The Response of Wild Fish to Municipal Wastewater Effluent Exposures at Sites in Canada

by

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AUTHOR'S DECLARATION

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

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Abstract

Aquatic receiving environments have long been used to dilute municipal wastewater effluents (MWWE) which are the largest discharge by volume into the aquatic environment in Canada. These treated effluents are a complex mixture of environmental contaminants that includes natural and synthetic hormones, pharmaceuticals, industrial chemicals, nutrients and ammonia. Discharge of MWWE may lead to serious problems in aquatic environments such as eutrophication, hypoxia as well as increased occurrence of disease and toxicity in resident aquatic biota. Reproductive impairment in fish has also been widely reported in association with exposure to wastewaters. Recently, concerns have been raised about the potential for municipal effluents to cause endocrine disruption in fish and other organisms. The effects of MWWE on fish and fish populations in Canada are currently poorly understood. The overall objective of this thesis is to contrast the impact of MWWE discharged into two Canadian rivers on sentinel fish species across levels of biological organization ranging from biochemical responses to changes at the fish community level. Results from these studies support the development of robust effects-based biological monitoring approaches to assess the effectiveness of regulations and remedial actions for minimizing the effects of MWWE.

Understanding the temporal changes in physiological and reproductive parameters across the annual cycle of a sentinel species is necessary to optimize biomonitoring programs. The annual variability in terms of survival, reproduction and energy storage in the Greenside Darter (*Etheostoma blennioides*), a potential sentinel species for the Grand River, Ontario, was documented at a reference site across two years. Variation in energy storage and reproductive development indicated by somatic indices (i.e., relative organ size) and steroid production suggest that biomonitoring can be optimized for this species by sampling in late fall or early spring (pre-spawning). With this new knowledge, field studies conducted with small bodied species, including Rainbow Darter (*E. caeruleum*), Brook Stickleback (*Culaea inconstans*) and Fathead Minnow (*Pimephales promelas*) have demonstrated

that, when sampled during the appropriate season (e.g., pre-spawning), they can be used as effective biomonitoring tools to detect fish responses associated with exposure to MWWEs.

Two sentinel fish species, Rainbow Darter and Greenside Darter, were then used to examine the impact of two MWWE discharges on fish in the Grand River, Ontario, relative to reference sites in two seasons (fall and early spring). Fish responses, in terms of energy storage (condition factor, liver size), energy utilization (gonadosomatic indices) and reproduction (in vitro sex steroid production, cellular development and intersex) were assessed at each site. Both sentinel species were longer and heavier downstream of the wastewater outfalls. However, these larger fish did not demonstrate consistent increases in condition and liver somatic indices. MWWE-exposed male Rainbow and Greenside Darters had impaired capacity to produce androgens in vitro, lower gonadosomatic indices and altered sperm cell staging. Exposed female fish also had impaired capacity to produce estrogens in vitro, however, they did not demonstrate differences in oocyte development. Male Rainbow and Greenside Darters collected downstream of both MWWE discharges showed increased incidence of intersex (33 - 100%) in contrast to very low occurrences of this condition in upstream agricultural and urban reference sites. This increased incidence of intersex coincided with reductions in gonadosomatic indices and capacity to produce steroids, demonstrating the ability of MWWE to alter the reproductive systems of these fish. The fish communities downstream of the the MWWE outfalls demonstrated differences in abundance, diversity, and species composition when compared to reference sites. MWWE exposed sites had few of the darter species that dominate the fish community at reference sites. More mobile fish species such as suckers (*Catostomidae spp.*) and sunfish (*Centrarchidae spp.*) were more common downstream of the outfalls, with occurances becoming more pronounced downstream of the second sewage discharge.

Wascana Creek, Saskatchewan, downstream of the wastewater treatment plant for the City of Regina can be up to 100% treated municipal wastewater. Brook Stickleback and Fathead Minnow

exhibited delayed spawning and altered gonadal development downstream of the wastewater outfall. Exposed male Fathead Minnows were feminized, having lower expression of secondary sexual characteristics (i.e., loss of nuptial tubercles, dorsal pad, and dorsal fin dot) and induction of the female egg-yolk precursor protein, vitellogenin. Fathead Minnows also showed cellular damage to the gills and kidneys. These responses indicate exposure to a variety of environmental contaminants in the effluent such as ammonia as well as endocrine disruptors.

The potential effect of MWWE discharges in these two Canadian watersheds on fish responses was demonstrated across various levels of biological organization including reduced sex steroid production, altered gonadal development, reduction in gonadosomatic indices, delayed spawning, and changes in fish assemblages. An effects-based monitoring approach using sentinel species can be successfully applied to detect changes associated with MWWE outfalls, as long as sampling of sentinel species is conducted during optimal time periods (i.e., when somatic indices are maximized and variability among individuals is minimized). MWWE can impair the reproductive potential of fish beyond a threshold where impacts are expressed at higher levels of organization such as populations or communities. It is essential to make mechanistic linkages between responses at different levels to determine the overall potential impact of effluents on fish. The collection of responses across multiple levels of biological organization can complement and support development of biomonitoring approaches that are focused at the population and community levels such as those being proposed for MWWE in Canada.

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Chapter 1

General Introduction

Adverse effects of municipal wastewater on fish have been documented at almost every level of biological organization (Figure 1.1). However, most studies examine the chemical concentration or biological effects at only one or two levels of biological organization (e.g., gene expression and/or organ size). Recently, research has focused on using genomics, bioinformatics and modeling approaches in concert with whole animal testing of well-studied fish species such as Fathead Minnow (*Pimephales promelas*) to identify response linkages across biological levels of organization (Ankley et al., 2010). This approach involves the development of predictive models to derive adverse outcome pathways (AOP) for compound-specific risk assessments. This approach has limitations as these models are subject to high uncertainty due to limited data availability and variability among studies and are limited in scope as they intended to be a tool for risk assessment of a single chemical and not mixtures such as municipal wastewater effluent (MWWE) (Kramer et al., 2011).

Despite the diversity of publications few studies have identified effects of MWWE across multiple levels of biological organization. Our poor understanding of these linkages currently limits our ability to accurately predict effects on fish in the receiving environment. This thesis is a series of field studies using population and community level responses in fish, as well as physiological endpoints and histopathology, to better understand responses of exposure to MWWEs among the different levels of biological organization. Understanding the potential impacts of MWWE discharges in Canada is necessary to effectively interpret and validate future assessments and monitoring programs.

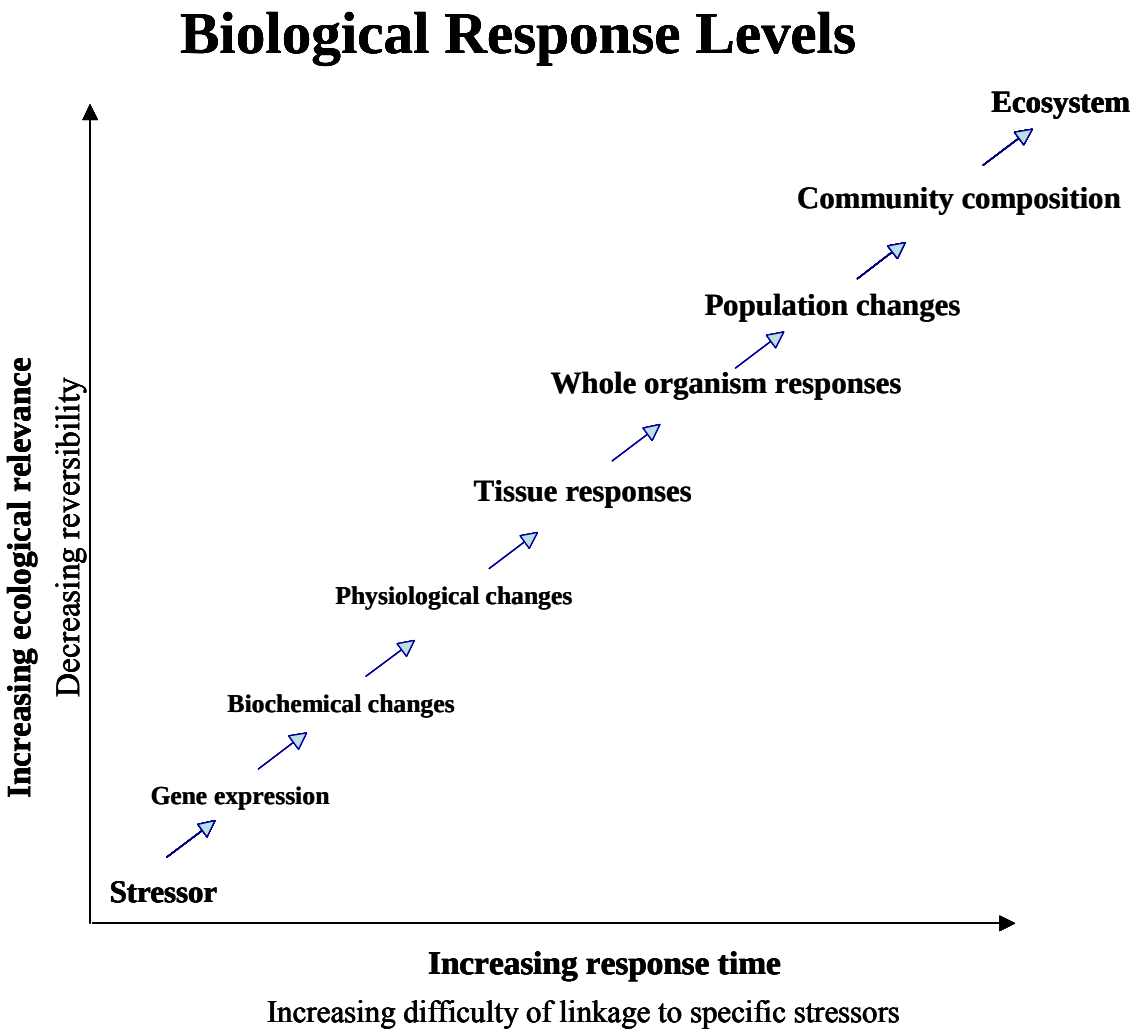

Figure 1.1. The progression of increasing response times and ecological relevance in relation to levels of biological organization. As ecological complexity and relevance increases, the response time increases and there is a decreasing ability to respond and reverse the effect (modified from Walker et al., 2001).

1.1 Municipal wastewater effluent

Wastewater treatment is a complex series of processes that involves several steps to remove solids, nutrients and contaminants (Metcalf & Eddy Inc. et al., 2004). Although the principles are similar, the actual configurations of wastewater treatment facilities vary considerably and the level of treatment and disinfection varies among municipalities across Canada (Chambers et al., 1997; Holeton et al., 2011). Wastewater treatment normally starts with primary treatment, which is the removal of large solids, such as debris, garbage, gravel, and sand, with a screen and settling tank. Secondary treatment is designed to remove the organic matter with bacteria and aerobic digestion followed by more settling of suspended solids (clarifiers). A proportion of the material settled out from this process is usually diverted back to the primary settling tanks to seed bacteria back into the system. The remaining solids are diverted to digesters where anaerobic bacteria utilize the organic material present in the sludge as a food source and produce carbon dioxide and methane gas. When present, tertiary treatment removes additional dissolved or suspended substances, but it is variable from plant to plant. For example, some tertiary treatment is targeted at removal of phosphorous to reduce enrichment of receiving waters while others remove ammonia, metals and organics to reduce effluent toxicity. The final effluent is then often disinfected using chlorine, ultra violet (UV) radiation, or ozone (Metcalf & Eddy Inc. et al., 2004).

Significant investments in wastewater treatment have been made to reduce the impacts of biochemical oxygen demand (BOD), total suspended solids (TSS) and nutrients released in MWWE. Despite these improvements, degradation of the quality of receiving waters remains an issue across Canada (Holeton, et al., 2011). In addition, recent concerns have been raised about a wide variety of "emerging" contaminants in MWWE that may be biologically active (Daughton and Ternes, 1999). MWWE discharges have been identified as the major source of "emerging" environmental contaminants in the aquatic receiving environment around the globe (Purdom et al., 1994; Tyler et al.,

1996; Harries et al., 1997; Ternes et al., 1999; Servos et al., 2003; 2005). As treatment facilities have not been designed to remove these emerging contaminants of concern, many are discharged in the final effluent or are present in biosolids. Emerging chemicals detected in Canadian wastewater and surface waters include pharmaceuticals and personal care products (PPCP) such as anti-inflammatories (ibuprofen, naproxen), lipid regulators (gemfibrozil), antiepileptics (carbamazepin), antidepressants (fluoxetine) (Metcalfe et al., 2003; Lee et al., 2005; 2007; Lishman et al., 2006; Tanna, 2012), natural hormones such as 17β-estradiol and estrone (Servos et al., 2005), and industrial compounds such as bisphenol-A (Lee et al., 2004; Mohapatra et al., 2011) and nonylphenol (Lee and Peart, 1995; Servos et al., 2003; Gross et al., 2004). Although many of these chemicals are short-lived in the environment, they are considered "pseudo-persistent" because they are being used and discharged into the aquatic environment on a continual basis (Sumpter and Johnson, 2005). PPCPs are of particular interest because these products are designed to be biologically active and elicit or suppress physiological responses in humans or target species (livestock, etc.) (Purdom et al., 1994; Knacker et al., 2010). Many of these emerging chemicals detected in effluents and surface water can alter the function of the endocrine system, resulting in impacts on growth and reproduction in fish at very low concentrations (Tyler et al., 1996; Jobling et al., 1998; Jobling et al., 2002ab; Blazer et al., 2006; Jobling and Tyler, 2006; Jobling et al., 2006; Vajda et al., 2008; Vajda et al., 2011; Tanna, 2012).

1.2 Biological responses

Fish have various compensatory mechanisms to deal with changes in their environment. These include mobilization and reallocation of energy resources to meet the increased energy demand of the physiological processes that maintain homeostasis. Chronic exposure to industrial or municipal wastewater effluents may lead to adverse effects in fish by causing a reallocation of energy resources at the expense of normal growth, reproduction, organ function, and cellular integrity. Exposure to an effluent may impair gene expression that prevents the biosynthesis of a protein that can ultimately alter

physiology and the development of organs. Physiological changes occur over relatively short time periods (weeks to months). Research involving physiological endpoints can aid in the determination of cause-effect relationships between exposure and the responses by fish (Munkittrick et al., 1991; 1992). Alterations in synthesis of key proteins (vitellogenin), histopathology and sex steroid levels may be predictive of whole body responses in fish (growth, energy use and energy expenditure) (Munkittrick et al., 2000). A better understanding of how physiological and reproductive endpoints respond to MWWEs and how changes in these endpoints relate to potential responses at higher levels of organization is needed to use these endpoints in biological monitoring programs.

Evaluation of fish responses is often conducted in an effects-based assessment where fish are collected immediately downstream of a discharge and are compared to those from an upstream reference site or reference lake (Fish Survey Expert Working Group, 1997). This approach is the foundation of Environment Canada's Environmental Effects Monitoring Program (EEM), which focuses on the assessment of the effects rather than on the identification of the pollutants and aims to provide ecologically-relevant results for decision makers. The Canadian Fisheries Act requires that fish, fish habitat and human use of the fisheries resource are protected. The Adult Fish Survey (AFS) in the EEM program is one approach to evaluate the effectiveness of environmental regulations and remedial activities on fish. By using fish species, this approach can detect change over time and space and provide a wide variety of population-level response data, ranging from changes in recruitment to individual-level measurements such as survival, growth, reproduction and energy storage.

In order to conduct effective biological monitoring programs, study designs must consider the reproductive cycle of the sentinel species to be evaluated. Barrett and Munkittrick (2010) conducted a review of Canada's EEM program to develop recommendations for the design of monitoring programs. Their review revealed that 72% of EEM studies using small-bodied fish for the fish surveys underestimated the magnitude of the impact on the receiving environment by conducting their surveys

at inappropriate times in the reproductive cycle of those fish. They concluded that conducting fish collections during a period when growth among individuals is highly variable reduces the power of surveys to detect differences among sites. Therefore it is imperative to take into consideration the reproductive profile of a sentinel species for efficient environmental monitoring. This also emphasizes the importance of knowing the life history of the sentinel species when interpreting site-specific monitoring programs. Despite the limitations, Environment Canada's EEM Program has been successfully applied to evaluate existing effluent regulations under the Fisheries Act in the pulp and paper and metal mining sectors (Kilgore et al., 2005).

The Adult Fish Surveys (AFS) in the EEM program evaluates the health of fish in aquatic environments receiving industrial effluent. In recent years, these surveys have utilized several small-bodied fish species including Johnny Darter, *Etheostoma nigrum* (McMaster et al., 2002), Trout-Perch, *Percopsis omiscomaycus* (Gibbons et al., 1998b; Tetreault, 2003), Slimy Sculpin, *Cottus cognatus* (Tetreault et al. 2003; Gray and Munkittrick, 2005; Brasfield, 2007), Pearl Dace, *Semotilus margarita* (Tetreault et al., 2003), and Mummichog, *Fundulus heteroclitus* (Dube and MacLatchy, 2001). These small fish have several characteristics which make them excellent candidates as sentinel species including a relatively short life span (i.e., they respond to effects on shorter time scale), sufficient abundance and increased certainty of exposure (i.e., reduced mobility) (Gray et al., 2002). The reduced mobility of small fish species allows for the collection of fish at multiple sites upstream of the area of exposure as well as downstream prior to another input discharging into the river allowing the separation of effects from multiple inputs. The reduced mobility of most small fish will increase confidence in site fidelity and the ability to detect effects among sites. Unfortunately, little is often known of the reproductive biology of most small fish species and research is required to optimize the most appropriate times to sample these species.

Although the EEM approach has been proposed for MWWEs across Canada, its ability to detect population-level responses in fish exposed to MWWEs has not been tested. Fish respond to MWWE in a variety of ways. Studies of Roach, *Rutilus rutilus* (Jobling et al., 1998), Flounder, *Platichthys flesus* (Allen et al., 1999), Gudgeon, *Gobio gobio* (van Aerle et al., 2001), White Sucker, *Catostomus commersoni* (Hinck et al., 2009), and Smallmouth Bass, *Micropterus dolomieu* (Iwanowicz et al., 2009) collected downstream of MWWEs reported that exposed fish had larger body mass indicating increased investment in energy storage compared to fish at upstream reference sites. Decreases in liver size (energy storage) have also been reported in wild Flounder in UK sewage estuaries (Allen et al., 1999). Roach (Jobling et al., 1998), Flounder (Allen et al., 1999), Bream, *Abramis brama* L. (Hecker et al., 2002), White Sucker (Vajda et al., 2008) and Smallmouth Bass (Iwanowicz et al., 2009) exposed to MWWE have also demonstrated reduction in energy utilization (gonadal development). These recent studies provide evidence to suggest that there are widespread effects of MWWE at the whole organism level. This emphasizes the need to research the potential for subsequent population-level responses in fish exposed to MWWE.

