Response of wild fish to municipal wastewater treatment plant upgrades

by

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Examining Committee Membership

The following served on the Examining Committee for this thesis. The decision of the Examining Committee is by majority vote.

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AUTHOR'S DECLARATION

This thesis consists of material all of which I authored or co-authored: see Statement of Contributions included in the thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

I understand that my thesis may be made electronically available to the public.

Statement of Contributions

This thesis is a series of four data chapters each written in manuscript format (Chapter 2-5). The first two data chapters are published and the last two are prepared for submission to refereed journals. I am the first author and have written each manuscript. Mark Servos is the anchor author and has provided support, direction, and review of each. There are several co-authors on these manuscripts who have made the following contributions:

Chapter 2

Keegan A Hicks, Heather A Loomer, Meghan LM Fuzzen, Sonya Kleywegt, Gerald R Tetreault, Mark E McMaster, Mark R Servos. 2017. δ15N tracks changes in the assimilation of sewage-derived nutrients into a riverine food web before and after major process alterations at two municipal wastewater treatment plants, Ecological Indicators (72) 747-758.

Heather Loomer provided a historical data set, and Meghan Fuzzen and Gerald Tetreault provided archived samples. I collected and analyzed samples, designed/ran the experiment, statistically analyzed and interpreted the data, and wrote the manuscript. All authors participated in the discussions of the results and review of the manuscript.

Chapter 3

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Meghan Fuzzen and Gerald Tetreault provided the historical biological data sets. Emily McCann, Maricor Arlos, and Leslie Bragg were responsible for method development and analyses of all effluent pharmaceutical and total estrogenicity data. I collected and analyzed the new biological data, statistically analyzed and interpreted all data, and wrote the manuscript. All authors participated in the discussions of the results and review of the manuscript.

Chapter 4:

Keegan A Hicks, and Mark R Servos. Site fidelity and movement of a small-bodied fish species, the rainbow darter (Etheostoma Caeruleum): implications for environmental effects assessment. Prepared for submission to a scientific journal.

I designed and coordinated the field work, developed the tagging approach, analyzed and interpreted the data, and wrote the manuscript. Mark Servos provided direction and assisted in the review of the manuscript.

Chapter 5:

Keegan A Hicks, Gerald R Tetreault, Adam Yates, Mark E McMaster, Mark R Servos. Using riverine fish communities to detect impacts from municipal wastewater treatment plant effluents. A case study in the Grand River watershed, Ontario. Prepared for submission to a scientific journal.

Gerald Tetreault provided a historical data set for fish communities and also developed the fish community protocol. Adam Yates provided a historical data set on benthic macroinvertebrates. I designed and coordinated the new field collections of fish, invertebrate, and habitat data. I did the data analyses, interpretation, and wrote the manuscript. All authors participated in the discussions of the results and review of the manuscript.

Abstract

Impacts on aquatic biota residing near municipal wastewater treatment plant (MWWTP) outfalls have been documented globally. These impacts may be directly or indirectly associated with elevated contaminants such as nutrients, metals, suspended solids (SS), biochemical oxygen demanding matter (BOD), pharmaceuticals, and personal care products. A variety of effects have been well documented in the Grand River watershed of southern Ontario below the outfalls of the MWWTPs of the cities of Kitchener and Waterloo. Responses in wild fish have been reported at multiple levels of biological organization, ranging from altered gene expression to changes in fish communities, as well as changes in nutrient cycling within the aquatic food web. The most consistently observed effect has been high occurrences and severe cases of intersex (ova-testes) in the male rainbow darter (*Etheostoma Caeruleum*); this finding represents one of the worst examples of pollution-caused intersex reported anywhere in the world.

Primarily in response to the introduction of new effluent quality standards, the Region of Waterloo has invested millions of dollars to upgrade several of its MWWTPs including the facility servicing Kitchener, creating a unique opportunity to conduct a before-and-after study. The main objective of this thesis was to assess if treatment upgrades, which were targeted at conventional contaminants (i.e., ammonia, BOD, SS, and chloride), effectively remediated the responses previously reported in wild fish downstream of the MWWTP. To test this, historical, archived, and new data collections were used to assess changes at multiple levels of biological organization, including changes in nutrient cycling in the aquatic food web, reproductive effects in the male rainbow darter (e.g., intersex), and changes in fish community composition. For comparative purposes, responses in rainbow darter were also examined at numerous reference sites and below the smaller Waterloo MWWTP, which did not undergo any major upgrades during the study period.

The treatment upgrades at the Kitchener MWWTP (which included nitrifying activated sludge) improved the overall quality of the effluent; these improvements included reductions in nutrients (total ammonia), pharmaceuticals, and total estrogenicity (E2eq). In

contrast, the Waterloo MWWTP had deteriorating effluent quality, with ammonia levels increasing over the course of the study. Changes in effluent quality at both the Kitchener and Waterloo MWWTPs were detected in the downstream aquatic food webs using stable isotope ratios ($\delta^{15}N$ and $\delta^{13}C$). Patterns of $\delta^{15}N$ in a primary consumer (benthic invertebrate) and a secondary consumer (rainbow darter) reflected the exposure to MWWTP effluents and changes in nutrient cycling in response to the changing effluent quality. A major reduction in intersex in the male rainbow darter below the Kitchener MWWTP outfall was also associated with the improvements in effluent quality. Rates of intersex were reduced by as much as 70% in the first year post-upgrade and dropped to near background levels within three years. Detecting change in fish communities below MWWTP outfalls (including before and after the upgrades) was more challenging. While subtle changes were detected (e.g., increases in pollution-tolerant species below the MWWTP outfalls), these could not be directly associated with MWWTP effluents because they were confounded by a watershed gradient (e.g., stream size). Fish communities were highly variable both spatially and temporally, limiting our ability to associate changes with local environmental conditions (i.e., effects of MWWTP outfalls).

Although rainbow darter has been used as a sentinel species for detecting impacts of MWWTP effluents in many studies, little is known about its movement patterns. Elevated intersex was observed historically at the near-field upstream site of the Kitchener MWWTP outfall, leading to a hypothesis that wastewater-exposed fish may be moving upstream. To inform the interpretation of responses in rainbow darter as a sentinel species, a mark-and-recapture study was conducted at an upstream reference site to better understand their movement. Although the majority of fish (85%) had high site fidelity, a small proportion of fish moved considerable distances (up to 975 m). This study confirmed that there is potential for some fish to move and thereby confound the interpretation of near-field upstream sites that are not physically separated from the sites below the MWWTP outfall. The decline in intersex in rainbow darter after the upgrades at the site immediately upstream of the Kitchener outfall supports the view that at least some of the responses seen at this site were probably associated with fish movements.

Overall, this thesis advances our understanding of the impacts of MWWTP effluents on wild fish and their response to improved effluent quality (i.e., treatment). The relatively simple (conventional) upgrades at the Kitchener MWWTP resulted in improvements in the aquatic receiving environment, indicating that more advanced treatment may not be required to address these effects of concern. However, other impacts may be occurring that were not measured in this study. The results drawn from this thesis may have implications for future wastewater management strategies for other MWWTPs across Canada and around the globe. In addition, these studies may provide insight into key biological endpoints that could be useful for future biomonitoring programs for MWWTP effluents.

Acknowledgements

This PhD has been an incredible experience and throughout it I have met many great people who have helped along the way. I would first like to thank Mark Servos. I could not have asked for a more supportive supervisor, who provided great guidance, advice, and opportunities throughout this thesis.

The studies in this thesis are completely field-based and therefore I constantly required assistance. I am thankful for all the hard working technicians, summer students, and co-op students that have been involved throughout the years. They tolerated long hours and extreme weather conditions. A special thanks to Jessie Cunningham who has contributed significantly in the field and lab throughout the studies in my thesis. She rarely missed a fish and made great histology slides. Hadi Dhiyebi has also been a key player in the field and was able to keep all the equipment working properly. I can only hope that netting has not created any permanent back damage! Leslie Bragg was also very supportive, whether helping in the field or making sure I have all the supplies and equipment that I needed.

Gerald Tetreault was valuable throughout my thesis. He was particularly crucial at the beginning of my studies to get me started and to discuss ideas. He has also assisted me many times with field work. I would also like to thank my other committee members Heidi Swanson and Simon Courtenay who have provided valuable feedback and ideas for my studies. Mark McMaster has also made himself available to discuss ideas and review manuscripts. I would also like to acknowledge Jennifer Thomas who copyedited parts of this thesis.

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Chapter 1

Introduction

Municipal wastewater treatment plants (MWWTPs) are the largest point sources of water pollution in Canada (Chambers et al., 1997). These facilities release a diversity of chemicals and other materials into aquatic receiving environments, leading to a relatively continuous exposure of downstream aquatic ecosystems. Treatment has traditionally targeted conventional contaminants, including suspended solids, metals, and nutrients (phosphorous and nitrogen compounds), as well as pathogenic bacteria (Chambers et al., 1997). High biological oxygen demand associated with MWWTP effluents can create conditions of low dissolved oxygen in the receiving environment (Lijklema et al., 1993). High loads of nutrients (e.g., nitrogen and phosphorous) can cause excessive growth of primary producers (e.g., macrophytes and periphyton), which can lead to eutrophication and consequently may create hypoxic/anoxic conditions in the environment (Chambers and Prepas, 1994). Contaminants of emerging concern (CECs), which include endogenous hormones, pharmaceuticals, and personal care products, have received considerable attention (Daughton and Ternes, 1999; Metcalfe et al., 2003; Servos et al., 2005; Lishman et al., 2006). These chemicals can have subtle effects on biological functions (such as the endocrine system) that can alter the growth, development, and reproduction of aquatic organisms (Tyler et al., 1998; Mills and Chichester, 2005). As a result of their complexity, MWWTP effluents have the potential to cause a number of changes in the aquatic environment either directly (through acute and chronic toxicity to organisms) or indirectly (e.g., by physically altering habitat), and these changes can occur across multiple trophic levels (Chambers et al., 1997; Kilgour et al., 2005). Impacts from MWWTP effluents on biota, including fish, have been well documented across Canada (Holeton et al. 2011).

1.1 Impacts from MWWTP effluents on multiple levels of biological organization

Impacts of municipal wastewater have been reported in organisms across many levels of biological organization and trophic levels (McMaster, 2001). Responses detected at lower levels of organization include molecular responses such as the expression of *vtg* (vitellogenin; an egg yolk protein) in male fish (Jobling et al., 1998; Adeogun et al., 2016) and changes in circulating sex steroid hormones (Jobling et al., 1998; Hecker et al., 2002; Blazer et al., 2012). Histopathological effects associated with MWWTP effluents have also been widely reported, particularly intersex (simultaneous presence of male and female gonad tissue) condition in male fish (Jobling et al., 1998; Bjerregaard et al., 2006; Woodling et al., 2006; Blazer et al., 2007; Tetreault et al., 2011; Abdel-moneim et al., 2015). Changes in organ weight (e.g., gonad and liver size) and body size (condition factor) have also been observed and are indicators of changes in energy allocation and storage (Vajda et al., 2008; Iwanowicz et al., 2009). Impacts at the population level include changes in sex ratios (Jeffries et al., 2008; Vajda et al., 2008) and reduced fertilization success (Jobling et al., 2002; Fuzzen et al., 2015). One of the most convincing studies to demonstrate population-level effects was the exposure of a whole lake to 5 ng/L of 17α-ethynylestradiol (EE2), an environmentally relevant concentration of an estrogenic compound commonly found in MWWTP effluents. After three years of exposure, reproduction ceased in a population of fathead minnows (*Pimephales promelas*), which resulted in recruitment failure (Kidd et al., 2007).

Changes linked to MWWTP effluents have also been documented at higher levels of biological organization (e.g., fish communities), although it is increasingly difficult to demonstrate an association between changes at these higher levels and a particular contaminant or stressor (Figure 1.1). Community-level responses to MWWTP effluents may include changes in diversity, species richness, or trophic guilds or increases in pollution-tolerant species (Porter and Janz, 2003; Winger et al., 2005; Ra et al., 2007; Yeom et al., 2007). The excessive nutrients released by MWWTPs provide additional food sources to primary producers and consumers and can increase primary and secondary productivity (deBruyn et al., 2003). The transfer of sewage-derived nutrients can be detected throughout entire food webs from primary producers to fish using stable isotope ratios of nitrogen (deBruyn and Rasmussen, 2002). It is evident that MWWTP effluents can have an impact on the receiving environment at all levels of biological organization, from changes detected at the molecular level to changes in nutrient cycling within the aquatic food web. To mitigate these effects, considerable investments have been made into MWWTP facilities to improve the quality of effluents released into the environment.

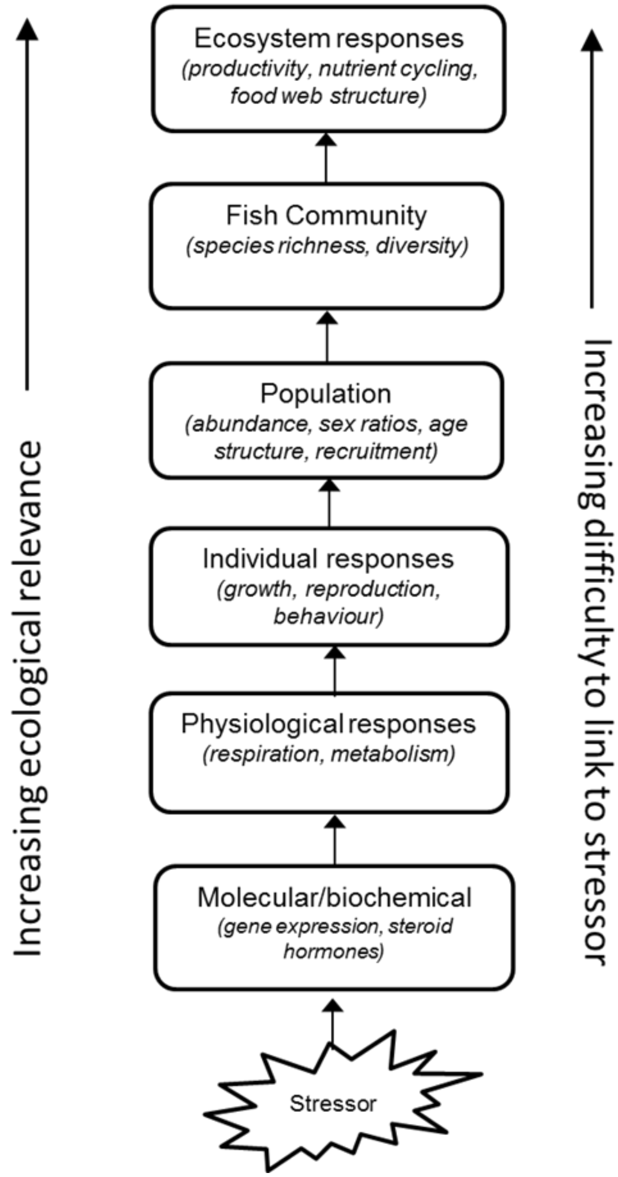

Figure 1.1 Potential effects of contaminants across increasing levels of biological organization. It is often easier to link effects from contaminants at lower levels of biological organization (e.g., biochemical, physiological). Establishing cause-effect relationships at higher levels of organization (e.g. population and community) becomes increasingly difficult. (Modified from Clements, 2000).

1.2 Wastewater management and guidelines

In Canada, MWWTPs operate at varying levels of treatment; they range from facilities that release raw sewage to state-of-the-art treatment plants (Figure 1.2; Environment and Climate Change Canada, 2016a; Holeton et al., 2011).

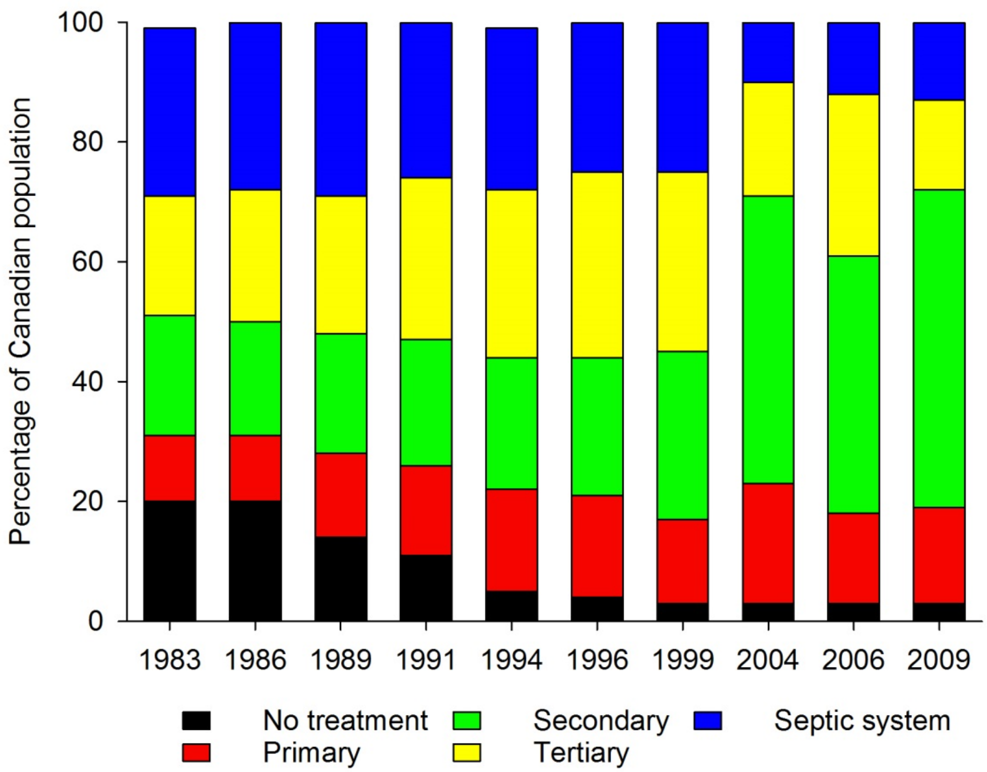

Figure 1.2 Percentage of Canadians serviced by wastewater treatment facilities with no treatment, primary treatment, secondary treatment, tertiary treatment, and septic systems. In 2006 and earlier, the definition of tertiary treatment included any advanced treatment. The definition was narrowed in 2009 to refer to tertiary treatment processes only. Data retrieved by Environment Canada and Climate Change: https://www.ec.gc.ca/indicateurs-indicators/default.asp?lang=En&n=2647AF7D-1 (accessed on December 21, 2016).

The sophistication of treatment is generally related to the population served, with larger populations usually having more advanced treatment plants (Holeton et al., 2011). Although the design of MWWTPs and their processes are diverse, MWWTPs are generally classified according to the level of treatment they provide. Preliminary and primary treatment involves the removal of large debris, suspended solids, and organic matter (George et al., 2003). Secondary treatment involves the use of biological processes including variations of activated sludge systems to break down organic matter and suspended solids (George et al., 2003). There are varying levels of secondary treatment, with some only targeting the removal of BOD and others also targeting the removal of nutrients (e.g., nitrogen and/or phosphorous; George et al., 2003). Disinfection is also normally included in a plant with secondary treatment. Tertiary treatment is variable, but it typically involves the removal of residual suspended solids, and it may also include the removal of nutrients as well as some disinfection processes (George et al., 2003). The design and operation of MWWTPs can alter the efficiency with which they remove both conventional and contaminants of emerging concern (CECs). For example, solid retention time (SRT) has been identified as an important parameter related to removal of different contaminants including CECs (Salveson et al., 2012).

The management of MWWTP effluents and their associated risks is complex and can involve many jurisdictions at the municipal, provincial, and federal levels. The federal government of Canada recently (2012) published the *Wastewater Systems Effluent Regulations* (WSER; SOR/2012-139) under the *Fisheries Act* (Government of Canada, 2012). These are national standards for MWWTPs but apply only to facilities across Canada receiving at least 100 m^3 of influent a day and discharging their effluent into natural environments. These standards, which came into effect in January 2015, include mandatory minimum effluent quality standards for biological oxygen demand, suspended solids, chlorine, and un-ionized ammonia (Table 1.1).

Table 1.1 Canadian Wastewater Systems Effluent Regulation standards

cBOD[1]	$\leq$ 25 mg/L
SS[2]	$\leq$ 25 mg/L
Total residual chlorine	$\leq$ 0.02 mg/L
Un-ionized ammonia	$\leq$ 1.25 mg/L

1 Carbonaceous biological oxygen demand
2 Total suspended solids

To achieve these standards, the secondary level of treatment (or equivalent) will probably be needed at a minimum. The majority of Canadians are already being served by a MWWTP with secondary treatment or greater. Treatment has improved significantly since the 1980s (Figure 1.2; Holeton et al., 2011), and the percentage of Canadians served by secondary treatment or greater will increase with the implementation of the new regulations. While it is important to have set minimum standards in Canada for MWWTP facilities, the effectiveness of these targets and associated treatment infrastructure (e.g., secondary treatment) are not well known, and no formal biological monitoring program is in place to assess the standards' suitability for protecting the aquatic receiving environments, especially against the subtle effects of emerging contaminants (e.g., endocrine disruption).

1.3 Biological monitoring

Due to the complexity of MWWTP effluents, it is difficult to design effective biological monitoring programs, and very little guidance is available. As impacts from MWWTP effluents can cross several trophic levels and levels of biological organization (Kilgour et al., 2005), choosing appropriate endpoints is challenging. Scientific focus is required to address specific hypotheses in monitoring programs because measuring numerous endpoints simultaneously is very resource intensive (Dale and Beyeler, 2001). In Canada, there is well-developed guidance for monitoring the effects of industrial discharges (pulp and paper and metal mining) on receiving aquatic environments, which is a requirement under the federal *Fisheries Act* and termed the Canadian Environmental Effects Monitoring (EEM) Program (Walker et al., 2002). This is an effects-based program that monitors effluent effects at exposed and reference sites in one or two sentinel fish species, benthic macroinvertebrates (BMI; indicator of fish habitat), and fisheries resources (Walker et al., 2002). Endpoints measured in fish include age, energy use (size at age, gonad size), energy storage (condition, liver size), and reproductive endpoints (e.g., fecundity). BMI are monitored for abundance, richness, and diversity. While the EEM program was developed specifically for industrial emissions, its endpoints are not specific to pulp and paper or metal mining effluents but rather are biological indicators that would respond to multiple stressors

(Dube, 2003). This type of program has been proposed for biological monitoring of MWWTP effluents in the future. However, several limitations exist with this program, such as the lack of multiple reference sites, the lack of consideration of natural variability, and the relevance of endpoints (Munkittrick et al., 2010). In addition, the effectiveness of using EEM endpoints to detect impacts from MWWTP effluents is not well understood (Tetreault et al. 2011).

Criteria for biological monitoring programs to assess impacts from MWWTP effluents have been recommended by Kilgour et al. (2005). They suggested the use of BMI and sentinel fish species surveys as the basis of biomonitoring, similar to the approach used in the EEM program. They designed their recommendations around ensuring healthy fish communities. Where appropriate and feasible, Kilgour et al. (2005) recommended assessing fish communities directly, as this is the highest trophic level, and if this level is protected one can assume that lower trophic levels are also protected (Karr, 1981). However, the assessment of fish communities is often very difficult, and only severe responses, such as the loss of a non-rare species, are typically detectable. It is also not always feasible to reliably measure community endpoints because of the receiving water characteristics (e.g., habitat, accessibility) and associated high cost. Therefore, Kilgour et al. (2005) recommended using surrogate endpoints at lower trophic levels (e.g., sentinel fish, BI, or primary producers); different levels of impairment within these lower trophic levels would trigger different levels of concern (e.g., warnings level vs. severe level effect) that the fish community may be impaired.

Although extensive research exists on the impacts of MWWTPs, selecting the appropriate endpoint(s) to use for a biological monitoring program is difficult because of the complexity of the receiving environments and potential effects. Ideally, the appropriate endpoint(s) would be sensitive, would exhibit low natural variability, and would be ecologically relevant (Matono et al., 2012). A better understanding of which endpoints exhibit these traits would be valuable for the design of future biomonitoring programs for MWWTP effluents.

1.4 Impacts of MWWTPs in the Grand River watershed

The Grand River watershed is the largest watershed draining into the northern part of Lake Erie, with an area of approximately 6800 km^2. There are multiple pressures on the watershed, including agriculture (along 71% of the extent of the watershed), flood control dams, several urban centres, and a growing population of over 1 million people (Loomer and Cooke, 2011). The watershed also assimilates point source inputs from 30 MWWTPs of varying sizes (Figure 1.3).

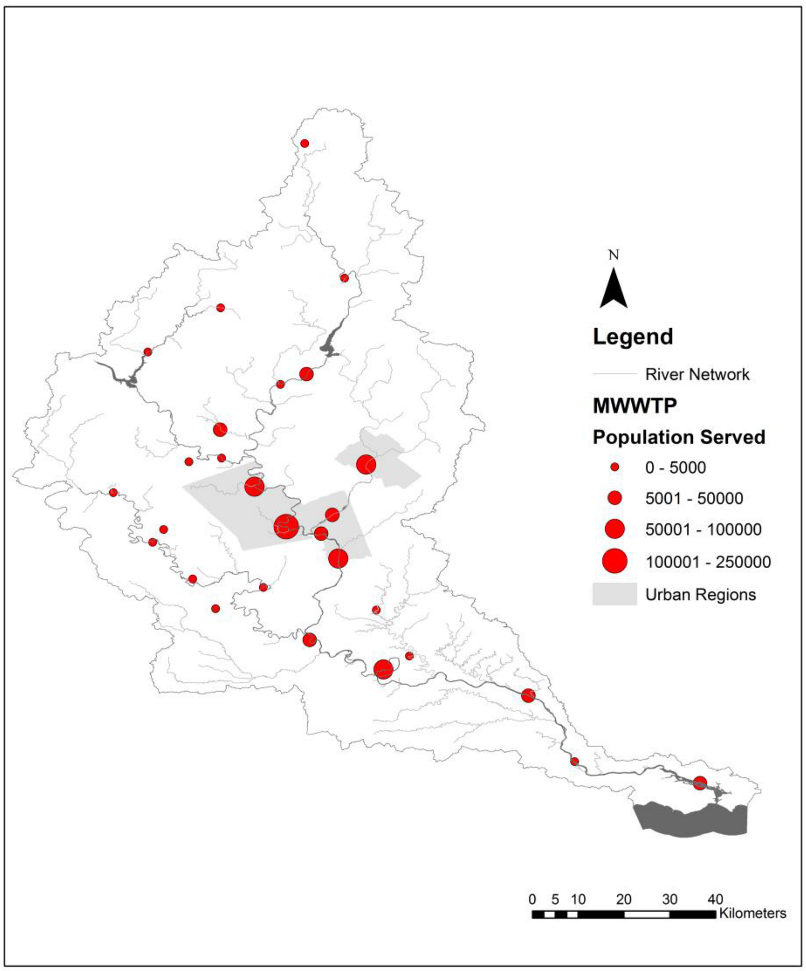

Figure 1.3 Map illustrating urbanization and municipal wastewater treatment plants located across the Grand River watershed of southern Ontario.

The two largest MWWTPs are secondary conventional activated sludge treatment plants that service the cities of Waterloo and Kitchener, with a combined population >340,000 people. The water quality downstream of these MWWTP outfalls has historically been generally poor, with high loads of chloride and nutrients, including levels of un-ionized ammonia above the Ontario provincial water quality objective (0.0165 mg/L; Loomer and Cooke, 2011). The Kitchener MWWTP effluents have also been associated with hypoxic conditions below the outfall (Loomer and Cooke, 2011; Venkiteswaran et al., 2015). Other water quality concerns include pharmaceuticals, personal care products, and endocrine disruptors, which are commonly associated with MWWTP effluents (Metcalfe et al., 2003; Servos et al., 2005) and have been detected in the Waterloo and Kitchener effluents and the receiving surface waters (Arlos et al., 2015).

Studies conducted in Grand River have associated impacts on wild fish with poor water quality below both the Waterloo and Kitchener MWWTP outfalls. These impacts have primarily been studied in the rainbow darter (*Etheostoma caeruleum*), a native species of southern Ontario. This species was considered an ideal study subject because rainbow darter are highly abundant (Tetreault et al., 2013), they are short lived (thus any impacts on them will be recent) (Beckman, 2002), and they are thought to have limited mobility (Tetreault et al., 2011). Impacts below the MWWTP outfalls have primarily been observed in males at multiple levels of biological organization. These effects include increased expression of vitellogenin (Bahamonde et al., 2014; Fuzzen et al., 2016), decreased steroid hormone production (e.g., 11-ketotestosterone) (Tetreault et al., 2011; Fuzzen et al., 2016), delayed sperm development (Fuzzen et al., 2016), and high incidences and severe cases of intersex (Tetreault et al., 2011; Tanna et al., 2013; Bahamonde et al., 2015b; Fuzzen et al., 2015; Fuzzen et al., 2016). For all these endpoints, intersex was the most consistent effect observed across multiple years and seasons (Fuzzen et al., 2016). It was speculated that these reproductive effects were due to compounds with estrogenic or antiandrogenic activity (Tanna et al., 2013; Arlos et al., 2015; Fuzzen et al., 2016).

Fish communities have also been assessed across this same stretch of river. Below the Kitchener and Waterloo outfalls, there tended to be a decrease in the abundance and diversity of fish compared with upstream sites (Tetreault et al., 2013). Downstream sites also showed a shift of community composition from predominantly darter species (insectivores) to sucker species (more tolerant omnivores; Tetreault et al., 2013). According to Kilgour et al. (2005), this level of impact at the fish community level would indicate a warning level for potential impacts. However, the conclusions from the studies conducted by Tetreault et al. (2013) were speculative, as there was high variability and thus very few significant differences were detected. Although these fish community responses are consistent with MWWTP effluent exposure, changes in habitat and/or natural gradients may have been confounding factors. The use of fish communities as an indicator to detect changes associated with MWWTP effluents requires further investigation.

Changes in the cycling of nutrients within the aquatic food webs below the Kitchener and Waterloo MWWTPs were investigated previously using stable isotopes ratios (e.g., $\delta^{15}N$ and $\delta^{13}C$; Loomer et al., 2015). Delta^{15}N in fish and BMI below the Kitchener outfall were significantly lower compared to the immediate upstream sites (Loomer et al., 2015). This indicated that the food web is deriving nutrients from a different source or process associated with nitrogen cycling below the Kitchener MWWTP. It was demonstrated in a separate study that primary producers below both the Kitchener and Waterloo outfalls were incorporating effluent ammonia as their main source of nitrogen (Hood et al., 2014). Therefore, stable isotope ratios have been demonstrated to be an important tool for assessing changes in nutrient cycling and examining the assimilation of sewage-derived nutrients into aquatic food webs. In addition, site-specific stable isotope ratios can also help infer effluent exposure and help link impacts to local environmental conditions.

In summary, changes below the Kitchener and Waterloo MWWTPs have been detected in a sentinel fish species (across levels of biological organizations), in the fish community composition, and in the processing of nutrients within the food web. These changes have been linked directly to the poor water quality associated with the MWWTP outfalls. The Region of Waterloo has recently invested millions of dollars in infrastructure upgrades to improve the effluent quality at both the Kitchener and Waterloo MWWTPs. This was done primarily in response to concerns about environmental protection and the need to meet revised national effluent quality regulations, which now include objectives for un-ionized ammonia (Region of Waterloo, 2007). The major planned upgrade at both secondary treatment plants was to convert them from carbonaceous activated sludge treatment to nitrifying activated sludge treatment to enhance the removal of total ammonia (as well as other effluent parameters). However, as a result of construction delays, only minor infrastructure upgrades have been completed to date at the Waterloo MWWTP. Major infrastructure upgrades were implemented in August of 2012 at the Kitchener MWWTP. A schematic diagram of the Kitchener MWWTP with its major upgrades is provided in Figure 1.4.

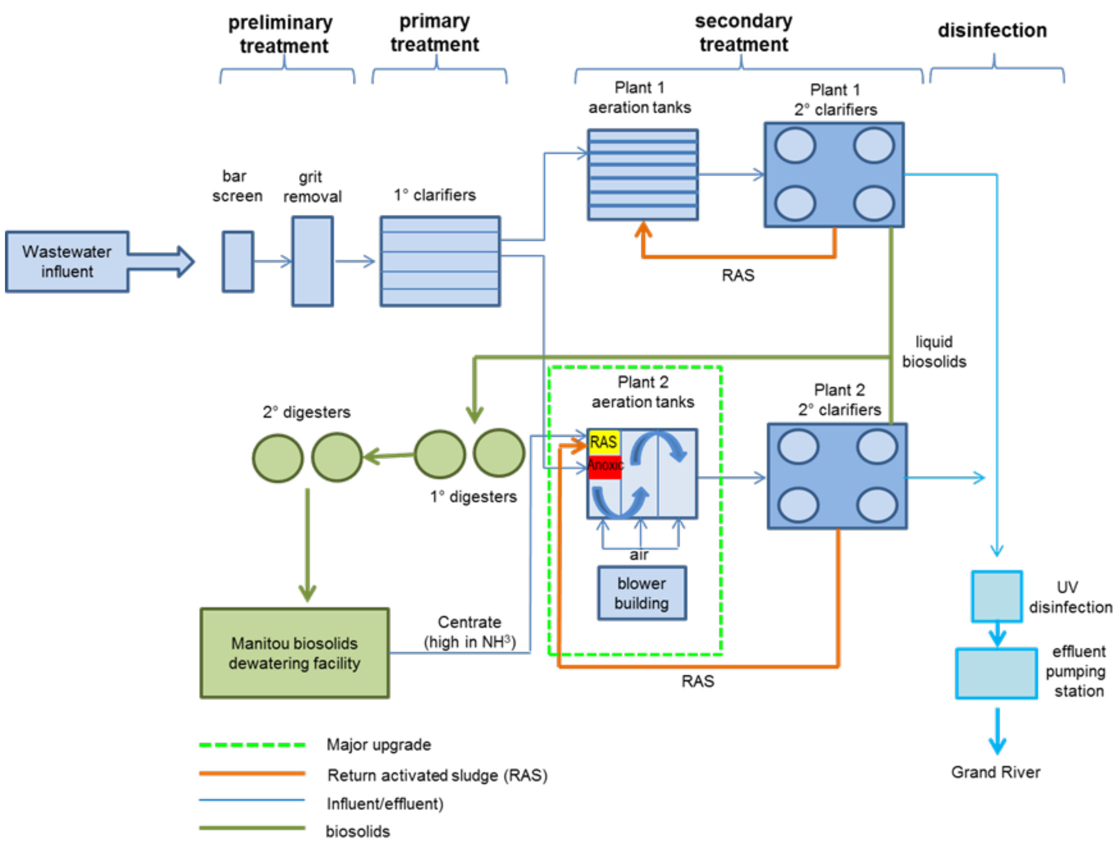

Figure 1.4 Diagram of the Kitchener wastewater treatment plant, which is a conventional activated sludge secondary treatment facility. The major planned upgrades began in August 2012 (green dotted line) and included a new RAS (return activated sludge) zone to treat the centrate coming from the biosolids dewatering facility. Mechanical aerators from both plant 1 and plant 2 were replaced with more efficient fine bubbler aeration. The treatment plant was fully nitrified by January 2013.

These upgrades are expected to improve the overall effluent quality and therefore the river water quality of the receiving environment. It would be valuable to understand whether these investments in upgrades at the MWWTPs to meet new stringent effluent quality objectives are sufficient and effective at remediating the local effects previously observed in wild fish.

1.5 Research objectives

The major goal of this thesis was to investigate the responses of wild fish to the MWWTP upgrades in the Grand River. To test specific questions related to the upgrades, this thesis used historical data and archived samples collected in the years before the upgrade (years 2007, 2010–2012) and compared them with samples collected after the upgrade (2013–2015). The upgrades at the Kitchener MWWTP began in August 2012, and by January 2013 the treatment plant was fully nitrified (Bicudo et al., 2016). The fact that upgrades at the smaller Waterloo MWWTP were delayed provided a unique opportunity to contrast one treatment plant that was improving (Kitchener) with another (Waterloo) that was not, all within the same watershed. The thesis addressed four main objectives with each presented as an individual chapter (Chapters 2–5).

Objective 1: The first objective is related to the nutrient cycling below the Kitchener and Waterloo MWWTPs. While the Kitchener effluent was improving (decreased ammonia), the quality of effluent at the Waterloo MWWTP was in fact getting worse, with increasing ammonia concentrations. Therefore, the first objective of this thesis was to assess whether these changes in effluent quality could be tracked throughout the aquatic food web, including rainbow darter (Chapter 2).

Objective 2: Nitrification is known to be associated with the removal of many contaminants (in addition to ammonia), including pharmaceutical and personal care products (Suarez et al., 2010b). It was speculated that nitrification would enhance the removal of estrogenic compounds (McAdam et al., 2010) (e.g., estrone, estradiol, and 17α-ethynylestradiol) that are hypothesized to be linked to the reproductive impacts observed in wild fish below the

MWWTPs in the Grand River (Fuzzen et al., 2016). Therefore, the second objective was to assess whether intersex incidence and severity in rainbow darter was reduced in wild fish in response to the MWWTP upgrades (Chapter 3).

Objective 3: Over many years before the upgrades, intersex occurrence and severity were highest below the Kitchener MWWTP outfall. However, elevated occurrences and severity of intersex were frequently observed at the immediate upstream site (i.e., 1 km above the Kitchener outfall). This led to the hypothesis that previously exposed fish may be moving to the upstream site. Therefore, the third objective was to assess the site fidelity and movement of the rainbow darter in the Grand River (Chapter 4). As it was important to minimize alterations to the main study sites, this movement study was conducted in a series of riffle habitats in the upper watershed.

Objective 4: The last objective of this thesis was to examine fish communities as a potential endpoint for detecting effects from MWWTP effluents. As indicated earlier, changes in richness and diversity were detected in a previous published study. However, it was difficult to directly associate these observed changes with specific stressors (MWWTP effluents) because of the associated high variability and confounding factors. Therefore, the fourth objective of this thesis was to assess whether changes in fish communities can be detected downstream of MWWTPs (relative to numerous reference sites), and to assess whether changes could be detected in response to the Kitchener MWWTP upgrades through the use of historical data (collected before the upgrades) (Chapter 5).

The final chapter of the thesis (Chapter 6) integrates observations from the various components of the study, provides insights into the impacts of MWWTPs, and makes recommendations for future studies.

Chapter 2

$\delta^{15}N$ tracks changes in the assimilation of sewage-derived nutrients into a riverine food web before and after major process alterations at two municipal wastewater treatment plants

2.1 Chapter summary

Stable isotopes ratios of nitrogen and carbon ($\delta^{15}N$ and $\delta^{13}C$) were used to assess the changes in exposure and assimilation of sewage-derived nutrients in an aquatic food web following changes in effluent quality over an 8 year period at two municipal wastewater treatment plants (MWWTPs) that discharge to the Grand River, in southern Ontario. Upgrades at the Kitchener MWWTP started in late 2012 to enhance nitrification, while the Waterloo MWWTP had a series of construction issues at the plant that resulted in a deterioration of its effluent quality over the study period (2007–2014). Fish (rainbow darter, *Etheostoma caeruleum*) and primary consumers (benthic invertebrates) were sampled in the receiving waters associated with each outfall. Upgrades at the Kitchener MWWTP resulted in improved effluent quality with total annual ammonia output dropping by nearly sixfold (583–100 t), while the Waterloo MWWTP increased its total annual ammonia output by nearly fourfold (135–500 t) over the duration of the study. Downstream of the Kitchener MWWTP, the reduction in total ammonia output negatively correlated with changes in $\delta^{15}N$ of rainbow darter from being depleted (prior to the upgrade) to reflecting signatures similar to those at the upstream reference site. The biota downstream of the Waterloo MWWTP showed the opposite trend, going from slightly enriched, to being depleted relative to the upstream reference sites. $\delta^{13}C$ was consistently higher downstream of both MWWTPs regardless of changing effluent quality, and annual variability in $\delta^{13}C$ was associated with annual river discharge. In a laboratory based dietary switch study conducted with rainbow darter, the isotope half-life in muscle (29 days for $\delta^{15}N$ and 33 days for $\delta^{13}C$) were determined and these rapid changes were consistent with responses in muscle of wild fish. This is a unique study that was able to contrast two MWWTPs in the same watershed as they underwent major changes in treatment processes. Stable isotopes were very effective as a tool to trace the changes in aquatic biota due to changes in wastewater effluent quality, both improvements and deterioration over time.

2.2 Introduction

Municipal wastewater treatment plants (MWWTPs) discharge among the highest volumes of effluent compared to other industries in Canada (Chambers et al., 1997). MWWTP effluents contain a mixture of chemicals including total suspended solids (TSS), nutrients (phosphorous and nitrogen products), metals, and pharmaceuticals and personal care products (Chambers et al., 1997; Daughton and Ternes, 1999; Metcalfe et al., 2003; Lishman et al., 2006). Environmental impacts associated with municipal MWWTP effluents released into aquatic environments have been associated with eutrophication and oxygen depletion (Gücker et al., 2006; Carey and Migliaccio, 2009; Kiedrzyńska et al., 2014), endocrine disruption (Jobling et al., 1998; Tetreault et al., 2011), impacts on fish assemblages (Tetreault et al., 2013), and alterations of food webs (deBruyn et al., 2003).

Stable isotope ratios of carbon ($\delta^{13}C$) and more commonly nitrogen ($\delta^{15}N$) have successfully been used to track the exposure and assimilation of sewage-derived nutrients into aquatic food webs (deBruyn and Rasmussen, 2002; Morrissey et al., 2013; Loomer et al., 2015). Wastewater constituents enter the aquatic food web through ingestion of particulate organic matter by consumers or through the uptake of sewage-derived inorganic nutrients by primary producers (Tucker et al., 1999). $\delta^{15}N$ measured in organisms exposed to MWWTP effluent will depend on the treatment processes utilized at the plant, final effluent quality, and the characteristics of the receiving environment. Organisms exposed to secondary or greater treated effluent typically results in enriched $\delta^{15}N$ values (Gaston et al., 2004; Morrissey et al., 2013; Robinson et al., 2016). This is because nitrification and denitrification processes associated with secondary treatment tend to result in the accumulation of the heavier nitrogen isotope, ^{15}N (Heaton, 1986). A lack of nitrification and denitrification processes (e.g. in raw sewage or primary treated effluent) usually result in an accumulating pool of ammonia depleted in ^{15}N and when released into the receiving environment, primary producers will preferentially take up $^{14}NH_4$ over $^{15}NH_4$ (Birgand et al., 2007), resulting in organisms depleted in ^{15}N (deBruyn and Rasmussen, 2002; Gaston and Suthers, 2004; Daskin et al., 2008). The carbon discharged from MWWTP effluent is primarily terrestrial in origin which has a relatively constant $\delta^{13}C$ value of about -28 ‰, hence it is possibly discriminated from aquatically derived (autotrophic) sources which can range between -40 and -20 ‰ (France, 1995).

The Grand River watershed is the largest drainage basin in southern Ontario, Canada, which flows into the northeastern part of Lake Erie. This watershed assimilates effluent from 30 MWWTPs serving almost one million people. The largest MWWTPs, Kitchener and Waterloo, (collectively serving >370,000 people in 2014), both use secondary conventional activated sludge processes, and discharge into the central reaches of the Grand River. The effluents from these MWWTPs have been historically associated with poor water quality in the receiving environment including hypoxic river conditions (Venkiteswaran et al., 2015), unionized ammonia concentrations above the provincial water quality objective (> 0.0165 mg/L) (Loomer and Cooke, 2011), and the presence of elevated levels of selected pharmaceuticals (Arlos et al., 2015). Impacts on fish downstream of these MWWTPs include the feminization (Tetreault et al., 2011; Tanna et al., 2013; Bahamonde et al., 2015b) and reduced reproductive success (Fuzzen et al., 2015) of male rainbow darter (*Etheostoma caeruleum*). A study conducted in 2007 by Loomer et al. (2015), documented changes in $\delta^{13}C$ and $\delta^{15}N$, in rainbow darter and primary consumers exposed to these MWWTP effluents in the Grand River. Exposure to the poorly treated (non-nitrifying) Kitchener effluent resulted in a decrease in $\delta^{15}N$, while exposure to the effluent at the Waterloo MWWTP (higher quality effluent with partial nitrification at the time) resulted in little to no change compared to immediate upstream site (Loomer et al., 2015). Major planned upgrades at both the Kitchener and Waterloo MWWTPs created a unique opportunity to examine how changes in effluent quality impacted the stable isotope ratios of fish (rainbow darter) and primary consumers (benthic invertebrates).

The major planned upgrades at the Waterloo and Kitchener MWWTPs were to convert them from carbonaceous activated sludge treatment (primarily for BOD removal) to fully nitrifying activated sludge. In August 2012, the Kitchener MWWTP had initiated its upgrades for nitrification, and by January 2013 it achieved full nitrification. Nitrification was achieved by retrofitting the current MWWTP with return activated sludge (RAS) reaeration and replacing the old aeration system with more efficient fine bubblers (Table 2.1) (Bicudo et al., 2016). At the same time, the Waterloo MWWTP initiated upgrades, but a number of changes and construction issues led to a decrease in effluent quality (e.g. increasing total ammonia) over several years. Similar to the Kitchener MWWTP, the Waterloo MWWTP was retrofit with RAS reaeration in 2014; however, fine bubblers had not been installed to achieve full nitrification (Table 2.1) (Region of Waterloo, 2016).

The primary objective of the present study was to assess how the changing effluent quality at two MWWTPs altered the stable isotope ratios ($\delta^{15}N$ and $\delta^{13}C$) throughout an aquatic food web, using two trophic levels, primary consumers (benthic invertebrates) and a secondary consumer (rainbow darter). The rainbow darter was selected for this study since it had been used as a sentinel species in a variety of recent biomonitoring studies in the Grand River (Tetreault et al., 2011; Tanna et al., 2013; Bahamonde et al., 2015). Using new collections, archived samples, and previously published data, the patterns of stable isotopes in rainbow darter and selected primary consumers collected adjacent to the Waterloo and Kitchener MWWTPs were assessed before and after the process changes (2007-2014). There were two specific research questions addressed in this study. The first question was to test whether a difference could be detected in $\delta^{15}N$ and $\delta^{13}C$ in fish and primary consumers before and after the Kitchener MWWTP upgrade and in the years the Waterloo MWWTP had deteriorating effluent quality. The second question was to test whether any changes in $\delta^{15}N$ and $\delta^{13}C$ could be linked to changing effluent quality. To help with the interpretation of the isotope data, a laboratory-based diet switch experiment was conducted with rainbow darter to estimate the relative isotopic turnover rate in muscle and liver tissues. The contrasting changes in effluent quality at the Kitchener and Waterloo MWWTPs, with either improvements or deteriorations over time, provided a unique opportunity to follow these changes, and how they may alter the flow of nutrients in a riverine food web.

2.3 Materials and methods

2.3.1 Sampling sites

Sampling sites selected for this study were based on previously published or unpublished studies related to the impacts of MWWTPs on rainbow darter in the Grand River, Ontario, Canada (Tetreault et al., 2011; Bahamonde et al., 2015; Fuzzen et al., 2015; Loomer et al., 2015). These sites were selected due to their proximity to the Kitchener and Waterloo MWWTP outfalls and to represent similar riffle/run habitats (Figure 2.1). This study comprised a total of nine sites all located on the Grand River and spanning a distance of 60 km from the furthest upstream to the furthest downstream site (Figure 2.1). These sites were sampled between 2007–2014 in spring and/or fall seasons, however fish and primary consumer samples from archived collections (2007 to spring 2013) were not always available for all sites in every year/seasons due to different study objectives (Table S2.1).

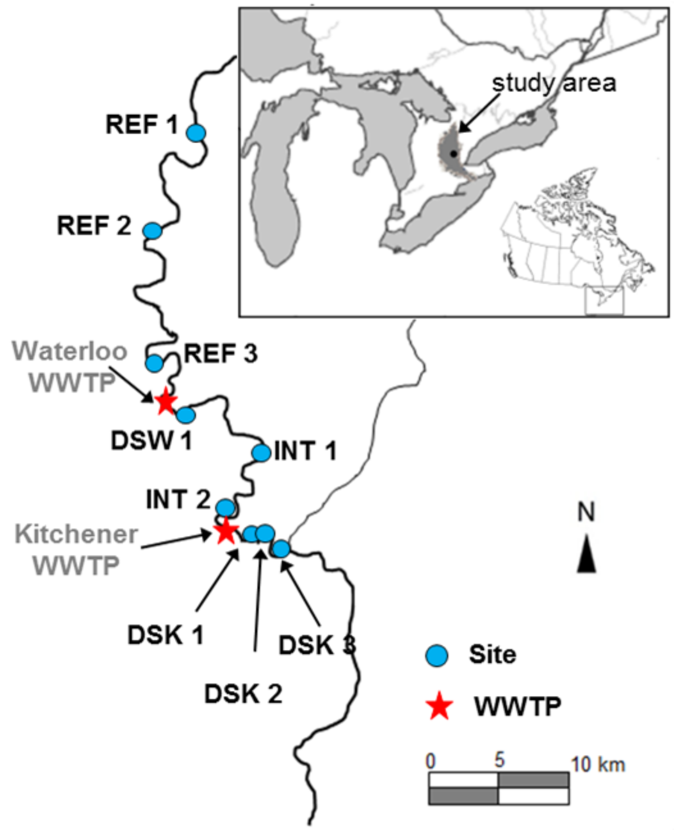

Figure 2.1 Map of the sampling sites along the Grand River, Ontario, where fish and primary consumers were collected annually during the fall and spring periods of 2007–2014. Reference sites include two non-urban reference sites (REF 1 and REF 2) and one urbanized reference site (REF 3). The exposure sites consist of one near-field exposure site downstream of the Waterloo WWTP (DSW 1) and two sites located farther downstream (INT 1 and INT 2) but upstream of the Kitchener outfall. Three exposure sites were sampled downstream of the Kitchener WWTP outfall (DSK 1, DSK 2, and DSK 3).

