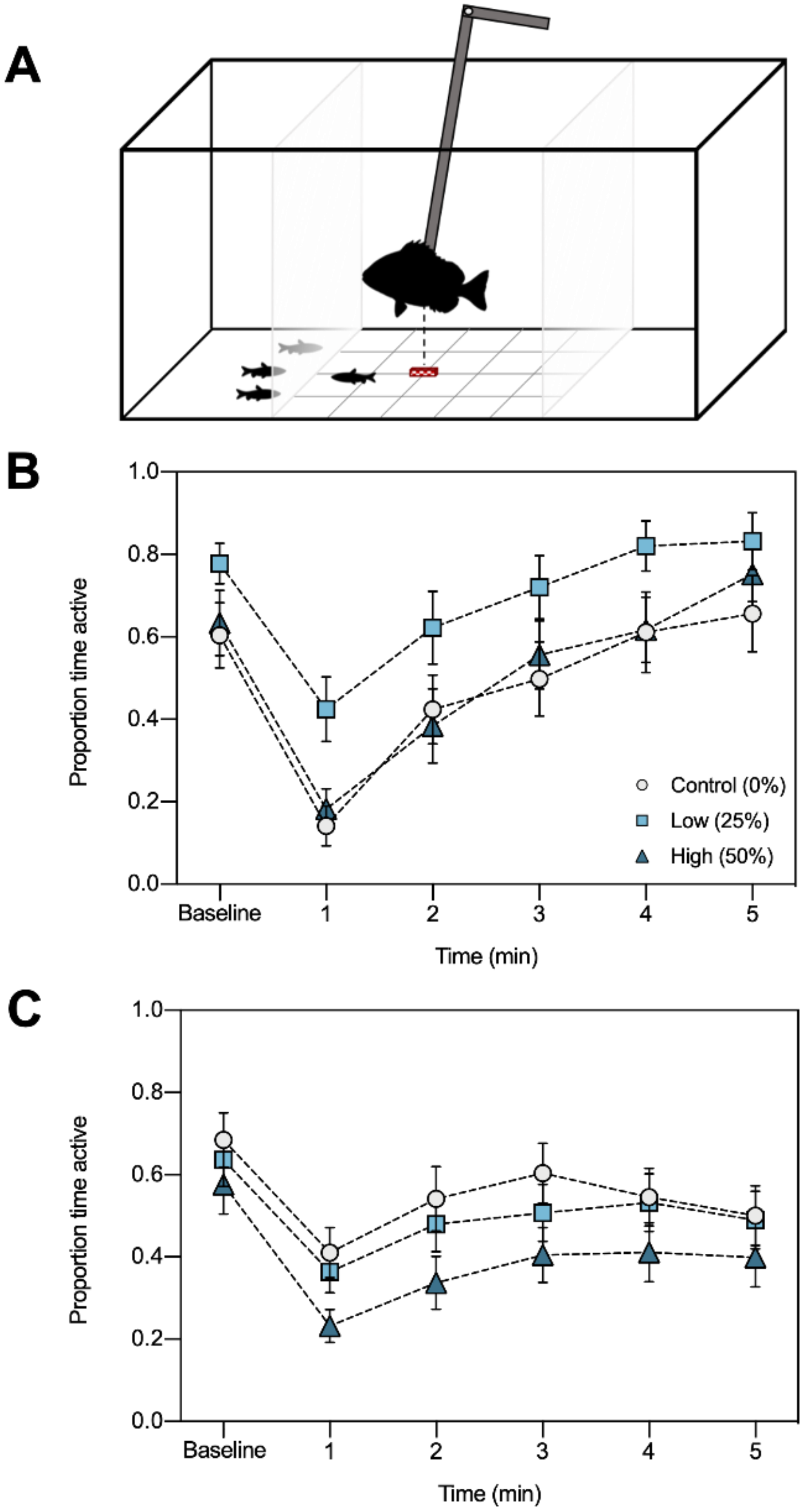


Figure 5.4. (A) Foraging assay showing fish interacting with food item and (**B**) Mean ± SEM (**A**) Number of times focal fish interacted with food item at 20°C and 4°C.


A
B
1.0
0.8
0.6
0.4
0.2
0.0
Proportion time active
Baseline
1
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5
Time (min)
○ Control (0%)
■ Low (25%)
▲ High (50%)
C
1.0
0.8
0.6
0.4
0.2
0.0
Proportion time active
Baseline
1
2
3
4
5
Time (min)

Figure 5.5. (**A**) Predator response assay showing model-predator striking centre of arena and mean ± SEM proportion of time active post-predator attack at **(B)** 20°C and (**C**) 4°C. Baseline indicates activity prior to predator attack (during foraging assay). Activity post-predator attack is presented in 1-min bins.

5.5 Discussion

In this study, we exposed fathead minnow to different levels of wastewater effluent at 20 °C and 4 °C. We demonstrate that wastewater effluent exposure can have multiple physiological and behavioural impacts on fish, and that these impacts can vary depending on the exposure temperature. We show that exposure to wastewater effluent has metabolic costs in fathead minnow, demonstrated by increases in standard metabolic rate, but only at 20 °C. We also found that wastewater effluent exposure resulted in fish taking longer to leave the safety of a shelter (a boldness measure) at 20 °C and that fish performed fewer social interactions at 4 °C. Additionally, we confirmed the presence of several contaminants of emerging concern that are often detected in wastewater effluents (e.g., venlafaxine, fluoxetine, caffeine, carbamazepine, triclosan, and diclofenac) and are associated with significant impairments in various physiological and behavioural endpoints (Nassef et al., 2010; Martin et al., 2017; McCallum et al., 2017b; Mehdi et al., 2019; Parrott and Metcalfe, 2018; Li et al., 2020; Thompson and Vijayan, 2021).

5.5.2 Metabolic costs of wastewater exposure

Exposure to wastewater effluent posed a metabolic cost on fish, demonstrated by higher standard metabolic rates, but only at 20 °C. The observed increase in metabolic demands in response to wastewater effluent exposure supports a number of previous studies across multiple species, including bluegill sunfish (Du et al. 2018, 2019) and rainbow darter (Etheostoma caeruleum; Mehdi et al., 2017). Increases in metabolic demands in response to contaminant exposure can create metabolic tradeoffs between detoxification and basal processes (e.g., growth and reproduction; Handy et al., 1999; Scott and Sloman, 2004). Such energetic tradeoffs can potentially be detrimental to the health and fitness of exposed fish, especially if energetic demands are not met by sufficient energetic supply (i.e., increased food consumption). It is important to note that we did not observe an increase in foraging rate in fish exposed to wastewater effluent. This may possibly be because the food provided in our foraging assay did not sufficiently mimic a natural food source or was not sufficiently attractive to the fish. Moreover, fish may have perceived the food item presented (in a cassette) as a novel object rather than food. Previous studies have suggested that food consumption is reduced in fish exposed to contaminants (e.g., dieldrin; Beyers et al., 1999 and fluoxetine; Mennigen et al., 2010). Additionally, we observed that fish exposed to wastewater at 20 °C were less inclined to emerge from their refuge. This is of particular concern as fish require additional energetic supply to sustain their increased energetic demands; however, if wastewater exposure is limiting their propensity to forage or their boldness, then fish exposed in the wild may suffer significant fitness costs.

The observation that standard metabolic rates were not increased by wastewater exposure at 4 °C does not suggest that fish will necessarily suffer fewer detrimental effects in the wild during colder periods of the year. Interestingly, our previous work has demonstrated that fish often congregate near wastewater plumes during the winter, presumably seeking warmer environments as temperatures downstream of WWTPs can be as much 9 °C warmer than in upstream sites during the winter (Mehdi et al., 2021). The increased likelihood of contaminant exposure when residing in wastewater plumes, the greater metabolic costs of wastewater exposure in thermally-enhanced environments, and the fact that the quality of the effluent is often poorer in the winter due to poorer degradation and increased human consumption of PPCPs (Vieno et al., 2005; Gardarsdottir et al., 2010; Ter Laak et al., 2010; Sui et al., 2011; Yu et al., 2013; Suda et al., 2014; Mehdi et al., 2021) may collectively lead to higher metabolic costs of exposure during the winter than in the summer.

5.5.2 Behavioural effects of wastewater exposure

Fish exposed to wastewater effluent showed modest behavioural effects. At 20 °C, exposed fish were less bold, as reflected by slower emergence from their refuge during behavioural assays. This could indicate that wastewater effluent exposure reduces an individual's tendency to take risks and explore novel environments (Wilson et al., 1994; Wilson and Stevens, 2005; Wilson and Godin, 2009). In the presence of predators, dampened boldness may decrease predation risk and therefore mortality (Dugatkin, 1992). However, in predator-free or low predator abundance systems, dampened boldness may prevent fish from exploiting novel environments, food sources, and mating opportunities, potentially reducing growth and reproduction (Persson and Greenberg, 1992; Brodin et al., 2013). Although boldness is often associated with many other behaviours (e.g., exploration, dispersal, foraging; Fraser et al., 2001; Rehage and Sih, 2004; Wilson and Stevens, 2005), in our study we did not see any clear effects of wastewater effluent exposure on foraging or anti-predator responses.

At 4 °C, we observed that fish exposed to high concentrations of wastewater effluent were less socially interactive with a shoal compared to those that were not exposed. Reduced sociability may increase predation risk, as vigilance against predators decreases when in isolation and an individual's likelihood of being preyed upon increases (dilution effect, Magurran, 1990). Additionally, if fish follow each other to good foraging areas, then reduced sociability may make it more difficult for fish to locate food sources (Pitcher et al., 1982), which may be especially concerning for fish exposed to wastewater during the winter – when food is scarce. Interestingly, previous studies that have examined the exposure effects of a contaminant commonly found in wastewater effluents, fluoxetine, have not found any detectable effects on sociability (McCallum et al., 2017b; Meijide et al., 2018; Martin et al., 2019). This, however, is the first study to examine how sociability is affected by whole wastewater effluent, which better reflects the realistic exposures to chemical mixtures that animals are likely to experience in the wild. Although we did detect some wastewater effluent exposure effects on behaviour in the cold, we had initially predicted that the effects would be stronger than those observed. The mild behavioural impacts observed in the cold could partly be due to lowered contaminant uptake linked to metabolic depression and decreased gill ventilation (Capkin et al., 2006; Buckman et al., 2007; Noyes et al., 2009).

5.5.3 Conclusions

Our study took a unique approach of examining the impacts of wastewater effluent, by focusing on non-reproductive endpoints and by exploring these endpoints at two different temperatures, 4 °C and 20 °C. Our findings suggest that the impacts of wastewater effluent exposure are dependent on temperature. It would be imperative to validate these findings in the field in future studies, and to consider the potential interactive effects of seasonal differences in temperature and effluent quality, as wastewater in the winter is often of poorer quality than in the summer (Mehdi et al., 2021). We believe that our findings will strengthen our understanding of ecotoxicology during the winter, a season that is rarely studied in ecotoxicological research (Larocque et al., 2020; McMeans et al., 2020; Mehdi et al., 2021).

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Chapter 6: General Discussion

6.0 Thesis aims revisited

My thesis aimed to: (i) characterize the impacts of wastewater effluents on fish communities in summer and winter; (ii) identify the impacts of wastewater effluents on fish prey communities (benthic macroinvertebrates and zooplankton) in summer and winter; (iii) assess the effects of wastewater effluents on various water quality parameters in summer and winter; (iv) explore the effects of realistic concentrations of wastewater effluent exposure on the physiology and behaviour of an ecotoxicological model species, the fathead minnow, under laboratory-controlled simulated summer (20°C) and winter (4°C) conditions. Altogether, these aims allowed me to further understand the impacts of one of the largest sources of aquatic pollution on aquatic biota; and doing so across multiple trophic levels and multiple levels of biological organization. In this final chapter of my thesis, I summarize my main research outcomes, interpret my research findings across my data chapters and the broader body of literature, and provide suggestions for future research directions.

6.1 Thesis summary

Investigations of ecotoxicological impacts across seasons, particularly during winter, have long been neglected, underexplored, and perhaps even underappreciated. In **Chapters 2 – 5** of my thesis, I demonstrated that conducting ecotoxicological research in winter can reveal “ecological surprises” that would have otherwise remained unknown had research only been conducted during warmer periods of the year. In **Chapter 2**, I demonstrated that effluent quality and water quality in effluent-receiving environments are poorer in winter than in summer at both the Dundas and Woodward WWTPs, similar to studies from other systems (Vieno et al. 2005; Sui et al., 2011; Yu et al., 2013; Kot-Wasik et al., 2016). I demonstrated an overall degradation to water quality, as indicated by higher levels of ammonia, nitrate, nitrite, and biochemical oxygen demand in winter. Also, the concentrations of several classes of contaminants of concern (e.g., venlafaxine, fluoxetine, caffeine, carbamazepine, triclosan, and diclofenac) were all higher in winter effluents compared to summer effluents. In addition to the higher concentrations of contaminants and overall poorer water quality in winter, I showed through long-term temperature monitoring that WWTPs are a source of thermal pollution, but only during winter, where temperatures downstream of wastewater outfalls were ~8 – 9°C warmer than in reference sites. Further, in **Chapter 2**, I showed that fish communities differ greatly along the effluent gradients of the Dundas and Woodward WWTPs. This was demonstrated by higher abundance, richness, and diversity near the outfall sites, but only during the winter. Beta diversity was also affected by proximity to wastewater outfalls, where dissimilarities in fish communities were highest between the sites nearest and farthest from the outfalls of both WWTPs and in both seasons. In **Chapter 3**, I investigated how effluents from the Dundas and Woodward WWTPs impact benthic macroinvertebrate communities using artificial substrates incubated in sites along the effluent gradients of the Dundas and Woodward WWTPs during summer and winter. I found no support for a universal “effect of wastewater” on benthic macroinvertebrate communities, as the impacts of the Dundas and the Woodward WWTPs were in opposite directions to one another in both seasons. At the larger Woodward WWTP, benthic macroinvertebrate abundance was higher and diversity was lower at sites downstream of the outfall compared to the upstream reference sites, and this was true in both seasons. However, at the smaller Dundas WWTP, abundance was lowest and diversity was highest near the outfall in both seasons. Community composition in sites along the effluent gradients of both WWTPs differed significantly in both seasons, where benthic macroinvertebrate communities of sites closest to the outfalls were the most dissimilar from communities of sites farthest away. Additionally, while I found strong seasonal differences in overall benthic macroinvertebrate abundance and diversity (both lower in winter), seasonality was not a significant factor in how benthic macroinvertebrate communities responded to wastewater. In **Chapter 4**, I further investigated the impacts of wastewater effluents on aquatic communities in summer and winter, using zooplankton as an indicator of lower trophic level communities. Zooplankton communities were largely dominated by rotifers (i.e., microzooplankton), in both summer and winter. At the Woodward WWTP, macrozooplankton abundance, richness, and diversity all increased with distance from the outfall in summer; while in winter, apart from diversity, the opposite patterns were observed. At the Dundas WWTP, only macrozooplankton richness appeared to increase with distance from the outfall during winter. Rotifer abundance and dominance were highest near the outfalls at both WWTPs, especially so during winter. Moreover, sites closest to the outfall were the most compositionally distinct from sites farthest away, especially so in summer. In my final data chapter, **Chapter 5**, I evaluated how wastewater effluent exposure affects fathead minnow physiology and behaviour under two acclimation temperatures, 20°C and 4°C, simulating summer and winter conditions, respectively. I found that at 20°C, wastewater effluent exposure increased resting metabolic rate (at 25 and 50%) and haematocrit (at 25 and 50%) and reduced boldness (at 50%); whereas at 4°C, wastewater effluent exposure did not affect metabolic rate, but it did reduce sociability (at 50%).

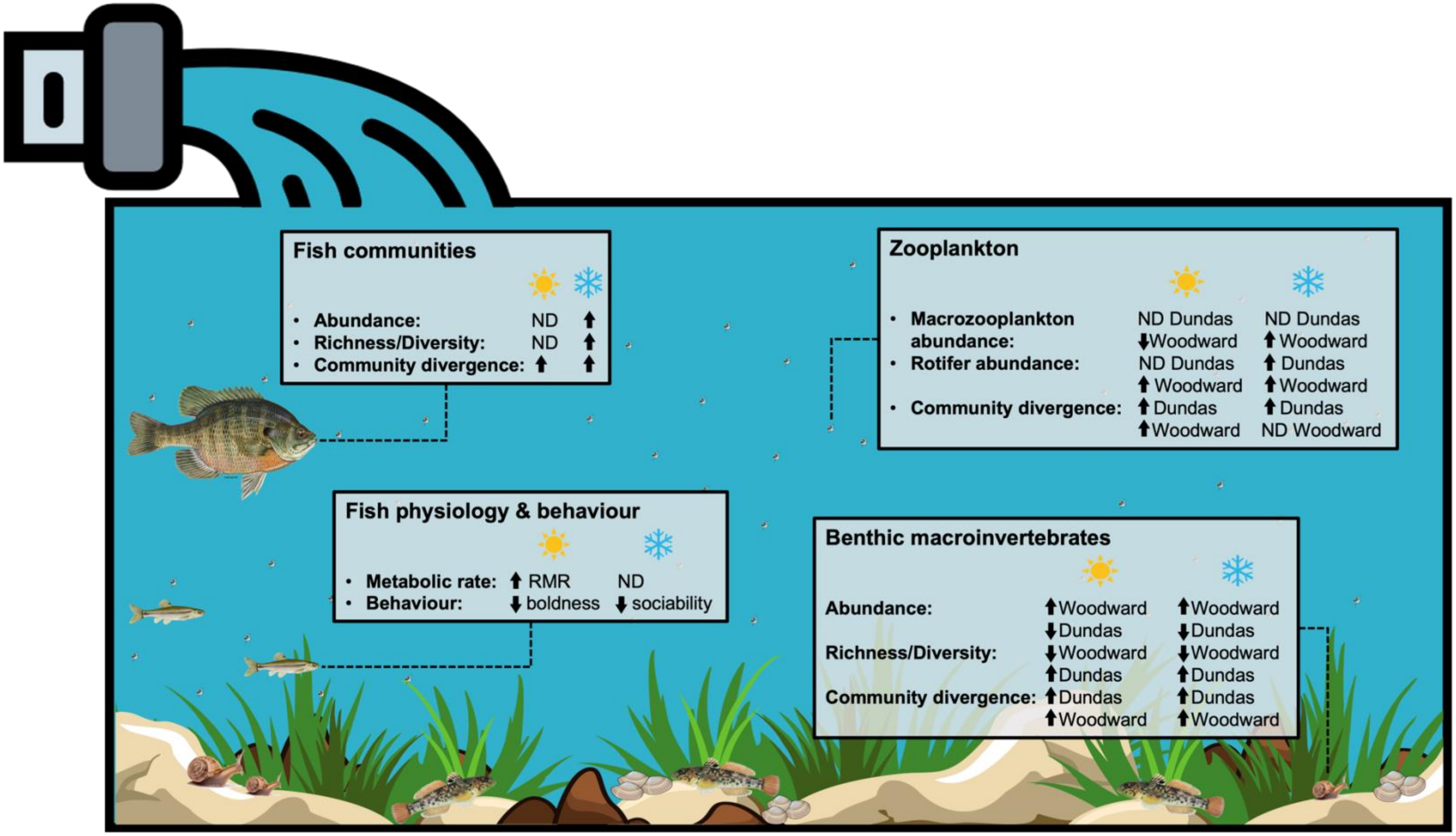

Figure 6.1. A graphical thesis summary depicting the effects of wastewater on fish communities, benthic macroinvertebrate communities, zooplankton communities, and fish physiology (resting metabolic rate; RMR) and

behaviour in summer (sun icon) and winter (snowflake icon). Community effects are interpreted as proximate versus distant from effluent outfalls. Physiology and behaviour effects are interpreted as wastewater exposure versus control. Arrows indicate direction of the effect and “ND” indicates no difference.

6.2 The importance of incorporating seasonality in ecotoxicological research

Seasonality, or at the very least, thermal variation, has been recognized to be an important variable to consider when determining the toxicity of chemicals on aquatic life. As reviewed by Noyes et al., (2009), numerous studies have demonstrated the importance that temperature plays in the toxicity of chemicals, with the general consensus being that increases in temperature likely enhance the toxicity of contaminants, as contaminant uptake often increases at higher temperatures in ectotherms. However, studies incorporating thermal variation have often ignored winter conditions; as such, we know little about how the toxicity of contaminants is influenced by colder weather and other climatic conditions brought on by winter. In general, winter limnological and ecotoxicological research has long been undervalued, as evidenced by statements like “suboptimal field conditions” or “non-growing season”. This has contributed to the general thought that winter ecology is unimportant or insignificant relative to warmer periods of the year, when most fieldwork is conducted (Powers and Hampton, 2016). However, underneath all that ice and misconception lies a dormant story, very much worth telling. In this thesis, I presented an argument for why winter ecotoxicological research is fundamental for our understanding and evaluation of aquatic pollution; as doing so may reveal “ecological surprises” or unexpected findings that may shape and influence future management decisions as well as stimulate further questions on the impacts of wastewater pollution.

In **Chapter 2**, I demonstrate how water quality parameters can vary drastically between summer and winter. Although the observed differences in effluent quality are not novel and have been demonstrated in previous studies before (e.g., Vieno et al. 2005; Sui et al., 2011; Yu et al., 2013; Kot-Wasik et al., 2016), my research is the first to connect the differences in water quality parameters to observable ecological responses – fish communities (**Chapter 2**), benthic macroinvertebrate communities (**Chapter 3**), and zooplankton communities (**Chapter 4**). The lack of studies connecting seasonal changes in effluent quality to ecological change is concerning for several reasons. First, given that the vast majority of ecotoxicological research is conducted during warmer periods of the year and/or at spring/summer exposure temperatures, our understanding about the toxicological effects of various contaminants of concern (e.g., fluoxetine and 17α-ethinylestradiol) is therefore inherently biased and incomplete. Second, if the vast majority of our ecotoxicological research ignores winter conditions, then policy-driven decisions within conservation management contexts are also likely to be biased and not based on complete ecological realism. For example, in **Chapter 2**, we found that the concentrations of nitrogenous waste products, particularly ammonia, tended to be significantly higher in winter than in summer. One might think that evaluating the toxic effects of ammonia at winter concentrations would suffice. However, that would preclude complete ecological relevance as the toxicity of contaminants, like ammonia, can differ significantly between summer and winter exposure conditions. For instance, the toxicity of ammonia, as measured by LC_{50}, is higher at colder temperatures for several fish species in the Laurentian Great Lakes (Environment Canada, 1999). Therefore, ecotoxicological assessments of contaminants must be based on realistic exposure conditions, not only in terms of environmentally-relevant concentrations of contaminants but also environmentally-relevant exposure conditions (e.g., temperature, photoperiod, pH).

The notion that changes in effluent quality can manifest into ecological change is supported by several previous studies. For example, multiple studies have documented high levels (70-100%) of intersex in male rainbow darter (*Etheostoma caeruleum*) downstream of WWTPs in Kitchener and Waterloo, ON, Canada (Tetreault et al., 2011; Tanna et al., 2013; Bahamonde et al., 2015; Fuzzen et al., 2015; Fuzzen et al., 2016). Following upgrades at one of the WWTPs (Kitchener), intersex incidence was significantly reduced (<10%; Hicks et al., 2017). While these studies did not evaluate the effects of seasonality per se, they do however present strong evidence that changes in effluent quality (e.g., reductions in nutrients and PPCPs) can result into measurable ecological change. Analogously, I argue that seasonal changes in effluent quality should be evaluated when determining the impacts of WWTPs on aquatic life.

6.3 Importance of incorporating multiple levels of biological organization in ecotoxicological research

A central goal of my thesis was to evaluate the impacts of wastewater effluent exposure in summer and winter using methodologies drawn from multiple levels of biological organization. Doing so enhances our ability to understand the impacts of environmental disturbances more holistically, as linking change from one level to the next is a fundamental tenet in ecological applicability. This was best exemplified in **Chapter 5**, where I demonstrated that wastewater effluent exposure poses a metabolic cost (increase in standard metabolic rate) in fathead minnow, but only under summer-like exposure temperatures (20°C). Had this study been done in isolation (i.e., without supplementary field evaluations), it would have appeared as if wastewater effluent exposure does not pose a threat to fishes in winter. However, such conclusions would be incorrect and misguided. Interestingly, research presented in **Chapter 2** demonstrates that fish often congregate near wastewater outfalls during winter, likely seeking the warm effluent that WWTPs discharge. Therefore, fish exposed to wastewater effluents during winter are likely experiencing exposures at spring-like temperatures (10 – 15°C), where uptake of contaminants is likely to be higher than at near-freezing temperatures. Moreover, the effects of wastewater effluent exposure may be further exacerbated not only because downstream temperatures can increase contaminant uptake rates, but also because the effluent itself is often of poorer quality during winter (Vieno et al., 2005; Gardarsdottir et al., 2010; Ter Laak et al., 2010; Sui et al., 2011; Yu et al., 2013; Suda et al., 2014).

6.4 Importance of incorporating multiple trophic levels in ecotoxicological research

In my thesis, I evaluated the effects of wastewater effluents on aquatic organisms across multiple trophic levels. I found that the effects of wastewater are not generalizable across trophic levels; for example, fish community responses to wastewater were not completely congruent with benthic macroinvertebrate community responses. This incongruency in responses across trophic levels further supports the idea that holistic assessments of environmental disturbances must not only involve evidence from multiple levels of biological organization but also from multiple trophic levels for several reasons. First, species within and across trophic levels can vary considerably with their responses to contaminants of concern (Fleeger et al., 2003). Such differences in responses to contaminants is further complicated by the fact that species' sensitivity to contaminants, either within or across trophic levels, is often contaminant-dependent. Fishes, for example, are more sensitive to endocrine disrupting chemicals (EDCs) than invertebrates (Caldwell et al., 2012; Brown et al., 2014). Second, the effects of contaminant exposure on a species are largely influenced by the route of exposure. Exposures via direct routes (i.e., waterborne) can differ in their effects when compared to exposures via contaminated food (i.e., trophic cascade). That is because when a contaminant is released into aquatic environments, via WWTPs for example, it is degraded and/or biotransformed, which may alter its original form, possibly altering its toxicological effects (Nunes et al., 2020). Furthermore, aquatic organisms found in contaminated environments may become contaminant vectors, potentially allowing contaminants to bioaccumulate in food webs and/or transfer up trophic cascades (Zuccato et al., 2000; Kosjek et al., 2007; Kummerer, 2009). Third, contaminants may affect aquatic organisms not just through direct exposure routes but also through indirect changes in food web interactions (e.g., changes in predation pressures and inter- and intraspecific competition; Fleeger et al., 2003). Additionally, contaminants like wastewater effluents can alter nutrient and oxygen levels in aquatic environments which may alter ecosystem function (Fleeger et al., 2003). Furthermore, contaminant-induced changes in the physiology (e.g., changes in metabolic demand) or behaviour (e.g., changes in grouping or foraging/grazing behaviours) of exposed aquatic organisms can further alter species abundances and community composition in contaminated environments (Fleeger et al., 2003). Cumulatively, exposure to contaminants may exert direct and indirect effects on populations and communities across multiple trophic levels, making comprehensive ecosystem-wide studies necessary for fully understanding the effects of contaminants on aquatic biota.

6.5 Are wastewater outfalls ecological traps?

Here, I revisit the idea of an ecological trap and attempt to answer the question of whether wastewater outfalls act as an ecological trap. As noted previously, an ecological trap forms when an organism makes a maladaptive habitat choice based on cues that were once reliable of habitat quality

(Dwernychuk and Boag, 1972; Schlaepfer et al., 2002). Organisms often rely on direct and indirect cues emitted from a habitat to assess its current and future state. Over time, these decisions become adaptive because animals' survival and reproductive success depend on them (Williams and Nichols, 1984). However, when there is a sudden change in an environment, likely as a result of anthropogenic disturbance, these decisions no longer correlate with survival and reproductive success, and can therefore be maladaptive (Tinbergen, 1958; Levins, 1968).

Wastewater outfalls create conditions that may be perceived as beneficial or preferential for organisms, but are likely maladaptive. **In Chapter 2**, I outlined how wastewater outfalls can thermally-enhance receiving environments, raising water temperatures by as much as 9°C, but only during winter. Fish and other aquatic organism may select to reside near warm wastewater plumes as dictated by their thermal optima; therefore, such habitats may be perceived as thermal refugia during winter (Cooke et al., 2000, 2004; Brodersen et al., 2011). Another potential perceived benefit of living near wastewater outfalls is food availability. In **Chapters 3** and **4**, I outlined how prey communities are affected by wastewater inputs in summer and winter. These studies indicated that wastewater outfalls are indeed high in food availability, as predicted by the high nutrient concentrations in the effluents of the two WWTPs sampled. This high abundance of food availability was consistent in summer and winter. As a matter of fact, downstream of the Woodward WWTP, rotifers were found at significantly higher densities in winter compared to summer. The consistent availability of food is further supported by previous studies demonstrating increased growth and body condition in fishes downstream of wastewater outfalls (Chambers et al., 1997; McMaster et al., 2005; Brown et al., 2011; Tetreault et al., 2011; Nikel et al., 2021). Therefore, the combination of a steady supply of nutrients, high food availability, and thermal enhancement may create enticing environments for fishes and other aquatic organisms to reside in, particularly during winter, when conditions elsewhere are harsh. However, as stated throughout this thesis, living in such highly contaminated environments comes at a cost.

The costs of living in effluent-receiving environments come from the effects of wastewater exposure demonstrated by numerous studies. These effects manifest across multiple levels of biological organization, including altered mRNA transcription (Bahamonde et al., 2014; Garcia-Reyero et al., 2011), changes in endogenous hormone levels (Pottinger et al., 2013), increased incidence of male feminization rates (Jobling et al., 1998; Harris et al., 2011; Bahamonde et al., 2015; Fuzzen et al., 2015), increased metabolism and increased energetic demands (Du et al., 2018, 2019; Mehdi et al., 2018; **Chapter 5**), and abnormal aggressive and courtship behaviours (McCallum et al., 2017b; Saaristo et al., 2014). Such effects can have significant costs on survival and reproduction, implying potential consequences to the sustainability of vulnerable populations in habitats impacted by wastewater effluents. The well-documented costs of wastewater effluent exposure suggest that indeed, wastewater outfalls are an ecological trap, especially during winter.

While there is ample evidence suggesting that wastewater exposure poses a fitness cost on aquatic organisms, there are also studies that suggest the effects of wastewater exposure are minimal. For example, round goby (*Neogobius melanostomus*) exposed to wastewater *in situ* had little to no alterations in behaviour or physiology, although exposure reduced survival (McCallum et al., 2017). Similarly, killifish (*Fundulus heteroclitus*) exposed to wastewater showed no evidence of metabolic costs, although hypoxia tolerance was disrupted (Lau et al., 2021). Above all else, it is hard to quantify the number of studies that have shown "no to little effects" of wastewater exposure, as not only do these studies receive less attention than studies with strong effects, but importantly, researchers often fail to publish null results ("*the file drawer problem*"; Rosenthal, 1979). Furthermore, it is well known that species within a trophic level and most certainly across different trophic levels differ in their tolerance, resilience, and sensitivity to contaminants (Fleeger et al., 2003). Therefore, it may be rather crude to assume that wastewater outfalls are an ecological trap for all organisms, as some species may tolerate and even flourish in effluent-receiving environments while others may suffer. Therefore, the classification of wastewater outfalls as an ecological trap must be a more nuanced one, as we must take into consideration the individual species' costs and benefits of residing in wastewater plumes across seasons.

6.6 Thesis limitations

In this thesis, I conducted field and laboratory studies in extraordinary conditions (e.g., freezing or near-freezing temperatures) while applying established field and laboratory sampling techniques (e.g., electrofishing, trapping, artificial substrate incubation, respirometry, and behavioural testing). Drawing on previously established methods allowed me to provide new insights into how such methods can be retrofitted and adapted to work in a multitude of scenarios, such as investigating whether wastewater outfalls are an ecological trap for aquatic organisms in summer and winter. While these methodological approaches were appropriate, certain constraints may limit the conclusions that can be drawn. Below, I outlined the most important limitations from the aforementioned data chapters, and in the following section, I explored how some of these limitations can be transformed into future directions (Section 6.7).

Conducting the field study portions of this thesis in the Hamilton Harbour watershed allowed me to collect an important dataset and of one particular significance to the remedial action plan (HHRAP, 2022). However, despite the importance of conducting ecological research in an Area of Concern, there were a few limitations that I had to overcome or acknowledge. The sampling sites in our field studies lack the homogeneity that we see in other systems, particularly riverine systems like the Grand River, ON, Canada. In Appendix C of this thesis, I highlight the differences in habitat characteristics between each of the ten sites that I sampled in **Chapters 2 – 4**. The differences in depth, substrate type, flow, and other factors may play a role in shaping aquatic communities (Gorman and Karr, 1978). However, while I recognize that this is a limitation of these studies, realistically, no alternatives could have been employed if our goal was to sample within the Hamilton Harbour watershed.

Another limitation to my studies was the geographical location of my sampling sites. Particularly, differences in site order between the smaller Dundas WWTP and the larger Woodward WWTP was a challenge and a potential drawback of my study systems that I had to account for either statistically and/or when interpreting the results. At the Dundas WWTP, I had five sites that were all downstream of one another, and the effects of the effluent were understood to be that of a gradient over a geographical distance between each site from the outfall. However, at the Woodward WWTP, I had to contend with the fact that the sites are ordered differently, such that three sites were downstream of the outfall and two were upstream. Having upstream sites at the Dundas WWTP was not a possibility since the WWTP outfall was located at the head of the stream. Similarly, downstream reference sites could not be sampled at the Woodward WWTP, as any downstream sampling sites after the third site (W3 or WDS3) would have been in Hamilton Harbour proper, where sites would have varied drastically from where my sampling took place in Red Hill Creek.