Exposure to MWWE has been correlated with deformities in the testicular tissue of fish (Jobling et al., 2002b). These changes in male fish include altered gamete production, malformations of the reproductive duct(s) preventing release of gametes (Vajda et al., 2008), fibrosis and inhibition of testicular development (Woodling et al., 2006). Effects observed in female fish include advanced oocyte development (Douxfils et al., 2007) and asynchronous ovarian development (Woodling et al., 2006; Vajda et al., 2008). It is imperative to investigate how changes in gonadal development can influence the reproductive success of fish, and how these alterations in gonadal development may lead to impacts at the population level.

There also is the potential for adverse effects of treated wastewater on non-reproductive organs such as the kidney. Studies have documented that tubules of the kidney tissue of fish exposed

to the active ingredient in the birth control pill, 17α-ethynylestradiol (EE2), were enlarged, had intertubular edema and hyaline degeneration of the proximal tubules. These effects lead to inflammation and leakage across the glomeruli resulting in malfunction of the kidneys and eventual renal failure (Länge et al., 2001; Zaroogian et al., 2001; Palace et al., 2006). These effects were attributed to the filtration by the kidneys of elevated concentrations of the large vitellogenin molecule. Other deformities detected in the kidneys of fish exposed to wastewater include cellular degeneration or vacuolar alterations of the renal tubules (Bucher and Hofer, 1993; Palace et al., 2006). These adverse effects are of particular concern in effluent-dominated streams where municipal and/or industrial wastewater effluents are discharged and may comprise the majority of the flow (Brooks et al., 2006).

1.2.1 Responses to emerging contaminants

In environments receiving MWWEs, a variety of impacts have been detected on reproduction and development in fish that are mediated by changes in the function of the endocrine system (Fent et al., 2006). An endocrine-disrupting chemical (EDC) as defined by the U.S. Environmental Protection Agency (EPA) is an exogenous compound that interferes with the production, release, transport, metabolism, binding, action or elimination of natural hormones responsible for maintaining homeostasis and regulation of developmental processes (Kavlock et al., 1996). A class of EDCs which are often studied in MWWE are estrogenic compounds as they can cause alterations in reproduction and development along the hypothalamic-pituitary-gonadal (HPG) axis, in particularly through the estrogen receptor (ER) (Ankley et al., 2010). This allows the extrapolation across vertebrate test species and to human health as the HPG axis is highly conserved across vertebrate classes (Ankley et al, 2009). The production of gonadal sex steroids is the result of a complex chain of events as fish integrate information from external cues, which stimulate hormonal responses within the hypothalamic

region of the brain (McMaster et al., 1995ab). Regulation of these processes involves the stimulation and release of gonadotropin (GtH) from the pituitary gland into the blood stream. Gonadotropins are transported through the blood stream to the ovaries or testes to stimulate the production of sex steroids leading to gonadal growth (Ankley et al, 2009). This system is normally controlled by a negative feedback system, which halts when the sex steroids produced are circulated back to the hypothalamic region. If this negative feedback mechanism is impaired, the ability of the gonads to produce steroids is also impaired, and if the impairment persists, it can result in altered gonadal development (Van Der Kraak et al., 1992).

The measurement of circulating sex steroids in the plasma of fish has been used as a reproductive endpoint to evaluate the impact of an effluent on fish (McMaster et al., 1995b; Van Der Kraak et al., 1992). This information provides greater insight into the reproductive status of the fish under study and potential mechanisms of action of compounds of concern. Reductions in gonadal development and alterations in the expression of secondary sexual characteristics in wild fish exposed to pulp mill effluent have been positively correlated with decreased circulating plasma levels of sex steroids (McMaster et al., 1996; 2005). As it is difficult to obtain enough plasma to determine circulating levels of sex steroids in small fish species, McMaster et al. (1995a) developed an in vitro bioassay to measure the production of sex steroids by the gonadal tissue of fish. Typically, the basal production of steroids is measured as well as some form of stimulated steroid production to provide further information about the integrity and maximal capacity of the tissue to produce steroid hormones. Research is needed to determine the basal capacity of gonadal tissues of fish exposed to MWWE to produce sex steroids in vitro as well as the capacity of gonadal tissue to respond to a chemical messenger to increase production.

An exogenous compound binding to the estrogen receptor is the key initiating event for impairment of reproduction and development (Ankley et al., 2010). Activation of ER receptors in the

liver results in synthesis of vitellogenin (VTG) which is then transported through the blood to be sequestered in the gonads and VTG forms the major constituent of yolk, which is subsequently utilized by a growing embryo as a food source. This protein is not produced by male fish under normal conditions due to the absence of circulating estrogens. However, if exposed to an estrogenic compound a male fish will produce the VTG protein to levels found in female fish (Harries et al. 1996; 1997). Conversely, the inhibition of E2 by an estrogen antagonists binding to ER results in reduced circulating VTG, resulting in reduced oocyte development and fecundity in female fish (Ankley et al., 2010). The knowledge of this mechanism has lead to VTG becoming a biomarker for exposure to estrogenic contamination in the environment (Purdom et al., 1994; Sumpter and Jobling, 1995; Jobling et al., 1998; Allen et al., 1999; Hecker et al., 2002; Kavanagh et al., 2004; Jefferies et al., 2008; Vajda et al., 2008; Iwanowicz et al., 2009). In a study of two prairie rivers in Alberta, male fish exposed to MWWE demonstrated increases in VTG expression and there was a female-biased sex ratio in the population suggesting severe endocrine disruption was occurring (Jefferies et al., 2008). Male Fathead Minnows exposed to EE2 in a whole lake experiment had 9000-fold higher concentrations of VTG than fish captured in the same lake prior to EE2 additions (Palace et al., 2002). Fathead Minnows from this same exposed lake also demonstrated altered oogensis in females, oocytes in male testicular tissue (intersex), and subsequent loss of recruitment leading to near extinction of the population (Kidd et al., 2007).

Exposure of fish to estrogenic compounds in municipal wastewater has also been associated with the occurrence of intersex (Jobling et al., 1998). Substantive evidence has been documented to support the hypothesis of estrogenic compounds contributing to the cause of intersex in exposed fish, however the relationship of induction of VTG and the presence of intersex in males still needs to be developed (Jobling et al., 2006). Intersex has been reported in a variety of wild fish species collected downstream of MWWEs in the UK, Europe, and North America (Jobling et al., 1998; Sole et al., 2003; Bjerregaard et al., 2006; Hinck et al., 2009; Vajda et al., 2011). Although many studies have measured

elevated levels of vitellogenin and reported the intersex condition in male fish exposed to wastewater effluent, intersex is poorly correlated with plasma vitellogenin in the same fish suggesting that compounds may be active through additional pathways that result in reproductive impairment in fish (Jobling et al., 2006; Ankley et al., 2010). Although feminization responses in fish exposed to MWWE are well documented, the impact of long-term exposure of fish populations is not known. However, recent studies of chronic exposure to estrogenic MWWE have demonstrated population-level effects such as sex reversal and impaired breeding performance of feminized male fish (Harris et al. 2011; Lange et al. 2011).

The effects of MWWEs on endocrine disruption in fish have not previously been studied in a comprehensive way in river systems in Canada. Considering that municipal wastewater is such a large source of potential endocrine disrupting contaminants in the Canadian aquatic environment, it is crucial that the potential impacts to fish and fish populations be more clearly defined.

1.2.2. Responses to poor water quality

The degradation of water quality in the receiving environment is strongly linked to increased urbanization, and is especially pronounced when MWWE from a treatment plant servicing a large urban population discharges into a small receiving environment (Brooks et al., 2006; Waiser et al., 2011ab). Historically, impacts of continuous discharges of MWWEs include the increase in toxic chemicals such as ammonia, eutrophication due to increased nutrient inputs, and increased BOD. The effects of eutrophication on aquatic biota have been demonstrated downstream of MWWE discharges in the United Kingdom (Harries et al., 1996), the United States (Koplin et al., 2002; Blazer et al., 2007) and Canada (McMaster et al., 2005; Jefferies et al., 2008). In most Canadian rivers, nutrient enrichment leads to increased primary productivity (macrophyte and algal growth) and potential food availability. Decomposition of in-stream organic matter and nocturnal respiration of abundant aquatic

vegetation during the summer consumes oxygen and leads to decreased dissolved oxygen (DO) levels resulting in hypoxia, defined as 0 to 2.0 mg/L DO (Thomas et al., 2007).

Hypoxia has been shown to alter endocrine function in fish, likely through the hypothalamus-pituitary-gonadal-liver (HPGL) axis (Thomas et al, 2007). Fish collected from a hypoxic area demonstrated reduced secretion of gonadotropins, altered steroid levels, reduced liver ERα mRNA and vitellogenin, altered gonadal development leading to lower fecundity and impaired spermatogenesis, reduced gonadosomatic index and skewed sex ratios (Thomas et al, 2007). This cascade of effects is influenced by the rate-limiting step of secretion of hypothalamic serotonin, which requires molecular oxygen. A second site along the HPGL axis that may also be influence by hypoxia is the oxygen-dependant cytochrome P450 enzyme CYP19, resulting in reduced aromatase activity which is responsible for converting testosterone to 17β-estradiol (Shang et al., 2006). Disruption at this location in the HPGL axis would result in a similar cascade of effects.

In a freshwater fish, ammonia is eliminated from the blood via passive diffusion through the gills (branchial ammonia excretion) or through the conversion to urea in the kidneys (Randall and Wright, 1987). In an environment receiving MWWE, elevated environmental ammonia levels may reduce excretion and even result in a net uptake of ammonia by the fish. Accumulation of ammonia in tissues is deleterious to fish. The effects of chronic exposure to environmentally relevant concentrations of ammonia include irregular gill ventilation and morphology (Person-Le Ruyet et al., 1997), reduction growth rate of fish (Fairchild et al., 2005), hyperplasia of the gill epithelium and fused lamellae (Soderberg et al., 1984). Ammonia and nitrate, which are often elevated in wastewater effluents, may also alter reproduction in vertebrates by inhibiting gonadotropin-induced synthesis of androgens, however, this has not been demonstrated in freshwater teleosts (Guillette and Edwards, 2005). The potential for cumulative impacts of nutrient enrichment from agricultural and domestic

sources has also been identified as an area in need for research in Canada (Chambers et al., 1997) and continues to be an issue (Holeton et al., 2011).

Treated MWWEs have the potential to have negative effects on higher levels of biological organization in the aquatic ecosystem (Brown et al. 2011; Lange et al. 2011). Effects on fish communities from exposure to MWWEs have included changes in abundance (Dyer and Wang, 2002; Porter and Janz, 2003; Ra et al., 2007; Yeom et al., 2007), decreased diversity (Ra et al., 2007), and increased proportion of tolerant species and omnivorous, opportunistic fish (Ra et al., 2007; Yeom et al., 2007). A study by Wickert (1995) demonstrated the return of previously extirpated fish to receiving waters following reductions in nutrient loading and BOD resulting from upgrades to sewage treatment plants. The impacts of MWWE on aquatic organisms in the environment need to be understood in order to define potential environmental risks and to formulate appropriate remedial actions on a site-specific basis (Knacker et al., 2010). Increasing our understanding of the impacts of MWWE on aquatic organisms allows the potential environmental risks of MWWE to be better defined and to provide environmental managers with better information to develop remedial actions.

1.3 Thesis objectives

The overall objective of this thesis is to examine the impact of MWWE on the performance of wild fish in Canadian environments. To accomplish this, field studies were conducted in two urban Canadian rivers, the Grand River and Wascana Creek. The Grand River watershed in southern Ontario is representative of a complex Canadian receiving environment servicing many municipalities. This watershed is expected to have a 38% increase in population by 2036 which may further threaten water quality (Environmental Commissioner of Ontario, 2010). In addition to eutrophication, oxygen levels in the river downstream of one of the major municipal sewage treatment plants frequently fall below 4 mg/L resulting in seasonal hypoxic conditions. Wascana Creek, in southern Saskatchewan, is a small

stream, which receives treated sewage effluent from the City of Regina and surrounding area. During the winter months this effluent comprises 100% of the creek flow and concentrations of ammonia are consistently above guidelines for the protection of fish during early life stages and sensitive aquatic species. Wascana Creek is therefore an effluent-dominated system that is well suited for studying aquatic organism responses to the variety of environmental contaminants discharged in MWWEs.

This thesis is comprised of a series of field studies involving the measurement of population- and community-level responses, as well as physiological and histopathology endpoints, to better understand the relationships of fish responses to MWWE exposure among different levels of biological organization.

This research is composed of four phases.

1. The first phase was to generate knowledge of the temporal changes in physiological and reproductive parameters across the annual cycle of a sentinel species that is needed to determine the optimal period for collection in studies evaluating the impact of MWWE on fish (Chapter 2).
2. Incorporating the new knowledge generated in Chapter 2, fish exposed to MWWE in selected Canadian receiving environments were studied to determine the specific physiological and reproductive responses in exposed fish (Chapters 3 and 4).
3. Adverse effects of MWWE on the histopathology of respiratory and excretory organs of exposed fish were examined (Chapter 4).
4. Knowledge gained from Chapters 2, 3 and 4 were used to link reproductive impairment of sentinel species exposed to MMWE with changes in fish community assemblages downstream of MWWE discharges (Chapter 5).

Chapter 2

Description of seasonal patterns of the reproductive development of the Greenside Darter (*Etheostoma blennioides*)

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Tetreault G.R., Bennett, C.J., Servos, M.R., McMaster, M.E. 2011. Description of seasonal patterns of the reproductive development of the Greenside Darter (*Etheostoma blennioides).* Prepared for scientific journal submission.

2.1 Summary

This study describes seasonal variability of key endpoints in sentinel species in order to aid in the interpretation of effects and design effective monitoring programs. Sentinel fish species collections can provide a wide variety of population-level information, ranging from changes in recruitment to individual-level measurements of survival, growth, reproduction, and energy storage. The objective of this study was to characterize the seasonality of whole body fish measures of a wild reference population of the Greenside Darter, *Etheostoma blennioides*. It is a small, benthic, spring multi-spawning species, common throughout the mid-west of the United States of America and south-western Ontario, Canada. We observed and described the seasonal changes in gonadosomatic index (GSI), liver somatic index (LSI), condition factor, and in vitro gonadal production of estradiol (E2) and testosterone (T) in females and T and 11-ketotestosterone (11KT) in males. This study provides evidence for collecting Greenside Darters 4 to 6 six weeks pre-spawn in order to achieve temporal stability, minimum variability, maximum statistical power, maximum value in GSI, and maximum steroid production capacity. This information can be used to maximize the potential to detect effects of effluent exposure on fish populations.

Keywords: reproductive strategy, reproductive profile, recrudescence, Greenside Darter

2.2 Introduction

There has been extensive research on basic life history of economically and commercially important fishes for such as *Esox sp.* (Craig, 2008; Edeline et al., 2009), *Stizostedion canadense* (Haxton and Findlay, 2009), *Perca flavescens* (Kocovsky et al., 2010), *Oncorhynchus spp.*, and *Salvelinus spp.* (Mobrand et al., 1997). Much less information is available on basic life history traits or reproductive development of small forage fishes. However, over the last decade many of these fishes have been used to evaluate the impact of anthropogenic activities on the aquatic environment. Environmental monitoring using fish species can provide a wide variety of information including changes of survival, growth, reproduction and energy storage (Environment Canada, 2011). Conducting biological monitoring for the purpose of evaluating the responses of biota to effluents when biological growth is suboptimal will reduce the statistical power of the study and will impede the ability to detect impacts in the receiving environment.

The Greenside Darter (*Etheostoma blennioides*) is one of the most abundant small fish species in the Grand River making it an excellent candidate to evaluate the impact of municipal wastewater. The Greenside Darter was listed as a Species of Special Concern under Schedule 3 of COSEWIC (Committee on the Status of Endangered Wildlife in Canada) (COSEWIC, 2006). The COSEWIC recovery strategy was based on the Sydenham River, Ontario, where the Greenside Darter is native (Dextrase, 2005). It was introduced into the Grand River ecosystem in approximately 1990 and has increased its distribution throughout the watershed. With new information on its range and distribution collected over the last several years, the Greenside Darter was delisted as a species of Special Concern by COSEWIC in November of 2006 (COSEWIC, 2006). The increased distribution of this species in the Grand River indicates that these populations would be able to withstand the minor collection pressure of this study. The general biology and distribution of the Greenside Darter is well described, however there is a knowledge gap related to the storage, utilization and allocation of energy resources

during its reproductive cycle. This information is necessary to determine the optimal time to sample this species to minimize parameter variability, maximize statistical power and decrease the sample size required for environmental monitoring.

Small-bodied fish have numerous advantages for use in monitoring programs and have been used frequently in recent environmental monitoring and research programs in Canada. These species include Johnny Darter, *Etheostoma nigrum* (McMaster et al., 2002), Trout-Perch, *Percopsis omiscomaycus* (Gibbons et al., 1998b, Tetreault et al., 2003), Spoonhead Sculpin, *Cottus ricei* (Gibbons et al., 1998a), Slimy Sculpin *Cottus cognatus*, Pearl Dace *Semotilus margarita* (Tetreault et al., 2003), and Mummichog, *Fundulus heteroclitus* (Dube and MacLatchy, 2001). The reduced mobility of small-bodied fish species allows for the collection of independent samples of fish at multiple sites upstream and downstream of an area of exposure allowing the separation of effects from multiple inputs within a very narrow geographic range. However, life history information necessary to interpret data collected on small-bodied fish is limited.

Effects-based assessment is an approach to evaluate the impact of an anthropogenic activity on fish collected immediately downstream of a discharge, by comparing them to fish from an upstream reference site or reference lake (Fish Survey Expert Working Group, 1997). This approach is the foundation of Environment Canada's Environmental Effects Monitoring (EEM) Program, which focuses on the effects observed in aquatic biota rather than on the identification of the pollutants in a receiving environment. The EEM program is designed to provide ecologically relevant results to decision makers.