Two of the nine sites (REF 1 and REF 2) in this study were in non-urban environments, outside of the Kitchener and Waterloo city limits. These sites were included in this study to characterize any change related to urbanization and also to characterize spatial heterogeneity typical of a river system (Vannote et al., 1980). The first urban reference site (REF 3) is located 5 km above the Waterloo MWWTP outfall. The first near-field exposure site (DSW 1) is located 1 km downstream from the Waterloo MWWTP outfall. There are two sites located further downstream from the Waterloo MWWTP but upstream of the Kitchener MWWTP. INT 1, which is 12 km downstream from the Waterloo MWWTP outfall and INT 2 which is located 19 km downstream from the Waterloo MWWTP outfall and 1 km upstream from the Kitchener

MWWTP outfall. There are three additional exposure sites each located at 0.5 km (DSK 1), 1.5 km (DSK 2), and 5 km (DSK 3) downstream of the Kitchener MWWTP outfall.

2.3.2 Effluent characterization and river discharge

To assess changes in effluent quality at the Kitchener and Waterloo MWWTPs, total annual tonnage of ammonia and nitrate released on site was obtained through the Environment Canada National Pollutant Release Inventory (Environment and Climate Change Canada, 2015b). These data were also used to test for any associations between annual changes in ammonia tonnage and δ^{15}N measured in biota. Additional plant characterizations (population served, effluent flow, and effluent quality) at the Kitchener and Waterloo MWWTPs from 2007–2014 were provided by the Region of Waterloo (2016) (Table S2.2).

Daily river discharge in the Grand River was obtained to assess if any relationships existed with annual river discharge and annual variability in stable isotope ratios. Relationships between annual δ^{13}C in biota and discharge was of particular interest as river flows will drive CO_2 supply and possibly relate to annual δ^{13}C values in biota (Finlay et al., 1999). These data were available for all the years of this study (2007–2014) and were obtained by the Water Survey of Canada at one flow gauge just above the Kitchener MWWTP outfall (station: 02GA048) (Environment and Climate Change Canada, 2015c)

2.3.3 Fish collection and primary consumers

The rainbow darter is a small-bodied, highly abundant and widely distributed species found throughout the Grand River watershed and is thought to have limited mobility (Tetreault et al., 2011). Rainbow darter are benthic and their diet consists of benthic invertebrates including members of the groups Chironomidae, Trichoptera, Ephmeroptera and Isopoda (Robinson et al., 2016). Rainbow darter were collected consistently across all years at the selected sites in the spring and/or fall using backpack electrofishing (Smith Root LR-24). Normally 20 males and 20 females were collected from each site per sampling event and 3 to 20 fish (normally 8; Table S2.1) were sub-sampled per site for stable isotope analysis. To limit variability, the size range of the fish selected for isotope analysis was minimized (5.6 ± 0.91 cm, *n*=574) to reduce any variability related to fish size. After being euthanized by a blow to the head and spinal severance, fish bodies were placed in bags, transported on ice, and stored at -20°C until further analysis. All fish were handled according to protocols approved by the University of Waterloo animal care committee (AUPP #10-17).

Primary consumers (benthic invertebrates) were also included in this study as an additional trophic level in the aquatic food web. They were sampled less frequently than the rainbow darter and only in the years 2007, 2013 and 2014. Details on the collection of primary consumers in 2007 are provided in Loomer et al. (2015). The most abundant taxonomic groups collected in 2007 included Ephemerellidae, *Stenonema* sp., *Asellus* sp., Chironomidae, Elmidae, Hydropsychidae, Simuliidae, *Physella* sp., and Sphaeriidae. In the fall of 2013 and 2014, only members of the family Hydropsychidae (Trichoptera) were sampled as they were highly abundant, available at all sites, and are known to be part of the rainbow darter diet in the Grand River watershed (Robinson et al., 2016). In this two-year period, all primary consumers were sampled using a D-frame kick net. Samples collected in 2007 were held on ice in the field prior to being stored at -20°C. Live organisms collected in 2013 and 2014 were held overnight in a petri-dish with filtered river water to allow enough time to clear their gut content then frozen at -20°C.

2.3.4 Diet switch study

Rainbow darter (n = 80) were collected in July (2014) from the wild in the upper Grand River of southern Ontario, near the town of Grand Valley by backpack electrofishing. Benthic invertebrates from the families Hydropsychidae (*n* = 40) and Heptageniidae (*n* = 15) were also collected at the site to get a stable isotope ratio baseline of their original diet. Fish were brought back to the lab in coolers on aerators and transferred randomly to 20 L tanks (6-7 fish/tank) in a flow through AHAB (Aquatic Habitats) system with 10% water replacement every 24 h. Throughout the study water temperature was maintained at 18ºC reflecting summer conditions of their natural habitat with day and night cycles continuous at 12 h light and 12 h dark. Fish were fed to satiation twice a day with frozen bloodworms (San Francisco Bay Brand, Inc), which had an isotope signature different from their original diet. Prior to the experiment, eight fish were immediately sacrificed to get a baseline isotope estimate for both muscle and liver tissue. Fish were sampled more frequently in the first month (day 3, 7, 10, 14, 21, 28) as the isotope turnover was expected to be a first order process. This was followed by biweekly sampling (day 42 and 54) and the last fish were sampled on day 84. During each sampling event, 4 males and 4 females were randomly selected from tanks, sacrificed, and fish length, weight, liver weight and gonad weight were recorded. The exception was at 84 d where only 6 fish (4 males and 2 females) remained. At each time point, liver tissue was transferred to a cryovial and fish bodies were placed in bags, both stored at –20^{o}C until being further processed.

2.3.5 Stable isotope analysis

A skinless piece of epaxial dorsal muscle tissue was removed from one side of each fish collected from either the field or the diet switch study. Fish muscle tissue, whole liver (from the diet switch study) or a whole invertebrate were freeze dried, and ground into a fine homogenous power using a ball and mill grinder (Retsch MM2000, 1996). Homogeneous powdered tissues were weighed (0.25 – 0.30 mg) into tin capsules. Carbon and nitrogen elemental composition (%) and stable isotope ratio composition (δ^{13}C and δ^{15}N) was determined through combustion conversion of sample material to gas through a 4010 Elemental Analyzer (Costech Instruments, Italy) coupled to a Delta Plus XL (Thermo-Finnigan, Germany) continuous flow isotope ratio mass spectrometer (CFIRMS). The δ^{13}C and δ^{15}N are corrected delta values reported in per mil (‰) against the standards Vienna Pee Dee Belemnite and atmospheric nitrogen, respectively. All benthic invertebrate δ^{13}C values were lipid normalized using the formula published in Post (2002). The C:N ratio of rainbow darter collected in the field was considered low (3.6 ± 0.4, n = 262) and consistent between fish, thus a lipid correction had minimal effect on the interpretation of the data. However, δ^{13}C values in rainbow darter muscle from the diet switch study were lipid normalized, as this reduced the variability among fish. Liver tissue data from the diet switch study was also lipid-normalized since the C:N ratios were highly variable (7.2 ± 2.6, n = 81). A subset of the samples (n = 55) for both fish tissue and benthic invertebrates were run in duplicate. The mean (± SE) difference between replicates was 0.12 ± 0.02‰ and 0.20 ± 0.03‰, for δ^{13}C and δ^{15}N, respectively.

2.3.6 Statistics and data analysis

To test whether there was a difference in mean δ^{15}N and δ^{13}C before and after process alterations at the Waterloo or Kitchener MWWTPs, it was only sensible to compare the immediate upstream site (REF 3 or INT 2) with their associated downstream site (DSW 1, DSK 1, DSK 2 or DSK 3). This is due to the spatial change naturally associated with rivers (Vannote et al., 1980) and the processing of nitrogen and carbon along the river gradient that is naturally associated with changing stable isotope ratios (Rounick and Winterbourn, 1986). The additional non-urban reference sites (REF 1 and REF 2) and intermediate site (INT 1) are included in this study to characterize this spatial change. Stable isotope values for fish and primary consumers are reported in the text as the absolute difference between the immediate upstream site and its associated downstream site (downstream delta value – upstream delta value). Two-way ANOVAs, with factors site and year, were computed separately for each combination of immediate upstream site and their associated downstream site (i.e., REF 3 vs. DSW 1; INT 2 vs. DSK 1; INT 2 vs. DSK 2; and INT 2 vs. DSK 3). Analyses for each combination of sites were done separately because of the unbalanced design (not all sites were sampled each year). To assess spatial change in $\delta^{15}N$ and $\delta^{13}C$, one-way ANOVAs were computed for each year to test for spatial differences across all sites. All one-way and two-way ANOVAs were analyzed for fish and primary consumers for both spring and fall seasons. Pairwise comparisons were computed with a Tukey's post-hoc test. The assumption of equal variance was often not met (even with transformation); therefore to reduce the risk of a type 1 error due to heterogeneous variance, alpha was set to 0.01 to assess statistical significance.

To test whether annual changes in $\delta^{15}N$ at downstream sites could be associated with changing effluent quality, Pearson correlations were computed with mean annual $\delta^{15}N$ in biota and annual ammonia tonnages released on site. Similarly, annual variability in $\delta^{13}C$ (at both reference and exposure sites) was tested against the annual median 6 month river discharge using Pearson correlations. Due to small sample sizes, Pearson correlations were limited to data sets with six or more years ($n \geq 6$), which excludes all primary consumers and spring data sets with rainbow darter.

To monitor any changes in health of the rainbow darter throughout the diet switch study, somatic indices including condition factor (k = body weight/length^3 x 100), gonadosomatic index (GSI = gonad weight/body weight x 100), and liver somatic index (LSI = liver weight/body weight x 100) were computed. Changes in k, GSI and LSI throughout the study were assessed using a one-way ANOVA. Isotopic turnover rate and half-life in muscle and liver tissue were estimated using a one compartment model as described in Hobson and Clark (1992) where isotopic change is expressed as an exponential function of time (equation one).

$$[1]\ f = y + a*e^{(-bt)}$$

Where f is the isotopic value (‰) of the organism at time t (days), y is the isotopic value (‰) after equilibration with the new diet, a is the difference in isotopic value between the initial isotopic value (at time 0) and y, and b is the derived constant (turnover rate/day). The constant (b) can be entered into equation two to yield the half-life.

$$[2]\ \text{half-life} = \ln(2)/b$$

A one-way ANOVA was used to test the difference in isotopic turnover rates between males and females as well as tissue types (muscle and liver). All data were analyzed and plotted using SigmaPlot version 13.

2.4 Results

2.4.1 Effluent quality

Annual tonnage of total ammonia and total nitrate released from the Kitchener and Waterloo MWWTPs between 2007–2014 indicated that effluent quality (based on ammonia) had changed during these years (Figure 2.2). Nitrate was inversely related to the ammonia and is a strong indicator of the degree of nitrification occurring at the MWWTPs. In pre-upgrade years (2007–2011), the Kitchener MWWTP released 500–600 t/year of total ammonia. Total ammonia levels began to drop in 2012 (beginning of upgrades) and by 2013, had dropped sixfold (100 t/year) relative to 2011 (pre-upgrade). Total ammonia loading increased slightly in 2014, possibly due to process upsets from additional upgrades. In contrast to the Kitchener MWWTP, the total annual tonnage of ammonia at the Waterloo MWWTP increased by as much as 3.7 fold between 2007 and 2012, and ranged from 135 t/year in 2008 to 500 t/year in 2012; reaching levels similar to the Kitchener MWWTP before it was upgraded. There was a slight decrease in ammonia tonnage in 2014, likely due to the installation of return activated sludge reaeration (to treat the centrate); however, proper aeration was not installed thus full nitrification was not achieved. Other than ammonia and nitrate, there were no other major changes in effluent quality measured at either of the MWWTPs during the period of this study (Table S2.2).

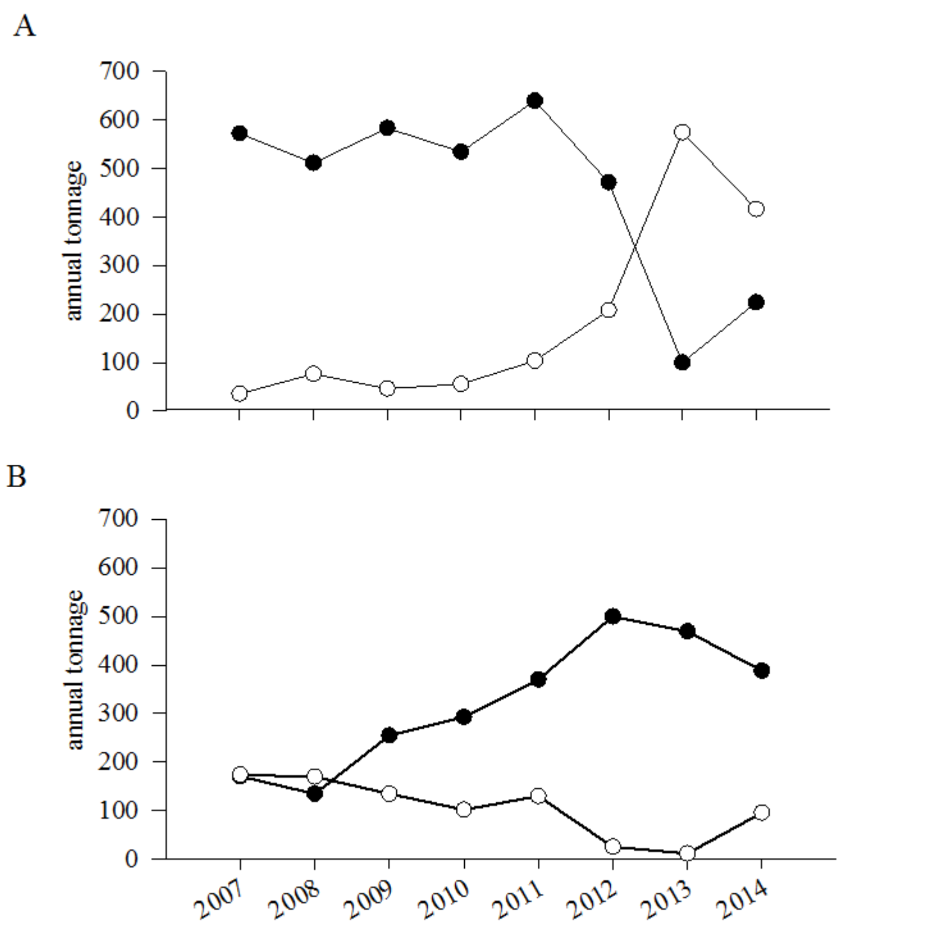

Figure 2.2 Annual tonnage of total ammonia and nitrate (ion in solution at pH ≤ 6) released from the (A) Kitchener and (B) the Waterloo MWWTPs from 2007–2014. Closed circles represent ammonia and open circles represent nitrate. This data was provided by Environment and Climate Change Canada National Pollution Release Inventory: https://www.ec.gc.ca/inrp-npri/ (accessed September 2015).

2.4.2 Stable isotope ratio of $\delta^{15}N$

Prior to the Kitchener MWWTP upgrades, including the year of the initial upgrades (2012), $\delta^{15}N$ values of rainbow darters were consistently lower (3.9–9.7 ‰, $p < 0.001$) downstream (DSK 1 and DSK 2) compared to the immediate upstream site (INT 2; Figure 2.3 A and C) in both fall and spring. $\delta^{15}N$ values at the third, and furthest site downstream of Kitchener (DSK 3), were higher than either of the two preceding, exposed sites (DSK 1 or DSK 2), indicating a slight recovery. Primary consumers followed the same trend as rainbow darter in 2007, the only pre-upgrade year they were sampled (spring and fall 2007; Figure 2.3 B and D). In the fall, $\delta^{15}N$ at DSK 1 was 6.8 ‰ lower ($p < 0.001$) relative to the immediate upstream site

(INT 2). Spring primary consumer data showed a similar trend, however with greater variability, likely driven by variability in the different species of primary consumers collected representing different feeding regimes. The furthest downstream site (DSK 3) again mirrored the same pattern observed in rainbow darter (Figure 2.3).

Unfortunately, Hydropsychidae were not available at all sites when primary consumers were sampled in 2007. However, they were available for sites REF 2, DSW 1, and DSK 1, and indicate that they do follow the same patterns as the pooled primary consumers (Figure 2.3 B). This provided a level of confidence that valid comparisons could be made between pooled primary consumers and Hydropsychidae at least in terms of their patterns in isotopic signatures between the different years.

After the upgrade at the Kitchener MWWTP (2013–2014), there was a large shift in $\delta^{15}N$ values in rainbow darter and primary consumers at the downstream sites (Figure 2.3). In the fall assessments post-upgrade, $\delta^{15}N$ values in both fish and primary consumers at all three exposure sites (DSK 1, DSK 2, DSK 3), were no longer different than $\delta^{15}N$ values at the immediate upstream site (INT 2; Figure 2.3 A and B). The only exception in the fall was at DSK 3, where for the first time, rainbow darter $\delta^{15}N$ values were higher than INT 2 by 2.1 ‰ ($p = 0.01$).

The shift in $\delta^{15}N$ values in biota collected in the spring during post upgrade years was not as obvious (Figure 2.3 C and D). In the spring of 2013 (approximately 8 months post-upgrade), the $\delta^{15}N$ value of rainbow darter at DSK 1 and DSK 2 were still significantly lower by 5.1 ‰ and 4.3 ‰ ($p < 0.001$), respectively, compared to INT 2. In the spring of 2014, the $\delta^{15}N$ value in rainbow darter at DSK 1 was still significantly lower by 2 ‰ ($p < 0.01$); however, DSK 2 was no longer different than INT 2. The third exposure site (DSK 3) was not measured in spring 2013 or 2014. Primary consumers sampled in the spring of 2014 still showed lower $\delta^{15}N$ values at DSK 1 (1.3 ‰) and DSK 2 (1.6 ‰) compared to INT 2 (Figure 2.3 D) but were not significant. The degree of difference, however, was not at the same magnitude observed prior to the upgrades in either spring 2007 (4.5 ‰) or fall 2007 (6.8 ‰; Figure 2.3 B and D). Additional supportive statistics are provided in supplementary data (Table S2.3).

Rainbow darter downstream of the Waterloo MWWTP (DSW 1) in the years of better effluent quality (2007–2010) were not significantly different from the immediate upstream site (REF 3), but showed a pattern of $\delta^{15}N$ values being slightly higher in both spring and fall seasons (0.6–1.3 ‰) (Figure 2.3 A and C). When the effluent quality decreased (2011–2014), rainbow darter at DSW 1 had $\delta^{15}N$ values that shifted 2.3–3.5 ‰ ($p < 0.001$) lower than REF 3 in the fall. The same trend of decreasing $\delta^{15}N$ values at DSW 1 was observed in the spring, however, only significantly lower by 4.9 ‰; ($p < 0.001$) in 2014.

Primary consumers followed a similar trend as rainbow darter. In years of better effluent quality (2007–2010), they showed a pattern of $\delta^{15}N$ values either being higher or not different from REF 3 (fall 2007 [5.3 ‰; $p < 0.001$]; spring 2007 [0.6 ‰; $p = 0.211$]). In years of poor effluent quality (2011–2014) they had $\delta^{15}N$ values lower than REF 3, ranging from 5.1–5.6 ‰ ($p < 0.001$) (Figure 2.3B and D). Additional supportive statistics are provided in supplementary data (Table S2.4).

In the fall of 2013 and 2014, an additional site (INT 1) was sampled 11 km further downstream of DSW 1 and 7 km upstream of INT 2. In rainbow darter, the $\delta^{15}N$ values are significantly higher by 6.6 ‰ ($p < 0.001$) in 2013 and 4.6 ‰ ($p < 0.001$) in 2014, indicating that the ammonia discharged from the Waterloo MWWTP had been processed within the 11 km of river from the MWWTP outfall. The same pattern is seen in primary consumers, however to a much lower degree than rainbow darter. In fall 2013, primary consumers at INT 1 had $\delta^{15}N$ values slightly higher by 1.8 ‰ ($p < 0.001$) and in 2014 they were higher by 1.2 ‰ ($p = 0.003$).

Higher annual tonnage of ammonia released at the Kitchener or Waterloo MWWTP correlated negatively with $\delta^{15}N$ values of rainbow darter collected in the fall at the exposed sites (DSK 1, DSK 2, DSW 1; Figure 2.4). Pearson correlations were limited to fall fish where there were reasonable sample sizes. Primary consumers followed a similar trend at sites downstream of the Waterloo and Kitchener MWWTP, where years with higher ammonia tonnages appeared to be associated with lower $\delta^{15}N$ values (Figure 2.3 B and D); however, it was difficult to make any conclusions with only 2 or 3 years of data.

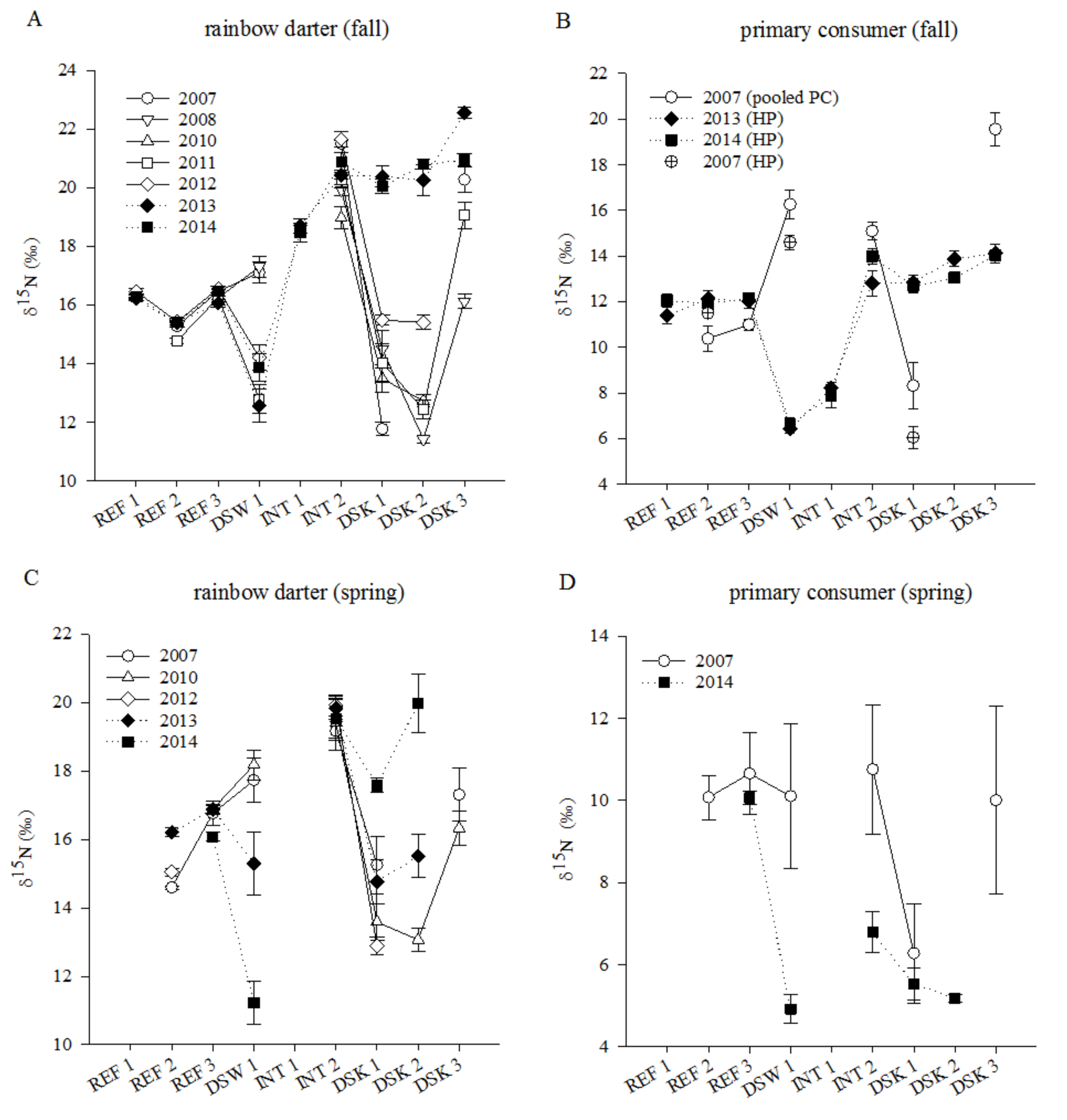

Figure 2.3 $\delta^{15}N$ (mean ± SE) of rainbow darter muscle tissue collected in the (A) fall and (C) spring and primary consumers (PC) collected in the (B) fall and (D) spring above and below the Waterloo and Kitchener MWWTP in 2007–2014. The open symbols and solid lines represent the years 2007–2012 (pre-upgrades at the Kitchener MWWTP) and the solid symbols and dotted line represent the years 2013–2014 (post upgrades at the Kitchener MWWTP). Open circles with a cross (B) represents the family Hydropsychidae (HP) only.

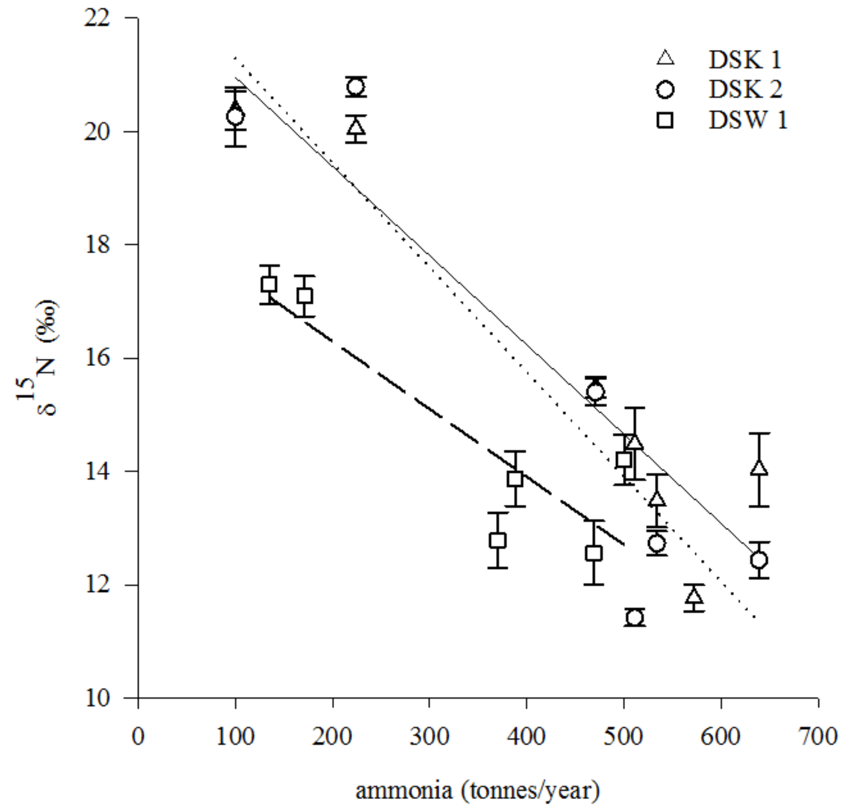

Figure 2.4 Relationship between total annual ammonia released on site (t/year) and $\delta^{15}N$ in rainbow darter collected in the fall at downstream sites from 2007–2014 where there were six or more years of data ($n \geq 6$). Annual ammonia tonnages from the Kitchener MWWTP are tested against $\delta^{15}N$ values from DSK 1 (triangles) and DSK 2 (circles), while annual ammonia tonnages from the Waterloo MWWTP are tested against $\delta^{15}N$ from DSW 1 (squares). A line of best fit is included where there was a significant correlation and is indicated by a dotted line (DSK 1; r = -0.94; $p < 0.05$, $n = 7$), a solid line (DSK 2; r = -0.93; $p < 0.05$; $n = 6$) or a dashed line (DSW; r = -0.88; $p < 0.05$, $n = 6$).

2.4.3 Stable isotope ratio of $\delta^{13}C$

Final effluent quality, whether it was decreasing (Waterloo MWWTP) or increasing (Kitchener MWWTP), did not affect the patterns of $\delta^{13}C$ values of either rainbow darter or primary consumers, over the years (Figure 2.5 A–D). The first exposure site immediately downstream of the Kitchener (DSK 1) and Waterloo (DSW 1) MWWTPs had consistent patterns of higher $\delta^{13}C$ values in both rainbow darter and primary consumers relative to their corresponding immediate upstream sites, INT 2 and REF 3, across all years and seasons (Table S2.6 and S2.7). Downstream of the Waterloo MWWTP (DSW 1), rainbow darter $\delta^{13}C$ values were higher by 0.8–1.4 ‰ in the fall and 0.3–1.0 ‰ in the spring, whereas primary consumers

$\delta^{13}C$ values were higher by 1.6–4.9 ‰ in the fall and 0.6–1.7 ‰ in the spring relative to REF 3. Downstream of the Kitchener outfall (DSK 1), rainbow darter $\delta^{13}C$ values were higher by 1.1–2.7 ‰ in the fall and 0.9–1.8 ‰ in the spring; whereas primary consumer $\delta^{13}C$ values were higher by 1.3–3.9 ‰ in the fall and 1.1–2.1 ‰ in the spring relative to the reference site (INT 2) (Table S2.6 and S2.7).

The degree of difference between DSK 1 and INT 2 was higher in pre-upgrade years compared to post-upgrade years for rainbow darter and primary consumers sampled in both the fall and spring (Table S2.5). This coincided with the wetter years (Figure 2.6), making it difficult to separate out changes that could be related to annual discharge verses process changes at the MWWTPs. Similar patterns are distinct at other sites (e.g. REF 1, REF 2, and REF 3; Figure 2.5) suggesting that these changes are likely related to natural processes in the river and are less likely to be related to the MWWTP upgrades.

Based on the Pearson correlations, there is evidence to suggest that the $\delta^{13}C$ values in rainbow darter are associated with the year they were sampled and the median six month discharge (Figure 2.7). There were consistent negative correlations between $\delta^{13}C$ values in fish collected in the fall with median six month flow, where drier years had higher $\delta^{13}C$ values and wetter years had lower $\delta^{13}C$ values. These relationships were only significant for rainbow darter at the sites further downstream including INT 2, and the first two sites below the Kitchener MWWTPs (DSK 1 and DSK 2). Correlations were limited to fall fish where sample sizes were greater ($\geq$ 6 years).

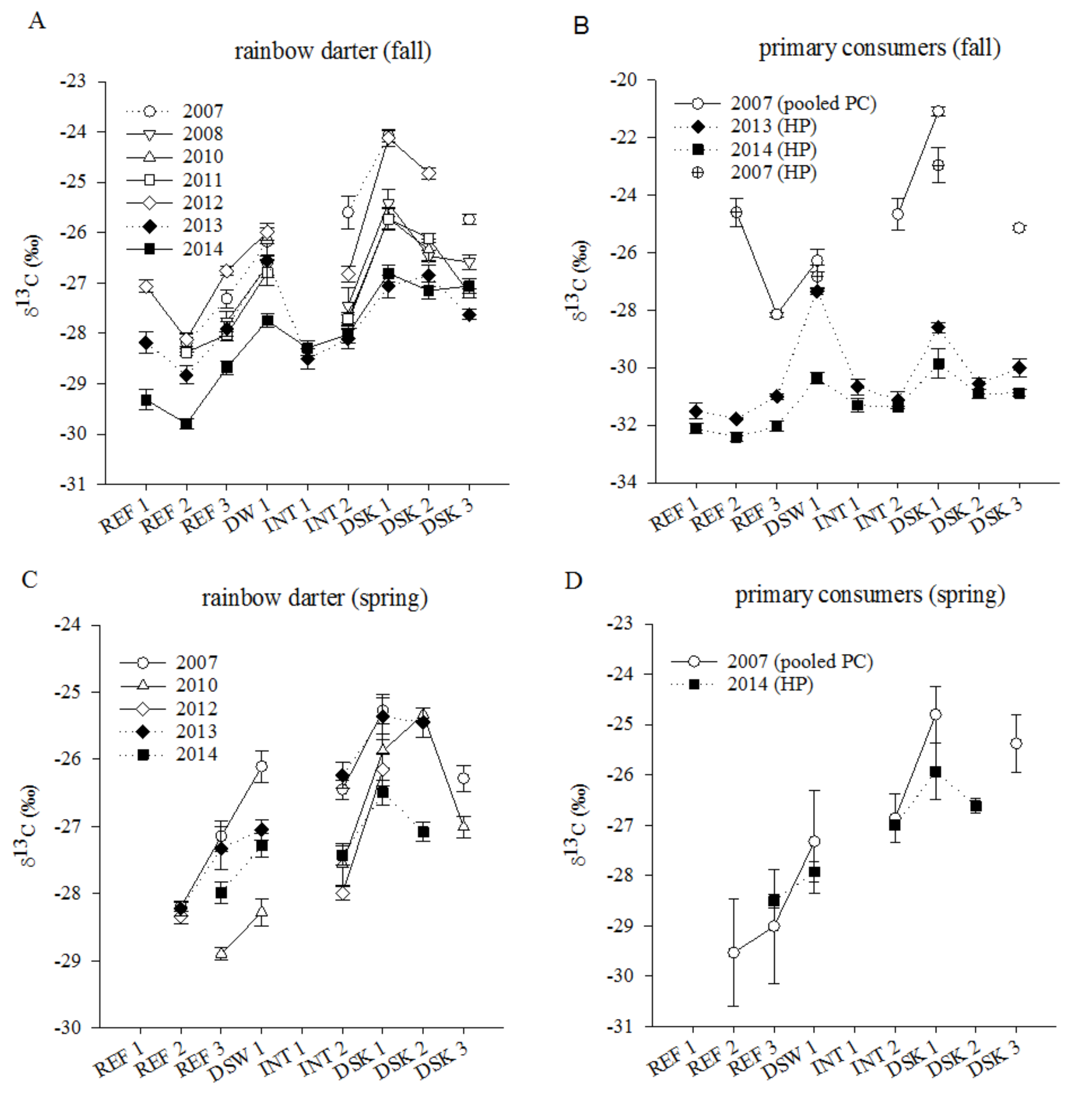

Figure 2.5 $\delta^{13}C$ (mean ± SE) of rainbow darter muscle tissue collected in (A) the fall and (C) the spring (C) and primary consumers (PC) collected in (B) the fall and (D) the spring above and below the Waterloo and Kitchener WWTP in 2007–2014. The open symbols and solid lines represent the years 2007–2012 (pre-upgrades at the Kitchener MWWTP) and the solid symbols and dotted line represent the years 2013–2014 (post upgrades at the Kitchener MWWTP). Open circles with a cross (B) represents the family Hydropsychidae (HP).

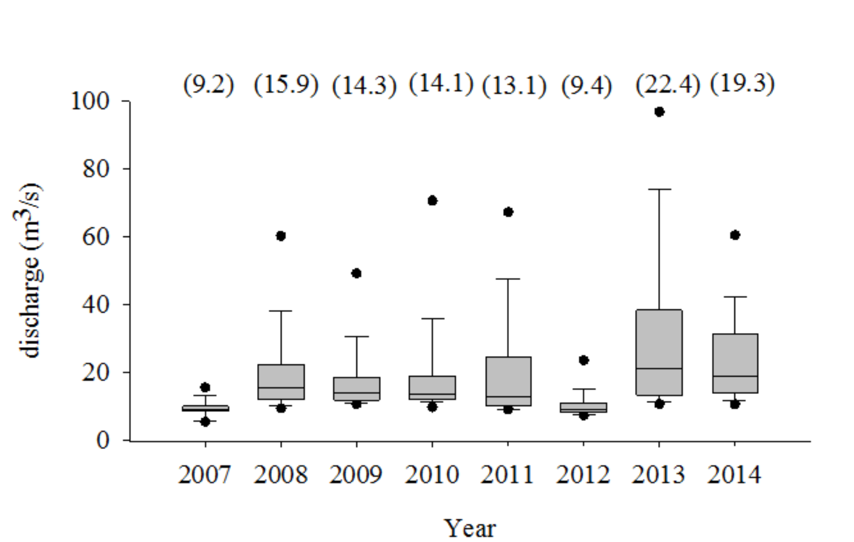

Figure 2.6 Boxplots representing the annual median six month (May–October) river discharge (m^3/s) at a flow station located upstream of DSK 1. Horizontal lines represent the median (also provided in brackets above each box plot), boxes are the 25th and 75th percentiles, whiskers are the 10th and 90th percentiles, and outliers (solid dots) represent the 5th and 95th percentiles. Data was provided by the Water Survey of Canada: https://wasteroffice.ec.gc.ca/ (accessed November, 2015).

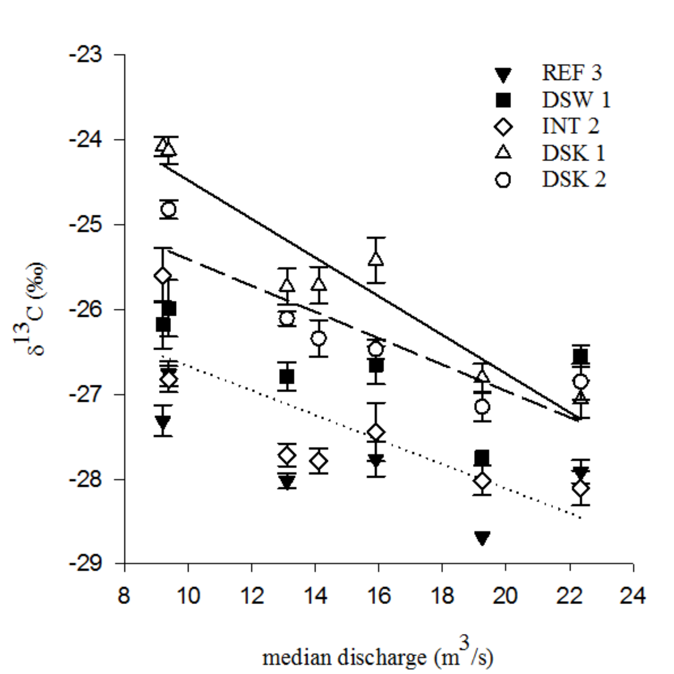

Figure 2.7 Relationship between median river discharge (m^3/s) and $\delta^{13}C$ in rainbow darter collected in the fall from 2007–2014 for sites with six of more years of data ($n \geq 6$). Sites with a significant correlation are indicated by open symbols and a line of best fit with either a dotted line (INT 2; r = -0.795, p = 0.033, n = 7), a solid line (DSK 1; r = -0.953, p < 0.001, n = 7), or a dashed line (DSK 2; r = -0.884, p = 0.020, n = 6). Sites that had no significant correlations are indicated by closed symbols and include REF 3 (r -0.733, p = 0.098, n = 6) and DSW 1 (r = -0.618, p = 0.191, n = 6).

2.4.4 Diet switch study

Rainbow darter muscle and liver tissues shifted isotopic signatures toward values representative of their new diet during the 84-day diet switch study (Figure 2.8). Throughout the experiment the condition factor did not change, however, liver size increased in both males and females throughout most of the study, indicating that the rainbow darter were sufficiently feeding (Table S2.7). The new diet fed to rainbow darter during the diet switch study had a large difference in isotope composition ($\delta^{13}C$: -22.67 ± 0.10 ‰; $\delta^{15}N$: 1.32 ± 0.13 ‰) compared to the original rainbow darter diet ($\delta^{13}C$: -29.41 ± 0.11 ‰; $\delta^{15}N$: 8.97 ± 0.16 ‰) and rainbow darter baseline (Figure 2.8). The direction of the isotopic shift was opposite for $\delta^{15}N$ and $\delta^{13}C$, revealing patterns of depletion and enrichment, respectively. Thus the data were fit to the appropriate models to estimate isotopic turnover rates and half-life. Males and females were pooled together to estimate isotope turnover rate, because they did not differ significantly for either muscle ($\delta^{15}N$, p = 0.865; $\delta^{13}C$, p = 0.205) or liver ($\delta^{15}N$, p = 0.876; $\delta^{13}C$, p = 0.392). Muscle tissue turnover rates for $\delta^{15}N$ and $\delta^{13}C$ were estimated to be 0.024 ± 0.044 ‰/d (half-life = 29 d) and 0.021 ± 0.006 ‰/d (half-life = 33 d), respectively. Liver tissue turnover rate for $\delta^{15}N$ was estimated to be 0.044 ± 0.015 ‰/d (half-life = 16 d) nearly double that of muscle, though not significantly different (p = 0.273). Liver tissue turnover rate estimated for $\delta^{13}C$ was 0.059 ± 0.015 ‰/d (half-life = 12 d) which was nearly triple the rate compared to muscle tissue (p = 0.04).

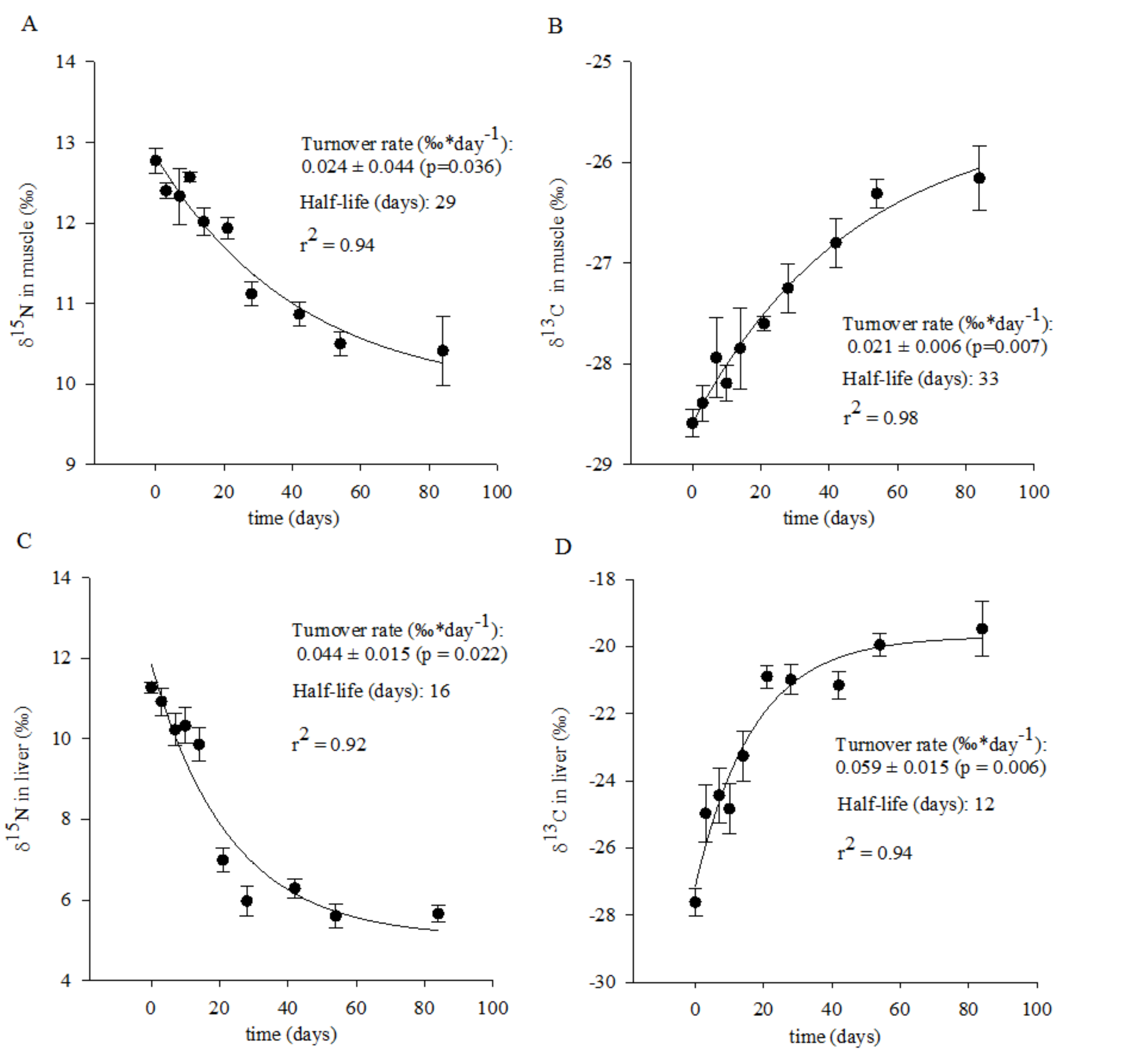

Figure 2.8 Isotopic turnover rate ($\delta^{15}N$ and $\delta^{13}C$) in muscle (A and B) and liver (C and D) as an exponential function of time during an 84 day diet switch study with rainbow darter. Each point represents the mean ± SE of 6–8 fish. $\delta^{13}C$ values for muscle and liver were lipid corrected using the formula from Post (2002). Data from $\delta^{15}N$ is fitted to an exponential decay, single, 3 parameter model and $\delta^{13}C$ is fitted to an exponential rise to a maximum, single, 3 parameter model. Turnover rate constant (± SE) and associated *p* value, half-life, and coefficient of variation (r^2) are represented in each figure.

2.5 Discussion

2.5.1 Change in $\delta^{15}N$

The upgrades at the Kitchener MWWTP, which resulted in increased nitrification, considerably reduced the amount of ammonia (and increased nitrate) in the final effluent. This change in effluent quality was associated with higher $\delta^{15}N$ values of both fish (rainbow darter) and primary consumers in the receiving environment relative to years prior to the upgrades. This is also consistent with higher $\delta^{15}N$ values often associated with secondary or greater treatment plant outfalls (Wayland and Hobson, 2001; Morrissey et al., 2013; Robinson et al., 2016) rather than reflecting that of a primary treatment plant where $\delta^{15}N$ has been shown to usually be depleted in ^{15}N (deBruyn and Rasmussen, 2002).

The observation that effluent quality (total ammonia) was associated with $\delta^{15}N$ provides further evidence that the sewage-derived nutrients were being assimilated by aquatic organisms directly below the Kitchener and Waterloo MWWTPs. This is likely a result of the trophic transfer of the nitrogen signature from NH_4 from the MWWTPs to the rainbow darter. A study by Hood et al. (2014) measured $\delta^{15}N$ of effluent (NH_4 and NO_3), river water, and macrophytes (*Potamogeton* spp.) along the Grand River above and below the Kitchener and Waterloo MWWTPs prior to the MWWTP changes (2007–2009). The $\delta^{15}N$ values they measured in macrophytes downstream of both Waterloo and Kitchener showed similar patterns as primary consumers and fish in the current study during that same period. Hood et al. (2014) also demonstrated that the macrophytes in close proximity to the MWWTPs were incorporating effluent NH_4, providing further evidence that sewage-derived nutrients were being incorporated into the food web.

The changes in $\delta^{15}N$ that occurred in biota downstream of the Kitchener MWWTP (after the upgrades) were likely a result of the enhanced nitrification process. Nitrifying bacteria are able to convert NH_4 to NO_3 and preferentially, convert $^{14}NH_4$ over $^{15}NH_4$ (Heaton, 1986), resulting in an effluent with a larger proportion of ^{15}N. Other processes in MWWTPs such as volatilization and denitrification also favour the lighter stable isotope ($^{14}NH_3$ and $^{14}NO_3$) further increasing the $\delta^{15}N$ signature in MWWTP effluents (Heaton, 1986). A higher proportion of $^{15}NH_4$, which primary producers will preferentially take up in the presence of both NH_4 and NO_3 (Birgand et al., 2007), will result in higher $\delta^{15}N$ values.