Aside from field work limitations, in **Chapter 5**, I had to limit the scope of the laboratory exposure study to a double factorial design. In this final study, I wanted to simulate wastewater exposure under summer and winter conditions. There were several factors that were omitted (e.g., seasonal photoperiod, use of summer versus winter effluents, continuous exposure to fresh effluent, unlimited versus limited food rations) that if employed, would have enhanced the ecological validity of this study and potentially increased the strength of the conclusions drawn from it. These factors are all linked to onset of winter and would have made my study design more elaborate and more ecologically relevant. However, these factors would have also been difficult to simulate in a laboratory setting and would have added further complexity to the study, potentially rendering it useless if these factors were not controlled for properly. Another limitation of this study was the limited number of temperatures to which fish were exposed. Given that sites downstream of effluent outfalls experience thermal pollution during winter, it would have been ideal to expose fathead minnow to wastewater at similar temperatures to those observed in **Chapter 2** (10 – 15°C).

6.7 Future directions

Based on the findings of my thesis, there are numerous possibilities for future research. Those include tracking fish movements in and out of wastewater plumes across seasons, conducting natural experiments to better quantify the costs and benefits of wastewater exposure in summer and winter, and studying the impacts of wastewater in more homogenous environments, unlike the Hamilton Harbour watershed.

6.7.1 Fish movement in and out of wastewater plumes

In **Chapter 2**, I show that fish communities are influenced by proximity to wastewater outfalls in summer and in winter; such that communities near wastewater outfalls have higher abundance, higher richness, higher diversity, and a greater degree of dissimilarity relative to communities farther away. While that kind of research shows us cumulatively how wastewater shapes and influences fish communities, it would be pertinent to study fine scale movements of fish in and out of wastewater plumes over multiple seasons to better understand how and why fish choose to live in such environments. As of now, we do not know whether fish are staying near wastewater outfalls in the winter and/or if fishes are migrating towards effluent outfalls from nearby sites. Conducting fine scale telemetry studies using established methods such as acoustic telemetry or mark-recapture studies (Cooke et al., 2013) corroborated with long-term temperature monitoring will allow us to ascertain if fishes are actively seeking these environments as a form of behavioural thermoregulation (Golovanov, 2006). Previous studies have successfully implemented fixed antenna array and a continuous scanning coded receiving system to monitor the movement of radio-tagged smallmouth bass (*Micropterus dolomieu*) in a thermal discharge canal on Lake Erie during winter (Cook et al., 2004). Fish were found to spend the majority of winter near the tempering pumps, where water was warmest. I believe similar telemetry techniques could be employed at our study sites to investigate fish movements as a function of thermal variation across seasons, but with the inclusion of wastewater exposure as an additional factor. Such study would further support our hypothesis of wastewater outfalls acting as an ecological trap for aquatic organisms. It would also add a finer level of detail of a "*cause-and-effect relationship*", particularly if it is combined with continuous temperature and effluent quality monitoring.

6.7.2 Evaluating the effects of wastewater effluents on aquatic organisms using natural experiments

By the end of 2022, the upgrades to the Woodward WWTP should be finalized (City of Hamilton, 2022). One of those upgrades includes the relocation of the effluent outfall from its current location (W1 or WS1) to one of our upstream sites (W4 or WUS2). The relocation of the effluent discharge site will present an exciting opportunity to compare aquatic communities before and after the outfall relocation. This would allow for my current thesis findings to act as a baseline to which future studies can be compared to. I believe this is a rare opportunity that is worth seizing, as it will provide further support to our hypothesis of wastewater acting as an ecological trap. Following the outfall relocation process, if findings at the new outfall site are similar to those at the original outfall site, where wastewater outfalls supported high abundance of fish and prey communities, then we would have an additional line of evidence that fish do indeed choose to live in contaminated environments, likely for the perceived benefits of thermal enhancement and increased productivity during winter.

6.7.3 Impacts of wastewater effluents in homogenous streams

One of the major limitations of the field component of this study was the lack of homogeneity of habitat characteristics across my sampling sites. I believe it would be beneficial to study the effects of habitat characteristics on various biodiversity metrics (e.g., total abundance, species richness, species relative abundance) using advanced statistical techniques (e.g., Fourth Corner and RQL analysis). Such techniques would further tease apart differences linked to habitat characteristics versus differences linked to proximity to wastewater outfalls. Furthermore, conducting community assessments similar to those outlined in my thesis but in a watershed with more homogenous sampling sites would allow us to better differentiate between differences linked to site habitat differences and site proximity to a WWTP outfall. For example, there are currently 30 WWTPs that discharge their effluents into the Grand River, the longest river in southwestern Ontario. While there are considerable differences along the ~280 km stretch of the river, the consistency in habitat characteristics in this system exceeds that of Desjardins Canada, Cootes Paradise Marsh, and Red Hill Creek (all within the Hamilton Harbour watershed). Additionally, sampling along 30 different WWTPs would allow for more robust sampling points. It would also allow for the opportunity to sample different kinds of WWTPs, offering greater variation in the size of WWTPs and the treatment technologies employed.

6.8 General conclusion

My PhD research investigated the effects of municipal wastewaters on the community composition of fishes, benthic macroinvertebrates, and zooplankton in Hamilton Harbour in summer and winter. I also studied the effects of wastewater exposure on the physiology and behaviour of fathead minnow under temperatures simulating summer and winter conditions. My thesis underscores the importance of incorporating seasonal and/or thermal variation in ecotoxicological research and provides evidence that support the "*ecological trap hypothesis*" of wastewater outfalls during winter. More work is now needed to evaluate how and why aquatic organisms choose to reside in such contaminated environments. Future research should further investigate the relative costs and benefits of living in effluent-dominated environments using laboratory and field manipulation studies. While the winter ecology of freshwater systems has been vastly understudied, interest is rapidly growing. I hope my thesis encourages the amalgamation of winter ecology and ecotoxicology, as environmental disturbances do not happen in a void; therefore, we should not study them in one. Importantly, research that enhances ecological relevance by incorporating seasonality can improve the direction and precision of remediation strategies in restoring impacted ecosystems, protecting water quality, and creating conservation policies that will ensure the sustainability of aquatic habitats.

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Appendix A: A comparison of passive and active gear in fish community assessments in summer versus winter

1.0 Abstract

Fish populations and communities are monitored using a variety of sampling gears, each with their own inherent biases. Gear biases can arise from a number of factors, such as fish species characteristics (e.g., body shape/size, physiology, and behaviour), species habitat requirements, as well as the abiotic characteristics of sites sampled. Such factors and their effects on gear selectivity are also heavily influenced by seasonality. Consequently, understanding the effects of seasonal changes on gear selectivity is of vital importance, especially during the winter—a season seldom studied in freshwater systems. Here, we compared the selectivity, efficiency, and degree of biodiversity in fish communities sampled using three gear types: minnow traps, Windermere traps, and electrofishing during summer and winter in Hamilton Harbour, ON, Canada. Catch per unit effort was similar among gear types in the summer, whereas in the winter, minnow traps captured the most fish. Electrofishing samples were the most species rich and species diverse, but only during the summer. Additionally, sampling efficiency and the number of different species encountered was highest when all gear types were used in combination, followed by electrofishing alone, Windermere traps alone, and minnow traps alone in both seasons. Each gear type differed in its selectivity for certain species, which was further influenced by seasonality. This resulted in the fish communities caught within each gear type being dissimilar from one another.

Our study highlights the importance of understanding gear type selectivity, particularly under different climatic conditions, and outlines the importance of incorporating multiple gear types in ecological assessments of fish populations and communities.

2.0 Introduction

Fish population monitoring and community assessment are a cornerstone for fisheries management, conservation, and ecology. The accurate assessment of fish populations and communities is an essential yet extremely challenging task, as fish can occupy large and diverse habitats, move long distances, and be over- or underrepresented when monitored using certain sampling gears (MacKenzie et al., 2002; Elphick, 2008; Dextrase et al., 2014). Selecting the appropriate sampling gear, or gear type, is one of the most crucial decisions fish biologists and resource managers consider when conducting fish community and population assessments, as there is a great variety of gear types available, each with their own advantages and disadvantages (Portt et al., 2006; Bernhardt and Palmer, 2011; Jähnig et al., 2011). Gear types are often classified into two broad categories: (i) active gear and (ii) passive gear. Active gear has to be moved or activated by the sampler in order to catch fish (e.g., electrofishing, seine netting, and trawling; Portt et al., 2006; Winger et al., 2010). In contrast, passive gear is left out for a period of time before being retrieved, relying solely on the animal’s movement and interaction towards it for capture to occur (e.g., minnow traps, Windermere traps, fyke nets, and gill nets; Hamley, 1975; Lagler, 1978; Portt et al., 2006; He and Pol, 2010). The usage of different gear types can lead to vast differences in fish population and community estimates, as each gear type has inherent biases and selectivity towards certain species, sexes, sizes, and habitats (Murphy and Willis, 1996; Ruetz III et al., 2007).

Fish sampling gear varies in a number of ways, including size, shape, period of deployment/activation, and usage of bait (Murphy and Willis, 1996; Portt et al., 2006). Such variety allows researchers to choose from a wide selection of gear types depending on targeted species of interest, habitat characteristics, and labour and gear cost considerations (Murphy and Willis, 1996; Diana et al., 2006). For example, fyke nets are well-suited for the capture of small-bodied mobile fishes, whereas electrofishing is well suited for the capture of sedentary fishes and is less size-selective (Hubert, 1996; Reynolds, 1996; Chick et al., 1999; Dolan and Miranda, 2003; Breen and Ruetz, 2006; Ruetz et al., 2007). Consequently, gear type selection can yield unforeseen biases in population and community assessments, especially if only a single sampling gear type is employed in a study, resulting in partial representation of the true community or population (Murphy and Willis, 1996). Gear type biases can influence our confidence in critical studies of fish assemblages, including those utilised in conservation efforts of endangered species, control and management of invasive species, and assessments of fish communities in response to anthropogenic disturbances (e.g., pollution, habitat modification, and climate change; Brandner et al., 2013; McCallum et al., 2019;

Mehdi et al., 2021). For instance, in a study comparing the selectivity of electrofishing, trawling, seining, and drift netting in several large rivers draining into the North Sea and Baltic Sea, researchers found that electrofishing on average yielded higher biodiversity metrics, while species composition differed significantly across each sampling method (Zajicek and Wolter, 2018). Similarly, a study comparing the effectiveness of six different gear types (seine nets, hoop nets, trap nets, Windermere traps, minnow traps, and electrofishing) found staggering differences in the abundance, species richness, and species composition of fish caught between each gear type (Lapointe et al., 2006). Numerous additional studies have demonstrated the differences in fish population and community estimates that can arise from inherent gear type biases. However, despite such biases, single gear type sampling techniques continue to be employed in fish population and community studies, resulting in certain species being over- or underestimated; thereby hampering accurate population and community estimates.

Although gear type selectivity is widely recognised as a hindrance in fish community research, one issue that has received little attention is how seasonal changes in catch are influenced by gear selectivity. As mentioned before, species characteristics, species life history traits, species habitat requirements, sampling site characteristics (e.g., water depth, flow, clarity, temperature, and substrate type), and even inter- and intraspecies interactions can all contribute to gear type selectivity (Penczak and Jakubowski, 1990; Hubert and Fabrizio., 2007; Hubert et al., 2012). However, fish capture and sampling gear encounter rates are largely dependent on fish activity, which in turn is strongly modulated by seasonality (Rudstam et al., 1984; Wilson et al., 2011; Olsen et al., 2012). Most species have distinct seasonal movements and behaviours as their habitat requirements, spawning activity, food availability, and physiology change considerably across seasons (Hurst, 2007; McMeans et al., 2020). At higher latitudes, winters bring cold temperatures, shortened photoperiods, ice cover, hypoxia, and limited food availability (Shuter et al., 2012). Most fish species respond to the onset of such winter conditions with pronounced reductions in movement, foraging, growth, reproduction, as well as constriction of their home range (Hurst, 2007; McMeans et al., 2020). This is largely driven by reductions in metabolic rate, typical of ectothermic organisms responding to winter conditions. During winter, fish remain dormant for long periods of time in order to conserve energy; therefore, their movement and likelihood of being captured by certain sampling gear would presumably decline (Hurst, 2007; Shuter et al., 2012; McMeans et al., 2020). In contrast, during the spring and summer when environments are warmer and more productive, fish are generally more active, explorative, and have higher energy demands met by greater metabolic scopes (Hasley et al., 2015). As such, fish are more likely to encounter and be caught by sampling gear, specifically passive gear types. Understanding the effects of seasonal changes on gear selectivity is of critical importance, especially in temperate and polar regions where winter is a dominant season, yet remains vastly understudied (McMeans et al., 2020).

Research of this kind can further enhance and guide fisheries management, especially when most decisions have historically been based on research conducted during warmer periods of the year.

Our objective was to compare the selectivity and efficiency of three gear types on fish communities during summer and winter in shallow streams flowing into Hamilton Harbour, ON, Canada. Using minnow traps (passive gear), Windermere traps (passive gear) and boat electrofishing (active gear), we compared the abundance, richness, diversity, community composition, and the selectivity and species discovery rate (i.e., efficiency) of each gear type in the summer and winter. We predicted that overall abundance, richness, and diversity of fish samples would be lower during the winter, given lower fish mobility. Based on previous studies, we also predicted that these metrics would differ across gear types, with electrofishing being the most successful and efficient, especially during the winter, when fish activity is subdued, making fish less likely to encounter passive gear types. We further hypothesised that our gear types would capture distinct fish communities, as each gear type is inherently biased towards certain fish species characteristics (e.g., body shape/size, habitat preference, physiology, and behaviour). Furthermore, we predicted that the species makeup of communities captured within each gear type would differ between summer and winter, as gear type species selectivity is likely to differ across seasons.

3.0 Methods

3.1 Study area

We sampled fish communities in Hamilton Harbour (ON, Canada), a large freshwater embayment situated at the western end of Lake Ontario. Due to historical degradation caused by anthropogenic development, Hamilton Harbour is listed as one of 43 Areas of Concern under the Great Lakes Water Quality Agreement (2012). The present study was focused on shallow (<2 m) streams and wetlands that flow into the harbour. Sampling took place at two different areas of the harbour, with five sampling sites in each area. First, we sampled along Red Hill Creek, which flows from Albion Falls on the Niagara Escarpment and discharges into the eastern end of Hamilton Harbour at the Windermere Basin. This area is heavily degraded and industrialised, with clear signs of anthropogenic modifications and shoreline alterations (McCallum et al., 2019; Mehdi et al., 2021). Second, we sampled sites located on the western end of the harbour, along Desjardins Canal, West Pond, and Spencer Creek. These sites are within Cootes Paradise Marsh, the largest wetland in western Lake Ontario. The marsh is a protected nature sanctuary, known for its rich biodiversity, use as an important migratory waterfowl stopover site, and fish nursery habitats (Leslie and Timmins, 1992; Smith and Chow-Fraser, 2010). Despite the considerable biodiversity found within Cootes Paradise Marsh, it is among the most degraded wetlands in Lake Ontario due to poor water quality and its hypereutrophic state (Chow-Fraser, 2006; Thomasen and Chow-Fraser, 2012). These particular sampling sites were initially targeted for accessibility and are part of a long-term research program (McCallum et al., 2019; Mehdi et al., 2021; Nikel al., 2021). See Supplementary Fig. 1 for a map of the sampling sites.

3.2 Sampling techniques

Fish communities were sampled during the summer (July and August) and winter (November, December, and March) of 2018 and 2019. Fish were sampled using a combination of passive (minnow traps and Windermere traps) and active (electrofishing from a boat) gears. These sampling gear types were chosen as they have been commonly used in shallow systems with different habitat types and were also used in previous research conducted at our study sites (McCallum et al., 2019; Mehdi et al., 2021; Nikel et al., 2021). Additionally, the gear types selected in our study offer novel insight into gear selectivity in the shallow zones of Hamilton Harbour. Ongoing monitoring efforts in Hamilton Harbour have largely been performed using electrofishing from large vessels, a method that cannot be utilised in shallow systems (Boston et al., 2016). At each site, and on each sampling event, 10 black minnow traps (wall height = 16.7 cm; entry hole diameter = 2.10 cm, trap length = 40.5 cm; mesh diameter = 0.76 cm), each baited with ∼20 g of corn, were deployed from land ∼10 m apart from one another. Two meshed Windermere traps (wall height = 66.0 cm; entry hole diameter = 17.5 cm, trap length = 96.0 cm; mesh diameter = 0.30 cm), each baited with ∼100 g of corn, were also deployed from land ∼10 m away from the first and last minnow traps. Minnow and Windermere traps were deployed on ropes extending ∼5 m from shore. Traps were retrieved

24 h post-deployment. Additionally, at each site, two 50 m transects (within 5 m from shore) were sampled from a boat using a portable electrofishing unit (1.5-KVA Electrofisher, Smith-Root Inc.). All gear types were deployed at the same depth (see Supplementary Table 3). Sites on the eastern end of Hamilton Harbour were sampled five times in the summer and three times in the winter. Sites on the western end were sampled four times in the summer and three times in the winter. Sampling was always performed during daytime, between 0800 and 1400 h on weather permitting days. On each field date, all five sites on either end of Hamilton Harbour were sampled using all three techniques, with the exception of one of the summer dates, when electrofishing could not be performed due to heavy rainfall during sampling on the east end of the harbour. See Supplementary Table 1 for additional field sampling information.

During each sampling event, we measured the following water quality parameters: temperature, dissolved oxygen (YSI ProODO), pH, salinity, conductivity, and total dissolved solids (Oakton multiparameter Testr) at each site (see Supplementary Table 2). Habitat characteristics were also assessed at each site based on a subset of the metrics used in the Qualitative Habitat Evaluation Index (QHEI; Taft and Koncelik, 2006; Strickland et al., 2010) and following a previously described protocol by McCallum et al. (2019). Habitat metrics taken included: total water depth, water clarity (Secchi depth), substrate type, sediment particle size, shoreline slope, degree of sinuosity, degree of anthropogenic modifications (i.e., physical modifications of the shore-water interface), riparian zone width, degree of estimated bank erosion, and the presence of any aquatic plants (see Supplementary Table 3).

Fish collected at each sampling site were transported in dark-coloured, aerated bins to shore, where they were counted and identified to species level. At each site, fish caught using minnow traps or Windermere traps or electrofishing were pooled and measured with other fish caught with the same respective gear type. The standard and total lengths (mm) and body mass (g) of the first 15 individuals of a given species caught at each site were individually measured, while the remaining fish were only counted and batch-weighed. This was done to reduce processing time and handling stress. Native fishes were immediately returned to their site of collection, while invasive fish were euthanised with an overdose of benzocaine (small fishes; < 10 cm) or by a lethal cephalic blow (large fishes; > 10 cm), as required by the Ontario Ministry of Natural Resources and Forestry (2015). All fish were handled in accordance with approved animal utilisation protocols from McMaster University's Animal Research Ethics Board (AUP 17−12-45).

3.3 Statistical analysis

All statistical analyses were performed using R (version 3.6.2, R Core Team, 2019). Prior to any analysis of fish communities, fish count data were mean-standardised for each gear type (multi-gear mean standardisation; following Gibson-Reinemer et al., 2017) to allow the different gear types to be compared to one another. Catch per unit effort (CPUE) was calculated per trap deployed for minnow traps and Windermere traps and per shock-second for electrofishing. Fish abundance (in total number and biomass), species richness (number of identified species), species diversity (Shannon-Weiner Index), proportion of benthic species, proportion of invasive species (invasive: not native to the Great Lakes), proportion of tolerant species (tolerant: able to respond and adapt to disturbances and perturbations in its environment as defined by Eakins (2018)), and proportion of resilient species (resilient: able to recover and double its population within <1.4 years following exploitation as defined by Eakins (2018)) were analysed using permutation linear mixed effects models (PLMM; n = 5000 iterations) with gear type and season as main effects, and sampling period within each season and sampling site as random effects (lme4 and predictmeans packages; Bates et al., 2015; Luo et al., 2020). Overall morphological differences (total length and body mass) between fish caught in each gear type were analysed in a similar manner, while including fish species as a random effect. Tukey's HSD post-hoc tests were used to identify significant pairwise differences between each gear type within a season. Fish community compositional differences among gear types and between seasons were visualised using unconstrained principal coordinate analysis (PCoA) biplots performed on Bray-Curtis dissimilarity matrix, with 80 % confidence ellipses overlaid on top of each gear type (Oksanen et al., 2019). Differences in gear type and seasonal community compositions were further analysed using permutation ANOVAs with 5000 permutations (Vegan package; Oksanen et al., 2019). Fish communities were further examined using similarity percentages (SIMPER) analysis to determine which species were driving compositional differences within each gear type. Permutation tests (n = 5000) were used to identify gear type significant differences in CPUE of these characteristic species within each season. Although all species were included in the model, only those that contributed > 5% to the total abundance were analysed. Indicator species analysis (ISA) was used to examine which species were indicative of each gear type within each season. Indicator species values (ISVs), ranging from 0 (absent from all samples) to 1 (present in all samples within gear type), were generated to determine which species are considered "true" indicators and are consistently present within a certain gear type. Permutation tests (n = 5000) were also carried out to determine which species were significant indicators. Species accumulation curves (SACs) were used to determine gear type efficiency and measured if the sample size (number of sampling sites) was large enough to adequately characterise the communities caught within each gear type during each season (McCune and Grace, 2002). Each sampling effort (site) in our SACs was conducted on the species richness found in 10 minnow traps, two Windermere traps, or two electrofishing transects. SACs were generated for each gear type within each season from random permutations of the data (n = 5000) to determine the average number of new species found and standard deviations for each increase in sampling effort (specaccum function, Vegan package; Oksanen et al., 2019). SACs, for each individual gear type and for all gear types combined, were compared by calculating the initial slope (averaged across efforts 1 through 5). We also compared the amount of effort needed for each gear type when used individually versus in combination to reach a cut-off of >1% of new species discovered per effort standardised by the maximum amount of potential species that can be caught using each method. SACs were based only on sampling events when all three gear types were employed. Data are reported as means ± standard error (SE) unless otherwise stated, and in all analyses, α was set to 0.05.

4.0 Results

4.1 Abundance, richness, and diversity

Across all sites and sampling events, we caught 4226 fish (3658 in the summer and 568 in the winter) composed of 27 unique species (Table 1; Fig. 1). Minnow traps captured 706 fish (506 in the summer and 200 in winter) composed of 20 species. Windermere traps captured 1258 fish (1128 in the summer and 130 in winter) composed of 23 species. Electrofishing captured 2268 fish (2024 in the summer and 244 in winter) composed of 26 species.

Overall, fish abundance (number of fish) was greatly reduced in the winter compared to the summer (PLMM, $t = -3.28$, $p < 0.01$; Fig. 2A). In the summer, fish abundance was not significantly different across gear types (CPUE, PLMM, $F = 0.04$, $p = 0.41$; Fig. 2A). However, during the winter, gear type played a critical role in determining the number of fish caught (PLMM, $F = 12.35$, $p < 0.001$), with minnow traps more successful at capturing fish than either Windermere traps

($t = 3.84$, $p < 0.001$; Fig. 2A) or electrofishing ($t = 4.78$, $p < 0.001$; Fig. 2A). Windermere traps and electrofishing were equally successful at capturing fish during the winter ($t = 0.36$, $p = 0.93$). Biomass did not differ between seasons (PLMM, $t = 1.47$, $p = 0.12$) nor across gear types (PLMM, $F = 0.08$, $p = 0.27$).

Similar to abundance, species richness and species diversity (Shannon-Weiner Index) were greatly reduced during the winter compared to the summer (PLMM, t(Richness) = -4.44, $p < 0.001$; t(Diversity) = -3.99, $p < 0.01$; Fig. 2B; Fig. 2C). In the summer, species richness and species diversity varied significantly by gear type (PLMM, F(Richness) = 14.89, $p < 0.001$; F(Diversity) = 12.16, $p < 0.001$; Fig. 2B; Fig. 2C), with catches by minnow traps being considerably less species rich and less species diverse than catches from Windermere traps (t(Richness) = -3.72, $p < 0.001$; t(Diversity) = -3.58, $p < 0.01$; Fig. 2B; Fig. 2C) and electrofishing (t(Richness) = -5.39, $p < 0.001$; t(Diversity) = -4.81, $p < 0.001$; Fig. 2B; Fig. 2C). During the summer, the catch yielded by Windermere traps and electrofishing did not vary significantly with respect to species richness or species diversity (t(Richness) = 1.20, $p = 0.46$; t(Diversity) = 0.78, $p = 0.72$). In contrast to the summer, species richness and species diversity did not differ across gear types during the winter (PLMM, F(Richness) = 0.71, $p = 0.46$; F(Diversity) = 0.42, $p = 0.63$; Fig. 2B; Fig. 2C).

4.2 Gear type species discovery rate

As sampling effort (number of sites) increased, the species accumulation curves for all gear types individually and cumulatively (all three gear types together) began to reach the asymptote in both summer and winter. This indicated that all gear types were able to adequately sample their respective fish communities, while also demonstrating significant differences in the total number of species that can potentially be caught by each gear type (Fig. 3). The species encounter rate as sampling effort increased was highest when all gear types were used in combination, followed by electrofishing alone, Windermere traps alone, and then minnow traps alone. This pattern was observed during both the summer and winter. Additionally, the initial species discovery slope (averaged across efforts 1 through 5) was greatest when all gear types were used in combination (2.22(Summer), 2.30(Winter)), followed by electrofishing alone (2.12(Summer), 1.87(Winter)), Windermere traps alone (1.91(Summer), 1.26(Winter)), and then minnow traps alone (1.19(Summer), 1.07(Winter)) in both seasons. In the summer, the effort needed to reach an increase of <1% in species richness was overall higher in the winter than in the summer, with an average effort of ~26 sites needed in the winter compared to ~22 sites in the summer. In the summer, the effort needed to reach an increase of <1% in species richness was 26 sites for minnow traps alone, 18 sites for Windermere traps alone, 28 sites for electrofishing alone, and 14 sites for when all gear types were used in combination. In the winter, the effort needed to reach an increase of <1% in species richness was >30 sites for minnow traps alone, 29 sites for Windermere traps alone, 23 sites for electrofishing alone, and 19 sites for when all gear types were used in combination.

4.3 Fish species characteristics and morphology

The proportion of benthic to benthopelagic and pelagic fishes caught was similar between seasons (PLMM, $t = 0.93$, $p = 0.86$; Fig. 4A). In both summer and winter, gear type significantly influenced the proportion of benthic fishes caught (PLMM, F(Summer) = 19.63, $p < 0.001$); F(Winter) = 15.10, $p < 0.001$; Fig. 4A). In both seasons, minnow trap catches consisted of a greater proportion of benthic fishes compared to catches using Windermere traps (t(Summer) = 5.01, $p < 0.001$; t(Winter) = 3.02, $p < 0.01$) and electrofishing (t(Summer) = 5.91, $p < 0.001$; t(Winter) = 5.48, $p < 0.001$). In the summer, the proportion of benthic fishes caught using Windermere traps did not differ from that of electrofishing ($t = 0.20$, $p = 0.98$), while in the winter, Windermere traps tended to catch more benthic fishes than electrofishing ($t = 2.25$, $p = 0.07$).

The proportion of non-native fishes did not differ between seasons (PLMM, $t = 1.03$, $p = 0.14$; Fig. 4B). During the summer, the proportion of non-native fishes was influenced by gear type (PLMM, $F = 13.35$, $p < 0.001$; Fig. 4B), with a higher proportion of non-native fishes caught by minnow traps compared to those caught using both Windermere traps ($t = 3.68$, $p < 0.01$) and electrofishing ($t = 5.05$, $p < 0.001$). Windermere traps and electrofishing did not differ in the proportion of non-native fishes caught during the summer ($t = 0.87$, $p = 0.66$). In the winter, the proportion of non-native fishes caught did not differ across all three gear types (PLMM, $F = 2.01$, $p = 0.14$; Fig. 4B).

The proportion of tolerant to intermediate and intolerant fishes caught did not differ between seasons (PLMM, $t = 0.10$, $p = 0.93$; Fig. 4C). However, gear type significantly influenced the proportion of tolerant species caught during both seasons (PLMM, $F(\text{Summer}) = 21.94$, $p < 0.001$; $F(\text{Winter}) = 16.09$, $p < 0.001$; Fig. 4C). Fishes caught using electrofishing were generally more tolerant than those caught in Windermere traps ($t(\text{Summer}) = 3.73$, $p < 0.001$; $t(\text{Winter}) = 3.70$, $p = 0.001$) and minnow traps ($t(\text{Summer}) = 6.41$, $p < 0.001$; $t(\text{Winter}) = 5.37$, $p < 0.001$) in both seasons. Windermere traps caught more tolerant fishes than minnow traps, but only significantly so during the summer ($t(\text{Summer}) = 2.37$, $p = 0.049$; $t(\text{Winter}) = 1.64$, $p = 0.24$).

The proportion of highly resilient fishes to those of medium or low resilience was not significantly affected by season (PLMM, $t = 0.23$, $p = 0.63$; Fig. 4D) nor gear type (PLMM, $F = 0.72$, $p = 0.49$; Fig. 4D); however, a significant interaction was detected, indicating gear type differences in the degree of resilient species caught were seasonally-dependent (PLMM, $F = 6.49$, $p = 0.002$; Fig. 4D). During the summer, more resilient species were caught using Windermere traps than via electrofishing ($t = 3.75$, $p = 0.001$). Similarly, Windermere traps tended to catch more resilient fishes than minnow traps ($t = 2.28$, $p = 0.06$). The proportion of resilient fishes caught using either minnow traps or electrofishing was similar

($t = 1.14$, $p = 0.49$). In the winter, the proportion of resilient fishes caught did not differ across gear types (PLMM, $F = 2.03$, $p = 0.14$; Fig. 4D).

To assess gear type size selectivity, we compared the differences in body size (estimated by total length in mm) of the most commonly caught species across our three gear types. These commonly caught species included: brown bullhead, goldfish, white sucker, green sunfish, pumpkinseed sunfish, bluegill sunfish, white perch, round goby, yellow perch, common logperch, and fathead minnow; see Supplementary Table 5. Overall, fish caught in the winter were larger than fish caught in the summer (PLMM, $t = 7.42$, $p < 0.001$). Additionally, average body size of fish caught depended on which gear type was utilised (PLMM, $F = 10.86$, $p < 0.001$). During the summer, electrofishing caught larger fish than both minnow traps ($t = 2.33$, $p = 0.05$) and Windermere traps ($t = 3.78$, $p < 0.001$). In contrast, fish caught in minnow traps and Windermere traps were of similar sizes ($t = 0.74$, $p = 0.74$). During the winter, electrofishing caught larger fish than minnow traps ($t = 4.89$, $p < 0.001$) but not Windermere traps ($t = 1.82$, $p = 0.17$). Furthermore, Windermere traps caught fish of larger sizes than minnow traps ($t = 3.11$, $p < 0.01$). See Supplementary Table 7 for a detailed breakdown of body size by species. Similarly, body mass of the most commonly caught fishes was greater in the winter than in the summer (PLMM, $t = 5.25$, $p < 0.001$). Additionally, body mass of fish varied significantly based on which gear type they were caught in (PLMM, $F = 15.2$, $p < 0.001$). During both seasons, minnow traps selected for fishes with smaller body mass than electrofishing (t(Summer) = -2.41, $p = 0.04$; t(Winter) = -2.41,

$p = 0.04$) but not Windermere traps ($t(\text{Summer}) = -1.81$, $p = 0.17$; $t(\text{Winter}) = -1.75$, $p = 0.19$). However, fish caught in Windermere traps and electrofishing did not differ significantly in their body mass in either season ($t(\text{Summer}) = 0{,}62$ $p = 0.81$; $t(\text{Winter}) = 1.48$, $p = 0.31$).

4.4 Community composition

The composition of fish communities differed significantly between seasons (PERMANOVA, $F(\text{Season}) = 4.33$, $p < 0.001$; Fig. 5) and across gear types (PERMANOVA, $F(\text{Gear type}) = 6.94$, $p < 0.001$; Fig. 5). During both seasons, fish communities caught in minnow traps appeared to be most dissimilar from fish communities caught using electrofishing (PCOA; Fig. 5). Similarity percentage analysis revealed that differences in key species contributing to the overall dissimilarity across gear types were as follows during the summer: minnow traps were more successful at catching brook stickleback than were Windermere traps, and minnow traps also caught more round goby than electrofishing; Windermere traps were more successful at catching rudd compared to electrofishing. During the winter: minnow traps caught more round goby and brown bullhead than Windermere traps and electrofishing (Table 2).

In both seasons, fish communities caught within each gear type were identifiable by the presence of a number of indicator species (see Supplementary Table 5). In the summer, fish communities caught using minnow traps were identifiable by only round goby; Windermere trap fish communities were identifiable by brook stickleback, rudd, and spottail shiner; electrofishing fish communities were only identifiable by gizzard shad. In the winter, minnow trap fish communities were once again identifiable by round goby; electrofishing fish communities were identifiable by smallmouth bass; no indicator species were detected for fish communities caught using Windermere traps.