Sampling fish at inappropriate times, such as outside of the peak gonadal development stage, may hinder the detection of possible reproductive effects. In an attempt to clarify some of the variability in fish responses to pulp and paper mill effluents, Barrett and Munkittrick (2010) conducted a literature review to obtain details on the biology of fish species used in EEM studies in Canada and

to recommend sampling times based on periods of temporal stability, minimum variability, and maximum value in gonadosomatic indices (Barrett and Munkittrick, 2010). This study revealed that 72% of EEM studies using small-bodied fish were not conducted at the appropriate time of the reproductive cycle, resulting in an underestimation of the magnitude of impacts of industrial effluents on the receiving environment. The designs of environmental monitoring programs can be optimized using sampling times recommended based on reproductive strategies and life history characteristics of the sentinel species. Standardizing sampling times based on reproductive strategies will reduced the variability among studies and allows comparison of data between studies.

Population characteristics of fish species have been used to infer the status of aquatic environments and this approach has been applied in the evaluation of point-source impacts such as pulp and paper effluents (McMaster et al., 2002), metal mining discharges (Environment Canada, 2011), oil sands development (Tetreault et al., 2003), nonpoint sources from agriculture (Gray et al., 2005) and urban areas (Fitzgerald et al., 1999). These studies involve monitoring sentinel species to obtain estimates of energy storage through body condition and liver somatic indices, estimates of investment in gonad development and growth, and estimates of survival based on age structure (Munkittrick et al., 2000). Recent studies involving non-lethal sampling methods have incorporated population length-frequency distributions to identify age-class structure, growth rates and recruitment success, especially in small fish with fewer age classes (Grey et al., 2002).

Although the basic natural history of the Greenside Darter has been investigated (Fahy, 1954; Scott and Crossman, 1998), changes in growth, reproduction and survival of this sentinel species are not well known. The objective of this study was to characterize the reproductive seasonality of a wild reference population of the Greenside Darter to provide guidance as to the most appropriate time to collect this species for efficient environmental monitoring of municipal wastewater and to facilitate

interpretation of monitoring data. This study also provides new information for this species that could be useful in recovery programs in other regions.

2.3 Materials and methods

Greenside Darters were collected from the Eramosa River (43° 32’ 88”N, 80° 10’ 9”W), in southern Ontario, Canada, on a monthly basis from September 2005 to January 2007. The Eramosa River is part of the Speed-Eramosa subbasin which drains 780 km^2 on the eastern side of the Grand River watershed and flows through a largely forested and wetland (34%) land cover with water quality and temperature capable of sustaining trout populations (M. Anderson, pers. comm.). Fish collections were unable to take place in January and February 2006 due to ice cover, nor in October 2006 due to high water levels. Greenside Darters were collected by sampling flowing runs and riffles (approximately 1.1–1.5 m/s) approximately 0.5 to 0.75 m deep with boulder/cobble substrates, between the hours of 09:00 and 12:00 h. During collection events, water surface samples were collected and measured by standard methods with a YSI 650MDS meter with 600 QS Sonde for temperature, conductivity, DO, and pH. Fish were collected using a backpack electrofishing unit (Smith-Root Type 12-D or Halltech HT-2000). Stunned fish were removed using dip nets (approximately 0.5 cm mesh size) held downstream of the electrofishing unit to collect fish swept downstream. Fish were transported live to the on-site laboratory trailer in aerated buckets, transferred to an aerated tank, and held for no more than 1 h prior to sampling to standardize holding time across sampling periods among months. During each sampling session, attempts were made to collect the first 100 individuals to establish annual length-frequency distributions in this population of darters (females, male and immature fish inclusive). Catch per unit effort (CPUE) per sampling event is expressed as the number of fish collected as a function of the number of electrofishing seconds for that event (Table 2.1). Female and male darters were sexed externally by the presence of a genital papilla

(Miller, 1968). A sub-sample of 20 female and 20 male adult darters were randomly selected to be sacrificed for the fish health assessment endpoints of condition factor (k) [(body weight / length^3)*100], liver somatic index (LSI) [(liver weight / body weight)*100], and gonadosomatic index (GSI) [(gonad weight / body weight)*100]. Each fish was rendered unconscious by concussion, euthanized by spinal severance, and fork length (±1.0 mm) and body weight (±0.01 g) were measured. The internal organs were removed and the gonads (±0.01 g) and liver (±0.01 g) were weighed. A sub-sample of gonadal tissue was taken from 10 adults of each sex, weighed and placed in incubation media for analysis of in vitro steroid hormone production (McMaster et al., 1995a). All fish were handled according to the University of Waterloo's and Environment Canada's Animal Care Committee Protocols (AUP 02-24, 08-08 and AUP-810; respectively).

2.3.1 In vitro steroid production

Incubations of gonadal tissues were used to estimate the steroid-producing potential of fish during each sampling period. Replicate samples of 20 $\pm$ 2 mg of ovarian tissue from females and 20 $\pm$ 2 mg of testicular tissue from males were incubated in nutrient media. Depending on the mass of tissue available, replicates of ovarian tissue were either unstimulated (basal, media alone) or were treated with 5 μL of 40 μM forskolin solubilized in ethanol to stimulate steroid production. Due to low mass of testicular tissue in this species (~1% body weight), all steroid levels for males are based on stimulated replicates. Gonadal tissues were incubated for an 18h period at 16-18°C, and then the media was drawn off the incubation wells and frozen in liquid nitrogen for transport to the laboratory. Concentrations of testosterone (T) (both sexes), 17β-estradiol (E2) (females) and 11-ketotestosterone (11KT) (males) released into the media during the incubation period were quantified by radioimmunoassay (RIA) (McMaster et al., 1995a).

2.4 Statistical analyses

Analysis of fish data were conducted between months for both sexes separately. As this study was trying to investigate the variability in fish indices and steroid production between successive months over a reproductive cycle, comparisons were only conducted between the preceding and the following month (adjacent months) for any given month. For example, parameters for October were only compared to those in September and November for monthly differences. Length frequency distributions (0.5 cm size classes) were compared between months using the two-sample Kolmogorov-Smirnov test (Gray et al., 2002). Differences in fish length and body weight were evaluated using analysis of variance (ANOVA). Fish condition, LSI and GSI were evaluated using analysis of covariance (ANCOVA). Data were log-transformed to ensure a normal distribution of the biological data as biological measures usually occur on an exponential scale (Environment Canada, 2002). Tukey's post-hoc test was then used when $p<0.05$ to identify differences between adjacent months. Non-parametric Kruskal-Wallis tests were used to compare in vitro steroid production between adjacent months. Regression analysis was conducted on gonad data plotted against body weight to evaluate variability (R^2) in gonadal development within months (Barrett and Munkittrick, 2010; Barrett et al., 2010). Reduced variability in this relationship would increase the power of detecting site or month differences if they existed. All data analyses were conducted using SYSTAT 12.0 statistical software (SYSTAT, 2007).

2.5 Results

Water quality parameters were taken during each sampling event (Table 2.1). The highest recorded surface water temperature was in July (22^{o}C) and the lowest recorded water temperature was in January (0.38^{o}C). The highest measured conductivity in Eramosa River was also in January

Table 2.1. Water quality parameters and fishing effort measured during monthly fish collections from September 2005 to January 2007 on the Eramosa River, Ontario.

Month	Year	Water Temp (^{o}C)	Conductivity (μS/cm)	Dissolved Oxygen (mg/L)	pH	CPUE (fish/sec)*100
September	2005	18.20	631	NA	8.08	4.00
October	2005	4.23	577	27.7	9.26	5.27
November	2005	6.00	617	NA	7.17	4.92
December	2005	4.70	593	NA	7.71	4.56
March	2006	2.30	577	40	9.12	4.44
April	2006	4.70	522	45.1	9.3	3.52
May	2006	11.66	570	45.1	9.32	4.67
June	2006	19.60	574	45.1	9.42	1.17
July	2006	22.00	583	55.3	8.92	4.09
August	2006	17.90	609	54.3	9.16	3.66
September	2006	14.27	590	41	9.34	3.63
October	2006	NA	NA	NA	NA	NA
November	2006	6.9	596	18.6	9.55	4.92
December	2006	2.65	537	15.5	9.21	2.79
January	2007	0.38	640	22.7	9.33	3.16

(640 μ/Seimens) however conductivity did not differ significantly among months. CPUE was standardized to the number of fish per electrofishing seconds collected because a minimum of 100 individual darters were not collected every month. CPUE was lowest in June when the abundance of post-spawning fish was low and surface water temperatures were approximately 20^{o}C (Table 2.1). CPUE was highest in the October-November time period when fish may be actively foraging prior to ice-over, and again in the March-May period prior to spawning when fish may be seeking appropriate spawning habitat or suitable mates (Table 2.1). The lack of a significant change in CPUE from the beginning of the study to the end indicates that the study design had no impact on this population of darters.

Monthly length-frequency distributions of 100 individual Greenside Darters demonstrate a normal distribution pattern of length across the four periods of the reproductive cycle (Figure 2.1). Age determination was based on age structure from Bunt et al. (1998). The pre-spawning collections revealed two distinct age classes. The first are likely offspring from the previous spawning season (3.8-4.6 cm), and adults (>5 cm) who will spawn that year (Figure 2.1a). The darter population collected during the spawning season appears to be comprised of mostly adult fish (5.2-7.2 cm) (Figure 2.1b). Fish collections from July appear to provide evidence for the first appearance of young of the year (YOY) Greenside Darters (Figure 2.1c), however none were found the following month. In November (Figure 2.1d), we observed two distinct size-classes (YOY (3.6-5.0 cm) and adults $\geq$ 5.8 cm). These size-classes appear to persist over the winter period (Figure 2.1a). From September through to May we see gradual integration of the YOY class into the rest of the population. Over the winter, growth of that age-class is slow and has yet to integrate into the rest of the adult population.

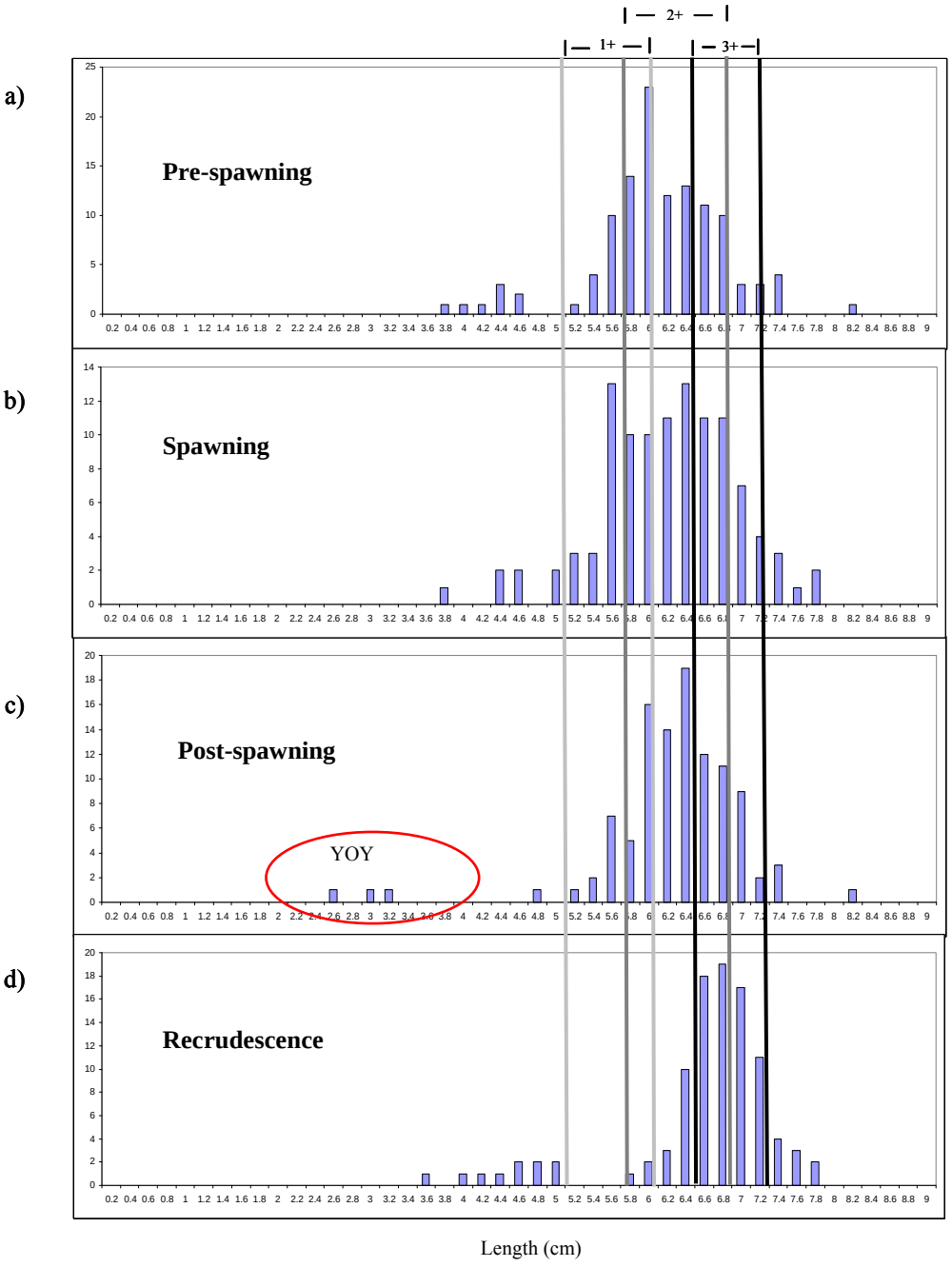


Figure 2.1. Length-frequency distribution (number of individuals / total fish length) of Greenside Darter collected during the annual cycle (a) pre-spawning (March), (b) spawning (May), (c) post-spawning (July) and (d) during recrudescence (November) in the Eramosa River, Ontario.

2.5.1 Parameters of fish health

Female darters had the lowest average length and weight in June (5.94 $\pm$ 0.14 cm, 2.02 $\pm$ 0.16 g; respectively), however fish length or weight did not differ significantly between the months of June through to December ($p \geq 0.056$) (Table 2.2). Female length increased significantly from March to April ($p \leq 0.001$), before decreasing again in May ($p \leq 0.001$). Male Greenside Darters did not demonstrate any statistically significant differences in length or body weight between adjacent months ($p \geq 0.121$).

Male condition was lowest in December 2005 (0.919 $\pm$ 0.01) while female condition was lowest in October of 2005 (0.937 $\pm$ 0.01 %) (Figure 2.2). Condition of male darters decreased significantly in December 2005 compared to November 2005 ($p=0.001$). Female darters increased again between March and April 2006 ($p \leq 0.001$), before decreasing in May ($p \leq 0.001$) prior to spawning in June 2006. Condition of darters of both sexes was significantly increased in January 2007 compared to condition observed in December 2006 ($p \geq 0.001$). There were no other significant differences in condition for female or male darters between adjacent months ($p \geq 0.086$).

Liver size, which is an indicator of available energy stores, change dramatically in the Greenside Darter throughout the year (Figure 2.3). Livers in female darters are at their lowest size after spawning in July (0.858 $\pm$ 0.11 %). Female liver size gradually increased from October 2005 to April 2006, reaching its maximum in April (3.90 $\pm$ 0.17 %) ($p \leq 0.011$). Male liver size increase was not as dramatic, and was lowest in September 2006 (0.57 $\pm$ 0.06 %) and similar to the female profile, peaks in April (1.62 $\pm$ 0.13%). The only statistical difference between months in male liver sizes occurred between October and November 2005 ($p=0.001$) and between April and May 2006 ($p=0.008$).

Table 2.2. Monthly, length, weight, liver and gonad weight of female and male Greenside Darters (*E. blennioides*) from the Eramosa River collected from September 2005 to January 2007. Values are reported as mean ± SE. * indicates significant difference in parameter from the preceding month.