Although the $\delta^{15}N$ values in biota below the Kitchener MWWTP were higher after the upgrade compared to the previous years, they did not become more enriched relative to upstream values as was observed in other studies with secondary or more advanced treatment (Morrissey et al., 2013; Robinson et al., 2016). This may be for a number of reasons. The immediate upstream site from the Kitchener MWWTP (INT 2) had relatively high $\delta^{15}N$ values for a river system (19–22 ‰, Heaton, 1986). This could be a result of natural nitrification/denitrification processes along the length of the river (Seitzinger et al., 2002) in addition to other possible anthropogenic factors. For example, there is a weir in close proximity to INT 2 (1.5 km upstream) possibly creating a small reservoir effect. This could enhance the nitrification and denitrification processes resulting in ^{15}N enriched inorganic nitrogen (Marty et al., 2008). There are also several golf courses adjacent to the river that may be inputting organic fertilizers, which generally have highly enriched signatures (+20 ‰), compared to inorganic fertilizers which are closer to 0 ‰ (Heaton, 1986). The Grand River watershed also supports the most intensive agriculture in southern Ontario. With the addition of inputs from several other MWWTPs, the central Grand River has continuously high nitrogen loads (Loomer and Cooke, 2011). Those high loads could be influencing $\delta^{15}N$ values of biota in the central region of the Grand River that are on the upper end (15–22 ‰) for aquatic systems (Heaton, 1986). The saturation of the surface waters with nitrogen, along with the recent improvements in effluent quality, may cause the minimal effect of the Kitchener MWWTP effluent on $\delta^{15}N$ in the biota downstream in later years of the study.

Changes in $\delta^{15}N$ below the Kitchener MWWTP was most evident in the fall of post-upgrade years (2013 and 2014), and less evident in the spring. The improvements in effluent quality started in August of 2012 and the effluent was not fully nitrified until January of 2013. The $\delta^{15}N$ in rainbow darter caught in the spring of 2013 at DSK 1 had not yet fully reflected the Kitchener MWWTP upgrades (i.e., the values were still lower relative to the upstream site). This is likely because the upgrade occurred after the major growth period of the rainbow darter, thus they were reflecting their diet from spring/summer 2012 (prior to the upgrade). This has been illustrated in a study with whitefish (*Coregonus lavaretus*) where muscle tissue $\delta^{15}N$ values had reflected their diet from the previous spring and summer months, the period in which they were growing (Perga and Gerdeaux, 2005).

It wasn't until fall 2013 that rainbow darter collected downstream of the Kitchener MWWTP reflected the same values as those in the immediate upstream site. However, in spring of 2014, rainbow darter $\delta^{15}N$ had decreased again. This was likely due to a process upset at the Kitchener MWWTP in the first five months (January–May) of 2014 which resulted in increased effluent ammonia concentrations (Bicudo et al., 2016). In addition, even though rainbow darter likely are not growing much in the winter months, biological activity at the MWWTPs (nitrification and denitrification) is likely reduced during this time compared to summer months resulting in nitrogen species (NH_4 and NO_3) being more depleted. This was demonstrated in Jordan et al. (1997) where effluent NH_4 had $\delta^{15}N$ values less enriched in winter months (+8 to +11 ‰) compared to summer months (+13 to +19 ‰) at a MWWTP in Falmouth (Cape Cod), Massachusetts, USA.

The Waterloo MWWTP was expected to have upgrades completed during the time period of the study but construction delays and other changes resulted in a deterioration of the effluent quality. The effluent quality began to decrease in 2009, which coincided with the commencement of dewatering the biosolids. The resulting centrate, which is high in ammonia, was recycled back into the treatment system. Centrate, in addition to other construction issues on site resulted in the output of ammonia being almost fourfold higher. This change in effluent quality was associated with a shift in rainbow darter and primary consumer $\delta^{15}N$ from being either not different or slightly enriched in ^{15}N (relative to upstream) to being highly depleted in ^{15}N; reflecting the characteristics of exposure to effluent from a primary treated MWWTP (deBruyn and Rasmussen, 2002).

The $\delta^{15}N$ values among fish (replicates) within a year are much more variable at the sites downstream of wastewater outfalls than in upstream reference sites. One factor may be that fish are moving in and out of the MWWTP effluent plume. On several occasions at DSK 1, there were fish with $\delta^{15}N$ signatures outside the population mean by as much as 8 ‰ or nearly 3 standard deviations. The diet switch study estimated that it would take approximately 29 days for one half of $\delta^{15}N$ in rainbow darter muscle tissue to turn over when eating a diet with a different stable isotope signature (based on turnover rate of 0.0239 ‰/d). Therefore a fish with a signature outside the normal range may have recently (< month) moved into the plume and did not have enough time to reach a steady state with their new diet. Unfortunately, stable isotope ratios in the liver were not measured in the wild fish, as this could have provided a more accurate time line of their exposure to the effluent plume, as the liver has a higher turnover rate in the rainbow darter (0.0436 ‰/d). The higher rate in liver compared to muscle has been observed in other fish species and is attributed to the higher metabolic activity in liver (Vander Zanden et al., 2015).

This is one of the first studies to track the changes in the assimilation of sewage-derived nutrients in a freshwater food web before and after changes to MWWTPs. However, it has previously been documented in marine system studies. In an estuarine environment in eastern Australia (Moreton Bay) $\delta^{15}N$ signatures of aquatic organisms changed in response to a MWWTP upgrade involving biological nutrient removal (Pitt et al., 2009). This is a much more advanced upgrade than the current study, whereby biological nutrient removal also includes denitrification processes in addition to nitrification thus reducing both nitrate and ammonia. The removal of up to 80% of total ammonia and nitrate in the effluent resulted in $\delta^{15}N$ values in both filamentous algae and shore crabs reflecting the upstream environment after the upgrade (Pitt et al., 2009). Another study on Moreton Bay (Australia) linked reduced sewage-derived nitrogen assimilated by in situ red macroalga using $\delta^{15}N$ with several of the MWWTP upgrades in the region (Costanzo et al., 2005). Similarly, Tucker et al. (1999) observed the recovery of $\delta^{15}N$ value in marine sediments towards values more similar to that of the natural marine environment after improved disposal practices and sewage upgrades into the Boston Harbor, USA. The current study is consistent with the previous studies on marine systems, where the upgrade at the Kitchener MWWTP resulted in $\delta^{15}N$ values in primary consumers and rainbow darter reflecting the upstream conditions. In addition to the upgrade at the Kitchener MWWTP, this study also documented the Waterloo MWWTP producing lower quality effluent over the course of the study. It is clear from the biplots for both fish and primary consumers (Figure 2.9), that the nitrogen stable isotope, but not carbon, was a good indicator to separate out effluent quality. Poor effluent quality from both Kitchener and Waterloo MWWTP had more depleted values (fish =12–16 ‰; primary consumers = 6–8 ‰) compared to the higher quality effluent being more enriched (fish =17–20 ‰; primary consumers = 13–16 ‰; Figure 2.9).

2.5.2 Changes in $\delta^{13}C$

Multiple years of data, at sites immediately downstream both MWWTPs (DSW 1 and DSK 1), indicate that sewage-derived carbon sources are entering the aquatic food web, as $\delta^{13}C$ values are constantly higher relative to immediate upstream sites for both primary consumers and fish. The reason for this enrichment was likely due to the input of terrestrially derived carbon which has an enriched carbon signature compared to aquatic sources (France, 1995). This was similar to the finding by Robinson et al. (2016), who also saw enriched $\delta^{13}C$ in primary consumers and rainbow darter exposed to tertiary treated effluent. This was not the case for Morrissey et al., (2013) who was not able to distinguish benthic invertebrates exposed to secondary treated effluent from reference sites. Other studies show inconsistencies (Wayland and Hobson, 2001; deBruyn and Rasmussen, 2002; Freedman et al., 2012), which is likely a result of variable receiving environments and differences in life histories of species under study.

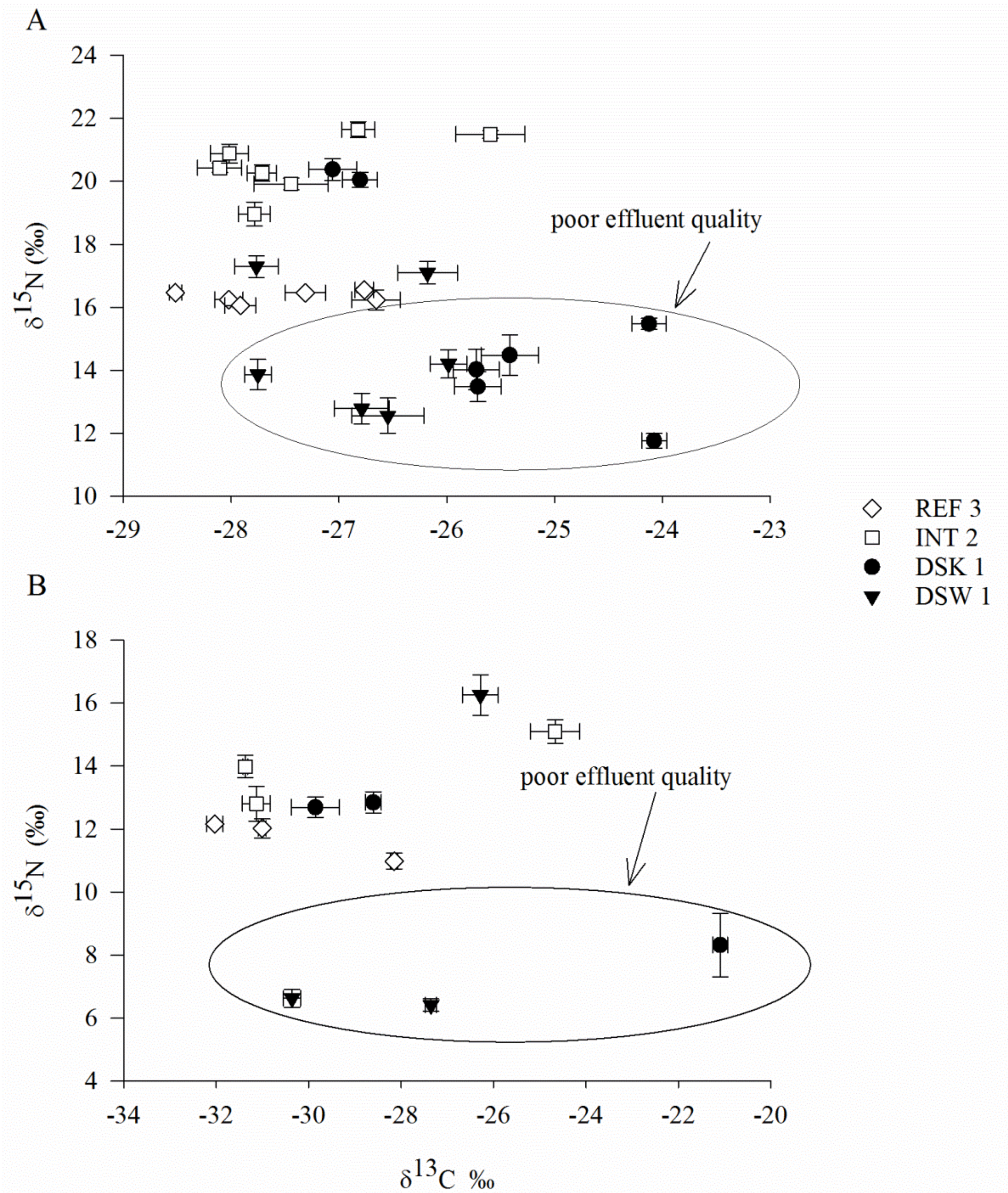

Figure 2.9 Stable isotope biplots of $\delta^{15}N$ and $\delta^{13}C$ for (A) rainbow darter and (B) primary consumers for the sites immediately downstream of the Kitchener (DSK 1; solid circle) and Waterloo (DSW 1; solid triangle) MWWTPs. Also plotted are the sites immediate upstream of the Kitchener MWWTP (INT 2; open square) and the Waterloo MWWTP (REF 3; open diamond). The biplot is showing the shift in the means (±SE) of $\delta^{15}N$ and $\delta^{13}C$ across all years, with a circle around the downstream sites when the effluent quality was poor at both the Kitchener (2007–2012) and Waterloo (2011–2014) MWWTPs.

It is clear from the carbon isotope data set that there are consistent site differences and annual differences which are likely caused by a combination of natural and anthropogenic factors. For example, fish at REF 1 in the fall are consistently more enriched in ^{13}C compared to REF 2. This is likely a result of REF 1 being in closer proximity to a bottom draw dam, which are known to release CO_2, FPOM and CPOM that are enriched in ^{13}C (Angradi, 1994). Other factors that may be contributing to differences between sites include the natural change in carbon inputs (allochthonous verses autochthonous) and CO_2 processing along a river gradient (Vannote et al., 1980; Finlay, 2001). It is clear that variability between years was to some extent driven by annual flow patterns. Lower flows would reduce CO_2 supply and increase the boundary layer effect resulting in a decrease in the discrimination against $^{13}CO_2$ of primary producers (Finlay et al., 1999). Other studies have demonstrated that there is a strong negative relationship between water velocity and $\delta^{13}C$ in primary consumers and herbivores (Finlay et al., 1999; Singer et al., 2005; Rasmussen and Trudeau, 2007). Rasmussen and Trudeau (2010) further demonstrated that the effect of velocity on $\delta^{13}C$ in algae was transmitted up the food chain to salmonids through trophic transfer. Our findings are similar for rainbow darter, although the relationships were only significant at sites further downstream in the urban gradient, after the Waterloo and Kitchener MWWTPs, suggesting that dilution of the MWWTP effluent may be a factor. Other factors that may contribute to the annual variability may include temperature (Power et al., 2003), variability in time of sampling (Murchie and Power, 2004), annual fluctuations in carbon inputs from natural and anthropogenic sources (MWWTPs; agricultural inputs), and potential immigration of rainbow darter.

2.5.3 Conclusion

This was a unique study that assessed the changes in exposure and assimilation of sewage-derived nutrients into a riverine aquatic food web following either improved or deteriorated effluent quality from two MWWTPs. There was a direct link between effluent quality and the assimilation of nutrients from primary consumers to fish. The improved effluent quality at the Kitchener MWWTP was associated with changes in $\delta^{15}N$ from being depleted to reflecting reference conditions in primary consumers and ultimately fish (rainbow darter). The treatment issues at the Waterloo MWWTP resulted in increased ammonia concentrations which was associated with the opposite trend as $\delta^{15}N$ became more depleted in both primary consumers and fish. The shift in $\delta^{15}N$ associated with the MWWTP changes reflects the high turnover rates estimated in the diet switch study. $\delta^{13}C$ continued to be higher at the immediate downstream sites from both MWWTPs and it did not appear to be affected by changes in effluent quality but instead by annual river discharge. This study illustrated that measuring stable isotopes in aquatic food webs would be a useful tool to include in future studies or biomonitoring programs that are assessing impacts from municipal wastewater treatment plants. They can be used to assess the quality of effluent, but also the exposure and assimilation of sewage-derived nutrients into entire aquatic food web, which could also be used to infer exposure to other sewage-derived contaminants.

Chapter 3

Reduction of intersex in a wild fish population in response to major municipal wastewater treatment plant upgrades

3.1 Chapter summary

Intersex in fish downstream of municipal wastewater treatment plants (MWWTPs) is a global concern. Consistent high rates of intersex in male rainbow darter (*Etheostoma caeruleum*) have been reported for several years in the Grand River, in southern Ontario, Canada, in close proximity to two MWWTPs. The larger MWWTP (Kitchener) recently underwent upgrades that included the conversion from a carbonaceous activated sludge to nitrifying activated sludge treatment process. This created a unique opportunity to assess whether upgrades designed to improve effluent quality could also remediate the intersex previously observed in wild fish. Multiple years (2007–2012) of intersex data on male rainbow darter collected before the upgrades at sites associated with the MWWTP outfall were compared with intersex data collected in post-upgrade years (2013–2015). These upgrades were associated with a reduction from 70-100% intersex incidence (pre-upgrade) to <10% in post-upgrade years. Although the cause of intersex remains unknown, indicators of effluent quality including nutrients, pharmaceuticals, and estrogenicity improved in the effluent after the upgrades. This study demonstrated that investment in MWWTP upgrades improved effluent quality and was associated with an immediate change in biological responses in the receiving environment. This is an important finding considering the tremendous cost of wastewater infrastructure.

3.2 Introduction

Feminization of male fish in association with municipal wastewater treatment plant (MWWTP) outfalls has been reported on a global scale (Jobling et al., 1998; Bjerregaard et al., 2006; Tetreault et al., 2011; Blazer et al., 2012). Intersex (ova-testis) has been one of the most commonly reported effects observed in male fish downstream of MWWTPs (Bahamonde et al., 2013). This is concerning as severe cases of intersex have been associated with reduced reproductive success (Jobling et al., 2002; Harris et al., 2011; Fuzzen et al., 2015). The feminization of male fish has been associated with compounds such as natural hormones (17ß-estradiol [E2] and estrone [E1]), synthetic estrogen (17α-ethynylestradiol [EE2]) (Desbrow et al., 1998), and industrialized products that mimic estrogens, including alkylphenolic chemicals (Sheahan et al., 2002), which are routinely measureable in MWWTP effluents. Recent studies have suggested that additional compounds may also cause feminization of male fish, possibly through other pathways, including those with anti-androgenic activity (Jobling et al., 2009). EE2 added to a whole lake at an environmentally relevant concentration (~5 ng/L) resulted in near extirpation of a fish population (Kidd et al., 2007) followed by changes to the whole ecosystem (Kidd et al., 2014). Changes in reproductive endpoints, including intersex in male fish, were observed in multiple species in this whole lake experiment (Palace et al., 2006; Kidd et al., 2007). Thus, intersex is a prevalent and a biologically relevant marker of exposure to endocrine-disrupting compounds (EDCs) in MWWTP outfalls.

To protect aquatic species from potential deleterious effects of EDCs, it is necessary to reduce their exposure. The removal of compounds with estrogenic properties from MWWTPs depends on the plants' operational processes (Andersen et al., 2003; Servos et al., 2005; McAdam et al., 2010). Enhanced wastewater treatment, including nitrifying activated sludge, has been shown to be effective at reducing the estrogenicity (natural and synthetic estrogens) of the final effluent and the associated endocrine disruption in fish exposed to it under laboratory conditions (Filby et al., 2010; Baynes et al., 2012) or in fish caged in the effluent outfalls (Barber et al., 2012). However, it is difficult to extrapolate the findings from these studies to the recovery of free-living fish as these studies are typically short (do not cover the entire life cycle), and laboratory studies do not accurately reflect associated environmental conditions. Although numerous studies have documented endocrine responses in fish exposed to MWWTP outfalls, to our knowledge, no studies have documented the recovery of reproductive endpoints in free-living fish in receiving waters in response to MWWTP upgrades.

The Grand River watershed in southern Ontario, Canada, is the largest watershed that drains into Lake Erie. The area has a growing population of nearly 1 million people (Loomer and Cooke, 2011). The rainbow darter (*Etheostoma caeruleum*), a native species of southern Ontario, has been studied extensively along a 60 km section of the central Grand River that includes two major MWWTP outfalls, Kitchener and Waterloo (Figure 3.1) (Tetreault et al., 2011; Tanna et al., 2013; Bahamonde et al., 2014; Bahamonde et al., 2015a; Bahamonde et al., 2015b; Fuzzen et al., 2015). This sentinel species was selected for several reasons: they are highly abundant, are gonochoristic and sexually dimorphic, are short lived (5 years)(Beckman, 2002), and are thought to have limited mobility (Tetreault et al., 2011). The rainbow darter grow rapidly in their first summer and are sexually mature at 1 year of age (Crichton, 2016). They spawn asynchronously, with females laying multiple egg clutches each spring. Multiple studies indicated that the male rainbow darter in close proximity to the Waterloo and Kitchener

MWWTPs were being feminized, with evidence of altered gene expression, vitellogenin induction (Bahamonde et al., 2014; Bahamonde et al., 2015a), reduced steroid production (Tetreault et al., 2011), and reduced gonad size (Tetreault et al., 2011). The most consistent effect observed across multiple years and seasons was intersex in the male rainbow darter, with up to 100% incidences at sites below the Kitchener MWWTP (Fuzzen et al., 2016). High intersex severity scores were associated with the Kitchener effluent outfall, including many cases where the gonad had greater than 50% ovarian tissue (Bahamonde et al., 2015b; Fuzzen et al., 2015), with instances of macroscopic intersex (e.g., Figure 3.2). Severe cases of intersex in the male rainbow darter were previously associated with altered gene expression (Bahamonde et al., 2015a) and reduced fertilization success (Fuzzen et al., 2015).

Major infrastructure upgrades were implemented at the Kitchener MWWTP to improve treatment efficiency and effluent quality. These included converting the plant from carbonaceous activated sludge (primarily the removal of biological oxygen demand (BOD)) to nitrifying activated sludge to enhance the removal of ammonia. Upgrades were initiated in mid-2012, and full nitrification was achieved by early 2013 (Bicudo et al., 2016). Although upgrades at the Waterloo MWWTP were planned during this period, construction delays at this MWWTP resulted in only minimal treatment changes and poor effluent quality over the course of the study period (Hicks et al., 2017).

The objective of this study was to determine if major treatment plant upgrades at the Kitchener MWWTP would effectively alleviate the intersex previously observed in wild rainbow darter. It was hypothesized that the implementation of treatment upgrades, including nitrification, would decrease total effluent estrogenicity and intersex occurrence in wild male rainbow darter at downstream sites. To test this hypothesis, the study took advantage of the established baseline data (4 years before the upgrades) on intersex in the male rainbow darter, collected in both spring and fall seasons, and compared these with data collected in three additional fall seasons and two additional spring seasons after the Kitchener MWWTP was upgraded. These data were examined in conjunction with measurements of effluent quality in terms of nutrients, select pharmaceuticals, and total estrogenicity over the same time period.

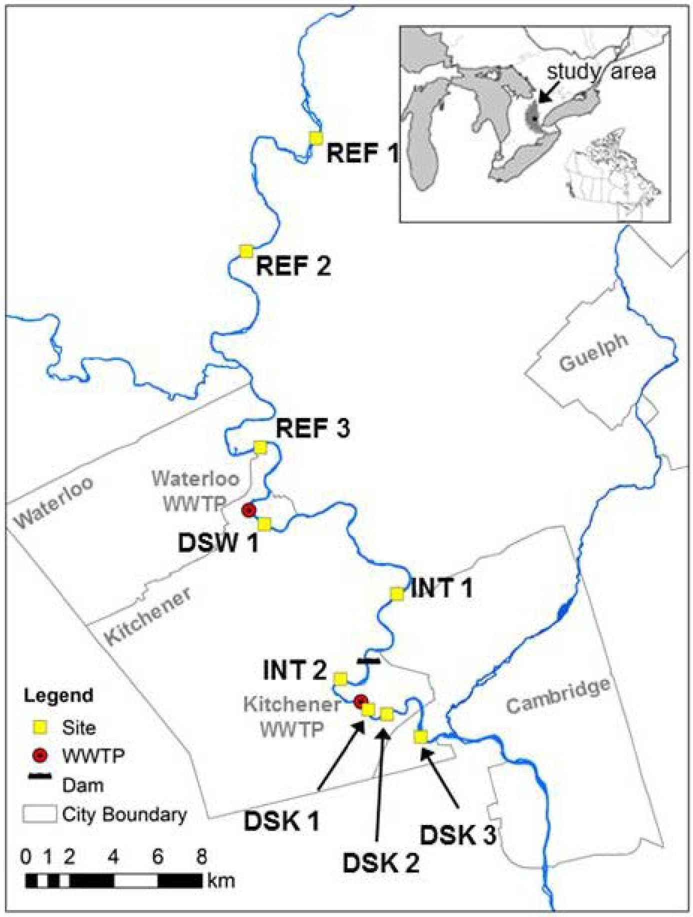

Figure 3.1 Map of sampling sites along the Grand River, Ontario, where fish were collected in multiple years during the fall and spring periods of 2007–2015. There were three upstream reference sites (REF), four downstream sites (DSW, DSK), and two sites located between the Kitchener and Waterloo wastewater treatment plant (WWTP) outfalls (INT). A full description of the sites is provided in the materials and methods section.

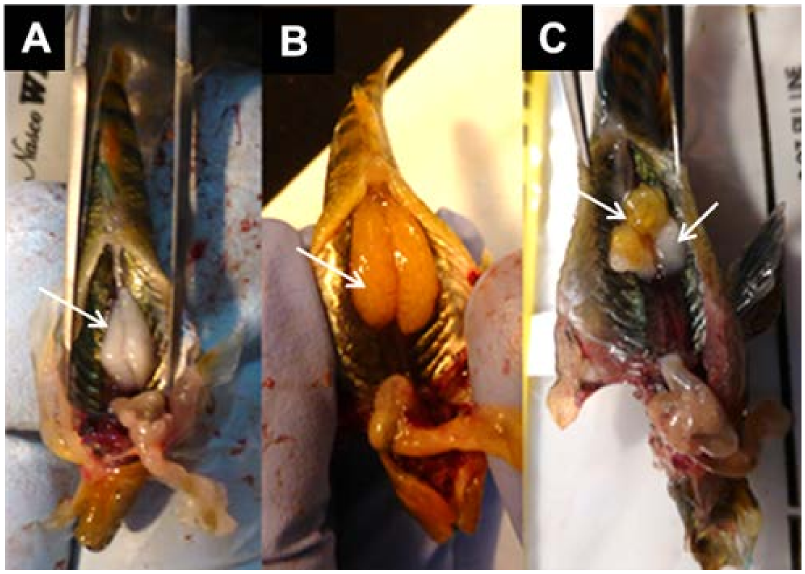

Figure 3.2 (A) Normal male, (B) normal female, and (C) a severely intersexed male rainbow darter. Arrows point to gonad tissue (testis or ovaries). The intersex male caught in close proximity to the Kitchener MWWTP outfall reveals macroscopic intersex (presence of both testicular and ovarian tissues).

3.3 Materials and methods

3.3.1 Description of sites

Nine sites in close proximity to the Kitchener and Waterloo MWWTP outfalls were sampled in multiple years between 2007 and 2015 in spring and/or fall seasons (Figure 3.1). Sites were selected to represent similar riffle/run habitats. Two upstream non-urban reference sites (REF 1 and REF 2) and one urbanized reference site (REF 3) were included in addition to one near-field exposure site downstream of the Waterloo MWWTP (DSW 1) and two sites located farther downstream (INT 1 and INT 2), but upstream of the Kitchener outfall. Three sites were sampled downstream of the Kitchener MWWTP outfall (DSK 1, DSK 2, and DSK 3). The exact location (GPS coordinates) and distances between sites are provided in Table S3.1.

3.3.2 Effluent characterization and river water quality

Data for traditional effluent quality parameters including monthly total ammonia, nitrate, total Kjeldahl nitrogen (TKN), BOD, total phosphorous (TP), and total suspended solids (TSS) were provided by the Region of Waterloo for the Waterloo and Kitchener MWWTPs for the duration of the study (2007–2015). Effluent quality was also assessed on the basis of the removal of select pharmaceuticals and total estrogenicity at the Waterloo MWWTP (2010–2015) and the Kitchener MWWTP before (2010 to July 2012), during (August 2012 to January 2013), and after the upgrades (2013–2015). Effluent samples from both the Kitchener and Waterloo MWWTPs were collected, preserved, extracted, and analyzed for three pharmaceuticals (ibuprofen, naproxen, and carbamazepine) following the protocols outlined in Arlos et al. (2015). The yeast estrogen screen (YES) assay was used to assess total estrogenicity quantified in estradiol equivalence (E2eq) following the method described by Arlos et al. (2016). Dissolved oxygen (DO) concentrations in river water below the Kitchener MWWTP were provided by the Grand River Conservation Authority.

3.3.3 Fish collection and processing

Historical data on rainbow darter in proximity to the Kitchener and Waterloo MWWTPs before the Kitchener upgrades (collected between 2007 and 2012) were included in several earlier studies (Tetreault et al., 2011; Tanna et al., 2013; Bahamonde et al., 2014; Bahamonde et al., 2015b; Fuzzen et al., 2016). Fuzzen et al. (2015) reported on the first post-upgrade data set; these data were collected in spring 2013, 9 months after the Kitchener MWWTP upgrades. Rainbow darter were sampled again in the fall of 2013, 2014, and 2015 and in the spring of 2015 at the same sites and in the same manner. Briefly, rainbow darter were collected in riffle/run habitats at the selected sites by backpack electrofishing (Smith Root LR-24). A target sample size of 20 males and 20 females was established to collect fish to analyze all sampling endpoints, including intersex, somatic indices, and additional endpoints for other research studies. For each sampling event, rainbow darter were held in well-aerated buckets until they were sampled on site in a portable laboratory. Fish were rendered unconscious by concussion and then euthanized by spinal severance. Fish total length (± 0.1 cm), weight (± 0.01 g), gonad weight (± 0.001 g) and liver weight (± 0.001 g) were recorded. A single testis lobe from a subset of the male rainbow darter was transferred to Davidson's solution for 48 hours and stored in 70% ethanol before being processed for histology. All fish were handled in accordance with the approved University of Waterloo animal care protocols (AUPP# 10-17 and 14-15).

3.3.4 Histology

Gonad tissues were dehydrated and embedded in paraffin wax. Embedded samples were microtomed at a thickness of 5 µm, put on slides with slide mount, and stained with hematoxylin and eosin. A minimum of 40 sections per fish were scanned for intersex at 100x magnification using a Leica DM100 light microscope. Two parameters were calculated for each site at each sample date. The first parameter was intersex incidence, which is the percentage of male rainbow darter with intersex (based on presence or absence of oocytes). The second parameter was intersex severity, which was based on the scoring index adopted from Bahamonde et al. (2015b) using the number and development stage of oocytes in addition to the proportion of ovarian tissue to testicular tissue. From 2007 to 2015, an average of 18 male rainbow darter were sampled each year for intersex incidence and severity but this number ranged from 5 to 68 depending on the availability of archived samples (Table S3.2).

3.3.5 Data analysis and statistics

A BACI (before-after control-impact) design was used to test for differences in intersex incidence and severity between upstream and downstream sites before and after the upgrades [two-way ANOVA with factors site (upstream vs. downstream) and period (pre-upgrade vs. post-upgrade) with year as the replicate]. This was completed separately for each combination of downstream site and a single reference site (REF 3), where there were at least 3 years pre-upgrade and 3 years post-upgrade. The third reference site (REF 3) was selected because it is the only reference in the urbanized region above both the Waterloo and Kitchener MWWTPs. This resulted in four two-way ANOVAs, one for intersex incidence and one for severity for each of the following pairs (fall data only): DSK 1 versus REF 3 and DSK 2 versus REF 3. For the remaining sites in the fall and for all of the spring collections, differences in intersex incidence and severity among years within sites were tested. Differences in intersex incidence were tested with the Fisher’s exact test, while differences in intersex severity were tested with the Kruskal–Wallis test with Dunn’s pairwise comparison (with individual fish as replicates). The relationship between intersex incidence and severity across all seasons and years was assessed with linear regression. Fish body weight, total length, liver weight, and gonad weight were used to calculate condition factor (k = body weight/length^3 x 100), gonadosomatic index (GSI = gonad weight/body weight x 100), and liver somatic index (LSI = liver weight/body weight x 100).

These data are provided to support interpretation of the intersex and were not compared statistically (Figure S3.1). Annual changes in effluent nutrient concentrations (ammonia and nitrate) at both MWWTPs (Kitchener and Waterloo) as well as river DO concentrations were assessed with a Kruskal–Wallis test with Dunn's pairwise comparison. Changes in pharmaceuticals and E2eq across different time points were also assessed for both the Waterloo and Kitchener effluent using one-way ANOVAs with Tukey's pairwise comparisons. Pharmaceutical and E2eq data from the Kitchener MWWTP were pooled into three categories: pre-upgrade, during upgrades, and post-upgrade. All data were plotted and tested with SigmaPlot version 13 using $\alpha < 0.05$.

3.4 Results and discussion

3.4.1 Effluent characterization before and after MWWTP upgrades

Before the upgrades (2007–2012), the Kitchener MWWTP lacked nitrification primarily because of inefficient aeration and short solids retention time (SRT; < 2 d; Hicks et al. 2017). The upgrades, which included more efficient aeration and higher SRT (> 5 d), significantly improved the removal of ammonia, resulting in a decrease in the median annual ammonia concentration from 25 mg/L to 2–6 mg/L in post-upgrade years (Figure 3.3). In contrast, ammonia concentrations in the Waterloo MWWTP increased over the course of the study, with concentrations in 2013 reaching levels similar to those in the Kitchener MWWTP before the upgrades (Figure 3.3). A partial upgrade at Waterloo was implemented in 2014 to treat the centrate (elevated in ammonia), derived from the centrifugation of the biosolids (Hicks et al. 2017); however, full nitrification was not achieved and ammonia concentrations remained high in the final effluent (> 20 mg/L; Figure 3.3). The 2014 upgrade also resulted in a decrease in both BOD and TSS (Table S3.3). Nitrate at both MWWTPs was inversely related to ammonia concentrations and was a good indicator of the degree of nitrification.

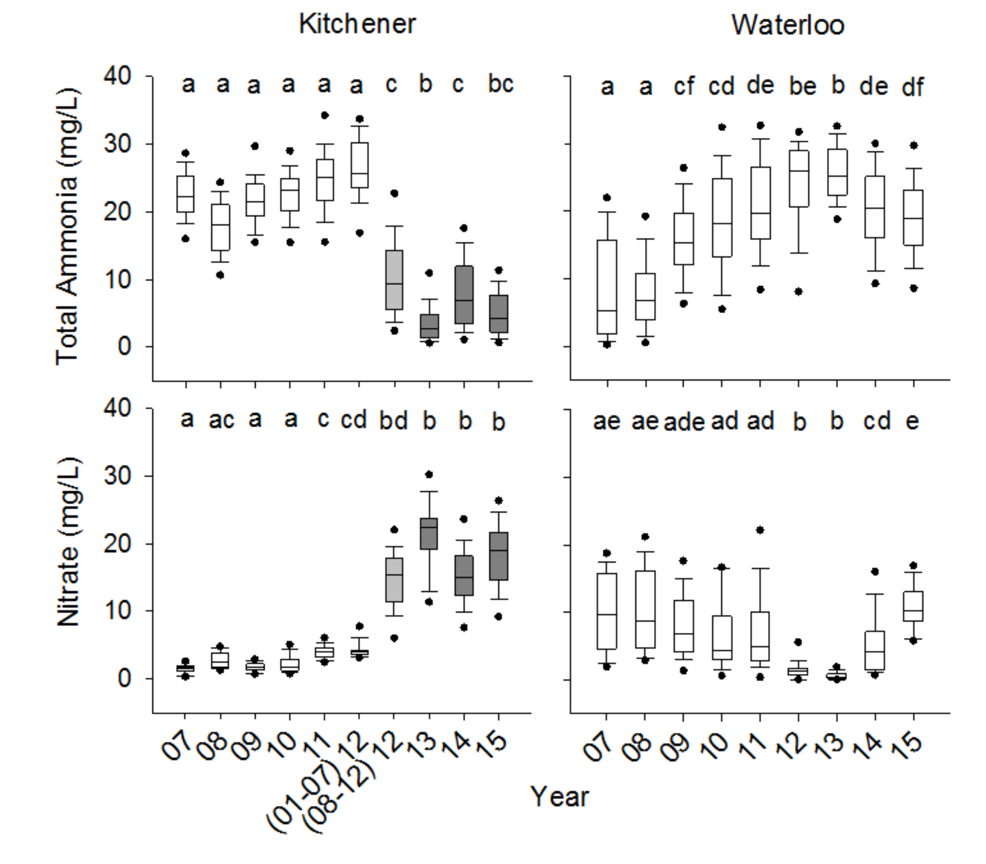

Figure 3.3 Total ammonia (top panel) and nitrate (bottom panel) for both the Kitchener (left) and Waterloo (right) MWWTPs from 2007 to 2015. For Kitchener only, the white boxes indicate pre-upgrade years (up until July 2012), light grey indicates the period during the upgrades (Aug–Dec 2012), and dark grey indicates post-upgrade years (2013–2015). Black dots represent the upper 95% and lower 5%. Boxplots that do not share a letter in common are significantly different at $p < 0.05$. Boxplots are represented by weekly measurements ($n = 52$) with the exception of Kitchener from 2013 to 2015 and Waterloo from 2014 to 2015 where the frequency of measurements was increased ($n = 153–158$).

Additional effluent characterizations included the measurement of select pharmaceuticals (indicators of treatment quality) and total estrogenicity (in E2eq) (Figure 3.4). Before the upgrades, when nitrification was lacking at the Kitchener MWWTP, both ibuprofen (IBU) and naproxen (NPX) concentrations were significantly higher than after the implementation of nitrification (IBU: one-way ANOVA, F = 20.2, df = 32, $p < 0.001$; NPX: one-way ANOVA, F = 10.5, df = 32, $p < 0.001$). This was not surprising, as these compounds have high biotransformation potential (Salveson et al., 2012). IBU concentrations were up to 135 fold higher and NPX concentrations were up to 20 fold higher before the upgrades. In contrast, compounds that have low biotransformation potential and low sorption rates onto solids typically have slow removal rates, and more advanced treatment is needed to achieve removal (Salveson et al., 2012). Therefore, it is not surprising that carbamazepine was more persistent and not affected by the upgrades (Figure 3.4; one-way ANOVA, F = 3.0, df = 32, p = 0.08). The pattern of pharmaceuticals at the Waterloo MWWTP was variable and reflected the lack of nitrification over the years (Figure 3.4). This study is consistent with previous studies that have demonstrated that nitrification and extended SRT are associated with greater removal of pharmaceuticals (Salveson et al., 2012; Luo et al., 2014).

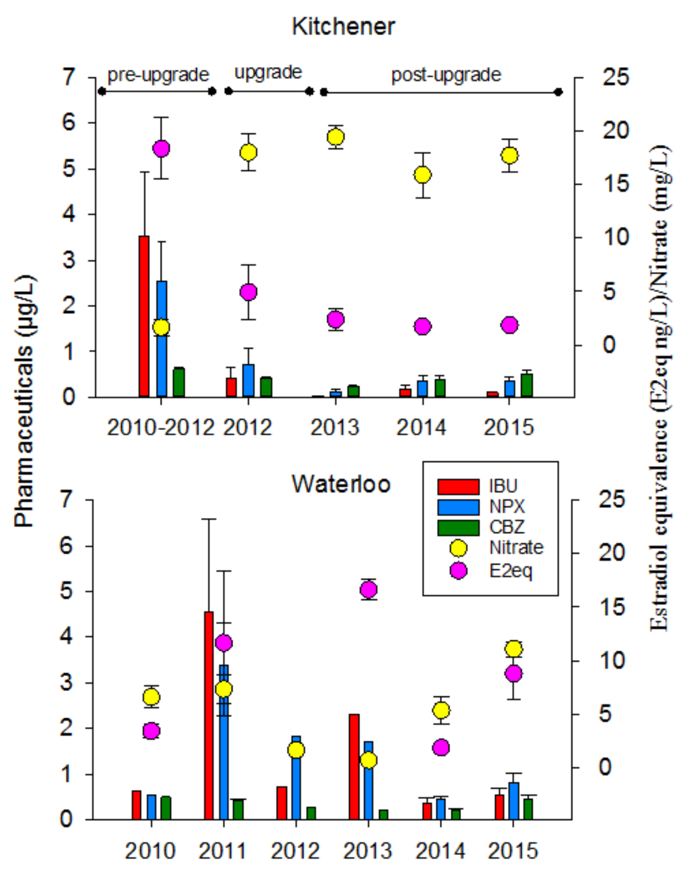

Figure 3.4 Effluent characterization for the Kitchener (top) and Waterloo (bottom) MWWTPs between 2010 and 2015. For Kitchener only, the pre-upgrade years are 2010 to July 2012; the period during the upgrades is August to December 2012, and the post-upgrade years are 2013 to 2015. The bars represent three pharmaceuticals (ibuprofen (IBU), naproxen (NPX), and carbamazepine (CBZ)), the pink filled circles represent estradiol equivalence (E2eq), and the yellow filled circles represent nitrate. All parameters are represented by the means (±SE) of multiple sample points (days) with the exception of pharmaceuticals in 2010, 2012, and 2013 at Waterloo, where only one sample point (one day) was available. Otherwise, the sample sizes range from 2 to 9, where each replicate represents one event (day) sampled in triplicate. Sample sizes are provided in Table S4.

Total effluent estrogenicity (E2eq) was also assessed at both MWWTPs. At the Kitchener MWWTP, there was a significant reduction from 18 ng/L E2eq before the upgrades to < 2 ng/L E2eq (one-way ANOVA, F = 17.6, df = 20, p = < 0.001) in post-upgrade years. These values are similar to those in other studies that have quantified E2eq in secondary treated effluent (Matsui et al., 2000; Svenson et al., 2003; Filby et al., 2010). Although the reduced estrogenicity is probably associated with the changes in effluent treatment, influent was not measured during the study so a change in the source cannot be ruled out. The population was increasing over the years of the study so MWWTP inputs were probably increasing (Table S3.3). The E2eq at Waterloo was usually lower than at Kitchener in pre-upgrade years (Figure 3.4).

Natural and synthetic estrogens could have contributed to E2eq in the effluents (Desbrow et al., 1998). An attempt was made to quantify E1, E2, and EE2 in this study; however, matrix effects resulted in the data failing quality assurance, probably because of low selectivity (unit resolution) in the LS-MS/MS method. Nitrifying activated sludge have been shown to be associated with the removal of estrogenic compounds (including E1, E2, and EE2) with 90–99% efficiency (Andersen et al., 2003; McAdam et al., 2010; Suarez et al., 2010a). This has mainly been attributed to biodegradation processes (Verlicchi et al., 2012), which are favourable under nitrifying conditions (Salveson et al., 2012). It has also been shown that the longer the solids retention time, the greater the removal of estrogenic compounds: an SRT of > 5 d is typically associated with enhanced removal (Clara et al., 2005; Servos et al., 2005). The higher aeration and SRT (going from < 2 d to > 5 d) at the Kitchener MWWTP, which resulted in nitrifying conditions after the upgrades, is probably also contributing to a more diverse biological community in the treatment system and therefore a reduction in many contaminants as well as in total estrogenicity in the final effluent.

3.4.2 Intersex before and after MWWTP upgrades

The main objective of this study was to evaluate whether the high occurrence (70–100%) of observed intersex in the wild male rainbow darter downstream of MWWTPs would be reduced following major infrastructure upgrades to improve effluent quality. The implementation of nitrification at the Kitchener MWWTP corresponded with a distinct decrease in the incidence and severity of intersex in wild rainbow darter (Figure 3.5). At the second downstream site (DSK 2), intersex incidence had already decreased from 100% (in fall 2012) to 29% (a 71%

reduction) in the first fall season (2013) after the upgrades. In contrast, the decrease at the first site immediately downstream of the Kitchener MWWTP (DSK 1) was more gradual from 2013 to 2015. By the third fall season after the upgrades (2015), intersex incidence had decreased to 9% (DSK 1) and 14% (DSK 2). Similarly, intersex severity scores also decreased gradually in post-upgrade years downstream of the Kitchener MWWTP. The mean intersex score at DSK 1 and DSK 2 before the upgrades ranged from two to three, with maximum scores of six (including visible eggs). By fall 2015 (3 years post-upgrade), the mean intersex scores were less than one, the lowest mean score recorded at these sites below the Kitchener MWWTP outfall since these studies began in 2007. The decrease in intersex in post-upgrade years in the fall was also supported by spring data collections, where intersex incidence and severity was at its lowest in spring 2015 at all three sites below the Kitchener MWWTP (Figure S3.2). Supporting statistics for comparing years within sites for both intersex incidence and severity are provided in Tables S3.5 (fall) and S3.6 (spring).

A BACI analysis was used to assess whether sites below the Kitchener MWWTP (DSK 1 and DSK 2) returned to reference conditions after the upgrades. The analyses revealed significant interaction between factors (upstream vs. downstream x pre-upgrade vs. post-upgrade). For the test between DSK 2 (second site downstream of the Kitchener MWWTP) and REF 3, pairwise comparisons for the interactions revealed significant differences in intersex incidence before the upgrades ($p < 0.001$) but not after ($p = 0.226$). The finding was similar for intersex severity, indicating that both intersex incidence and severity at DSK 2 are returning to reference conditions. The test for DSK 1 (first site below the Kitchener MWWTP) and REF 3 also revealed a significant difference in intersex severity before the upgrades ($p < 0.001$) but not after ($p = 0.129$), thus also indicating that this site is returning to reference conditions. Interestingly, there was a difference in intersex incidence between DSK 1 and REF 3 both before ($p < 0.001$) and after the upgrades ($p < 0.034$), possibly indicating that intersex incidence at DSK 1 is taking longer to recover than intersex severity. Intersex incidence was lower at DSK 1 in post-upgrade years than in pre-upgrade years ($p = 0.001$); however, it might be that intersex incidence was taking longer to recover than intersex severity. This would not be surprising, since with decreasing exposure, severity could be decreasing more rapidly than incidence. It is interesting to note that across all sites, years, and seasons, intersex incidence was positively correlated with severity ($r^2 = 0.88$; df = 82, $p < 0.001$). Additional supporting statistics for the two-way ANOVAs (BACI analysis) are provided in Table S3.7–S3.10.

Mean severity scores at the furthest downstream site (DSK 3) were highly variable among the years, but by fall 2015, it was at its lowest ever reported, with a maximum score of two (Figure 3.5). This site is approximately 5 km downstream from the Kitchener MWWTP outfall, where the effluent would be more evenly distributed and diluted across the river (Arlos et al., 2014). Although intersex incidence was slightly elevated at this site relative to the immediate upstream reference site (INT 2), intersex severity was similar to that at the sites below Waterloo (DSW 1, INT 1) and never as severe as at the sites immediately below the Kitchener outfall.

Intersex below the Waterloo MWWTP occurred less frequently and was less severe than at the sites below the Kitchener MWWTP throughout the study period (Figure 3.5). Intersex incidence ranged from 7 to 40% with no significant differences among years in either the spring (Fisher's exact, $p = 0.237$) or fall (Fisher's exact, $p = 0.204$). Similarly, intersex severity did not differ among years in either the spring (Kruskal–Wallis, H = 3.203, $p = 0.202$) or fall (Kruskal–Wallis, H = 5.596, $p = 0.347$), where the mean scores were consistently less than one every year. The maximum severity score reported at this site was four; however, this score was infrequently observed. An additional site located 12 km downstream of the Waterloo outfall (INT 1) that was sampled less frequently beginning in 2013 had similar trends to DSW 1, with intersex incidence ranging from 12 to 33%. The Waterloo MWWTP services 100,000 fewer people and produces 40% less volume of effluent (Table S3.3) than the Kitchener MWWTP, and the receiving environments of the Waterloo and Kitchener effluent outfalls have similar river flows (Loomer and Cooke, 2011). This lower loading is a possible explanation for the reduced impacts at this site compared with the sites below Kitchener before the upgrades.

Intersex was infrequent at the reference sites (REF 1, REF 2, and REF 3). Incidence averaged 7.3 ± 1.2% (mean ± SE), ranging from 0 to 20% over the study period, and severity scores were low at these sites. There were no differences between years within reference sites for either intersex incidence or severity (Table S3.5 and S3.6). It is unknown whether intersex at these sites was due to anthropogenic stressors or a natural phenomenon. It is not unusual to find intersex at reference sites, especially when the sites are not free of anthropogenic influences. A review on intersex in teleost fish by Bahamonde et al. (2013) noted that other studies reported 0.5-55% intersex incidence at reference sites.