Table 1. Fish species characteristics and abundances from all sampling events. The native vs. non-native status of each species in Ontario (native/non-native) is based on the Ontario Ministry of Natural Resources and Forestry (2015) criteria. Tolerance describes a species ability to respond and adapt to disturbances and perturbations in its environment following Eakins (2018). Resilience describes a species recovering capacity and its doubling time following exploitation (low > 4 years, medium 1.4–4 years, high < 1.4 years; Froese and Pauly, 2020). Habitat is based on where in the water column each species is typically found (Eakins, 2018). Abundance catch data is the number of individuals caught of each species using each of the three gear types (minnow traps, Windermere traps, and electrofishing) in summer and winter (summer | winter). Catch abundance data are cumulatively represented from all sampling events within each season.

		Species Characteristics				**Catch Abundance**		
Species	**Family**	**Native/Non-native**	**Tolerance**	**Resilience**	**Habitat**	**Minnow Traps**	**Windermere Traps**	**Electrofishing**
Ambloplites rupestris (Rock bass)	Centrarchidae	Native	Intermediate	Medium	Benthopelagic	0 | 0	0 | 6	0 | 0
Ameiurus nebulosus (Brown bullhead)	Ictaluridae	Native	Intermediate	Medium	Benthic	34 | 37	71 | 9	192 | 13
Amia calva (Bowfin)	Amiidae	Native	Intermediate	Low	Benthopelagic	1 | 0	0 | 0	1 | 13
Carassius auratus (Goldfish)	Cyprinidae	Non-native	Tolerant	Medium	Benthopelagic	25 | 0	42 | 1	280 | 5
Catostomus commersonii (White sucker)	Catostomidae	Native	Tolerant	Low	Benthic	5 | 2	27 | 0	146 | 13
Culaea inconstans (Brook stickleback)	Gasterosteidae	Native	Intermediate	High	Benthopelagic	6 | 6	40 | 0	3 | 5
Cyprinus carpio (Common carp)	Cyprinidae	Non-native	Tolerant	Medium	Benthopelagic	1 | 0	1 | 0	8 | 16
Dorosoma cepedianum (Gizzard shad)	Clupeidae	Non-native	Tolerant	Medium	Pelagic	2 | 3	0 | 19	166 | 6
Esox lucius (Northern pike)	Esocidae	Native	Intermediate	Low	Benthopelagic	0 | 0	3 | 0	14 | 3
Labidesthes sicculus (Brook silverside)	Atherinopsidae	Native	Intermediate	High	Pelagic	0 | 0	0 | 0	1 | 2
Lepisosteus osseus (Longnose gar)	Lepisosteidae	Native	Tolerant	Low	Benthopelagic	0 | 0	0 | 0	1 | 0
Lepomis cyanellus (Green sunfish)	Centrarchidae	Native	Tolerant	Medium	Benthopelagic	19 | 26	77 | 18	161 | 75
Lepomis gibbosus (Pumpkinseed sunfish)	Centrarchidae	Native	Intermediate	Medium	Benthopelagic	5 | 6	33 | 9	67 | 5
Lepomis macrochirus (Bluegill sunfish)	Centrarchidae	Native	Intermediate	Medium	Benthopelagic	50 | 15	330 | 19	283 | 10
Luxilus cornutus (Common shiner)	Cyprinidae	Native	Intermediate	Medium	Benthopelagic	0 | 0	8 | 2	38 | 0
Micropterus salmoides (Largemouth bass)	Centrarchidae	Native	Tolerant	Low	Benthopelagic	3 | 0	32 | 0	105 | 15
Morone americana (White perch)	Moronidae	Non-native	Intermediate	Low	Benthopelagic	38 | 0	82 | 2	4 | 0
Neogobius melanostomus (Round goby)	Gobiidae	Non-native	Intermediate	Medium	Benthic	283 | 84	61 | 20	46 | 2
Notropis atherinoides (Emerald shiner)	Cyprinidae	Native	Intermediate	High	Benthopelagic	0 | 2	9 | 2	10 | 5
Notropis hudsonius (Spottail shiner)	Cyprinidae	Native	Intermediate	Medium	Benthopelagic	3 | 5	148 | 2	18 | 3
Perca flavescens (Yellow perch)	Percidae	Native	Intermediate	Medium	Benthopelagic	14 | 3	8 | 7	65 | 13
Percina caprodes (Common logperch)	Percidae	Native	Intolerant	Medium	Benthic	14 | 1	10 | 1	9 | 12
Pimephales notatus (Bluntnose minnow)	Cyprinidae	Native	Intermediate	Medium	Benthopelagic	1 | 1	8 | 1	56 | 3
Pimephales promelas (Fathead minnow)	Cyprinidae	Native	Tolerant	High	Benthopelagic	2 | 8	74 | 7	282 | 15

Pomoxis nigromaculatus (Black crappie)	Centrarchidae	Native	Tolerant	Medium	Benthopelagic	0 | 0	2 | 0	10 | 1
Scardinius erythrophthalmus (Rudd)	Cyprinidae	Non-native	Tolerant	Low	Benthopelagic	0 | 1	62 | 5	53 | 9
Semolitus atromaculatus (Creek chub)	Cyprinidae	Native	Intermediate	Medium	Benthopelagic	0 | 0	0 | 0	5 | 0

Table 2. Similarity percentages (SIMPER) analysis showing the contribution of key species to the overall dissimilarity between different gear types. Average A and Average B represent the gear-standardised catch per unit effort (abundance) for each species of the pair of gear types being compared. Only species that contributed $\geq$ 5% to the overall abundance are shown. Bolded averages indicate significant differences ($p < 0.05$).

	Summer					Winter				
Comparison	**Total dissimilarity**	**Species**	**Average A**	**Average B**	**Contribution**	**Total dissimilarity**	**Species**	**Average A**	**Average B**	**Contribution**
A. Minnow Traps B. Windermere Traps	90.16%	*Lepomis macrochirus* (Bluegill sunfish)	0.13	0.48	10.8	90.45%	*Neogobius melanostomus* (Round goby)	**0.37**	**0.04**	**23.47**
		Culaea inconstans (Brook stickleback)	**0.02**	**0.06**	**6.93**		*Ameiurus nebulosus* (Brown bullhead)	**0.15**	**0.02**	**14.07**
		Lepomis cyanellus (Green sunfish)	0.05	0.11	6.71		*Lepomis cyanellus* (Green sunfish)	0.11	0.04	10.59
							Lepomis macrochirus (Bluegill sunfish)	0.06	0.04	9.04
							Pimephales promelas (Fathead minnow)	0.03	0.02	5.26
A. Minnow Traps B. Electrofishing	91.93%	*Neogobius melanostomus* (Round goby)	**0.73**	**0.04**	**19.39**	94.73%	*Neogobius melanostomus* (Round goby)	**0.37**	**0.002**	**20.12**
		Lepomis macrochirus (Bluegill sunfish)	0.13	0.22	10.85		*Lepomis cyanellus* (Green sunfish)	0.1	0.09	14.07
		Carassius auratus (Goldfish)	0.06	0.17	8.1		*Ameiurus nebulosus* (Brown bullhead)	**0.15**	**0.02**	**13.37**
		Lepomis cyanellus (Green sunfish)	0.05	0.13	7.6		*Lepomis macrochirus* (Bluegill sunfish)	0.06	0.02	6.41
		Ameiurus nebulosus (Brown bullhead)	0.06	0.14	7.49		*Pimephales promelas* (Fathead minnow)	0.03	0.02	5.42
		Catostomus commersonii (White sucker)	0.01	0.13	6.15					
		Pimephales promelas (Fathead minnow)	0.005	0.25	5.9					
A. Windermere Traps B. Electrofishing	89.51%	*Lepomis macrochirus* (Bluegill sunfish)	0.48	0.22	12.03	91.63%	*Lepomis cyanellus* (Green sunfish)	0.04	0.09	17.35
		Lepomis cyanellus (Green sunfish)	0.11	0.13	9.02		*Neogobius melanostomus* (Round goby)	0.04	0.002	9.09
		Carassius auratus (Goldfish)	0.06	0.17	7.59		*Lepomis macrochirus* (Bluegill sunfish)	0.04	0.02	8
		Ameiurus nebulosus (Brown bullhead)	0.1	0.14	7.55		*Perca flavescens* (Yellow perch)	0.02	0.02	6.71
		Pimephales promelas (Fathead minnow)	0.11	0.25	7.03		*Pimephales promelas* (Fathead minnow)	0.02	0.02	6
		Catostomus commersonii (White sucker)	0.04	0.13	6.35		*Ameiurus nebulosus* (Brown bullhead)	0.02	0.02	5.83
		Scardinius erythrophthalmus (Rudd)	**0.09**	**0.05**	**5.7**					
		Micropterus salmoides (Largemouth bass)	0.05	0.09	5.1					

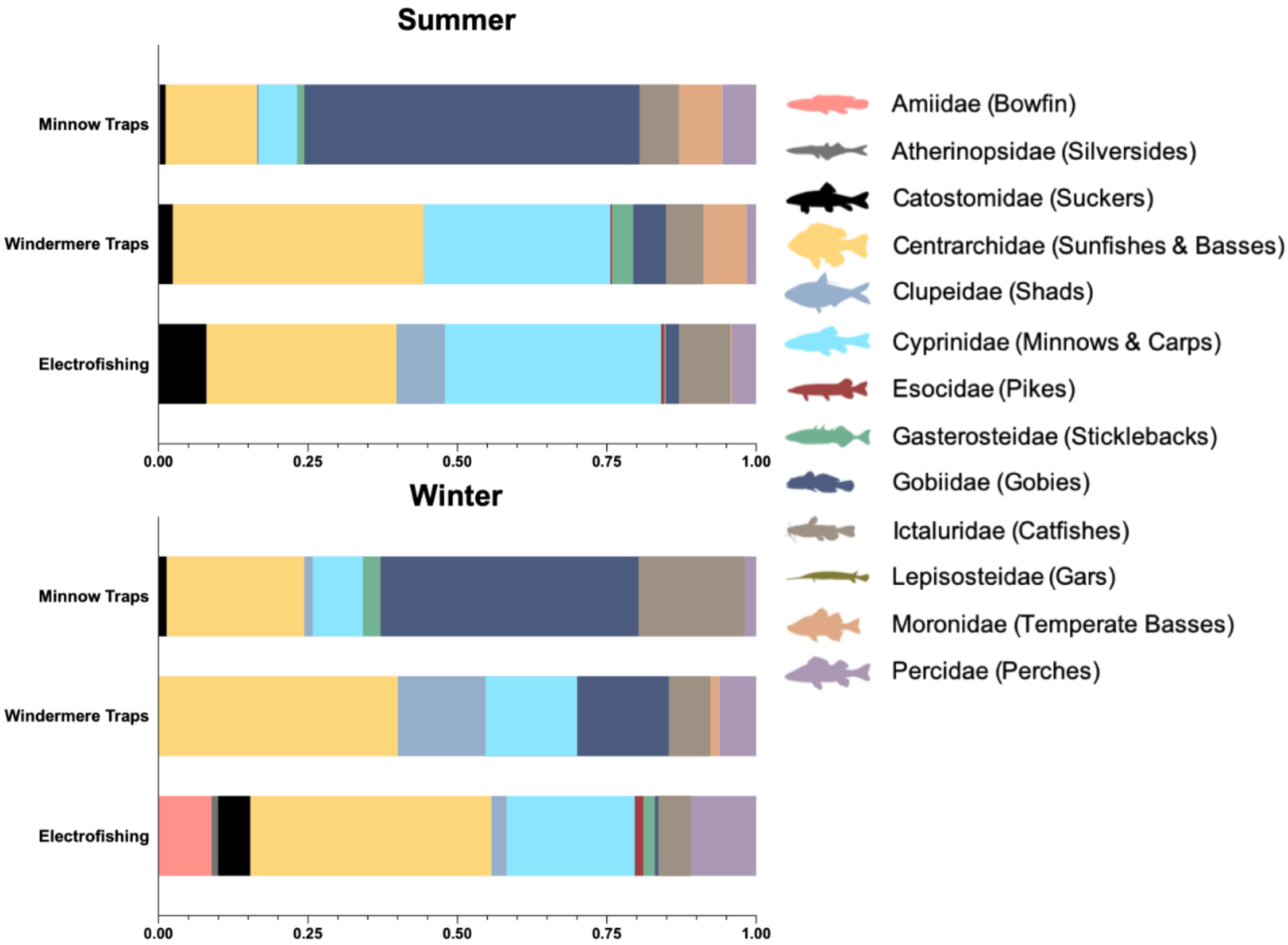
Summer
Minnow Traps
Windermere Traps
Electrofishing
0.00
0.25
0.50
0.75
1.00
Winter
Minnow Traps
Windermere Traps
Electrofishing
0.00
0.25
0.50
0.75
1.00
Amiidae (Bowfin)
Atherinopsidae (Silversides)
Catostomidae (Suckers)
Centrarchidae (Sunfishes & Basses)
Clupeidae (Shads)
Cyprinidae (Minnows & Carps)
Esocidae (Pikes)
Gasterosteidae (Sticklebacks)
Gobiidae (Gobies)
Ictaluridae (Catfishes)
Lepisosteidae (Gars)
Moronidae (Temperate Basses)
Percidae (Perches)

Figure 1. Fish family composition broken down by season and gear type. Proportions based on gear-standardised catch per unit effort of all sampling events within each season. See Table 1 for species within each family group and see Supplementary Fig. 2 for fish species composition within each gear type and season.

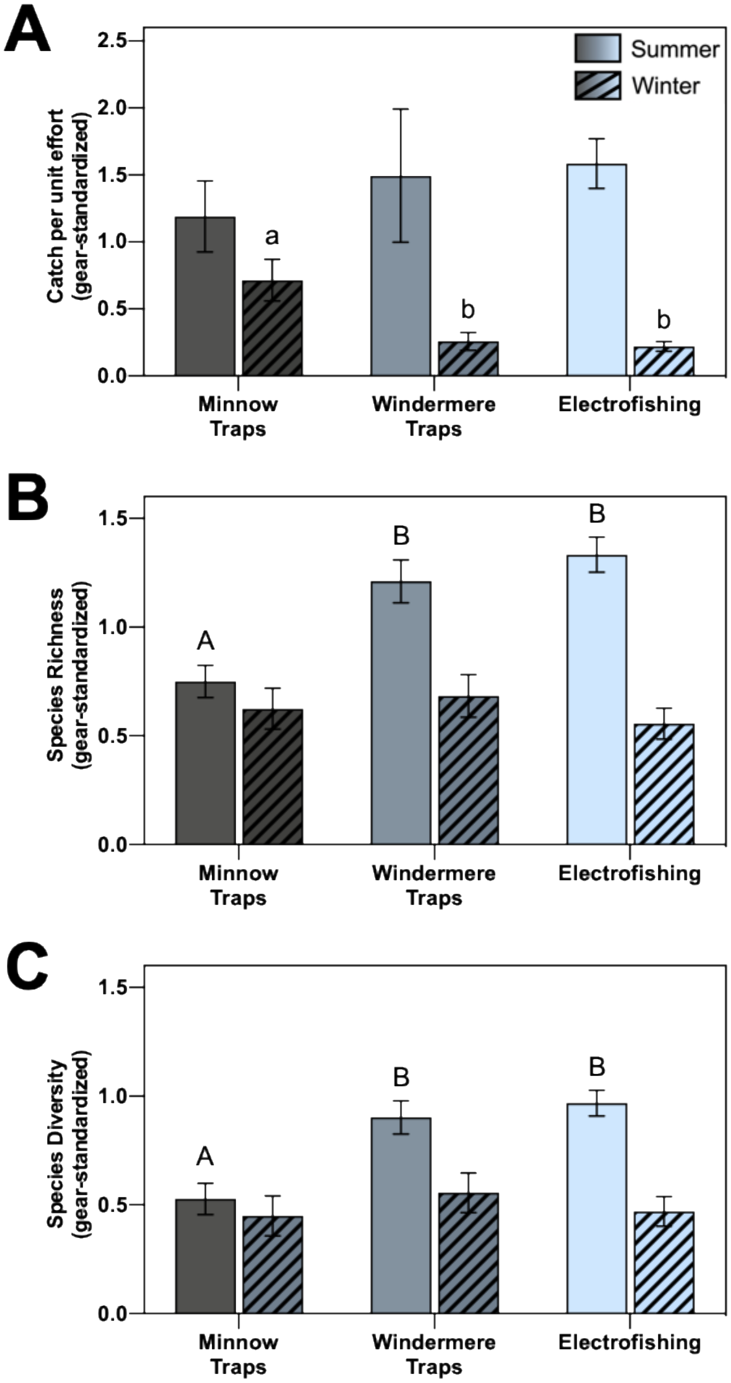

Figure 2. Mean (± SE) gear-standardised **(A)** abundance, **(B)** species richness, **(C)** species diversity of fish caught using minnow traps, Windermere traps, and electrofishing in the summer (solid) and winter (hatched). Different uppercase letters indicate significant pairwise differences between gear types in the summer, while different lowercase letters indicate significant pairwise differences between gear types in the winter.

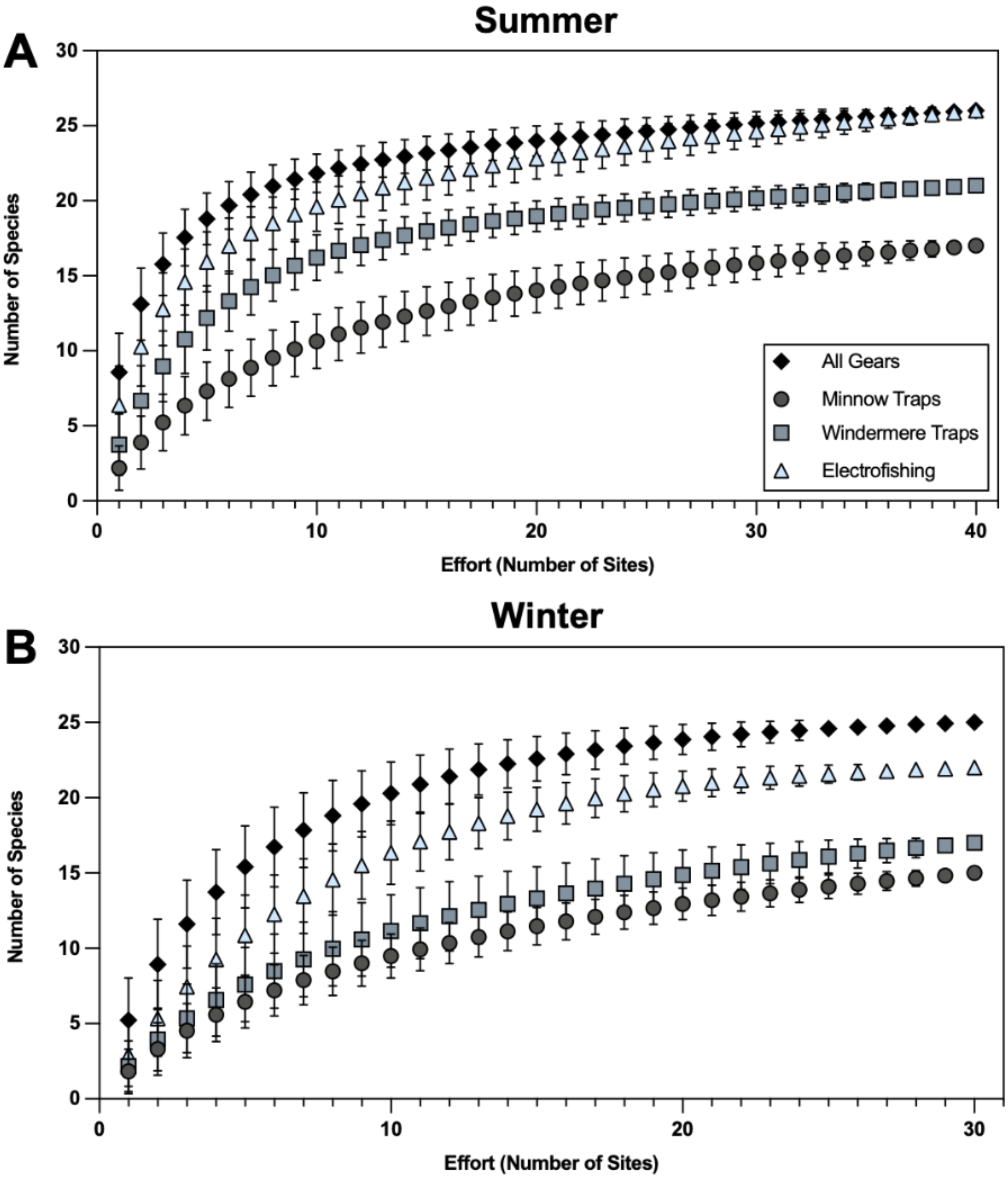

Figure 3. Species accumulation curves for all gears combined and each gear type individually in the **(A)** summer and **(B)** winter in all sampling sites across different events. Each data point represents the average richness achieved for each level of effort ± standard deviation. The efforts needed to reach ≤1% change in species richness in each gear type within each season are: minnow traps ($26_{\text{(Summer)}}$, $>30_{\text{(Winter)}}$); Windermere traps ($18_{\text{(Summer)}}$, $29_{\text{(Winter)}}$); electrofishing ($28_{\text{(Summer)}}$, $23_{\text{(Winter)}}$); all gears ($14_{\text{(Summer)}}$, $19_{\text{(Winter)}}$).

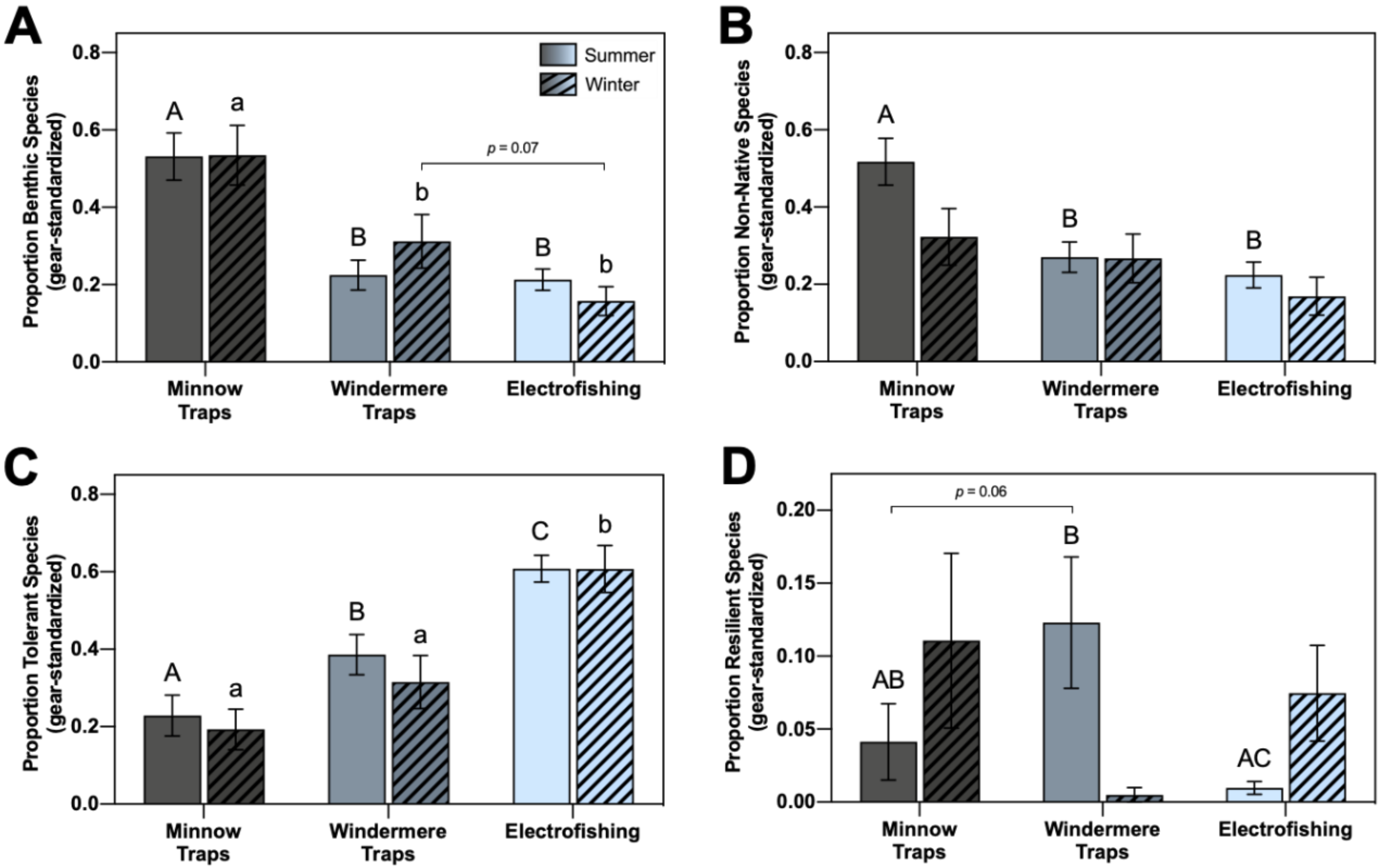

Figure 4. Mean (± SE) gear-standardised **(A)** proportion of benthic species, **(B)** proportion of non-native species, **(C)** proportion of tolerant species, and **(D)** proportion of resilient species of fish caught using minnow traps, Windermere traps, and electrofishing in the summer and winter. Different uppercase letters indicate significant pairwise differences between gear types in the summer. Different lowercase letters indicate significant pairwise differences between gear types in the winter.

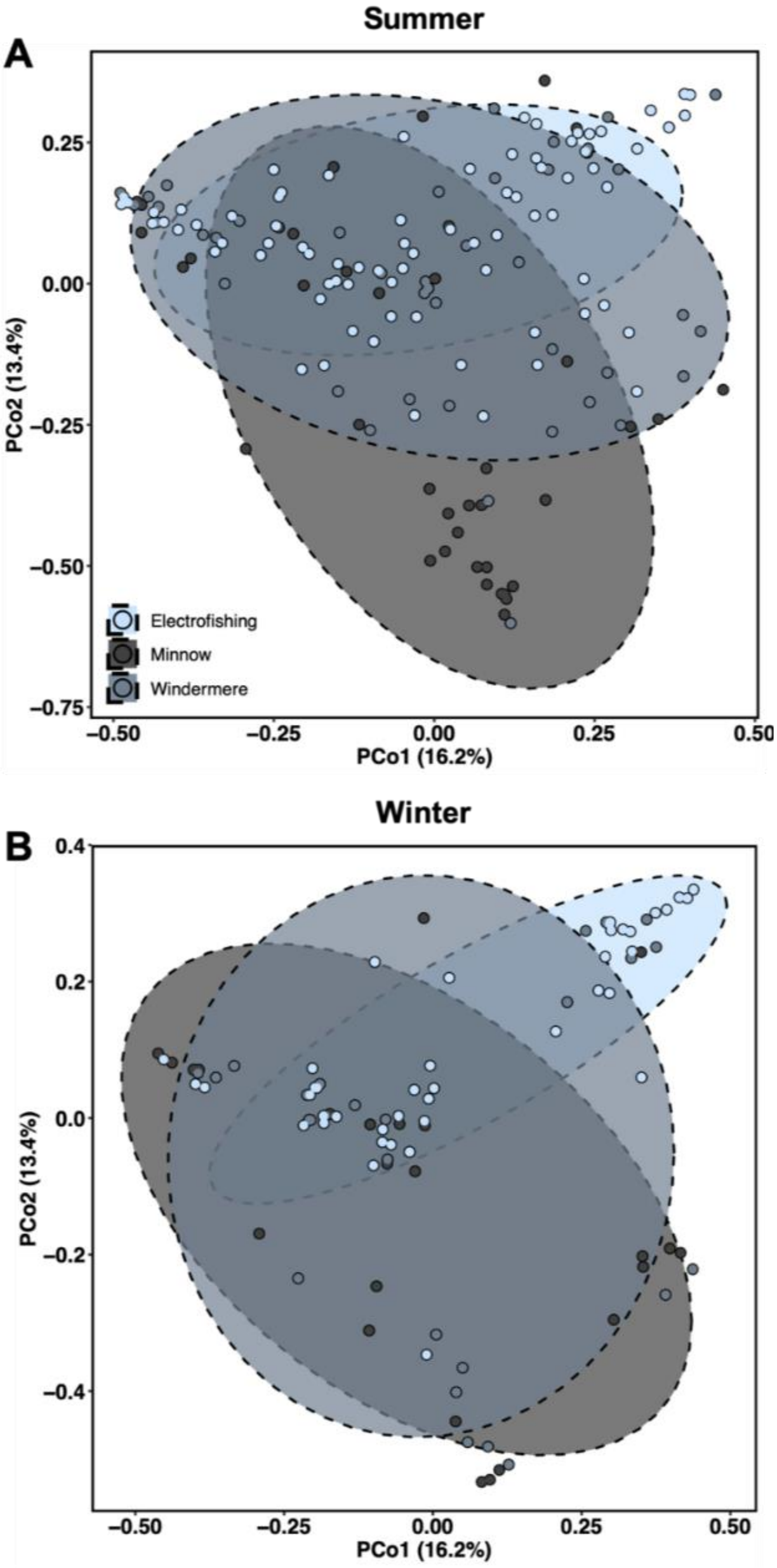

Figure 5. Principal coordinate analysis (PCoA) ordination output of fish community compositions with 80% confidence ellipses overlaid on each gear type in the **(A)** summer and **(B)** winter.

5.0 Discussion

Our study explored how certain gear types (minnow traps, Windermere traps, and electrofishing), when used in shallow aquatic systems, differed in their ability to catch fish, their efficiency, and their selectivity, with a specific focus on how these parameters are further modulated by seasonality. We found that minnow traps were more successful at catching fish (per unit effort) in comparison to Windermere traps and electrofishing, but only during the winter. This was contrary to our predictions, as we expected active gear type (e.g., electrofishing) to be more successful at catching fish, especially during the winter, when fish are generally less active and therefore the rate at which traps are encountered would presumably be lowest (Hurst, 2007; McMeans et al., 2020). The increased capture success of minnow traps during the winter may be due to more fish occupying lower depths (i.e., beyond the range of electrofishing from a boat), perhaps even burrowing under substrate during colder months of the year. As such, fish during the winter may encounter benthic-oriented sampling gear types (e.g., minnow traps) more frequently than pelagic-oriented ones (e.g., electrofishing). Additionally, electrofishing is more successful at catching fish closer to the surface as opposed to lower depths, where current lines attenuate and get more dispersed—possibly not reaching fish that concentrate at lower depths during the winter (SFCC, 2007; Larocque et al., 2020). Likewise, benthic fishes may be more likely to escape an electrofishing shock by burrowing into the substrate, perhaps making their relatively low abundance in our electrofishing samples less surprising.

Moreover, responses of fish to an electrofishing shock are modified by temperature, where fish are less responsive in the cold, making their capture by electrofishing more difficult than during the summer (SFCC, 2007). Furthermore, the addition of bait (food) in our passive gear types may have contributed to the increased capture success of fish in minnow traps during the winter, as food is often scarce and limited during the winter (Shuter et al., 2012; Speers-Roesch et al., 2018).

Unlike abundance, the richness and diversity of species we collected were highest in electrofishing hauls, although this was only the case during the summer. Minnow traps and Windermere traps are typically considered to be more selective than electrofishing, as they are both closer to the substrate, limited by the size of their entry holes, and are mostly encountered and entered by small-bodied mobile fishes (Weaver et al., 1993). In our study, electrofishing often selected for larger fishes and fish with a wider range of body sizes than when using either minnow traps or Windermere traps (see Supplementary Table 4). The entry hole size in minnow traps and Windermere traps results in size-selectivity, thereby limiting fishes of larger body sizes (e.g., common carp, northern pike, and longnose gar) from being caught. In support of our results, previous studies have also demonstrated that species richness and species diversity (Shannon-Weiner Index) are often higher in active gear types (e.g., electrofishing and seining) as they are less selective and are therefore able to catch a wider variety of species compared to passive gear types (e.g., minnow traps and Windermere traps; Lapointe et al., 2006).

As predicted, fish community assemblages differed significantly across gear types and between seasons. In both seasons, fish communities caught using minnow traps were most dissimilar to those caught using electrofishing. This was expected as minnow traps are often highly selective for benthic and benthopelagic small-bodied fishes, while electrofishing is less selective overall (Weaver et al., 1993). Minnow trap catches were dominated by benthic species (~53 %) during both seasons in comparison to Windermere trap and electrofishing catches. Additionally, minnow traps were more successful at catching invasive species (a category mostly dominated by round goby). The higher proportion of invasive fishes caught in minnow traps could be because our study sites are highly abundant with round goby (McCallum et al., 2019; Mehdi et al., 2021), a benthic invasive species that was rarely caught in our electrofishing samples. A number of previous studies have used minnow traps to track and monitor the spread and persistence of invasive species, such as round goby (Young et al., 2010; McCallum et al., 2014; Bose et al., 2018; McCallum et al., 2018). Conversely, a study investigating the effects of sampling techniques on round goby population assessments found improved round goby catchability using electrofishing compared to minnow traps (Brandner et al., 2013). It is however important to note that the minnow trap deployment period used by Brandner et al. (2013) was only 20 min long, while in our study, traps were left out for 24 h, suggesting that deployment time length plays a significant role in catch success. Additionally, round goby are nocturnal feeders; hence, overnight deployment is likely to yield higher catch success than daytime deployment (Johnson et al., 2008). Moreover, deeper waters (>1.5 m) and impaired water clarity will both act to reduce the efficacy of electrofishing in catching benthic fishes (e.g., round goby and brown bullhead) since these individuals cannot be seen by the netter. Differences in species characteristics, study design, and overall composition reported here highlight the importance of using multiple gear types in fish community and population assessments, as usage of a single gear type might result in under- or overestimation of certain species, possibly leading to inaccurate community and/or population estimates (Portt et al., 2006; Pope et al., 2010). This may be especially critical in studies monitoring species of concern (e.g., invasive species or endangered species), where inaccurate population estimates can be consequential for the management decisions they are meant to inform.