Month	Year	Sex	Length (cm)	Weight (g)	Gonad (g)	Liver (g)
September	2005	Female	6.92 ± 0.08	2.95 ± 0.23	0.062 ± 0.003	0.021 ± 0.002
		Male	6.84 ± 0.06	3.19 ± 0.07	0.028 ± 0.002	0.019 ± 0.002
October	2005	Female	6.65 ± 0.09	2.75 ± 0.09	0.096 ± 0.002 *	0.035 ± 0.003 *
		Male	6.76 ± 0.10	3.06 ± 0.15	0.037 ± 0.001	0.026 ± 0.002
November	2005	Female	6.46 ± 0.05	2.67 ± 0.05	0.123 ± 0.006 *	0.047 ± 0.004 *
		Male	6.52 ± 0.09	2.83 ± 0.13	0.027 ± 0.003	0.02 ± 0.002 *
December	2005	Female	6.18 ± 0.05	2.27 ± 0.06	0.121 ± 0.008	0.050 ± 0.003
		Male	6.07 ± 0.07	2.18 ± 0.11	0.020 ± 0.002	0.023 ± 0.002
March	2006	Female	6.08 ± 0.05	2.30 ± 0.07	0.183 ± 0.023	0.088 ± 0.007
		Male	6.39 ± 0.10	2.48 ± 0.14	0.022 ± 0.003	0.034 ± 0.005
April	2006	Female	6.39 ± 0.05 *	2.98 ± 0.08	0.44 ± 0.029 *	0.115 ± 0.007
		Male	6.52 ± 0.10	2.79 ± 0.14	0.030 ± 0.004	0.043 ± 0.002
May	2006	Female	6.15 ± 0.07 *	2.47 ± 0.08	0.329 ± 0.020	0.084 ± 0.011
		Male	6.38 ± 0.13	2.65 ± 0.18	0.027 ± 0.004	0.026 ± 0.004 *
June	2006	Female	5.94 ± 0.14	2.02 ± 0.16	0.061 ± 0.005 *	0.031 ± 0.004 *
		Male	6.50 ± 0.19	2.72 ± 0.28	0.014 ± 0.006 *	0.027 ± 0.004
July	2006	Female	6.23 ± 0.05	2.45 ± 0.05	0.011 ± 0.001 *	0.022 ± 0.003 *
		Male	6.44 ± 0.10	2.69 ± 0.13	0.005 ± 0.002	0.017 ± 0.003
August	2006	Female	6.43 ± 0.04	2.76 ± 0.05	0.027 ± 0.001 *	0.023 ± 0.001
		Male	6.77 ± 0.10	3.07 ± 0.17	0.010 ± 0.0023	0.019 ± 0.002
September	2006	Female	6.42 ± 0.11	2.81 ± 0.05	0.072 ± 0.004	0.031 ± 0.002
		Male	6.67 ± 0.09	3.09 ± 0.12	0.041 ± 0.003	0.021 ± 0.002
November	2006	Female	6.69 ± 0.04	3.07 ± 0.07	0.160 ± 0.009	0.072 ± 0.004
		Male	6.75 ± 0.13	3.25 ± 0.17	0.038 ± 0.002	0.039 ± 0.003
December	2006	Female	6.78 ± 0.07	3.23 ± 0.13	0.195 ± 0.012 *	0.076 ± 0.004 *
		Male	6.85 ± 0.09	3.45 ± 0.16	0.038 ± 0.003	0.036 ± 0.003
January	2007	Female	6.81 ± 0.05	3.38 ± 0.08	0.245 ± 0.018 *	0.092 ± 0.007
		Male	7.03 ± 0.07	3.74 ± 0.13	0.040 ± 0.003	0.052 ± 0.023

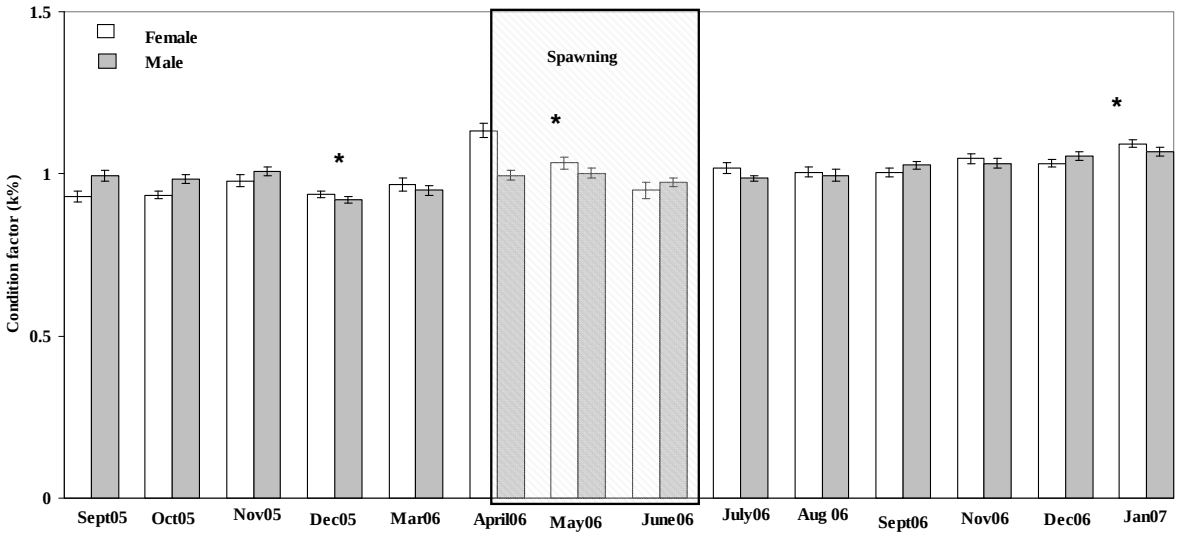


Figure 2.2. Seasonal differences in condition of female and male Greenside Darter (*E. blennioides*) collected from Eramosa River, Ontario. * indicates significant difference between sites from the preceding month within a sex.

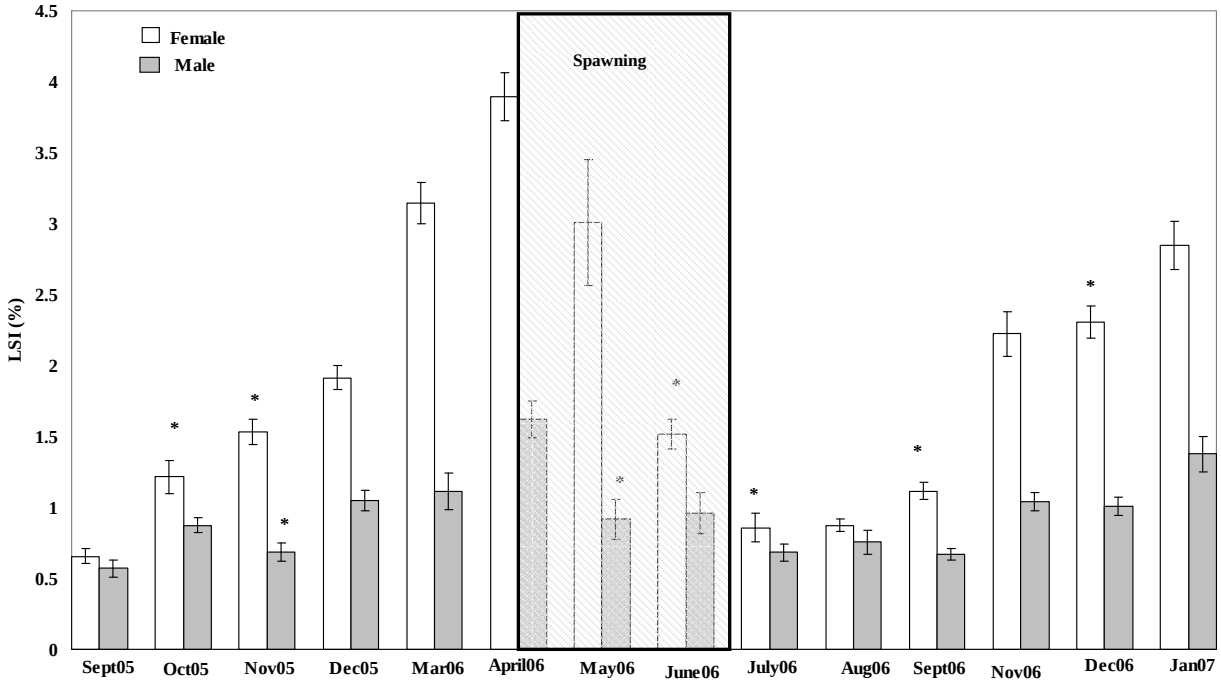


Figure 2.3. Seasonal differences in liver somatic index of female and male Greenside Darter (*E. blennioides*) collected from Eramosa River, Ontario. * indicates significant difference between sites from the preceding month within a sex.

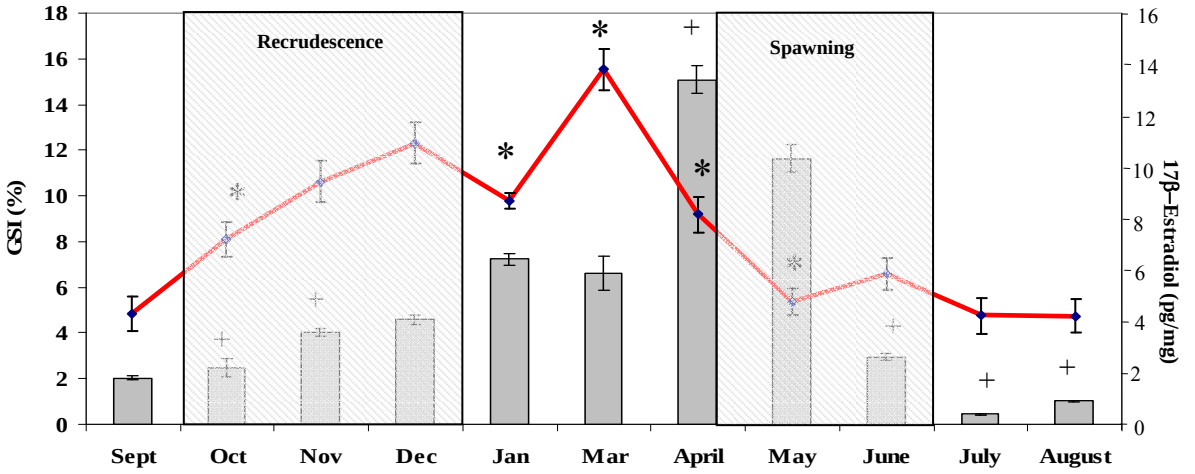
Female ovarian size was lowest in July after spawning (0.592 $\pm$ 0.44 %) (Figure 2.4). Gonad development began to gradually increase between October to January ($p \leq 0.020$). Ovarian tissue increases to maximum size in April (15.09 $\pm$ 0.58 %), just prior to spawning in May. Male gonad size was lowest post-spawning in July and August (0.243 $\pm$ 0.12 %, and 0.297 $\pm$ 0.05 %, respectively) and increased rapidly starting in late August into early September, reaching maximum size (1.345 ± 0.09 %) in October (Figure 2.5). Testes size decreased somewhat after October and maintained this same size (~1.13 %) over the winter months until April (1.02 %). Male gonad sizes decreased in May (0.90 %), which coincided with presumed spermatocyte maturation prior to spawning, and then continued to gradually decrease throughout the spawning period until July.

Regression analysis indicated that the relationship between gonad size and body weight was strongest for female fish collected in January 2007 (R^2=0.934), followed by September 2006 (R^2=0.734) and April 2006 (R^2=0.721) (Table 2.3). Regression analysis also revealed large variability in this relationship in collections conducted within the same month but in different years as the variability of fish collections in September, November, and December 2005 (R^2=0.233, R^2=0.449, and R^2=0.327; respectively) were significantly greater than those conducted in September, November, and December of 2006 (R^2=0.734, R^2=0.694, and R^2=0.720; respectively). The relationship of the male gonad weight in relation to body weight was more variable than in females; with the strongest relationship observed in post-spawning fish in July (R^2=0.782) when GSI is low, followed by November and December 2006 (R^2=612, and R^2=0.569; respectively). Variability in male GSI between months within the year was minimal as the maximum change in GSI throughout this study was 1.15% with the lowest GSI observed post-spawning in August 2006 (0.24 + 0.18 %) and the maximum GSI observed during gonadal recrudescence in November 2006 (1.39 + 0.09 %) (Table 2.3).

Table 2.3. Regression analysis output (R^2) from data in Table 2.2 of gonad weight (dependant) plotted against weight and length (independent) and GSI (± SE) by month from September 2005 to January 2007 to evaluate gonad variability. * indicates months when the variability in gonad size between individuals for both sexes is low and the preferred months to collect Greenside Darters.

Month	Year	Sex	Gonad vs. Body Weight	R^2	GSI [% (+SE)]
September	2005	Female	y = 0.0124x + 0.024	0.233	2.03 + 0.09
October	2005		y = 0.0019x + 0.093	0.007	3.47 + 0.20
November	2005		y = 0.0315x + 0.028	0.449	4.05 + 0.17
December	2005		y = 0.0404x + 0.009	0.327	4.40 + 0.25
March	2006		y = 0.08x - 0.007	0.473	7.74 + .045
April	2006		y = 0.1681x - 0.052	0.7208 *	15.09 + 0.58 *
May	2006		y = 0.0582x + 0.163	0.188	11.64 + 0.63
June	2006		y = 0.035x - 0.014	0.700	2.73 + 0.25
July	2006		y = 0.0135x - 0.022	0.328	0.48 + 0.06
August	2006		y = 0.0113x - 0.004	0.226	1.00 + 0.06
September	2006		y = 0.038x - 0.034	0.734	2.57 + 0.09
November	2006		y = 0.0831x - 0.107	0.6935 *	4.83 + 0.24 *
December	2006		y = 0.1125x - 0.171	0.7195 *	6.07 +0.29 *
January	2007		y = 0.1207x - 0.154	0.9336 *	7.23 +0.26 *
September	2005	Male	y = 0.007x + 0.002	0.114	0.84 + 0.06
October	2005		y = 0.0092x - 0.025	0.411	1.24 + 0.05
November	2005		y = 0.0144x - 0.0171	0.544	0.87 + 0.07
December	2005		y = 0.0091x + 0.004	0.442	1.05 + 0.17
March	2006		y = 0.0105x - 0.010	0.497	1.06 + 0.07
April	2006		y = 0.1465x + 0.602	0.179	0.72 + 0.07
May	2006		y = 0.0118x - 0.008	0.5247 *	1.02 +.0 90 *
June	2006		y = 0.0088x - 0.010	0.171	0.90 + 0.10
July	2006		y = -0.005x + 0.020	0.7815 *	0.48 + 0.21
August	2006		y = 0.194x - 0.301	0.359	0.24 + 0.18
September	2006		y = 0.0133x - 0.0003	0.180	0.30 + 0.05
November	2006		y = 0.0101x + 0.002	0.6122 *	1.39 + 0.09 *
December	2006		y = 0.0146x - 0.015	0.5685 *	1.07 + 0.04 *
January	2007		y = 0.0167x - 0.022	0.448	1.08 + 0.05 *

a)



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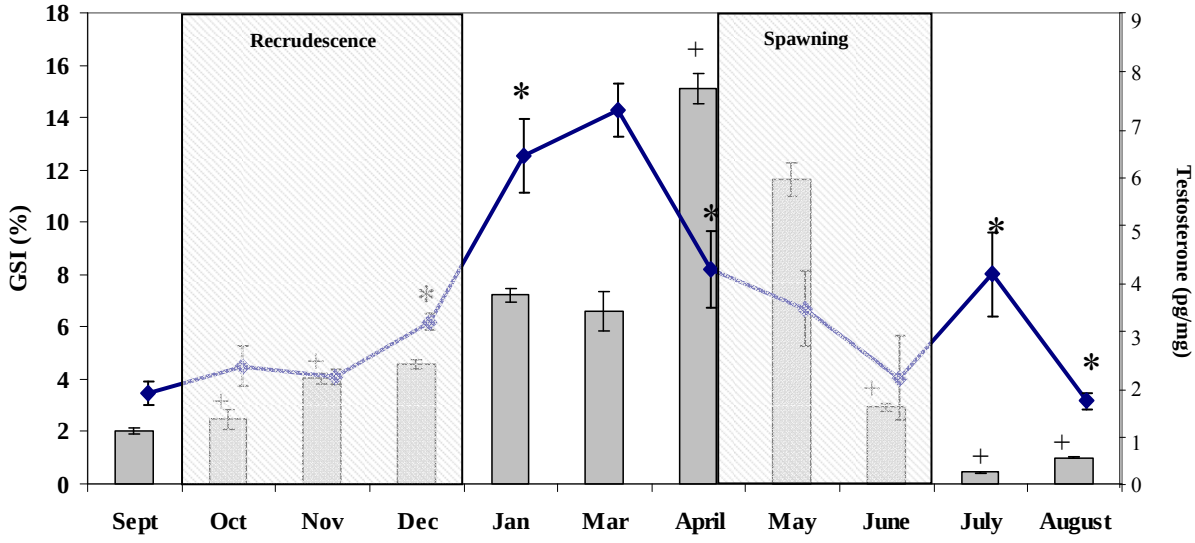
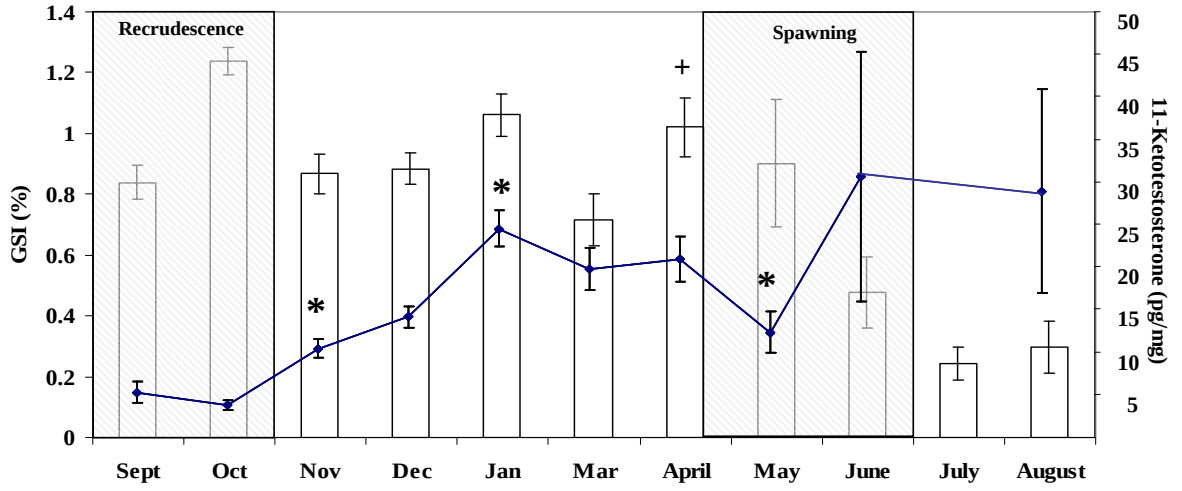


Figure 2.4. Female Greenside Darter (*E. blennioides*) seasonal differences in gonadosomatic index (GSI) (bars) and in vitro steroid production (line) of (a) 17β-estradiol and (b) testosterone collected from Eramosa River, Ontario. $^{+}$ indicates significant difference in GSI from the preceding month; * indicates significant difference in steroid production from the preceding month.

a)



b)

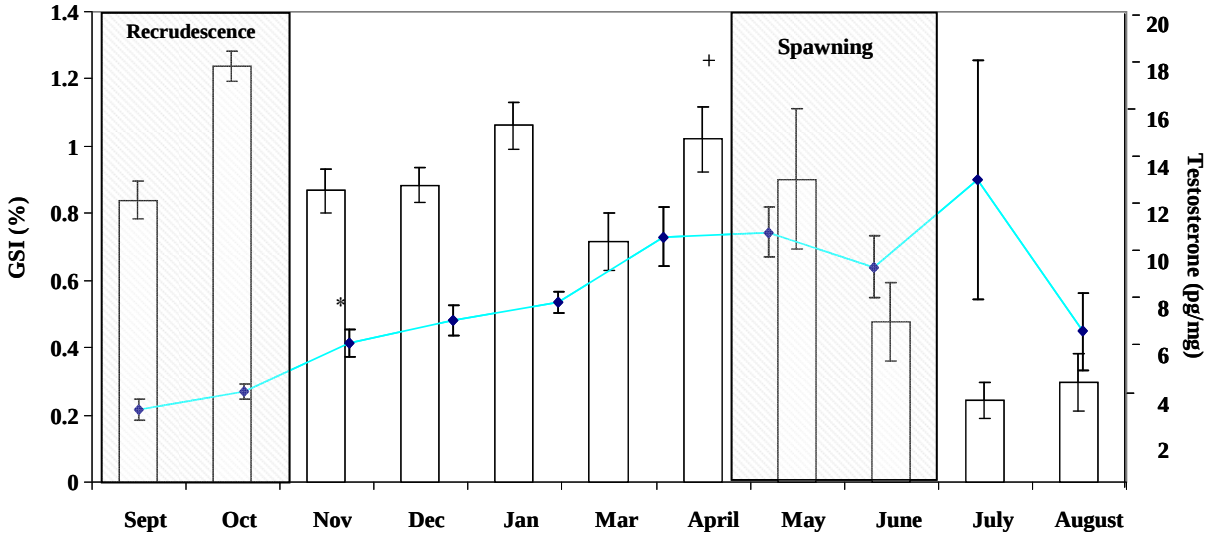


Figure 2.5. Male Greenside Darter (*E. blennioides*) seasonal differences in gonadosomatic index (GSI) (bars) and in vitro steroid production (line) of (a) 11-ketostestosterone and (b) testosterone collected from Eramosa River, Ontario. $^{+}$ indicates significant difference in GSI from the preceding month; * indicates significant difference in steroid production from the preceding month.