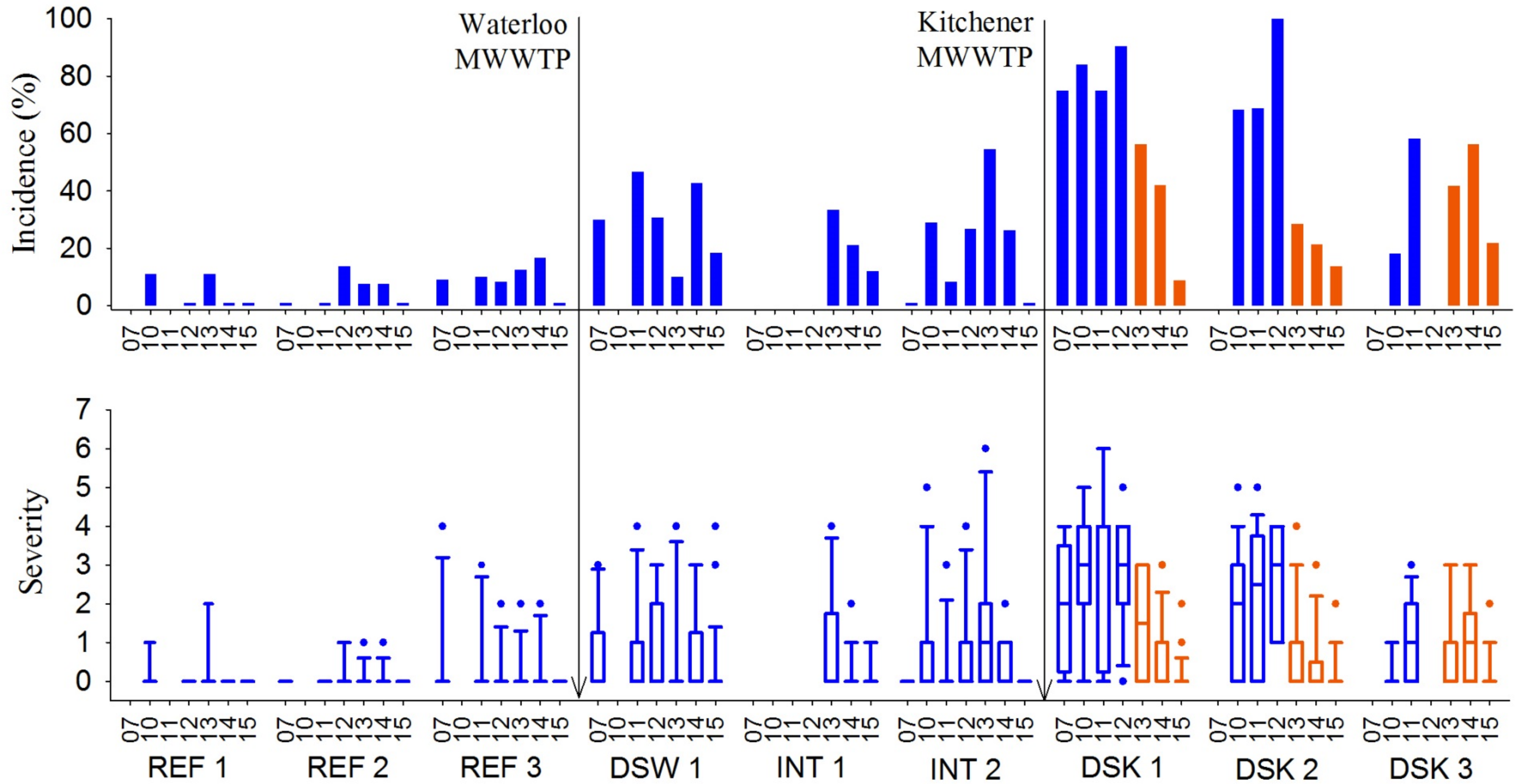

Figure 3.5 Intersex incidence (top panel) and severity (bottom panel) for fish collected in the fall in 2007 and 2010–2015. Sites are arranged from upstream (REF 1) to downstream (DSK 3), with the black arrows indicating the inputs of MWWTP effluents. Orange bars and orange box plots indicate post-upgrade years (2013–2015) below the Kitchener MWWTP. Sample sizes are provided in Table S2.

The site immediately above the Kitchener MWWTP outfall (INT 2) had highly variable rates of intersex incidence, ranging between 0 and 55%, with a maximum severity score of six, which was normally only ever observed below the Kitchener MWWTP. This site is 19 km downstream of the Waterloo MWWTP outfall, which may have contributed to the intersex observed at this site. However, a more plausible explanation may be that the rainbow darter are moving between sites as there are no physical barriers in this section of the river and INT 2 is a short distance (1 km upstream) from the Kitchener MWWTP outfall. It is interesting to note that intersex incidence and severity also significantly decreased at this site after fall 2013, mirroring the period of the upgrades. Hicks et al. (2017) previously showed site-specific stable isotope signatures ($\delta^{15}N$ and $\delta^{13}C$) in rainbow darter at the same sites as the current study, suggesting that although most fish have high site fidelity some fish may move across larger spatial scales (among closely situated sites). More knowledge on the movement patterns of rainbow darter is needed to better interpret these data.

3.4.3 Potential causative agents of intersex

The implementation of nitrification at the Kitchener MWWTP dramatically improved the plant's overall effluent quality in terms of observed concentrations of nutrients, concentrations of pharmaceuticals, and total estrogenicity. This corresponded to a reduction in the occurrence and severity of intersex at sites below the Kitchener MWWTP. The exact cause of intersex in this study is still not known, although strong evidence in the literature suggests that these types of responses are related to natural (E1/E2) and synthetic (EE2) estrogens (Desbrow et al., 1998) as well as to some industrial contaminants such as bisphenol A (Metcalfe et al., 2001) and alkylphenols (Balch and Metcalfe, 2006). Recent studies have also suggested that chemicals such as metformin (an anti-diabetic) detected in MWWTP effluents can cause intersex in fathead minnows (*Pimephales promelas*) (Niemuth and Klaper, 2015). Jobling et al., (2009) have also suggested that chemicals acting as anti-androgens may be contributing to some intersex found downstream of MWWTP outfalls in England. Two anti-androgens, the microbial agents triclosan and chlorophene, have been measured in both the Kitchener and Waterloo MWWTP effluents (Arlos et al., 2015). Hypoxia has also been suggested as a mechanism for endocrine disruption (i.e., oxygen levels reduced below 1.0 mg/L) (Wu et al., 2003). The excessive nutrients released into the Grand River have historically caused severe oxygen sags downstream of the Kitchener outfall, where mean summer daily DO levels were well below the recommended objective of 4 mg/L and were as low as 1.2 mg/L in the early morning before the upgrades (Loomer and Cooke, 2011). After the upgrades, daily summer DO never dropped below 6 mg/L, and median values were the highest in post-upgrade years (Figure S3.3). Multiple possible chemicals or conditions might have worked through various pathways or mechanisms to cause the intersex observed in this study.

Advanced treatment technologies (e.g., granular activated carbon (GAC), chlorine dioxide (ClO_2), and ozonation) have been demonstrated to reduce effluent estrogenicity and associated endocrine disruption in laboratory-exposed fish compared with conventional activated sludge (Filby et al., 2010; Baynes et al., 2012). Baynes et al. (2012) found that nitrifying activated sludge processes (e.g., nitrification) were less effective at removing estrogenic compounds and reducing associated intersex and vitellogenin induction in laboratory-exposed roach. More advanced treatment (GAC) was required to completely remove intersex. A study by Barber et al. (2012) demonstrated that an upgrade from a trickling filter to nitrifying activated sludge was sufficient to reduce total effluent estrogenicity and associated endocrine disruption (as measured by its effects on vitellogenin induction, sperm abundance, gonad size, and secondary sexual characteristics) in caged fish. The current study further supports that nitrifying activated sludge can be an effective and perhaps sufficient upgrade for removing many estrogenic compounds and reducing their associated biological effects such as intersex.

3.4.4 Manifestation of intersex in the rainbow darter

The timing and duration of exposure to EDCs and the resulting manifestation of intersex in fish is still poorly understood (Abdel-moneim et al., 2015). The recovery of the rainbow darter population from intersex after the MWWTP upgrades suggests that adult rainbow darter can recover quickly from past exposure to EDCs. This is demonstrated by the decrease in intersex incidence (up to 71% reduction) in the first year post-upgrade, which eventually declined to levels similar to those observed at reference sites. If exposure during early life stages (e.g., gonad differentiation) caused intersex to be manifested during the darters' entire lifetime, a rapid decrease in intersex in older fish (with life expectancy of about 5 years; Beckman, 2002) would not be expected. The largest (i.e., oldest) fish did not show a tendency to retain high intersex in the years after the upgrades (Figure S3.4). Unfortunately, rainbow darter were not aged for this study, but a consistent range in lengths was always sampled from the population and the majority of the fish sampled (Figure S3.4) were probably 2 or more years old based on studies on rainbow darter growth conducted by Crichton (2016) in the Grand River. Other studies support the hypothesis that fish can recover from exposure to EDCs. For example, zebrafish (*Danio rerio*) (including adults) exposed to environmentally relevant concentrations of EE2 have been observed to recover from endocrine-disrupting effects at multiple levels of biological organization including gene expression, protein production (vitellogenin induction), proportion of gonad cell types, gonad size, growth, and sex ratios (Van den Belt et al., 2002; Baumann et al., 2014; Luzio et al., 2016). The recovery of the wild rainbow darter from intersex in the Grand River and zebrafish in the laboratory is in contrast to the findings of Liney et al., (2005), who suggested that intersex induced by municipal wastewater effluent in early life stage roach (*Rutilus rutilus*) was permanent. However, the manifestation of intersex in roach was based on the presence of an ovarian cavity in male fish and not ova-testis as in this study. Similarly, Schwindt et al. (2014) suggested that fathead minnow populations may not recover from exposure to EE2, including potential transgenerational effects. Therefore, there are studies that document cases where exposure to EDCs may either be irreversible or reversible, and this may depend on species sensitivities, the duration (exposure and recovery) and type of exposure (compound specific versus whole effluents), and the manifestation of the effect in question. Most studies on the recovery from exposure to EDCs are laboratory based, and field observations may involve many confounding factors. Additional studies are needed to further understand how different chemicals, effluents, and species of fish may respond to altered EDC exposure.

The time of the year in which adult fish are exposed to EDCs may also be important in determining the manifestation of intersex. In the first spring (2013) immediately following the upgrades, intersex incidence and severity remained high at DSK 1 and DSK 2 (Figure S3.2). This was probably because the Kitchener MWWTP still had poor effluent quality in the previous summer (June–July 2012), before the initial upgrade in August 2012. The summer is the post-spawning period of the rainbow darter, when they build their gonads (recrudescence) for the next spring. The following post-spawning period (summer 2013) would have been the first full period of recrudescence in post-upgrade effluent, and this coincided with reduced intersex in the fall of 2013. This suggests that the manifestation of intersex may be related to the exposure to EDCs during a critical window of each year, such as the post-spawning period when germ cell proliferation is occurring in the gonads (Devlin and Nagahama, 2002). It has been suggested that there is a window of sensitivity during which exposure to EDCs can induce intersex in the early life stages of fathead minnows (van Aerle et al., 2002). Liney et al. (2005) were also able to induce intersex in roach when exposure occurred during the critical window of germ cell proliferation in early life stages. Intersex has also been induced in post-spawning adult roach exposed to MWWTP effluents (Baynes et al., 2012), but not in adult roach where the testes were fully mature (Rodgers-Gray et al., 2000), further supporting the theory of a window of sensitivity. For the rainbow darter, further studies are needed to validate whether intersex can be induced in post-spawning adults.

3.4.5 Conclusion

This is a unique study with an important finding that investments in treatment infrastructure at MWWTPs can improve ecosystem health. The results of this study suggest that the relatively conventional treatment plant upgrades at the Kitchener MWWTP reduced exposure to contaminants or conditions that had previously induced the severe intersex condition in fish. The recovery of the rainbow darter from high intersex incidence and severity below the Kitchener MWWTP outfall suggests that wild fish can recover from previous exposure to EDCs. This study complements work in the laboratory as well as the whole lake exposures conducted at the Experimental Lakes Area (Kidd et al., 2007) that predict that chemicals typically found in MWWTP effluents can cause histological responses in fish. Fortunately this study also demonstrates that improved treatment (targeted at conventional parameters) can greatly reduce the effects in the environment. This study has implications for wastewater management at other sites around the globe in that it confirms that treatment upgrades can reduce biologically relevant indicators of EDC responses in wild fish in a relatively short period of time.

Chapter 4

Site fidelity and movement of a small-bodied fish species, the rainbow darter (*Etheostoma caeruleum*): implications for environmental effects assessment

Hicks, K.A., and Servos, M.R. Site fidelity and movement of a small-bodied fish species, the rainbow darter (*Etheostoma caeruleum*): implications for environmental effects assessment. Prepared for submission to a scientific journal.

4.1 Chapter summary

Small-bodied fish species are commonly used for environmental effects assessments because they are short lived, abundant, and they mature early. Although they are generally considered to be less mobile than larger bodied species, relatively little is known about their movement patterns. In this study, we tagged 3001 rainbow darter (<76 mm) in the upper Grand River of southern Ontario with visible implant alpha tags and elastomer in three riffles. A total of 565 fish were recaptured over four recapture events (including spawning and non-spawning periods) over a spatial extent of 1900 m. Rainbow darter demonstrated high site fidelity with a median movement of 5 m and with 85% staying within the same riffle in which they were tagged. Most movements occurred during their spawning period, where males had a tendency to move longer distances (up to 975 m). There was also a bias in the direction of movement which was dependent on the recapture season. Overall, the high site fidelity of the rainbow darter makes them a good candidate sentinel species for environmental effects monitoring.

4.2 Introduction

Fish have widely been used to assess the impacts of point and nonpoint source pollutants. Indicators of fish health such as age, growth, energy storage, and reproduction are recommended endpoints in monitoring programs such as the Canadian Environmental Effects Monitoring (EEM) program (Munkittrick et al., 2010). Key factors in selecting a sentinel species include the potential exposure of the species to the contaminant(s) of interest, the species' abundance, and its relevance to the study area and research objectives (Munkittrick and McMaster, 2000). Life history, including life-span, age to maturation, spawning time, and position within the food web, should also be considered when selecting a sentinel species (Munkittrick and McMaster, 2000). Mobility is another important factor, as this will determine how well the fish reflect their local environmental conditions (Barrett and Munkittrick, 2010).

In the EEM program, larger bodied fish species have more commonly been chosen than smaller bodied species (<150 mm at maturity) as a sentinel species because their life histories are generally well known (Munkittrick et al., 2010). However, small-bodied species have many advantages over larger bodied species because they mature early, they are short lived (and thus any environmental impact is recent), they are generally more abundant and easier to capture, and they are considered less mobile and therefore better represent their local environment

(Munkittrick et al., 2010). The use of small-bodied fish species in the EEM program increased from 10% in the first EEM cycle to 33% by the third EEM cycle (Barrett et al., 2010). Small-bodied fish species have also been successfully used to assess impacts from the Alberta oil sands (Tetreault et al., 2003), pulp and paper mills (Gibbons et al., 1998), nonpoint sources of pollution such as agriculture (Gray and Munkittrick, 2005), and municipal wastewater (Tetreault et al., 2012; Fuzzen et al., 2016). Although small-bodied fish species have advantages over larger bodied fish species relatively little is known about their basic life history, and although they are expected to be less mobile (Minns, 1995) there is generally a lack of knowledge about their movement patterns.

Methods for assessing movement of small-bodied fish species are limited (Lucas and Baras, 2000). Electronic tags such as passive integrated transponders (PIT) have primarily been used only with larger bodied species (e.g., fish >120 mm; Adams et al 1998). With advances in technology, PIT tags have been successfully used with some smaller bodied fish species such as salmon parr (>84 mm; Roussel et al. 2000), cyprinids (>113 mm; Bolland et al. 2009), and sculpin (>55 mm; Breen et al. 2009). Because of the costs, logistics, and effort associated with using PIT tags, the movement patterns in small-bodied fish species have primarily been studied using mark–recapture techniques, such as fin clips (Reed, 1968), or by marking the fish with externally visible dyes (Brown and Downhower, 1982) or coloured biocompatible plastics (Weston and Johnson, 2008; Phillips and Fries, 2009). Chemical analysis, with stable isotopes, for example, has also been used to assess the site fidelity of small-bodied fishes (Gray et al., 2004).

The rainbow darter (*Etheostoma caeruleum*), a small-bodied species (<75 mm), has recently been used in several biomonitoring studies to assess the impacts of municipal wastewater in the Grand River watershed in southern Ontario (Tetreault et al., 2011; Bahamonde et al., 2015b; Fuzzen et al., 2016). This benthic species primarily lives in riffle/run habitats. The fish are short lived, reaching a maximum age of 5 years (Beckman, 2002), are sexually mature at age 0+, and spawn in the spring (Winn, 1958). Recent work in the Grand River watershed revealed reproductive impacts including high rates and severe cases of intersex (ova-testis) in the male rainbow darter at sites below two municipal wastewater treatment plants (MWWTP; Kitchener and Waterloo; Fuzzen et al., 2016). The impacts were attributed to contaminants such as endocrine-disrupting compounds present in the MWWTP effluents (Fuzzen et al., 2016). The same phenomenon (though at lower rates) was also consistently observed in an urban upstream site in close proximity (1–2 km) to the Kitchener MWWTP outfall. There are no barriers between these sites, leading to the hypothesis that exposed fish from downstream sites may be moving to the upstream reference site, especially during the spring.

Very few studies have been published on movement of darters and other small-bodied fish species, and to our knowledge no studies have examined the movement patterns of the rainbow darter. The objective of the current study was to assess the movement and site fidelity of the rainbow darter in the Grand River watershed using a mark–recapture method. Movement patterns were contrasted between sexes and recapture events (seasons), and related to fish size (total length). A better understanding of the mobility of the rainbow darter will help to interpret the biological responses of this fish when it is used as a sentinel species for environmental effects assessment. In addition, it will enhance our understanding of the ecology of this small-bodied fish species, which are part of fish assemblages in many North American rivers.

4.3 Materials and methods

4.3.1 Site description

This study was conducted in the upper portion of the Grand River watershed (43°54’56” N, 80°19’11” W), in an agriculturally rich area 1 km upstream of the town of Grand Valley in southern Ontario (Figure 4.1). At the study site the river is 4th order with wetted widths ranging from 5 to 15 m and maximum depths in riffle habitat of 0.5 m during summer conditions. The substrate in the riffles was predominantly cobble with little stream vegetation. To characterize river discharge during the sampling periods, we used daily river discharge data from the Grand River Conservation Authority (https://maps.grandriver.ca/data-monitoring.html) collected at a flow gauge 7 km upstream of the site. The study site had a total length of 1900 m. It consisted of three core riffles, each 50–100 m in length, which were separated by runs, pools, and/or other riffles. The core riffles (with a total area of 2700 m^2) were each divided into 5 x 5 m plots, making a total of 108 sampling plots.

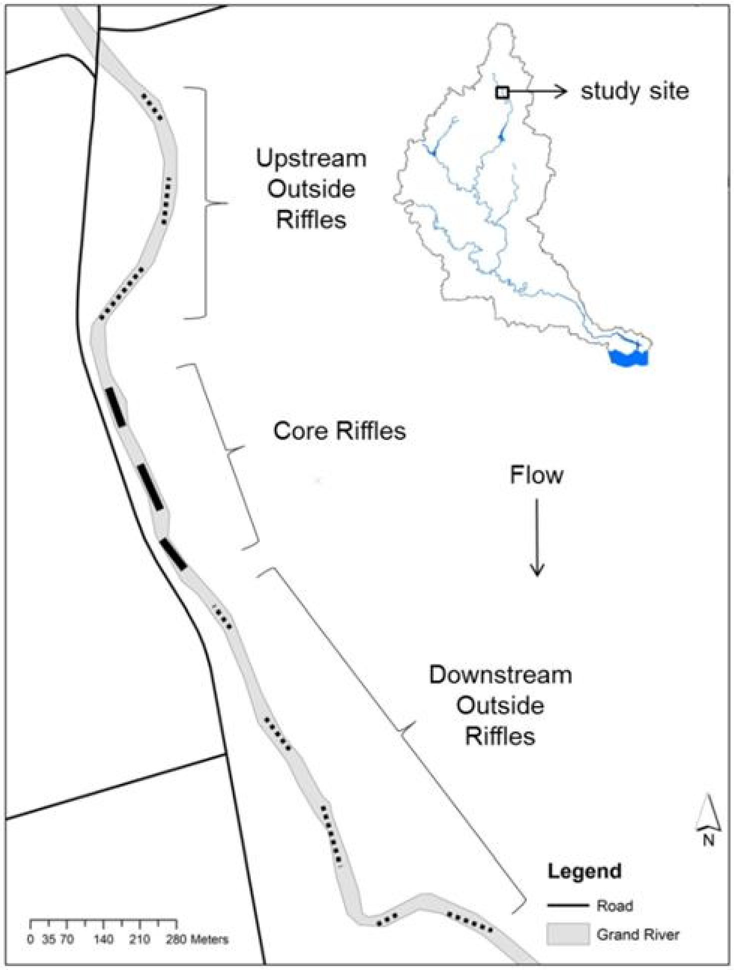

Figure 4.1 Map of the study site in the upper part of the Grand River watershed, in southern Ontario. The three core riffles (where rainbow darter tagging took place) span a distance of 350 m; each riffle is 50 to 100 m in length. The whole area, including the additional outside riffles, spans a total distance of 1900 m.

4.3.2 Fish collections

Fish were tagged in three separate sampling periods in July, August, and November 2014. Rainbow darter were captured by backpack electrofishing (Smith-Root, LR24). Each 5 x 5 m plot was electrofished with two passes and with two netters working in the upstream direction. Electrofishing effort (catch per unit effort) was recorded for all plots, and to maintain consistency the same person electrofished throughout the study. Fish caught in each 5 x 5 m plot were placed in separate aerated buckets and, if necessary, in coolers to maintain river water temperature. In an on-site laboratory (trailer), fish sex, total length (TL; ±1.0 mm), and weight (±0.001 g) were recorded before the fish were tagged.

4.3.3 Fish tagging and recapture events

All fish were tagged subcutaneously with visible implant elastomer (VIE, Northwest Marine Technology, Inc.) using a 0.3 cc injection syringe. Fish $\geq$42 mm were tagged mid-ventrally and fish <42 mm were tagged beneath the second dorsal fin with a 3- to 4-mm long mark. This was to differentiate between fish that were probably young of the year (YOY) and adults (Crichton, 2016). Placement of tags (dorsally verses ventrally) has previously been shown to have no effect on retention rate in darters (*Percina* spp. and *Etheostoma* spp.) (Roberts and Angermeier, 2004; Phillips and Fries, 2009). Four different florescent VIE colours were used (blue, red, yellow, and pink), with a unique colour assigned to the fish caught in each of the two shorter core riffles and two colours assigned to the fish caught in the longer middle core riffle (the middle core riffle was 100 m long; the bottom and top 50 m each received a different colour of VIE). All fish $\geq$42 mm were also individually tagged with a regular sized (1.2 x 2.7 x 0.13 mm thick; 0.5 mg), fluorescent visible implant (VI) alpha tag (Northwest Marine Technology, Inc.), which had a unique alphanumeric code (Figure 4.2). VI alpha tags were injected with a VI alpha injector needle (V2.0; Northwest Marine Technology, Inc.) beneath the translucent tissue covering the pectoral girdle. All fish were maintained in aerated buckets for at least one hour after tagging and were assessed for health and tag retention. No mortalities were recorded during this recovery period. All fish were returned to the middle of the plot from which they were captured.

There were four recapture events. These included the last two tagging events in 2014 (Augusts and November) and two periods in 2015 (May and August). The recapture efforts in August and November 2014 only involved fishing in the three core riffles. The May and August 2015 recapture events were focused solely on recaptures, thus fishing effort was extended to areas between the core riffles and to the outside riffles (Figure 4.1) in both the upstream and downstream directions. Two electrofishing passes were always completed for between and outside plots. The effort at additional outside riffles continued in either the upstream or downstream direction until two consecutive riffles had no recaptures, for a total distance of 946 m (2910 m^2) and 605 m (3490 m^2) in the downstream and upstream directions, respectively.

During the recapture events, all fish were checked for tags using an ultraviolet light to enhance detection of both types of fluorescent tags (VIE and VI alpha). The TL and weight of all recaptured fish were recorded and then the fish were returned to their site of recapture. All fish were handled in accordance with the approved University of Waterloo animal care protocols (AUPP# 10-17 and 14-15).

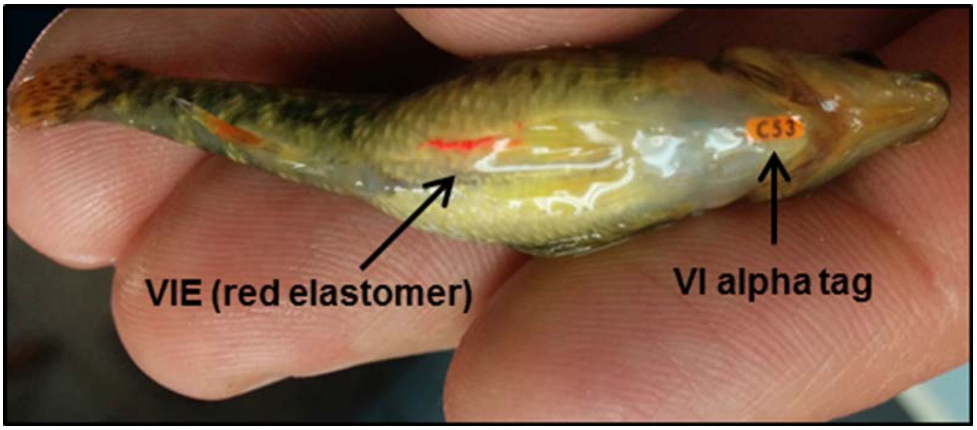

Figure 4.2 A gravid female rainbow darter recaptured during the May 2015 recapture event. The double tagging approach is visible with the VIE (visible implant elastomer) tag located mid-ventrally and the VI (visible implant) alpha tag located in the translucent tissue of the pectoral girdle containing a unique alphanumeric code.

4.3.4 Data analysis and statistics

The movements of recaptured fish that had VI alpha tags were quantified to the nearest 5 m for both longitudinal (upstream and downstream) and lateral (to adjacent plots) movements. For longitudinal movements, fish that were caught in the same plot or in a plot adjacent to the one from which they were originally tagged were assigned a value of 0 m (i.e., no upstream/downstream movement). Lateral movements were assigned a value of 0 m (no lateral movement), 5 m, or 10 m; 10 m was the maximum because movements were quantified from the middle of each plot, and the maximum stream width was 15 m.

Recaptured fish that only had VIE tags (either small fish [<42 mm], or fish that had lost their VI alpha tags) were categorized as either staying in or leaving their original tagging riffle. For fish that stayed in their riffle, small-scale movement could not be quantified further, but they were assigned a value of ±50 m. For fish that moved to another riffle, a conservative estimate (shortest distance) was estimated on the basis of the location where the fish was recaptured and the distance between riffles.

As a result of discrepancies in the data (for recaptured fish with and without VI alpha tags), three different data sets were created to analyze and test specific questions (detailed below). It should also be noted that additional fishing effort at outside riffles was only performed during the 2015 recapture events; this difference in fishing effort between the 2014 and 2015 recapture events introduced biases into the data set. For statistical purposes, we removed this bias (by removing from our analysis the recaptured fish that were caught with additional fishing effort) when we compared movements across sampling events. SigmaPlot version 13 was used to analyze and plot the data, with a significance level of $p < 0.05$.

All recaptured fish (data set 1)

Data set 1 (n = 565) was used to compare the proportions of fish that moved outside their original tagging riffle with the proportions of those that stayed. Chi-square analysis was used to test if there was a difference in the proportion of fish that left and those that stayed between recapture events and between sexes. In addition, the difference in mean TL between fish that moved riffles and those that stayed was assessed with two sample t-test performed separately for males and females.

Fish with VI alpha tags + fish with VIE only where a conservative movement estimate could be quantified (data set 2)

Data set 2 (n = 229) was used to create histograms to visualize movement patterns of rainbow darter. Histograms were plotted for pooled data (all recapture events) and for each individual recapture event. Downstream displacement (movement) was assigned a negative value. This data set was also used to assess the relationship between absolute fish movement and fish TL for males and females separately, using Spearman's rank correlation. A two-sample Kolmogorov–Smirnov test was used to test if there were differences in the movement distributions of males and females (pooled across recapture events). Finally, Chi-square analysis was used to assess if there was a tendency toward either upstream or downstream movement for each of the recapture events and for data pooled across all recapture events.

All Fish with VI alpha tags (data set 3)

Data set 3 (n = 170) was used to estimate median longitudinal movement for males, females, and both sexes (pooled data) across recapture events. To test if there were differences in median movement between recapture events, a Kruskal–Wallis test was performed with Dunn's pairwise comparison. A Mann–Whitney rank sum test was used to test if there were any differences between the movements of males and females during each of the recapture events. Chi-square analysis was used to test if there were any differences in the proportion of lateral fish movements (0 m, 5 m, and 10 m) between recapture events and if there were any differences between males and females. For this data set, all comparisons among recapture events were done between only the first 3 recapture events; the last recapture event (August 2015) was excluded because of the small number of fish with VI alpha tags that were recaptured at this time.

4.4 Results

A total of 3001 (2773 tagged with both VIE and VI alpha tags) fish were tagged throughout the study periods; 565 of them were recaptured over the four recapture events (Table 4.1). The recapture rate averaged 6.2% and ranged from 4.9% to 8.8% for the different recapture events (Table 4.1). Nine of the 565 recaptures were only tagged with VIE tags (fish < 42 mm) and the remaining 556 were originally tagged with both VIE and VI alpha tags. Out of these 556 recaptures, thirty percent (170 fish) retained their VI alpha tags, where the retention rate had decreased over the period of the study. Tagging fish with both VI alpha tags and VIE tags did not appear to affect growth as the majority of the recaptured fish had a larger TL and weight at the time of recapture than at the time of tagging (Figure S4.1), indicating that the fish were still growing. In addition, reproduction was probably not affected by the double-tag approach, as fish recaptured in the May 2015 recapture event (spawning season) appeared to be reproducing normally, as evidenced by the presence of eggs and milt in females and males, respectively.

Table 4.1 Summary of tagging/recapture dates, number of fish tagged and recaptured, fishing effort, and river discharge.

				No. of fish tagged				No. of recaptures						fishing effort		
Week	Date	No. Unmarked (core riffles)	No. unmarked (outside riffles)	Total No. tagged (>4.2 cm)*	No. females (>4.2 cm)*	No. males (>4.2 cm)*	No. <4.2 cm **	Total no. recaptures	No. males	No. females	Total no. with alpha tag	recapture rate (%)	Alpha tag retention (%)	Core riffles (h) (2830 m^2)	Outside riffles (h) (6400 m^2)	Mean (SE) hourly flow m^3/s
0	July, 2014	1368	-	1290	648	642	78	-			-	-	-	13.8	-	2.1 ± 0.09
3	Aug., 2014	724	-	657	301	356	67	79	50	29	62	5.8	78.5	13.0	-	2.1 ± 0.10
18	Nov. -Dec., 2014	909	-	826	431	395	83	184	109	75	35	8.8	19.0	13.8	-	2.8 ± 0.04
42	May, 2015	569	2061	-	-	-	-	156	82	74	67	5.2	42.9	13.0	23.1	0.8 ± 0.02
56	Aug.-Sept., 2015	751	3288	-	-	-	-	146	53	93	6	4.9	4.1	11.2	23.8	0.7 ± 0.02
	Total			2773	1380	1393	228	565	294	271	170					

* Rainbow darter tagged with both alpha tags and coloured elastomer

** Rainbow darter tagged only with coloured elastomer

Overall, the majority (85%) of fish stayed within the riffle in which they were originally tagged (data set 1; Figure 4.3). There was a statistical difference in the proportion of fish that moved from their original tagging riffle among the four recapture events ($X^2 = 52.7$; d.f. = 3, $p < 0.001$). May 2015 was the only recapture event that was statistically different from the rest, with 19% of the recaptures having moved to a different riffle (Figure 4.3); for the other recapture events, this proportion ranged from 1% to 7%. The 15% (87 fish) that had moved outside their original riffle (pooled data) had an absolute median movement of 165 m, and the majority (67%) of these fish were males.

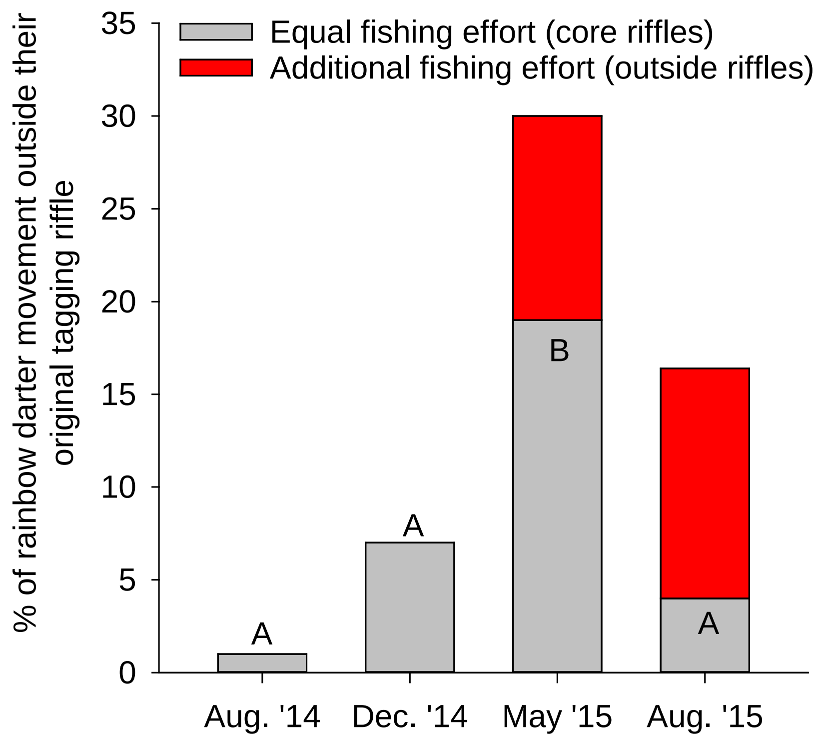

Figure 4.3 The proportion of recaptured rainbow darter that had moved outside the riffle in which they were originally tagged (data set 1). The grey part of the bar indicates the proportion of those fish that were caught in the core riffles and with equivalent fishing effort. The proportion of fish caught with the additional fishing effort in outside riffles, which took place in May and August 2015 only, is represented in red. Bars that do not share a letter in common indicate a significant difference (X^2, $p < 0.05$) in the proportion of fish that moved outside their original riffle for data represented in grey only.

The overall distribution of longitudinal movement in rainbow darter was leptokurtic (kurtosis = 10.139; skewness = –2.245), demonstrating higher peaks and longer tails than would be expected in a normal distribution (kurtosis = 3) (data set 2; Figure 4.4). The majority of recaptured fish demonstrated high site fidelity, with an absolute median movement of 5 m throughout the study. Seventy percent of the recaptured fish that had VI alpha tags remained within ±5 m of the plot in which they were originally tagged. There were also four fish that were recaptured twice after tagging that had remained within ±5 m of their original tagging plot, further illustrating high site fidelity, even after multiple recaptures. Although there were very few recaptured fish in the last recapture event (August 2015) that had retained their VI alpha tag, 5 out of the 6 were still within ±5 m of their original tagging location, and 4 of these fish had been tagged in July or August of the previous year, demonstrating high site fidelity over the 12 or 13 months after tagging.

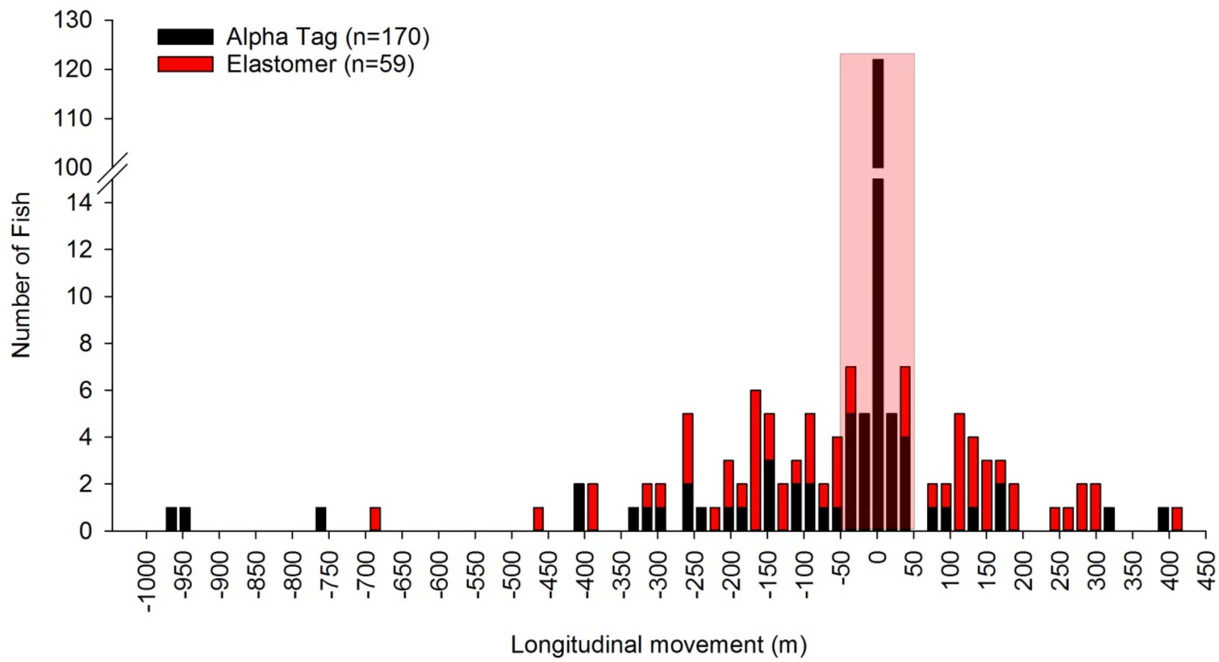

Figure 4.4 Pooled frequency distribution of rainbow darter movement for all sampling periods (data set 2). Negative numbers indicate downstream movement. Black bars indicate recaptured fish with VI alpha tags, and red bars are recaptured fish that had lost their VI alpha tags but had moved riffles, thus movement could be quantified on the basis of the presence of a VIE tag. The red box indicates additional rainbow darter (*n* = 336) that remained in their riffle (–50 to 50 m) but had lost their VI alpha tag, thus movement could not be quantified further.

Although the majority of recaptured fish had high site fidelity, a small proportion of fish moved greater distances. A total of 69 fish were recorded moving >100 m. The maximum distances recorded were 975 m downstream and 420 m upstream, with both of these extremes being logged during the May 2015 recapture event (data set 2; Figure 4.5). Some of the larger movements occurred over a short period after tagging, for example, one fish had moved 130 m within 3 weeks of being tagged.

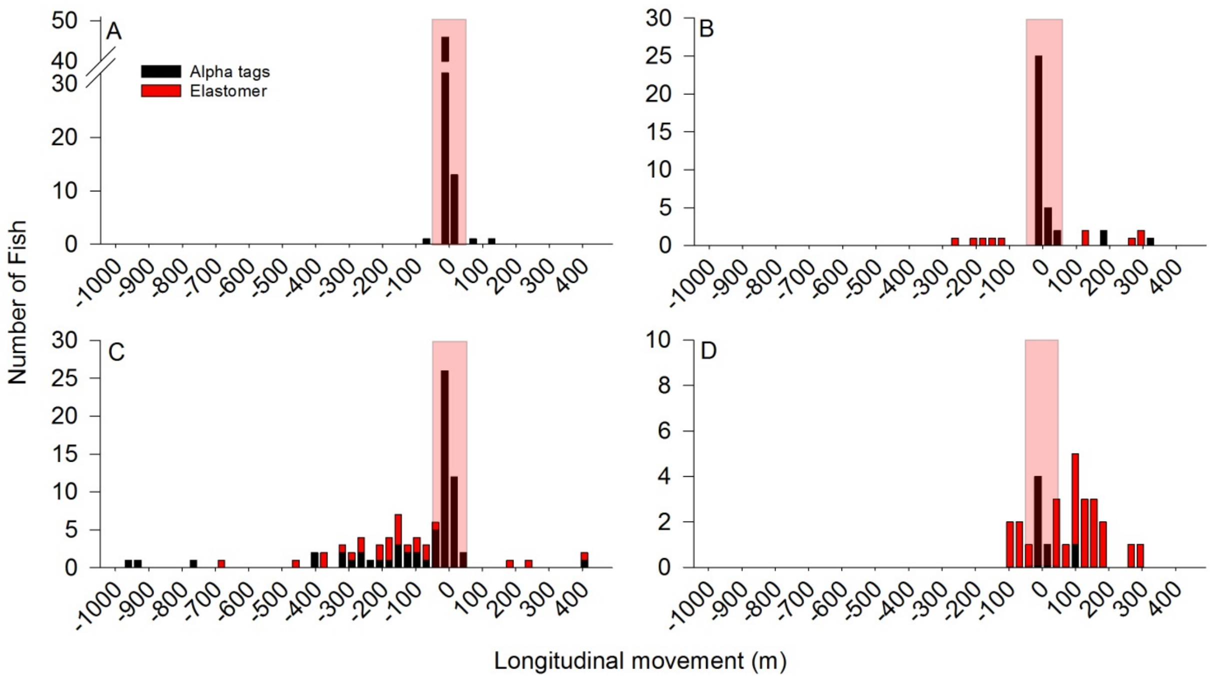

Figure 4.5 Frequency distribution of rainbow darter at four sample time points (data set 2): (A) August 2014, (B) November/December 2014, (C) May 2015, and (D) August/September 2015. Black bars represent recaptured fish with VI tags and red bars represent recaptured fish that had lost their VI alpha tag but had moved riffles. The red box indicates additional rainbow darter that remained in their riffle (–50 to 50 m) but had lost their VI alpha tags, thus movement could not be quantified further. The number of fish represented by the red box are as follows: A, n = 17; B, n = 139; C, n = 63; and D, n = 117.

The tendency in the direction of longitudinal movement differed between some recapture events. In the first recapture event (3 weeks after tagging, August 2014), only 6 fish moved in either the upstream or downstream direction, with the remaining 56 not moving at all; thus, there was no tendency to move in either direction. In the November 2014 recapture event, for those fish that had moved (>0 m), there was a tendency toward upstream movement (X^2 = 4.689, d.f. = 1, p = 0.030). In contrast, fish caught during the May 2015 recapture event had a tendency toward downstream movement ($X^2 = 15.454$, d.f. = 1, $p < 0.001$). There was no statistical difference ($X^2 = 3.471$, d.f. = 1, $p = 0.062$) in movement during the August 2015 recapture event; however, more fish (74%) had moved in the upstream direction. When all data were pooled across recapture events, there was no tendency toward either upstream or downstream movement ($X^2 = 0.160$, d.f. = 1, $p = 0.689$). There were no differences in the median distance moved upstream versus downstream in any of the recapture events (Mann–Whitney rank sum test, $p > 0.05$).

The majority of fish (72%) showed no lateral movement throughout the study (data set 3; Figure 4.6). There were no differences between recapture events in the proportion that showed no lateral movement (0 m) and lateral movements of 5 m and 10 m ($X^2 = 3.000$, d.f. = 4, $p = 0.558$). Similarly, there were no differences in lateral movement between males and females ($X^2 = 1.098$, d.f. = 2, $p = 0.577$).

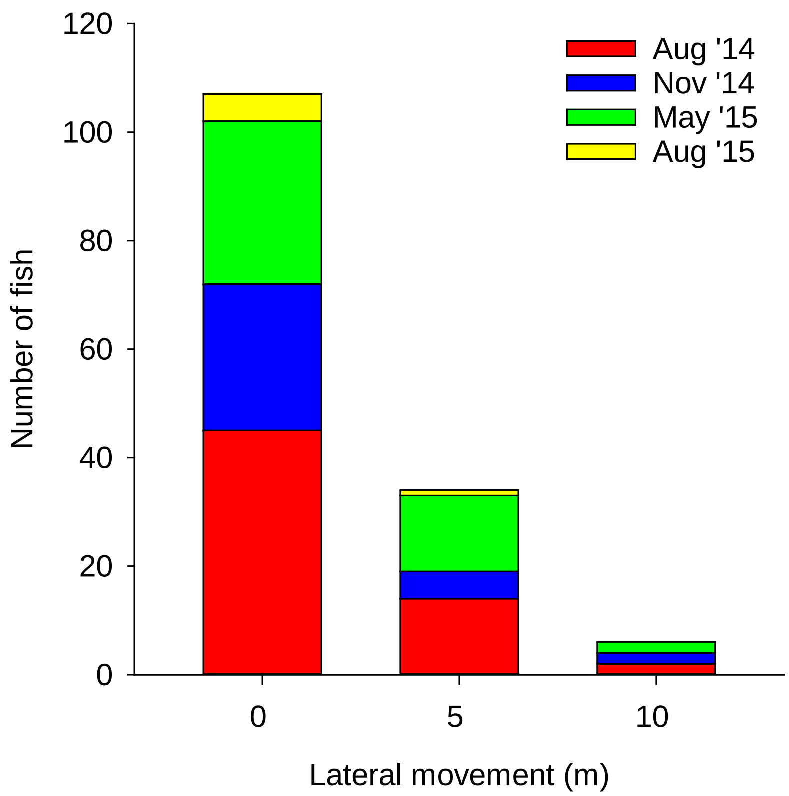

Figure 4.6 Lateral movement of rainbow darter in August 2014 (red), November 2014 (blue), May 2015 (green), and August 2015 (yellow) (data set 3). Fish were categorized as either having no movement (0 m) or lateral movements of 5 or 10 m.

The absolute median movements for each of the recapture events are presented in Table 4.2. The median movement ranged from 0 m in both the August and November (2014) recapture events to 10 m in the May 2015 recapture event. This difference in the May 2015 recapture event was primarily driven by males who had a significantly higher absolute median movement than females (Mann–Whitney rank sum test, $p = 0.009$; data set 3; Figure 4.7). No other differences between male and females were observed in the other recapture events (Mann–Whitney rank sum test, $p > 0.05$; data set 3; Figure 4.7). In addition, males and females (pooled across recapture events) did not show any differences in their distribution of movements (Kolmogorov–Smirnov test, $p > 0.05$).

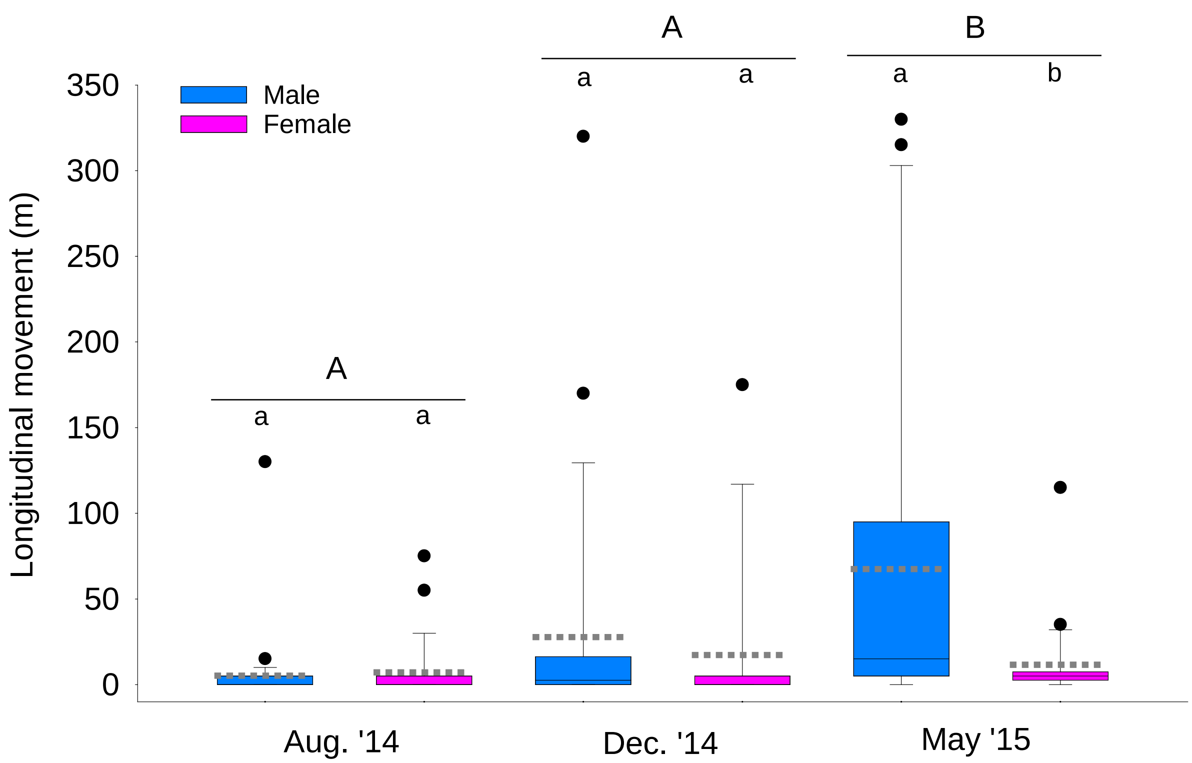

Figure 4.7 Boxplots of the absolute movement of males (blue) and females (pink) for each time point for VI alpha tags only with fishing effort bias removed (data set 3). Boxplots show median (solid black line), mean (dotted line), 25th and 75th percentiles (box), 10th and 90th percentiles (whiskers), and outliers (black dots). Recapture events (time points) that do not share an uppercase letter in common indicate a significant different in median movement (pooled males and females) ($p < 0.05$). Male and female boxplots within recapture events that do not share a lowercase letter in common indicate a significant difference between male and female movement ($p < 0.05$).

Table 4.2 Summary of absolute median (mean ± SEM) movement of rainbow darter for pooled and individual recapture events (data set 3), excluding the last recapture event because of the small number of fish that retained their VI alpha tag.