While using multiple gear types seems ideal when conducting fish community and population assessments, it is worth noting that employing multiple gear types can be time consuming and labour- and cost-intensive, especially during the winter, when working conditions are often sub-optimal. In our study, we found that when all three gear types were combined, the species discovery rate was highest, implying that more unique species are captured and a more holistic and accurate view of the community can be achieved when multiple gear types are used simultaneously. This was further highlighted as each gear type demonstrated targeted selectivity for certain species. In addition, gear type species selectivity differed between seasons, indicating that the species selectivity of different gear types can be modulated by seasonal changes in biotic and abiotic factors. We found that a number of species were more likely to be caught by certain gear type(s) over others. For example, minnow traps were effective at capturing round goby in both the summer and winter. Similarly, Windermere traps were specialised at capturing spottail shiner, brook stickleback, and rudd during the summer; in the winter, no species were exclusively captured by Windermere traps. Electrofishing was the optimal gear type for capturing gizzard shad during the summer and largemouth bass during the winter. Such differences in gear type selectivity further highlight the importance of using multiple gear types in fish community assessments, as several species in our study were caught almost exclusively by one gear type. Additionally, the seasonal changes in selectivity shed light on the importance of incorporating fish life history traits and the interactions between biotic and abiotic factors when considering which gear type(s) to use in fish population and community surveys conducted across seasons.

Overall, our study demonstrated that minnow traps, Windermere traps, and electrofishing differ considerably in their catchability, selectivity, and efficiency. We clearly demonstrate how these parameters may be modulated by seasonality, a variable rarely considered in gear type selection research. In the summer, abundance did not vary among gear types, whereas in the winter, minnow traps captured the most fish per unit effort. Fish communities caught using electrofishing were the most species rich and species diverse, but this pattern was only apparent during summer sampling. Furthermore, we observed a high degree of species selectivity within each gear type, where fish communities differed significantly from one another, depending on which gear type was used. In addition, gear types differed in key species caught within each season. Understanding and accurately assessing fish populations and communities during winter is an important yet challenging task. Our findings contribute to the recent focus on characterising seasonal differences (Larocque et al., 2020; McMeans et al., 2020; Mehdi et al., 2021), which is especially important since our current knowledge on winter ecology in higher latitudinal regions is lacking, partly due to the challenges associated with field sampling during that time of year. In conclusion, we recommend the usage of multiple gear types; specifically, a combination of active and passive gears. A combination gear type approach would allow researchers to gain a more holistic and accurate view of the fish community or population surveyed, especially if surveys are conducted across seasons, where gear type selectivity can change drastically.

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Supplementary Materials for Appendix A: A comparison of passive and active gear in fish community assessments in summer versus winter

Supplementary Figure 1: Map of sampling sites

Supplementary Figure 2: Fish species composition broken down by season and gear type

Supplementary Table 1: Dates and fish sampling details for each sampling event

Supplementary Table 2: Sampling site water quality parameters

Supplementary Table 3: Habitat characteristics of the sampling sites

Supplementary Table 4: Morphological differences of the most common fishes caught using all gear types

Supplementary Table 5: Results of indicator Species Analysis (ISA)

Figure S1. Maps of sampling sites in **(A)** West Hamilton Harbour and **(B)** East Hamilton Harbour. Maps generated in Google Earth Pro 7.3.2.5776, imagery date 06/30/2018 and accessed on 24/02/2020.

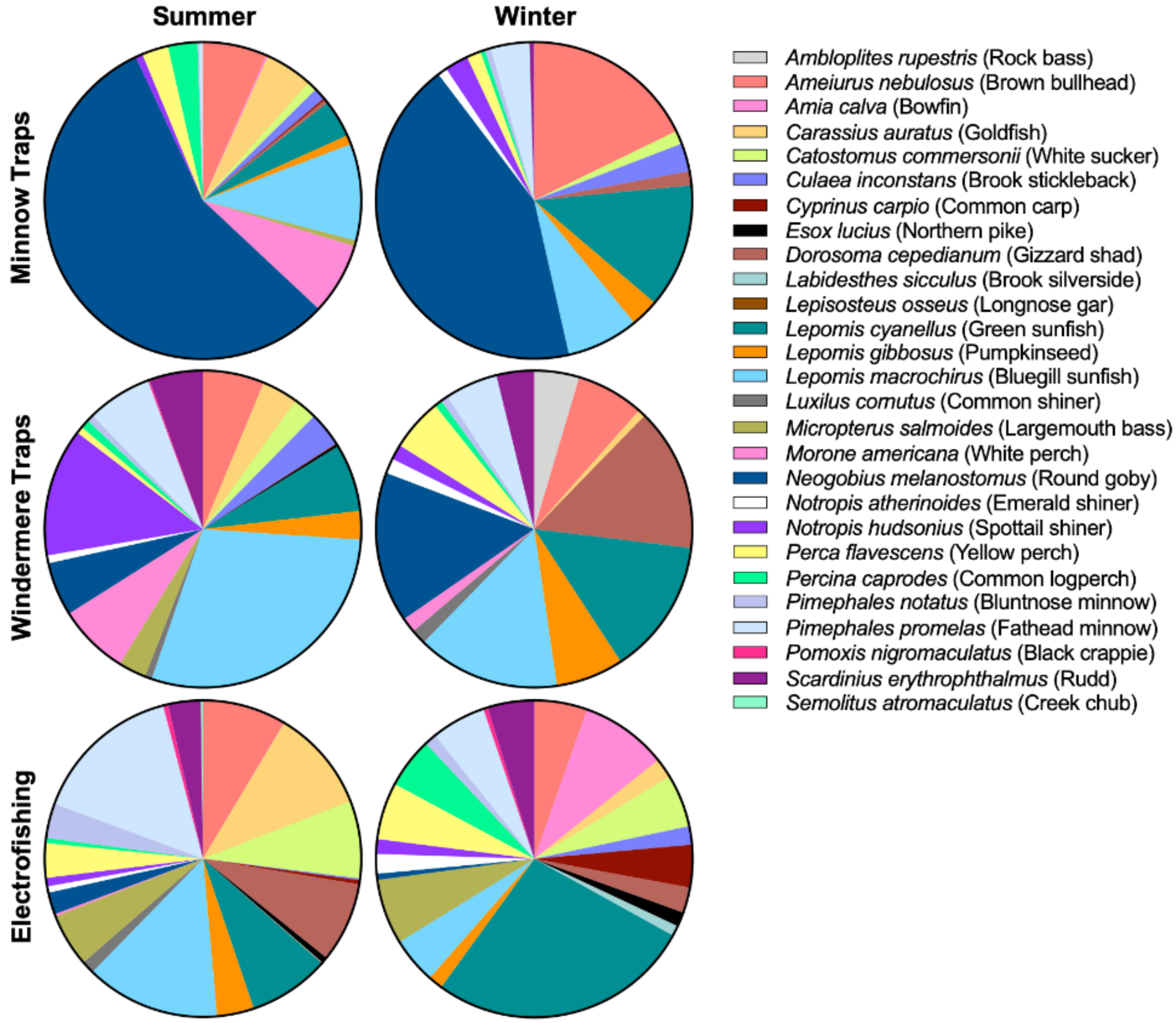

Figure S2. Fish species composition broken down by season and gear type. Proportions based on gear-standardised catch per unit effort of all sampling events in summer and winter.

Table S1. Dates of each field event. Number of minnow traps and Windermere traps deployed, and the average number of electrofishing shock-seconds used at each sampling event.

Date	Minnow traps	Windermere traps	Electrofishing
July 7, 2018	50 traps	10 traps	168 sec / 7-8 A / 141-212 V
July 17, 2018	49 traps	10 traps	151 sec / 8-9 A / 110-211 V
August 14, 2018	50 traps	10 taps	143 / 6-8 A / 177-212 V
August 21, 2018	49 traps	10 traps	NA
August 22, 2018	45 traps	9 traps	138 sec / 6 A / 177-247 V
March 26, 2019	47 traps	10 traps	211 sec / 5-6.5 A / 106-282 V
March 29, 2019	50 traps	10 traps	210 sec / 6 A / 141 V
July 16, 2019	50 traps	10 traps	229 sec / 6 A / 141 V
July 23, 2019	50 traps	10 traps	136 sec / 6 A / 141 V
August 2019	50 traps	10 traps	147 sec / 6 A / 141 V
August 2019	50 traps	10 traps	181 sec / 7 A / 177 V
November 2019	49 traps	10 traps	130 sec / 6 A / 177-247 V
December 2019	50 traps	10 traps	171 sec / 6 A / 177 V
December 2019	48 traps	10 traps	171 sec / 6 A / 141-177 V
December 2019	50 traps	10 traps	215 sec / 6 A / 141-177 V

Table S3. Habitat characteristics of the sampling sites in Hamilton Harbour following a subset of the habitat metrics used in the Qualitative Habitat Evaluation Index (QHEI; Taft and Koncelik, 2006; Strickland et al. 2010). Aquatic vegetation was only recorded during the summer as it was absent during the winter. Total depth and clarity are reported as means ± standard error for summer | winter. Habitat characteristics have been previously published in McCallum et al. (2019). Sites represented by their coordinates and named 1 – 5 (West Hamilton Harbour) and 6 – 10 (East Hamilton Harbour).

	West HH Site 1 (43.266492, -79.942999)	**West HH Site 2 (43.268398, -79.936095)**	**West HH Site 3 (43.270933, -79.928665)**	**West HH Site 4 (43.277102, -79.911483)**	**West HH Site 5 (43.271681, -79.906462)**
Aquatic habitat					
Bottom substrate (% coverage)	85% cobble 15% boulder	90% sand 10% gravel	100% silt	100% silt	100% silt
Bottom slope	> 45°	> 25°	< 15°	< 15°	< 15°
Shoreline habitat					
Sinuosity	None, straight	None, straight	Low, 1 curve	Low, 1 curve	Low, 1 curve
Anthropogenic modifications	Recent, recovering	Recent, recovering	None	None	None
Shoreline quality	Urban, industrial, residential trail	Urban, industrial, residential trail	Forest, wetland	Forest, wetland	Forest, wetland
Bank erosion	Moderate (25-50%)	Moderate (25-50%)	Low (0-25 %)	Low (0-25 %)	Low (0-25 %)
Riparian width	Narrow (5-10 m)	Moderate (10-50 m)	Wide (>50 m)	Wide (>50 m)	Wide (>50 m)
Additional measures					
Total depth (cm) Summer | Winter	189±51.7 | 164±1.73	190±59.7 | 142±18.9	81.3±34.5 | 46.7±2.88	99.3±21.0 | 73.3±15.3	96.3±41.1 | 46.7±2.88
Clarity (%) Summer | Winter	45.0±13.6 | 92.5±7.57	34.8±9.33 | 79.2±18.1	70.7±25.8 | 100±0	50.1±15.0 | 100±0	29.6±2.26 | 82.6±10.9
Aquatic vegetation	Algae, lesser/greater duckweed, milfoil	Filamentous algae, lesser/greater duckweed, milfoil	Filamentous algae, lesser/greater duckweed, milfoil, water lily	Lesser/greater duckweed, liverwort, water lily, potamogeton	Lesser/greater duckweed, milfoil, water lily
Site comments	Human-made canal, erosion-prevention logs, bank built of cobble	Human-made canal, undercut banks, overhanging *phragmites*	Natural site, cattail stands between site and shore	Fencing near water flowering rush between site and shore	Natural site, cattail stands between site and shore

Table S3 (continued)

	East HH Site 6 (43.251786, -79.764808)	**East HH Site 7 (43.257001, -79.768042)**	**East HH Site 8 (43.263196, -79.773479)**	**East HH Site 9 (43.262828, -79.775787)**	**East HH Site 10 (43.266645, -79.778757)**
Aquatic habitat					
Bottom substrate (% coverage)	50% cobble 50% silt	50% cobble 50% silt	100% cobble	50% cobble 50% boulder	50% cobble 50% boulder
Bottom slope	≥ 45°	≥ 45°	≥ 45°	≥ 45°	≥ 25°
Shoreline habitat					
Sinuosity	None-straight	None-straight	Low	Low-moderate	Low
Anthropogenic modifications	Recent, recovering	Recent, recovering	Recent, recovering	Recent	Recent
Shoreline quality	Urban, industrial	Urban, industrial	Urban, industrial	Urban, industrial	Urban, industrial
Bank erosion	Low	Low	Low	Low	Low
Riparian width	Narrow (5-10 m)	Narrow (5-10 m)	Narrow (5-10 m)	Very narrow (<5 m)	Very narrow (<5 m)
Additional measures					
Total depth (cm) Summer | Winter	138±24.0 | 78.3±10.1	131±19.7 | 98.3±6.66	210±25.5 | 192±6.0	215±15.7 | 155±23.6	210±29.2 | 142±13.0
Clarity (%) Summer | Winter	35.2±12.1 | 66.7±16.7	34.6±8.62 | 60.5±5.59	58.0±8.77 | 63.2±3.85	52.2±10.9 | 76.1±10.3	45.3±1.47 | 66.1±17.4
Aquatic vegetation	Lesser duckweed, water smartweed	Lesser duckweed, water smartweed	Algae on rocks, pondweed	Algae on rocks, Canada waterweed, lesser/greater duckweed, smartweed	Floating pondweed, lesser duckweed, pondweed, smartweed
Site comments	Near large highway, modified shoreline	Near large highway, cemented shoreline	Near large highway, modified shoreline	Modified shoreline, bank shaping	Modified shoreline, bank shaping

Table S4. Mean ± SE and range (min – max) of total length (TL) and body mass of the most common fishes caught using all gear types (minnow traps, MT; Windermere traps, WT; electrofishing, EF). Only measured individuals and not those batch-weighed were analysed (see methods section).

	Gear Type	**TL (mm)** **Body Mass (g)**	**Sample Size (n)**
Ameiurus nebulosus (Brown bullhead)	MT	55.3 ± 4.28 (34.0 - 135) | 63.3 ± 1.71 (49.8 - 87.0) 3.41 ± 1.07 (0.420 – 27.0) | 3.21 ± 0.259 (1.59 – 7.91)	33 | 30
	WT	66.6 ± 8.38 (26.3 - 370.) | 102 ± 15.2 (62.6 - 189) 30.6 ± 12.6 (0.330 – 648) | 25.1 ± 12.1 (2.76 – 151)	71 | 13
	EF	78.5 ± 5.2 (17.5 - 230.) | 99.3 ± 7.04 (68.0 - 129) 15.2 ± 2.86 (0.074 – 162) | 12.2 ± 2.07 (3.41 – 22.8)	102 | 12
Carassius auratus (Goldfish)	MT	57.8 ± 3.32 (31.8 - 94.0) | - 3.59 ± 0.701 (0.450 – 13.2) | -	25 | -
	WT	58.5 ± 3.65 (29.8 - 120.) | - 4.69 ± 1.04 (0.450 – 26.5) | -	42 | -
	EF	66.2 ± 2.45 (15.8 - 320.) | 238 ± 45.53 (114 - 320.) 9.40 ± 3.46 (0.091 – 497) | -	147 | 5
Catostomus commersonii (White sucker)	MT	70.4 ± 7.57 (52.0 - 92.4) | 146 ± 1.5 (144 - 147) 3.73 ± 1.13 (1.28 – 7.24) | 28.0 ± 0.450 (27.5 – 28.4)	5 | 2
	WT	58.6 ± 4.31 (30.1 - 135) | - 3.55 ± 1.58 (0.226 – 41.2) | -	26 | -
	EF	109 ± 4.94 (19.0 - 340.) | 140. ± 13.2 (80.1 - 211) 27.7 ± 3.86 (0.020 – 448) | 33.9 ± 8.07 (5.18 – 81.9)	144 | 13
Lepomis cyanellus (Green sunfish)	MT	53.0 ± 3.48 (38.6 - 109) | 51.4 ± 3.18 (9.70 - 102) 3.30 ± 1.21 (0.941 – 24.8) | 3.74 ± 0.95 (0.774 – 20.4)	19 | 26
	WT	63.5 ± 3.1 (24.8 - 139) | 88.7 ± 10.8 (30.6 - 156) 7.57 ± 1.25 (0.220 – 54.4) | 24.1 ± 6.25 (0.412 – 79.1)	78 | 18
	EF	71.7 ± 2.29 (18.5 - 149) | 95.1 ± 3.47 (33.0 - 146) 10.9 ± 0.950 (0.050 – 79.3) | 21.6 ± 2.06 (0.210 – 64.3)	159 | 70
Lepomis gibbosus (Pumpkinseed sunfish)	MT	62.8 ± 10.72 (39.4 - 105) | 47.5 ± 0.920 (45.1 - 50.7) 6.05 ± 3.97 (1.64 – 21.9) | 1.53 ± 0.118 (1.13 – 1.85)	5 | 6
	WT	59.1 ± 3.89 (39.1 - 141) | 102.7 ± 10.0 (48.4 - 129) 5.01 ± 1.32 (0.950 – 32.4) | 27.0 ± 5.88 (1.83 – 50.8)	32 | 9
	EF	72.6 ± 3.42 (36.7 - 150.) | 72.4 ± 11.8 (47.7 - 117) 10.1 ± 1.44 (0.836 – 44.8) | 10.4 ± 6.24 (1.84 – 35.2)	65 | 5
Lepomis macrochirus (Bluegill sunfish)	MT	37.0 ± 1.16 (5.80 - 51.0) | 43.1 ± 1.70 (32.2 - 52.0) 0.985 ± 0.083 (0.13 – 2.72) | 1.24 ± 0.162 (0.370 – 2.25)	50 | 15
	WT	39.0 ± 2.66 (20.8 - 165) | 74.9 ± 8.65 (30.0 - 135) 3.11 ± 1.30 (0.093 – 83.8) | 10.7 ± 3.64 (0.220 – 48.9)	82 | 16
	EF	44.9 ± 2.50 (16.2 - 162) | 56.4 ± 13.4 (4.70 - 169) 5.96 ± 1.31 (0.040 – 91.1) | 11.5 ± 9.67 (0.682 – 98.5)	163 | 10
Morone americana (White perch)	MT	71.2 ± 0.950 (60.5 - 76.2) | - 4.74 ± 0.210 (2.67 – 6.27) | -	19 | -
	WT	87.9 ± 7.86 (42.9 - 200.) | 107 ± 8.1 (99.0 - 115) 16.3 ± 6.00 (0.904 – 120.) | 17.8 ± 6.80 (11.0 – 24.6)	25 | 2
	EF	127 ± 34.6 (68.8 - 210.) | - 57.8 ± 37.6 (4.26 – 164) } -	4 | -
Neogobius melanostomus (Round goby)	MT	78.8 ± 1.34 (9.50 - 140.9) | 86.5 ± 2.93 (30.7 - 132) 7.29 ± 0.328 (0.810 – 21.6) | 11.9 ± 1.18 (0.270 – 37.9)	182 | 69
	WT	56.4 ± 3.15 (18.0 - 107) | 56.5 ± 3.11 (32.5 - 95.0) 3.62 ± 0.543 (0.030 – 16.2) | 2.58 ± 0.495 (0.378 – 10.6)	61 | 23
	EF	70.1 ± 3.48 (20.8 - 117) | 66.5 ± 3.50 (63.0 - 70.0) 6.28 ± 0.870 (0.070 – 31.1) | 4.00 ± 0.700 (3.30 – 4.70)	46 | 2

Table S4. Continued

Perca flavescens (Yellow perch)	MT	70.0 ± 6.94 (45.0 - 152) | 73.1 ± 12.6 (48.4 - 90.1) 5.21 ± 2.40 (1.21 – 36.0) | 4.55 ± 1.71 (1.40 – 7.30)	14 | 3
	WT	74.1 ± 10.5 (50.3 - 143) | 95.5 ± 11.4 (69.9 - 152) 4.93 ± 2.20 (1.20 – 20.0) | 10.6 ± 4.22 (2.73 – 33.6)	8 | 7
	EF	71.1 ± 3.37 (47.9 - 174) | 114 ± 12.9 (71.3 - 180.) 5.62 ± 1.46 (0.714 – 65.0) | 22.4 ± 8.12 (2.74 – 75.8)	51 | 10
Percina caprodes (Common logperch)	MT	69.4 ± 1.65 (55.1 - 81.0) | - 2.59 ± 0.207 (1.40 – 4.60) | -	13 | -
	WT	72.4 ± 2.75 (59.7 - 91.2) | - 2.95 ± 0.579 (1.50 – 7.64) | -	10 | -
	EF	59.5 ± 3.05 (39.6 - 74.4) | 76.5 ± 3.39 (63.3 - 100.) 1.85 ± 0.206 (0.940 – 3.25) | 4.20 ± 0.788 (1.57 – 9.80)	13 | 12
Pimephales promelas (Fathead minnow)	MT	53.2 ± 2.55 (50.6 - 55.7) | 59.6 ± 2.01 (49.5 - 69.7) 1.54 ± 0.010 (1.53 – 1.55) | 1.99 ± 0.232 (0.760 – 2.90)	2 | 8
	WT	48.3 ± 1.31 (31.6 - 59.0) | 50.1 ± 1.07 (47.8 - 56.0) 1.34 ± 0.161 (0.040 – 5.61) | 1.04 ± 0.115 (0.770 – 1.70)	34 | 7
	EF	32.1 ± 0.770 (14.5 - 63.5) | 46.4 ± 2.19 (35.3 - 62.0) 0.423 ± 0.038 (0.009 – 2.38) – 0.873 ± 0.174 (0.340 – 2.20)	152 | 14

Table S5. Fish species determined as indicators of each gear type (minnow traps, MT; Windermere traps, WT; electrofishing, EF) and their respective indicator species values (ISV) as per Indicator Species Analysis. Bolded ISVs and gear types resemble a significant ($p < 0.05$) indicator species for that gear type.

	Summer		**Winter**	
	Indicator Species Value (ISV)	**Indicator gear type(s)**	**Indicator Species Value (ISV)**	**Indicator gear type(s)**
Ambloplites rupestris (Rock bass)	-	-	0.27	WT
Ameiurus nebulosus (Brown bullhead)	0.51	MT, WT, EF	**0.54**	**MT**
Amia calva (Bowfin)	0.14	MT	0.31	EF
Carassius auratus (Goldfish)	0.54	MT, WT, EF	0.27	WT, EF
Catostomus commersonii (White sucker)	0.57	WT, EF	0.39	MT, EF
Culaea inconstans (Brook stickleback)	**0.43**	**WT**	0.3	MT, EF
Cyprinus carpio (Common carp)	0.26	MT, EF	0.31	EF
Dorosoma cepedianum (Gizzard shad)	**0.33**	**EF**	0.27	MT, WT, EF
Esox lucius (Northern pike)	0.30	WT, EF	0.27	EF
Labidesthes sicculus (Brook silverside)	0.11	EF	0.16	EF
Lepisosteus osseus (Longnose gar)	0.11	EF	-	-
Lepomis cyanellus (Green sunfish)	0.66	MT, WT, EF	0.6	MT, WT, EF
Lepomis gibbosus (Pumpkinseed sunfish)	0.48	WT, EF	0.4	MT, WT
Lepomis macrochirus (Bluegill sunfish)	0.58	MT, WT, EF	0.52	MT, WT
Luxilus cornutus (Common shiner)	0.29	WT, EF	0.27	WT
Micropterus salmoides (Largemouth bass)	0.54	WT, EF	**0.35**	**EF**
Morone americana (White perch)	0.31	MT, WT	0.27	WT
Neogobius melanostomus (Round goby)	**0.71**	**MT**	**0.65**	**MT**
Notropis atherinoides (Emerald shiner)	0.20	WT, EF	0.25	MT, WT, EF
Notropis hudsonius (Spottail shiner)	**0.47**	**WT**	0.29	MT, WT, EF
Perca flavescens (Yellow perch)	0.43	MT, WT, EF	0.36	MT, WT, EF
Percina caprodes (Common logperch)	0.37	MT, WT	0.35	EF
Pimephales notatus (Bluntnose minnow)	0.32	EF	0.21	MT, WT, EF
Pimephales promelas (Fathead minnow)	0.52	WT, EF	0.39	MT, WT, EF
Pomoxis nigromaculatus (Black crappie)	0.16	WT, EF	0.16	EF
Scardinius erythrophthalmus (Rudd)	**0.48**	**WT**	0.34	WT, EF
Semolitus atromaculatus (Creek chub)	0.11	EF	-	-

Appendix B: The impacts of wastewater effluent on fish, benthic macroinvertebrates, and zooplankton biomass in summer and winter

1. Introduction and motivation

In this section of my thesis, I explored how fish biomass and benthic macroinvertebrate biomass are influenced by proximity to wastewater outfalls in summer and winter. This study will allow us to examine the amount of energy (biomass) moving between trophic levels within the food webs found in effluent-receiving environments. It will also allow us to examine community responses across different trophic levels using biomass as our indicator of interest rather than abundance. Finally, by examining the biomass ratio between predator communities (fishes) and prey communities (benthic macroinvertebrates and zooplankton), we can determine the efficiency of energy transfer across trophic levels and potentially identify indicators of concern if energy transfer lacks the efficiency found in undisturbed ecosystems. Such data could prove useful for understanding how wastewater outfalls support the high abundance of fishes, benthic macroinvertebrates, and zooplankton in both summer and winter. The results presented below are still preliminary and are not ready for proper analyses.

2. Methods

Fish biomass was based on body mass measurements taken of fish collected in the field. Briefly, after fishes were identified to species level, they were measured (total and standard lengths) and weighed (0.01 g). This was only done on the first 15 individuals of a given species, sampling technique (minnow traps, Windermere traps, and electrofishing), and site. The remaining fish caught were batch-weighed. Total biomass of all individuals caught on a sampling date were combined by mean-standardizing the effort of the sampling technique used. Benthic macroinvertebrate biomass was calculated by measuring all the individuals of each family within a site, season, and rock basket using a fine analytical scale (0.001 g). The sum biomass of each family within each site and season was then calculated and analyzed.

Preliminary statistical analyses were performed on the biomass data collected using linear models with proximity to the outfall and season being the two predictor variables.

3. Results:

3.1 Fish biomass

At the Dundas WWTP (Fig. 1A), fish biomass did not vary between seasons (lm, $t_{(1,31;\ \mathrm{Season})} = 0.43$, $p = 0.67$). Similarly, biomass was not influenced by proximity to the outfall in either season (lm, $t_{(1,18;\ \mathrm{Summer})} = 1.29$, $p = 0.21$; $t_{(1,13;\ \mathrm{Winter})} = 0.04$, $p = 0.97$). At the Woodward WWTP (Fig. 1B), fish biomass was ~16% higher in winter than in summer (lm, $t_{(1,41;\ \mathrm{Season})} = 2.68$, $p = 0.01$). Biomass decreased with distance from the outfall during winter (lm, $t_{(1,24;\ \mathrm{Winter})} = -2.35$, $p = 0.04$) but not during summer (lm, $t_{(1,24;\ \mathrm{Summer})} = 1.21$, $p = 0.24$).

3.2 Benthic macroinvertebrate biomass

At the Dundas WWTP (Fig. 3A), benthic macroinvertebrate biomass did not vary between seasons (lm, $t_{(1,40;\ \text{Season})} = 0.46$, $p = 0.65$). Biomass increased with distance from the outfall in winter (lm, $t_{(1,24;\ \text{Winter})} = 5.47$, $p < 0.001$) but not in summer (lm, $t_{(1,24;\ \text{Summer})} = 1.21$, $p = 0.24$). At the Woodward WWTP (Fig. 3B), benthic macroinvertebrate biomass was ~29% higher in summer than in winter (lm, $t_{(1,41;\ \text{Season})} = 2.65$, $p = 0.01$). Biomass tended to increase with distance from the outfall in summer (lm, $t_{(1,24;\ \text{Summer})} = 2.00$, $p = 0.06$)and significantly decrease with distance from the outfall in winter (lm, $t_{(1,24;\ \text{Winter})} = -2.85$, $p = 0.01$).

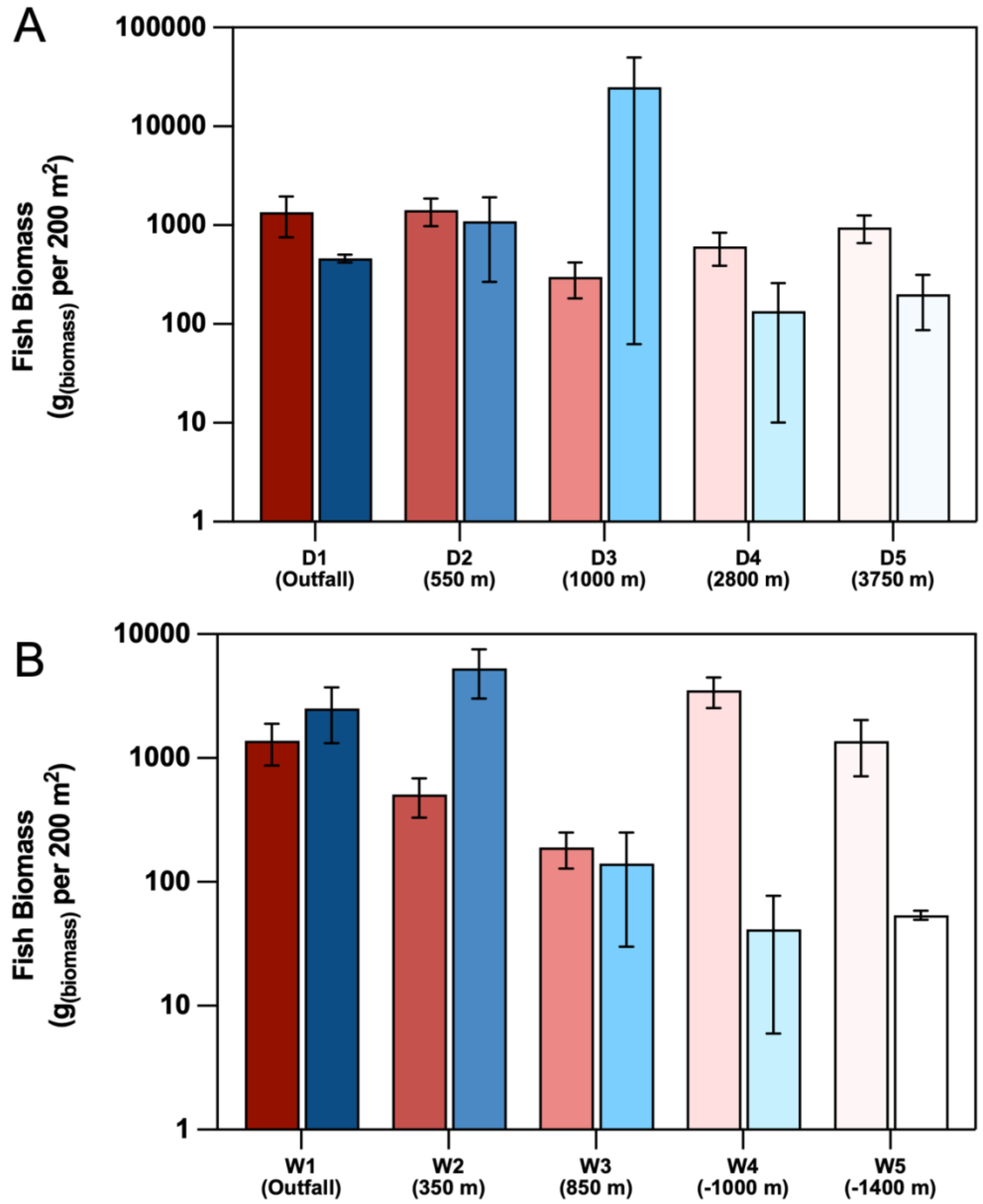

Figure 1. Mean ± SEM fish biomass (on a log scale) for the **(A)** Dundas and **(B)** Woodward WWTPs in summer and winter. Biomass calculated as the average weight of all fish collected using minnow traps, Windermere traps, and electrofishing in a 200 m^2 transect per site. In all figures, summer data are represented in red and winter data are represented in blue; the intensity of the colours signify the proximity to the outfall (darker colours being closest and light colours being most distant). All data are presented as per basket (N = 3-4 samples/site).