2.5.2 In vitro sex steroid production

Similar to the profile of ovarian tissue, basal 17β-estradiol (E2) production was at its lowest levels in the post-spawning months of July and August, however these levels were not significantly different from the adjacent months of June ($p=0.413$) and September ($p=0.943$), respectively) (Figure 2.4a). E2 levels gradually increase 2.5 times from September (4.09 $\pm$ 0.65 pg/mg) to December (10.46 $\pm$ 0.75 pg/mg). E2 levels in October were higher than those in September ($p=0.018$). Although E2 continued to increase from October to December there were no differences between months ($p\geq0.202$), then E2 levels decreased in January (8.32 $\pm$ 0.30 pg/mg) ($p=0.003$). E2 levels reached their maximum in March (13.19 $\pm$ 0.76 pg/mg), one month prior to ovarian tissue reaching its maximum size in April, then E2 levels decrease dramatically in April (7.78 $\pm$ 0.65 pg/mg) and May (4.51 $\pm$ 0.51 pg/mg) prior to spawning ($p<0.001$, and $p=0.011$; respectively). Stimulated E2 production followed a similar profile to basal production (data not shown). Female testosterone (T) production also demonstrated a similar seasonal steroid production profile, however the variability in T production was greater (Figure 2.4b). T levels were lowest in August (1.70 $\pm$ 0.15 pg/mg) and September (1.86 $\pm$ 0.23 pg/mg). Similar to E2, T production gradually increased from September (1.86 $\pm$ 0.23 pg/mg) throughout the fall and winter (October, 2.36 $\pm$ 0.39; November, 2.16 $\pm$ 0.14; December, 3.20 $\pm$ 0.16; and January, 6.35 $\pm$ 0.69 pg/mg) (Sept-Oct $p=0.308$; Oct-Nov $p=0.282$, Nov-Dec $p\leq0.001$; Dec-Jan $p\leq0.001$; Jan-Mar $p=0.373$) until it reached its maximum in March (7.22 $\pm$ 0.51 pg/mg) (Mar-April, $p\leq0.001$). From April (4.19 $\pm$ 0.73 pg/mg) to June (2.14 $\pm$ 0.80 pg/mg) T production gradually decreases through the pre-spawning/spawning period (March-April $p\leq0.001$; April-May $p=0.423$; May-June $p=0.273$). T production increases slightly in July (4.10 $\pm$ 0.80 pg/mg) before decreasing again in August ($p\leq0.41$).

Although male gonad sizes were lowest in the summer months of June, July and August, both 11-ketotesterone (11KT) and T production reached their maximum levels in June (30.39 $\pm$ 13.30 pg/mg, and 13.16 $\pm$ 4.90 pg/mg, respectively) (Figure 2.5ab). As a result of the low gonad size in July, steroid data from male darters were not available. Conversely, when testes mass was highest in September-October, T and 11KT production were at their lowest (3.74 $\pm$ 0.43 pg/mg, and 7.50 $\pm$ 1.19 pg/mg, respectively). From October through to January, 11KT levels gradually increase (Oct-Nov ($p< 0.001$), Nov-Dec ($p=0.063$), Dec-Jan ($p=0.002$)), and stabilized between March and April (20.61 $\pm$ 2.23, and 21.62 $\pm$ 2.39 pg/mg, respectively) ($p=0.962$) before decreasing in May prior to spawning (13.84 $\pm$ 2.18 pg/mg) ($p=0.004$). Although testes size does not change dramatically from October to just prior to spawning in May and June, T levels gradually increased, though they were not statically different between adjacent months ($p\leq 0.160$) except between October and November ($p=0.017$). The highest T levels occurred during spawning in June (13.16 $\pm$ 4.90 pg/mg) (Figure 2.5b).

2.6 Discussion

This study describes seasonal patterns in the way the Greenside Darter stores and assimilates energy for use in growth and reproduction. Although energy stores in terms of body condition of fish of both sexes remained relatively stable, there were slight decreases during the winter months (December to March) and during the spring spawning period (June). These results are intuitive since fish should have limited foraging activity during the overwintering period with low water temperatures (December to March), as well as during the spawning period (June) when they are actively seeking mates. Conversely, energy storage in terms of hepatic tissue was dynamic in both sexes. Liver sizes increased gradually during the fall months (September-December) and reach maximum levels in April just prior to spawning, and were minimal post-spawning in July to August when energy stores would have been depleted.

Development of gonad tissue followed dynamic patterns, which can be separated into three distinct periods (spawning (mid-April to the end of June); recrudescence (October through to January); and maturation (March to April)). There was evidence of spawning in late April as Greenside Darter ovarian tissue (GSI) reached its maximum size in early April and had already begun to decrease by the beginning of May. Spawning events as determined by rapid loss of gonad size continued throughout June and ceased in July when GSI was at its lowest. This is consistent with observations made by Fahy (1954) who observed Greenside Darter spawning at night in a wild population of darters and in a laboratory controlled experiment from mid-April for a 5-week period ending in June.

Ovarian production of T and E2 were at the lowest levels after spawning, in August. E2 levels rose significantly from September to October and continued to increase throughout recrudescence with ovarian tissue reaching the maximum steroid production in March. One month later in April, ovarian growth was at its maximum when E2 production was significantly declining. This is consistent with patterns of plasma levels of E2 in other teleosts as the steroid pathway is diverted to the production of other hormones involved in maturation and ovulation. In the White Sucker, E2 production tapers off while 17α-20β-dihydroxy 4-pregnen-3-one is up-regulated in the final stages of gonad ovulation and maturation (Van Der Kraak et al., 1992). The increase in the production of T in Greenside Darter during recrudescence was less dramatic, which may be a result of the T produced being rapidly converted to E2 by the cytochrome P450 enzyme aromatase responsible for biosynthesis of estrogens. Similar to the observation of E2 levels, the greatest rate of T production occurred in March, which is the month prior to maximal ovarian size.

Similar to female gonad development, relative male testicular size (GSI) was lowest in the post-spawning months (July to August). Recrudescence in male darters occurred in September when male GSIs increased dramatically (349%) from August and reached their maximum GSI in October. This increase in testicular tissue corresponded with elevated production of 11KT, the major androgen,

in July and August. Throughout the remainder of the reproductive cycle, the GSI stabilized around 0.80 to 0.90 % until the spawning period began and the mass of the testicular tissue decreased from April to June. Although production of T and 11KT increased gradually from October to March, levels of both steroids appeared to stabilize in the early spring. Maximum steroid production capacity and lower variability between individuals was observed in April, which strengthens our recommendation to conduct biological monitoring using the Greenside Darter in the early spring. Knowledge of sex steroid production in small-bodied fish species is limited. Therefore, the ability to measure these steroids (E2, T, 11KT) in the Greenside Darter make it a suitable candidate as a sentinel species to evaluate the potential for endocrine disruption in fish exposed to municipal wastewater.

A recent re-evaluation of a large database of biological monitoring data from the Canadian EEM program has evaluated the timing of fish collection for the purpose of evaluating the impact of industrial effluent discharges on aquatic organisms in the Canadian receiving environment (Barrett et al., 2010). In the fall, spring-spawning sentinel species are undergoing gonad recrudescence, which may result in greater within site variability in measured parameters and reduced ability to detect differences in fish health between reference and exposed sites (Barrett et al., 2010). In order to provide guidance for environmental monitoring programs, Galloway and Munkittrick (2006) documented seasonal changes in parameters such as condition, relative liver size and relative gonad size throughout the year in four fish species. Their study suggested the most appropriate time to collect small-bodied fish species in order to minimize variability for monitoring purposes is just prior to their first spawning event. However, additional basic biological research was recommended for each species in order to fully interpret their responses to exposure effluent. Reduced variability of measured endpoints will in turn reduce the sample size of fish needed to achieve the required statistical power, which will also reduce the possibility of over sampling wild populations of fishes (Barrett et al., 2010).

The recommended sampling time outlined in Barrett and Munkittrick (2010) for a spring multiple-spawner with few spawns such as the Iowa Darter (*E. exile*) and Johnny Darter (*E. nigrum*) was four to six weeks pre-spawn which corresponds to late March to mid-April depending on local conditions. Although the Greenside Darter is not listed in their review, it is in the same genus with the same reproductive strategy as some of those listed, and shows a similar pattern of gonadal development; it should therefore be sampled at the same recommended time. Data described in this study clearly demonstrate that sampling four to six weeks pre-spawn or in late fall, are optimal sampling period for the Greenside Darter since this is when female Greenside Darters obtain the maximum GSI, GSI is temporally stable, and variability among individuals is relatively low.

Bunt et al. (1998) described age-classes of Greenside Darter using scales for a subsample of individuals collected during and after the spawning season. They found that fish age-classes corresponding to length were; 1+ (50 - 60 mm); 2+ (58 - 68 mm); 3+ (66 – 73 mm); and 4+ (> 73 mm). By using these criteria, the majority of the Eramosa River population is comprised of two to three year old individuals with only a few individuals reaching age four (Figure 2.1). This study reported young of the year (YOY) (2.6 - 3.2 cm) Greenside Darters first appearing in July, whereas Fahy (1954) collected the earliest YOY in August.

The purpose of this study was to describe the yearly cycle of the Greenside Darter in southern Ontario in terms of survival, reproduction and energy storage. The early spring or late fall observations of energetic storage, utilization and sex steroid production indicate when the annual levels of natural variability among individuals within a site were at their lowest and thus the most appropriate time within the reproductive cycle to collect Greenside Darters for the purposes of biomonitoring. Although this species was previously designated as a Species of Special Concern under COSEWIC, it now occupies a wide distribution in the Grand River watershed of southern

Ontario, Canada (Beneteau et al., 2009), making it an excellent candidate as a sentinel species to monitor the environmental health of the receiving waters in that watershed.

2.7 Acknowledgements

This project was funded through grants from the Canadian Environmental Protection Act and Health Canada to MEM. Technical support was provided through Environment Canada (W. Ribble, S. Spina) at the National Water Research Institute and the Waterloo Aquatic Toxicology and Ecosystem Remediation Laboratory at the University of Waterloo (WATERL). WATERL received support from an NSERC Discovery Grant, the Canadian Water Network and the Canada Research Chair in Water Quality Protection at the University of Waterloo to MRS. The invaluable help of field assistants, graduate students, and visiting scholars is greatly appreciated.

Chapter 3

Intersex and reproductive impairment of wild fish exposed to multiple municipal wastewater discharges.

3.1 Summary

The Grand River watershed in Ontario, Canada, receives and assimilates the outflow of 30 municipal wastewater effluent (MWWE) discharges which are a mixture of domestic and industrial wastes. The purpose of this study was to investigate the impact of multiple sewage discharges on populations of wild fish. In field studies, responses of fish populations and individual fish responses in terms of condition factor and liver somatic index, and reproduction (*in vitro* sex steroid production, gonadosomatic indices, histology (cellular development and intersex)) were assessed upstream and downstream of two municipal discharges. Greenside Darters, *Etheostoma blennioides*, and Rainbow Darters, *E. caeruleum*, collected downstream of two municipal wastewater plants had the potential to be larger (longer and heavier) when compared to reference fish regardless of sex. Exposed fish were not assimilating additional anthropogenic resources into energy storage (increased condition and liver somatic index). Impacts on ovarian development appeared to be minor, with no differences in gonad growth, steroid production or cellular development. MWWE exposed male fish were experiencing impairment in the capacity to produce testosterone and 11-ketotestosterone in vitro, and in cellular development (GSI, intersex). Male darters of both species collected in the upstream agricultural region demonstrated no evidence of intersex whereas the urban reference sites had incidences of intersex of up to 20%. Rates of intersex were elevated downstream of both MWWE discharges studied (33% and $\geq$ 60%, respectively). Lower rates of intersex at the intermediate sites, followed by increases downstream of the second MWWE discharge, suggest that fish populations have the potential to recover prior to exposure to the second MWWE effluent discharge. Pre-spawning darters demonstrated dramatically higher incidences of intersex in the spring at both urban reference sites (33 & 50%, respectively), which increased downstream of the near-field and far-field exposure sites (60 & 100%, respectively). These findings suggest that the compounds released in MWWE exert a greater effect on the reproductive system of male fish. These effects may become more pronounced as

projected human population growth in this watershed will require the aquatic environment to assimilate an increasing amount of MWWE waste.

Keywords: intersex, reproductive impairment, fish responses, municipal wastewater effluent

3.2 Introduction

Municipal wastewater effluent (MWWE) is a mixture of liquid wastes, solids, debris and chemicals discharged from residential, institutional, commercial and industrial sources. It is a matrix of environmental contaminants that includes natural and synthetic hormones, pharmaceuticals (Ternes, 1999; Metcalfe et al., 2003, Servos et al., 2003, 2005), and industrial chemicals (Bennie, 1999; Servos et al., 1999; Mikaelian et al. 2002; Kavanagh et al., 2004). The input of MWWE organic material into the aquatic receiving environment can result in eutrophication and lower dissolved oxygen content (Chamber et al., 1997; Cooke, 2006). MWWE can also contain ammonia, inorganic chloramines, and textile mill effluents (CEPA, 1999).

Previous studies evaluating the effects of MWWE effluent on wild fish in the United Kingdom discovered an intersex condition (female oocytes in male testicular tissue) in male Roach collected downstream of sewage treatment plants (Jobling et al., 1998). These results stimulated studies aimed at investigating the intersex condition in wild fish collected downstream of MWWE discharges in the UK, Europe and the U.S.A. (Table 3.1). The intersex condition has also been observed in Canadian waters in Lake Whitefish (*Coregonus clupeaformis*) in the St. Lawrence River, Quebec (Mikaelian et al., 2002), and in wild White Perch (*Morone americana)* exposed to domestic and industrial effluents in two large bays of Lake Ontario (Kavanagh et al., 2004). The Grand River watershed, in southern Ontario, Canada, supports a large human population in a water-limited environment. This system receives effluent from 30 municipal discharges as well as other multiple point and nonpoint source inputs resulting in water quality that is complex, dynamic and nutrient-enriched (Cooke, 2006). Thus, the conditions that induce intersex or other forms of reproductive disruption in fish may exist in this watershed.

Table 3.1. Review of studies by country where the intersex condition (female oocytes in male testicular tissue) has been observed in feral fish collected downstream of a sewage discharge.

Country	Species	Common name	Reported Intersex in Males (%)	Study
Belgium	*Gobio gobio*	*Gudgeon*	5 to 20	Douxfils et al. 2007
	Barbatula barbatula	*Stoneloach*	11	Douxfils et al. 2007
Canada	*Coregonus clupeaformis*	*Lake Whitefish*	1.2 to 11.7	Mikaelian et al. 2002
	Morone americana	*White Perch*	22 to 83	Kavanaugh et al. 2004
	Neogobius melanostomus	*Round Gobies*	13	Marentette et al. 2010
Denmark	*Rutilus rutilus*	*Roach*	4.5 to 6.5	Bjerregaar et al. 2006
France	*Platichthys flesus*	*Flounder*	8	Minier et al. 2000
Germany	*Abramis brama L*	*Bream*	0.5 to 6	Hecker et al. 2002
Italy	*Barbus plebejus*	*Barbell*	50	Viganò et al. 2001
Netherlands	*Abramis brama*	*Bream*	0.37	Vethaak et al. 2002
	Platichthys flesus	*Flounder*	0	Vethaak et al. 2002
Spain	*Cyprinus carpio*	*Carp*	18 to 50	Sole et a. 2002, 2003
United Kingdom	*Rutilus rutilus*	*Roach*	16 to 100	Jobling et al. 1998, 2002a
	Platichthys flesus	*Flounder*	0.2	Allen et al. 1999
	Gobio gobio	*Gudgeon*	6 to 15	van Aerle et al. 2001
	Escox lucius	*Pike*	14 to 26	Vine et al. 2005
USA	*Catostomus commersoni*	*White Sucker*	4 fish; 18 to 22	Woodling et al. 2006; Vajda et al. 2008
	Micropterus spp.	*Black Bass*	9	Hinck et al. 2006
	Micropterus dolomieu	*Smallmouth Bass*	0 to100; 44; 82 to100	Blazer et al. 2007; Hinck et al. 2009; Iwanowicz et al. 2009
	Micropterus salmoides	*Largemouth Bass*	44; 23	Hinck et al. 2009; Iwanowicz et al. 2009
	Ictalurus punctatus	*Channel Catfish*	50	Hinck et al. 2009

The presence of the intersex condition could influence the reproduction, distribution, and abundance of Canadian native fish in this river as described for wild fish populations in other countries. Biological endpoints such as changes in growth, reproduction and survival are directly relevant to the fish population dynamics and change over relatively short time periods (weeks to months), are linked closely to physiological indicators, enabling researchers to trace cause-effect relationships (Munkittrick et al., 1991). By testing the biochemical responses (individual effects) of forage fish collected downstream of MWWE discharges, an evaluation of the physiological homeostasis of exposed fish can be conducted to assess if the aquatic environment as represented by fish health is effected in a deleterious manner. Alterations in physiological responses (sex steroid levels (McMaster et al., 1995b; 1996) and histopathology (cellular development) (Jobling et al., 2002b) may respond sooner than whole body responses in the fish population (i.e., growth, energy use and energy expenditure (Munkittrick et al., 1991)) following increased MWWE inputs into the receiving environment. In order to predict the impact of future human development, it is also important to learn if these reproductive endpoints are sufficiently sensitive to detect subtle responses by fish exposed to additional discharges in this system. The primary objective of this study was to determine the prevalence of intersex condition and reproductive disruption present in wild fishes exposed to multiple MWWE discharges in a system with limited surface water available for dilution.