Recapture Event	median (mean ± SEM) m
August 2014	0 (6.0 ± 2.5)
November 2015	0 (23.9 ± 11.1)
May 2015	10 (109.6 ± 25.1)
August 2015	NA
Overall median	5 (50.9 ± 10.8)

The range in TL of recaptured fish over all of the recapture events was 3.6–7.6 cm, with a mean (± standard deviation) of 61 ± 7 mm for males and 60 ± 5 mm for females. There were no differences in the mean TL between fish that moved or stayed in their original tagging riffle for either males or females (*t*-test, $p > 0.05$). Overall, TL was a poor predictor of distance moved (data set 2; Figure 4.8).

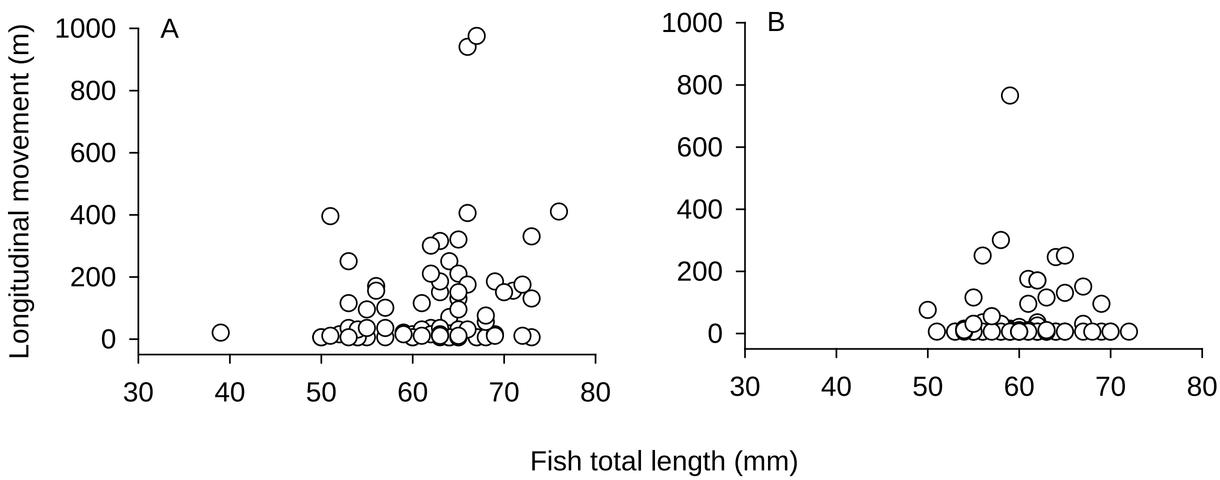

Figure 4.8 The relationship between absolute distance moved (m) and fish total length for both (A) males and (B) females, where movement is >0 m. Data were pooled across all 4 sampling periods (data set 2). A Spearman's rank order correlation was used to assess the relationship between total length and displacement for males ($r = 0.207$, $p = 0.053$, $n = 88$) and females ($r = -0.03$, $p = 0.795$, $n = 75$).

4.5 Discussion

The major objective of this study was to assess the site fidelity of the rainbow darter by using a unique dual tagging mark–recapture method. Site fidelity was examined in a relatively undisturbed river reach across four recapture events occurring over three different seasons including spawning (spring) and non-spawning periods (summer and fall). The majority of the rainbow darter in this study had a small home range (median = 5 m) and remained in the same riffle in which they were tagged. Fish that moved tended to travel in either the upstream or downstream direction depending on the season, which probably had to do with searching for spawning habitat or foraging for food. Males were more likely to move than females and to travel greater distances than females, primarily during the spawning period. There was very little lateral movement, and fish size was a poor predictor of fish movement. Overall, this study demonstrated that rainbow darter generally have high site fidelity, but a small proportion of the population can move considerable distances (up to 975 m).

4.5.1 Site fidelity of the rainbow darter

Findings from the limited number of studies that have used mark–recapture methods to assess darter movement fit the general pattern and conclusions observed with the rainbow darter in the Grand River. High site fidelity was the general observation reported among multiple darter species in several geographic regions (Reed, 1968; Scalet, 1973; Mundahl and Ingersoll, 1983; Freeman, 1995; Roberts and Angermeier, 2007; Roberts et al., 2008; Dammeyer et al., 2013). A movement distribution that is leptokurtic is common among darters (Roberts and Angermeier, 2007; Dammeyer et al., 2013) and other stream fish (Skalski and Gilliam, 2000), where a large proportion of fish have a small home range and a smaller proportion show more movement, sometimes referred to as the mobile and static dichotomy (Roberts et al., 2008). The movements of rainbow darter, along with other darter species, are generally consistent with the restricted movement paradigm, a theory that purports that adult stream fish are sedentary (Gowan et al., 1994).

Mark–recapture studies are not the only method to assess the movement and site fidelity of fish. A less labour-intensive but less direct method to assess animal movement has been the use of stable isotopes (Hobson, 1999). For example, Gray et al. (2004) found differences in site-specific isotope signatures ($\delta^{15}N$ and $\delta^{13}C$) in the slimy sculpin (*Cottus cognatus*) across agricultural and forested sites in Little River, New Brunswick. They concluded that the distinct isotope signatures suggested limited movement and high site fidelity. This finding was later confirmed in a mark–recapture study that slimy sculpin do display high site fidelity (Cunjak et al., 2005). Similarly, stable isotopes ($\delta^{15}N$ and $\delta^{13}C$) have also been measured in the rainbow darter across several sites in the Grand River (the same watershed as in the current study) in close association with two municipal wastewater treatment plants (MWWTPs); that study revealed site-specific isotope signatures (Hicks et al., 2017). Rainbow darter collected in fall and spring showed distinct differences in $\delta^{15}N$ downstream of both the Waterloo and Kitchener MWWTP outfalls (Hicks et al., 2017). In addition, there was a distinct difference across the river below the outfalls until the effluent was fully mixed, again suggesting minimal lateral movement of rainbow darter during the summer (Loomer, 2009). This further supports the notion that rainbow darter probably do not have a large home range, at least for most of the year.

Part of the reason the present study was conducted was to assess the utility of the rainbow darter as a sentinel species to monitor the impacts of wastewater in the central reaches of the Grand River, in close proximity to the Kitchener and Waterloo MWWTPs. Several fish upstream of the outfall (1 km) were found to have very high expression of intersex, while fish collected even further upstream (separated by a dam) had a much lower occurrence and severity of intersex (Fuzzen et al. 2016). The elevated rates of endocrine disruption (intersex) reported by Fuzzen et al. (2016) are typically associated with sewage, and these findings were thought to possibly be due to rainbow darter movement. In the current study, there were only two cases (out of 565) where rainbow darter had moved almost 1 km, representing less than 1% of the recaptures. The number of fish that travelled far may be under-represented because of the low recapture rates; however, another possibility is that the movement of rainbow darter may be dependent upon factors such habit quality and complexity. This has been illustrated with *Etheostoma podostemone*, where microhabitat diversity has been negatively correlated with fish movement (Roberts and Angermeier, 2007). As suggested in other studies, the habitat in urbanized river reaches is probably of lower quality and complexity than in non-urban reaches (Wang et al., 2001), possibly leading to lower rates of site fidelity (Albanese et al., 2004). In addition, sewage outfalls decrease water quality, and this may result in unfavourable conditions (e.g., low dissolved oxygen and elevated ammonia concentrations) that cause fish to emigrate (Lucas and Baras, 2001). Before 2012, the Kitchener WWTP effluent resulted in low oxygen and elevated ammonia concentrations downstream of the outfall (Hicks et al., 2017). However, three years after process upgrades were implemented at the Kitchener WWTP, there were no highly intersex fish in the upstream (or downstream) site (Hicks et al., 2016), suggesting that before the upgrades highly intersex fish (likely exposure to sewage) were probably coming from downstream. The results of the current study suggest that although most rainbow darters remain within a small home range, there is potential for some fish to move and confound interpretation of near-field upstream sites that are not physically separated. Further studies are needed to assess the link between rainbow darter movement and habitat/water quality.

4.5.2 Limiting bias in mark-recapture studies

There are several limitations of mark–recapture studies. One is the inherent biases associated with the study design (Gowan et al., 1994; Albanese et al., 2003). For example, shorter distances are typically sampled more often than longer distances, and the probability of detecting movement decreases with distance (Albanese et al., 2003). It has been demonstrated that when the spatial extent of the study is increased, the study subjects are reported to have moved greater distances and greater maximum distances are detected (Albanese et al., 2003; Schwalb et al., 2011). Study duration has also been shown to be positively correlated with maximum distance moved in darter species (Schwalb et al., 2011). In the present study, median distance moved was probably underestimated in the first two recapture events, as the recapture area only covered the original three core riffles in which tagging had taken place. This bias would have been reduced in the last two recapture events, where the total recapture area was extended to riffles 946 m downstream and 605 m upstream. In this study we maximized our efforts spatially, with a spatial extent that was greater than most other movement studies on small–bodied fish species (reviewed in Schwalb et al. 2011).

A second limitation of mark–recapture studies is that they generally have low recapture rates. The mean recapture rate in this study was 6.2%, which is similar to that of other mark–recapture studies with darters and other small-bodied fish species (reviewed in Schwalb et al. 2011). The low recapture rate could be due to several reasons including tag loss, fish mortality, and fishing efficiency (Gowan et al., 1994). Tag loss was probably not a reason for the low recapture rate in this study especially because a dual tagging approach was used. VIE tags have been demonstrated to have high retention rates (88%–100%) in darter species, including a 100% retention rate in the rainbow darter during a 58-day laboratory study (Weston and Johnson, 2008). In addition, although the retention rate of VI alpha tags was low, no fish were ever recaptured that had a VI alpha tag but had lost their VIE tag. Mortality caused by tagging was also probably not a reason for our low recapture rates, as both VIE tags (Roberts and Angermeier, 2004; Coombs and Wilson, 2008; Phillips and Fries, 2009) and VI alpha tags (Turek et al., 2014) have been demonstrated to be associated with low mortality rates in several small-bodied fish species and juvenile fish. In fact, rainbow darter that maintained both VIE and VI alpha tags were generally longer and heavier than at the time of their initial capture (Figure S4.1), indicating that the dual tagging approach probably did not stop growth. In addition, in the last recapture event, 5 recaptured fish had survived 13 months after being tagged with both VIE and VI alpha tags, further suggesting that mortality associated with tagging was probably minimal. Fishing efficiency (or fish escapement) may have also been a reason for our low recapture rates. The abundance of rainbow darter (fish per square meter) was quite variable in the three core riffles (Table S4.1) over the five time periods, with instances where there was up to a threefold difference. This difference may be real, or it may due to variability in fishing efficiency because of changes in environmental conditions such as river discharge (Table 4.1) or changes in water temperature across the seasons (Speas et al., 2004).

4.5.3 The use of VI alpha tags in small-bodied fish

The retention rate of VI alpha tags dropped from 78% retention at three weeks post-tag to 4% retention at 38–56 weeks post-tag, with a mean of 30% over the course of the study. VI alpha tags typically have lower retention rates than VIE tags (Summers et al., 2006) and have predominantly been used in larger bodied fish species (>100 mm), where the tags are inserted in the translucent tissue of fish eyelids (Turek et al., 2014). More recently, 91% to 100% of juvenile fish >84 mm including ecocides and salmonids have successfully retained their VI alpha tags in a four-week laboratory study. To our knowledge the current study is the first attempt at individually tagging a small-bodied fish species (42–76 mm) with VI alpha tags to track movements. The low retention rate in rainbow darter suggests that the use of VI alpha tags in small-bodied species (<76 mm) may be appropriate for short-term studies (e.g., 1 month); however, at least using the methodology in this study, it would not be recommended for longer studies (e.g., 1 year in duration) or studies requiring high retention rates.

4.5.4 Movement of the rainbow darter

In previous studies, the longest recorded movement from an etheostomid was 500 m in the *Etheostoma flabellare* (Roberts and Angermeier, 2007). In the current study with rainbow darter, four fish were recorded moving >600 m, and up to 975 m within 6 months of tagging. Although the majority of the recaptured fish had high site fidelity, 12% (69 fish) moved >100 m. The maximum movement reported for the rainbow darter in this study was also greater than that for most other small-bodied fish species such as sculpin and larger darter species (Percina spp.) (reviewed in Schwalb et al. 2011). Again, this may have to do with study design, as this study had a much greater spatial extent than other studies assessing movement of small-bodied fishes (reviewed in Schwalb et al. 2011).

The factors responsible for fish movement in this study are unknown; however, they probably included spawning, seeking refuge (e.g., from high temperatures), predator avoidance, habitat quality (e.g., food availability), and population densities (Lucas and Baras, 2001). The greatest distances moved were observed during the May 2015 recapture event, which coincided with the spawning period. This is similar to findings with *Etheostoma fonticola* (which spawn year round) in which movement was greatest during peak reproductive seasons (Dammeyer et al., 2013). Male rainbow darter from the spring recapture event also moved greater distances than females, which is probably related to their reproductive behaviours (Winn, 1958). Also, the tendency in movement in the spring period was clearly downstream. This is contrary to other findings, where darters have been observed moving primarily in the upstream direction during spawning periods (Winn, 1958; Ingersoll et al., 1984). This difference may be related to local habitat conditions. The tendency in movement was opposite during the non-spawning recapture events. Upstream tendency in movement among darters during non-spawning season is common and possibly related to foraging for food or seeking refuge from low flow or high temperatures (Winn, 1958; Mundahl and Ingersoll, 1983; Roberts and Angermeier, 2007).

There did not appear to be an association between fish total length (TL) and distance moved in the present study. This finding is consistent with the relatively weak relationships that have been observed for some other darter species such as the fantail (*Etheostoma flabellare*) and riverweed darter (*E. podostemone*) (Roberts and Angermeier, 2007), but it is contrary to findings for some additional species. For example, Roberts and Angermeier (2007) found a negative correlation between movement and TL for the Roanoke darter (*Percina roanoka*), whereas Dammeyer et al. (2013) found that larger fountain darters (*E. fonticola*) were likely to move longer distances. In the current study, the size of the fish was not a factor; for example, a YOY fish (38 mm) moved 205 m (downstream) three months after being tagged.

4.5.5 Conclusion

This study confirms that rainbow darter have high site fidelity, following a leptokurtic distribution that is common among stream fish. This study also indicates that rainbow darter are capable of moving at least 975 m within a short time frame (<6 months), providing evidence that etheostomids may be more mobile than originally thought. Not surprisingly, the greater movements were observed during the spawning period, with males moving more frequently than females and at greater distances. This study supports the use of the rainbow darter for environmental effects assessment, as these fish will probably reflect local site-specific environmental conditions because of their small home range. However, their small-scale movements must be considered when designing studies and selecting sites for assessing environmental impacts.

Chapter 5

Using riverine fish communities to detect impacts from municipal wastewater treatment plant effluents. A case study in the Grand River watershed, Ontario

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5.1 Chapter summary

Fish communities are a desirable indicator to use for environmental impact assessments because they are ecologically relevant and socially significant. This study assessed the use of riverine fish community structure as biological indicators for detecting impacts from municipal wastewater treatment plant (MWWTP) effluents in the Grand River watershed of southern Ontario. A variety of reproductive effects were previously observed in a sentinel fish species, rainbow darter (*Etheostoma caeruleum*), associated with two MWWTPs in this watershed. The goal of this study was to assess whether these impacts in a sentinel fish species translated to community-level effects. Fish communities were assessed with a standardized electrofishing protocol in two years (2013 and 2014) at sites above and below the MWWTPs. This data set was combined with a historical data set from 2007 and 2008, providing data for a total of four years. For comparative purposes, changes in benthic macroinvertebrate (BMI) communities were also included in the study (2012–2014). Both fish and BMI communities at sites below the MWWTPs consistently had greater abundances of pollution-tolerant species and lower abundances of pollution-intolerant species than at sites above the MWWTPs, and these changes were most evident in drier years (less dilution). Changes in BMI species composition were more consistent and sensitive to MWWTP effluents, and these changes were linked with local water quality. In contrast, it was more difficult to associate changes in fish communities with MWWTP effluent exposure, and potential effects were confounded by a natural river gradient. Considerable variability in fish and BMI communities was observed at a larger spatial scale across the watershed.

5.2 Introduction

Municipal wastewater treatment plant (MWWTP) effluents contribute an array of contaminants to the aquatic receiving environment including total suspended solids, nutrients, metals, pharmaceuticals, and personal care products (Daughton and Ternes, 1999; Metcalfe et al., 2003; Lishman et al., 2006; Holeton et al., 2011). There are over 3000 treatment plants across Canada, and they discharge a higher volume of effluent than almost any other industry (Chambers et al., 1997). Impacts of MWWTP effluents on the aquatic receiving environment have been documented at multiple trophic levels (primary consumers, benthic invertebrates, and fish), and impacts on fish have been widely studied, with effects documented at multiple levels of biological organization ranging from gene expression to fish communities (Karr et al., 1985; Wichert, 1995; Chambers et al., 1997; Porter and Janz, 2003; Tetreault et al., 2013; Fuzzen et al., 2016).

Healthy fish communities are often associated with well-functioning and sustainable aquatic ecosystems (Kilgour et al., 2007). Hence, fish communities have been recommended as a biological endpoint to include in programs to monitor the environmental impacts of various stressors, including MWWTPs (Kilgour et al., 2005). For aquatic ecosystem management purposes, fish communities are generally considered a relevant endpoint for protection because of their dependency on lower trophic levels (Karr, 1981). Although highly relevant, investigations into the impacts on fish communities are difficult to conduct because of their high spatial and temporal variability (Munkittrick et al., 2010). Changes resulting from processes that drive natural variability in fish communities are difficult to discriminate from changes associated with anthropogenic stressors (Clements et al., 2012). Despite these challenges, assessments at the community level are considered desirable because of their ecological relevance and social significance (Clements and Rohr, 2009).

Several different approaches have been applied to analyzing fish community data to detect environmental impacts (Fausch et al., 1990). Methods include the use of indicator taxa or guilds (presence-absence), indices of species riches, diversity, and abundance (Fausch et al., 1990), as well as multi-metric indices such as the index of biotic integrity (Karr, 1981). In addition, multivariate approaches have been recommended as sensitive approaches for detecting effects on fish communities (Kilgour et al., 2004). There are pros and cons to each of these methods, and often a combination of these approaches are recommended.

The Grand River watershed in southern Ontario is a highly impacted watershed that is dominated by agricultural land (70%) and several urban centres supporting a population of approximately 1 million people (Loomer and Cooke, 2011). Within the watershed, there are 30 MWWTPs, the two largest servicing the cities of Kitchener and Waterloo with a combined population of > 340,000 people. Impacts on wild fish, including the rainbow darter (*Etheostoma caeruleum*), have been documented for several years at sites below the Kitchener and Waterloo MWWTP outfalls. These impacts include effects detected across many levels of biological organization ranging from gene expression (Bahamonde et al., 2014), to steroid production (Fuzzen et al., 2016), histopathological changes (intersex; ova-testis) (Tetreault et al., 2011;

Bahamonde et al., 2015b; Fuzzen et al., 2016), and possible effects at the population level (Fuzzen et al., 2015). Changes in species richness in the fish community have also been reported along with a reduced abundance of rainbow darter and an increase in more pollution-tolerant species (Tetreault et al., 2013). However, changes observed in the fish communities were highly variable and could not be directly associated with water quality because of confounding factors such as habitat (Tetreault et al., 2013). Therefore, it is still not well understood whether the effects associated with MWWTP effluents observed in the wild fish populations translate to community-level responses.

This study assessed spatial and temporal changes in fish communities in the Grand River watershed relative to water quality and habitat. Fish communities in riffle habitats were examined using a standardized electrofishing method across several seasons and years (2013–2014). Changes in benthic macroinvertebrate (BMI) communities, a well-established indicator of water quality (Cairns and Pratt, 1993), were contrasted with changes observed in fish communities. Major infrastructure upgrades at the Kitchener MWWTP were implemented in late 2012 that improved the quality of the effluent as well as the receiving river water (Hicks et al., 2016; Hicks et al., 2017). Thus, a potential change in fish community composition in response to the treatment upgrades was assessed by comparing the data collected in the present study with the historical data sets of Tetreault et al. (2013). To better understand variability in fish communities across spatial scales, data gathered at additional sites in rural areas of the Grand River were contrasted with data collected in the urbanized reaches.

5.3 Materials and methods

5.3.1 Description of sites

A total of 15 sites were selected across the Grand River watershed (Figure 5.1). Seven sites were located in the upper portion of the watershed where the land use is primarily agricultural. Sites 1–3 were located in Four Mile Creek, and sites 4–7 were located in the upper portion of the Grand River. The remaining eight sites were located in the central portion of the Grand River. Sites 8 and 9 were rural sites, while sites 10–15 were located in the urbanized reaches of the cities of Kitchener and Waterloo. Sites 10 and 12 were located above the Waterloo MWWTP and Kitchener MWWTP, respectively. Site 11 was below the Waterloo MWWTP and there were three sites below the Kitchener MWWTP (sites 13–15; Figure 5.1).

All sites were selected on the basis of their similarity in mesohabitats (e.g., wadeable riffles with maximum depths of 0.5 m) and their accessibility. Each site was approximately 150 to 300 m in length, with varying wetted widths (Table 5.1).

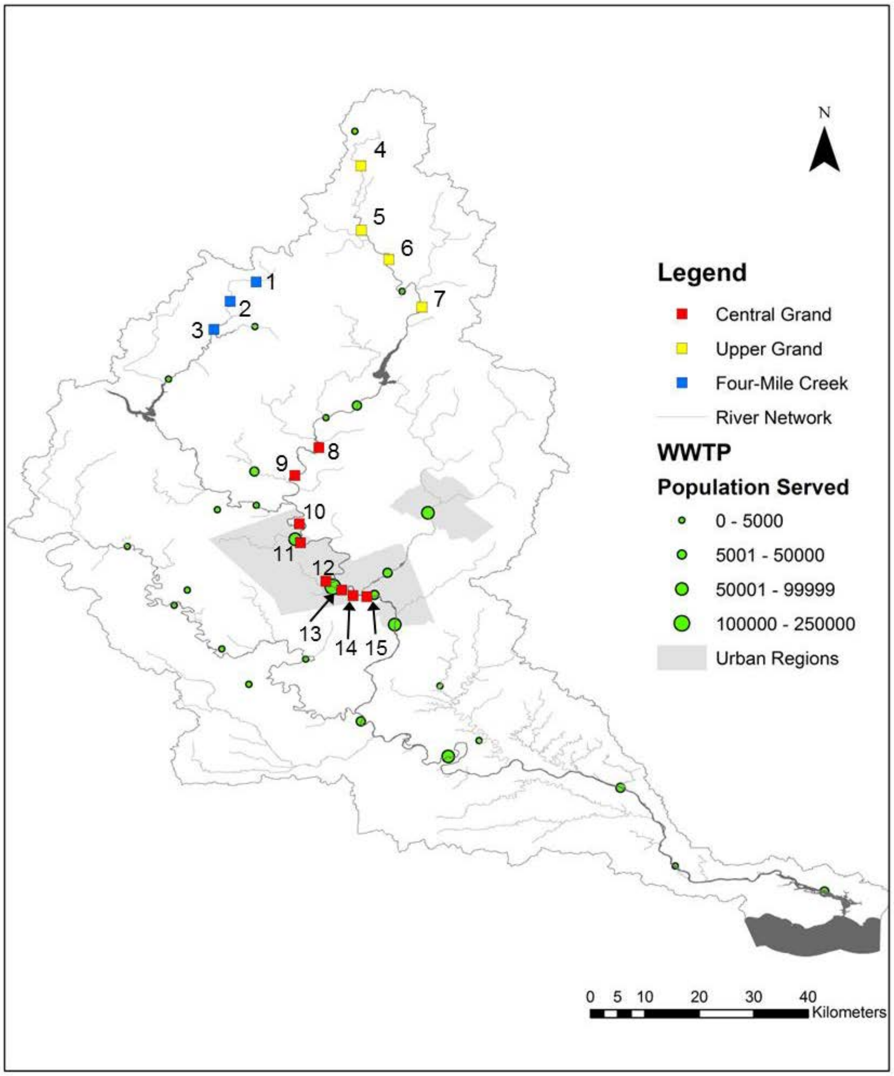

Figure 5.1 Map of sampling sites in the Grand River watershed. Sites (filled squares) were located in Four Mile Creek (blue; sites 1–3), the upper Grand River (yellow; sites 4–7), and the central Grand River (red; sites 8–15). The river network shows river orders of three or greater (Strahler system).

Table 5.1 Summary of site information including geographic description (GPS coordinates, stream order, land use), general habitat (US EPA/QHEI), specific habitat conditions, and water quality.

Site code	GPS Coordinates		Surrouding land use	Stream Order[a]	US EPA* /260	QHEI** /90	Discharge[b] (m^3/s)	Wetted[c] width (m)	Velocity[c] (m/s)	Depth[c] (m)	Geomorphology				% Algae[c]	% Plant[c]	Water chemistry				
	Latitude (N)	Longitude (W)									substrate diameter[d]	% gravel[c]	% cobble[c]	% bolder[c]			summer °C[e]	SC[cf] (µS/cm)	pH[c]	DO[c] (mg/L)	NH_4[c] (mg/L)
4 Mile Creek																					
1	43°54'15.73"	80°32'48.52"	AG	3	200	60.0	0.18	4.9	0.20	0.35	5.0	48.9	48.6	0.0	30.8	6.2	20.0	432.8	7.9	8.5	0.16
2	43°52'20.88"	80°35'23.29"	AG	3	208	67.5	0.26	7.9	0.19	0.28	8.0	47.8	49.7	0.8	30.3	0.7	21.1	421.5	8.1	9.1	0.13
3	43°49'33.87"	80°36'59.77"	AG	4	205	60.5	0.81	10.7	0.25	0.20	12.0	17.5	78.3	3.3	21.6	1.7	21.6	430.5	8.3	10.3	0.17
Upper Grand River																					
4	44° 5'47.45"	80°22'25.61"	AG	4	223	59.5	0.95	7.1	0.40	0.34	7.3	37.1	57.8	0.8	0.3	15.8	21.2	511.1	8.0	8.0	0.06
5	43°59'23.46"	80°22'23.63"	AG	4	169	61.0	0.86	11.7	0.27	0.28	8.0	31.4	61.1	0.3	9.6	1.1	21.9	494.1	7.0	8.9	0.08
6	43°56'31.10"	80°19'38.63"	AG	4	204	62.0	3.15	21.1	0.38	0.33	12.0	16.9	70.0	12.8	0.4	2.0	21.9	296.0	8.4	9.2	0.03
7	43°51'45.15"	80°16'23.12"	AG	4	216	62.5	5.49	26.5	0.26	0.38	13.0	17.8	80.9	1.2	2.3	1.6	21.7	375.4	8.3	10.7	0.04
Central Grand River																					
8	43°37'52.32"	80°26'34.30"	AG	5	222	68.5	15.27	42.4	0.52	0.23	8.0	45.3	54.7	0.0	46.1	1.6	20.2	438.6	8.5	9.2	0.03
9	43°35'9.44"	80°28'49.40"	AG	5	185	49.0	8.04	38.4	0.46	0.35	6.8	44.7	55.3	0.0	46.7	2.9	21.0	440.3	8.2	8.2	0.04
10	43°30'17.05"	80°28'30.79"	UR	6	190	62.0	13.73	58.9	0.41	0.30	6.3	68.6	31.4	0.0	43.9	11.2	21.7	484.2	8.5	10.8	0.05
11	43°28'24.69"	80°28'23.83"	UR/MWWTP	6	nd	nd	nd	64.7	0.38	0.34	nd	43.6	42.8	0.3	74.2	2.2	21.7	843.9	8.0	7.0	1.19
12	43°24'36.05"	80°25'54.70"	UR	6	202	64.5	16.64	63.4	0.46	0.34	9.0	59.4	40.6	0.0	57.5	8.3	22.4	571.8	8.2	9.4	0.08
13	43°23'45.51"	80°24'20.16"	UR/MWWTP	6	174	54.0	14.51	65.1	0.27	0.28	8.0	33.9	66.1	0.0	59.4	9.3	22.2	677.3	8.2	10.7	0.10
14	43°23'17.95"	80°23'12.39"	UR/MWWTP	6	226	69.0	18.49	120.0	0.35	0.41	6.0	39.2	61.1	0.0	37.9	51.9	22.5	664.8	8.3	8.8	0.11
15	43°23'5.64"	80°21'50.90"	UR/MWWTP	6	200	58.5	11.25	140.0	0.28	0.34	6.5	57.2	35.6	0.0	39.7	35.4	22.7	643.1	8.5	12.2	0.13

a Horton-Strahler system
b June 2013 (at low flow)
c Mean (n = 3) of three sampling events in July '13, August '13 and August '14. Each sampling event is the mean of the subsites (n = 6)
d Median substrate diameter (n = 150) derived from the median axis of substrate at the beginning, middle, and end of the sampling site
e Mean daily summer temperature from June-September (data loggers)
f SC = specific conductivity
AG = agriculture; MWWTP = municipal wastewater treatment plant; UR = urban
nd: no data
* USA Environental Protection Agency habitat assessment guidelines in the Rapid Bioassessment Protocols for Wadeable Streams
**Ohio Environmental Protection Agency Qualitative Habitat Evaluation Index

5.3.2 Fish collection and processing

Fish communities were assessed in five different sampling events (Table S5.1), which included four periods in 2013 (May, July, August, and November) and one period in 2014 (August). Additional sites were added after May 2013 and only a select few sites were sampled in November 2013 because of weather conditions (i.e., ice formation on water). Fish communities were assessed using a standardized electrofishing protocol developed by Tetreault et al. (2013). Electrofishing has been previously demonstrated to be an efficient method in wadeable streams (Poos et al., 2007). Each site was divided into 15 subsites; each subsite was100 m^2 in area. During each sampling event, six subsites (out of 15) were randomly selected for electrofishing. Species accumulation plots demonstrated that this was a suitable sample size (Figure S5.1). Each subsite was electrofished in a single pass for 300 shocking seconds using a zigzag pattern, with two netters on either side of the backpack electrofisher (Smith-Root model LR-24) moving in an upstream direction. All sampling took place in the morning (7 am – 12 pm). The electrofishing settings were standardized across all sites/seasons by adjusting the frequency and voltage to reach a maximum power output of 125 watts. The average settings were as follows: power of 95 watts (range 55–125 watts), frequency of 40 Hz (30–60 Hz), and voltage of 248 volts (160–375 volts). A constant duty cycle of 25% was maintained throughout the study.

Fish from each subsite were collected in well-aerated buckets maintained at river water temperature. In an onsite mobile laboratory (trailer), fish were identified by species, they were sexed (if possible), and their total (or fork) length (±0.1 cm) and weight (±0.01 g) were recorded before they were returned to the river. Any unidentified specimens were collected and preserved for identification with a dissecting microscope at a later date. All fish were handled in accordance with the approved University of Waterloo animal care protocol (AUPP #10-17).

5.3.3 Benthic macroinvertebrate collections

As an indicator of fish habitat and water quality, BMI communities were also collected at a subset of the sites in 2012 and 2013, and at all of the sites in 2014 (Table S5.1). Sampling took place in fall (October/November), which is the optimum time to sample BMI because of their larval stage and emergence. BMI were collected with a D-frame net (400 µm mesh) during a 3-min kick in riffle habitats (where fish had been sampled) following the Canadian Aquatic

Biomonitoring Network (CABIN) protocol (Environment and Climate Change Canada, 2012d). Samples were preserved in 95% ethanol. For analysis, BMI were subsampled from each site until there were at least 300 individuals. These individuals were then identified to the lowest taxonomic level possible. Although some individuals were identified to the genus level, some could only be identified to the family level; thus, to prevent their exclusions, all taxa were analyzed at the family level. Resolution at the family level has been considered effective (Bailey et al., 2001).

5.3.4 Habitat assessment

Several habitat parameters were assessed throughout the study (Table 5.1). Temperature data loggers (HOBO Tidbits or water temperature Pro) were installed at each of the 15 sites from June to December 2013, where water temperatures were logged every eight hours. A subset of the sites (sites 1, 3–8, and 13) also had specific conductivity (µS/cm) recorded every eight hours (HOBO water temperature Pro). To assess changes in discharge (m^3/s) across the watershed over the course of the study (2013–2014), mean daily discharge was retrieved for four of the 15 sites (sites 4, 7, 9, and 13) from the Water Survey of Canada (Environment and Climate Change Canada, 2015c) and the Grand River Conservation Authority. Mean daily discharge, water temperature, and conductivity (for the select sites and months) are provided in Supplementary Information (Figures S5.2–S5.4).

Discharge was also manually measured at each of the sites using a Swoffer model 3000 (Swoffer Instruments, Inc.) in June 2013 (low flow conditions). A general habitat assessment was also conducted in June 2013 at each of the sites using the Ohio Environmental Protection Agency (EPA)'s Qualitative Habitat Evaluation Index (QHEI; Rankin, 2006) and the USA EPA's habitat assessment guidelines in the Rapid Bioassessment Protocols for Wadeable Streams (Barbour et al., 1999). In addition, median substrate diameter (using the median axis) was recorded (June 2013) based on a 150 pebble count throughout the beginning, middle, and end of the site. During each of the fish community sampling events, a habitat assessment (including depth and velocity) was completed for each of the six selected subsites. The % of dominant substrates, % algae cover (filamentous/brown), and % of aquatic macrophytes were recorded by visual observation. Water quality parameters at each of the subsites were also recorded using an YSI professional plus handheld multi meter (YSI, Inc.); these included pH, specific conductivity (µS/cm), dissolved oxygen (mg/L), temperature (°C), and total ammonia (mg/L).

5.3.5 Data analysis and statistics

The main comparisons of fish community data were based on the summer collections because the summer sampling events had lower water flows (Figure S5.2), higher abundances of fish (Table S5.2), and lower within-site variability (homogeneity of multivariate dispersion [Anderson, 2006], Figures S5.5 and S5.6). In addition, historical data were available for summer collections in 2007 and 2008 (Tetreault et al., 2013). The analysis of the data was divided into two parts: 1) detecting changes in fish and BMI communities at sites associated with MWWTPs in the central Grand River (sites 8–15) using current and historic data sets, and 2) detecting changes in fish and BMI communities across larger spatial scales (sites 1–15).

5.3.5.1 Fish and BMI classification and univariate indices

Fish and BMI communities were primarily analyzed with multivariate statistics; however, some univariate matrices and other descriptive parameters were assessed. Each fish species caught was characterized on the basis of tolerance (ability to adapt to disturbances/stress; Eakins, 2016); resilience (ability to withstand exploitation – double time based on fecundity and age to maturation; Froese and Pauly, 2016); and vulnerability (predisposition to predation/catchability; Froese and Pauly, 2016). Fish were also classified on the basis of their diet (Eakins, 2016; Froese and Pauly, 2016). Fish classifications are provided in Table S5.2. Fish communities were also described by abundance (catch per unit effort), species richness, and Simpson's diversity index. Indices calculated for BMI communities included %EPT (Ephemeroptera, Plecoptera, and Trichoptera), %chironomidae, and the Hilsenhoff biotic index (Hilsenhoff, 1987; Merritt and Cummins, 2008). BMI communities were also characterized by their composition of functional feeding groups (grazers, collectors, shredders, and predators).

5.3.5.2 Data treatment for multivariate analyses

For multivariate analyses (including visual representation and statistics) all species abundance data (fish and BMI) were square-root transformed on the basis of shade plots (Clarke et al., 2014). Bray-Curtis similarity was the chosen resemblance matrix for all multivariate analyses on species abundance data. Environmental parameters used to explain patterns in fish and BMI communities (discussed below) were assessed for normality using scatterplots (draftsman plots). Variables including %gravel, %cobble, %boulder, %algae, and %plant were highly skewed, thus they were fourth-root transformed while the remaining variables were left untransformed. After transformation, these data were normalized (z-scores) before further analysis (discussed below).

5.3.5.3 Detecting changes in fish communities and BMI below MWWTPs (sites 8–15)

Significant differences in fish community composition across regions, sites (within region), and sampling events were tested with a nested three-factor permutational multivariate analysis of variance (PERMANOVA; Anderson, 2001) with the historical and current data sets combined (with overlapping sites only; sites 9–14). Subsite was the lowest level of replication (n = 6). F or the first factor region (fixed), the central Grand River was divided into three spatial regions, which were the upper, middle, and lower parts, where each region is separated from the others by several kilometres (Figure 5.1). The second factor was site (fixed; nested within region) and included site 9 (upper region), sites 10 and 11 (middle region), and sites 12–14 (lower region). The third factor was sampling event (fixed; including events from 2007, 2008, 2013 [July and August], and 2014). The analysis used type III (partial) sums of squares and p values were obtained using 9999 permutations under a reduced model. This test was designed in this manner because 1) it allowed for the comparisons between regions, to account for any changes along the spatial gradient; 2) it tested for changes between sites, including sites above and below a MWWTP within their respective region; and 3) it tested for any changes between regions or sites (within region) over multiple sampling events (e.g., changes before and after the Kitchener MWWTP upgrades). Since there was a significant interaction with the main effects (site (region) x sampling event), pairwise comparisons were computed for each site x site combination (within region) by sampling event. This included the comparison of upstream verses downstream sites within the middle region (comparison of sites upstream and downstream of the Waterloo MWWTP) and lower region (sites above and below the Kitchener MWWTP). In addition, pairwise comparisons were computed for pairs of sampling events for each site.

An nMDS ordination plot on centroids (n = 6) was used to visualize the changes in fish communities across sites and to support the PERMANOVA model. All central Grand River sites

(sites 8–15) were included in the nMDS plot regardless of any overlap between sites in the historical and current data sets. Vectors (represented by species) that correlated with the nMDS axes were also included to determine which species were the main ones driving the dissimilarities. Those species that correlated highly with the nMDS axes (i.e., Pearson correlation coefficients (r) $\geq$ 0.5) were further assessed with nMDS bubble plots, to better visualize the change in relative abundance of those species across sites and years.

Changes in BMI were not assessed with PERMANOVA because of the lack of replication. Patterns in BMI in the central Grand River were assessed with an nMDS ordination plot. Bubble plots were also constructed with taxa that correlated highly with the nMDS axis (r > 0.6) to visualize the change in relative abundance of those taxa driving dissimilarities between sites.

5.3.5.4 Patterns in fish and BMI communities at a larger spatial scale

Fish community data collected in the summers of 2013 and 2014 (July 2013, August 2013, and August 2014) were used to assess patterns in fish communities across multiple regions of the Grand River watershed (4 Mile Creek, upper Grand River, and central Grand River). This assessment included both univariate indices (abundance, richness, and diversity) and multivariate (nMDS ordination) approaches. To link the composition of fish communities to environmental variables, the BIOENV procedure was used (Clarke and Ainsworth, 1993). This calculates the Spearman rank correlations between the fish community similarity matrix and subsets of spatial and environmental variables to define the variable or combination of variables that best explain the community matrix. For the analysis, fish community data were averaged over the three summer sampling events and Bray-Curtis was chosen as the similarity matrix. All environmental and spatial (e.g., latitude) variables from Table 5.1 were included in the analysis except for the USEPA/QHEI generic habitat assessments and total discharge since these data were not available for all sites. Two BMI indices were also included in the analysis: %EPT and %chironomidae. Finally, all variables were first screened for collinearity using draftsman plots (i.e., scatterplots of all pairwise comparisons). Wetted width, stream order, and latitude were highly correlated (Pearson correlation coefficients, r > 0.9); thus, only latitude was retained in the analysis to represent all three as a spatial variable. The BIOENV procedure was also assessed with the BMI community data set from 2014 using the same environmental variables (except %EPT and

%chironomidae). All multivariate analyses were completed using PRIMER (and PERMANOVA^{+} software) Version 7.

5.4 Results and discussion

5.4.1 Detecting impacts of MWWTP effluents on fish and BMI communities

Differences in fish community composition were detected in the central Grand River over the four-year period (Figure 5.2). The dissimilarities between the sites upstream and downstream of MWWTPs were evident and consistent between both historical and current data sets. These changes are supported by the PERMANOVA model (Table 5.2) that revealed not only spatial differences (i.e., between the regions) but also differences between sites within regions. The degree of differences between upstream and downstream sites (within the regions) was dependent on the sampling event (Table 5.2; interaction between site (region) x sampling event). Pairwise comparisons within most years revealed that fish communities below the Waterloo and Kitchener MWWTPs were consistently different from those in the associated upstream sites (Table 5.3). The degree of difference between upstream and downstream sites in the lower portion of the Grand River (e.g., near the Kitchener MWWTP) was usually greater in the historical data sets than in the current data set (i.e., greater t-values, Table 5.3). The summer of 2014 was the only year where no differences were observed between the sites immediately downstream and upstream of the Kitchener MWWTP (site 12 vs. site 13) (Table 5.3), which was two years after the Kitchener MWWTP upgrades. The smaller degree of change (or lack of difference altogether) between these sites may suggest a possible response to the treatment plant upgrades.

Fish communities demonstrated high temporal variability. This is illustrated with the pairwise comparisons between sampling events at individual sites (Table 5.4). It was often difficult to replicate the same results for the same site, and this was particularly true when comparing historical and current data sets. It is very evident in Figure 5.2 that the historical and current data sets differ at the upstream (reference) sites as the data sets group differently. This finding may represent a real difference between the years at the upstream sites, or it may indicate that different personnel have difficulty replicating similar habitats at individual sites.

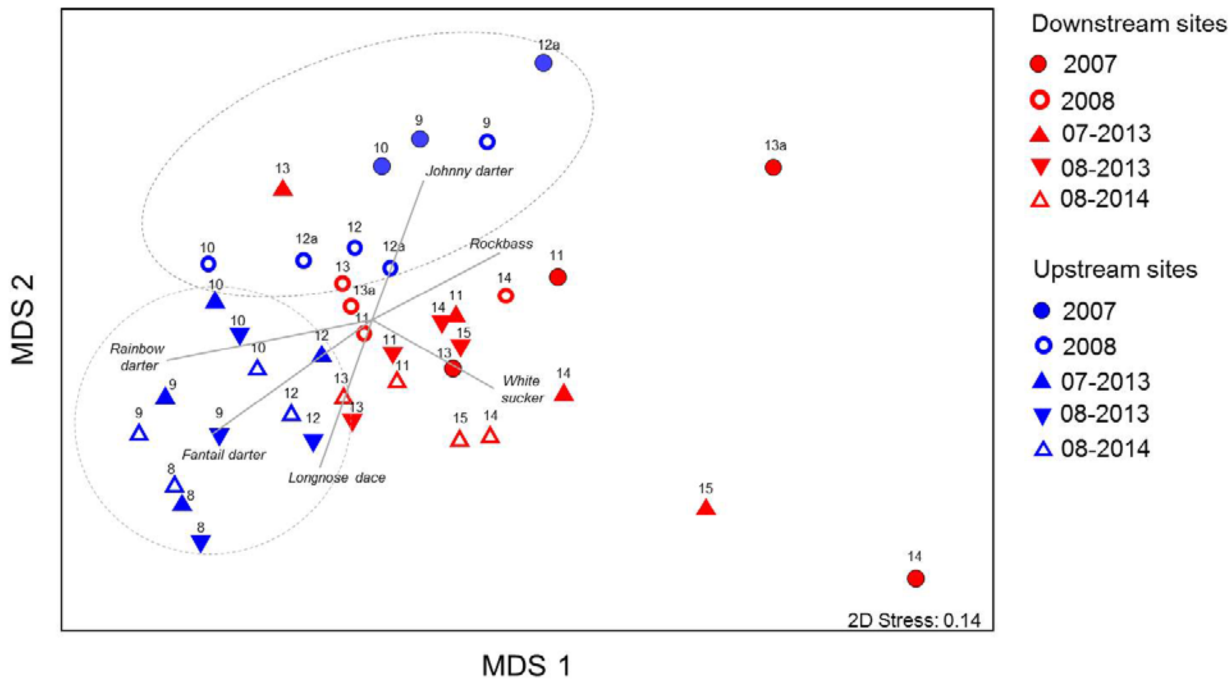

Figure 5.2 Non-metric multidimensional scaling ordination plot on a Bray-Curtis similarity for fish community species abundance data (square-root transformed) in the central Grand River for the summers of 2007, 2008, 2013, and 2014. Each point represents a site centroid (*n* = 6 subsites). Numbers represent the site codes (8–15). A letter "a" beside a number code indicates a different site (from the historical data set) sampled in close proximity to the associated code. The vectors are Pearson correlations of individual fish species with the axes (MDS 1 and MDS 2) where $r \geq 0.5$. The grey circles highlight the separation in upstream (reference) sites between 2007/2008 and 2013/2014.

Table 5.2 PERMANOVA results for the analysis of fish community data (including historical and current data sets) with the main effects of region, site (region), and sampling event.

Source	df	MS	Pseudo-*F*	*p*	% variation*	Sqrt (component of variation)**
Region	2	10,450	11.92	0.0001	8.2	13
Sampling event	4	9,449	10.78	0.0001	13.5	17
Site (region)	3	10,813	12.34	0.0001	16.1	18
Region X Sampling event	8	3,745	4.27	0.0001	12.3	16
Site (region) X Sampling event	11	2,187	2.50	0.0001	9.9	14
Residuals	145	876			39.9	29
Total	173					

*Percentage of total variation attributed by the different sources in the model

**Variation in Bray-Curtis units

Table 5.3 PERMANOVA pairwise comparisons for pairs of sites within regions by sampling event.

Region	Sampling event	Pairs of sites	*t* value	*p*
Mid	2007	10 vs 11*	2.68	0.0022
Mid	2008	10 vs 11*	1.98	0.0076
Mid	July 2013	10 vs 11*	2.88	0.0026
Mid	August 2013	10 vs 11*	1.86	0.016
Mid	August 2014	10 vs 11*	1.77	0.011
Low	2007	13* vs 14*	2.49	0.001
Low	2008	12 vs 13*	2.407	0.005
Low	2008	12 vs 14*	2.386	0.0026
Low	2008	13 vs 14*	2.457	0.0023
Low	July 2013	12 vs 13*	1.83	0.0065
Low	July 2013	12 vs 14*	1.671	0.0131
Low	July 2013	13* vs 14*	2.003	0.014
Low	August 2013	12 vs 13*	2.016	0.005
Low	August 2013	12 vs 14*	1.463	0.015
Low	August 2013	13 vs 14*	1.616	0.0033
Low	August 2014	12 vs 13*	1.2377	0.1585
Low	August 2014	12 vs 14*	2.132	0.0021
Low	August 2014	13* vs 14*	2.226	0.0019

* indicates a site below a MWWTP

Table 5.4 PERMANOVA pairwise comparisons for pairs of sampling events within sites

Region	Site	Pairs of sampling events	*t*	*p*
Upper	9	07, 08	1.8965	0.002
Upper	9	07, 7-13	4.2471	0.0017
Upper	9	07, 8-13	3.8711	0.0024
Upper	9	07, 8-14	3.8106	0.0028
Upper	9	08, 7-13	3.2661	0.0027
Upper	9	08, 8-13	3.0141	0.0025
Upper	9	08, 8-14	2.9715	0.0021
Upper	9	7-13, 8-13	1.0549	0.3281
Upper	9	7-13, 8-14	1.3672	0.1057
Upper	9	8-13, 8-14	1.3618	0.1094
Mid	10	07, 08	2.942	0.0018
Mid	10	07, 7-13	3.0715	0.0028
Mid	10	07, 8-13	2.7337	0.0027
Mid	10	07, 8-14	2.549	0.0069
Mid	10	08, 7-13	1.9451	0.0068
Mid	10	08, 8-13	2.3394	0.0081
Mid	10	08, 8-14	2.7505	0.0046
Mid	10	7-13, 8-13	1.1066	0.3089
Mid	10	7-13, 8-14	1.5942	0.0386
Mid	10	8-13, 8-14	0.95293	0.5057
Mid	11*	07, 08	2.0711	0.006
Mid	11*	07, 7-13	1.307	0.1351
Mid	11*	07, 8-13	1.6058	0.0451
Mid	11*	07, 8-14	1.5274	0.0486
Mid	11*	08, 7-13	1.6199	0.0391
Mid	11*	08, 8-13	1.2967	0.1498
Mid	11*	08, 8-14	1.4254	0.106
Mid	11*	7-13, 8-13	1.2939	0.153
Mid	11*	7-13, 8-14	1.3812	0.0994
Mid	11*	8-13, 8-14	1.2827	0.1355
Low	12	08, 7-13	2.0083	0.008
Low	12	08, 8-13	2.5765	0.0022
Low	12	08, 8-14	2.5842	0.0053
Low	12	7-13, 8-13	1.1665	0.2552
Low	12	7-13, 8-14	1.3112	0.1439
Low	12	8-13, 8-14	1.6261	0.0202
Low	13*	07, 08	2.0273	0.008
Low	13*	07, 7-13	2.2341	0.0022
Low	13*	07, 8-13	1.7782	0.0234
Low	13*	07, 8-14	1.768	0.0035
Low	13*	08, 7-13	1.713	0.0344
Low	13*	08, 8-13	3.1451	0.0023
Low	13*	08, 8-14	2.9133	0.0027
Low	13*	7-13, 8-13	2.5763	0.0026
Low	13*	7-13, 8-14	2.2887	0.0018
Low	13*	8-13, 8-14	2.0514	0.0035
Low	14*	07, 08	2.4172	0.0023
Low	14*	07, 7-13	2.034	0.002
Low	14*	07, 8-13	2.3361	0.0028
Low	14*	07, 8-14	2.5511	0.0022
Low	14*	08, 7-13	1.4864	0.1088
Low	14*	08, 8-13	0.98837	0.5202
Low	14*	08, 8-14	2.6289	0.0023
Low	14*	7-13, 8-13	0.82629	0.6562
Low	14*	7-13, 8-14	1.8683	0.0023
Low	14*	8-13, 8-14	1.4763	0.0053

* indicates a site below a MWWTP

* indicates a site below a MWWTP

Dissimilarities in fish communities across the central Grand River were primarily driven by six species (Figure 5.3) that range in their level of tolerance to pollution (Table S5.2). They were *Etheostoma flabellare* (fantail darter), *Catostomus commersoni* (white sucker), *Etheostoma caeruleum* (rainbow darter), *Etheostoma nigrum* (Johnny darter), *Rhinichthys cataractae* (longnose dace), and *Ambloplites rupestris* (rockbass). It is evident from the bubble plots (Figure 5.3) that at downstream sites, fantail darter, classified as intolerant, decreased in abundance. In some years, there was also a decrease at downstream sites in the abundance of the rainbow darter, another species classified as intolerant. In contrast, white sucker (an omnivore classified as tolerant) increased in abundance at downstream sites. *Cyprinus carpio* (common carp), which is also a pollution-tolerant species, were commonly associated with white sucker and downstream sites (see shade plot, Figure S5.7). The increase in omnivores and tolerant species is consistent with other studies that have assessed fish communities below MWWTP outfalls (Ra et al., 2007; Yeom et al., 2007).