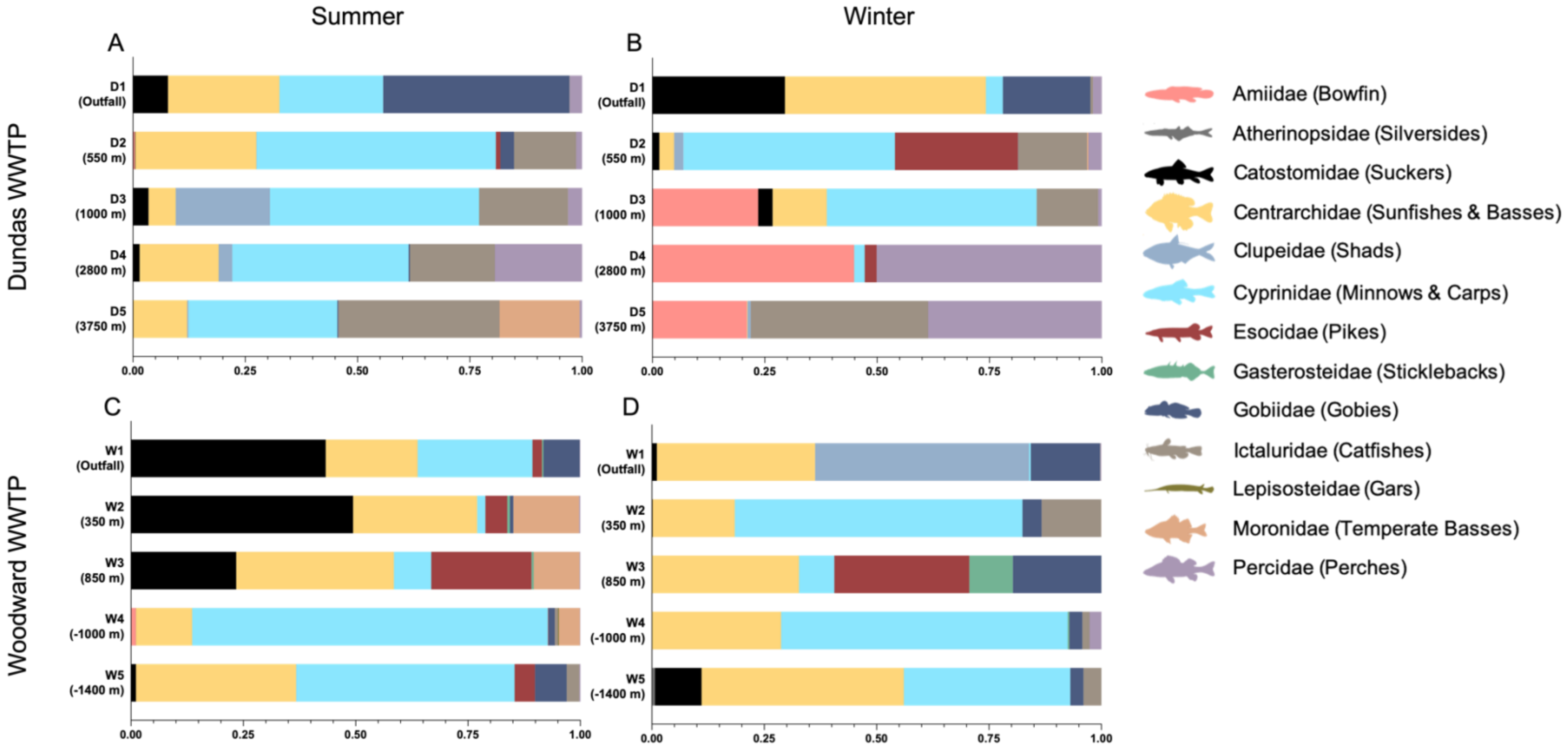

Figure 2. Proportions of fish families from the Dundas WWTP sampling sites in summer **(A)** and winter **(B)**, and from the Woodward WWTP sampling sites in summer **(C)** and winter **(D)**. Proportions based on mean gear-standardized biomass catch from all sampling gears across a 200 m^2 transect of all sampling events within each season.

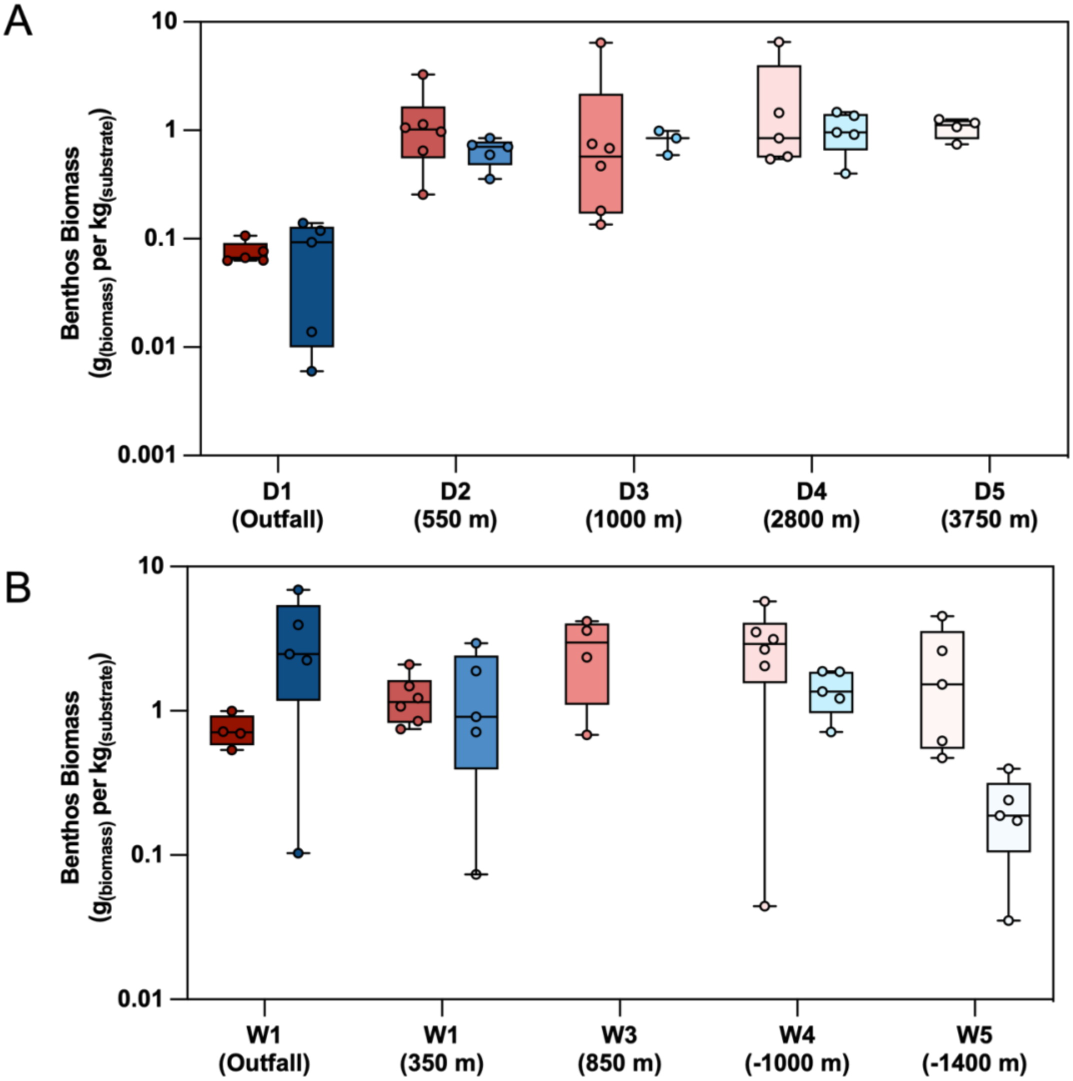

Figure 3. Benthic macroinvertebrate biomass (on a log scale) for the **(A)** Dundas and **(B)** Woodward WWTPs in summer and winter. Biomass calculated as the average weight of all benthic macroinvertebrates collected per kg of substrate deployed. Box plots show the median and inter-quartile range, whiskers show minimum and maximum values, and individual data points are jittered to improve visualization. In all figures, summer data are represented in red and winter data are represented in blue; the intensity of the colours signify the proximity to the outfall (darker colours being closest and light colours being most distant). All data are presented as per basket (N = 3-6 samples/site).

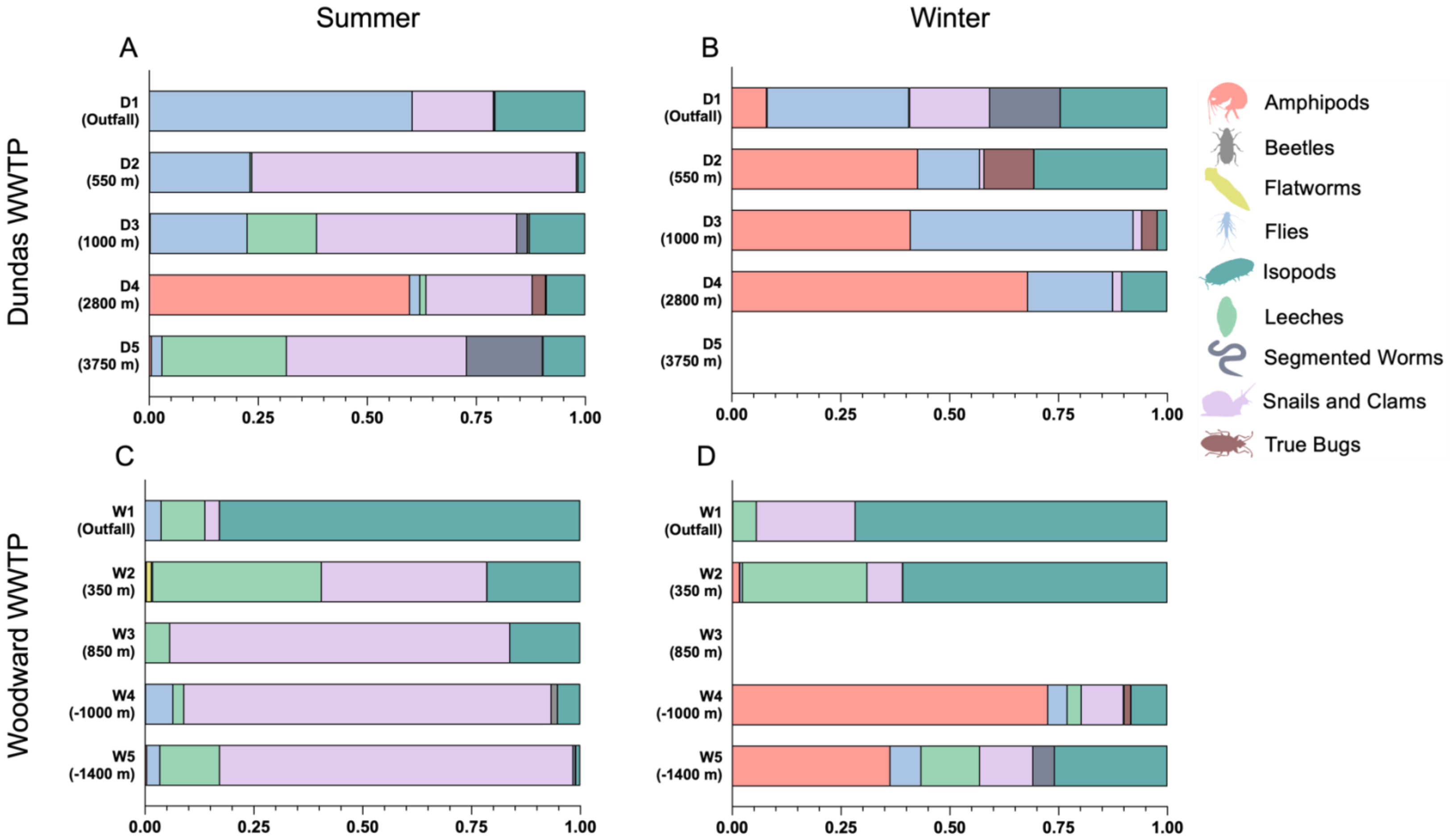

Figure 4. Proportions of different types of benthic macroinvertebrates from the Dundas WWTP sampling sites in summer **(A)** and winter **(B)**, and from the Woodward WWTP sampling sites in summer **(C)** and winter **(D)**. Proportions based on total biomass of macroinvertebrates within each site and season (all basket replicates combined per site).

Table 1. Fish species characteristics and abundances from all sampling events. The native vs. non-native status of each species in Ontario (native/non-native) is based on the Ontario Ministry of Natural Resources and Forestry (2015) criteria. Tolerance describes a species' ability to respond and adapt to disturbances and perturbations in its environment following Eakins (2018). Resilience describes a species recovering capacity and its doubling time following exploitation (low > 4 years, medium 1.4–4 years, high < 1.4 years; Froese and Pauly, 2020). Habitat is based on where in the water column each species is typically found (Eakins, 2018). Biomass calculated as the average weight ± SEM of each fish species collected using minnow traps, Windermere traps, and electrofishing in a 200 m^2 transect per site.

Species	Family	Native/Non-native	Tolerance	Resilience	Habitat	Dundas WWTP									
						D1 (Outfall)		D2 (550 m)		D3 (1000 m)		D4 (2800 m)		D5 (3750 m)	
						Summer	Winter	Summer	Winter	Summer	Winter	Summer	Winter	Summer	Winter
Ambloplites rupestris (Rock bass)	Centrarchidae	Native	Intermediate	Medium	Benthopelagic	0 ± 0	31.342 ± 31.342	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Ameiurus nebulosus (Brown bullhead)	Ictaluridae	Native	Intermediate	Medium	Benthic	0.436 ± 0.436	2.218 ± 2.218	236.31 ± 217.31	62.871 ± 43.929	102.526 ± 93.573	2.612 ± 1.695	140.131 ± 108.855	0 ± 0	457.347 ± 265.75	23.488 ± 17.787
Amia calva (Bowfin)	Amiidae	Native	Intermediate	Low	Benthopelagic	0 ± 0	0 ± 0	7.529 ± 7.529	0 ± 0	0 ± 0	26 ± 26	0 ± 0	115.136 ± 115.136	0 ± 0	83.879 ± 83.879
Carassius auratus (Goldfish)	Cyprinidae	Non-native	Tolerant	Medium	Benthopelagic	4.927 ± 4.927	10.682 ± 10.682	254.472 ± 133.214	23.093 ± 14.361	41.802 ± 6.899	637.168 ± 637.168	2.012 ± 1.671	0 ± 0	67.346 ± 23.745	0 ± 0
Catostomus commersonii (White sucker)	Catostomidae	Native	Tolerant	Low	Benthic	58.327 ± 30.925	141.386 ± 39.136	0.895 ± 0.571	5.633 ± 5.633	5.307 ± 3.226	3.539 ± 3.539	4.825 ± 4.005	0 ± 0	0.057 ± 0.057	0 ± 0
Culaea inconstans (Brook stickleback)	Gasterosteidae	Native	Intermediate	High	Benthopelagic	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Cyprinus carpio (Common carp)	Cyprinidae	Non-native	Tolerant	Medium	Benthopelagic	642.48 ± 642.48	0 ± 0	136.661 ± 136.661	0 ± 0	0 ± 0	24179.808 ± 24179.808	201.631 ± 201.631	0 ± 0	37.77 ± 37.77	0 ± 0
Dorosoma cepedianum (Gizzard shad)	Clupeidae	Non-native	Tolerant	Medium	Pelagic	0 ± 0	0 ± 0	0.244 ± 0.244	56.492 ± 56.492	50.773 ± 30.18	0 ± 0	30.707 ± 29.273	0 ± 0	2.253 ± 1.341	2.897 ± 2.897
Esox lucius (Northern pike)	Esocidae	Native	Intermediate	Low	Benthopelagic	0 ± 0	0 ± 0	25.136 ± 25.136	752.966 ± 752.966	0 ± 0	0 ± 0	0 ± 0	0.359 ± 0.359	0 ± 0	0 ± 0
Labidesthes sicculus (Brook silverside)	Atherinopsidae	Native	Intermediate	High	Pelagic	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Lepisosteus osseus (Longnose gar)	Lepisosteidae	Native	Tolerant	Low	Benthopelagic	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Lepomis cyanellus (Green sunfish)	Centrarchidae	Native	Tolerant	Medium	Benthopelagic	164.764 ± 50.454	69.559 ± 35.505	15.807 ± 15.785	1.771 ± 0.936	1.458 ± 0.925	0 ± 0	1.978 ± 1.978	0 ± 0	11.718 ± 10.111	0 ± 0
Lepomis gibbosus (Pumpkinseed)	Centrarchidae	Native	Intermediate	Medium	Benthopelagic	46.024 ± 19.123	0.713 ± 0.713	23.532 ± 23.532	20.034 ± 20.034	0 ± 0	0 ± 0	12.579 ± 12.579	0 ± 0	12.516 ± 10.208	0 ± 0
Lepomis macrochirus (Bluegill sunfish)	Centrarchidae	Native	Intermediate	Medium	Benthopelagic	1.147 ± 1.072	33.134 ± 25.042	154.863 ± 83.353	44.744 ± 43.882	0.603 ± 0.32	1.057 ± 1.057	34.715 ± 20.987	0 ± 0	107.158 ± 64.157	0.635 ± 0.635
Luxilus cornutus (Common shiner)	Cyprinidae	Native	Intermediate	Medium	Benthopelagic	1.806 ± 1.043	0 ± 0	1.98 ± 1.98	0.295 ± 0.295	0.693 ± 0.693	7.334 ± 7.334	10.883 ± 10.883	0 ± 0	0 ± 0	0 ± 0
Micropterus salmoides (Largemouth bass)	Centrarchidae	Native	Tolerant	Low	Benthopelagic	34.527 ± 11.51	63.528 ± 42.792	48.472 ± 25.433	0 ± 0	12.319 ± 7.958	0 ± 0	5.199 ± 1.817	0 ± 0	4.154 ± 2.902	0 ± 0
Morone americana (White perch)	Moronidae	Non-native	Intermediate	Low	Benthopelagic	0 ± 0	0 ± 0	0 ± 0	8.255 ± 8.255	0 ± 0	0 ± 0	0 ± 0	0 ± 0	158.706 ± 146.275	0 ± 0
Neogobius melanostomus (Round goby)	Gobiidae	Non-native	Intermediate	Medium	Benthic	352.446 ± 171.308	96.162 ± 69.101	29.244 ± 6.481	1.265 ± 1.265	0 ± 0	0 ± 0	1.986 ± 1.447	0 ± 0	1.862 ± 1.862	0 ± 0
Notropis atherinoides (Emerald shiner)	Cyprinidae	Native	Intermediate	High	Benthopelagic	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.649 ± 0.649	0 ± 0
Notropis hudsonius (Spottail shiner)	Cyprinidae	Native	Intermediate	Medium	Benthopelagic	3.466 ± 2.371	0 ± 0	0.13 ± 0.09	0.4 ± 0.4	9.369 ± 9.174	0 ± 0	1.356 ± 1.215	0.116 ± 0.116	0.42 ± 0.42	0 ± 0
Perca flavescens (Yellow perch)	Percidae	Native	Intermediate	Medium	Benthopelagic	12.302 ± 8.264	0 ± 0	10.601 ± 7.541	2.595 ± 1.456	6.41 ± 4.177	6.211 ± 4.938	59.817 ± 29.394	7.027 ± 7.027	3.606 ± 1.695	90 ± 55.748
Percina caprodes (Common logperch)	Percidae	Native	Intolerant	Medium	Benthic	4.747 ± 2.741	9.194 ± 7.492	0 ± 0	4.802 ± 2.409	1.779 ± 1.779	0 ± 0	1.24 ± 0.55	6.47 ± 6.47	1.166 ± 0.682	0 ± 0
Pimephales notatus (Bluntnose minnow)	Cyprinidae	Native	Intermediate	Medium	Benthopelagic	0 ± 0	3.482 ± 1.862	0.369 ± 0.214	0 ± 0	0 ± 0	0 ± 0	1.201 ± 1.201	0 ± 0	0 ± 0	0 ± 0
Pimephales promelas (Fathead minnow)	Cyprinidae	Native	Tolerant	High	Benthopelagic	1.286 ± 0.893	1.292 ± 1.292	0.394 ± 0.394	0.923 ± 0.923	0.115 ± 0.115	0.289 ± 0.289	0 ± 0	0 ± 0	0.099 ± 0.099	0 ± 0
Pomoxis nigromaculatus (Black crappie)	Centrarchidae	Native	Tolerant	Medium	Benthopelagic	0 ± 0	0 ± 0	0.161 ± 0.161	0 ± 0	1.913 ± 1.913	5.42 ± 5.42	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Scardinius erythrophthalmus (Rudd)	Cyprinidae	Non-native	Tolerant	Low	Benthopelagic	21.614 ± 20.512	0 ± 0	472.081 ± 298.817	110.685 ± 47.454	64.933 ± 64.933	21.754 ± 18.915	102.33 ± 83.248	5.992 ± 5.992	87.842 ± 36.999	0 ± 0
Semolitus atromaculatus (Creek chub)	Cyprinidae	Native	Intermediate	Medium	Benthopelagic	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0

Table 1. Continued

						Woodward WWTP									
						W1 (Outfall)		W2 (350 m)		W3 (850 m)		W4 (-1000 m)		W5 (-1400 m)	
Species	Family	Native/Non-native	Tolerance	Resilience	Habitat	Summer	Winter	Summer	Winter	Summer	Winter	Summer	Winter	Summer	Winter
Ambloplites rupestris (Rock bass)	Centrarchidae	Native	Intermediate	Medium	Benthopelagic	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Ameiurus nebulosus (Brown bullhead)	Ictaluridae	Native	Intermediate	Medium	Benthic	0 ± 0	3.744 ± 3.744	0 ± 0	100.242 ± 100.242	0 ± 0	0 ± 0	35.623 ± 35.623	1.818 ± 1.818	11.365 ± 11.365	2.242 ± 2.242
Amia calva (Bowfin)	Amiidae	Native	Intermediate	Low	Benthopelagic	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	47.92 ± 35.835	0 ± 0	0 ± 0	0 ± 0
Carassius auratus (Goldfish)	Cyprinidae	Non-native	Tolerant	Medium	Benthopelagic	26.818 ± 26.818	0 ± 0	0.23 ± 0.23	0 ± 0	3.331 ± 2.418	0 ± 0	141.177 ± 120.84	0 ± 0	8.899 ± 8.017	0 ± 0
Catostomus commersonii (White sucker)	Catostomidae	Native	Tolerant	Low	Benthic	448.718 ± 206.376	23.822 ± 23.822	318.014 ± 201.828	12.577 ± 12.577	35.204 ± 22.394	0 ± 0	0.622 ± 0.622	0 ± 0	5.065 ± 4.805	4.688 ± 4.688
Culaea inconstans (Brook stickleback)	Gasterosteidae	Native	Intermediate	High	Benthopelagic	4.101 ± 2.001	0 ± 0	2.18 ± 1.094	0 ± 0	1.491 ± 1.36	4.052 ± 2.163	0 ± 0	0.406 ± 0.406	0 ± 0	0 ± 0
Cyprinus carpio (Common carp)	Cyprinidae	Non-native	Tolerant	Medium	Benthopelagic	594.121 ± 518.67	0 ± 0	0 ± 0	4842.609 ± 2428.536	0 ± 0	0 ± 0	3045.547 ± 1039.631	0 ± 0	774.477 ± 774.477	0 ± 0
Dorosoma cepedianum (Gizzard shad)	Clupeidae	Non-native	Tolerant	Medium	Pelagic	0 ± 0	1771.044 ± 1217.725	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.162 ± 0.162	0 ± 0	0.411 ± 0.411	0 ± 0
Esox lucius (Northern pike)	Esocidae	Native	Intermediate	Low	Benthopelagic	17.836 ± 17.836	0 ± 0	12.584 ± 12.584	0 ± 0	79.562 ± 76.787	108.748 ± 108.748	4.628 ± 4.628	0 ± 0	20.324 ± 17.357	0 ± 0
Labidesthes sicculus (Brook silverside)	Atherinopsidae	Native	Intermediate	High	Pelagic	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.047 ± 0.047	0.363 ± 0.363
Lepisosteus osseus (Longnose gar)	Lepisosteidae	Native	Tolerant	Low	Benthopelagic	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	7.937 ± 7.937	0 ± 0	0 ± 0	0 ± 0
Lepomis cyanellus (Green sunfish)	Centrarchidae	Native	Tolerant	Medium	Benthopelagic	135.266 ± 85.018	508.359 ± 117.562	52.311 ± 21.951	219.622 ± 51.668	35.686 ± 9.602	7.484 ± 4.067	39.252 ± 22.109	32.055 ± 32.055	113.313 ± 65.035	19.054 ± 18.155
Lepomis gibbosus (Pumpkinseed)	Centrarchidae	Native	Intermediate	Medium	Benthopelagic	0 ± 0	38.028 ± 10.652	21.811 ± 17.109	46.355 ± 37.557	2.702 ± 2.702	1.846 ± 1.846	60.733 ± 27.982	0 ± 0	41.243 ± 15.625	2.022 ± 1.222
Lepomis macrochirus (Bluegill sunfish)	Centrarchidae	Native	Intermediate	Medium	Benthopelagic	33.845 ± 31.49	3.815 ± 3.815	4.792 ± 4.792	16.387 ± 13.993	3.478 ± 3.478	0.569 ± 0.569	23.893 ± 18.237	0.314 ± 0.314	20.12 ± 13.207	5.68 ± 4.77
Luxilus comutus (Common shiner)	Cyprinidae	Native	Intermediate	Medium	Benthopelagic	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.511 ± 0.511	0 ± 0	0.585 ± 0.372	0 ± 0
Micropterus salmoides (Largemouth bass)	Centrarchidae	Native	Tolerant	Low	Benthopelagic	11.261 ± 11.261	0 ± 0	0 ± 0	0 ± 0	10.46 ± 6.118	7.778 ± 4.026	12.596 ± 12.596	0 ± 0	2.256 ± 2.256	0 ± 0
Morone americana (White perch)	Moronidae	Non-native	Intermediate	Low	Benthopelagic	0 ± 0	0 ± 0	76.377 ± 64.216	3.691 ± 3.691	11.941 ± 11.941	0 ± 0	30.266 ± 30.266	0 ± 0	0 ± 0	0 ± 0
Neogobius melanostomus (Round goby)	Gobiidae	Non-native	Intermediate	Medium	Benthic	93.942 ± 38.291	160.834 ± 47.776	4.228 ± 2.437	52.074 ± 20.3	0 ± 0	6.668 ± 6.478	14.317 ± 7.061	0.201 ± 0.201	58.899 ± 31.358	1.375 ± 1.001
Notropis atherinoides (Emerald shiner)	Cyprinidae	Native	Intermediate	High	Benthopelagic	0 ± 0	1.534 ± 1.534	0 ± 0	0 ± 0	0 ± 0	0.716 ± 0.716	0.317 ± 0.317	1.679 ± 1.679	1.086 ± 1.086	6.156 ± 6.156
Notropis hudsonius (Spottail shiner)	Cyprinidae	Native	Intermediate	Medium	Benthopelagic	2.589 ± 2.505	0 ± 0	0 ± 0	1.98 ± 1.98	2.34 ± 1.718	2.215 ± 1.673	1.188 ± 1.039	1.443 ± 1.443	0.915 ± 0.566	5.838 ± 4.266
Perca flavescens (Yellow perch)	Percidae	Native	Intermediate	Medium	Benthopelagic	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.157 ± 0.157	0 ± 0	0.948 ± 0.948	2.95 ± 2.95	0 ± 0	0 ± 0
Percina caprodes (Common logperch)	Percidae	Native	Intolerant	Medium	Benthic	3.475 ± 3.475	4.661 ± 4.661	1.291 ± 1.291	0 ± 0	0 ± 0	0 ± 0	0.551 ± 0.551	0 ± 0	1.006 ± 1.006	0 ± 0
Pimephales notatus (Bluntnose minnow)	Cyprinidae	Native	Intermediate	Medium	Benthopelagic	0.663 ± 0.463	0 ± 0	2.5 ± 2.5	0 ± 0	0.619 ± 0.545	0 ± 0	0.054 ± 0.054	0.671 ± 0.671	0 ± 0	0.3 ± 0.3
Pimephales promelas (Fathead minnow)	Cyprinidae	Native	Tolerant	High	Benthopelagic	10.177 ± 9.412	1.141 ± 1.141	4.333 ± 2.519	3.925 ± 3.925	2.786 ± 2.143	0.611 ± 0.305	17.184 ± 15.551	0 ± 0	6.041 ± 4.546	6.294 ± 3.281
Pomoxis nigromaculatus (Black crappie)	Centrarchidae	Native	Tolerant	Medium	Benthopelagic	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Scardinius erythrophthalmus (Rudd)	Cyprinidae	Non-native	Tolerant	Low	Benthopelagic	0 ± 0	0 ± 0	0 ± 0	1.527 ± 1.527	0 ± 0	0 ± 0	33.851 ± 33.851	0 ± 0	303.775 ± 303.775	0 ± 0
Semolitus atromaculatus (Creek chub)	Cyprinidae	Native	Intermediate	Medium	Benthopelagic	0 ± 0	0 ± 0	9.2 ± 9.2	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0

Table 2. Mean ± SEM biomass of each benthic macroinvertebrate family collected in all rock baskets per site and season.

			Dundas WWTP									
			D1 (Outfall)		D2 (550 m)		D3 (1000 m)		D4 (2800 m)		D5 (3750 m)	
Organism Type	Order	Family	Summer	Winter	Summer	Winter	Summer	Winter	Summer	Winter	Summer	Winter
Amphipods	Amphipoda	Gammaridae	0.003 ± 0.002	0.003 ± 0.002	0.002 ± 0.568	0.568 ± 0.127	0.568 ± 0.127	0.668 ± 0.302	0.127 ± 0.668	1.429 ± 0.437	0.668 ± 0.302	-
Isopods	Isopoda	Asellidae	0.029 ± 0.009	0.029 ± 0.009	0.009 ± 0.364	0.364 ± 0.138	0.364 ± 0.138	0.029 ± 0.019	0.138 ± 0.029	0.188 ± 0.056	0.029 ± 0.019	-
Leeches	Arhynchobdellida	Erpobdellidae	0.001 ± 0.001	0.001 ± 0.001	0.001 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	-
	Rhynchobdellida	Glossiphoniidae	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	-
Seg. Worms	Haplotaxida	Naididae	0.03 ± 0.03	0.03 ± 0.03	0.03 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	-
Flatworms	Tricladida	Planariidae	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	-
Snails	Basommatophora	Physidae	0.002 ± 0.002	0.002 ± 0.002	0.002 ± 0	0 ± 0	0 ± 0	0.034 ± 0.022	0 ± 0.034	0.053 ± 0.025	0.034 ± 0.022	-
		Planorbidae	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	-
		Lymnaeidae	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	-
	Heterostropha	Valvatidae	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	-
	NA	Snails w/ No Shell	0 ± 0	0 ± 0	0 ± 0.007	0.007 ± 0.007	0.007 ± 0.007	0 ± 0	0.007 ± 0	0 ± 0	0 ± 0	-
	Neotaenioglossa	Hydrobiidae	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	-
	Venerida	Sphaeriidae	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	-
Beetles	Coleoptera	Dryopidae	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	-
		Haliplidae	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	-
		Hydrophilidae	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	-
		Elmidae	0 ± 0	0 ± 0	0 ± 0.001	0.001 ± 0	0.001 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	-
		Dytiscidae	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	-
True Bugs	Hemiptera	Gerridae	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	-
		Veliidae	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	-
		Corixidae	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	-
		Belostomatidae	0 ± 0	0 ± 0	0 ± 0.169	0.169 ± 0.079	0.169 ± 0.079	0.07 ± 0.07	0.079 ± 0.07	0 ± 0	0.07 ± 0.07	-
Flies	Diptera	Chironomidae	0.001 ± 0.001	0.001 ± 0.001	0.001 ± 0.005	0.005 ± 0.002	0.005 ± 0.002	0.003 ± 0.002	0.002 ± 0.003	0.012 ± 0.006	0.003 ± 0.002	-
		Ceratopogonidae	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	-
		Tabanidae	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	-
	Ephemeroptera	Caenidae	0 ± 0	0 ± 0	0 ± 0.007	0.007 ± 0.004	0.007 ± 0.004	0 ± 0	0.004 ± 0	0 ± 0	0 ± 0	-
		Ameletidae	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	-
	Odonata	Libellulidae	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	-
		Coenagrionidae	0.004 ± 0.002	0.004 ± 0.002	0.002 ± 0.135	0.135 ± 0.009	0.135 ± 0.009	0.506 ± 0.144	0.009 ± 0.506	0.018 ± 0.005	0.506 ± 0.144	-
		Calopterygidae	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	-
		Corduliidae	0.079 ± 0.049	0.079 ± 0.049	0.049 ± 0.037	0.037 ± 0.037	0.037 ± 0.037	0 ± 0	0.037 ± 0	0 ± 0	0 ± 0	-
		Aeshnidae	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	-
	Plecoptera	Nemouridae	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.002 ± 0.002	0 ± 0	-
	Trichoptera	Psychomyiidae	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.06 ± 0.06	0 ± 0.06	0 ± 0	0.06 ± 0.06	-
		Hydropsychidae	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	-
		Phryganeidae	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.249 ± 0.205	0 ± 0.249	0 ± 0	0.249 ± 0.205	-
		Odontoceridae	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.05 ± 0.039	0 ± 0	-
	Megaloptera	Sialidae	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	-
		Corydalidae	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.29 ± 0.223	0 ± 0	-
	Lepidoptera	Pyralidae	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	-