3.3 Materials and methods

3.3.1 Treatment plant and site description

The Grand River watershed is located in southern Ontario and is the largest drainage basin on the northern shore of Lake Erie (Figure 3.1). This watershed supports a population of approximately 925,000 inhabitants, and also has intensive agricultural activity (~70% agricultural landscape).

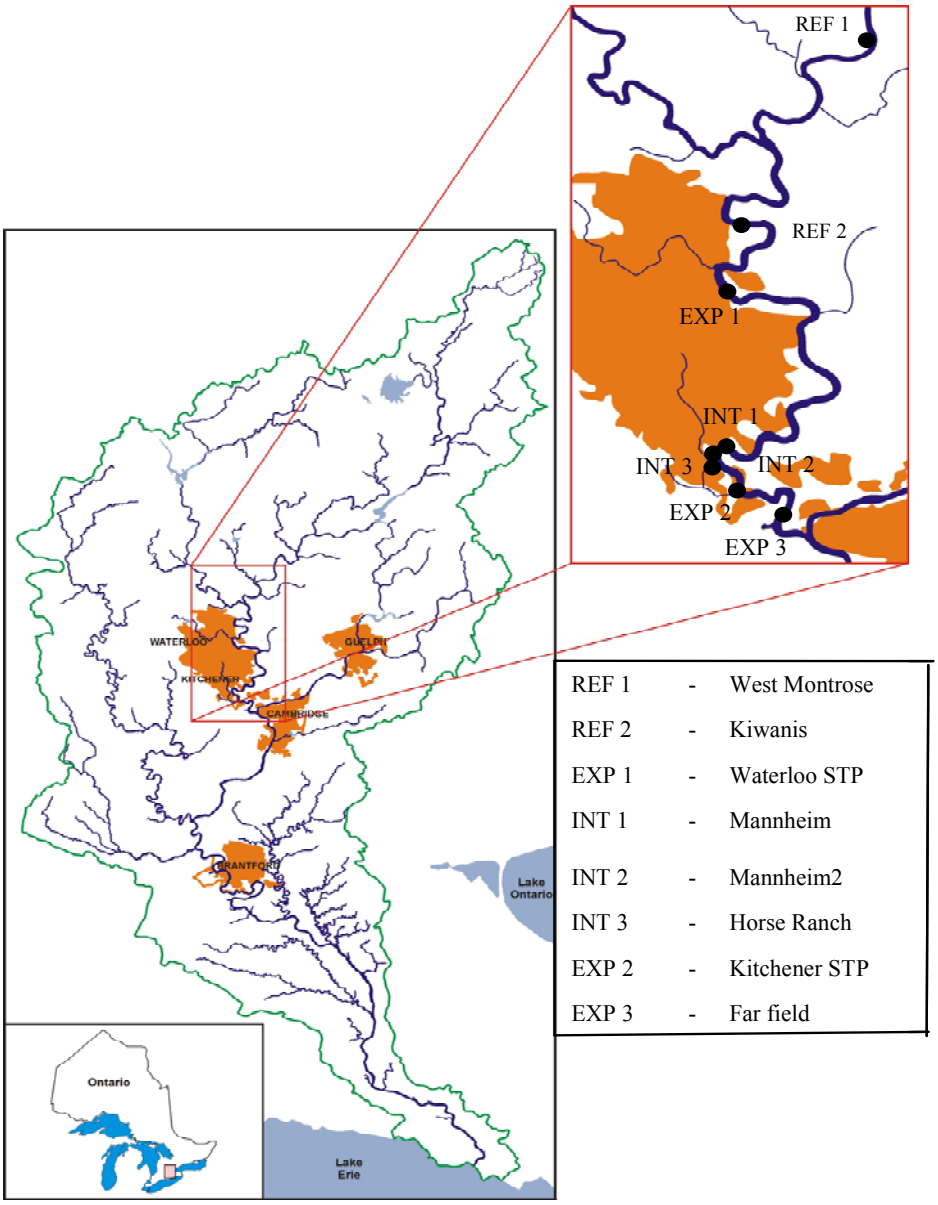


Figure 3.1. Sites in the Grand River where Greenside and Rainbow Darters were collected in the fall of 2007 and the spring of 2009 upstream/downstream of the municipal wastewater treatment facilities servicing the Municipality of Kitchener-Waterloo, Ontario, Canada.

Throughout the watershed, nutrient levels are consistently elevated and downstream of the Kitchener sewage treatment plant (STP) oxygen levels frequently fall below the Ontario Provincial Water Quality Guideline of 4 mg/L dissolved oxygen at night during the summer season (Cooke, 2006). The municipality of Kitchener-Waterloo is serviced by two secondary-conventional activated sludge treatment plants, which treat different sources and amounts of wastewater and therefore produce effluent of differing quality and composition (Table 3.2). In the fall of 2007, wild fish collections were conducted at two reference sites upstream of the municipality of Waterloo, West Montrose (REF 1) and Kiwanis (REF 2), and at an intermediate reference site (INT 1) which is located between the two sewage treatment plant sites (Waterloo STP (EXP 1) and Kitchener STP (EXP 2)). Field collections in the spring of 2009 were focused on the intermediate sites upstream (Mannheim (INT 2) and Horse Ranch (INT 3)) and immediately downstream (EXP 2) and far field (EXP 3) of the STP discharge for the city of Kitchener (Table 3.3).

3.3.2 Field collections and fish health

Preliminary field studies identified Greenside Darters (*Etheostoma blennioides*) and Rainbow Darters (*E. caeruleum*) as abundant and spatially distributed throughout the watershed, making them desirable sentinel species for this study. Both darter species were collected by sampling faster runs and riffles (approximately 1.1–1.5 m/s) approximately 0.5 to 0.75 m deep with boulder/cobble substrates, between 09:00 and 12:00 h. Fish were collected using a backpack electrofisher (Smith-Root Type 12-D or Halltech HT-2000). Stunned fish were removed using dip nets (approximately 0.5 cm mesh size) held downstream of the electrofishing unit to collect fish swept downstream. Fish were transported live to the on-site laboratory in aerated buckets, transferred to an aerated tank, and held for no more than 1 h prior to sampling to standardize holding time across sampling periods between sites. A sub-sample of 20 female and 20 male adult darters were randomly selected to be sacrificed for

Table 3.2. Characteristics of the final effluent from the municipal wastewater treatment facilities servicing the cities of Kitchener and Waterloo from April to September of 2007 (2007 Ontario Clean Water Agency Performance Assessment Report - Wastewater Treatment Plant; Waterloo and Kitchner (K. Chow personal communication)).

Effluent characteristics	Waterloo	Kitchener
Population served	105,100	185,000
Avg. Suspended Solids (kg/d)	881	465
Avg. Biological Oxygen Demand (kg/d)	152	656
Avg. Ammonia Load (kg/d)	188	1560
Avg. Nitrate Load (kg/d)	238	99
Total N/person (kg/d)[a]	>4.05(10^3)	>8.9(10^3)
Yearly Ammonia/Nitrate	0.99	15.00

[a] = Daily Total Nitrogen (kg) loading per person.

Table 3.3. General description and site coordinates of reference and exposure sampling areas for Greenside Darter (GD) and Rainbow Darter (RD) collections in the Grand River, Ontario conducted in November 2007 and April 2009.

Site	Year	Site Description	Latitude (N)	Longitude (W)
REF 1	2007 (GDS, RD)	Upstream urban development	43° 35' 07.54"	80° 28' 54.08"
REF 2	2007 (GDS, RD)	Urban/upstream STP	43° 30' 17.41"	80° 28' 28.61"
EXP 1	2007 (GDS, RD)	Downstream STP - Near field	43° 28' 24.69"	80° 28' 23.99"
INT 1	2007 (GDS, RD)	Urban/upstream STP	43° 24' 42.26"	80° 28' 00.42"
INT 2	2007 (GSD, RD), 2009 (RD)	Urban/upstream STP	43° 24' 30.34"	80° 28' 20.25"
INT 3	2007 (GSD, RD), 2009 (RD)	Urban/upstream STP	43° 24' 06.45"	80° 28' 43.73"
EXP 2	2007 (GSD, RD), 2009 (RD)	Downstream STP - Near field	43° 23' 49.40"	80° 28' 54.23"
EXP 3	2009 (RD)	Downstream STP - Far field	43° 23' 08.49"	80° 28' 08.63"

fish health assessment endpoints of condition factor [k=(body weight/length^3)*100], gonadosomatic index [GSI=(gonad weight/body weight)*100], and liver somatic index [LSI = (liver weight/body weight)*100]. Each fish was rendered unconscious by concussion, euthanized by spinal severance, and total length (±1.0 mm), and body weight (±0.01 g) were measured. The internal organs were removed and the gonads (±0.001 g) and liver (±0.001 g) were weighed. A sub-sample of gonad tissue was taken from 10 adults of each sex, weighed and placed in incubation media for analysis of in vitro steroid hormone production capacity (McMaster et al., 1995a). An additional sub-sample of gonad tissue was taken for histological analysis for ovarian and testicular development as described below. The fish survey of pre-spawning darters in April 2009 only involved Rainbow Darters as Greenside Darter were in low abundance in the river at that time of the year.

3.3.3 Stable isotope analysis

Stable isotope analysis has been used to confirm site fidelity in small-bodied fish species as well as to provide some insight into the impact of anthropogenic sources of nutrients in aquatic biota (Gray et al., 2004; Loomer, 2008). In order to ensure the fish in this study were exposed to the MWWE, stable isotope signatures in darter muscle tissue were measured. Dorsal muscle tissue was sampled from Greenside Darters from each site and preserved in liquid nitrogen, and stored at -20^{o}C until freeze-dried. The finely ground powder prepared from the fish collections was weighed (0.2 ± 0.05 mg) into tin cups and analyzed for stable isotope signatures (δ^{13}C and δ^{15}N) and ‰ elemental composition using a Delta Plus Continuous 26 Flow Stable Isotope Ratio Mass Spectrometer (Thermo Finnigan / Bremen-Germany) coupled to a Carlo Erba Elemental Analyzer (CHNS-O EA1108-Italy). Analysis was performed by the Environmental Isotope Lab at the University of Waterloo (Drimmie and Heemskerk, 2005).

3.3.4 In vitro steroid production

Due to the low volume of blood in small fish species, it is difficult to obtain enough plasma to determine circulating levels of sex steroids. As a surrogate to circulating levels of sex steroids, an in vitro bioassay to measure the production of sex steroids by the gonadal tissue of fish was developed (McMaster et al. 1995a). Incubations of gonadal tissues were used to estimate the steroid-producing potential of fish. Replicate samples of 20 $\pm$ 2 mg of ovarian tissue from females and testicular tissue from males were incubated in nutrient media. Sub-samples from a single ovary were either unstimulated (basal, media alone) or were treated with 5 μL of 40 x 10^{-6}M forskolin solubilized in ethanol to stimulate steroid production. As Rainbow Darter tissue did not responding to forskolin treatment in 2007, spring 2009 Rainbow Darter gonad tissue was stimulated with 10 IU/mL human Chorionic Gonadotropin (hCG). Due to the low mass of testicular tissue of Rainbow Darters in April 2009 (<1% body weight), all steroid levels for males represent stimulated replicates from both testes per fish. Gonad tissues were incubated for a 24 h period at 16°C, and then the media was drawn off the incubation wells and frozen in liquid nitrogen for transport to the laboratory. The number of replicates for each basal and stimulated treatment was dependent on availability of tissue. Concentrations of testosterone (T) (both sexes), 17β-estradiol (E2) (females) and 11-ketotestosterone (11KT) (males) released into the media during the incubation period were quantified by radioimmunoassay (RIA) procedures (McMaster et al., 1995a).

3.3.5 Histological analysis

Gonad sections were examined for histological evidence of alterations in gonad development between upstream reference areas and sites downstream of STP discharges. A section of testicular tissue from each male fish was processed according to standard histological methods (fixation in Davidson's solution and embedded in paraffin). Testes were sectioned using a microtome, placed on glass slides and stained with hematoxylin and eosin before being coverslipped. The entire testes tissue

was sampled into sections of 4-5 µm thickness with no more than 15 µm spacing between sections to maximize the potential to detect identifiable oocytes in each section. All sections from each male fish were scanned for intersex (presence of female oocytes). Five males from each site were selected to evaluate cellular development. From each of these fish, five random images at 40x magnification were collected and analyzed using applications written for the Northern Eclipse (v8.0) software package (Empix Imaging, 2006). A 391-point grid was stamped onto each image and the cell types under each grid point were scored. Recorded cell types were spermatogonia, spermatocyte, spermatid, and spermatozoa. The relative proportions of each cell type within a fish were calculated and used for analysis.

Cross sections of an ovary from 10 female fish from each site were processed according to standard histological methods (fixation in Davidson's solution and embedded in paraffin). Using a microtome, six to twelve thin sections (5-8 µm thickness) were produced, placed on glass slides and stained with hematoxylin and eosin before coverslipping. Images of entire cross sections of each ovary were prepared by stitching image fields at 2.5x magnification. All cell types containing a visible nucleus within these cross sections were scored and reported. The size of vitellogenic cells were calculated by tracing using Northern Eclipse (v8.0) applications. Recorded cell types were primary, cortical alveolar and vitellogenic oocytes. The relative proportions of each cell type and the size of vitellogenic cells were used for analysis.

3.3.6 Statistical analyses

Analyses of fish data were conducted between sites with sexes separate. Differences in fish length and body weight were evaluated using analysis of variance (ANOVA). Condition factor, gonad size, and liver size were evaluated using analysis of covariance (ANCOVA). Data were subjected to a logarithmic transformation to ensure a normal distribution of the biological data as biological measures usually occur on an exponential scale (Environment Canada, 2002). Tukey's post-hoc tests were then

used when $p<0.05$ to identify site differences. Nonparametric Kruskal-Wallis tests were used to compare in vitro steroid production and histological staging. Stable isotope data were compared using T-tests, which assumed equal or unequal variances, depending on the results of the Levene's statistic. The error bars associated with the averages presented in all figures represent the standard error of the mean. All data analyses were conducted using SYSTAT 12.0 statistical software (SYSTAT, 2007).

3.4 Results

3.4.1 Stable isotope analysis

Stable carbon and nitrogen isotope analysis of samples collected in the fall of 2007 in the Grand River confirmed site fidelity for the Greenside Darter. Carbon isotope analysis of dorsal muscle demonstrated a gradual enrichment of $\delta C‰$ from the upstream REF 1 site to downstream of the EXP 2 site (Figure 3.2). Carbon isotope signatures were similar between fish from the REF 1 and REF 2 sites ($p=1.000$). Carbon signatures from the EXP 1 STP site were intermediate being similar to adjacent sites (REF 2 and INT 1, $p>0.054$), but significantly different than $\delta C‰$ from the REF 1 and EXP 2 STP sites ($p<0.001$). Nitrogen isotope signatures also demonstrated a gradual enrichment downstream from REF 1 through to INT 1. Contrary to our predictions of continued nitrogen enrichment downstream of the STP inputs, $\delta N‰$ values dropped dramatically downstream of the EXP 2 STP discharge (Figure 3.2). Again, the dorsal muscle from EXP 1 STP fish demonstrated intermediate $\delta N‰$ levels. Most sites were significantly different from each other in terms of nitrogen signature ($p \leq 0.006$), however fish from the REF 2 and EXP 1 sites were not ($p=1.000$).

3.4.2 Fish health

Female Greenside Darter collected downstream of both the EXP 1 and EXP 2 STPs in the fall of 2007 were longer and heavier when compared to fish collected from the REF 2 and INT 1 reference

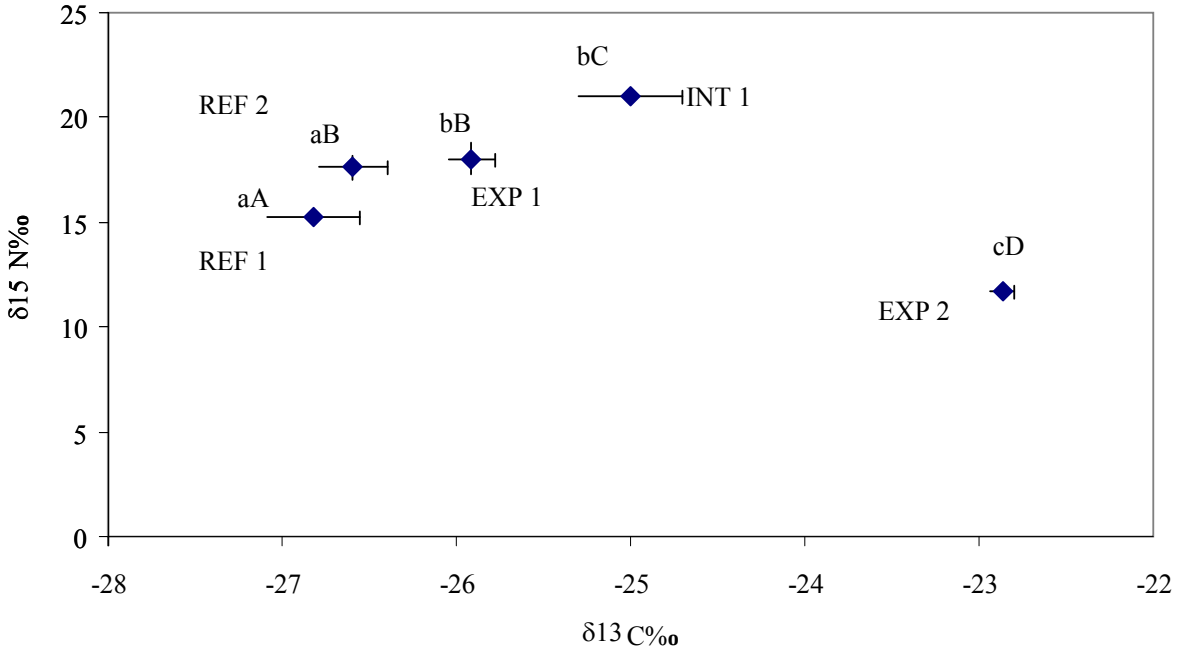


Figure 3.2. Grand River October 2007 Greenside Darter carbon & nitrogen stable isotopic signatures (‰). Statistically significant differences between sites are denoted by lower case letters for δ13C ‰ and upper case letters for δ15N ‰.

sites ($p \leq 0.033$), but female length in the exposed sites did not differ statistically from female Greenside Darter length collected from the REF 1 reference site ($p=0.092$) (Table 3.4). EXP 2 STP exposed female Greenside Darter also weighed significantly less than the REF 1 collections ($p=0.036$), but did not differ from the EXP 1 STP fish ($p=0.875$). Female Greenside Darter collected from the REF 2 site had significantly lower condition than fish from the REF 1 site ($p=0.031$), however there were no other significant site differences ($p=0.135$). Analysis of liver size relative to body weight for female Greenside Darter demonstrated no site differences among STP exposed fish relative to reference fish ($p \geq 0.419$) (Table 3.4). Female Greenside Darter collected downstream of the EXP 2 site had significantly smaller gonads relative to body size when compared to fish collected from all three reference sites ($p<0.032$), there were no other site differences ($p>0.109$) (Table 3.4).