Fish communities collected in 2007 at sites below the Kitchener MWWTP differed the most from those collected in any other year (Figure 5.2). This difference was probably driven by the low abundance of darter species (Figure 5.3). It is hypothesized that this change is due to the MWWTP effluent coupled with lower flows in 2007 (i.e., low dilution). The year 2007 was an extremely dry year, with a median summer discharge (above the Kitchener MWWTP outfall) of 9 m^3/s, compared with 16–22 m^3/s in 2008 and 2013–2014 (Chapter 2, Figure 2.6). The median dissolved oxygen (DO) was also lower in 2007 than in the other years, with daily mean DO falling more frequently below 4 mg/L (Chapter 3, Figure S3.3), the provincial water quality objective for Ontario (Loomer and Cooke, 2011). The data set from summer 2007 highlights the importance of understanding other environmental factors (e.g., low flow) and how they may exacerbate (or mitigate) the potential effects observed from the MWWTP outfalls.

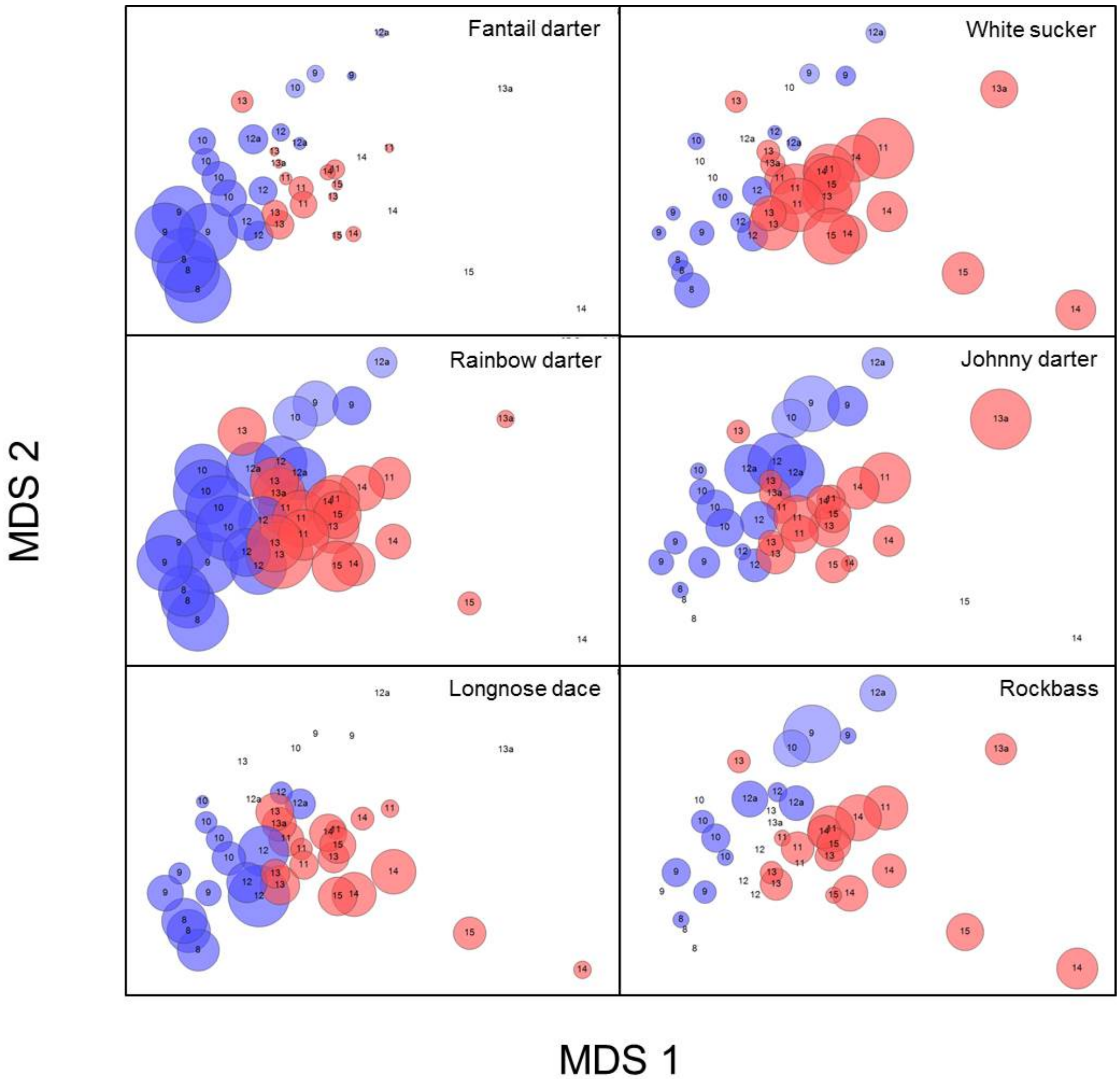

Figure 5.3 Non-metric multidimensional scaling ordination plot on a Bray-Curtis similarity for fish community species abundance data (square-root transformed) in the central Grand River for the summers of 2007, 2008, 2013, and 2014. Each plot is the same ordination (and the same as Figure 2) but is illustrated with a bubble plot for different species that correlated highly with the MDS axes. The size of each circle is proportional to the relative abundance of the species. Red symbols represent downstream sites and blue symbols represent upstream sites.

The patterns observed in benthic macroinvertebrates (BMI) mirror those observed in fish communities. The dissimilarities between sites upstream and downstream of the MWWTPs were most evident in 2012 (Figure 5.4), which was also a very dry year (similar to 2007), and the Kitchener MWWTP was still discharging poor-quality effluent for most of that year. Eight taxa were primarily responsible for driving the dissimilarities between upstream and downstream sites (Figure 5.5). Sites located below both MWWTPs (Kitchener and Waterloo) had lower abundances of pollution-intolerant taxa (e.g., Baetidae, Heptageniidae, Ephemerellidae, and Elmidae) and higher abundances of pollution-tolerant taxa (e.g., Simuliidae, Asellidae, Chironomidae, and Oligchaeta; Figure 5.5). The greater change observed in 2012 is further illustrated with the lower percentages of EPT taxa (Figure 5.6) and high Hilsenhoff biotic index scores, which classified the water quality as fairly poor (sites 13 and 14) to poor (site 11) on the basis of BMI composition (Table S5.3; Hilsenhoff, 1987). These changes observed in BMI were supported by Gillis et al. (2017) who found reduced abundances of sensitive mussel taxa below the MWWTPs in the central Grand River in both 2012 and 2014. The general patterns in BMI in this study are consistent with organic and nutrient enrichment in river systems (Kosmala et al., 1999; Rueda et al., 2002; Gücker et al., 2006; Robinson et al., 2016).

Detecting a response in BMI to the Kitchener MWWTP upgrades was confounded by the higher flows in the post-upgrade years (2013 and 2014). BMI communities at sites below the Kitchener MWWTP were more similar to those at the upstream sites in 2013 and 2014 (Figure 5.5). This was also illustrated with higher abundances of EPA taxa (compared with 2012; Figure 5.6), and lower Hilsenhoff biotic index scores with water quality classified as either good or fair (Table S5.3). This may indicate a possible recovery in response to either wetter conditions (Chapter 2, Figure 2.6) and/or better effluent quality due to the Kitchener MWWTP upgrades that took place in August 2012. Despite higher flows, the site below the Waterloo MWWTP still had relatively low abundances of EPT and high abundances of chironomidae (tolerant taxa) in 2014 compared with other sites, indicating that this site may still be impacted.

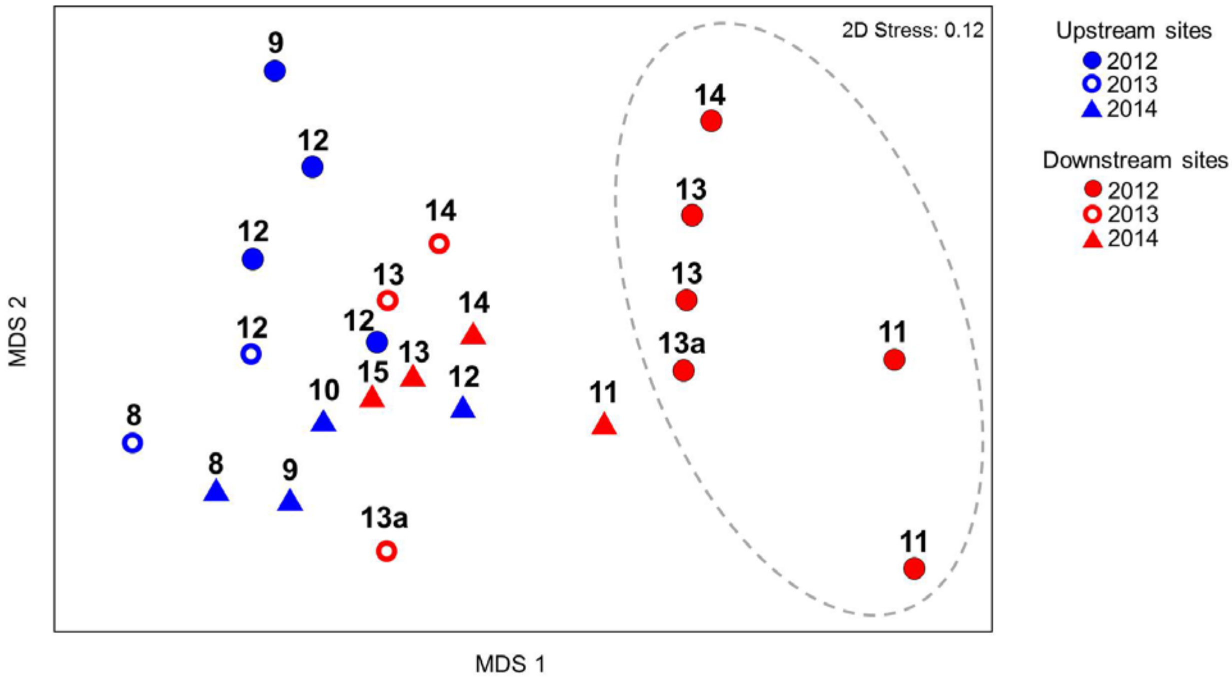

Figure 5.4 Non-metric multidimensional scaling ordination plot on a Bray-Curtis similarity for benthic invertebrate species abundance data (square-root transformed) in the central Grand River for fall sampling events in 2012–2014. Numbers represent site code. A letter “a” beside a number code indicates a different site (from the historical data set) sampled in close proximity to the associated code. The grey circle highlights the grouping of downstream sites in 2012.

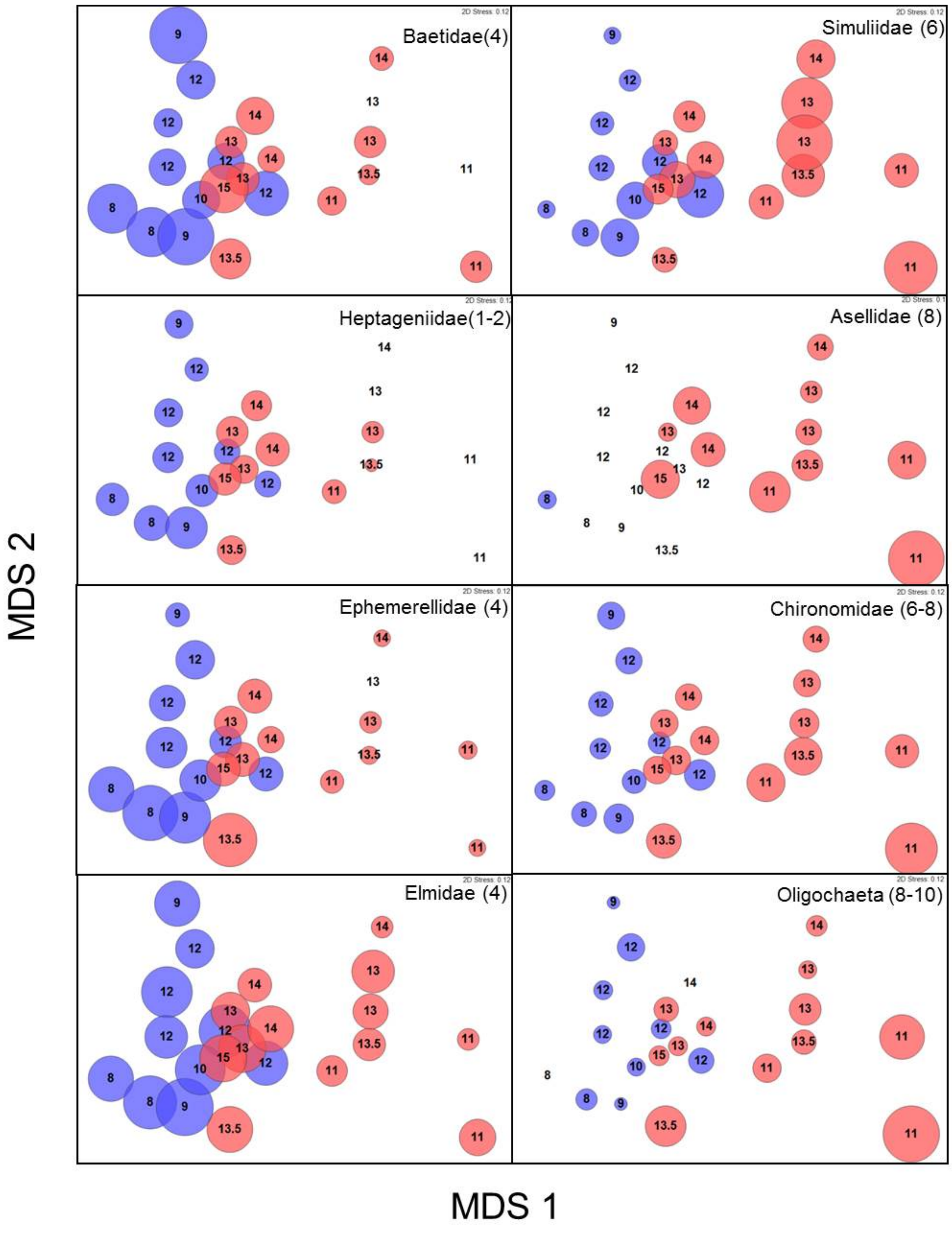

Figure 5.5 Non-metric multidimensional scaling ordination plot on a Bray-Curtis similarity for benthic invertebrate species abundance data (square-root transformed) in the central Grand River for falls 2012–2014. Each plot is the same ordination (and the same as Figure 4) but is illustrated with a bubble plot for different species that correlated with the 1st MDS axis. The size of the bubble (circle) is proportional to the relative abundance of the species. Species on the left are all more intolerant to pollution (HBI 1–4) and are negatively correlated with MDS1. Species on the right are more tolerant to pollution (HBI 6–10) and are positively correlated with the bottom axis. HBI = Hilsenhoff biotic index.

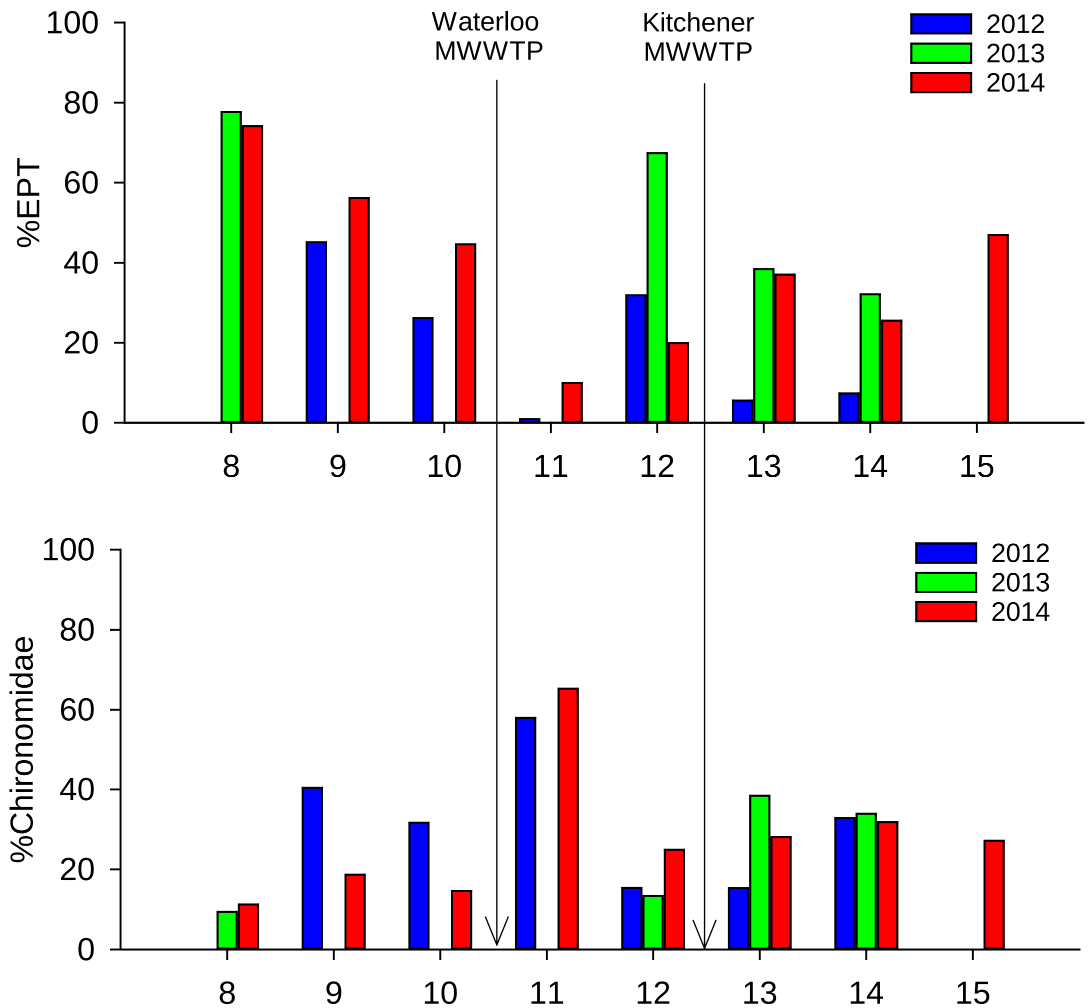

Figure 5.6 Composition of benthic invertebrate communities by %EPT (top graph) and %chironomidae (bottom graph) for samples collected in the falls of 2012–2014 at sites in the central Grand River. Sites are arranged from upstream (site 8) to downstream (site 15) and arrows indicate the input of the Waterloo and Kitchener MWWTPs.

This study highlights how communities may respond differently to contaminants under different environmental conditions (Clements et al., 2012). The degree of change detected in both fish and BMI communities because of MWWTP effluents depended on the year the communities were sampled and it was hypothesized that annual river discharge (i.e., the dilution of MWWTP effluent) probably influenced the degree of impact. This study also emphasizes the importance of collecting multi-year data, as the conclusions may differ from year to year (or season to season) depending on the environmental conditions. As the frequency of extreme weather conditions (e.g., droughts) is predicted to increase (Meehl and Tebaldi, 2004) the risk of MWWTP effluents to fish and BMI communities may increase. To better understand the effectiveness of the Kitchener facility upgrades in mitigating impacts, future studies should assess fish and BMI communities under extreme low flow conditions (such as those experienced in 2007 and 2012).

5.4.2 Changes in fish and BMI communities across larger spatial scales

It is difficult to attribute a change in fish communities to local environmental conditions (e.g., water quality; MWWTP) in a river system that is constantly changing at many different spatial scales (from the watershed scale, to the reach scale, to microhabitats; Frissell et al., 1986), including one-direction (e.g., latitudinal) changes in biotic and abiotic processes (Vannote et al., 1980). To better understand these types of changes in fish and BMI communities in the Grand River watershed, additional sites in rural parts of the watershed were added for comparison with sites in the urbanized reaches.

Spatial factors were the main determinant in explaining fish and BMI community composition across the three regions in the Grand River watershed (4 Mile Creek, upper Grand River, and central Grand River; Figure 5.7 and Figure 5.8). Sites located in close proximity were more similar and thus grouped closer together. Latitude, which was highly correlated with wetted width and stream order ($r > 0.9$), was the single best variable at explaining the patterns in the fish ($r = 0.64$; Table 5.5) and BMI communities ($r = 0.80$; Table 5.5). With the addition of other environmental variables, the models only slightly improved, with those variables usually being autocorrelated to some extent with latitude ($r = 0.50–0.68$). It is important to note that the watershed drains north to south; thus, several variables that correlated with latitude are those that are associated with natural river gradients. The fact that the composition of fish and BMI communities was associated with spatial variables is not unusual and the strength of this association often depends on the spatial extent of the study (Mykrä et al., 2007; Sály et al., 2011; Nakagawa, 2014). Only at much smaller scales (e.g., 100s of metres) do environmental variables become more important in describing community composition (Mykrä et al., 2007; Nakagawa, 2014).

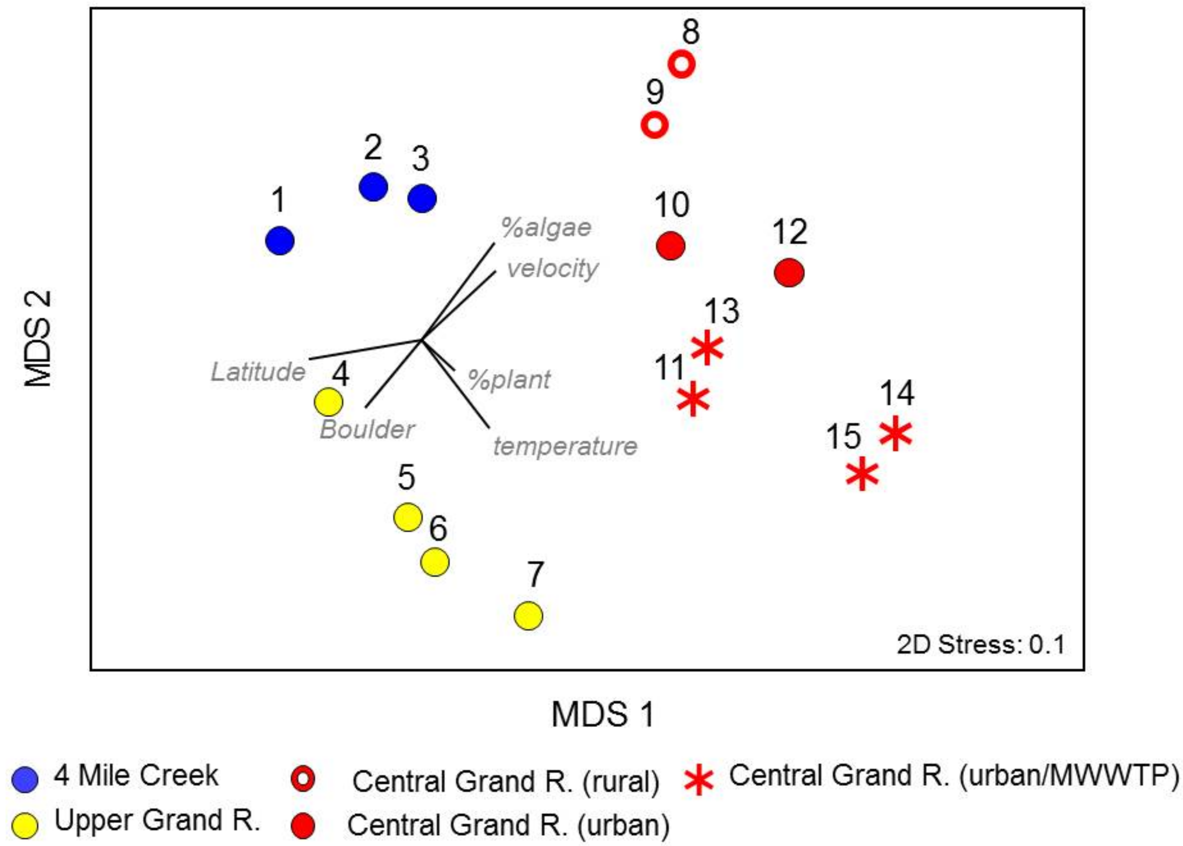

Figure 5.7 Non-metric multidimensional scaling ordination plot on a Bray-Curtis similarity for fish community species abundance data (square-root transformed). Each point represents the mean of three sampling events (July 2013, August 2013, and August 2014). The six variables in grey together best explain the patterns in fish communities (r = 0.70).

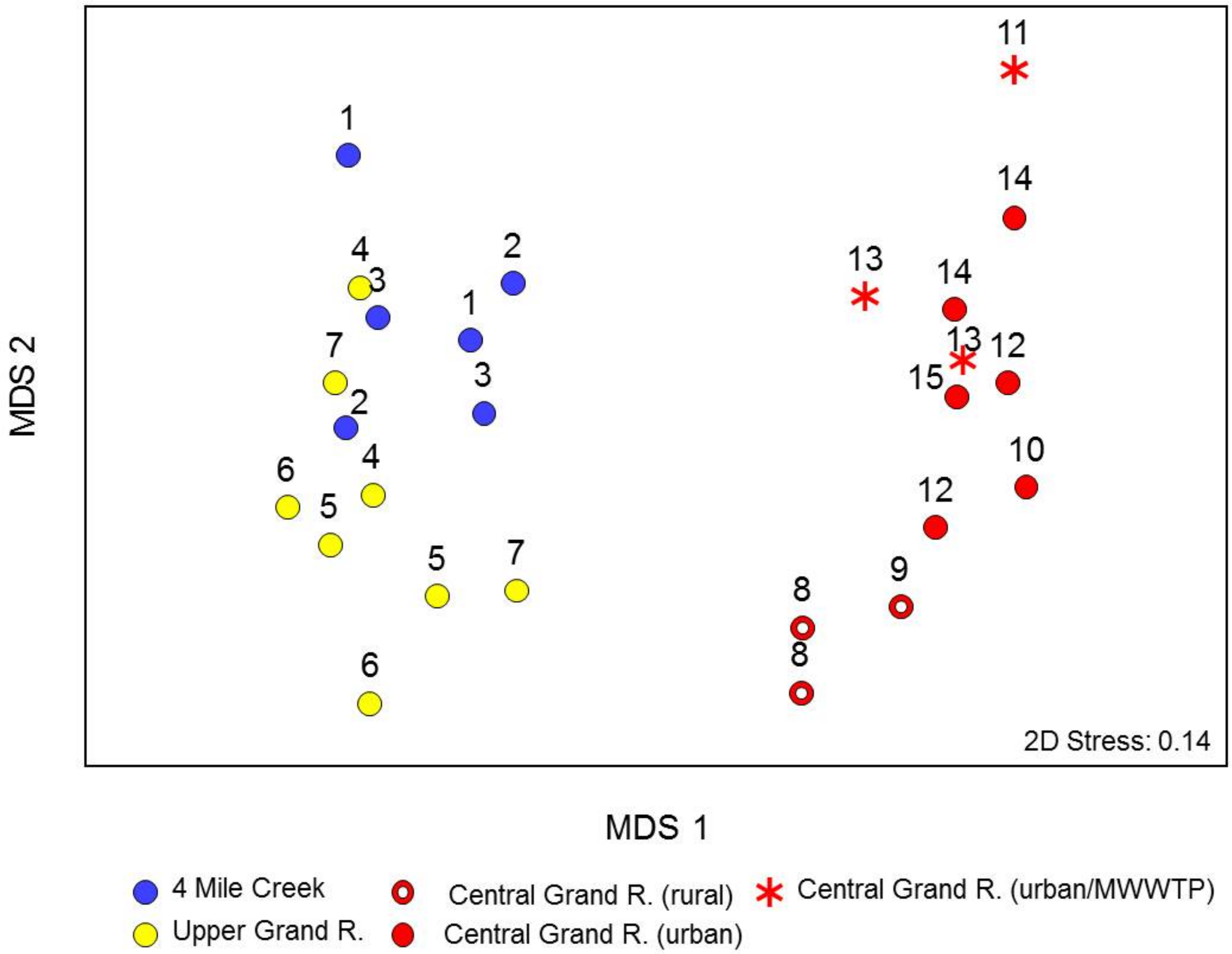

Figure 5.8 Non-metric multidimensional scaling ordination plot on a Bray-Curtis similarity for benthic macroinvertebrate species abundance data (square-root transformed) collected in fall 2013 and 2014. Each point is a single sampling event (3-min kick).

Table 5.5 Results from the BIOENV procedure for assessing the best combination of variables that most explain the patterns observed in fish and BMI communities. The analysis was completed on all sites (sites 1–15). Only the combinations of variables that reached the highest rank correlation are provided.

Number of variables	Spearman correlation	Variable(s)
Fish		
1	0.64	Latitude
2	0.68	Latitude, temp
3	0.67	Latitude, temp, %algae
4	0.69	Latitude, temp, %algae, %plant
5	0.69	Latitude, temp, %algae, %plant; velocity
6	0.70 ($p < 0.01$)	Latitude, temp, %algae, %plant; velocity, %bolder
BMI		
1	0.80	Latitude
2	0.78	Latitude, conductivity
3	0.81	Latitude, conductivity, %bolder
4	0.82 ($p < 0.01$)	Latitude, conductivity, %bolder, velocity

Table 5.6 Results from the BIOENV procedure for assessing the best combination of variables that most explain the patterns observed in fish and BMI communities. The analysis was completed on sites located in the central Grand River (sites 8–15). Only the combinations of variables that reached the highest rank correlation are provided.

Number of variables	Spearman correlation	Variable(s)
Fish		
1	0.71	Latitude
2	0.69	Temp, conductivity
3	0.75	Temp, conductivity, %plant
4	0.76 ($p < 0.01$)	Latitude, temp, %plant, %EPT
BMI		
1	0.81	NH4
2	0.85	NH4, temp
3	0.88	NH4, temp, pH
4	0.89 ($p < 0.01$)	NH4, temp, pH, latitude

Even within the central Grand River, there is a clear spatial gradient in fish community composition (Figure 5.7; Table 5.6). This makes it difficult to associate any impacts from MWWTPs with changes in fish communities. In contrast, patterns in BMI assessed at the smaller scale (i.e., the central Grand River) are associated with local environmental conditions (i.e., water quality conditions; Table 5.6). This is probably because there was a large enough change detected in BMI below the Waterloo MWWTP, which was associated with poor water quality (i.e., using ammonia as an indicator of water quality). This provides further evidence that changes in BMI below the Waterloo MWWTP (in 2014) are probably associated with the local water quality driven by MWWTP effluent. This demonstrates the use of BMI communities as a more sensitive indicator of the effects of MWWTP effluents than fish communities.

Detecting changes in fish and BMI communities across the larger spatial gradients using univariate metrics also proved useful. Replicable patterns in fish communities across the different parts of the watershed were evident with the measures of abundance (catch per unit effort), species richness, and diversity (Figure 5.9). Species richness was usually higher in the smaller order streams (order 3–4) in the rural parts of the watershed (sites 1–4) than in the higher order (order 5 or 6) central Grand River sites (sites 8–15). Species diversity and abundances were also changing across the sites. The most notable change in all measures appeared below the dam (Figure 5.9). This is a bottom-draw hydroelectric dam that creates a 12 km reservoir, interrupting the river continuum (Ward and Stanford, 1995). This dam creates downstream changes in DO and the reservoir acts as a source of many nutrients (De Baets, 2016). In addition, the bottom-draw dam releases cold water from the hypolimnion of the reservoir thus changing the natural thermal profile of the river. This is especially evident in the summer months, and the phenomenon is particularly influential in dry years (De Baets, 2016). In the summer of 2013, there was up to a 2.5°C change in the mean summer temperature between site 8 and site 15 (Table 5.1). Changes in water quality, flows, and other physiochemical conditions associated with the dam have undoubtedly created biological changes throughout the food web (McCartney, 2009). These changes, whether beneficial or detrimental, are probably responsible for some of the differences observed in the fish communities in the Grand River.

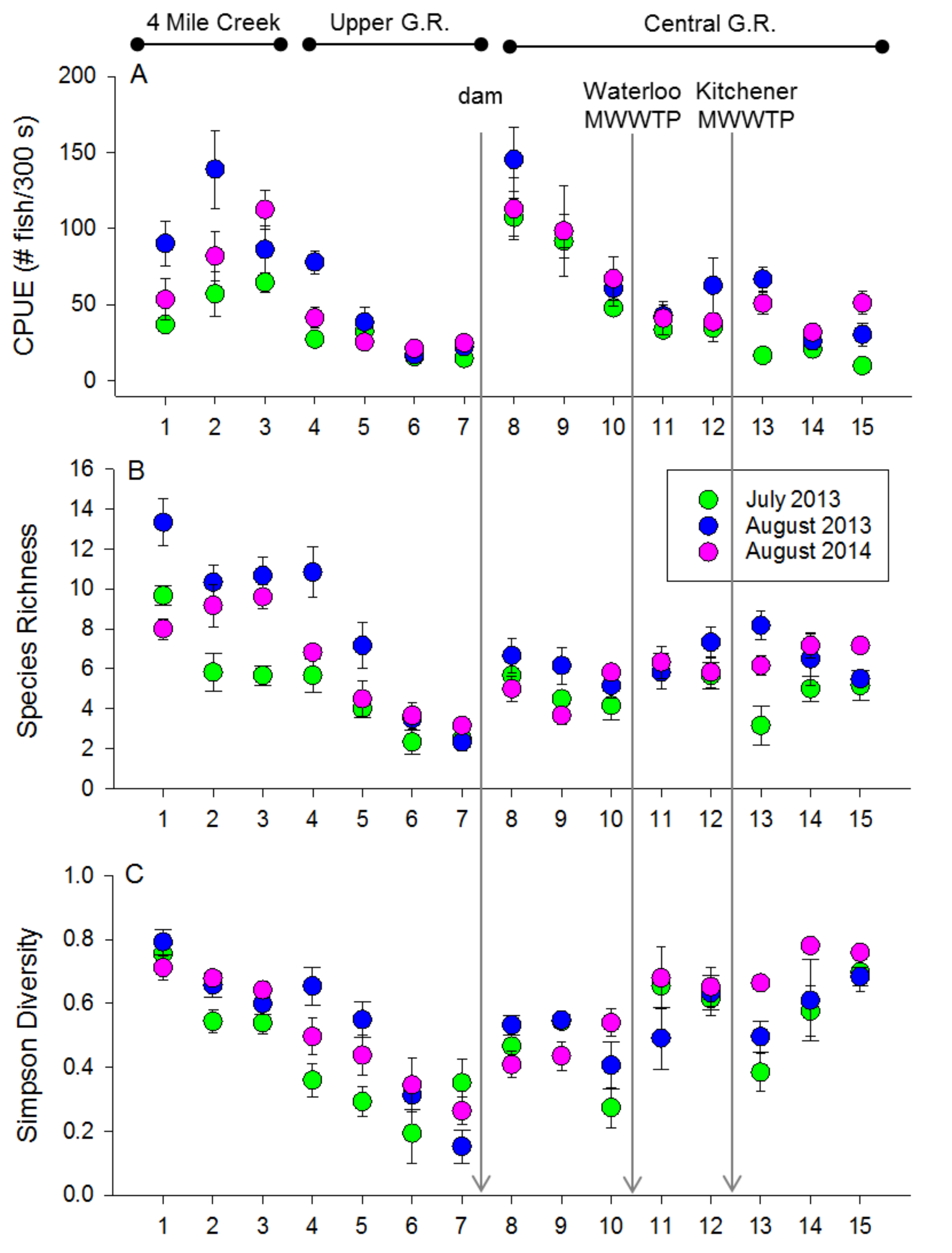

Figure 5.9 Fish community matrices including (A) catch per unit effort (CPUE; #fish/300 s), (B) species richness, and (C) Simpson's diversity index for Four Mile Creek (sites 1–3), the upper Grand River (sites 4–7), and the central Grand River (sites 8–15). Data are provided for the three summer sampling events, July 2013 (green), August 2013 (blue), and August 2014 (pink).

Changes in the composition of BMI functional feeding groups across the different regions of the watershed were evident and are probably influenced by both natural and anthropogenic factors (Figure 5.10). It has already been mentioned that changes in BMI across the larger spatial scale are primarily explained by watershed gradients (i.e., latitudinal changes). The dam may partly explain these spatial changes. When functional feeding groups were examined, the most obvious change was the increased abundances of collectors (gatherers/filter feeders) and decreased abundances of grazers in the central Grand River, beginning just after the dam (site 8). The increase in collectors below the dam is probably explained by the increase in fine particular organic matter often found below a dam, which changes the functional feeding group composition (Ward and Stanford, 1983). Natural processes are also probably contributing to the changes in functional feeding groups (e.g., the river continuum; Vannote et al., 1980).

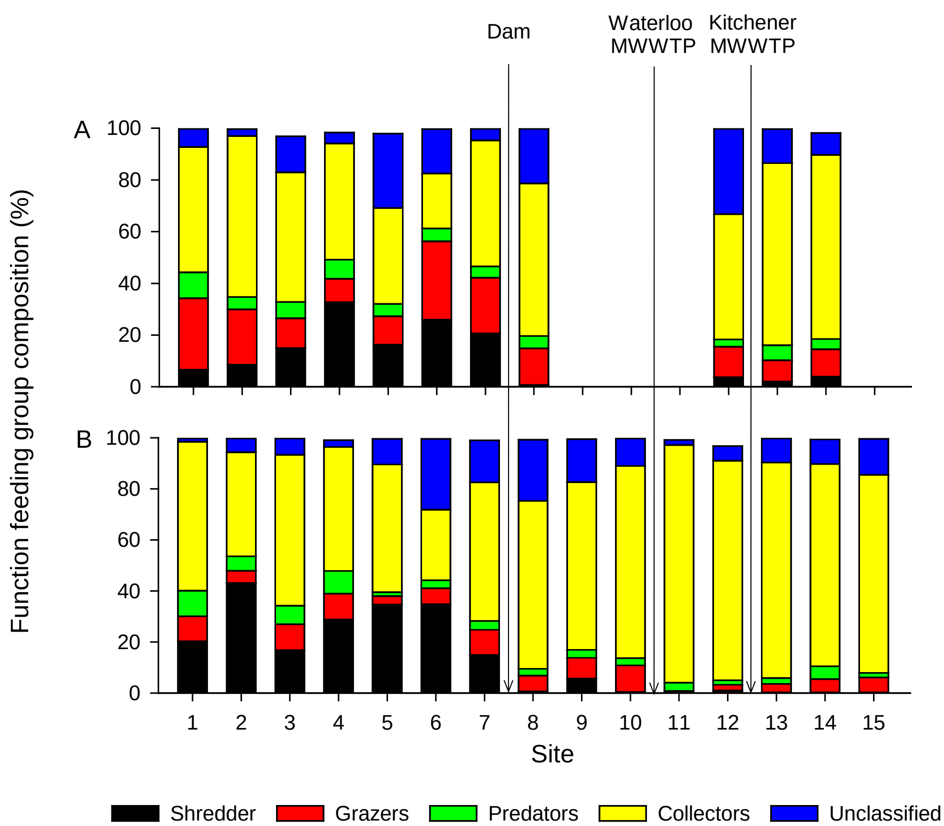

Figure 5.10 Functional feeding group composition for benthic macroinvertebrates collected in (A) 2013 and (B) 2014.

5.4.3 Confounding factors that limit our ability to detect change in fish communities

One of the objectives of the current study was to assess whether a change in fish communities could be detected below MWWTPs and whether a response could be detected after the Kitchener MWWTP upgrades. Effluent quality dramatically improved at the Kitchener MWWTP in the summer of 2012, when this plant was converted to a nitrifying activated sludge treatment plant. This resulted in improvements in effluent quality and downstream river water quality (including DO levels; see Chapter 3). These improvements were strongly linked to the recovery of several biological endpoints in wild rainbow darter populations, where a reduction in intersex was observed (see Chapter 3). However, despite these changes in river water quality and improvements in the health of the rainbow darter, a change in fish communities in post-upgrade years was not so evident. There are several possible explanations for this finding: 1) the MWWTPs may not have had a real effect on the composition of fish communities, 2) the magnitude of the improvements provided by the upgrades may not have been sufficient to produce a community response, 3) there may not have been enough time to detect a change, and/or 4) the methodology used in this study may not have been sensitive enough to detect a change. There are also a number of potential confounding factors (some discussed already) that may have limited our ability to associate changes with MWWTP effluents, including environmental and spatial factors and potential biases in the methodology.

Numerous environmental variables are changing along the 60 km river length of the central Grand River (sites 8–15). As indicated already, there is a bottom draw dam close to site 8 that is modifying the river's temperature profile (Figure S5.3). Wetted widths are also changing along this gradient, and even with an attempt at standardizing habitat across sites, increases in wetted width will change the diversity and complexity of habitats available (Vannote et al., 1980). From site 8 to site 15 there are changes in fish community composition including an increase in tolerant species (e.g., white suckers) as well as increases in fish diversity and decreases in total fish abundance. These changes may be due to the widening of the river and increases in the availability of habitat (38 m to 140 m).

Along the river gradient there are also the inputs of other rivers and streams (Figure 5.1). For example, a 5th order stream (Conestogo River) enters the Grand River just before site 10, and a 6th order stream (Speed River) enters at site 15. It is well known that river-stream connectivity can influence fish community composition (Osborne and Wiley, 1992; Wilkinson and Edds, 2001; Hitt and Angermeier, 2008). For example, Hitt and Angermeier (2008) found that river-stream connectivity influenced the presence of different trophic guilds (e.g., omnivore) and tolerant species, which confounded their ability to associate changes in fish communities with local environmental conditions. This may also have been the case in the current study. Although we saw a decreasing trend in intolerant species (e.g., darters) and increasing trend in tolerant species (e.g., white sucker) at downstream sites, this may simply be a factor of the natural gradients and river-stream connectivity present in the system. Teasing apart the changes in fish communities along the river gradient from the changes potentially associated with local water quality (i.e., presence of MWWTP effluent) is difficult to do in complex watersheds.

A potential factor that probably limited our ability to detect changes in fish communities is the standardized method used in this study. Electrofishing is considered one of the most efficient methods (Poos et al., 2007); however, the use of multiple methodologies may have increased our power to detect change. The methodology we used is standardized for riffle habitats and thus only characterizes riffle fish communities. Assessing multiple habitat types (e.g., pools) may have provided more insight into changes in fish communities. Although we attempted to standardize habitat in the current study, this is difficult to do in river systems that are normally associated with natural changes (Vannote et al., 1980). In addition, the fish communities characterized in this study were only those sampled closest to the banks, as the furthest distance out from the bank was only 10 m. At some sites, the wetted width was smaller than 10 m (e.g., upper Grand River and Four Mile Creek); thus, sampling was completed from bank to bank. This approach may have introduced more habitats and might be one of the reasons why species richness increased at the sites located in smaller order streams. There was also high variability associated with personnel, netters, and the electrofishing settings. For example, different people collected the data for the historical and current data sets used in this study. There was a clear separation of references sites between these two periods (Figure 5.3), which may be due to subtle changes in sampling technique from person to person or slight changes in site selection.

5.4.4 Conclusions

This study documented the changes in fish and BMI communities in urban areas in close proximity to MWWTP outfalls as well at a larger special scale (watershed) over multiple years. Changes downstream of MWWTP outfalls were most evident in dry years (low dilution). In other years, changes associated with MWWTP outfalls were subtle and difficult to separate from the changes associated with river gradients. Changes in response to the Kitchener MWWTP upgrades were not clear and were confounded by high flows in post-upgrade years. To confirm the effectiveness of the upgrades, further studies are needed under low-flow conditions (i.e., worst-case scenario). Assessing changes at the larger spatial scale (watershed) helped to better understand the gradients and spatial variability associated with both fish and BMI communities. High temporal variability was often associated with fish communities, which was probably triggered by subtle changes in habitat, annual variability in weather conditions (e.g., wet vs. dry years), and variability in personnel. It would be necessary to collect data over multiple years to capture this variability. Overall, the high temporal and spatial variability, along with other confounding factors, limited our ability to link changes in fish communities with local environmental conditions. To detect a change within this high variability, a large (severe) change in fish communities would be needed. In contrast, BMI communities appeared to be more sensitive indicators, and their associated changes appeared to link to local water quality conditions. This study has identified several challenges associated with fish communities that should be considered when they are used to assess environmental impacts. Other indicators, such as BMI communities and/or a sentinel fish species are probably more sensitive in detecting change and are thus recommended for use for an early warning in biomonitoring programs for MWWTP effluents.

Chapter 6

Conclusions and Recommendations

This thesis is a collection of research studies that assessed the responses of wild fish to municipal wastewater treatment plant (MWWTP) upgrades in the heavily impacted Grand River watershed of southern Ontario. The principal objective of this research was to evaluate the effectiveness of infrastructure upgrades at MWWTPs to remediate the impacts previously identified in wild fish. To address this objective, a collection of historical and archived data (before the upgrade), and new data (after the upgrade) were examined at multiple levels of biological organization and at multiple trophic levels. This work included studies on nutrient cycling within the aquatic food web (Chapter 2), reproductive impacts in individual fish (rainbow darter; Chapter 3), and changes in fish communities (Chapter 5). In addition, the site fidelity of the rainbow darter was investigated to better understand their utility as a sentinel fish species for environmental impact assessments (Chapter 4). This thesis has advanced our understanding of the biological impacts of MWWTP effluents and the changes in these impacts that occur in response to improved effluent quality. This work may have implications for future wastewater management strategies not only across Canada but globally as well. In addition, this thesis provides some insights into key indicators that may be useful for future biomonitoring programs for MWWTP effluents. The following major conclusions were drawn from the data chapters (Chapters 2–5) presented in this thesis.

Chapter 2: Stable isotope ratios ($\delta^{15}N$ and $\delta^{13}C$) provided evidence that sewage-derived nutrients were entering the aquatic food web below the MWWTP outfalls. Measurements of $\delta^{15}N$ in a primary consumer (benthic macroinvertebrates) and a secondary consumer (rainbow darter) enabled us to detect changes in effluent quality, whether it was improving (i.e., the Kitchener MWWTP upgrades) or deteriorating (i.e., the Waterloo MWWTP). Stable isotopes were an effective tool and serve as indicators of (1) exposure to sewage effluents and (2) disruption of nutrient cycling throughout the aquatic food web.

Chapter 3: The male rainbow darter responded to the Kitchener treatment upgrades, with reproductive impacts (i.e., intersex) reduced to near-background levels (reference conditions). This result was linked to improvements in the effluent quality (e.g., nutrients, pharmaceuticals, and total estrogenicity) and river water quality (e.g., dissolved oxygen). The relatively quick recovery suggests that rainbow darter may recover from past exposure to endocrine disrupting compounds.

Chapter 4: A study on the mobility of the rainbow darter advanced our understanding of the biology of this species and its potential as a sentinel species for environmental effects monitoring. Rainbow darter were confirmed to have high site fidelity, with the majority of fish moving no more than 5 m during a one-year period. However, a small proportion moved considerable distances, which was an unexpected finding for a darter (etheostomid) species. This study provided valuable insight into the interpretation of some impacts identified in the central Grand River associated with MWWTP outfalls and confirms that the rainbow darter is an ideal sentinel species.