Table 2. Continued

			Woodward WWTP									
			W1 (Outfall)		W2 (350 m)		W3 (850 m)		W4 (-1000 m)		W5 (-1400 m)	
Organism Type	Order	Family	Summer	Winter	Summer	Winter	Summer	Winter	Summer	Winter	Summer	Winter
Amphipods	Amphipoda	Gammaridae	0 ± 0	0 ± 0	0.012 ± 0.013	0.003 ± 0.003	0 ± 0	-	0.015 ± 0.011	2.078 ± 0.357	0.013 ± 0.008	0.124 ± 0.064
Isopods	Isopoda	Asellidae	1.237 ± 0.209	5.686 ± 2.224	0.495 ± 0.186	2.063 ± 0.876	0.536 ± 0.09	-	0.018 ± 0.011	0.227 ± 0.098	0.016 ± 0.01	0.086 ± 0.068
Leeches	Arhynchobdellida	Erpobdellidae	0.13 ± 0.056	0.223 ± 0.098	0.778 ± 0.312	0.38 ± 0.18	0.315 ± 0.271	-	0.065 ± 0.031	0.043 ± 0.032	0.201 ± 0.054	0.095 ± 0.082
	Rhynchobdellida	Glossiphoniidae	0 ± 0	0 ± 0	0.312 ± 0.226	0 ± 0	0.008 ± 0.008	-	0.084 ± 0.071	0.008 ± 0.008	0.064 ± 0.024	0 ± 0
Seg. Worms	Haplotaxida	Naididae	0.001 ± 0	0.001 ± 0.001	0.001 ± 0.001	0.001 ± 0.001	0 ± 0	-	0.001 ± 0.001	0.004 ± 0.002	0.028 ± 0.024	0.008 ± 0.003
Flatworms	Tricladida	Planariidae	0 ± 0	0 ± 0	0.046 ± 0.043	0.003 ± 0.002	0 ± 0	-	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Snails	Basommatophora	Physidae	0.041 ± 0.021	0.25 ± 0.06	0.247 ± 0.036	0.048 ± 0.027	0.044 ± 0.025	-	0.097 ± 0.05	0.031 ± 0.015	0.15 ± 0.074	0 ± 0
		Planorbidae	0 ± 0	0 ± 0	0.121 ± 0.058	0 ± 0	1.697 ± 1.644	-	0.089 ± 0.065	0 ± 0	0.056 ± 0.056	0 ± 0
		Lymnaeidae	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	-	0.007 ± 0.006	0.003 ± 0.003	0.052 ± 0.046	0 ± 0
	Heterostropha	Valvatidae	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.729 ± 0.195	-	0.001 ± 0.002	0.007 ± 0.007	0.014 ± 0.009	0 ± 0
	NA	Snails w/ No Shell	0.01 ± 0.01	0.099 ± 0.08	0.036 ± 0.025	0.002 ± 0.001	0 ± 0	-	0.001 ± 0.001	0 ± 0	0 ± 0	0 ± 0
	Neotaenioglossa	Hydrobiidae	0 ± 0	0 ± 0	0.432 ± 0.099	0.102 ± 0.045	2.061 ± 1.075	-	5.191 ± 1.185	0 ± 0	2.74 ± 1.469	0.008 ± 0.004
	Venerida	Sphaeriidae	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.02 ± 0.02	-	0.096 ± 0.048	0.235 ± 0.065	0.475 ± 0.183	0.048 ± 0.031
Beetles	Coleoptera	Dryopidae	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	-	0 ± 0	0 ± 0	0 ± 0	0 ± 0
		Haliplidae	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	-	0 ± 0	0 ± 0	0 ± 0	0 ± 0
		Hydrophilidae	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	-	0.01 ± 0.003	0.001 ± 0.001	0.003 ± 0.001	0 ± 0
		Elmidae	0 ± 0	0 ± 0	0.002 ± 0.002	0 ± 0	0 ± 0	-	0 ± 0	0 ± 0	0 ± 0	0 ± 0
		Dytiscidae	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	-	0 ± 0	0 ± 0	0 ± 0	0 ± 0
True Bugs	Hemiptera	Gerridae	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	-	0 ± 0	0 ± 0	0 ± 0	0 ± 0
		Veliidae	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	-	0 ± 0	0 ± 0	0 ± 0	0 ± 0
		Corixidae	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	-	0 ± 0	0 ± 0	0 ± 0	0 ± 0
		Belostomatidae	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	-	0 ± 0	0.057 ± 0.057	0 ± 0	0 ± 0
Flies	Diptera	Chironomidae	0.046 ± 0.021	0.006 ± 0.005	0.002 ± 0.001	0.004 ± 0.001	0 ± 0	-	0 ± 0	0.066 ± 0.028	0.002 ± 0.001	0.005 ± 0.004
		Ceratopogonidae	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	-	0 ± 0	0 ± 0	0 ± 0	0 ± 0
		Tabanidae	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	-	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	Ephemeroptera	Caenidae	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	-	0 ± 0	0 ± 0	0 ± 0	0 ± 0
		Ameletidae	0.001 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	-	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	Odonata	Libellulidae	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	-	0 ± 0	0 ± 0	0 ± 0	0 ± 0
		Coenagrionidae	0.007 ± 0.003	0.006 ± 0.004	0.004 ± 0.002	0.005 ± 0.004	0 ± 0	-	0.052 ± 0.02	0.025 ± 0.015	0.042 ± 0.02	0.002 ± 0.001
		Calopterygidae	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	-	0 ± 0	0 ± 0	0 ± 0	0 ± 0
		Corduliidae	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	-	0 ± 0	0 ± 0	0.015 ± 0.014	0.034 ± 0.032
		Aeshnidae	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	-	0 ± 0	0.029 ± 0.029	0 ± 0	0 ± 0
	Plecoptera	Nemouridae	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	-	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	Trichoptera	Psychomyiidae	0.004 ± 0.004	0 ± 0	0.005 ± 0.005	0 ± 0	0 ± 0	-	0 ± 0	0 ± 0	0 ± 0	0 ± 0
		Hydropsychidae	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	-	0 ± 0	0 ± 0	0.001 ± 0.001	0 ± 0
		Phryganeidae	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	-	0 ± 0	0 ± 0	0 ± 0	0 ± 0
		Odontoceridae	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	-	0 ± 0	0 ± 0	0 ± 0	0.001 ± 0.001
	Megaloptera	Sialidae	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	-	0 ± 0	0 ± 0	0.033 ± 0.015	0 ± 0
		Corydalidae	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	-	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	Lepidoptera	Pyralidae	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	-	0 ± 0	0 ± 0	0 ± 0	0.001 ± 0.001

Appendix C: Supplementary materials for Chapter 2

Table S1. Composite effluent water quality

Tables S2 – S5. Contaminants of emerging concern analysis parameters

Table S6 – S7. Habitat characteristics of sampling sites

Table S8. Fish species characteristics and abundances

Table S9 – S10. SIMPER analysis

Table S11. Water quality PCA loadings

1. Supplementary Results

Species tolerance, resilience, trophic level, and proportion of non-natives

Table S1. Water quality parameters measured on the 24-hr composite final treated effluent produced from the Dundas and Woodward WWTPs during both seasons. Not all parameters were measured in every sample. At the Woodward WWTP, TSS, BOD, TP, TKN, NH_3, NO_3, and NO_2 were measured in 22 samples in the summer and 22 samples in the winter, whereas *E. coli* was measured in 5 samples in the summer and 4 samples in the winter. At the Dundas WWTP, TSS, BOD, TP, TKN, NH_3, NO_3, NO_2, and *E. coli* were measured in 4 samples in the summer and 4 samples in the winter. Bolded averages indicate significant differences between seasons within each WWTP ($p < 0.05$).

	Dundas WWTP		Woodward WWTP	
	Summer	Winter	Summer	Winter
Total suspended solids (mg/L)	0.80±0	0.80±0	4.55±0.41	**12.1±1.20**
Biochemical oxygen demand (mg/L)	1.25±0.25	1.25±0.25	3.61±0.14	**7.27±0.82**
Total phosphorus (mg/L)	0.12±0.01	0.06±0	0.56±0.02	0.58±0.04
Total Kjeldahl nitrogen (mg/L)	0.6±0.07	0.65±0.04	1.66±0.08	3.22±0.31
Ammonia (mg/L)	0.04±0	0.06±0.01	0.60±0.09	**1.85±0.23**
Nitrate (mg/L)	16.7±0.38	16.9±1.04	**14.4±0.44**	6.65±0.79
Nitrite (mg/L)	0.08±0.01	0.21±0.09	0.17±0	**4.25±0.58**
Escherichia coli (CFU/100 mL)	10.0±0	9530±5210	28.0±13.2	**8250±2080**

Table S2. Liquid chromatography for positive and negative methods.

	Positive		Negative	
Column	2.1 mm x 50 mm x 1.8 µm Agilent ZORBAX Eclipse Plus C18			
Mobile phase A	Water with 5 mM ammonium acetate			
Mobile phase B	Methanol			
Injection volume (µL)	2		10	
Column temp (°C)	40		35	
Flow rate (mL/min)	0.3		0.3	
Stop time (min)	13		10.5	
Re-equilibration time (min)	4		4	
Gradient	Time (min)	% B	Time (min)	% B
	0	0	0	10
	0.5	0	0.5	10
	0.51	50	0.51	60
	8	100	8	100
	11	100	10	100
	11.1	0	10.5	10

Table S3. Triple quadruple source parameters for positive and negative methods.

	Positive	Negative
Gas Temp (°C)	250	300
Gas Flow (L/min)	5	5
Nebulizer (psi)	45	45
Sheath Gas Temp (°C)	400	250
Sheath Gas Flow (L/min)	12	11
Capillary (V)	3000	4500
Nozzle Voltage (V)	0	500
Cell Accelerator Voltage (V)	4	4

Table S4. Compound specific dynamic MRM parameters for positive method. All MS1 and MS2 were widest resolution except for sulfamethoxazole and caffeine which were unit resolution.

Compound Name	Precursor Ion	Product Ion	Ret Time (min)	Delta Ret Time	Fragmentor	Collision Energy	Detection Limit (ng/L)
ACE	152.1	65.1	4.45	1	100	34	25
	152.1	110.1	4.45	1	100	14	
ACE - d4	156.1	43.1	4.41	1	110	38	
	156.1	114.1	4.41	1	110	18	
ATEN	267.2	56.1	4.58	1	90	32	2.5
	267.2	145.1	4.58	1	90	24	
ATEN - d7	274.2	57.1	4.5	1	82	31	
	274.2	145.1	4.5	1	82	27	
SULF	254.1	92.1	4.68	1	96	30	5
	254.1	108	4.68	1	96	22	
SULF - d4	258.1	112	4.54	1	92	26	
	258.1	160	4.54	1	92	14	
SMZ	279.1	92.1	5.03	1	96	30	2.5
	279.1	124.1	5.03	1	96	26	
SMZ - d4	283.1	96.1	5.03	1	112	34	
	283.1	112.1	5.03	1	112	30	
CAFF	195.1	42.1	5.04	1	104	42	5
	195.1	138.1	5.04	1	104	18	
CAFF - d3	198.1	42.1	5.04	1	116	46	
	198.1	138.1	5.04	1	116	18	
TRIM	291.1	123	5.42	1	134	22	2.5
	291.1	230.1	5.42	1	134	22	
TRIM - d3	294.2	110	5.42	1	130	34	
	294.2	123.1	5.42	1	130	22	
desVEN	264.2	42.1	5.49	1	86	116	2.5
	264.2	58.1	5.49	1	86	16	
desVEN - d6	270.2	64.1	5.46	1	78	18	
	270.2	252.2	5.46	1	78	10	
e-CBZ	253.1	180.1	6.1	1	132	30	2.5
	253.1	236	6.1	1	132	6	
e-CBZ - d10	263.1	190.2	6.1	1	145	21	
	263.1	220.2	6.1	1	145	9	
VEN	278.2	58.1	6.41	1	78	16	2.5
	278.2	121	6.41	1	78	28	

VEN - d6	284.2	64.2	6.34	1	86	16	
	284.2	121.1	6.34	1	86	28	
LIN	407.2	126.1	6.54	1	122	28	5
LIN - d3	410	129.1	6.47	1	98	28	
CBZ	237.1	193.1	6.67	2	142	38	2.5
	237.1	194.1	6.67	2	142	14	
CBZ - d10	247.2	202.1	6.63	2	148	38	
	247.2	204.2	6.63	2	148	22	
p-ATOR	575.3	250.1	6.71	2	148	50	2.5
	575.3	440.2	6.71	2	148	18	
p-ATOR - d5	580.3	445.2	6.7	2	148	18	
ATRZ	216	43.1	7.02	2	106	46	2.5
	216	174.1	7.02	2	106	14	
ATRZ - d5	221	44.1	6.98	2	100	58	
	221	179.1	6.98	2	100	14	
o-ATOR	575.3	250.1	7.42	2	130	50	2.5
	575.3	440.2	7.42	2	130	22	
o-ATOR - d5	580.3	445.2	7.42	2	130	22	
NFLX	296.1	30.2	7.6	5	66	8	2.5
	296.1	134.1	7.6	5	66	0	
NFLX - d5	301.2	32.1	7.6	5	60	8	
	301.2	139.1	7.6	5	60	0	
FLX	310.1	44.1	7.64	5	66	12	2.5
	310.1	148.1	7.64	5	66	4	
FLX - d5	315.2	44.1	7.64	5	68	8	
	315.2	153.2	7.64	5	68	4	
ATOR	559.3	250.1	7.67	2	144	50	2.5
	559.3	440.2	7.67	2	144	19	
ATOR - d5	564.3	445.3	7.67	2	144	19	
MON	693.4	461.3	10.28	2	285	60	2.5
	693.4	675.3	10.28	2	285	40	

Table S5. Compound specific dynamic MRM parameters for negative method. All MS1 and MS2 resolution were widest.

Compound Name	Precursor Ion	Product Ion	Ret Time (min)	Delta Ret Time	Fragmentor	Collision Energy	Detection Limit (ng/L)
NPX	229.1	185.1	5.84	2	60	1	2.5
	229.1	169.1	5.84	2	60	30	
NPX - d3	232.1	173.1	5.83	2	48	8	
	232.1	171.1	5.83	2	48	28	
DCF	294	250.1	6.75	2	68	0	2.5
	294	214.1	6.75	2	68	12	
DCF - d4	298	254.1	6.8	2	80	0	
IBU	205.1	161.1	7	2	80	0	2.5
IBU - d3	208.1	164.2	6.94	2	78	0	
GFZ	249.2	127.1	8.11	2	70	5	2.5
	249.2	121.1	8.11	2	70	5	
GFZ - d6	255.2	121.1	8.11	2	52	8	
TCS	286.9	35.1	9.09	2	70	0	7.5
TCS - d3	289.99	35	9.09	2	90	9	

Table S6. Habitat characteristics of the Dundas WWTP sampling sites following a subset of the metrics used in the Qualitative Habitat Evaluation Index (QHEI; Taft and Koncelik, 2006; Strickland et al. 2010). Aquatic vegetation was only recorded during the summer as it was absent during the winter. Total depth and clarity are reported as means ± standard error for summer **|** winter. Habitat characteristics have been previously published in McCallum et al. (2019).

Dundas WWTP	**D1 (Outfall)**	**D2 (550 m)**	**D3 (1000 m)**	**D4 (2800 m)**	**D5 (3750 m)**
Aquatic habitat					
Bottom substrate	85% cobble	90% sand	100% silt	100% silt	100% silt
(% coverage)	15% boulder	10% gravel			
Bottom slope	> 45°	> 25°	< 15°	< 15°	< 15°
Shoreline habitat					
Sinuosity	None, straight	None, straight	Low, 1 curve	Low, 1 curve	Low, 1 curve
Anthropogenic modifications	Recent, recovering	Recent, recovering	None	None	None
Shoreline quality	Urban, industrial, residential trail	Urban, industrial, residential trail	Forest, wetland	Forest, wetland	Forest, wetland
Bank erosion	Moderate (25-50%)	Moderate (25-50%)	Low (0-25 %)	Low (0-25 %)	Low (0-25 %)
Riparian width	Narrow (5-10 m)	Moderate (10-50 m)	Wide (>50 m)	Wide (>50 m)	Wide (>50 m)
Additional measures					
Total depth (cm) Summer | Winter	189±51.7 | 164±1.73	190±59.7 | 142±18.9	81.3±34.5 | 46.7±2.88	99.3±21.0 | 73.3±15.3	96.3±41.1 | 46.7±2.88
Clarity (%) Summer | Winter	45.0±13.6 | 92.5±7.57	34.8±9.33 | 79.2±18.1	70.7±25.8 | 100±0	50.1±15.0 | 100±0	29.6±2.26 | 82.6±10.9
Aquatic vegetation	Algae, lesser/greater duckweed, milfoil	filamentous algae, lesser/greater duckweed, milfoil	Filamentous algae, lesser/greater duckweed, milfoil, water lily	Lesser/greater duckweed, liverwort, water lily, potamogeton	Lesser/greater duckweed, milfoil, water lily
Site comments	Human-made canal, erosion-prevention logs, bank built of cobble	Human-made canal, undercut banks, overhanging *phragmites*	Natural site, cattail stands between site and shore	Fencing near water flowering rush between site and shore	Natural site, cattail stands between site and shore

Table S7. Habitat characteristics of the Woodward WWTP sampling sites following a subset of the metrics used in the Qualitative Habitat Evaluation Index (QHEI; Taft and Koncelik, 2006; Strickland et al. 2010). Aquatic vegetation was only recorded during the summer as it was absent during the winter. Total depth and clarity are reported as means ± standard error for summer **|** winter. Habitat characteristics have been previously published in McCallum et al. (2019).

Woodward WWTP	**W1 (Outfall)**	**W2 (350 m)**	**W3 (850 m)**	**W4 (-1000 m)**	**W5 (-1400 m)**
Aquatic habitat					
Bottom substrate	100% cobble	50% cobble	50% cobble	50% cobble	50% cobble
(% coverage)		50% boulder	50% boulder	50% silt	50% silt
Bottom slope	≥ 45°	≥ 45°	≥ 25°	≥ 45°	≥ 45°
Shoreline habitat					
Sinuosity	Low	Low-moderate	Low	None-straight	None-straight
Anthropogenic modifications	Recent, recovering	Recent	Recent	Recent, recovering	Recent, recovering
Shoreline quality	Urban, industrial	Urban, industrial	Urban, industrial	Urban, industrial	Urban, industrial
Bank erosion	Low	Low	Low	Low	Low
Riparian width	Narrow (5-10 m)	Very narrow (<5 m)	Very narrow (<5 m)	Narrow (5-10 m)	Narrow (5-10 m)
Additional measures					
Total depth (cm) Summer | Winter	210±25.5 | 192±6.0	215±15.7 | 155±23.6	210±29.2 | 142±13.0	131±19.7 | 98.3±6.66	138±24.0 | 78.3±10.1
Clarity (%) Summer | Winter	58.0±8.77 | 63.2±3.85	52.2±10.9 | 76.1±10.3	45.3±1.47 | 66.1±17.4	34.6±8.62 | 60.5±5.59	35.2±12.1 | 66.7±16.7
Aquatic vegetation	Algae on rocks, pondweed	Algae on rocks, Canada waterweed, lesser/greater duckweed, smartweed	Floating pondweed, lesser duckweed, pondweed, smartweed	Lesser duckweed, water smartweed	Lesser duckweed, water smartweed
Site comments	Near large highway, modified shoreline	Modified shoreline, bank shaping	Modified shoreline, bank shaping	Near large highway, cemented shoreline	Near large highway and pedestrian bridge, cemented shoreline

Table S8. Fish species characteristics and abundances from all sampling events at the Dundas and Woodward WWTPs. Native/non-native describes a species origin and status in Ontario (following Ontario Ministry of Natural Resources, 2015). Tolerance describes a species capacity to respond and adapt to environmental perturbations and/or disturbances (following Eakins, 2018). Resilience describes a species recovering capacity and doubling time after exploitation (low > 4 years, medium 1.4–4 years, high < 1.4 years, Froese and Pauly, 2020). Trophic level describes where a species tends to operate in its respective food web (Froese and Pauly, 2020). Abundance data is shown as the number of individuals caught of each species in the summer and winter (summer/winter). Abundance data are cumulatively represented from all sampling events within each season.

	Species Characteristics				Dundas WWTP					Woodward WWTP				
	Native/ non-native	Tolerance	Resilience	Trophic level	D1 (Outfall)	D2 (550 m)	D3 (1000 m)	D4 (2800 m)	D5 (3750 m)	W1 (Outfall)	W2 (350 m)	W3 (850 m)	W4 (-1000 m)	W5 (-1400 m)
Ambloplites rupestris (Rock bass)	Native	Intermediate	Medium	3.4	0/1	0/5	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0
Ameiurus nebulosus (Brown bullhead)	Native	Intermediate	Medium	3.7	1/2	67/20	30/3	24/0	172/25	0/2	0/5	0/0	2/1	1/1
Amia calva (Bowfin)	Native	Intermediate	Low	3.8	0/0	1/0	0/1	0/8	0/4	0/0	0/0	0/0	1/0	0/0
Carassius auratus (Goldfish)	Non-native	Tolerant	Medium	2	2/1	187/2	53/3	4/0	78/0	1/0	1/0	11/0	8/0	2/0
Catostomus commersonii (White sucker)	Native	Tolerant	Low	2.8	51/8	3/1	10/1	7/0	1/0	51/3	37/1	12/0	1/0	5/1
Culaea inconstans (Brook stickleback)	Native	Intermediate	High	3.2	0/0	0/0	0/0	0/0	0/0	30/0	12/0	7/8	0/1	0/2
Cyprinus carpio (Common carp)	Non-native	Tolerant	Medium	3.1	1/0	1/0	0/14	1/0	1/0	2/0	0/2	0/0	4/0	0/0
Dorosoma cepedianum (Gizzard shad)	Non-native	Tolerant	Medium	3.1	0/0	1/22	124/0	38/0	3/2	0/4	0/0	0/0	1/0	1/0
Esox lucius (Northern pike)	Native	Intermediate	Low	4.1	0/0	1/1	0/0	0/1	0/0	2/0	1/0	8/1	1/0	4/0
Labidesthes sicculus (Brook silverside)	Native	Intermediate	High	3.4	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	1/2
Lepisosteus osseus (Longnose gar)	Native	Tolerant	Low	4.2	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	1/0	0/0
Lepomis cyanellus (Green sunfish)	Native	Tolerant	Medium	3.5	27/8	11/2	3/0	5/0	19/0	40/57	16/33	37/8	41/5	58/6
Lepomis gibbosus (Pumpkinseed)	Native	Intermediate	Medium	3.3	6/1	3/2	0/0	2/0	23/0	0/5	3/7	4/1	44/0	20/4
Lepomis macrochirus (Bluegill sunfish)	Native	Intermediate	Medium	3.2	6/9	22/6	3/1	38/0	360/2	14/12	9/7	2/1	131/2	78/4
Luxilus cornutus (Common shiner)	Native	Intermediate	Medium	2.8	5/0	28/1	1/1	5/0	0/0	0/0	0/0	0/0	5/0	2/0
Micropterus salmoides (Largemouth bass)	Native	Tolerant	Low	3.8	36/13	60/0	13/0	10/0	6/0	4/0	0/0	6/2	4/0	1/0
Morone americana (White perch)	Non-native	Intermediate	Low	3.1	0/0	0/1	0/0	0/0	117/0	0/0	4/1	1/0	2/0	0/0
Neogobius melanostomus (Round goby)	Non-native	Intermediate	Medium	3.3	184/42	19/1	0/0	2/0	1/0	78/37	7/14	1/7	44/1	54/4
Notropis atherinoides (Emerald shiner)	Native	Intermediate	High	2.8	0/0	0/0	0/0	0/0	1/0	8/2	0/0	0/1	8/1	2/5
Notropis hudsonius (Spottail shiner)	Native	Intermediate	Medium	2.1	6/0	2/1	7/0	5/1	2/0	131/0	0/1	9/2	3/1	4/4
Perca flavescens (Yellow perch)	Native	Intermediate	Medium	3.7	9/0	6/2	11/2	52/2	7/15	0/0	0/0	1/0	1/2	0/0
Percina caprodes (Common logperch)	Native	Intolerant	Medium	3.4	5/7	0/3	2/0	3/3	2/0	11/1	3/0	0/0	5/0	2/0
Pimephales notatus (Bluntnose minnow)	Native	Intermediate	Medium	2.7	0/3	5/0	0/0	1/0	0/0	17/0	35/0	6/0	1/1	0/1
Pimephales promelas (Fathead minnow)	Native	Tolerant	High	2.4	10/2	2/4	2/1	1/0	1/0	137/3	23/7	12/2	147/0	23/11
Pomoxis nigromaculatus (Black crappie)	Native	Tolerant	Medium	3.8	0/0	1/0	1/1	0/0	0/0	10/0	0/0	0/0	0/0	0/0

Scardinius erythrophthalmus (Rudd)	Non-native	Tolerant	Low	2.9	4/0	34/7	2/5	9/2	42/0	0/0	0/1	0/0	1/0	23/0
Semolitus atromaculatus (Creek chub)	Native	Intermediate	Medium	4	0/0	0/0	0/0	0/0	0/0	0/0	5/0	0/0	0/0	0/0

Table S9. Similarity percentages (SIMPER) analysis showing the contribution of key species to the overall dissimilarity of all the Dundas WWTP sampling sites relative to each other. Average A and Average B represent the gear-standardized catch per unit effort (abundance) for each species at the pair of sites being compared. Only species that contributed ≥ 5% to the overall abundance are shown. Bolded averages indicate significant differences ($p < 0.05$).

	Dundas WWTP									
	Summer					Winter				
Comparsion	Total dissimilarity	Species	Average A	Average B	Contribution	Total dissimilarity	Species	Average A	Average B	Contribution
A. D2 (550 m)	88.81%	*Ameiurus nebulosus* (Brown bullhead)	0.86	0.63	21	93.23%	*Ameiurus nebulosus* (Brown bullhead)	0.13	0.03	24.24
B. D3 (1000 m)		*Carassius auratus* (Goldfish)	0.3	**0.68**	16.49		*Scardinius erythrophthalmus* (Rudd)	0.05	**0.16**	16.82
		Micropterus salmoides (Largemouth bass)	**0.46**	0.18	10.78		*Pimephales promelas* (Fathead minnow)	0.002	0.05	8.27
		Scardinius erythrophthalmus (Rudd)	0.3	0.07	7.4		*Dorosoma cepedianum* (Gizzard shad)	0.89	0	7.3
		Neogobius melanostomus (Round goby)	0.09	0	6.59		*Lepomis cyanellus* (Green sunfish)	0.05	0	7.07
		Dorosoma cepedianum (Gizzard shad)	0.006	0.05	6.25		*Perca flavescens* (Yellow perch)	0.05	0.05	6.16
							Lepomis macrochirus (Bluegill sunfish)	0.11	0.05	5.76
A. D2 (550 m)	91.70%	*Ameiurus nebulosus* (Brown bullhead)	0.86	0.17	18.68	96.05%	*Percina caprodes* (Common logperch)	0.001	**0.15**	29.16
B. D4 (2800 m)		*Lepomis macrochirus* (Bluegill sunfish)	0.08	0.33	11.37		*Ameiurus nebulosus* (Brown bullhead)	0.13	0	19.51
		Micropterus salmoides (Largemouth bass)	0.46	0.04	9.48		*Scardinius erythrophthalmus* (Rudd)	0.05	0.005	8
		Carassius auratus (Goldfish)	0.3	0.002	8.67		*Dorosoma cepedianum* (Gizzard shad)	0.89	0	7.49
		Perca flavescens (Yellow perch)	0.03	**0.12**	8.67		*Lepomis cyanellus* (Green sunfish)	0.05	0	7.48
		Scardinius erythrophthalmus (Rudd)	0.007	0.15	6.83		*Amia calva* (Bowfin)	0	0.02	7.27
A. D2 (550 m)	88.42%	*Ameiurus nebulosus* (Brown bullhead)	0.86	**0.96**	22.62	94.57%	*Ameiurus nebulosus* (Brown bullhead)	0.13	**0.34**	32.23
B. D5 (3750 m)		*Lepomis macrochirus* (Bluegill sunfish)	0.08	**3.24**	16.81		*Perca flavescens* (Yellow perch)	0.05	**0.32**	26.05
		Carassius auratus (Goldfish)	0.3	0.36	11.53		*Dorosoma cepedianum* (Gizzard shad)	0.89	0.002	8.53
		Scardinius erythrophthalmus (Rudd)	0.3	0.22	9.04		*Scardinius erythrophthalmus* (Rudd)	0.05	0	6.53
		Morone americana (White perch)	0	**0.93**	8.51		*Lepomis cyanellus* (Green sunfish)	0.05	0	6.15
		Neogobius melanostomus (Round goby)	0.46	0.04	7					
A. D3 (1000 m)	90.51%	*Ameiurus nebulosus* (Brown bullhead)	0.63	0.17	15.28	98.91%	*Percina caprodes* (Common logperch)	0	**0.15**	33.45
B. D4 (2800 m)		*Carassius auratus* (Goldfish)	0.68	0.002	12.86		*Scardinius erythrophthalmus* (Rudd)	0.16	0.005	13.54
		Lepomis macrochirus (Bluegill sunfish)	0.04	0.33	12.19		*Ameiurus nebulosus* (Brown bullhead)	0.03	0	11.12
		Perca flavescens (Yellow perch)	0.09	**0.12**	12.16		*Amia calva* (Bowfin)	0.0006	**0.02**	8.8
		Dorosoma cepedianum (Gizzard shad)	**0.05**	0.02	9.83		*Pimephales promelas* (Fathead minnow)	0.05	0	7.74
							Perca flavescens (Yellow perch)	0.05	0	6.85
A. D3 (1000 m)	89.66%	*Ameiurus nebulosus* (Brown bullhead)	0.63	0.96	21.78	94.90%	*Perca flavescens* (Yellow perch)	0.05	**0.32**	29.5
B. D5 (3750 m)		*Lepomis macrochirus* (Bluegill sunfish)	0.04	**3.24**	17.42		*Ameiurus nebulosus* (Brown bullhead)	0.03	0.34	29.25
		Carassius auratus (Goldfish)	0.68	0.36	15.22		*Scardinius erythrophthalmus* (Rudd)	0.16	0	10.61
		Morone americana (White perch)	0	**0.93**	8.82		*Pimephales promelas* (Fathead minnow)	0.05	0	6.3
		Scardinius erythrophthalmus (Rudd)	0.07	0.22	6.81					
A. D4 (2800 m)	90.38%	*Lepomis macrochirus* (Bluegill sunfish)	0.33	**3.24**	22.5	96.61%	*Percina caprodes* (Common logperch)	**0.15**	0	29.03
B. D5 (3750 m)		*Ameiurus nebulosus* (Brown bullhead)	0.17	0.96	19		*Perca flavescens* (Yellow perch)	0.005	**0.32**	27.89
		Carassius auratus (Goldfish)	0.002	0.36	9.2		*Ameiurus nebulosus* (Brown bullhead)	0	0.34	22.46
		Morone americana (White perch)	0	**0.93**	8.86		*Amia calva* (Bowfin)	**0.02**	0.004	9.25
		Morone americana (White perch)	0.15	0.22	7.54					
		Perca flavescens (Yellow perch)	0.12	0.009	7.09					

Table S10. Similarity percentages (SIMPER) analysis showing the contribution of key species to the overall dissimilarity of all the Woodward WWTP sampling sites relative to each other. Average A and Average B represent the gear-standardized catch per unit effort (abundance) for each species at the pair of sites being compared. Only species that contributed $\geq$ 5% to the overall abundance are shown. Bolded averages indicate significant differences ($p < 0.05$).