Male Greenside Darter collected downstream of the EXP 2 STP discharge in the fall of 2007 were longer and heavier when compared to fish collected from the REF 2 and INT 1 reference sites ($p \leq 0.011$) (Table 3.4). Male fish collected at the EXP 1 STP site were also longer and heavier when compared to those collected from the INT 1 site ($p \leq 0.022$). There were differences between reference sites as INT 1 males were also shorter and lighter than male Greenside Darter collected at the upstream REF 1 site ($p<0.001$). Male Greenside Darter collected in the fall 2007 displayed no significant differences in condition among sites ($p=0.486$). Liver size relative to body size was greater in fish collected downstream of the EXP 2 STP when compared to male Greenside Darter collected from the INT 1 and REF 1 reference sites ($p \leq 0.023$). There were no other site differences with respect to liver size in male Greenside Darter in 2007 ($p \geq 0.286$) (Table 3.4). With respect to gonad size relative to body size, male Greenside Darter demonstrated no significant differences in the fall of 2007 among sites ($p \geq 0.055$). Female Rainbow Darters collected downstream of the EXP 2 STP site in the fall of 2007 were longer and heavier when compared to female Rainbow Darters collected from the REF 2

Table 3.4. Length, body weight, condition factor (k), liver somatic index (LSI), and gonadosomatic index (GSI) of Greenside and Rainbow Darters collected in the Grand River collected in 2007 and 2009. Values are reported as mean ± SE (n).

Year	Month	Species	Site	Sex	Length (cm)	Weight (g)	*k* [a]	LSI[b]	GSI[c]
2007	November	Greenside darter	REF 1	Female	7.95 ± 0.24 (20) a	6.03 ± 0.54 (20) a	1.09 ± 0.01 (18) a	2.22 ± 0.07 (20) a	4.40 + 0.15 (20) a
			REF 2		6.06 ± 0.16 (20) b	2.29 ± 0.25 (20) b	1.00 ± 0.2 (19) b	1.82 ± 0.09 (20) b	3.34 ± 0.17 (20) a
			EXP 1		7.46 ± 0.33 (17) a	4.83 ± 0.67 (17) ac	1.03 ± 0.02 (17) ab	2.01 ± 0. 09 (17) ab	3.85 ± 0.15 (16) a
			INT 1		6.08 ± 0.19 (31) b	2.63 ± 0.34 (31) b	1.05 ± 0.02 (31) ab	1.85 ± 0.07 (31) b	3.54 ± 0.11 (31) a
			EXP 2		7.05 ± 0.29 (20) a	4.11 ± 0.62 (20) c	1.04 ± 0.02 (20) ab	2.00 ± 0.06 (20) ab	3.10 ± 0.16 (20) b
			REF 1	Male	7.02 ± 0.34 (20) ac	4.46 ± 0.73 (20) ac	1.08 ± 0.02 (20) a	1.36 ± 0.05 (19) a	1.33 ± 0.12 (20) a
			REF 2		6.28 ± 0.08 (20) ab	2.65 ± 0.02 (20) ab	1.05 ± 0.02 (19) a	1.44 ± 0.07 (19) ab	1.41 ± 0.12 (19) a
			EXP 1		6.81 ± 0.21 (20) ac	3.64 ± 0.45 (20) ac	1.06 ± 0.02 (20) a	1.46 ± 0.07 (20) ab	1.42 ± 0.07 (20) a
			INT 1		5.82 ± 0.13 (32) b	2.64 ± 0.36 (34) b	1.07 ± 0.02 (32) a	1.33 ± 0.04 (31) a	1.16 ± 0.06 (32) a
			EXP 2		7.32 ± 0.14 (20) c	4.42 ± 0.38 (20) c	1.08 ± 0.02 (19) a	1.62 ± 0.07 (20) b	1.35 ± 0.08 (20) a
		Rainbow darter	REF 1	Female	5.71 ± 0.09 (20) a	2.07 ± 0.10 (20) abc	1.11 ± 0.02 (18) a	2.00 ± 0.14 (20) a	3.85 ± 0.12 (20) a
			REF 2		5.50 ± 0.11 (20) bc	1.92 ± 0.10 (20) b	1.14 ± 0.02 (20) a	1.77 ± 0.12 (20) a	3.53 ± 0.07 (20) ab
			EXP 1		5.79 ± 0.10 (20) ab	2.44 ± 0.14 (20) a	1.23 ± 0.02 (20) b	1.96 ± 0.09 (20) a	3.66 ± 0.11 (20) ab
			INT 1		5.28 ± 0.10 (20) c	1.69 ± 0.12 (20) c	1.11 ± 0.02 (20) a	1.82 ± 0.08 (20) a	3.55 ± 0.09 (20) ab
			EXP 2		5.83 ± 0.15 (20) a	2.63 ± 0.22 (20) a	1.27 ± 0.03 (20) b	2.20 ± 0.10 (20) a	3.43 ± 0.15 (19) b
			REF 1	Male	5.63 ± 0.14 (20) ac	2.22 ± 0.17 (20) ac	1.20 ± 0.02 (20) a	1.41 ± 0.07 (20) a	1.16 ± 0.05 (20) a
			REF 2		5.74 ± 0.12 (23) a	2.44 ± 0.20 (23) ac	1.22 ± 0.02 (22) a	1.25 ± 0.06 23) a	1.02 ± 0.05 (22) ab
			EXP 1		6.60 ± 0.14 (20) b	3.85 ± 0.31 (20) b	1.28 ± 0.03 (20) a	1.27 ± 0.07 (20) a	0.92 ± 0.05 (19) ab
			INT 1		5.15 ± 0.11 (20) c	1.61 ± 0.10 (20) c	1.16 ± 0.01 (20) a	1.24 ± 0.07 (20) a	1.18 ± 0.08 (20) a
			EXP 2		5.74 ± 0.20 (20) a	2.58 + 0.34 (20) ac	1.23 ± 0.03 (20) a	1.50 ± 0.08 (20) a	0.90 ± 0.08 (20) b
2009	April	Rainbow darter	INT 2	Female	4.72 ± 0.15 (20) a	1.42 ± 0.19 (20) a	1.26 ± 0.02 (19) a	4.45 ± 0.32 (20) a	12.29 ± 0.97 (20) a
			INT 3		5.03 ± 0.22 (15) a	1.87 ± 0.31 (15) a	1.30 ± 0.04 (14) a	4.80 ± 0.26 (15) a	13.56 ± 1.50 (15) a
			EXP 2		4.83 ± 0.12 (20) a	1.67 ± 0.19 (20) a	1.35 ± 0.03 (15) a	4.58 ± 0.25 (20) a	12.29 ± 1.04 (20) a
			EXP 3		4.61 ± 0.10 (20) a	1.24 ± 0.13 (20) a	1.21 ± 0.02 (20) b	3.74 ± 0.20 (20) a	11.87 ± 0.72 (19) a
			INT 2	Male	4.58 ± 0.10 (17) a	1.10 ± 0.08 (17) a	1.12 ± 0.02 (17) ab	1.84 ± 0.08 (15) a	1.59 ± 0.13 (17) a
			INT 3		4.72 ± 0.09 (20) a	1.21 ± 0.10 (20) a	1.09 ± 0.02 (19) a	2.08 ± 0.08 (17) a	1.58 ± 0.12 (20) a
			EXP 2		4.48 ± 0.09 (20) a	1.06 ± 0.08 (20) a	1.15 ± 0.02 (19) b	2.56 ± 0.12 (18) b	1.55 ± 0.10 (19) a
			EXP 3		4.74 ± 0.13 (11) a	1.21 ± 0.12 (11) a	1.10 ± 0.03 (11) a	2.07 ± 0.18 (10) a	1.75 ± 0.10 (11) a

[a] *k*, Condition factor = weight/length^3)×100

[b] LSI, Liver somatic index = (liver weight/body weight)×100.

[c] GSI, Gonadosomatic index = (gonad weight/body weight)×100.

and INT 1 reference sites ($p \leq 0.043$) (Table 3.4). Female fish collected at the EXP 1 STP site were also longer and heavier when compared to those collected from the INT 1 site ($p \leq 0.011$), which were also shorter than female Rainbow Darters collected at the upstream REF 1 site ($p < 0.001$). With respect to fish condition, female Rainbow Darters collected from both downstream MWWE sites were heavier with respect to body length when compared to the condition of female Rainbow Darters from all three reference sites ($p \leq 0.013$). Female Rainbow Darters did not demonstrate any differences among sites in liver size with respect to body size ($p \geq 0.232$) (Table 3.4). Gonad size with respect to body weight was lower in female Rainbow Darters collected downstream of the EXP 2 STP when compared to fish from the REF 1 reference site ($p = 0.022$). There were no other significant site differences in gonadosomatic index in female Rainbow Darters ($p \geq 0.401$) (Table 3.4). Male Rainbow Darters collected in the fall of 2007 downstream of the EXP 1 STP were longer and heavier than fish from the other four sites ($p < 0.002$). Male Rainbow Darters from the EXP 2 STP site were also longer and heavier when compared to male fish from the INT 1 reference site ($p < 0.031$) (Table 3.4). There were no site differences in the condition or in liver size with respect to body weight of male Rainbow Darters in the fall of 2007 ($p \geq 0.062$). Gonad size with respect to body size of male Rainbow Darters collected downstream of the EXP 2 STP was significantly reduced when compared to male fish from the REF 1 and INT 1 reference sites ($p < 0.039$) (Table 3.4). There were no other significant site differences in gonadosomatic index in male Rainbow Darters in 2007 ($p \geq 0.148$).

In April of 2009, Rainbow Darters were the only fish species present in sufficient abundance to evaluate the health of fish in the receiving environment. There were no significant differences among sites with respect to fish length or body weight of either sex ($p \geq 0.129$). Both female and male fish collected downstream of the EXP 2 STP discharge had greater condition when compared to fish collected at the INT 3 and EXP 3 sites ($p < 0.012$), while female Rainbow Darters collected at the EXP 2 site also had greater condition when compared to fish from the INT 2 reference site ($p = 0.003$) (Table

3.4). For liver size with respect to body weight there were no significant site differences in the liver somatic index of female Rainbow Darters ($p \geq 0.162$). Male Rainbow Darters collected from the EXP 2 STP site had larger livers with respect to body weight when compared to fish collected from the other three sites ($p \leq 0.020$). Neither female nor male Rainbow Darters collected in April of 2009 demonstrated any significant site differences in gonad size ($p \geq 0.253$) (Table 3.4).

3.4.3 In vitro steroid production

There were no site differences observed in basal or stimulated in vitro production of T by Greenside Darter ovarian tissue in the fall of 2007 ($p \geq 0.074$) (Table 3.5). Basal production of E2 was significantly lower in female Greenside Darter collected from the EXP 2 STP site when compared to all three reference sites ($p < 0.001$), but did not differ from basal E2 in fish from the EXP 1 STP site ($p = 0.116$), which were also significantly lower than levels observed from the REF 1 site ($p = 0.010$). No other site differences in basal E2 were observed ($p \geq 0.067$). Stimulated production of E2 by ovarian tissue was significantly lower in female Greenside Darter collected from the EXP 2 STP site when compared to all other sites ($p \leq 0.022$). Stimulated E2 production was also lower in fish from the EXP 1 STP site when compared to levels produced by INT 1 site fish ($p = 0.007$), but there were no other site differences ($p \geq 0.177$). There were no site differences in basal or forskolin-stimulated production of either E2 or T in female Rainbow Darters ($p \geq 0.308$), nor was there any significant increase by forskolin of either steroid at any of the sites ($p \geq 0.165$) (Table 3.5).

In the spring of 2009, ovarian tissues of pre-spawning female Rainbow Darters were analyzed for basal and hCG stimulated production of T and E2 in vitro. Basal and stimulated levels of T produced from fish at the EXP 3 site were significantly lower when compared to levels from fish

Table 3.5. Mean (±SE) basal and forskolin-stimulated in vitro production (pg/mg) of testosterone and 17β-estradiol by ovarian tissue from female Greenside and Rainbow Darters collected from reference and MWWE exposed sites in the Grand River from the fall of 2007 and the spring of 2009. Differences among sites ($p<0.05$) are denoted by different lowercase letters.

Year	Month	Species	Site	Testosterone		17β-Estraiol	
				basal	forskolin	basal	forskolin
2007	November	Greenside darter	REF 1	0.94 ± 0.13 a	1.83 ± 0.21 a	2.15 ± 0.19 a	3.61 ± 0.28 ab
			REF 2	0.67 ± 0.07 a	2.26 ± 0.20 a	1.59 ± 0.15 ab	3.17 ± 0.35 ab
			EXP 1	0.56 ± 0.09 a	1.80 ± 0.13 a	1.51 ± 0.16 bc	3.01 ± 0.16 a
			INT 1	0.76 ± 0.16 a	2.03 ± 0.15 a	3.95 ± 0.29 ab	3.95 ± 0.29 b
			EXP 2	0.65 ± 0.13 a	1.65 ± 0.23 a	0.96 ± 0.11 c	2.05 ± 0.26 c
		Rainbow darter	REF 1	1.43 ± 0.48 a	0.79 ± 0.33 a	4.52 ± 0.17 a	2.80 ± 0.74 a
			REF 2	1.37 ± 0.39 a	0.88 ± 0.24 a	3.35 ± 0.51 a	2.97 ± 0.45 a
			EXP 1	0.96 ± 0.21 a	0.89 ± 0.22 a	2.58 ± 0.41 a	2.59 ± 0.41 a
			INT 1	0.99 ± 0.28 a	1.06 ± 0.30 a	2.39 ± 0.63 a	2.39 + 0.63 a
			EXP 2	1.16 ± 0.28 a	0.90 ± 0.19 a	2.20 ± 0.43 a	1.72 + 0.31 a
2009	April	Rainbow darter	INT 2	0.38 ± 0.08 a	0.89 ± 0.14 a	0.74 ± 0.07 a	0.81 + 0.10 a
			INT 3	0.31 ± 0.09 ab	0.46 ± 0.14 b	0.65 ± 0.08 a	0.81 + 0.13 a
			EXP 2	0.38 ± 0.08 ab	0.71 ± 0.16 ab	0.49 ± 0.09 a	0.98 + 0.21 a
			EXP 3	0.18 ± 0.06 b	0.38 ± 0.13 b	0.44 ± 0.11 a	0.82 + 0.24 a

collected from the INT 2 site ($p \leq 0.030$). Stimulated T production was also significantly different between the reference sites with lower levels from fish at the INT 3 and EXP 3 sites when compared to the INT 2 site ($p \leq 0.006$). There were no other significant site differences in basal or stimulated T or E2 steroid production capacity in female Rainbow Darters ($p \geq 0.076$).

In the fall of 2007 there were no site differences in basal T production by male Greenside Darter, however males collected at the EXP 2 STP site had significantly lower forskolin-stimulated T production when compared to all other sites ($p \leq 0.036$) which did not differ from each other ($p \geq 0.062$) (Table 3.6). Male testicular tissue responded to forskolin stimulation at all sites however the increase was only statistically significant at the REF 2, EXP 1 and EXP 2 STP sites ($p \leq 0.047$). Basal 11KT production was significantly lower in male Greenside Darter collected at the EXP 2 STP and at REF 1 when compared to INT 1 males ($p=0.041$ and $p=0.006$; respectively). There were no other significant site differences in male Greenside Darter basal or forskolin-stimulated 11KT production ($p \geq 0.212$). Only INT 1 Greenside Darter testicular tissue responded significantly to forskolin stimulation ($p=0.025$). Due to low basal T and 11KT production by testicular tissue of Rainbow Darters in the fall of 2007, only stimulated T levels were compared statistically. Levels of 11KT produced by Rainbow Darters were below the detection limit of the assay and are not reported. Production of forskolin-stimulated T from males collected at both the EXP 1 and EXP 2 STP sites were significantly lower than steroid levels in fish from the REF 1 and INT 1 reference sites ($p \leq 0.006$), and EXP 1 STP levels were also significantly lower than those observed at the REF 2 reference site ($p=0.017$). In April 2009, pre-spawning male Rainbow Darters collected at the EXP 3 far field site had significantly lower stimulated T production when compared to male fish from all other sites ($p<0.022$). There were no other significant differences among sites ($p>0.307$) (Table 3.6). Stimulated 11KT production from male fish collected from both the near field (EXP 2) and far field (EXP 3) STP exposure sites was

Table 3.6. Mean (±SE) basal and forskolin-stimulated in vitro production (pg/mg) of testosterone and 11-ketotestosterone by testicular tissue from male Greenside and Rainbow Darters collected from reference and exposed sites in the Grand River from the fall of 2007 and forskolin-stimulated in vitro production in the spring of 2009. Significant differences among sites ($p<0.05$) are denoted by different lowercase letters. Levels of 11-ketotestosterone produced by Rainbow Darters in the fall of 2007 were below the assay's detection limit (ND). NA indicates sample Not Available.

Year	Month	Species	Site	Testosterone		11-Ketotestosterone	
				basal	forskolin	basal	forskolin
2007	November	Greenside darter	REF 1	1.59 ± 0.42 a	2.29 + 0.34 a	0.61 ± 0.08 a	0.98 ± 0.22 a
			REF 2	1.46 ± 0.35 a	2.66 ± 0.38 a	0.95 ± 0.44 ab	1.64 ± 0.99 a
			EXP 1	0.72 ± 0.18 a	1.96 ± 0.17 a	0.62 ± 0.27 ab	0.58 ± 0.11 a
			INT 1	1.72 ± 0.32 a	2.64 ± 0.28 a	1.27 ± 0.31 b	0.56 ± 0.05 a
			EXP 2	1.04 ± 0.21 a	1.0 ± 0.21 b	0.49 ± 0.11 a	0.34 ± 0.11 a
		Rainbow darter	REF 1	2.46 ± 0.75 a	3.84 ± 0.58 a	ND	ND
			REF 2	1.99 ± 0.0 a	3.17 ± 0.27 ab	ND	ND
			EXP 1	0.55 ± 0.0 a	1.83 ± 0.15 c	ND	ND
			INT 1	NA	3.86 ± 0.46 a	ND	ND
			EXP 2	0.74 ± 0.47 a	2.58 ± 0.62 bc	ND	ND
2009	April	Rainbow darter	INT 2	NA	1.23 ± 0.20 a	NA	1.57 ± 0.25 a
			INT 3	NA	0.75 ± 0.09 a	NA	0.82 ± 0.11 b
			EXP 2	NA	0.77 ± 0.10 a	NA	0.76 ± 0.12 b
			EXP 3	NA	0.51 ± 0.04 b	NA	0.70 ± 0.12 b

significantly lower when compared to levels observed in males from the INT 2 reference site ($p<0.041$), but was not significantly different from levels observed in males from the INT 3 reference site ($p\geq0.074$).