Chapter 5: Fish communities were highly variable, both temporally and spatially. Detecting changes in fish communities in response to MWWTP effluents (before and after the upgrades) was difficult and confounded by natural watershed gradients. Annual river discharge proved to be an important factor in assessing changes in fish communities below MWWTP outfalls. For example, there were greater changes below MWWTP outfalls in low-flow years (i.e., years with lower dilution). It was easier to associate changes in benthic macroinvertebrates with local water quality conditions than in fish communities.

The major findings of this PhD work can aid in establishing protocols for (1) field evaluations of MWWTP management strategies and (2) a Canada-wide MWWTP effluent biomonitoring program. This is discussed further below, along with recommendations for future research directions.

Effectiveness of improved wastewater management strategies

Overall, these studies indicate that the upgrades at the Kitchener MWWTP were effective in improving the conditions of the receiving aquatic environment. This conclusion was primarily supported by the chapters assessing nutrient cycling and reproductive impacts in wild fish (Chapters 2 and 3). The upgrades at the Kitchener MWWTP were relatively conventional and targeted conventional contaminants (e.g., nutrients), but they were also demonstrated to be effective at removing other compounds (e.g., those associated with estrogenicity) thought to be linked to endocrine disruption in wild fish. The results suggest that secondary treatment (with nitrifying activated sludge), which is the minimum treatment level required to comply with the national *Wastewater Systems Effluent Regulations* (i.e., secondary treatment or equivalent with specific targets for ammonia, total suspended solids, chloride, and biological oxygen demand) may be sufficient to improve local ecological conditions. Hence, more advanced treatment (e.g., granular activated sludge, ozone, membrane filtration), which comes at higher prices, higher energy demands, and higher CO_2 emissions (Jones et al., 2007), may not be needed. However, the choice of treatment method will be dependent on site-specific conditions and environmental goals. Improvements in wastewater management strategies in this case study (improved treatment) demonstrate what large impacts they can have on the aquatic receiving environment. While this has implications for other sites across Canada and even globally, it is particularly relevant for the Grand River watershed, which has a history of adverse effects from MWWTP outfalls. Large investments went into the Kitchener MWWTP upgrades, and this study demonstrated the effectiveness of these upgrades and the positive ecosystem outcomes they have achieved.

Intersex still persists in the urbanized region of the Grand River, which may be due to other MWWTP discharges or the cumulative effects of other upstream stressors (e.g., agriculture). In accordance with its *Wastewater Treatment Master Plan*, the Region of Waterloo has already begun to implement upgrades similar to those at the Kitchener MWWTP (i.e., nitrifying activated sludge) at other plants in the region (Region of Waterloo, 2007). This includes the Waterloo MWWTP, which is planned to be upgraded by 2018. In addition, further upgrades are being implemented at the Kitchener MWWTP that will not be completed until 2020. This includes building a whole new secondary treatment train to replace the oldest current treatment train. This will undoubtedly further improve treatment capacity and efficiency (e.g., greater solid retention times) and possibly further improve effluent quality. The Kitchener MWWTP also had an effluent pump diffuser installed in the Grand River in 2016, which will improve mixing of the effluent below the new outfall. Future studies should assess the reproductive impacts in the rainbow darter after the completion of both the Kitchener and Waterloo MWWTP upgrades to determine whether the remaining impacts will be remediated.

As reproductive effects (such as intersex) are strongly linked to estrogenic compounds present in MWWTP effluents, it is reasonable to assume that intersex in the Grand River will persist until total estrogenicity (E2eq) decreases to a particular threshold or benchmark value. The decrease in intersex at the Kitchener MWWTP was associated with a decrease in E2eq of approximately 2 ng/L in the final effluent. With the Kitchener MWWTP contributing on average 10% of the river flow, the estimated river water E2eq concentration would be only 0.2 ng/L. Since intersex has dropped to near background levels below the Kitchener MWWTP outfall, the value of 0.2 ng/L E2eq could be used as a site-specific benchmark for river water in the Grand River. This value is similar to the safe levels for aquatic environments reported elsewhere for total estrogenicity (Jarošová et al., 2014) and for individual compounds (e.g., estradiol [E2] and 17α-ethynylestradiol [EE2]) (Caldwell et al., 2012) derived primarily from chronic fish reproductive studies. The safe level of E2eq proposed here is only a rough estimate; future studies could refine this value by appropriately modelling concentrations of estrogenic compounds in the Grand River. With E2 and EE2 being considered for inclusion in the European Union Water Framework Directive (they are currently on the "watch list"), North America may follow similar regulatory provisions for these types of substances in the future. Field-based studies like this one conducted in the Grand River watershed may help support future water quality objectives for emerging contaminants in Canadian surface waters.

Recommendations for a biomonitoring program for MWWTP effluents

Studies in this thesis provide insights into biological indicators that may be appropriate (or that may be too complex) for a biomonitoring program for MWWTP effluents. Kilgour et al. (2005) suggested that fish communities are ecologically relevant indicators of a healthy and functioning aquatic ecosystem and protecting them is a major priority. Hence, fish communities must be measured directly where feasible (Kilgour et al. 2005). However, the complexities of fish communities make them challenging to use as an indicator for detecting change associated with a stressor, especially in multi-use watersheds like the Grand River.

The fish communities assessed in this thesis were highly variable across both spatial and temporal scales. Although some changes were observed in fish communities, it was difficult to associate these changes with MWWTP effluents, primarily because they were confounded by a watershed gradient. Other complicating issues were biases in methodology, subtle changes in habitat, and differences associated with application of methods (e.g., differences in personnel). It took considerable effort to sample fish communities in only one habitat type; to properly characterize the entire fish community, multiple habitats would need to be sampled. This would require considerably more effort and resources, and sampling in other habitats such as pools and non-wadeable waters may pose additional challenges. In addition, there is a lack of standardized protocols for many different habitat types and stream orders. Seasons also introduce different factors that need to be considered, such as the distribution of young-of-year, increased mobility during spawning periods, and changes in the effectiveness of gear types across seasons. The complexity of fish communities makes it difficult to characterize them and to link any changes observed in them with local environmental conditions. It would only be possible to conclusively establish a link between a change in a fish community and a change in local conditions if the change in the fish community was severe, but such changes are likely to be irreversible. Despite the advantages of examining fish communities, it may be preferable to use early warning indicator(s) that may signal a potential risk to fish communities for biomonitoring programs for MWWTPs or other stressors.

The Canadian Environmental Effects Monitoring (EEM) program, which monitors for the effects of industrial discharges (pulp & paper and metal mining), uses a sentinel fish species and benthic macroinvertebrate (BMI) communities as indicators to detect change. This may also be an appropriate approach to use to monitor for impacts from MWWTP effluents. BMI are used for programs like the EEM because they represent fish habitat and respond to changes in water quality across temporal scales of days to years. This was illustrated in the current study, where the responses of BMI to the Kitchener and Waterloo MWWTP effluents were consistent with responses to nutrient enrichment (Chapter 5). The methodologies to sample and analyze BMI are well established and used internationally in many biomonitoring programs. Therefore, BMI hold promise for use in a biomonitoring program for MWWTPs and may represent a better indicator of change than fish communities.

The EEM program uses a sentinel fish species as an indicator because it is a middle ground between an indicator of sensitivity at the biochemical/physiological level and an indicator that is relevant at the community level. Any change observed at the population level, including changes in energy, growth, or reproduction, puts the fish population at risk, and the effects, if recognized, can be reversed with management action (Munkittrick et al., 2010). A biomonitoring program for MWWTP effluents may apply a similar approach. The work in this thesis and other studies in the Grand River (e.g., Tetreault et al., 2011, Fuzzen et al., 2016) indicate that intersex is a particularly sensitive and consistent indicator of exposure to MWWTP effluents. Severe cases of intersex have also been associated with reduced reproductive success (Fuzzen et al., 2015); thus, intersex may also represent an early warning sign of a population-level risk. This thesis demonstrated that management action to improve effluent quality was associated with reduced intersex incidence and severity in the rainbow darter, thus reversing the effect and minimizing any potential risks to the population. This illustrates the effectiveness of intersex as an endpoint to use in a biomonitoring programs for MWWTP effluents as it is relevant (population-level responses), sensitive, reversible, and linked to specific mechanisms (endocrine disruption), and it could therefore be used as an early warning sign to trigger management action.

Studies on stable isotope ratios ($\delta^{15}N$ and $\delta^{13}C$) in the central Grand River also proved that these are a useful indicator to detect change below MWWTP outfalls. Changes in $\delta^{15}N$ in biota were related to treatment and effluent quality. This tool could be used in a biomonitoring program to better understand the exposure of a sentinel species or to better understand the assimilation of sewage-derived nutrients within the aquatic food web. It could also be used to help understand the spatial influence of wastewater outfalls. Under some circumstances, stable isotopes ratios could also help determine the relative site fidelity of a sentinel fish species. Stable isotopes have many applications and it may be useful to include them in a biomonitoring program for MWWTP effluents.

Additional recommendations for future research

Reproductive success in the rainbow darter has been reported to be reduced below the urbanized reaches of the central Grand River, including the section below the Kitchener MWWTP (Fuzzen et al., 2015). This was linked to severe cases of intersex. Now that intersex has been reduced, it would be beneficial to assess whether reproductive success has also improved in the central Grand River. Such a study would also further validate the use of intersex as an early warning indicator for population-level effects associated with endocrine disruption.

Patterns of rainbow darter movement are now better understood; however, this was an isolated study in the upper part of the Grand River watershed, with few cumulative stressors. It is unknown whether these patterns in movement are site specific or if they can be extrapolated to the central Grand River. Movement of fish will be triggered by many different factors including population density, spawning, food and habitat availability, and predators (Lucas and Baras, 2001). Movement may also be triggered by factors associated with urbanization or MWWTPs either indirectly (e.g., changes in habitat) or directly (e.g., ammonia or dissolve oxygen concentrations). Movement may be one of the reasons that there are lower incidences of intersex reported below the Waterloo MWWTP than below the Kitchener MWWTP. The effluent plume of the Waterloo outfall is much smaller; thus, fish may be able to escape exposure more easily. A comparison of the movement patterns of rainbow darter in a rural environment and in an urbanized environment would provide insight into the potential variability in patterns of movement and the various triggers that may cause those patterns.

Environmental conditions can alter the responses of aquatic organisms to anthropogenic effects. This was highlighted in Chapter 5, where only under extreme dry weather conditions could large responses in fish and BMI communities be detected below the MWWTPs. Future studies should assess the responses of fish and BMI communities below MWWTPs (post-upgrade) in a dry year(s). Similarly, intersex in the rainbow darter should also be assessed in post-upgrade years during a dry year when the exposure may be much greater. This would provide more information on the effectiveness of the upgrades and whether the effects are completely mitigated, even under extreme conditions. This type of study would support one of the mandates of the Grand River Watershed Water Management Plan, which is to improve water quality, river ecosystem health, and resilience of the watershed to climate change (GRWMP, 2014).

In conclusion, the research studies in this thesis all contribute to a common theme: the impacts of MWWTP effluents and the effectiveness of wastewater management strategies at mitigating those impacts. The thesis provides examples of biological endpoints that are both less and more sensitive at detecting impacts from MWWTP effluents and indicates which of these endpoints can recover following improved effluent quality. The complexities associated with detecting impacts in a watershed are also highlighted throughout the thesis; they include confounding factors often associated with field studies such as natural variability, environmental conditions, and cumulative anthropogenic and natural stressors. To help address these complexities, multiple years of biological data across multiple sites were assessed, illustrating the importance of having large data sets to better understand and characterize an aquatic system. It would be useful if additional studies were conducted in the Grand River watershed in the future to further build on this data set, which could be used as an invaluable example to help develop biomonitoring strategies for MWWTPs as well as for cumulative stressors. This study is a positive example of the effectiveness of wastewater management strategies. Future studies should continue in the Grand River to assess whether the system fully recovers.

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Appendix A

Supplementary data for Chapter 2

Table S2.1 Summary of all data used for this study including previously published data, historical (archived) samples, and newly collected samples. Each sample collection provides the year, season(s), which sites, and what biota were collected (rainbow darter (RD) and/or primary consumers (PC)). In brackets are the sample sizes for rainbow darter (total number of individuals) or benthic invertebrates (number of pooled individuals) used in the isotopic analysis.

Site	2007		2008	2010		2011	2012		2013		2014	
	springǂ	fallǂ	fall*	spring*	fall*	fall*	spring*	fall*	spring*	fall**	spring**	fall**
REF 1								RD (8)		RD (8)/PC (5)		RD (7)/PC (5)
REF 2	RD (9)/PC (3)	RD (3)/PC (8)				RD (12)	RD (12)	RD (8)	RD (8)	RD (8)/PC (4)		RD (8)/PC (5)
REF 3	RD (9)/PC (4)	RD (3)/PC (16)	RD (6)	RD (8)		RD (11)		RD (8)	RD (8)	RD (8)/PC (5)	RD (8)/PC (5)	RD (8)/PC (5)
DSW 1	RD (9)/PC (4)	RD (7)/PC (20)	RD (6)	RD (8)		RD (13)		RD (20)	RD (8)	RD (8)/PC (5)	RD (8)/PC (5)	RD (8)/PC (5)
INT 1										RD (8)/PC (5)		RD (8)/PC (5)
INT 2	RD (9)/PC (4)	RD (3)/PC (17)	RD (6)	RD (8)	RD (8)	RD (11)	RD (12)	RD (16)	RD (8)	RD(8)/PC (5)	RD (8)/PC (4)	RD (8)/PC (5)
DSK 1	RD (8)/PC (4)	RD (5)/PC (20)	RD (6)	RD (8)	RD (7)	RD (11)	RD (11)	RD (16)	RD (9)	RD (8)/PC (5)	RD (8)/PC (5)	RD (8)/PC (5)
DSK 2			RD (6)	RD (8)	RD (9)	RD (12)		RD (16)	RD (10)	RD (8)/PC (5)	RD (8)/PC (5)	RD (8)/PC (5)
DSK 3	RD (10)/PC (5)	RD (5)/PC (14)	RD (5)	RD (4)		RD (12)				RD (8)/PC (5)		RD (7)/PC (5)

ǂPreviously published (Loomer et al., 2015) and included in this study for pre-upgrade conditions

*Archived samples from previous studies with different study objectives (Tanna et al., 2013, Fuzzen et al., 2015) were included here for pre (2007-2012) and post-upgrade (2013) conditions

**Sample collected for this study to assess changes in post-upgrade years (Kitchener MWWTP) and changes in effluent quality at the Waterloo MWWTP

Table S2.2 Summary of the population served, mean annual daily flow, and composition of effluent from 2007 to 2014 for both the Kitchener and Waterloo MWWTPs (Region of Waterloo, 2016).

Annual mean	Population served		mean daily flow (m^3/day)		mean cBOD (mg/L)		mean TSS (mg/L)		mean TP (mg/L)		mean TAN (mg/L)		mean N (mg/L)	
	Waterloo	Kitchener	Waterloo	Kitchener	Waterloo	Kitchener	Waterloo	Kitchener	Waterloo	Kitchener	Waterloo	Kitchener	Waterloo	Kitchener
2007	120265	215247	41358	70051	4.86	6.82	8.66	7.85	0.42	0.66	9.50	22.50	10.01	1.47
2008	121413	219596	47562	74935	5.57	5.22	11.67	8.48	0.50	0.50	7.75	17.71	10.07	2.71
2009	124006	221223	45940	73002	4.26	8.97	8.09	10.51	0.28	0.52	16.00	21.54	8.11	1.76
2010	126029	226106	42007	64329	5.46	8.58	4.95	9.01	0.36	0.55	18.82	22.72	6.67	2.26
2011	127688	227761	45540	70443	5.42	7.44	6.85	11.48	0.34	0.51	20.73	24.63	7.21	4.02
2012	130987	229757	42104	65858	6.60	7.46	7.23	7.61	0.31	0.67	24.19	19.68	1.59	8.59
2013	134851	230922	48570	72433	9.29	5.07	8.35	8.61	0.36	0.55	25.62	3.72	0.65	21.58
2014	136179	234466	48242	70988	4.27	5.86	3.16	9.11	0.22	0.63	21.50	8.15	5.39	14.41

cBOD: carbonaceous biological oxygen demand
TSS: total suspended solids
TP: total phosphorous
TAN: total ammonia nitrogen
N: total nitrate

Table S2.3 A summary table of eight two-way ANOVAs, with factors year and site, for $\delta^{15}N$ values in rainbow darter and primary consumers computed separately for both spring and fall seasons. Due to an unbalanced design, two way ANOVAs are computed for each combination of exposure site (downstream of the Kitchener MWWTP; DSK 1, DSK 2 and DSK 3) and the immediate upstream (reference) site (INT 2). For each two-way ANOVA, summary statistics reported include the F-ratio (F) with degrees of freedom in brackets and the associated p-value (P) for each factor (year, site) and the interaction term (year x site). Differences in mean $\delta^{15}N$ values ($\delta^{15}N_{exposure\ site}$ - $\delta^{15}N_{reference\ site}$) are provided for each combination, where a negative value indicates a lower $\delta^{15}N$ value at the exposure site compared to the reference site. Where the interaction (year x site) term was significant, pairwise comparisons (between exposure and reference site within each year) were computed with a Tukey's post-hoc test. An asterix (*) indicates a significant difference between reference and exposure site for the given year at p<0.01.

site	DSK 1 X INT 2								DSK 2 X INT 2								DSK 3 X INT 2							
biota	rainbow darter				primary consumer				rainbow darter				primary consumer				rainbow darter				primary consumer			
season	fall		Spring		fall		spring		fall		spring		fall		spring		fall		spring		fall		spring	
two-way ANOVA	F	P	F	P	F	P	F	P	F	P	F	P	F	P	F	P	F	P	F	P	F	P	F	P
year	58.6 (6)	< 0.001	6.0 (4)	< 0.001	1.5 (2)	0.23	5.3 (1)	0.037	102.7 (5)	< 0.001	15.7 (2)	< 0.001	-	-	-	-	35.1 (4)	< 0.001	-	-	24.7 (2)	< 0.001	-	-
site	766.5 (1)	< 0.001	227.8 (1)	< 0.001	8.3 (1)	0.01	7.9 (1)	0.014	740.2 (1)	< 0.001	44.7 (1)	< 0.001	-	-	-	-	12.01 (1)	< 0.001	-	-	11.4 (1)	0.002	-	-
year x site	50.5 (6)	< 0.001	7.9 (4)	< 0.001	7.2 (2)	0.002	2.5 (1)	0.138	72.9 (5)	< 0.001	15.2 (2)	< 0.001	-	-	-	-	22.9 (4)	< 0.001	-	-	7.1 (2)	0.002	-	-

Difference in means between exposure site (DSK 1, DSK 2 and DSK 3) and reference site (INT 2)

	DSK 1 rainbow darter fall	DSK 1 rainbow darter Spring	DSK 1 primary consumer fall	DSK 1 primary consumer spring	DSK 2 rainbow darter fall	DSK 2 rainbow darter spring	DSK 2 primary consumer fall	DSK 2 primary consumer spring	DSK 3 rainbow darter fall	DSK 3 rainbow darter spring	DSK 3 primary consumer fall	DSK 3 primary consumer spring
Pre-upgrade												
2007	-9.7*	-3.9*	-6.8*	-4.5					-1.2	-1.9	4.5*	-0.8
2008	-5.4*				-8.5*				-3.8*			
2010	-5.5*	-5.8*			-6.2*	-6.3*				-3.1*		
2011	-6.8*				-7.8*				-1.2*			
2012	-6.2*	-7.0*			-6.2*							
Post-upgrade												
2013	0.0	-5.1*	0.0		-0.2	-4.3*	1.1		2.1*		1.3	
2014	-0.8	-2.0*	-1.3	-1.3	-0.1	0.5	-0.9	-1.6	0.1		0.0	

Table S2.4 A summary table of four two-way ANOVAs, with factors year and site, for $\delta^{15}N$ values in rainbow darter and primary consumers computed separately for both spring and fall seasons. Two-way ANOVAs are computed for the exposure site downstream of the Waterloo MWWTP (DSW 1) and its immediate upstream site (REF 3). For each two-way ANOVA, summary statistics reported include the F-ratio (F) with degrees of freedom in brackets and the associated p-value (P) for each factor (year, site) and the interaction term (year x site). Differences in mean $\delta^{15}N$ values ($\delta^{15}N_{exposure\ site}$ - $\delta^{15}N_{reference\ site}$) are provided for each combination, where a negative value indicates a lower $\delta^{15}N$ value at the exposure site compared to the reference site. Where the interaction (year x site) term was significant, pairwise comparisons (between exposure and reference site within each year) were computed with a Tukey's post-hoc test. An asterix (*) indicates a significant difference between reference and exposure site for the given year at p<0.01.

site	DSW 1 X REF 3							
biota	Rainbow darter				primary consumer			
season	fall		spring		fall		spring	
two-way ANOVA	F	P	F	P	F	P	F	P
year	8.1 (5)	< 0.001	24.1 (3)	< 0.001	33.8 (2)	< 0.001	10.0 (1)	0.007
site	34.9 (1)	< 0.001	8.5 (1)	0.005	10.7 (1)	0.002	9.7 (1)	0.008
year x site	8.3 (5)	< 0.001	15.8 (3)	< 0.001	53.3 (2)	< 0.001	6.3 (1)	0.025
Difference in means between exposure site (DWS 1) and reference site (REF 3)								
Good quality effluent								
2007	0.6		1.0		5.3*		-0.6	
2008	1.4							
2010			1.3					
Poor quality effluent								
2011	-3.5*							
2012	-2.4*							
2013	-3.5*		-1.6		-5.6*			
2014	-2.6*		-4.9*		-5.5*		-5.1*	

Table S2.5 A summary table of eight two-way ANOVAs, with factors year and site, for $\delta^{13}C$ values in rainbow darter and primary consumers computed separately for both spring and fall seasons. Due to an unbalanced design, two-way ANOVAs are computed for each combination of exposure site (downstream of the Kitchener MWWTP; DSK 1, DSK 2 and DSK 3) and the immediate upstream (reference) site (INT 2). For each two-way ANOVA, summary statistics reported include the F-ratio (F) with degrees of freedom in brackets and the associated p-value (P) for each factor (year, site) and the interaction term (year x site). Differences in mean $\delta^{13}C$ values ($\delta^{15}N_{exposure\ site}$ - $\delta^{15}N_{reference\ site}$) are provided for each combination, where a negative value indicates a lower $\delta^{13}C$ value at the exposure site compared to the reference site. Where the interaction (year x site) term was significant, pairwise comparisons (between exposure and reference site within each year) were computed with a Tukey's post-hoc test. An asterix (*) indicates a significant difference between reference and exposure site for the given year at p<0.01.

site	DSK 1 X INT 2								DSK 2 X INT 2								DSK 3 X INT 2							
biota	Rainbow darter				primary consumer				Rainbow darter				primary consumer				Rainbow darter				primary consumer			
season	Fall		spring		fall		spring		fall		spring		fall		spring		fall		spring		fall		spring	
Two-way ANOVA	F	P	F	P	F	P	F	P	F	P	F	P	F	P	F	P	F	P	F	P	F	P	F	P
year	42.9 (6)	< 0.001	14.0 (4)	< 0.001	72.7 (2)	< 0.001	1.5 (1)	0.25	36.3 (5)	< 0.001	24.7 (2)	< 0.001	-	-	-	-	30.2 (4)	< 0.001	-	-	56.6 (2)	< 0.001	-	-
site	241.8 (1)	< 0.001	77.7 (1)	< 0.001	30.2 (1)	< 0.001	10.0 (1)	0.01	177.5 (1)	< 0.001	46.2 (1)	< 0.001	-	-	-	-	21.0 (1)	< 0.001	-	-	1.8 (1)	0.2	-	-
year x site	501 (6)	< 0.001	1.9 (4)	0.12	2.3 (2)	0.11	1.1 (1)	0.32	3.4 (5)	0.01	11.4 (2)	< 0.001	-	-	-	-	2.1 (4)	0.1	-	-	2.3 (2)	0.12	-	-
Difference in means between exposure site (DSK 1, DSK 2 and DSK 3) and reference site (INT 2)																								
Pre-upgrade																								
2007	1.5*		1.2*		3.9		2.1										-0.1		0.2		-0.4		1.5	
2008	2.0*								1.0*								0.9*							
2010	2.1*		1.7*						1.4*		2.2*								0.6					
2011	2.0*								1.6*								0.5							
2012	2.7*		1.8*						2.0*															
Post-upgrade																								
2013	1.1*		0.9		2.2				1.3*		0.8		1.2				0.5				1.7*			
2014	1.2*		0.9*		1.3		1.1		0.9*		0.4		0.2		0.4		1.0*				0.4			

Table S2.6 A summary table of four two-way ANOVAs, with factors year and site, for $\delta^{13}C$ values in rainbow darter and primary consumers computed separately for both spring and fall seasons . Two-way ANOVAs are computed for the exposure site downstream of the Waterloo MWWTP (DSW 1) and its immediate upstream site (REF 3). For each two-way ANOVAs, summary statistics reported include the F-ratio (F) with degrees of freedom in brackets and the associated p-value (P) for each factor (year, site) and the interaction term (year x site). Differences in mean $\delta^{13}C$ values ($\delta^{15}N_{exposure\ site}$ - $\delta^{15}N_{reference\ site}$) are provided for each combination, where a negative value indicates a lower $\delta^{15}N$ value at the exposure site compared to the reference site. Where the interaction (year x site) term was significant, pairwise comparisons (between exposure and reference site within each year) were computed with a Tukey's post-hoc test. An asterix (*) indicates a significant difference between reference and exposure site for the given year at p<0.01.

site	DSW 1 X REF 3							
biota	rainbow darter				primary consumer			
season	fall		spring		fall		spring	
Two-way ANOVA	F	P	F	P	F	P	F	P
year	16.1 (5)	< 0.001	71.5 (3)	< 0.001	8.7 (2)	< 0.001	0.0 (1)	0.937
site	61.5 (1)	< 0.001	60.4 (1)	< 0.001	33.4 (1)	< 0.001	2.8 (1)	0.117
year x site	0.68 (5)	0.64	1.1 (3)	0.37	4.3 (2)	0.019	0.6 (1)	0.439
Difference in means between exposure site (DWS 1) and reference site (REF 3)								
Good quality effluent								
2007	1.1		1.0*		1.6		1.7	
2008	1.1							
2010			0.6					
Poor quality effluent								
2011	1.2*							
2012	0.8							
2013	1.4*		0.3		4.9*			
2014	0.9*		0.7		2.2*		0.6	

Table S2.7 Mean (± SE) condition factor, liver somatic index (LSI) and gonadal somatic index (GSI) for male and female rainbow darter from the diet switch study at each sampling event. An asterix (*) indicates a significant difference from a one-way ANOVA. Where there were significant differences, a Tukey's post-hoc test on pairwise comparisons were computed. Time points (day) that do not share a letter in common are significantly difference (p<0.05).

time	Condition		LSI		GSI	
(day)	females	Males	females*	males*	females	males
0	1.17±0.08	1.14±0.05	1.12±0.06[a]	1.02±0.05[a]	0.74±0.05	Not measureable
3	1.08±0.02	1.04±0.05	1.40±0.23[a]	1.35±0.14[ad]	0.63±0.05	0.11±0.04
7	1.07±0.06	1.19±0.06	1.30±0.08[ab]	1.58±0.14[abcd]	0.70±0.04	0.12±0.04
10	1.05±0.02	1.09±0.04	1.37±0.31[ab]	1.42±0.22[a]	0.65±0.14	0.21±0.10
14	1.12±0.05	1.17±0.03	1.67±0.26[ab]	1.47±0.26[a]	0.74±0.12	0.19±0.07
21	1.12±0.02	1.18±0.07	1.97±0.23[ab]	2.34±0.09[bc]	0.75±0.05	0.17±0.06
28	1.08±0.02	1.26±0.07	2.06±0.22[ab]	2.25±0.27[bc]	0.64±0.22	0.15±0.05
42	1.07±0.03	1.17±0.07	1.82±0.11[ab]	1.98±0.20[bcd]	0.91±0.03	0.12±0.09
54	1.22±0.03	1.23±0.02	2.38±0.04[b]	1.97±0.13[bcd]	1.10±0.08	0.40±0.17
84	1.09±0.05	1.18±0.04	1.78±0.35[ab]	1.89±0.07[bcd]	0.74±0.15	0.17±0.08

Appendix B

Supplementary data for Chapter 3

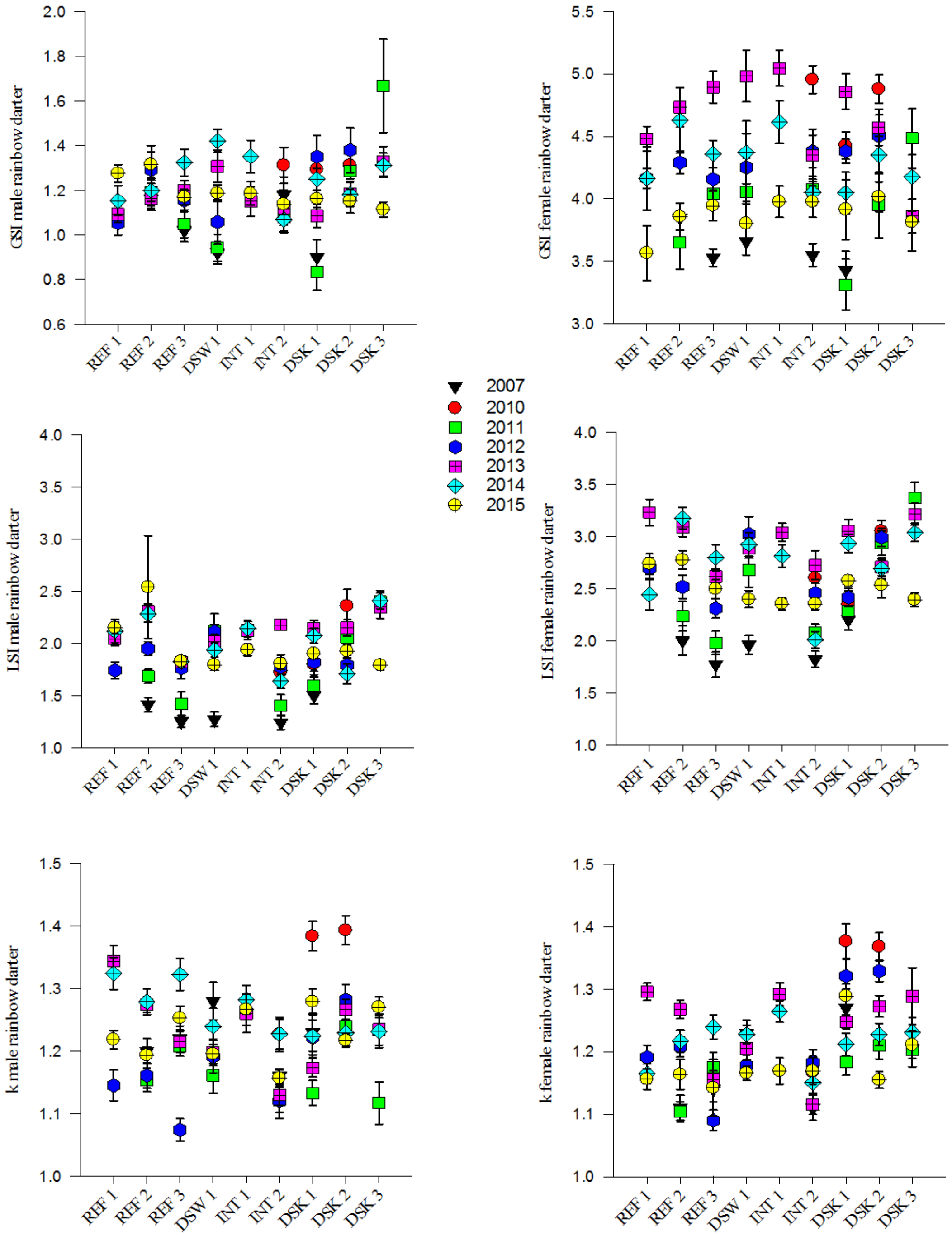


Figure S3.1 Gonadosomatic index (GSI), liver somatic index (LSI), and condition factor (k) in male and female rainbow darter before the upgrades to the municipal wastewater treatment plant (2007–2012) and after the upgrades (2013–2015). Each point represents the mean (± SE).

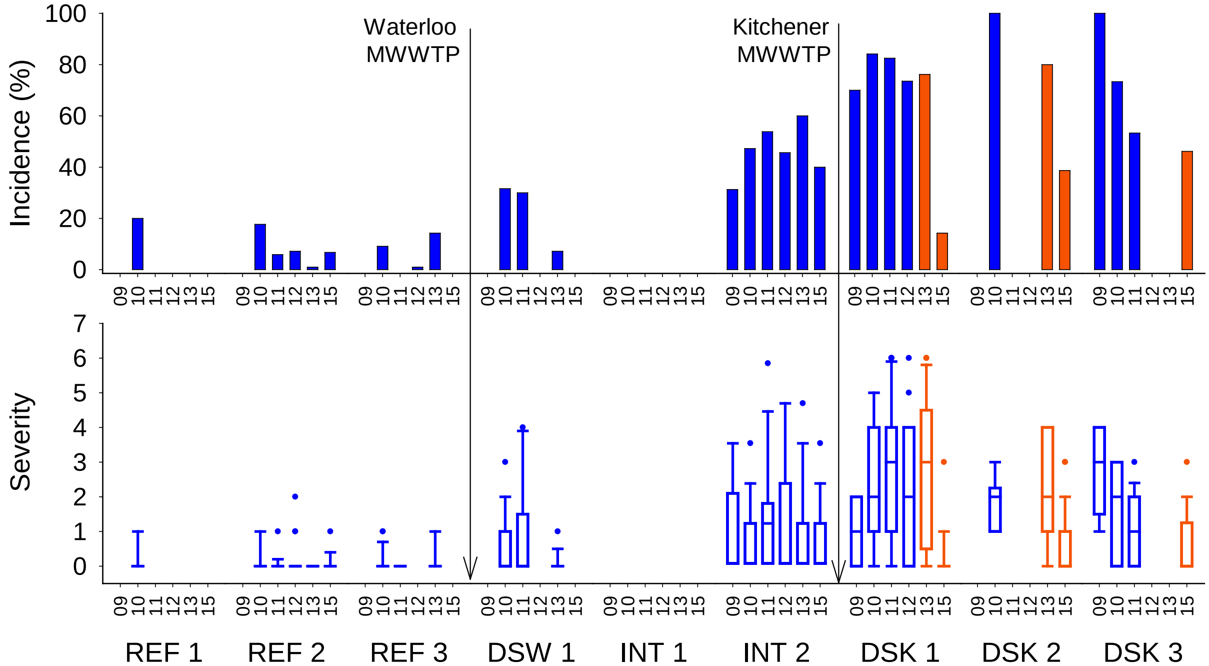


Figure S3.2 Intersex incidence (top panel) and severity (bottom panel) in male rainbow darter in the springs of 2009-2013 and 2015. Sites are in order from upstream (REF 1) to downstream (DSK 3) and the black arrows indicate the input of the MWWTP. Orange bars and box plots indicate post-upgrade years at the Kitchener MWWTP (2013–2015).

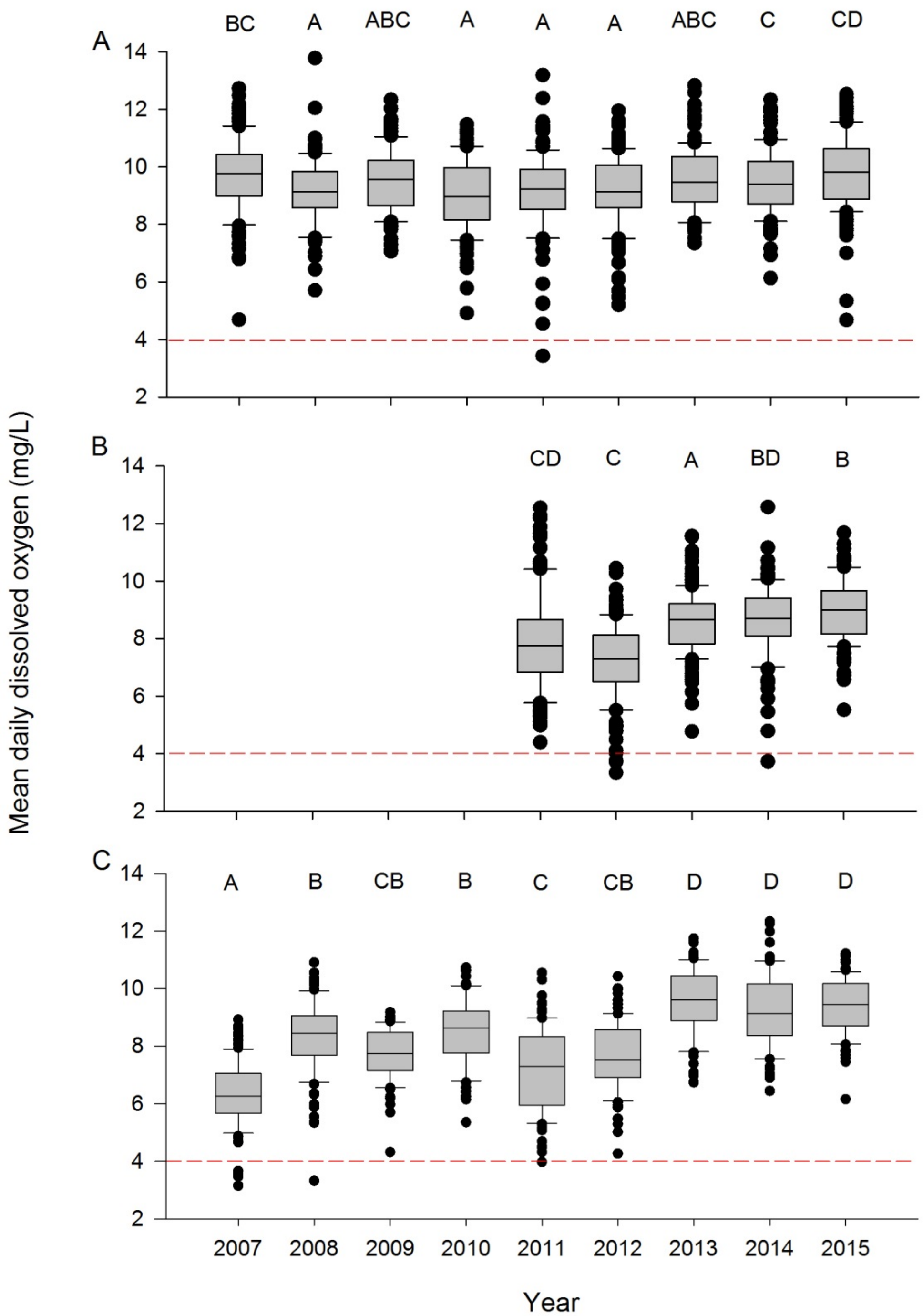


Figure S3.3 Boxplots of mean daily summer (June–September) dissolved oxygen concentrations upstream of the Waterloo MWWTP (A), between the Kitchener and Waterloo MWWTP (B), and downstream of the Kitchener MWWTP outfall (C) before (2007–2012) and after (2013–2015) the upgrade. The dashed line represents the Ontario provincial water quality guideline for dissolved oxygen for freshwater environments. Boxplots not sharing a letter in common are significantly different ($p < 0.05$). This figure was produced using information under licence with the Grand River Conservation Authority.

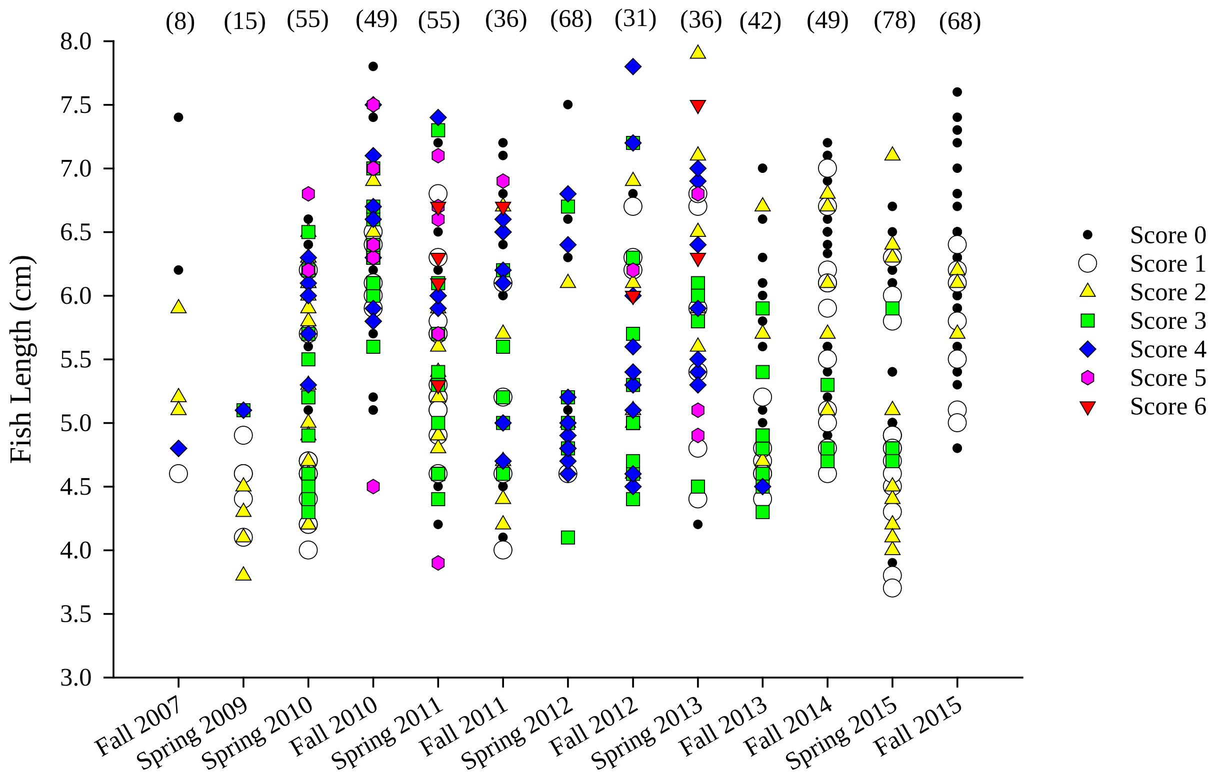


Figure S3.4 Length frequency and severity on intersex fish at the first three downstream sites (DSK 1, DSK 2, and DSK 3) before (2007–2012) and after (2013–2015) the upgrades at the Kitchener MWWTP. The brackets above each time point indicate the combined sample size for DSK1, DSK 2, and DSK 3. Fish are given different symbols based on their intersex severity scores, from a score of 0 (no intersex) to a score of 6 (severely intersexed).

Table S3.1 Description of each site, river distance between sites, and GPS coordinates.

Site description	Abbreviated name	Distance (km)	GPS coordinates	
			Latitude (N)	Longitude (W)
First upstream rural reference site	REF 1	0.0	43°37'52"	80°26'34"
Second upstream rural reference site	REF 2	11.0	43°35'07"	80°28'54"
Upstream urban reference site	REF 3	28.0	43°30'17"	80°28'28"
Waterloo MWWTP outfall	Waterloo MWWTP	33.0	43°28'46"	80°28'56"
Downstream Waterloo MWWTP	DSW 1	34.0	43°28'24"	80°28'23"
First site between Waterloo and Kitchener MWWTP	INT 1	45.0	43°26'41"	80°23'56"
Second site between Waterloo and Kitchener MWWTP	INT 2	52.0	43°24'35"	80°25'57"
Kitchener MWWTP outfall	Kitchener MWWTP	54.0	43°24'03"	80°25'12"
First site downstream Kitchener MWWTP	DSK 1	54.5	43°23'52"	80°24'56"
Second site downstream Kitchener MWWTP	DSK 2	55.5	43°23'45"	80°24'19"
Third site downstream Kitchener MWWTP	DSK 3	59.0	43°23'17"	80°23'12"

Table S3.2 Summary of sample sizes (number of individual males for histological analysis) for each site by year and season. Values in brackets () indicate the number of fish that had intersex. Footnotes indicate previously published data.

Site	2007		2008		2009		2010		2011		2012		2013		2014		2015	
	Spring	Fall[1]	Spring	Fall	Spring[1]	Fall	Spring[2]	Fall[3]	Spring[4]	Fall[5]	Spring[5]	Fall[5]	Spring[6]	Fall	Spring	Fall	Spring	Fall
REF 1							25(5)	9(1)				13(0)		9(1)		12(0)		20(0)
REF 2		10(0)					17(3)		17(1)	9(0)	42(3)	30(4)	26(0)	13(1)		13(1)	15(1)	18(0)
REF 3		11(1)					22(2)		14(0)	9(1)		12(1)	14(2)	16(2)		12(2)		20(0)
DSW 1		10(3)					19(6)		10(3)	15(7)		10(3)	14(1)	10(1)		14(6)		27(5)
INT 1														12(4)		19(4)		25(3)
INT 2		10(0)			16(5)		36(17)	38(11)	13(7)	12(1)	57(26)	14(4)	20(12)	11(6)		19(5)	25(10)	22(0)
DSK 1		8(6)			10(7)		19(16)	19(16)	40(33)	8(6)	68(50)	21(19)	21(16)	16(9)		16(7)	21(3)	23(2)
DSK 2							6(6)	19(13)		16(11)		10(10)	15(12)	14(4)		17(4)	31(12)	22(3)
DSK 3					5(5)		30(22)	11(2)	15(8)	12(7)				12(5)		16(9)	26(12)	23(5)

1 Tetreault et al., 2011
2 Tanna et al., 2013
3 Bahamonde et al., 2014
4 Bahamonde et al., 2015
5 Fuzzen et al., 2016
6 Fuzzen et al., 2015

Table S3.3 Population served, mean daily effluent flows, and effluent quality parameters from 2007 to 2015 for the Waterloo and Kitchener MWWTPs. All effluent parameters are represented by the annual means ± standard deviations. Effluent quality data are represented by weekly measurements throughout the year (n = 52) with the exception of Kitchener from 2013 to 2015 and Waterloo from 2014 to 2015, where measurements were taken approximately every second day (n = 153–158).

Year	Population served		mean daily flow (1000 m^3/day)		mean BOD (mg/L)		mean TSS (mg/L)		mean TP (mg/L)		mean TKN (mg/L)	
	Waterloo	Kitchener	Waterloo	Kitchener	Waterloo	Kitchener	Waterloo	Kitchener	Waterloo	Kitchener	Waterloo	Kitchener
2007	120265	215247	41.35 ± 5.07	70.09 ± 8.46	4.09 ± 2.74	6.81 ± 2.77	7.40 ± 6.51	7.76 ± 2.98	0.42 ± 0.18	0.65 ± 0.24	10.87 ± 8.49	27.24 ± 4.17
2008	121413	219596	47.62 ± 5.82	77.76 ± 8.39	5.46 ± 2.44	5.16 ± 3.07	11.57 ± 5.43	8.38 ± 5.68	0.50 ± 0.31	0.49 ± 0.30	10.87 ± 6.73	22.12 ± 6.05
2009	124006	221223	45.99 ± 5.20	73.08 ± 8.65	4.26 ± 1.69	8.93 ± 5.29	8.00 ± 4.65	10.47 ± 2.88	0.28 ± 0.13	0.52 ± 0.21	18.58 ± 7.16	25.92 ± 5.31
2010	126029	226106	42.00 ± 2.76	64.30 ± 3.25	5.20 ± 2.16	8.41 ± 4.80	4.98 ± 2.49	8.90 ± 4.01	0.35 ± 0.13	0.55 ± 0.30	23.98 ± 9.80	28.95 ± 6.74
2011	127688	227761	45.51 ± 7.41	70.38 ± 9.79	5.36 ± 2.81	7.32 ± 3.04	7.03 ± 4.08	7.62 ± 4.14	0.35 ± 0.13	0.50 ± 0.18	26.10 ± 9.23	29.65 ± 6.11
2012	130987	229757	41.97 ± 4.87	65.68 ± 3.97	6.53 ± 3.61	7.60 ± 5.45	7.21 ± 4.25	7.79 ± 3.95	0.31 ± 0.11	0.67 ± 0.31	29.05 ± 7.46	23.48 ± 11.36
2013	134851	230922	48.56 ± 5.59	72.43 ± 6.88	9.27 ± 4.27	5.02 ± 5.38	8.37 ± 4.27	8.65 ± 3.36	0.36 ± 0.12	0.56 ± 0.15	31.40 ± 5.51	5.39 ± 4.12
2014	136179	234466	49.37 ± 7.94	74.79 ± 7.99	4.05 ± 1.84	5.84 ± 2.30	3.25 ± 1.74	9.08 ± 3.88	0.22 ± 0.07	0.64 ± 0.16	27.15 ± 8.53	11.63 ± 6.91
2015	137347	238163	40.38 ± 4.14	68.57 ± 7.02	4.68 ± 1.78	5.55 ± 2.48	3.62 ± 2.74	7.41 ± 3.03	0.33 ± 0.12	0.62 ± 0.16	24.74 ± 6.46	7.54 ± 4.19

BOD: Carbonaceous Biological Oxygen Demand

TSS: Total Suspended Solids

TP: Total Phosphorous

TKN: Total Kjeldahl Nitrogen

Table S3.4 Summary of sample sizes (number of time points) for pharmaceuticals and total estrogenicity (E2eq).