	Woodward WWTP									
	Summer					Winter				
Comparsion	Total dissimilarity	Species	Average A	Average B	Contribution	Total dissimilarity	Species	Average A	Average B	Contribution
A. W2 (350 m)	93.01%	*Lepomis cyanellus* (Green sunfish)	0.18	0.72	18.8	90.88%	*Neogobius melanostomus* (Round goby)	0.31	0.2	26.44
B. W3 (850 m)		*Culaea inconstans* (Brook stickleback)	0.36	0.18	16.19		*Lepomis cyanellus* (Green sunfish)	0.14	0.1	20.42
		Catostomus commersonii (White sucker)	0.002	**0.08**	10.49		*Lepomis gibbosus* (Pumpkinseed)	0.2	0.05	9.59
		Neogobius melanostomus (Round goby)	0.13	0.006	7.15		*Culaea inconstans* (Brook stickleback)	0	0.06	9.41
		Carassius auratus (Goldfish)	0.0006	**0.23**	6.73		*Pimephales promelas* (Fathead minnow)	0.003	0.05	5.62
		Pimephales promelas (Fathead minnow)	0.08	0.24	6.67		*Lepomis macrochirus* (Bluegill sunfish)	0.23	0.0005	5.45
		Pimephales notatus (Bluntnose minnow)	**0.02**	0.002	6.25					
		Lepomis macrochirus (Bluegill sunfish)	0.02	0.06	5.85					
A. W2 (350 m)	93.08%	*Lepomis macrochirus* (Bluegill sunfish)	0.02	0.47	17.62	92.66%	*Lepomis cyanellus* (Green sunfish)	0.14	0.2	22.55
B. W4 (-1000 m)		*Neogobius melanostomus* (Round goby)	0.13	0.87	15.22		*Neogobius melanostomus* (Round goby)	0.31	0.08	17.04
		Lepomis cyanellus (Green sunfish)	0.18	0.23	12.31		*Lepomis macrochirus* (Bluegill sunfish)	0.23	0.17	10.3
		Pimephales promelas (Fathead minnow)	0.08	0.17	9.73		*Lepomis gibbosus* (Pumpkinseed)	0.2	0	7.84
		Culaea inconstans (Brook stickleback)	**0.36**	0	7.96		*Notropis atherinoides* (Emerald shiner)	0	0.02	6.95
							Ameiurus nebulosus (Brown bullhead)	0.23	0.02	5.68
A. W2 (350 m)	91.70%	*Neogobius melanostomus* (Round goby)	0.13	0.41	20.42	91.23%	*Neogobius melanostomus* (Round goby)	0.31	0.12	17.9
B. W5 (-1400 m)		*Lepomis cyanellus* (Green sunfish)	0.18	0.37	15.02		*Lepomis cyanellus* (Green sunfish)	0.14	0.05	15.81
		Lepomis macrochirus (Bluegill sunfish)	0.02	0.3	14.68		*Lepomis macrochirus* (Bluegill sunfish)	0.23	0.08	14.28
		Culaea inconstans (Brook stickleback)	0.36	0	9.11		*Pimephales promelas* (Fathead minnow)	0.003	**0.17**	12.7
		Pimephales promelas (Fathead minnow)	0.08	0.13	6.64		*Lepomis gibbosus* (Pumpkinseed)	**0.2**	0.08	12.05
		Scardinius erythrophthalmus (Rudd)	0	0.64	5.18					
A. W3 (850 m)	95.84%	*Lepomis macrochirus* (Bluegill sunfish)	0.06	0.47	17.97	94.16%	*Lepomis cyanellus* (Green sunfish)	0.1	0.2	20.4
B. W4 (-1000 m)		*Lepomis cyanellus* (Green sunfish)	0.72	0.23	15.72		*Neogobius melanostomus* (Round goby)	0.2	0.08	18.75
		Neogobius melanostomus (Round goby)	0.006	0.87	13.85		*Culaea inconstans* (Brook stickleback)	0.06	0.001	11.74
		Pimephales promelas (Fathead minnow)	0.24	0.17	10.58		*Notropis atherinoides* (Emerald shiner)	0.0003	0.02	8.98
		Lepomis gibbosus (Pumpkinseed)	0.09	**0.57**	9.16		*Lepomis macrochirus* (Bluegill sunfish)	0.0005	0.17	8.22
		Carassius auratus (Goldfish)	**0.23**	0.03	5.91		*Notropis hudsonius* (Spottail shiner)	0.009	0.08	6.6
		Culaea inconstans (Brook stickleback)	0.18	0	5.26					
A. W3 (850 m)	95.09%	*Neogobius melanostomus* (Round goby)	0.006	0.41	20.06	92.73%	*Neogobius melanostomus* (Round goby)	0.2	0.12	19.38
B. W5 (-1400 m)		*Lepomis cyanellus* (Green sunfish)	**0.72**	0.37	18.38		*Pimephales promelas* (Fathead minnow)	0.05	**0.17**	14.96
		Lepomis macrochirus (Bluegill sunfish)	0.06	0.3	15.48		*Lepomis cyanellus* (Green sunfish)	0.1	0.05	13.62
		Pimephales promelas (Fathead minnow)	0.24	0.13	6.46		*Lepomis macrochirus* (Bluegill sunfish)	0.0005	0.08	12.52
		Carassius auratus (Goldfish)	**0.23**	0.001	5.83		*Culaea inconstans* (Brook stickleback)	0.06	0.0009	10.12
		Lepomis gibbosus (Pumpkinseed)	0.09	0.09	5.49		*Lepomis gibbosus* (Pumpkinseed)	0.05	0.08	7.62
		Culaea inconstans (Brook stickleback)	0.18	0	5.49		*Notropis hudsonius* (Spottail shiner)	0.009	0.04	5.28
		Scardinius erythrophthalmus (Rudd)	0	0.64	5.07					
A. W4 (-1000 m)	87.58%	*Neogobius melanostomus* (Round goby)	0.87	0.41	22.79	92.73%	*Lepomis cyanellus* (Green sunfish)	0.2	0.05	16.82
B. W5 (-1400 m)		*Lepomis macrochirus* (Bluegill sunfish)	**0.47**	0.3	20.06		*Lepomis macrochirus* (Bluegill sunfish)	0.17	0.08	16.49
		Lepomis cyanellus (Green sunfish)	0.23	0.37	11.88		*Pimephales promelas* (Fathead minnow)	0	0.17	11.94
		Pimephales promelas (Fathead minnow)	0.17	0.13	8.81		*Neogobius melanostomus* (Round goby)	0.08	0.12	9.04
		Lepomis gibbosus (Pumpkinseed)	**0.57**	0.09	8.38		*Notropis atherinoides* (Emerald shiner)	0.02	0.01	8.6
		Scardinius erythrophthalmus (Rudd)	0.03	0.64	5.01		*Notropis hudsonius* (Spottail shiner)	0.08	0.04	7.06
							Lepomis gibbosus (Pumpkinseed)	0	0.08	5.73
							Pimephales notatus (Bluntnose minnow)	0.08	0.0007	5.68

Table S11. Proportion of variance explained and loadings for the first two principal components produced from the principal component analysis (PCA) on water quality parameters measured along the effluent gradient of both WWTPs during the summer and winter.

	Dundas WWTP				**Woodward WWTP**			
	Summer		**Winter**		**Summer**		**Winter**	
Measure	**PC1 (47.5%)**	**PC2 (18.3%)**	**PC1 (63.9%)**	**PC2 (15.9%)**	**PC1 (45.0%)**	**PC2 (37.6%)**	**PC1 (66.4%)**	**PC2 (18.0%)**
Conductivity	-0.39	0.26	-0.35	0.34	-0.09	0.47	0.20	-0.56
TDS	-0.39	0.26	-0.35	0.32	-0.10	0.47	0.23	-0.47
Salinity	-0.39	0.24	-0.35	0.31	-0.05	0.48	0.23	-0.54
pH	0.31	-0.03	0.35	0.20	0.35	0.19	0.28	0.14
Temperature	0.27	0.36	-0.29	-0.21	0.12	0.32	-0.32	-0.07
DO	0.01	-0.02	0.21	0.64	0.09	0.41	0.33	0.25
TAN	-0.13	0.45	-0.22	-0.42	-0.31	0.02	-0.28	-0.04
TNN	-0.38	-0.16	-0.37	0.07	-0.43	-0.05	-0.35	-0.19
TN	-0.38	-0.14	-0.38	0.06	-0.45	0.01	-0.35	-0.14
SRP	0.17	0.55	-	-	-0.43	-0.02	-0.35	-0.03
TP	0.19	0.35	-0.23	-0.13	-0.41	0.12	-0.35	-0.18

1. Supplementary results

1.1 Species tolerance, resilience, trophic level, and proportion of non-natives

1.1.1 Dundas WWTP

The proportion of non-native fish caught tended to be higher in the summer ($t = -2.26$, $p = 0.07$), but that trend was not affected by proximity to the outfall in either season ($t_{\text{(Summer)}} = -0.67$, $p = 0.51$; PLMM, $t_{\text{(Winter)}} = -1.50$, $p = 0.15$). The proportion of tolerant fish was slightly higher in the summer, although not significantly (PLMM, $t = -2.03$, $p = 0.09$). In the winter, more tolerant fish species were found near the outfall ($t_{\text{(Winter)}} = -2.64$, $p = 0.01$); this trend was also observed in the summer but it did not reach significance ($t_{\text{(Summer)}} = -1.89$, $p = 0.06$). In contrast, species resilience did not differe between seasons ($t = 0.13$; $p = 0.91$), nor was it affected by proximity to the effluent in either season ($t_{\text{(Summer)}} = 1.12$, $p = 0.27$; $t_{\text{(Winter)}} = 0.32$, $p = 0.76$). Finally, overall fish caught in the summer and winter were of similar trophic level ($t = 1.47$, $p = 0.20$). When broken down by season, fish caught far away from the outfall tended to be of higher trophic level than those nearby during the winter ($t_{(Winter)} = 2.68$, $p = 0.01$), however, that trend was not observed in the summer ($t_{(Summer)} = -0.76$, $p = 0.45$).

1.1.2 Woodward WWTP

The proportion of non-native fish caught was not significantly different between seasons ($t = 0.28$, $p = 0.79$). During the summer, a greater proportion of non-native species were caught far away from the outfall ($t_{\text{(Summer)}} = 2.03$, $p = 0.045$), while this trend was reversed during the winter ($t_{\text{(Winter)}} = -3.02$, $p < 0.01$).

Species tolerance was not affected by season ($t = 0.47$, $p = 0.66$), nor proximity to the outfall in either season ($t_{(\mathrm{Summer})} = -0.53$, $p = 0.60$; $t_{(\mathrm{Winter})} = 0.41$, $p = 0.41$). Fish caught in the winter tended to be more resilient, but that trend was not significant ($t = 2.00$, $p = 0.098$). Fish caught far away from the effluent tended to be more resilient in the winter ($t_{(\mathrm{Winter})} = 1.74$, $p = 0.09$), but not in the summer ($t_{(\mathrm{Summer})} = -0.23$, $p = 0.82$). Finally, fish caught in either season did not differ in trophic level ($t = 0.33$, $p = 0.76$). In the winter however, fish caught near the outfall were of higher trophic level and the average trophic level of fish decreased with distance ($t_{(\mathrm{Winter})} = -3.39$, $p < 0.01$), while no similar trend was observed in the summer ($t_{(\mathrm{Summer})} = 1.17$, $p = 0.25$).

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Appendix D: Supplementary materials for Chapter 3

S1.0 Habitat descriptions

Table S1

S2.0 Basket deployment and sample enumeration

Table S2

S3.0 Composite effluent quality

S4.0 Composite macroinvertebrate richness

Table S4

S5.0 SIMPER analysis

S5.1 Woodward WWTP

Table S4

S5.2 Dundas WWTP

Table S5

S1.0 Habitat descriptions

Table S1 Habitat characteristics of the Woodward WWTP and Dundas WWTP sampling sites following a subset of the metrics used in the Qualitative Habitat Evaluation Index (QHEI; Taft and Koncelik, 2006; Strickland et al. 2010). Aquatic vegetation was only recorded during the summer as it was absent during the winter. Total depth and clarity are reported as means ± SE for summer | winter. Sites ordered from left to right according to flow. These habitat characteristics have been previously published in research that examined fish communities at these sites (McCallum et al. 2019; Mehdi et al. 2021).

Woodward WWTP	**US1 (-1400 m)**	**US2 (-1000 m)**	**DS1 (Outfall)**	**DS2 (350 m)**	**DS3 (850 m)**
Aquatic habitat					
Bottom substrate (% coverage)	50% cobble 50% silt	50% cobble 50% silt	100% cobble	50% cobble 50% boulder	50% cobble 50% boulder
Bottom slope	≥ 45°	≥ 45°	≥ 45°	≥ 45°	≥ 25°
Shoreline habitat					
Sinuosity	None-straight	None-straight	Low	Low-moderate	Low
Anthropogenic modifications	Recent, recovering	Recent, recovering	Recent, recovering	Recent	Recent
Shoreline quality	Urban, industrial	Urban, industrial	Urban, industrial	Urban, industrial	Urban, industrial
Bank erosion	Low	Low	Low	Low	Low
Riparian width	Narrow (5-10 m)	Narrow (5-10 m)	Narrow (5-10 m)	Very narrow (<5 m)	Very narrow (<5 m)
Additional measures					
Total depth (cm) Summer | Winter	138±24.0 | 78.3±10.1	131±19.7 | 98.3±6.66	210±25.5 | 192±6.0	215±15.7 | 155±23.6	210±29.2 | 142±13.0
Clarity (%) Summer | Winter	35.2±12.1 | 66.7±16.7	34.6±8.62 | 60.5±5.59	58.0±8.77 | 63.2±3.85	52.2±10.9 | 76.1±10.3	45.3±1.47 | 66.1±17.4
Aquatic vegetation	Lesser duckweed, water smartweed	Lesser duckweed, water smartweed	Algae on rocks, pondweed	Algae on rocks, Canada waterweed, lesser/greater duckweed, smartweed	Floating pondweed, lesser duckweed, pondweed, smartweed
Site comments	Near large highway and pedestrian bridge, cemented shoreline	Near large highway, cemented shoreline	Near large highway, modified shoreline	Modified shoreline, bank shaping	Modified shoreline, bank shaping

Table S1. Continued

Dundas WWTP	**DS1 (Outfall)**	**DS2 (550 m)**	**DS3 (1000 m)**	**DS4 (2800 m)**	**DS5 (3750 m)**
Aquatic habitat					
Bottom substrate (% coverage)	85% cobble 15% boulder	90% sand 10% gravel	100% silt	100% silt	100% silt
Bottom slope	> 45°	> 25°	< 15°	< 15°	< 15°
Shoreline habitat					
Sinuosity	None, straight	None, straight	Low, 1 curve	Low, 1 curve	Low, 1 curve
Anthropogenic modifications	Recent, recovering	Recent, recovering	None	None	None
Shoreline quality	Urban, industrial, residential trail	Urban, industrial, residential trail	Forest, wetland	Forest, wetland	Forest, wetland
Bank erosion	Moderate (25-50%)	Moderate (25-50%)	Low (0-25 %)	Low (0-25 %)	Low (0-25 %)
Riparian width	Narrow (5-10 m)	Moderate (10-50 m)	Wide (>50 m)	Wide (>50 m)	Wide (>50 m)
Additional measures					
Total depth (cm) Summer | Winter	189±51.7 | 164±1.73	190±59.7 | 142±18.9	81.3±34.5 | 46.7±2.88	99.3±21.0 | 73.3±15.3	96.3±41.1 | 46.7±2.88
Clarity (%) Summer | Winter	45.0±13.6 | 92.5±7.57	34.8±9.33 | 79.2±18.1	70.7±25.8 | 100±0	50.1±15.0 | 100±0	29.6±2.26 | 82.6±10.9
Aquatic vegetation	Algae, lesser/greater duckweed, milfoil	filamentous algae, lesser/greater duckweed, milfoil	Filamentous algae, lesser/greater duckweed, milfoil, water lily	Lesser/greater duckweed, liverwort, water lily, potamogeton	Lesser/greater duckweed, milfoil, water lily
Site comments	Human-made canal, erosion-prevention logs, bank built of cobble	Human-made canal, undercut banks, overhanging *phragmites*	Natural site, cattail stands between site and shore	Fencing near water flowering rush between site and shore	Natural site, cattail stands between site and shore

S2.0 Basket deployment and sample enumeration

Table S2. Information about rock baskets that were deployed and retrieved at the Dundas WWTP and Woodward WWTP sites during summer and winter as well notes on subsampling information. Samples were mixed thoroughly prior to subsampling. If subsampling was performed, then a 2-minute rare taxa examination was completed on the whole sample to prevent rare taxa from being over- or under-estimated.

Season	Location	Deployment Date	Collection Date	Site	Baskets Deployed	Baskets/Samples Collected	Subsampling Notes
Summer	Dundas	July 10, 2018	September 4, 2018	DS1	6	5	N/A
				DS2	6	6	N/A
				DS3	6	6	N/A
				DS4	6	5	All were subsampled for all organisms (4 samples in quarters, 1 sample in halves)
				DS5	6	4	N/A
	Woodard	July 15, 2018	September 10, 2018	US1	6	4	N/A
				US2	6	6	N/A
				DS1	6	4	3 were subsampled for all organisms (2 samples in quarters, 1 sample in halves)
				DS2	6	6	2 were subsampled for all organisms (2 samples in quarters), 1 sample was subsampled for isopods only (in quarters)
				DS3	6	4	All 4 were subsampled for all organisms (4 samples in quarters)
Winter	Dundas	November 16, 2019	January 10, 2020	DS1	5	5	N/A
				DS2	5	5	N/A
				DS3	5	3	N/A
				DS4	5	5	N/A
				DS5	0	N/A	N/A
	Woodward	November 17, 2019	January 11, 2020	US1	5	5	N/A
				US2	5	5	N/A
				DS1	5	5	All 4 were subsampled for isopods only (4 samples in quarters)
				DS2	5	5	N/A
				DS3	5	0	N/A

S3.0 Composite effluent water quality

Table S3. Water quality parameters measured on the 24-hr composite final treated effluent produced from the Dundas and Woodward WWTPs during both seasons. Not all parameters were measured in every sample. At the Woodward WWTP, TSS, BOD, TP, TKN, NH_3, NO_3, and NO_2 were measured in 22 samples in the summer and 22 samples in the winter, whereas *E. coli* was measured in 5 samples in the summer and 4 samples in the winter. At the Dundas WWTP, TSS, BOD, TP, TKN, NH_3, NO_3, NO_2, and *E. coli* were measured in 4 samples in the summer and 4 samples in the winter. Bolded values indicate significant difference between seasons within each WWTP ($p < 0.05$).

	Dundas WWTP		Woodward WWTP	
	Summer	Winter	Summer	Winter
Total suspended solids (mg/L)	0.80±0	0.80±0	4.55±0.41	**12.1±1.20**
Biochemical oxygen demand (mg/L)	1.25±0.25	1.25±0.25	3.61±0.14	**7.27±0.82**
Total phosphorus (mg/L)	0.12±0.01	0.06±0	0.56±0.02	0.58±0.04
Total Kjeldahl nitrogen (mg/L)	0.6±0.07	0.65±0.04	1.66±0.08	3.22±0.31
Ammonia (mg/L)	0.04±0	0.06±0.01	0.60±0.09	**1.85±0.23**
Nitrate (mg/L)	16.7±0.38	16.9±1.04	**14.4±0.44**	6.65±0.79
Nitrite (mg/L)	0.08±0.01	0.21±0.09	0.17±0	**4.25±0.58**
Escherichia coli (CFU/100 mL)	10.0±0	9530±5210	28.0±13.2	**8250±2080**

S4.0 Benthic macroinvertebrate richness

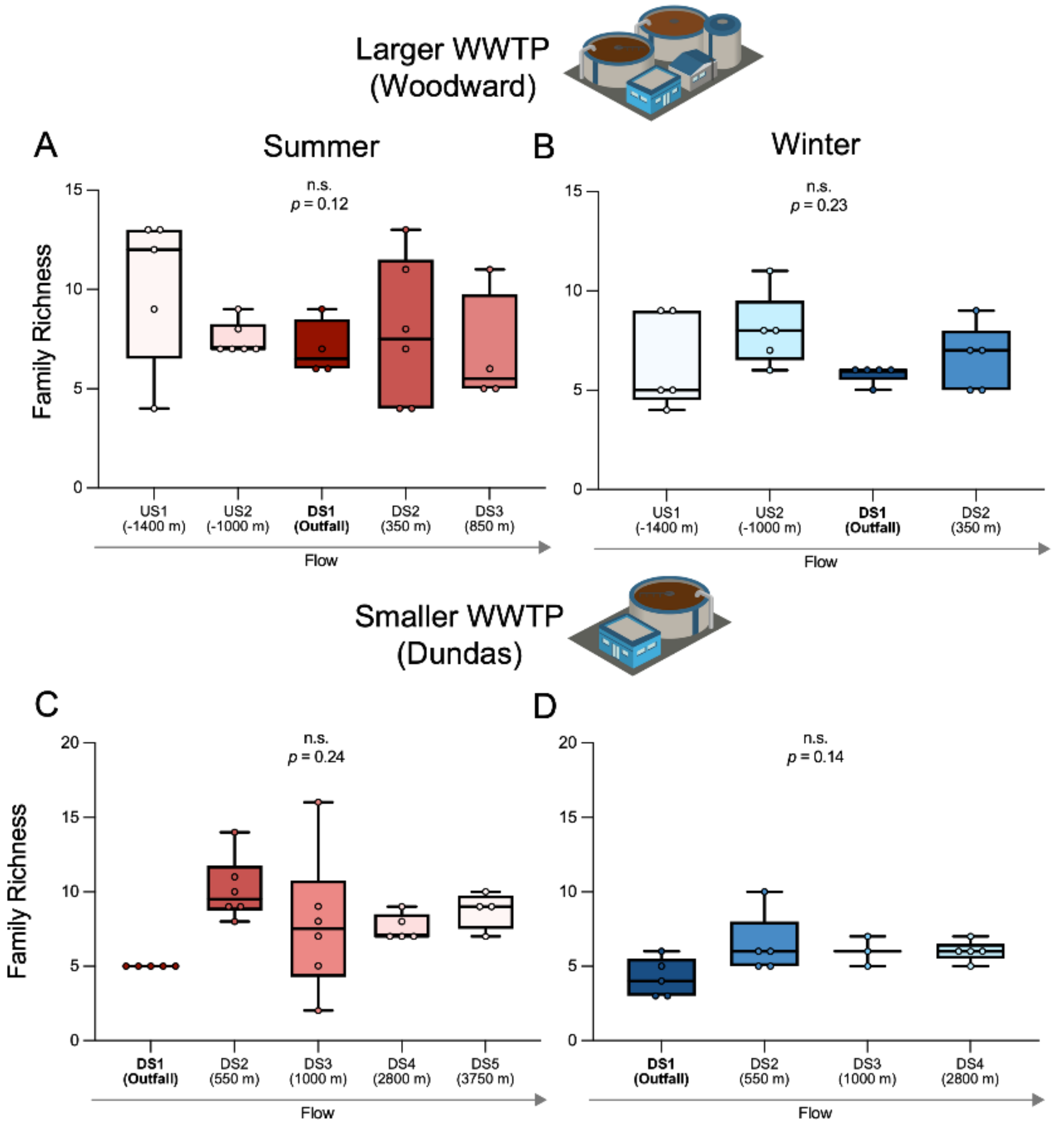

Figure S1. Boxplots showing family richness for the Woodward WWTP in the summer **(A)** and winter **(B),** and the Dundas WWTP in the summer **(C)** and winter **(D)**. Boxplots show the median and inter-quartile range, whiskers show minimum and maximum values, and individual data points are jittered to allow for greater readability. Summer data is represented in red and winter data is represented in blue; the intensity of the colours signify proximity to the outfall (darker colours being closest and light colours being most distant). All data are presented as per basket (N = 3 – 6 samples/site; see Supplementary Table S3 for sample sizes by site and season). Significant effects of proximity to the outfall in Dundas and differences between upstream and downstream sites at Woodward are indicated by (*$p \leq 0.05$), (**$p \leq 0.01$), (***$p \leq 0.001$), and (no significance, *n.s.*).

S5.0 SIMPER Analysis

S5.1 Woodward WWTP

Table S4. Similarity percentages analysis (SIMPER) indicating the relative contribution of family groups to overall dissimilarity of each Woodward site comparison. Average A and B are based on the abundance for each family group at the sites being compared. Sites are ordered according to proximity to outfall and contamination load. Only family groups that contribute ≥5% to overall abundance are shown. Bolded values indicate significant differences ($p < 0.05$).

	Woodward									
	Summer					Winter				
Comparison	Total dissimilarity	Family	Average A	Average B	Contribution	Total dissimilarity	Family	Average A	Average B	Contribution
A. DS1 (Outfall)	54.00%	*Asellidae*	1930	877	43.3	82.07%	***Asellidae***	**2190**	**182**	**80.5**
B. DS2 (350 m)										
A. DS1 (Outfall)	46.23%	*Asellidae*	1930	1040	22.1	NA				
B. DS3 (850 m)		*Valvatidae*	0.00	587	15.1					
A. DS1 (Outfall)	98.23%	***Asellidae***	**1930**	**6.83**	**90.1**	98.67%	***Asellidae***	**2190**	**9.40**	**86.6**
B. US2 (-1000 m)							*Gammaridae*	0.00	206	9.42
A. DS1 (Outfall)	97.33%	***Asellidae***	**1930**	**8.40**	**88.7**	98.97%	***Asellidae***	**2190**	**6.60**	**96.7**
B. US1 (-1400 m)										
A. DS2 (350 m)	61.39%	*Asellidae*	877	1040	27.6	NA				
B. DS3 (850 m)		***Valvatidae***	**0.00**	**587**	**20.6**					
		Hydrobiidae	20.8	171	5.32					
A. DS2 (350 m)	92.46%	***Asellidae***	**877**	**6.83**	**57.0**	93.71%	***Gammaridae***	**0.40**	**206**	**43.9**
B. US2 (-1000 m)		***Erpobdellidae***	**87.8**	**5.33**	**15.3**		*Asellidae*	182	9.40	36.1
		Glossiphonidae	**80.5**	**1.33**	**10.2**					
A. DS2 (350 m)	91.54%	***Asellidae***	**877**	**8.40**	**55.3**	90.67%	***Asellidae***	**182**	**6.60**	**70.3**
B. US1 (-1400 m)		***Erpobdellidae***	**87.8**	**12.6**	**13.7**		***Erpobdellidae***	**21.6**	**1.20**	**8.45**
		Glossiphonidae	**80.5**	**4.00**	**10.0**		*Gammaridae*	0.40	13.2	5.84

A. DS3 (850 m)	96.69%	*Asellidae*	1040	6.83	53.5	NA				
B. US2 (-1000 m)		***Valvatidae***	**587**	**0.17**	**29.7**					
		Hydrobiidae	**171**	**17.8**	**7.57**					
A. DS3 (850 m)	97.02%	*Asellidae*	1040	8.40	52.6	NA				
B. US1 (-1400 m)		***Valvatidae***	**587**	**0.20**	**29.3**					
		Hydrobiidae	**171**	**5.60**	**7.99**					
A. US2 (-1000 m)	66.36%	***Coenagrionidae***	**14.7**	**18.0**	**10.4**	81.96%	***Gammaridae***	**206**	**13.2**	**64.5**
B. US1 (-1400 m)		***Hydrobiidae***	**17.8**	**5.60**	**9.87**		***Chironomidae***	**20.8**	**3.20**	**6.01**
		Naididae	**1.33**	**23.0**	**9.40**					
		Asellidae	6.83	8.40	7.03					
		Erpobdellidae	5.33	12.6	6.49					
		Sphaeridae	**2.00**	**9.20**	**5.71**					

S5.2 Dundas WWTP

Table S5. Similarity percentages analysis (SIMPER) indicating the relative contribution of family groups to overall dissimilarity of each Dundas site comparison. Average A and B are based on the abundance for each family group at the sites being compared. Sites are ordered according to proximity to outfall and contamination load. Only family groups that contribute ≥5% to overall abundance are shown. Bolded values indicate significant differences ($p < 0.05$).

	Dundas									
	Summer					Winter				
Comparison	Total dissimilarity	Family	Average A	Average B	Contribution	Total dissimilarity	Family	Average A	Average B	Contribution
A. DS1 (Outfall)	61.69%	***Chironomidae***	**8.40**	**24.0**	**12.8**	89.02%	*Gammaridae*	1.20	44.2	40.8
B. DS2 (550 m)		*Asellidae*	19.0	16.3	10.7		*Coenagrionidae*	1.20	18.2	16.4
		Coenagrionidae	**27.8**	**14.5**	**10.4**		***Caenidae***	**0.40**	**15.8**	**13.4**
		Caenidae	**1.00**	**18.3**	**9.77**		***Asellidae***	**3.20**	**14.0**	**11.5**
		Gammaridae	0.40	9.50	5.79					
A. DS1 (Outfall)	74.62%	***Asellidae***	**19.0**	**120**	**40.2**	92.99%	*Gammaridae*	1.20	72.0	44.0
B. DS3 (1000 m)	_	***Coenagrionidae***	**27.8**	**12.5**	**13.2**		***Coenagrionidae***	**1.20**	**53.3**	**39.3**
A. DS1 (Outfall)	96.32%	***Gammaridae***	**0.40**	**1870**	**90.4**	94.53%	***Gammaridae***	**1.20**	**209**	**86.3**
B. DS4 (2800 m)										
A. DS1 (Outfall)	86.46%	***Asellidae***	**19.0**	**120**	**34.8**	NA				
B. DS5 (3750 m)		*Gammaridae*	0.400	40.0	11.5					
		Naididae	**1.60**	**37.3**	**10.3**					
		Erpobdellidae	**0.00**	**28.0**	**9.12**					
		Coenagrionidae	27.8	0.500	8.70					
A. DS2 (550 m)	73.84%	***Asellidae***	**16.3**	**120**	**33.3**	49.08%	*Coenagrionidae*	18.2	53.3	16.3
B. DS3 (1000 m)		***Chironomidae***	**24.0**	**6.00**	**9.10**		*Gammaridae*	44.2	72.0	16.0
		Caenidae	**18.3**	**0.500**	**7.06**		*Caenidae*	15.8	0.00	6.45
		Coenagrionidae	14.5	12.5	5.79		*Asellidae*	14.0	3.00	5.24
A. DS2 (550 m)	95.47%	***Gammaridae***	**9.50**	**1870**	**80.8**	64.69%	*Gammaridae*	44.2	209	48.8
B. DS4 (2800 m)							*Coenagrionidae*	18.2	2.40	5.03

A. DS2 (550 m)	82.22%	***Asellidae***	**16.3**	**120**	**30.9**	NA				
B. DS5 (3750 m)		***Naididae***	**0.17**	**37.3**	**9.60**					
		Gammaridae	9.50	40.0	8.85					
		Erpobdellidae	**5.17**	**28.00**	**7.64**					
		Chironomidae	24.00	0.25	6.61					
A. DS3 (1000 m)	96.76%	***Gammaridae***	**9.33**	**1868.80**	**85.41**	57.18%	*Gammaridae*	72.00	209.20	36.72
B. DS4 (2800 m)							*Coenagrionidae*	53.33	2.40	14.81
A. DS3 (1000 m)	56.55	*Asellidae*	119.83	119.75	19.90	NA				
B. DS5 (3750 m)		*Gammaridae*	9.33	40.00	8.38					
		Naididae	**2.50**	**37.25**	**8.02**					
		Erpobdellidae	**4.33**	**28.00**	**6.15**					
A. DS4 (2800 m)	93.94%	***Gammaridae***	**1868.80**	**40.00**	**80.77**	NA				
B. DS5 (3750 m)										

Appendix E: Supplementary materials for Chapter 4

Table of Contents

Table S1. Habitat characteristics of the Dundas WWTP and Woodward WWTP sampling sites following a subset of the metrics used in the Qualitative Habitat Evaluation Index (QHEI; Taft and Koncelik, 2006; Strickland et al. 2010). Aquatic vegetation was only recorded during the summer as it was absent during the winter. Total depth and clarity are reported as means ± SE for summer | winter. These habitat characteristics have been previously published in research that examined fish communities at these sites (McCallum et al. 2019; Mehdi et al. 2021).

Dundas WWTP	**D1 (Outfall)**	**D2 (550 m)**	**D3 (1000 m)**	**D4 (2800 m)**	**D5 (3750 m)**
Aquatic habitat					
Bottom substrate	85% cobble	90% sand	100% silt	100% silt	100% silt
(% coverage)	15% boulder	10% gravel			
Bottom slope	> 45°	> 25°	< 15°	< 15°	< 15°
Shoreline habitat					
Sinuosity	None, straight	None, straight	Low, 1 curve	Low, 1 curve	Low, 1 curve
Anthropogenic modifications	Recent, recovering	Recent, recovering	None	None	None
Shoreline quality	Urban, industrial, residential trail	Urban, industrial, residential trail	Forest, wetland	Forest, wetland	Forest, wetland
Bank erosion	Moderate (25-50%)	Moderate (25-50%)	Low (0-25 %)	Low (0-25 %)	Low (0-25 %)
Riparian width	Narrow (5-10 m)	Moderate (10-50 m)	Wide (>50 m)	Wide (>50 m)	Wide (>50 m)
Additional measures					
Total depth (cm) Summer | Winter	189±51.7 | 164±1.73	190±59.7 | 142±18.9	81.3±34.5 | 46.7±2.88	99.3±21.0 | 73.3±15.3	96.3±41.1 | 46.7±2.88
Clarity (%) Summer | Winter	45.0±13.6 | 92.5±7.57	34.8±9.33 | 79.2±18.1	70.7±25.8 | 100±0	50.1±15.0 | 100±0	29.6±2.26 | 82.6±10.9
Aquatic vegetation	Algae, lesser/greater duckweed, milfoil	filamentous algae, lesser/greater duckweed, milfoil	Filamentous algae, lesser/greater duckweed, milfoil, water lily	Lesser/greater duckweed, liverwort, water lily, potamogeton	Lesser/greater duckweed, milfoil, water lily
Site comments	Human-made canal, erosion-prevention logs, bank built of cobble	Human-made canal, undercut banks, overhanging *phragmites*	Natural site, cattail stands between site and shore	Fencing near water flowering rush between site and shore	Natural site, cattail stands between site and shore

Table S1. Continued

Woodward WWTP	**W1 (Outfall)**	**W2 (350 m)**	**W3 (850 m)**	**W4 (-1000 m)**	**W5 (-1400 m)**
Aquatic habitat					
Bottom substrate	100% cobble	50% cobble	50% cobble	50% cobble	50% cobble
(% coverage)		50% boulder	50% boulder	50% silt	50% silt
Bottom slope	≥ 45°	≥ 45°	≥ 25°	≥ 45°	≥ 45°
Shoreline habitat					
Sinuosity	Low	Low-moderate	Low	None-straight	None-straight
Anthropogenic modifications	Recent, recovering	Recent	Recent	Recent, recovering	Recent, recovering
Shoreline quality	Urban, industrial	Urban, industrial	Urban, industrial	Urban, industrial	Urban, industrial
Bank erosion	Low	Low	Low	Low	Low
Riparian width	Narrow (5-10 m)	Very narrow (<5 m)	Very narrow (<5 m)	Narrow (5-10 m)	Narrow (5-10 m)
Additional measures					
Total depth (cm) Summer | Winter	210±25.5 | 192±6.0	215±15.7 | 155±23.6	210±29.2 | 142±13.0	131±19.7 | 98.3±6.66	138±24.0 | 78.3±10.1
Clarity (%) Summer | Winter	58.0±8.77 | 63.2±3.85	52.2±10.9 | 76.1±10.3	45.3±1.47 | 66.1±17.4	34.6±8.62 | 60.5±5.59	35.2±12.1 | 66.7±16.7
Aquatic vegetation	Algae on rocks, pondweed	Algae on rocks, Canada waterweed, lesser/greater duckweed, smartweed	Floating pondweed, lesser duckweed, pondweed, smartweed	Lesser duckweed, water smartweed	Lesser duckweed, water smartweed
Site comments	Near large highway, modified shoreline	Modified shoreline, bank shaping	Modified shoreline, bank shaping	Near large highway, cemented shoreline	Near large highway and pedestrian bridge, cemented shoreline

Table S2. Water quality parameters measured on the 24-hr composite final treated effluent produced from the Dundas and Woodward WWTPs during both seasons. Not all parameters were measured in every sample. At the Woodward WWTP, TSS, BOD, TP, TKN, NH_3, NO_3, and NO_2 were measured in 22 samples in the summer and 22 samples in the winter, whereas *E. coli* was measured in 5 samples in the summer and 4 samples in the winter. At the Dundas WWTP, TSS, BOD, TP, TKN, NH_3, NO_3, NO_2, and *E. coli* were measured in 4 samples in the summer and 4 samples in the winter. Bolded means indicate significant difference between summer and winter within each WWTP.

	Dundas WWTP		Woodward WWTP	
	Summer	Winter	Summer	Winter
Total suspended solids (mg/L)	0.80±0	0.80±0	4.55±0.41	**12.1±1.20**
Biochemical oxygen demand (mg/L)	1.25±0.25	1.25±0.25	3.61±0.14	**7.27±0.82**
Total phosphorus (mg/L)	0.12±0.01	0.06±0	0.56±0.02	0.58±0.04
Total Kjeldahl nitrogen (mg/L)	0.6±0.07	0.65±0.04	1.66±0.08	3.22±0.31
Ammonia (mg/L)	0.04±0	0.06±0.01	0.60±0.09	**1.85±0.23**
Nitrate (mg/L)	16.7±0.38	16.9±1.04	**14.4±0.44**	6.65±0.79
Nitrite (mg/L)	0.08±0.01	0.21±0.09	0.17±0	**4.25±0.58**
Escherichia coli (CFU/100 mL)	10.0±0	9530±5210	28.0±13.2	**8250±2080**

Table S3. Simper analysis of all other sites

	Dundas WWTP									
	Summer					Winter				
Comparison	Total dissimilarity	Species	Average A	Average B	Contribution	Total dissimilarity	Species	Average A	Average B	Contribution
A. D2 (550 m) B. D3 (1000 m)	63.7%	Bosminidae	5.20	1.92	25.4	73.9%	Chydoridae	**4.89**	3.27	58.5
		Copepods	1.76	3.61	22.0		Copepods	1.19	1.09	14.4
		Daphnidae	1.68	0.877	8.17					
A. D2 (550 m) B. D4 (2800 m)	62.9%	Bosminidae	5.20	0.530	26.7	75.6%	Chydoridae	4.89	0.871	39.8
		Copepods	1.76	1.04	17.3		Copepods	1.19	1.35	29.4
		Daphnidae	1.67	0.152	7.71					
		Sididae	0.923	0.237	6.31					
		Chydoridae	**0.398**	0.199	5.00					
A. D2 (550 m) B. D5 (3750 m)	81.1%	Sididae	0.923	61.6	28.5	77.7%	Copepods	1.19	3.28	33
		Copepods	1.76	5.31	24.8		Chydoridae	4.89	0.492	29.4
		Bosminidae	5.20	0.123	17.6		Bosminidae	0.057	**0.568**	14.1
		Daphnidae	1.67	0.862	7.69					
A. D3 (1000 m) B. D4 (2800 m)	63.5%	Copepods	**3.61**	1.04	32.1	79.0%	Chydoridae	**3.27**	0.871	56.6
		Bosminidae	1.92	0.53	15.0		Copepods	1.09	1.35	21.1
		Daphnidae	0.867	0.152	5.95					
		Chydoridae	**0.696**	0.199	5.77					
A. D3 (1000 m) B. D5 (3750 m)	75.10%	Copepods	3.61	**5.31**	30.4	70.1%	Chydoridae	3.27	0.492	33.8
		Sididae	0.398	61.6	27.2		Copepods	1.09	3.28	25.3
		Bosminidae	1.92	0.123	7.75		Bosminidae	0	0.568	10.1
		Daphnidae	0.867	0.862	6.53					
A. D4 (2800 m) B. D5 (3750 m)	79.4%	Copepods	1.04	**5.31**	32.9	81.1%	Copepods	1.35	**3.28**	47.4
		Sididae	0.237	**61.6**	30.2		Bosminidae	0.013	**0.568**	19.2
		Daphnidae	0.152	0.862	7.12		Chydoridae	0.871	0.492	12.5
		Bosminidae	0.53	0.123	6.20					

Table S3. Continued

	Woodward WWTP									
	Summer					Winter				
Comparison	Total dissimilarity	Species	Average A	Average B	Contribution	Total dissimilarity	Species	Average A	Average B	Contribution
A. W2 (350 m) B. W3 (850 m)	72.8%	Bosminidae	1.85	**5.58**	29.4	77.2%	Chydoridae	1.74	2.77	51.1
		Chydoridae	0.085	0.298	14.8		Daphnidae	0.032	0.215	13.3
		Copepods	0.142	0.341	14.2		Copepods	0.13	0.127	9.62
		Daphnidae	0.114	**0.426**	12.0					
A. W2 (350 m) B. W4 (-1000 m)	88.6%	Sididae	0.043	**4.43**	43.1	74.4%	Chydoridae	1.74	0.313	50.4
		Copepods	0.142	1.97	26.4		Copepods	0.13	0.114	13.3
		Chydoridae	0.085	0.369	11.6		Bosminidae	0.032	0.085	6.35
A. W2 (350 m) B. W5 (-1400 m)	91.0%	*Copepods*	0.142	**6.43**	41.3	82.3%	Chydoridae	1.74	0.227	55.2
		Sididae	0.043	2.96	28.1		Copepods	0.13	0.028	18.3
		Chydoridae	0.085	1.31	15.7		Bosminidae	0.032	0	5.84
A. W3 (850 m) B. W4 (-1000 m)	86.3%	Sididae	0.014	Sididae	37.0	78.4%	Chydoridae	2.77	0.313	47.3
		Copepods	0.341	1.97	22.1		Daphnidae	0.215	0.057	17.9
		Bosminidae	5.58	0.028	14.5		Copepods	0.127	0.114	7.76
		Chydoridae	0.298	0.369	8.45		Bosminidae	0.025	0.085	5.33
A. W3 (850 m) B. W5 (-1400 m)	89.0%	Copepods	0.341	**6.43**	34.3	89.5%	Chydoridae	2.77	0.227	48.9
		Sididae	0.014	2.96	23.2		Daphnidae	**0.215**	0	26.3
		Chydoridae	0.298	1.31	14.2		Copepods	0.126	0.028	11.6
		Bosminidae	5.58	0.019	13.9					
A. W4 (-1000 m) B. W5 (-1400 m)	65.3%	Sididae	4.43	2.96	27.1	63.7%	Chydoridae	0.313	0.227	34.0
		Copepods	1.97	6.43	25.0		Copepods	0.114	0.028	16.6
		Chydoridae	0.369	1.31	11.2		Bosminidae	**0.085**	0	8.54

Table S4. Proportion of variance explained and loadings for the first two principal components produced from the principal component analysis (PCA) on water quality parameters measured along the effluent gradient of both WWTPs during the summer and winter.

	Dundas WWTP				**Woodward WWTP**			
	Summer		**Winter**		**Summer**		**Winter**	
Measure	**PC1 (47.5%)**	**PC2 (18.3%)**	**PC1 (63.9%)**	**PC2 (15.9%)**	**PC1 (45.0%)**	**PC2 (37.6%)**	**PC1 (66.4%)**	**PC2 (18.0%)**
Conductivity	-0.39	0.26	-0.35	0.34	-0.09	0.47	0.20	-0.56
TDS	-0.39	0.26	-0.35	0.32	-0.10	0.47	0.23	-0.47
Salinity	-0.39	0.24	-0.35	0.31	-0.05	0.48	0.23	-0.54
pH	0.31	-0.03	0.35	0.20	0.35	0.19	0.28	0.14
Temperature	0.27	0.36	-0.29	-0.21	0.12	0.32	-0.32	-0.07
DO	0.01	-0.02	0.21	0.64	0.09	0.41	0.33	0.25
TAN	-0.13	0.45	-0.22	-0.42	-0.31	0.02	-0.28	-0.04
TNN	-0.38	-0.16	-0.37	0.07	-0.43	-0.05	-0.35	-0.19
TN	-0.38	-0.14	-0.38	0.06	-0.45	0.01	-0.35	-0.14
SRP	0.17	0.55	-	-	-0.43	-0.02	-0.35	-0.03
TP	0.19	0.35	-0.23	-0.13	-0.41	0.12	-0.35	-0.18

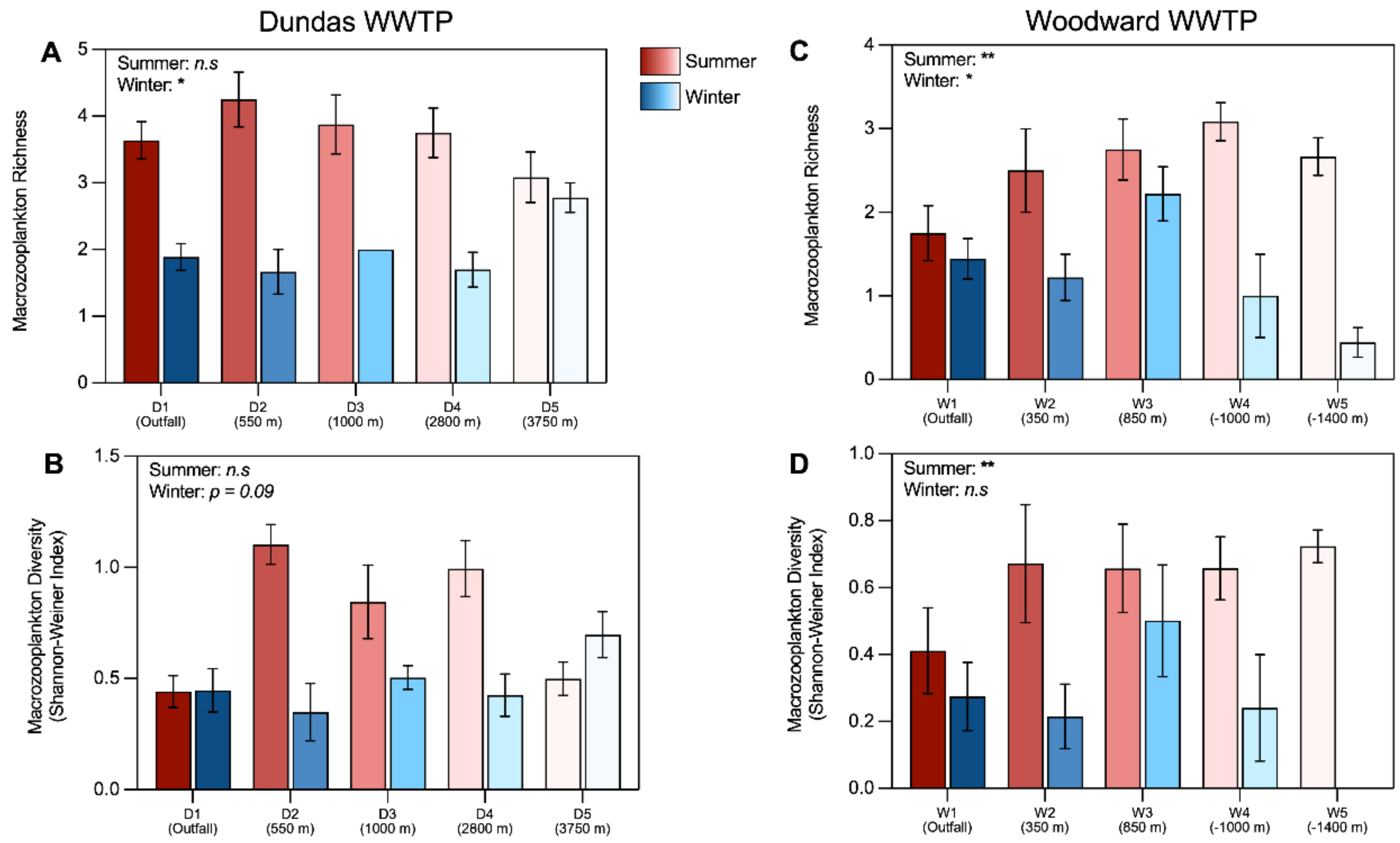

Figure S1. Mean (± SE) **(A, C)** macrozooplankton richness and **(B, C)** macrozooplankton diversity along the effluent gradients of the Dundas and Woodward WWTPs. Seasonal significant effects of proximity to the outfall are indicated by (*$p \leq 0.05$), (**$p < 0.01$), (***$p < 0.001$), and (no significance, *n.s.*).

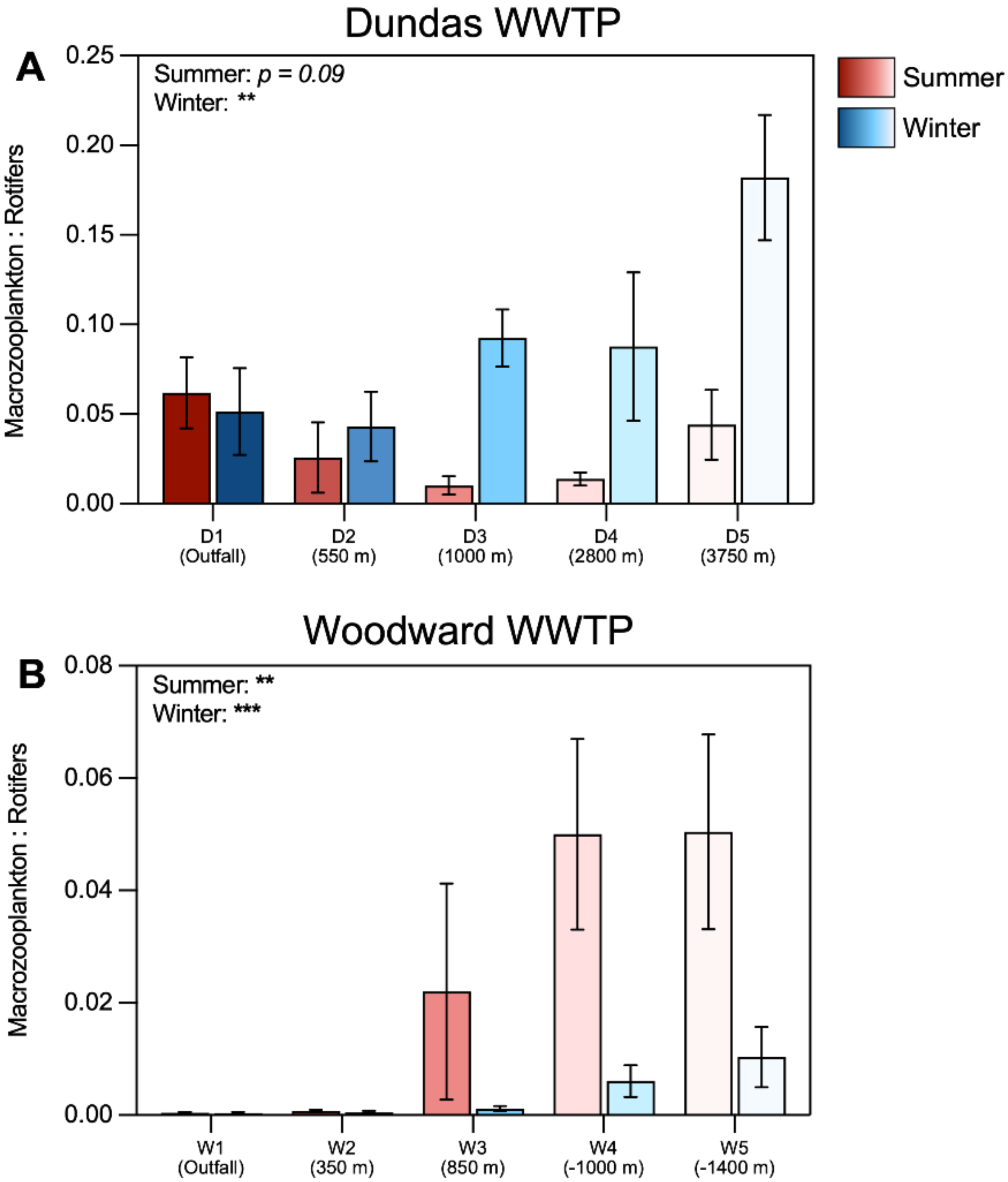

Figure S2. Mean (± SE) macrozooplankton to rotifer abundance ratio along the effluent gradients of the **(A)** Dundas and **(B)** Woodward WWTPs. At the Dundas WWTP, rotifers were more proportionally abundant than macrozooplankton in both seasons, however, macrozooplankton's relative abundance to rotifer abundance was greatly dependent on proximity to the outfall and season (PLMM, $t_{(\text{Distance x Seasonality})} = 2.03$, $p < 0.01$). In the winter, macrozooplankton relative abundance was higher in the reference sites than in sites closer to the outfall (PLMM, t = 1.81, p < 0.01). While in the summer, sites closer to the outfall appeared to have higher relative abundance of macrozooplankton to rotifers, but that did not reach significance (PLMM, $t = -1.61$, $p = 0.09$). At the Woodward WWTP, downstream sites had on average ~1.7 times more rotifers than the upstream sites in the summer and ~146 times more rotifers than the upstream sites in the winter. Rotifers were more abundant than macrozooplankton in both seasons, however, similar to the Dundas WWTP, their proportional abundance to macrozooplankton was dependent on both proximity to the outfall and seasonality (PLMM, $t_{(\text{Distance x Seasonality})} = 2.71$, $p < 0.01$). In the summer, sites upstream of the outfall had proportionally more macrozooplankton than sites downstream (PLMM, $t = 3.57$, $p = 0.01$). A similar trend was also observed in the winter (PLMM, $t = 3.01$, $p < 0.01$).

Appendix F: Supplementary materials for Chapter 5

Supplementary Table 1: Water quality measurements in exposure tanks

Supplementary Table 2: Water quality measurements in 24 h composite effluent samples

Supplementary Table 3: Concentrations of various classes of chemicals in the exposure tanks

Supplementary Table 4: Sample sizes for each endpoint analyzed

Supplementary Table 5: Morphometrics and haematocrit of fish post-exposure

Supplementary Methods: Representative statical model formulas

Table S1. Mean ± SEM of water quality measurements in exposure tanks. The following water quality parameters were measured: ammonia (NH_3), nitrite (NO_2^-), nitrate (NO_3^-), pH, conductivity, total dissolved solids (TDS), salinity, and dissolved oxygen. Water quality measures were measured once a week due to time constraints.

	Treatment	**NH_3 (ppm)**	**NO_2^- (ppm)**	**NO_3^- (ppm)**	**pH**	**Conductivity (µS/cm)**	**TDS (ppm)**	**Salinity (ppm)**	**Dissolved Oxygen (%)**
20°C	Control (0%)	<DL	<DL	5.28 ± 0.878	8.19 ± 0.052	369 ± 4.90	236 ±6.94	158 ± 4.83	94.7 ± 0.534
	Low (25%)	0.417 ± 0.083	0.083 ± 0.042	24.4 ± 1.76	8.18 ± 0.054	632 ± 4.52	404 ± 2.99	283 ± 2.27	93.8 ± 0.433
	High (50%)	1.06 ± 0.390	0.333 ± 0.083	46.7 ± 6.46	8.35 ± 0.044	933 ± 11.7	597 ± 8.98	433 ± 6.76	94.2 ± 0.372
4°C	Control (0%)	<DL	<DL	5.28 ± 0.773	8.18 ± 0.112	381 ± 5.29	244 ± 3.47	163 ± 2.32	95.7 ± 0.197
	Low (25%)	0.944 ± 0.056	1.11 ± 0.111	55.6 ± 6.48	8.06 ± 0.060	707 ± 8.41	452 ± 6.37	320 ± 4.79	95.2 ± 0.274
	High (50%)	1.56 ± 0.242	0.806 ± 0.176	47.8 ± 6.19	8.09 ± 0.128	1000 ± 14.5	640 ± 10.6	466 ± 6.24	95.3 ± 0.202

Table S2. Mean ± SEM of water quality measurements taken on 24 h composite effluent samples separated by sampling month. The following water quality parameters were measured: total suspended solids (TSS), biochemical Oxygen Demand (cBOD), total Kjeldahl nitrogen (TKN), ammonia and ammonium (NH_3 and NH_4^+), nitrite (NO_2^-), nitrate (NO_3^-), *Escherichia coli* (*E. coli*), conductivity, and chemical oxygen demand (COD). Measurements provided by the City of Hamilton.

Month (2019)	**TSS (mg/L)**	**cBOD (mg/L)**	**TP (mg/L)**	**TKN (mg/L)**	**NH_3 & NH_4^+ (mg/L)**	**NO_2^- (mg/L)**	**NO_3^- (mg/L)**	***E. coli* (CFU)**	**Conductivity (µhos/cm)**	**COD (mg/L)**
March	17.4 ± 2.95	7.77 ± 0.799	0.720 ± 0.040	7.12 ± 0.248	5.25 ± 0.325	0.340 ± 0.015	11.0 ± 0.565	$93\underline{0}0 \pm 2140$	1660 ± 92.8	37.5 ± 0.289
April	14.4 ± 1.58	6.37 ± 0.429	0.525 ± 0.027	4.78 ± 0.309	3.19 ± 0.319	0.291 ± 0.020	10.9 ± 0.536	$12100 \pm 15\underline{0}0$	1440 ± 52.1	28.3 ± 4.84
May	20.4 ± 1.51	7.80 ± 0.569	0.666 ± 0.029	3.01 ± 0.213	1.16 ± 0.110	0.272 ± 0.030	10.0 ± 0.514	8830 ± 6330	1350 ± 28.5	36.3 ± 2.59

Table S3. Mean ± SEM concentrations in [ng/L] of various classes of chemicals measured in the exposure tanks at 20°C and 4°C. Data presented as before | after water changes and/or wastewater effluent re-dosing. <DL indicate measurements below detection limits. All detection limit values can be found in Mehdi et al. (2021). A few compounds appeared to increase between the day wastewater was dosed and the day water changes were made. We believe this is due to possible conjugation of compounds not allowing us to detect its concentrations fully initially.

20°C

Class	**Chemical**	**Control (0%)**	**Low (25%)**	**High (50%)**
Lipid regulator	Gemfibrozil	0.437 ± 0.028 | 0.465 ± 0.028	2.57± 0.216 | 1.05 ± 0.436	4.73 ± 1.66 | 3.47 ± 1.68
	Atorvastatin	0.475 ± 0.475 | 0.200 ± 0.200	7.90 ± 0.994 | <DL	8.42 ± 8.42 | 2.22 ± 2.22
	p-hydroxy Atorvastatin	<DL| <DL	11.0 ± 0.830 | 3.37 ± 1.34	17.5 ± 12.2 | 10.5 ± 10.5
	o-hydroxy Atorvastatin	<DL| <DL	10.1 ± 0.581 | 1.74 ± 0.519	12.5 ± 10.3 | 5.89 ± 5.89
Anti-epileptic	Carbamazepine	0.180 ± 0.180 | 0.925 ± 0.499	12.1 ± 2.37 | 9.45 ± 1.30	23.3 ± 0.667 | 26.3 ± 0.518
Analgesic	Acetaminophen	<DL| <DL	<DL| <DL	0.725 ± 0.725 | <DL
Stimulant	Caffeine	12.7 ± 5.92 | 11.8 ± 6.06	41.0 ± 12.2 | 27.3 ± 6.00	54.2 ± 21.4 | 56.8 ± 11.2
Antibacterial	Triclosan	1.63 ± 0.844 | 2.17 ± 1.29	9.98 ± 3.47 | 2.07 ± 1.17	7.51 ± 2.90 | 4.29 ± 1.82
	Sulfamethazine	<DL| <DL	4.55 ± 0.763 | 4.50 ± 0.806	9.72 ± 2.98 | 11.8 ± 0.482
Antibiotic	Monensin	<DL| <DL	<DL| <DL	<DL| <DL
	Trimethoprim	0.197 ± 0.122 | 0.111 ± 0.111	5.69 ± 0.612 | 2.62 ± 0.597	8.19 ± 6.18 | 4.31 ± 4.31
	Lincomycin	<DL| <DL	0.627 ± 0.627 | 2.17 ± 1.20	1.28 ± 1.28 | 2.02 ± 2.02
	Sulfamethoxazole	<DL| <DL	17.7 ± 3.50 | 20.2 ± 4.07	37.0 ± 1.89 | 51.1 ± 8.01
Antidepressant	Fluoxetine	0.133 ± 0.133 | 0.129 ± 0.129	0.843 ± 0.091 | 0.220 ± 0.110	1.17 ± 0.497 | 0.205 ± 0.205
	Norfluoxetine	<DL| <DL	0.720 ± 0.178 | <DL	1.48 ± 0.758 | 0 ± 0
	Venlafaxine	0.346 ± 0.204 | 0.148 ± 0.148	20.0 ± 1.19 | 18.0 ± 1.97	49.7 ± 10.4 | 48.3 ± 2.40
	Desvenlafaxine		36.1 ± 3.69 | 34.1 ± 3.47	67.0 ± 24.3 | 82.2 ± 19.2
NSAID	Ibuprofen	0.620 ± 0.423 | 0.825 ± 0.490	46.8 ± 7.38 | 0.613 ± 0.613	71.9 ± 71.9 | 5.00 ± 5.00
	Naproxen	1.41 ± 0.845 | 1.14 ± 0.615	74.2 ± 9.66 | 53.4 ± 10.5	104 ± 102 | 1.85 ± 0.698
	Diclofenac	13.3 ± 4.32 | 13.2 ± 3.92	39.7 ± 1.66 | 42.5 ± 3.75	80.2 ± 1.89 | 92.2 ± 0.155
Herbicide	Atrazine	0.100 ± 0.100 | <DL	1.56 ± 0.072 | 1.37 ± 0.114	2.88 ± 0.947 | 3.42 ± 0.871

Table S3. Continued

		4°C		
Class	**Chemical**	**Control (0%)**	**Low (25%)**	**High (50%)**
Lipid regulator	Gemfibrozil	0.317 ± 0.172 | 0.126 ± 0.126	3.41 ± 0.255 | 3.64 ± 0.135	6.65 ± 0.385 | 5.87 ± 1.30
	Atorvastatin	0.309 ± 0.309 | 0 ± 0	4.47 ± 0.719 | <DL	20.3 ± 5.81 | 0.412 ± 0.412
	p-hydroxy Atorvastatin	<DL| <DL	7.75 ± 1.28 | <DL	26.6 ± 5.75 | 1.32 ± 1.32
	o-hydroxy Atorvastatin	<DL| <DL	7.26 ± 0.909 | 0.524 ± 0.524	24.6 ± 6.30 | 0.747 ± 0.747
Anti-epileptic	Carbamazepine	0.520 ± 0.266 | 0.578 ± 0.43	11.4 ± 1.29 | 10.9 ± 1.22	22.9 ± 2.08 | 20.5 ± 4.13
Analgesic	Acetaminophen	<DL| <DL	<DL| <DL	<DL| <DL
Stimulant	Caffeine	6.09 ± 3.16 | 4.78 ± 1.67	24.0 ± 2.13 | 10.3 ± 2.27	72.9 ± 37.9 | 18.9 ± 0.134
Antibacterial	Triclosan	2.34 ± 0.092 | 3.16 ± 0.239	8.28 ± 1.07 | 3.56 ± 0.271	14.5 ± 5.60 | 2.75 ± 0.839
	Sulfamethazine	2.03 ± 2.03 | 0.075 ± 0.075	5.79 ± 1.84 | 4.40 ± 0.187	14.1 ± 0.292 | 11.1 ± 3.99
Antibiotic	Monensin	<DL| <DL	<DL| <DL	<DL| <DL
	Trimethoprim	<DL| <DL	6.89 ± 1.10 | 6.74 ± 0.672	15.2 ± 1.98 | 13.1 ± 1.72
	Lincomycin	<DL| <DL	<DL| <DL	3.26 ± 0.616 | 5.93 ± 2.32
	Sulfamethoxazole	<DL| <DL	22.3 ± 2.48 | 21.2 ± 1.65	29.9 ± 1.93 | 28.6 ± 0.039
Antidepressant	Fluoxetine	0.130 ± 0.130 | <DL	0.847 ± 0.070 | 0.516 ± 0.031	1.81 ± 0.507 | 0.717 ± 0.044
	Norfluoxetine	<DL| <DL	<DL| <DL	0.987 ± 0.310 | <DL
	Venlafaxine	0.360 ± 0.210 | <DL	23.9 ± 3.96 | 25.1 ± 2.07	53.6 ± 7.67 | 45.3 ± 3.10
	Desvenlafaxine	0.463 ± 0.257 | <DL	38.8 ± 9.24 | 25.8 ± 0.064	88.8 ± 6.74 | 85.9 ± 10.7
NSAID	Ibuprofen	0.820 ± 0.671 | 0.313 ± 0.313	45.8 ± 5.25 | 21.6 ± 2.87	143 ± 49.8 | 58.3 ± 14.2
	Naproxen	1.39 ± 1.19 | 0.229 ± 0.125	71.1 ± 12.3 | 62.9 ± 11.2	185 ± 53.2 | 110. ± 4.91
	Diclofenac	9.10 ± 1.31 | 7.73 ± 4.02	49.8 ± 0.735 | 53.7 ± 0.413	79.6 ± 13.9 | 70.7 ± 3.03
Herbicide	Atrazine	<DL| <DL	1.38 ± 0.181 | 1.44 ± 0.107	3.42 ± 0.333 | 2.88 ± 0.439

Table S4. Sample sizes for each endpoint analyzed. Unequal sample sizes in the metabolic rate assays are due to technical issues (e.g., air bubbles in chamber). Behavioural assays were always conducted on fish prior to any respirometry experiments; not all fish that were used in behavioural assays were used for respirometry experiments due to equipment constraints. Unequal sample sizes in the behavioural assays are due to arena and camera technical issues (e.g., camera not recording). Finally, blood was not able to be extracted from all fish, therefore, haematocrit sample sizes are not always the same as the sample sizes for morphometrics. Survival measured in (%) during the exposure period.

		Metabolism			**Behaviour**				**Morphology**		**Survival (%)**
	Treatment	Resting MO2	Maximal MO2	Aerobic Scope	Boldness	Sociality	Foraging	Predator Response	Morphometrics	Haematocrit	Total
20°C	Control (0%)	12	12	12	22	20	20	20	37	37	71.2
	Low (25%)	12	12	12	24	24	24	22	34	34	65.4
	High (50%)	12	11	11	22	21	21	21	32	32	76.2
4°C	Control (0%)	7	10	7	27	26	26	25	39	36	68.4
	Low (25%)	10	9	9	22	22	22	22	30	28	53.6
	High (50%)	11	10	10	27	24	24	23	47	47	94.0

Table S5. Mean ± SEM of total length, standard length, body mass, body condition (Fulton's K) and haematocrit of the fish used in the experiment separated by sex, treatment, and acclimation temperature.

	Treatment	**Sex**	**Total length (mm)**	**Standard length (mm)**	**Body mass (g)**	**Condition (Fulton's K)**	**Haematocrit (%)**
20°C	Control (0%)	Male	60.5 ± 1.10	49.2 ± 0.982	1.73 ± 0.124	1.43 ± 0.053	21.5 ± 1.95
		Female	55.0 ± 0.794	45.0 ± 0.672	1.43 ± 0.088	1.52 ± 0.044	23.7 ± 1.84
	Low (25%)	Male	60.3 ± 1.16	48.9 ± 1.05	1.75 ± 0.122	1.46 ± 0.039	28.4 ± 1.91
		Female	59.1 ± 1.31	48.1 ± 1.13	1.75 ± 0.137	1.54 ± 0.050	26.4 ± 1.58
	High (50%)	Male	59.0 ± 1.80	48.2 ± 1.56	1.75 ± 0.226	1.44 ± 0.048	26.6 ± 2.90
		Female	60.8 ± 1.25	49.5 ± 1.21	1.91 ± 0.139	1.55 ± 0.035	28.5 ± 2.42
4°C	Control (0%)	Male	62.5 ± 1.59	51.1 ± 1.35	2.25 ± 0.183	1.64 ± 0.031	32.9 ± 1.55
		Female	59.5 ± 0.905	48.5 ± 0.771	1.94 ± 0.120	1.65 ± 0.036	31.7 ± 1.13
	Low (25%)	Male	60.0 ± 1.25	49.0 ± 1.05	1.98 ± 0.123	1.67 ± 0.043	33.9 ± 1.90
		Female	60.3 ± 1.51	49.4 ± 1.37	2.10 ± 0.200	1.67 ± 0.037	30.8 ± 1.67
	High (50%)	Male	60.0 ± 1.47	48.9 ± 1.21	1.96 ± 0.163	1.63 ± 0.035	34.5 ± 1.21
		Female	60.4 ± 0.797	49.3 ± 0.695	2.11 ± 0.114	1.71 ± 0.035	30.9 ± 1.19

Supplementary Methods

Representative statistical models of how data were analyzed. (i) Linear models were used to analyze metabolic rate, haematocrit, boldness, sociability (as measured by zonal scoring), and foraging (as measured by latency to interact with food item). (ii) Beta regressions were used to analyze the proportion of time the focal fish spent socially interacting with their shoal in the sociability assay. (iii) Negative binomial general linear models were used to analyze the number of times the focal fish interacted with the food item.

(i) Linear models:

```
LM <- lm(Response_Variable ~ (Temperature *
Wastewater_Concentration_Treatment) + Body_Mass + Sex + Start_Date), data
= Data_df)
```

(ii) Beta regressions:

```
BR <- betareg(Response_Variable ~ (Temperature *
Wastewater_Concentration_Treatment) + Body_Mass + Sex + Start_Date), data
= Data_df)
```

(iii) Negative binomial general linear models:

```
GLM <- glm((Response_Variable ~ (Temperature *
Wastewater_Concentration_Treatment) + Body_Mass + Sex + Start_Date), data
= Data_df, family = binomial)
```