MWWTP	Upgrade	Year (s)	Sample size*	
			Pharmaceuticals**	E2eq
Kitchener	Pre-upgrade	2010–July 2012	4	4
	During upgrade	Aug 2012–Feb 2013	5	2
	Post-upgrade	2013	7	9
		2014	7	2
		2015	10	4
Waterloo	na	2010	1	2
	na	2011	2	2
	na	2012	1	nd
	na	2013	1	3
	na	2014	9	2
	na	2015	9	4

*Each replicate represents a time point (day) sampled in triplicate
**Include Ibuprofen, Naproxen, and Carbamazepine
na = not applicable
nd = no data

Table S3.5 Summary statistics of Fisher's exact test (intersex incidence) and one-way ANOVA on ranks (intersex severity) for fall data. A significant difference is noted with an asterix (*) where $p < 0.05$.

Site	pairwise comparisons	% Incidence Fisher's exact p value	Severity (one-way ANOVA on ranks) test statistic (H)	DF	*p* value
REF 1	na	0.152	5.082	4	0.279
REF 2	na	0.580	5.220	5	0.390
REF 3	na	0.524	3.011	5	0.698
DSW 1	na	0.240	5.596	5	0.347
INT 1	na	0.285	3.131	2	0.209
INT 2	na	0.004*	19.206	6	0.004*
	07 vs. 10	0.093			1.000
	07 vs. 11	1.000			1.000
	07 vs. 12	0.125			1.000
	07 vs. 13	0.012*			0.634
	07 vs. 14	0.134			1.000
	07 vs. 15	na			1.000
	10 vs. 11	0.151			1.000
	10 vs. 12	1.000			1.000
	10 vs. 13	0.172			1.000
	10 vs. 14	0.770			1.000
	10 vs. 15	0.003*			0.810
	11 vs. 12	0.342			1.000
	11 vs. 13	0.027*			1.000
	11 vs. 14	0.363			1.000
	11 vs. 15	0.353			1.000
	12 vs. 13	0.228			1.000
	12 vs. 14	1.000			1.000
	12 vs. 15	0.021*			1.000
	13 vs. 14	0.238			1.000
	13 vs. 15	<0.001*			0.217
	14 vs. 15	0.016*			1.000
DSK 1		<0.001*	49.164	6	<0.001*
	07 vs. 10	0.616			1.000
	07 vs. 11	1.000			1.000
	07 vs. 12	0.300			1.000
	07 vs. 13	0.657			1.000
	07 vs. 14	0.211			1.000
	07 vs. 15	<0.001*			0.233
	10 vs. 11	0.616			1.000
	10 vs. 12	0.654			1.000
	10 vs. 13	0.132			0.227
	10 vs. 14	0.03*			0.005*
	10 vs. 15	<0.001*			<0.001*
	11 vs. 12	0.300			1.000
	11 vs. 13	0.657			1.000
	11 vs. 14	0.211			0.210

Site	pairwise comparisons	% Incidence Fisher's exact p value	Severity (one-way ANOVA on ranks) test statistic (H)	DF	*p* value
	11 vs. 15	<0.001*			0.003*
	12 vs. 13	0.024*			0.365
	12 vs. 14	0.003*			0.004*
	12 vs. 15	<0.001*			<0.001*
	13 vs. 14	0.742			1.000
	13 vs. 15	0.003*			0.405
	14 vs. 15	0.019*			1.000
DSK 2		<0.001*	37.349	5	<0.001*
	10 vs. 11	1.000			0.268
	10 vs. 12	0.068			1.000
	10 vs. 13	0.037*			0.102
	10 vs. 14	0.013*			0.01*
	10 vs. 15	<0.001*			1.000
	11 vs. 12	0.121			1.000
	11 vs. 13	0.067			0.268
	11 vs. 14	0.014*			0.082
	11 vs. 15	<0.001*			0.009*
	12 vs. 13	<0.001*			0.019*
	12 vs. 14	<0.001*			0.005*
	12 vs. 15	<0.001*			<0.001*
	13 vs. 14	1.000			1.000
	13 vs. 15	0.394			0.342
	14 vs. 15	0.658			1.000
DSK 3		0.066	10.323	4	0.035*
	10 vs. 11	na			0.592
	10 vs. 13	na			1.000
	10 vs. 14	na			0.648
	10 vs. 15	na			1.000
	11 vs. 13	na			1.000
	11 vs. 14	na			1.000
	11 vs. 15	na			0.455
	13 vs. 14	na			1.000
	13 vs. 15	na			1.000
	14 vs. 15	na			0.466

na: not applicable

Table S3.6 Summary statistics of Fisher's exact test (intersex incidence) and one-way ANOVA on ranks (intersex severity) for spring data. A significant difference is noted with an asterix (*) where $p < 0.05$.

		% Incidence	Severity (one-way ANOVA on ranks)		
Site	pairwise comparisons	Fisher's exact *p* value	test statistic (H)	DF	*p* value
REF 1	na	na	na	na	na
REF 2	na	0.136	6.646	4	0.156
REF 3	na	0.373	1.720	2	0.423
DSW 1	na	0.237	3.203	2	0.202
INT 1	na	na	na	na	na
INT 2	na	0.608	2.669	5	0.751
DSK 1		<0.001*	31.99	5	<0.001*
	09 vs. 10	0.633			0.639
	09 vs. 11	0.372			0.887
	09 vs. 12	1.000			1.000
	09 vs. 13	1.000			0.741
	09 vs. 15	0.004*			1.000
	10 vs. 11	1.000			1.000
	10 vs. 12	0.545			1.000
	10 vs. 13	0.698			1.000
	10 vs. 15	<0.001*			<0.001*
	11 vs. 12	0.380			1.000
	11 vs. 13	0.697			1.000
	11 vs. 15	<0.001*			<0.001*
	12 vs. 13	1.000			1.000
	12 vs. 15	<0.001*			<0.001*
	13 vs. 15	<0.001*			<0.001*
DSK 2		0.002*	12.746	2	0.002*
	10 vs. 13	0.526			1.000
	10 vs. 15	0.008*			0.047*
	13 vs. 15	0.012*			0.012*
DSK 3		0.046*	14.252	3	0.003*
	09 vs. 10	0.315			0.556
	09 vs. 11	0.114			0.053
	09 vs. 15	0.004*			0.015*
	10 vs. 11	0.200			0.525
	10 vs. 15	0.055			0.082
	11 vs. 15	0.751			1.000

na: not applicable

Table S3.7 Summary statistics of a two-way ANOVA (BACI analysis) with factors site (upstream vs. downstream) and period (pre-upgrade vs. post-upgrade) for intersex incidence at the sites DSK 1 and REF 3, with year as the level of replication.

Site	**Endpoint**	**Test**	**Source of Variation**	**DF**	**SS**	**MS**	**F**	***p***
DSK 1 vs. REF 3	Incidence	**Main test**	Pre X Post	1	1613.328	1613.328	9.575	0.013
			US X DS	1	7681.896	7681.896	45.593	<0.001
			Pre Post X US DS	1	1697.871	1697.871	10.077	0.011
		Pairwise comparisons		Diff. of means	*p*			
			US vs. DS (Pre)	72.03	<0.001			
			US vs. DS (Post)	25.961	0.037			
			Pre vs. Post (US)	0.581	0.958			
			Pre vs. Post (DS)	45.488	0.001			

Pre: Pre-upgrade
Post: Post-upgrade
US: Upstream site
DS: Downstream site

Table S3.8 Summary statistics of a two-way ANOVA (BACI analysis) with factors site (upstream vs. downstream) and period (pre-upgrade vs. post-upgrade) for intersex severity at the sites DSK 1 and REF 3, with year as the level of replication.

Site	Endpoint	Test	Source of Variation	DF	SS	MS	F	*p*
DSK 1 vs. REF 3	Severity	**Main test**	Pre X Post	1	3.343	3.343	16.998	0.003
			US X DS	1	7.172	7.172	36.471	<0.001
			Pre Post X US DS	1	2.541	2.541	12.921	0.006
		Pairwise comparisons		Diff. Of means	***p***			
				2.388	<0.001			
			US vs. DS (Pre)	0.606	0.129			
			US vs. DS (Post)	0.131	0.726			
			Pre vs. Post (US)	1.913	<0.001			

Pre: Pre-upgrade
Post: Post-upgrade
US: Upstream site
DS: Downstream site

Table S3.9 Summary statistics of a two-way ANOVA (BACI analysis) with factors site (upstream vs downstream) and period (pre-upgrade vs. post-upgrade) for intersex incidence at the sites DSK 2 and REF 3, with year as the level of replication.

Site	**Endpoint**	**Test**	Source of Variation	**DF**	**SS**	**MS**	**F**	***p***
DSK 2 vs. REF 3	Incidence	**Main test**	Pre X Post	1	2459.382	2459.382	21.353	0.002
			US X DS	1	4970.142	4970.142	43.152	<0.001
			Pre Post X US DS	1	2560.172	2560.172	22.228	0.002
		Pairwise comparisons		Diff. Of means	***p***			
				69.916	<0.001			
			US vs. DS (Pre)	11.49	0.226			
			US vs. DS (Post)	0.581	0.949			
			Pre vs. Post (US)	57.845	<0.001			

Pre: Pre-upgrade
Post: Post-upgrade
US: Upstream site
DS: Downstream site

Table S3.10 Summary statistics of a two-way ANOVA (BACI analysis) with factors site (upstream vs. downstream) and period (pre-upgrade vs. post-upgrade) for intersex severity at the sites DSK 2 and REF 3, with year as the level of replication.

Site	**Endpoint**	**Test**	Source of Variation	**DF**	**SS**	**MS**	**F**	***p***
DSK 2 vs. REF 3	Severity	**Main test**	Pre X Post	1	3	3	54.508	<0.001
			US X DS	1	3.707	3.707	67.349	<0.001
			Pre Post X US DS	1	2.266	2.266	41.169	<0.001
		Pairwise comparisons		Diff. Of means	***p***			
				1.981	<0.001			
			US vs. DS (Pre)	0.243	0.241			
			US vs. DS (Post)	0.131	0.514			
			Pre vs. Post (US)	1.869	<0.001			

Pre: Pre-upgrade
Post: Post-upgrade
US: Upstream site
DS: Downstream site

Appendix C

Supplementary data for Chapter 4

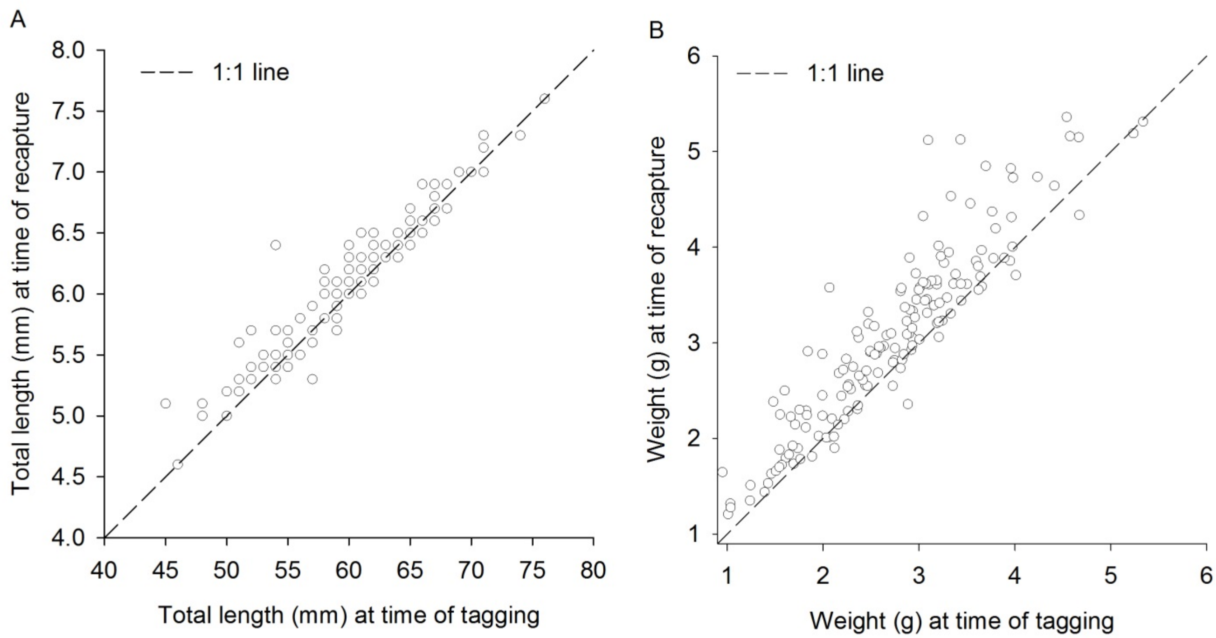


Figure S4.1 (A) Lengths and (B) weights of fish at time of tagging versus time of recapture. The dashed line represents a 1:1 relationship. Most fish were longer and heavier at the time of recapture than at the time they were tagged, indicating that tagging probably had no effects on fish growth.

Table S4.1 Mean (±SE) fish abundance for each core riffle (Core R.) during five different time points. One-way ANOVA (with Tukey post-hoc test) was computed for each riffle to test for differences in mean abundance across time points. Fish abundances at each time period that do not share a letter in common are significantly different ($p < 0.05$).

Sampling event	Fish abundance, fish/m^2		
	Core R. 1	Core R. 2	Core R. 3
July 2014	0.59 ± 0.06^{a}	0.47 ± 0.03^{a}	0.56 ± 0.04^{a}
August 2014	0.44 ± 0.04^{ab}	0.29 ± 0.03^{b}	0.25 ± 0.03^{bc}
November 2014	0.24 ± 0.04^{b}	0.42 ± 0.03^{a}	0.36 ± 0.08^{c}
May 2015	0.40 ± 0.05^{ab}	0.26 ± 0.03^{b}	0.17 ± 0.02^{b}
August 2015	0.36 ± 0.06^{b}	0.29 ± 0.03^{b}	0.42 ± 0.04^{c}

Appendix D

Supplementary data for Chapter 5

Table S5.1 Summary of data from sampling events for the current study and historical data for both fish communities and benthic macroinvertebrates (BMI).

Site	Fish community sampling events (2013–2014)					Historic fish community data*		BMI sampling events		
	May 2013	July 2013	Aug. 2013	Nov. 2013	Aug. 2014	Sept. 2007	Sept. 2008	Nov. 2012[#]	Nov. 2013[#]	Nov. 2014
4 Mile Creek										
1		✓	✓	✓	✓				✓	✓
2		✓	✓	✓	✓				✓	✓
3		✓	✓	✓	✓				✓	✓
Upper Grand River										
4		✓	✓	✓	✓				✓	✓
5	✓	✓	✓	✓	✓				✓	✓
6	✓	✓	✓	✓	✓				✓	✓
7	✓	✓	✓	✓	✓				✓	✓
Central Grand River										
8	✓	✓	✓	✓	✓				✓	✓
9		✓	✓		✓	✓	✓	✓		✓
10		✓	✓		✓	✓	✓			✓
11		✓	✓		✓	✓	✓	✓		✓
12	✓	✓	✓	✓	✓		✓	✓	✓	✓
13		✓	✓	✓	✓	✓	✓	✓	✓	✓
14	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
15	✓	✓	✓		✓					✓

*Historical samples that overlap with the current study sites (2013–2014) (Tetreault et al., 2013)

#Data provided by Adam Yates (Western University of Ontario)

BMI: benthic macroinvertebrates

Table S5.2 Summary of fish captured in the Grand River watershed including a list of fish families, species, and their abundances during each of the sampling events in 2013 and 2014. Also included are fish species tolerances (ability to adapt to disturbances), resilience (ability to withstand exploitation), vulnerability to predation, whether they are native or introduced, and their diet classification.

Family	Species	Common name	Code	Tolerance*	Resilience**	vulnerability**#	Native (N)/ introduced(I)	Function feeding group (adults)	May 13	July 13	Aug 13	Nov 13	Aug 14	Total number	Total %
Cyprinidae	*Notropis heterodon*	Blackchin Shiner	BCS	intolerant	high	low (14)	N	invertivore	0	12	0	0	0	12	0.07
	Rhinichthys atratulus	Blacknose Dace	BND	intermediate	high	low (23)	N	benthic invertivore	1	8	21	8	9	47	0.27
	Notropis heterolepis	Blacknose Shiner	BNS	intolerant	high	low (20)	N	invertivore/herbivore	0	31	0	0	0	31	0.18
	pimephales notatus	Bluntnose Minnow	BNM	intermediate	medium	low (22)	N	omnivore/detritovore	2	35	54	28	6	125	0.73
	Campostoma anomalum	Central Stoneroller	CSR	intermediate	medium	low (31)	N/I	benthic herbivore	1	20	444	165	212	842	4.92
	Cyprinus carpio	Common Carp	CC	tolerant	medium	moderate-high (46)	I	invertivore/detritivore	0	0	0	0	113	115	0.67
	Semotilus atromaculatus	Creek Chub	CRC	intermediate	medium	moderate (38)	N	generalist	0	12	81	0	43	136	0.79
	Notropis atherinoides	Emerald shiner	ES	intermediate	high	low (15)	N	invertivore/planktivore	1	0	0	0	0	1	0.01
	Nocomis biguttatus	Hornyhead Chub	HHC	intermediate	medium	moderate (37)	N/I	omnivore (invertivore/herbivore)	24	45	103	40	27	239	1.40
	Rhinichthys cataractae	Longnose Dace	LND	intermediate	medium	moderate (36)	N	benthic invertivore	30	173	263	37	228	731	4.27
	Nocomis micropogon	River chub	RC	intermediate	medium	moderate (40)	N/I	omnivore (planktivore/invertivore)	0	4	21	25	22	72	0.42
	Notropis rubellus	Rosyface shiner	RFS	intermediate	high	low (17)	N	generalist	5	51	37	18	33	144	0.84
	Cyprinella spiloptera	Spotfin Shiner	SFS	intermediate	medium	low (28)	N/I	invertivore/herbivore	0	0	3	0	0	3	0.02
	Luxilus spp	Common/Striped Shiner	CS/SS	intermediate	medium	low (16)	N/I	omnivore/invertivore	33	99	202	42	116	492	2.87
Percidae	*Percina maculata*	Blackside Darter	BSD	intermediate	medium	low (23)	N/I	benthic invertivore/piscavore	0	1	14	2	2	19	0.11
	Etheostoma flabellare	Fantail Darter	FT	intolerant	medium	low (26)	N	benthic invertivore	293	931	1224	215	1463	4126	24.10
	Etheostoma blennioides	Greenside Darter	GSD	intermediate	medium	low (25)	N/I	benthic invertivore	32	79	165	21	325	622	3.63
	Etheostoma nigrum	Johnny Darter	JD	tolerant	medium	low (14)	N	invertivore/detrivore	5	69	238	48	115	475	2.77
	Etheostoma microperca	Least Darter	LD	intolerant	high	low (14)	N	benthic invertivore	0	6	14	2	5	27	0.16
	Etheostoma caeruleum	Rainbow Darter	RBD	intolerant	high	low (17)	N	benthic invertivore	349	1928	2973	499	2197	7946	46.42
	Perca flavescens	Yellow Perch	YP	intermediate	medium	low-moderate (31)	N	invertivore/carnivore	0	0	0	2	0	2	0.01
Centrarchidae	*Lepomis macrochirus*	Bluegill	BG	intermediate	medium	low-moderate (33)	N	generalist	1	0	0	0	0	1	0.01
	Micropterus salmoides	Largemouth Bass	LMB	tolerant	low	moderate-high (45)	N/I	invertivore/carnivore	0	1	0	0	0	1	0.01
	Lepomis gibbosus	Pumpkinseed	PS	intermediate	medium	low-moderate (32)	N	invertivore/carnivore	1	0	4	0	0	15	0.09
	Ambloplites rupestris	Rockbass	RB	intermediate	medium	low-moderate (33)	N	invertivore/carnivore	20	58	81	2	45	206	1.20
	Micropterus dolomieui	Smallmouth Bass	SMB	intermediate	medium	moderate-high (50)	N/I	invertivore/carnivore	6	7	16	1	18	48	0.28
	Pomoxis annularis	White Crappie	WC	tolerant	medium	low-moderate (29)	N	invertivore/carnivore	0	0	0	2	0	2	0.01
Catostomidae	*Moxostoma erythrurum*	Golden Redhorse	GoRH	intermediate	low	high-very high (65)	N	benthic invertivore/herbivore	0	0	0	2	0	2	0.01
	Moxostoma valenciennesi	Greater Redhorse	GRH	intolerant	low	high-very high (68)	N	benthic invertivore/herbivore	1	0	0	1	1	3	0.02
	Hypentelium nigricans	Northern Hog Sucker	NHS	intermediate	low	high (62)	N	benthic invertivore/herbivore	1	9	35	8	2	55	0.32
	Catostomus commersoni	White Sucker	WS	tolerant	low	high (57)	N	benthic invertivore/herbivore/detritivore	49	169	120	37	52	427	2.49
Ictaluridae	*Ictalurus nebulorsus*	Brown bullhead	BBH	intermediate	medium	low-moderate(30)	N	benthic invertivore/carnivore/herbivore	3	6	0	0	3	12	0.07
	Noturus flavus	Stonecat	SCT	tolerant	medium	moderate (37)	N	benthic invertivore/carnivore/herbivore	5	47	25	15	17	109	0.64
Gasterosteidae	*Culaea inconstans*	Brook Stickleback	BS	intermediate	high	low (15)	N	planktivore/invertivore	0	2	0	2	3	7	0.04
Umbridae	*Umbra limi*	Central Mudminnow	CMM	tolerant	medium	low (13)	N	benthic invertivore/carnivore	0	4	0	2	1	7	0.04
Gobiidae	*Neogobius melanostomus*	Round Goby	RG	intermediate	medium	low-moderate (31)	I	benthic invertivore/carnivore	9	4	5	1	0	15	0.09
Salmonidae	*Salmo trutta*	Brown Trout	BT	intolerant	high	high (60)	I	invertivore/carnivore	1	0	0	0	0	1	0.01

*http://www.ontariofishes.ca/home.htm (access Nov 16, 2016). Eakins 2016

**http://www.fishbase.org/ (accessed Nov 16, 2016),

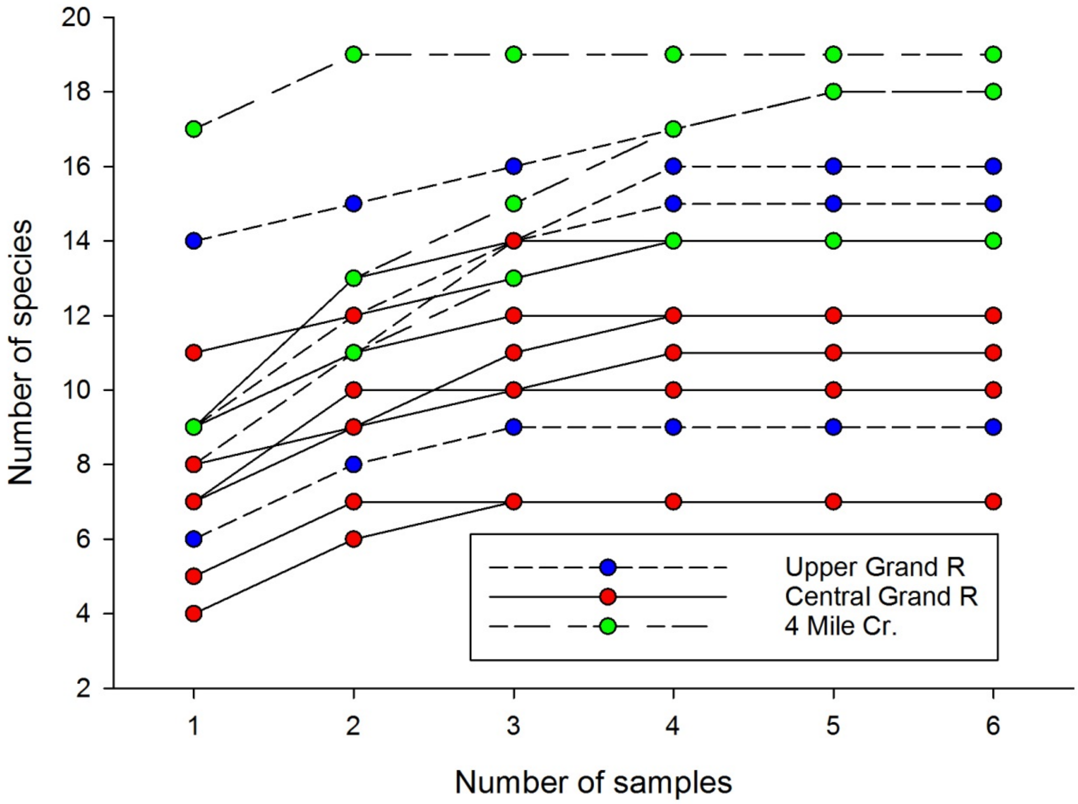
value in paratheses is the score of vulnerability out of 100, where high scores indicate high vulnerability to catchbility/predators.

Table S5.3 Hilsenhoff Biotic Index

Site	Hilsenhoff Biotic Index		
	2012	2013	2014
1		4.69	4.84
2		4.95	4.04
3		4.66	4.83
4		4.74	4.05
5		3.10	4.00
6		3.25	3.00
7		4.28	4.04
8		3.23	2.60
9	5.06		3.10
10			3.27
11	6.72*/7.22**		5.44
12	4.82*/4.24**	3.25	5.39
13	6.00*/6.07**	4.81	4.87
14	6.09	4.83	5.21
15			4.54

*Sampled in October 2012
**Sampled in November 2012
0-3.75 (excellent water quality)
3.76-4.25 (very good water quality)
4.26-5.0 (good water quality)
5.01-5.75 (fair water quality)
5.76-6.50 (fairly poor water quality)
6.51-7.25 (poor water quality)
7.26-10 (very poor water quality)

Figure S5.1 Species accumulation plots (number of species with increasing number of replicates) for 15 sites sampled in August 2013.



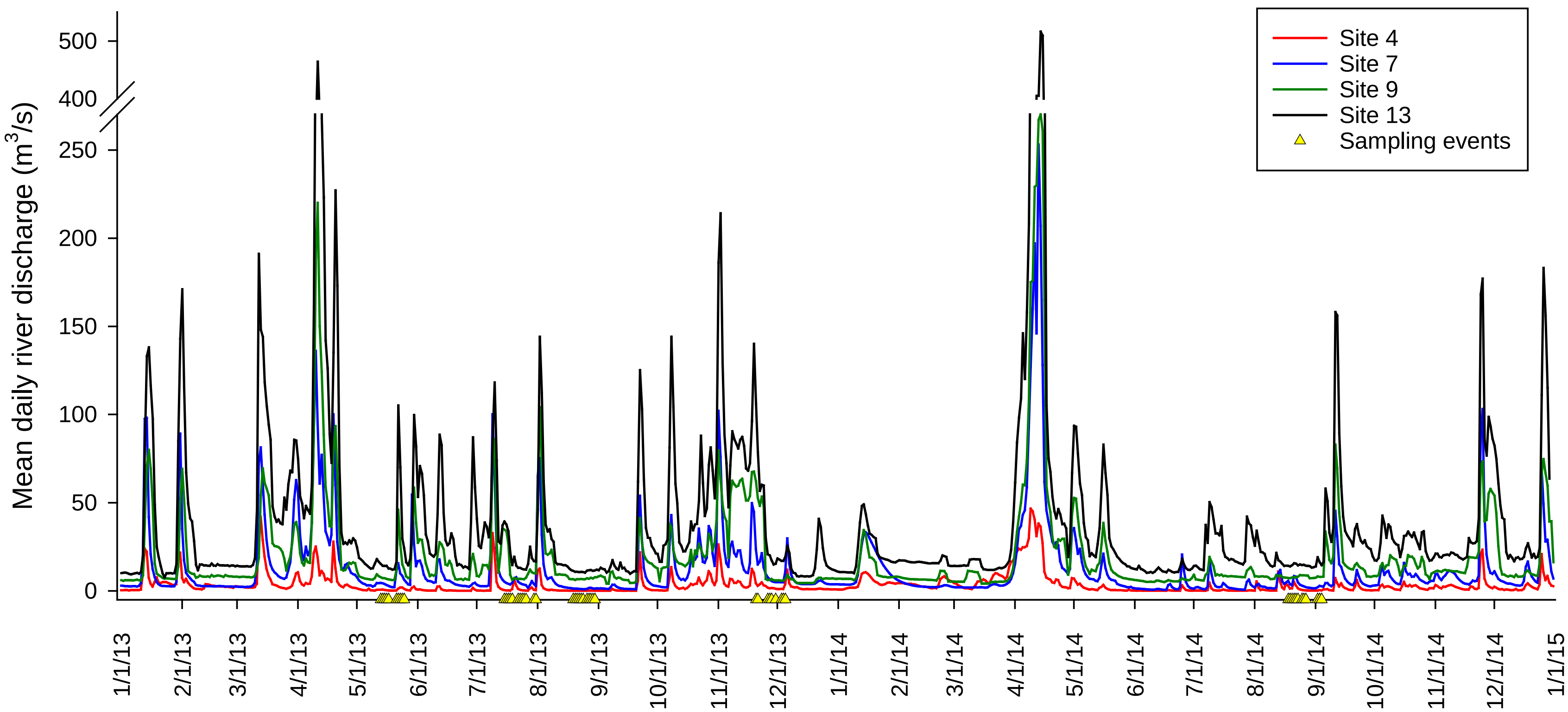


Figure S5.2 Mean daily discharge (m^3/s) for two sites in the upper Grand River (sites 4 and 7) and two sites in the central Grand River (sites 9 and 13). The yellow filled triangles indicate the sample days for each of the five sampling events between 2013 and 2014. Data provided by the Water Survey of Canada and Grand River Conservation Authority.

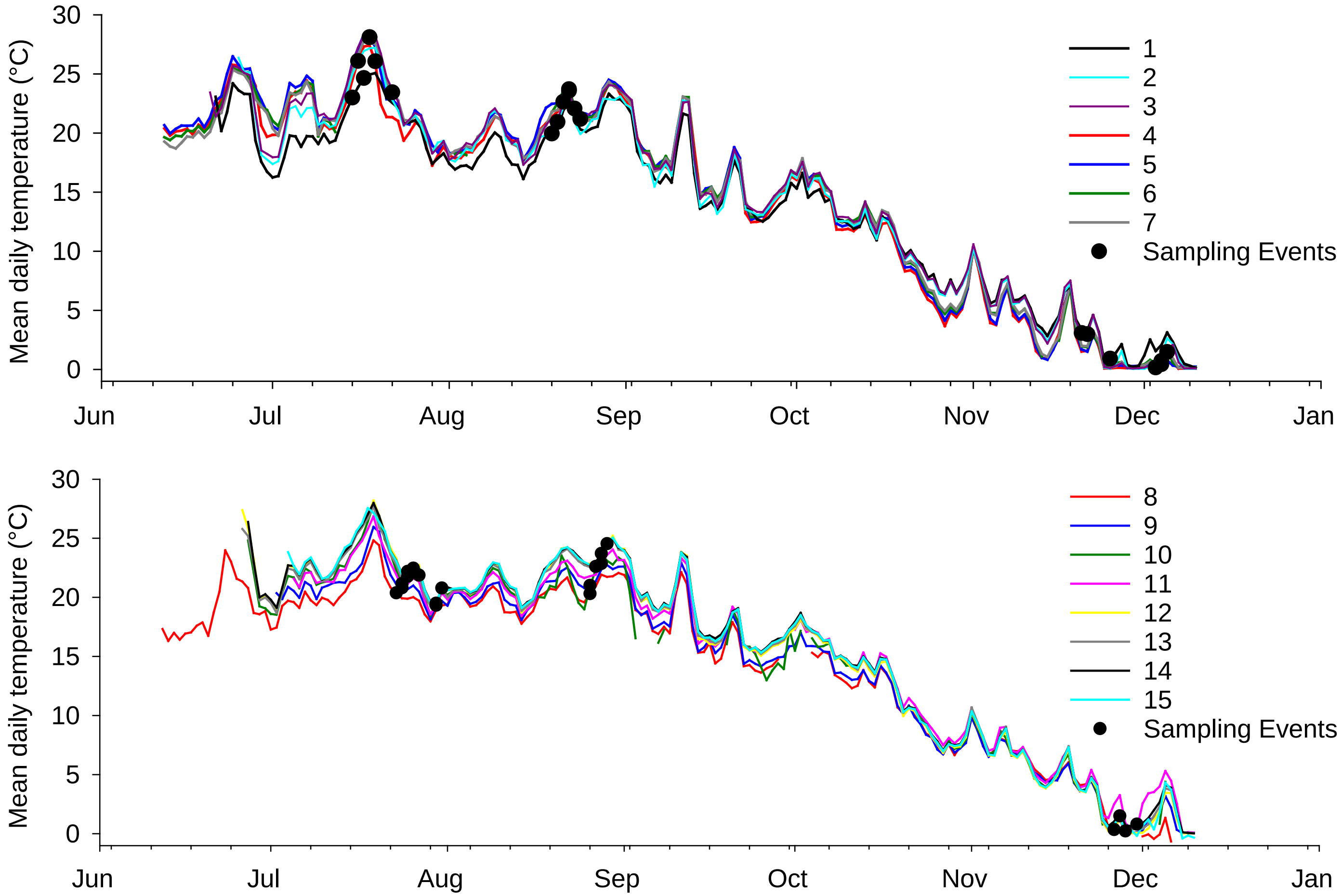


Figure S5.3 Mean daily temperature (^{o}C) from June to December 2013 for Four Mile Creek (sites 1–3) and the upper Grand River (sites 4–7) in the top panel and the central Grand River (sites 8–15) in the bottom panel. The black dots represent the sampling events for July 2013, August 2013, and November 2013. Data have been removed in instances where the logger appeared to be out of the water (e.g., reflecting air temperatures).

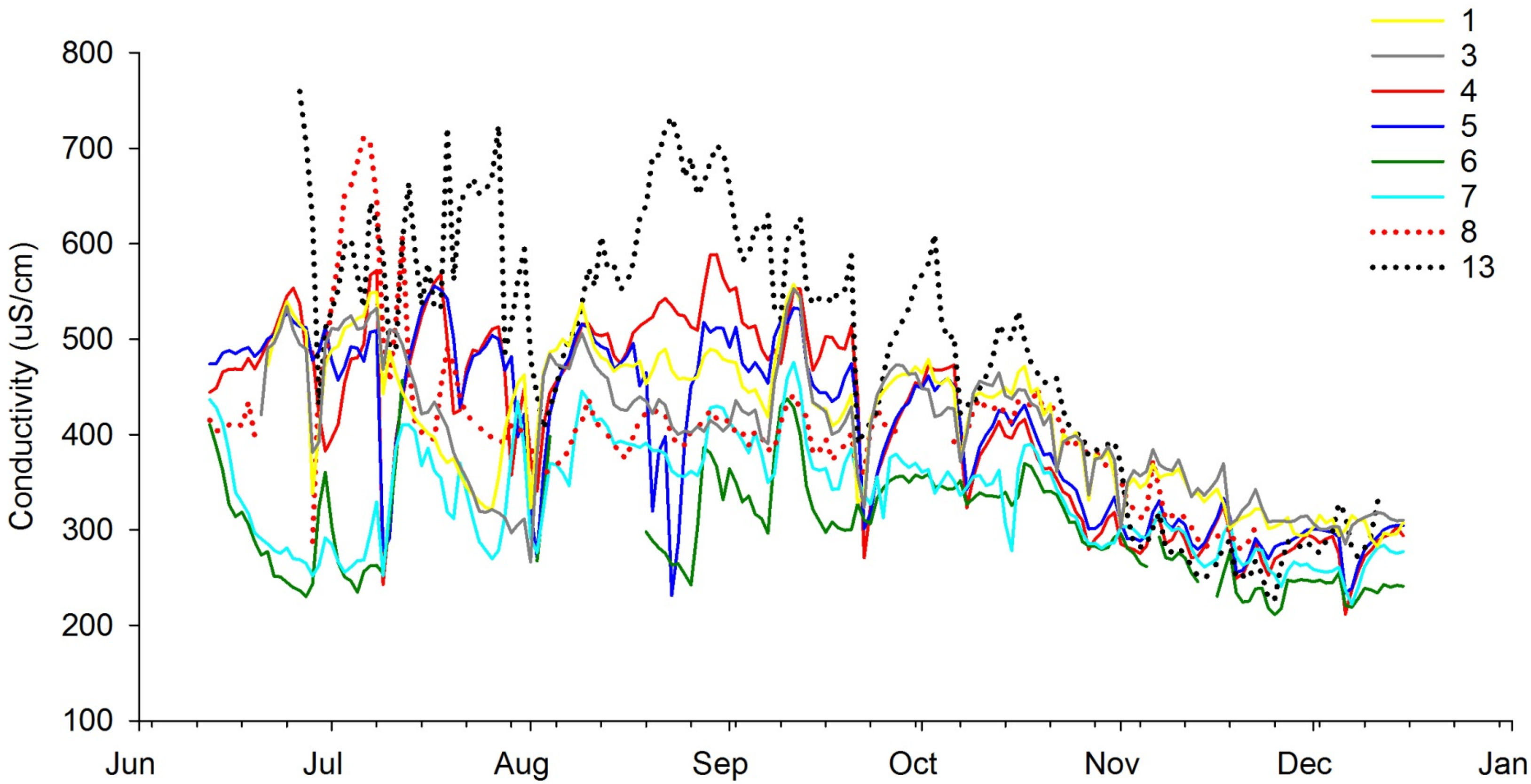


Figure S5.4 Mean daily specific conductance (µS/cm) from June to December 2013 for select sites throughout the Grand River watershed. Some data was removed in instances where the logger appeared to be out of the water (e.g., low conductivity values).

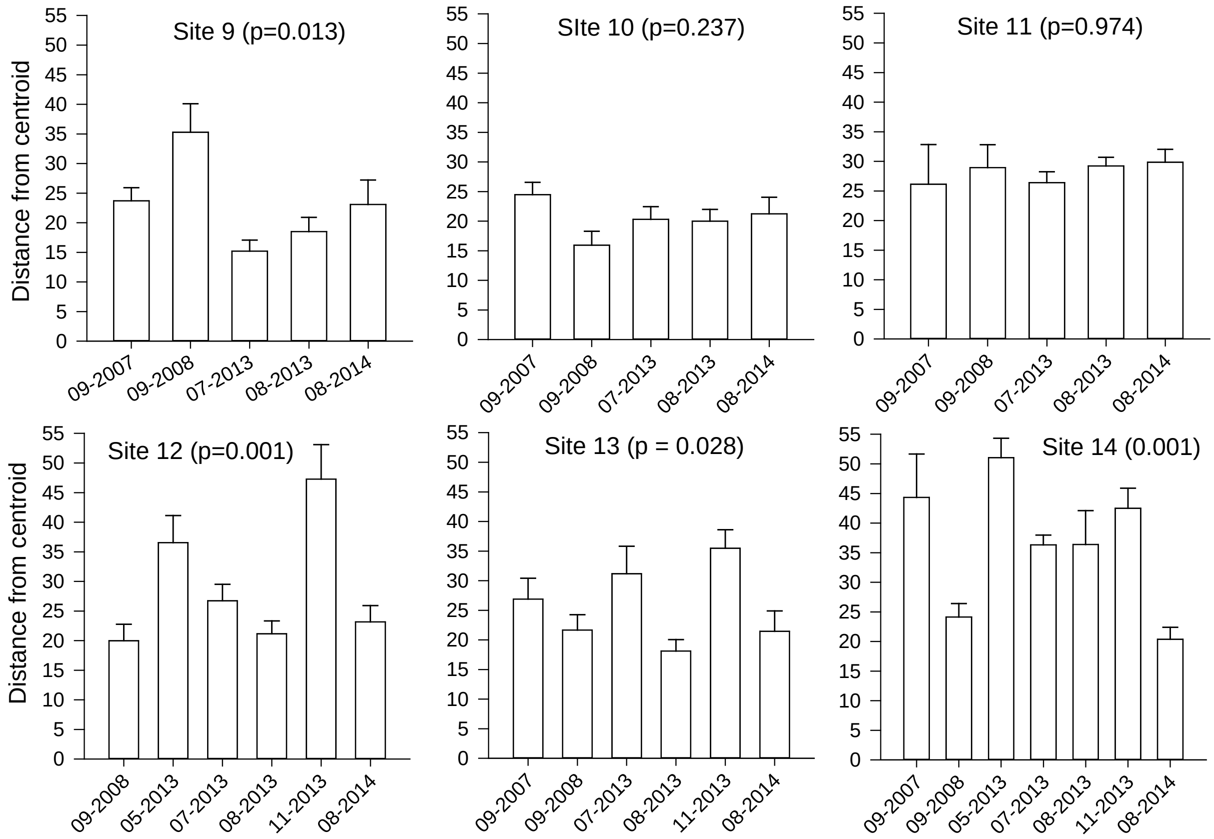


Figure S5.5 Homogeneity of multivariate dispersion (Bray-Curtis similarity on square-root transformed data) between sampling events within sites, including historical (2007–2008) and current data sets (2013–2014). Each bar represents the average Bray-Curtis distance to centroid (%) with subsite as the unit of replication (n = 6). Results of the test of homogeneity between sites are given for each site, where a p value < 0.05 indicates a significant difference in dispersion (variability) between sampling events.

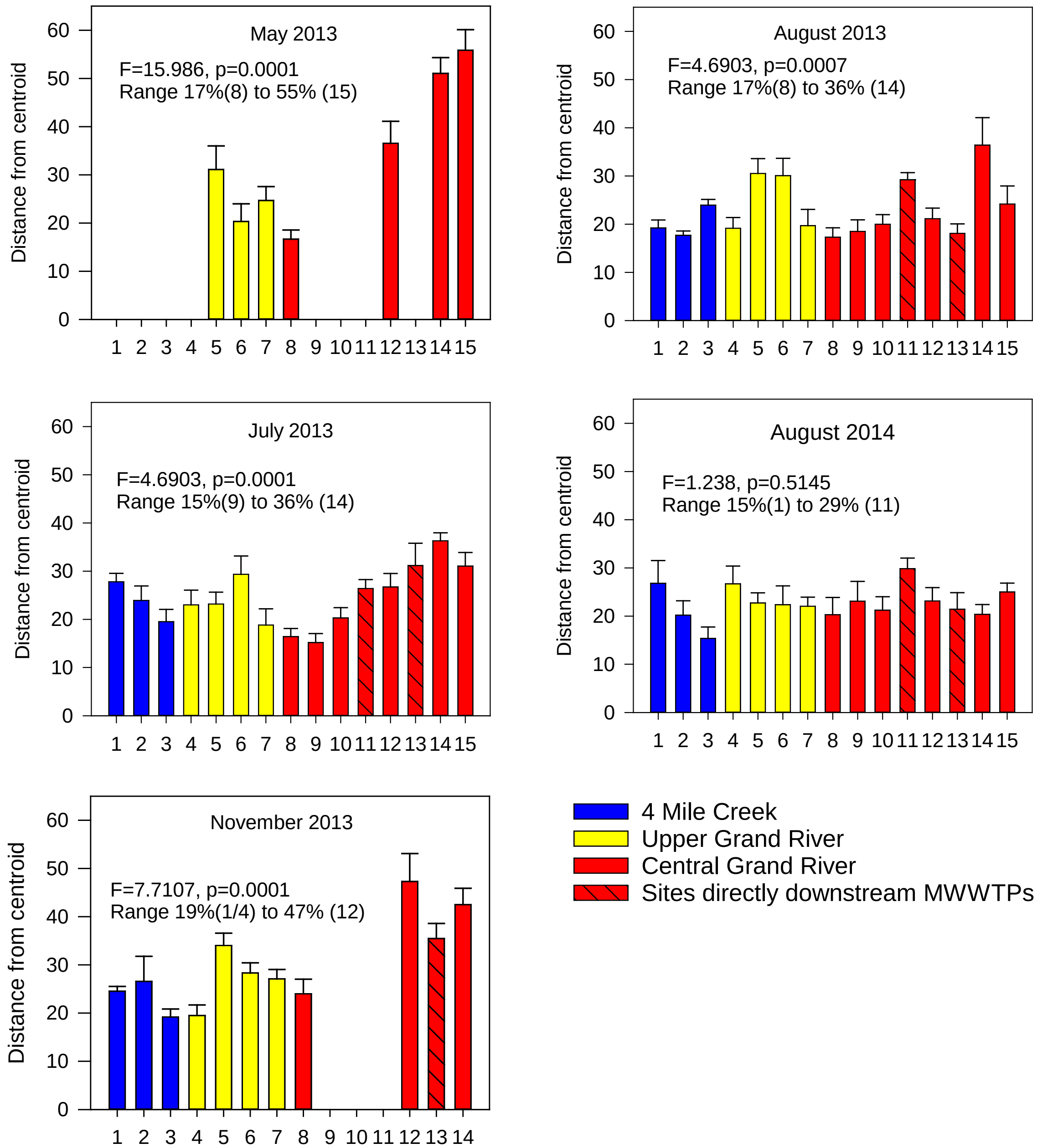


Figure S5.6 Homogeneity of multivariate dispersion (Bray-Curtis similarity on square root transformed data) between sites within sampling events. Each bar represents the average Bray-Curtis distance to centroid (%) with subsite as the unit of replication (n = 6). Results of the test of homogeneity between sites are given for each sampling event, where a p value < 0.05 indicates a significant difference in dispersion (variability) between sites.

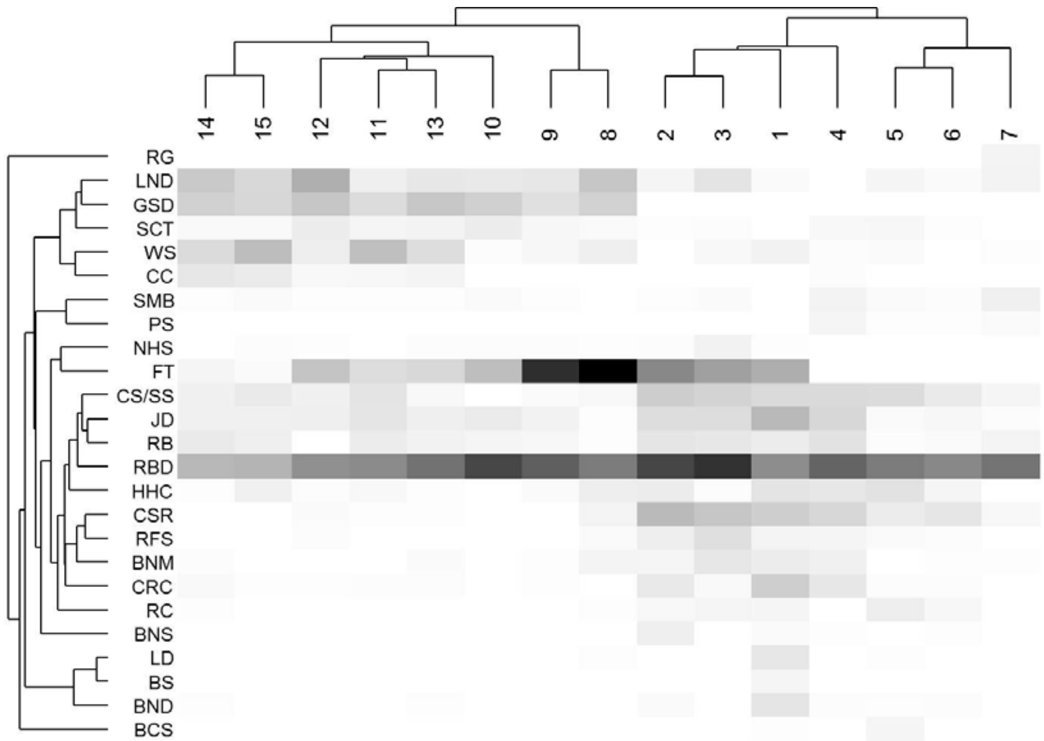


Figure S5.7 Shade plot showing the relative abundance (square-root transformed) of the top 25 fish species contributing to riffle fish communities at each of the 15 sites across the Grand River watershed. The intensity of shading is proportional to the relative fish abundances. The species abundance from each site was averaged over three summer sampling events. Fish groups (left) and sites (top) are ordered on the basis of hierarchical cluster analysis, where fish groups are based on index of association and sites by Bray-Curtis similarity.