3.4.4 Histopathology

Gonad sections were examined for alterations in gonadal development in fish collected downstream of STP discharges when compared to that of fish from reference sites. Male Greenside Darter collections from the REF 2, EXP 1 and EXP 2 sites had a significantly lower frequency of spermatogonia when compared to male Greenside Darter from the INT 1 site ($p\leq0.005$) (Figure 3.3). Spermatids were also in higher relative abundance in fish from the REF 2 and EXP 1 STP sites when compared to fish from the REF 1 site ($p=0.012$), and the presence of mature spermatozoa was higher in male Greenside Darter collected downstream of both MWWE discharges when compared to the frequency of this cell type in fish from REF 2 ($p=0.012$). Testicular tissue in male Rainbow Darters collected downstream of both the EXP 1 and EXP 2 STP discharge sites demonstrated higher frequency of the earlier cell stages (spermatogonia, and spermatocytes), and lower percentage of mature spermatozoa when compared to levels found in fish from the REF 1 and REF 2 sites ($p\leq0.030$) (Figure 3.3). The frequency of spermatogonia was also higher with lower numbers of spermatozoan cells in male testes from the INT 1 site compared to tissues from the REF 1 and REF 2 sites ($p\leq0.030$) (Figure 3.3). Histopathology of females of both darter species did not demonstrate any site differences in oocyte development, the frequency of the observed cell types or in the size of vitellogenic oocytes (data not shown). Male testicular tissue was also scanned for the presence of eggs (Figure 3.3 & 3.4). Male Greenside Darter and Rainbow Darters collected downstream of the EXP 1 STP site in the fall of 2007 had an intersex incidence rate of 33%, and the incidences of intersex in male fish collected downstream of the EXP 2 STP site were 60 (Greenside Darter) and 75% (Rainbow Darters),

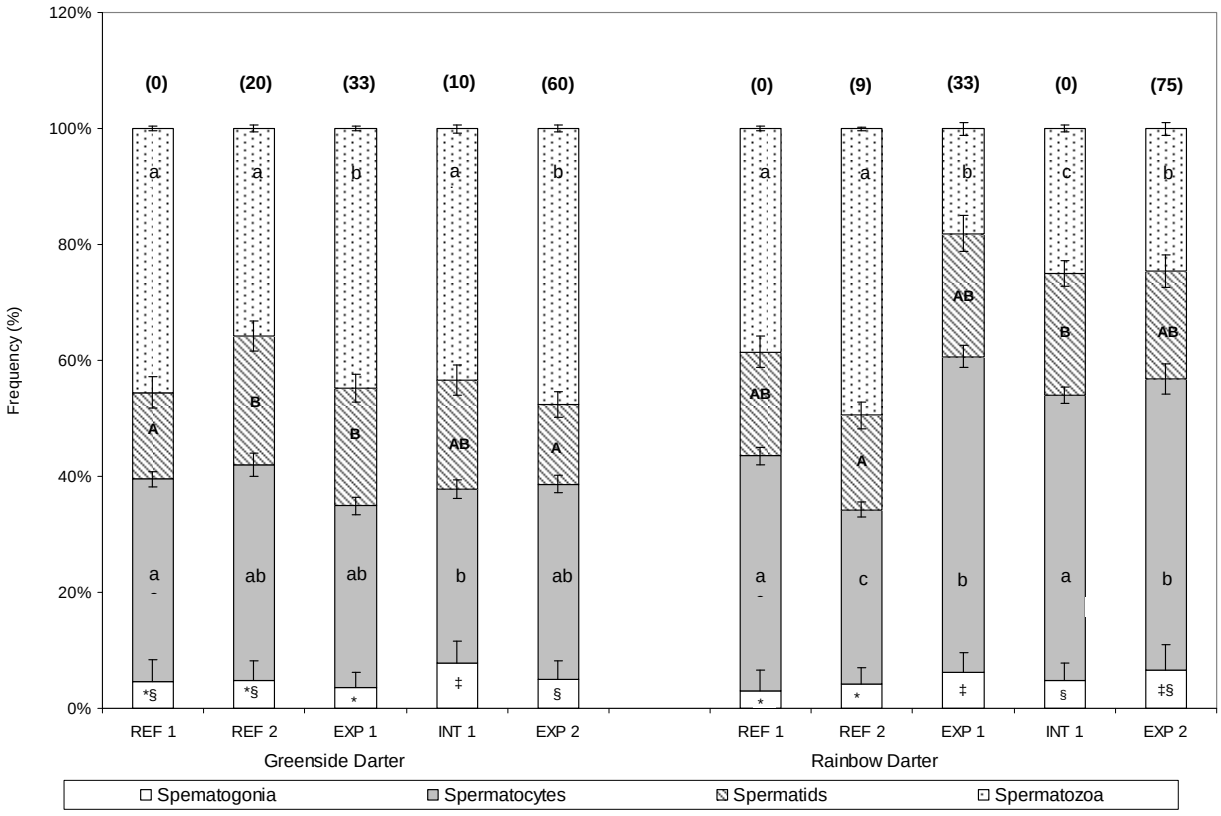
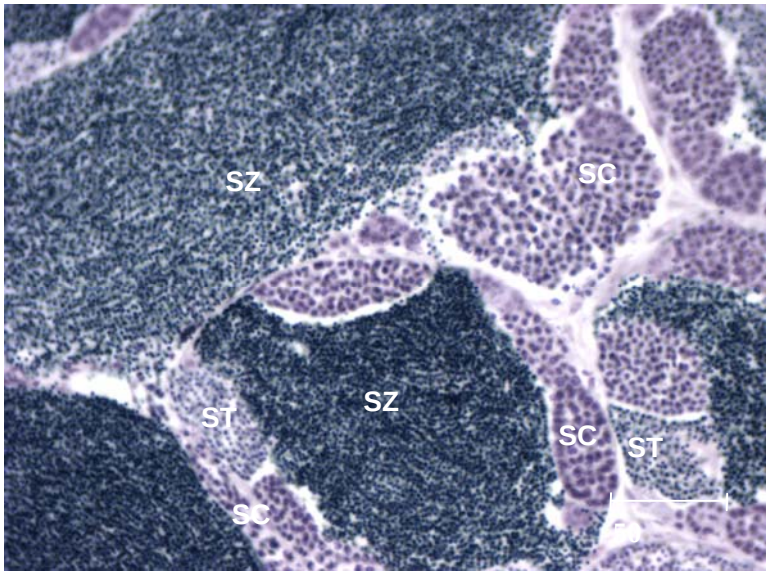


Figure 3.3. Histopathology of male Greenside and Rainbow Darters collected in the Grand River in the fall of 2007. Different letters and symbols denote statistically significant differences in frequency of cell type among sites. (%) indicates the frequency in which the intersex condition was identified in male fish at each site.

a)



b)

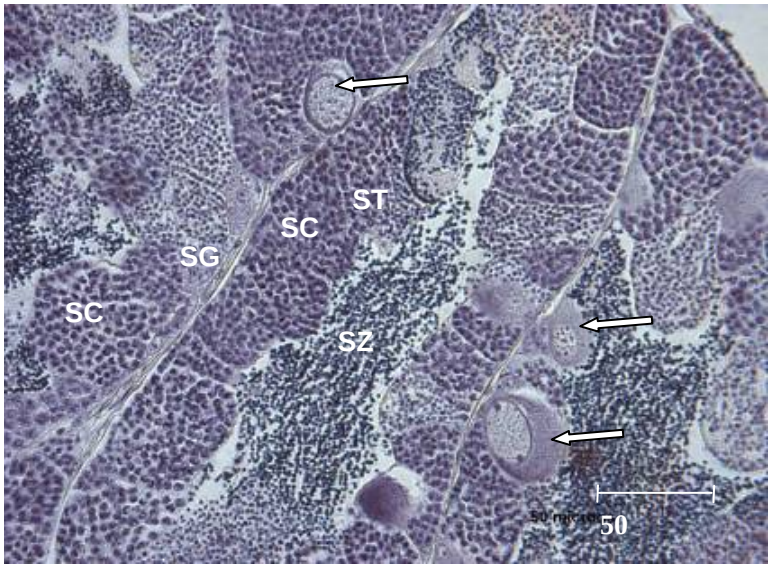


Figure 3.4. Gonadal tissue sections from male Rainbow Darter sampled in November, 2007. Testicular tissue from male darters collected from (a) the REF 1 reference site and (b) EXP 2 exposed STP site with primary-stage oocytes situated within the gonad tissue. Arrows indicate primary-stage oocytes among the testicular tissue clusters of spermatocytes (SC), spermatids (ST), spermatogonia (SG), and spermatozoa (SZ).

respectively (Figure 3.3). Intersex was observed in male Greenside Darter and Rainbow Darters collected from the REF 2 reference site (20 and 9%; respectively), and in male Greenside Darter collected from the INT 1 site (10%). Only the previtellogenic oocyte stage was observed in any of the intersex fish.

In a scan of testicular tissue from male Rainbow Darters collected in April 2009, the incidence of intersex in male fish collected downstream of the near field (EXP 2) and far field (EXP 3) STP sites were 60 and 100%, respectively. The intersex condition was also observed in male fish from both the INT 2 and INT 3 reference sites (33 and 50%, respectively). There were no sites differences in the relative abundance of the four cell types in male testes in April 2009 (data not shown). Although pre-spawning female Rainbow Darters collected in April 2009 downstream of the EXP 2 STP appeared to have a greater proportion of vitellogenic oocytes, and female Rainbow Darters from the EXP 3 site had considerably higher proportion of primary and fewer vitellogenic oocytes relative to the other sites, there were no statistically significant site differences with respect to the frequency of the four major developmental stages of oocytes (primary, cortical alveolar, vitellogenic, mature oocytes) ($p \geq 0.057$) (data not shown).

3.5 Discussion

In this study of the cumulative impacts of exposure of wild fish to two MWWE discharges, fish health, physiological responses and reproductive tissue development were examined. The impacts of point-source industrial discharges in Canada such as pulp and paper mill effluents have been well documented (McMaster et al., 2006) as well as the cumulative impact of MWWE adjacent to pulp mill effluent discharge on fish responses (Dube et al. 2005; McMaster et al. 2005), and industrial inputs on fish (Mikaelian et al., 2002; Kavanagh et al., 2004).

Assessing the effects of estrogenic chemicals in receiving environments downstream of STP discharges initially began in the mid 1990s (Purdom et al., 1994; Jobling et al., 1998). The current

study documents subtle effects of exposure to municipal wastewater effluents on small forage fish health in the Grand River, Ontario. In confirmation of predictions from other studies, nutrient enrichment responses such as increased energy storage in darters (condition factor, liver somatic index) were observed. Female and male darters of both species tended to be longer and heavier when collected at downstream MWWE exposed sites compared to fish collected from reference sites. Rainbow Darters demonstrated seasonal site differences with respect to fish condition between the spring and fall. Fish collected downstream of the MWWE discharges in the fall of 2007 were fatter relative to reference sites, however only male darters downstream of the second discharge were fatter in spring collections. The fish health parameters measured in this study indicate that both species of spring spawning darters collected downstream of the MWWE discharges in the fall have the potential to be larger (longer and heavier) than fish collected from the reference sites regardless of sex. Male Greenside Darters exposed to the second MWWE effluent (EXP 2) in the fall did have higher liver somatic indices indicating increased energy storage. This indicates that conditions in the aquatic environment receiving MWWE are capable of producing larger fish, however the fish are not assimilating the additional anthropogenic resources towards reproductive output. Our results are similar to previous studies that have measured health parameters of fish exposed to MWWE effluent. Although McMaster et al. (2005) observed increased condition and LSI in Longnose Sucker downstream of a MWWE discharge in Alberta, Canada, there is increasing evidence that exposure to MWWE effluent does not always manifest in increased fish condition. When it has been reported, wild fish collected downstream of MWWE tend to invest similar energy into body mass (Roach (Jobling et al., 1998); Gudgeon (van Aerle et al., 2001); White Sucker (Hinck et al., 2009); and Smallmouth Bass (Iwanowicz et al., 2009)). However, Allen et al. (1999) did report elevated LSI in wild Flounder in UK sewage estuaries. The river system examined by McMaster et al. (2005) is normally nutrient-limited therefore, the observed increases in fish growth and condition was attributed

to sewage effluent providing increased food availability. The current study documented increased condition and LSI in exposed fish in the spring collection of male Rainbow Darters downstream of the EXP 2 MWWE discharge. At this point and time, it is difficult to determine if these males were assimilating more energy from their environment (MWWE derived nutrients), or if they had simply failed to transform their internal energy reserves into gonad growth. These fish did have lower, albeit not statistically significant, GSI than fish from the other sites. An exception to the trend of increased condition following exposure to MWWE, was observed in male Carp collected in the spring downstream of a STP in Spain with reduced condition (Sole et al., 2002). In this study, researchers suggested that the lower fish condition could be attributed to either higher estrogenic exposure or the physiological state of the fish somehow affected this general health parameter.

In the current study, darters exposed to the second STP effluent at site EXP 2 consistently demonstrated reduced gonadal development relative to body weight. Female fish of both species and male Rainbow Darters demonstrated reduced GSI relative to upstream sites. The effects of exposure to MWWE on gonadal development in wild fish appear to be study-specific. Reduction in GSI of MWWE effluent exposed fish have been reported in Roach (Jobling et al., 1998), Flounder (Allen et al., 1999), Bream (Hecker et al., 2002) and White Sucker (Vajda et al., 2008). Gonadal development of Smallmouth Bass exposed to treated sewage in the Conococheague Creek (tributary of the Potomac River, USA) was reported to be 33-50% reduced when compared to reference populations (Iwanowicz et al., 2009). Pre-spawning female Bream collected downstream of major urban areas on the Elbe River, Germany, demonstrated lower GSI than fish from the control site which was attributed to an inhibition of maturation (Hecker et al., 2002). Significant reductions in GSI was also reported by van Aerle et al. (2001), however these Gudgeon collections occurred during various times of the year; therefore, further research is required to confirm if this effect was due to fish MWWE effluent exposure or if the selection of lake populations of Gudgeon as reference controls was appropriate. A

significant increase in GSI in MWWE exposed male and female wild Flounder was documented in Denmark (Bjerregaard et al., 2006). MWWE effluent is a complex matrix whose composition may vary seasonally and temporally (Harries et al., 1996; Alvarez et al., 2009), and as a result responses in exposed fish may also vary. McMaster et al. (2005) reported no significant impact on GSI of Longnose Sucker in the first year of their study, but reported MWWE exposed male sucker to have reduced GSI in the second year of their study. Jobling et al. (2002a) also reported that Roach collected in the spring did not differ in GSI, however, they measured gonad mass after stripping the fish of gametes for fertilization experiments. There were no alterations in GSI in either sex of two species of fish collected downstream of sewage treatment plants in Belgium (Douxfils et al., 2007).

An objective of this study was to investigate the potential for the intersex condition (female oocytes in male testicular tissue), which has been previously reported in wild fish exposed to MWWE effluents. In this study it was demonstrated that male darters collected downstream of multiple STP discharges had increased incidence of intersex compared to fish collected in urban stretches of the river at a distance from MWWE influences. Male darters of both species collected in the upstream agricultural region (REF 1) demonstrated no evidence of intersex whereas our urban reference and intermediate sites had incidences of intersex in Greenside Darters (20 and 10%, respectively); and in Rainbow Darters (9 and 0%, respectively). Noticeable differences in the rates of intersex between darter species may indicate differences in species sensitivity to endocrine disrupting compounds at these sites. Rates of intersex were also higher downstream of the first STP discharge studied; fish appeared to recover somewhat as intersex rates decreased at the intermediate site, but then increased again downstream of the second MWWE discharge. This suggests that fish populations have the potential to recover prior to exposure to the second MWWE effluent. Pre-spawning Rainbow Darters demonstrated dramatically higher incidence of male testicular oocytes in the spring compared to fall at both (upstream) intermediate urban reference sites (33 & 50%, respectively), and at the near-field

(EXP 2) and far-field exposure sites (EXP 3) (60 & 100%, respectively). It is probable that the higher incidence of intersex in the spring is due to effluent from the sewage treatment plants contributing a larger proportion of the mean flow of the river system during the winter months.

The intersex condition has been reported in wild fish collected downstream of sewage effluent in the UK, Europe, and the USA (Table 3.1). Mikaelian et al. (2002) discovered intersex in Lake Whitefish (*Coregonus clupeaformis*) in the St. Lawrence River, Quebec, Canada in the proximity of a sewage outfall, and Kavanagh et al. (2004) demonstrated high prevalence of intersex in wild White Perch (*Morone americana)* exposed to domestic and industrial effluents in two large bays of Lake Ontario, Canada. White Perch sampled in Cootes Paradise, a freshwater estuary of Hamilton Harbour, receives municipal sewage treatment effluent and had the highest incidence of intersex in this study (Kavanagh et al., 2004). Recently, a greater proportion of non-reproductive males, feminization of the male urogenital papilla and intersex has also been observed in the invasive Round Goby (*Neogobius melanostomus*) from sites in Hamilton Harbour (Marentette et al., 2010). Due to the presence of other major industries in these larger systems, a case must be built to associate causation of this intersex with MWWE exposure in these studies. Lake Whitefish demonstrating intersex were also exposed to a variety of agricultural and industrial practices in the St. Lawrence system impairing the ability to identify causation (Mikaelian et al., 2002).

Stable isotope analysis in this study confirmed site fidelity of darters and also provided some insight into the effects of anthropogenic sources of nutrients on aquatic biota. Loomer (2008) demonstrated lower variability in isotope signatures in the fall when compared to the spring collections, therefore the results presented in this study were collected at a period with limited variability to provide an optimal representation of the carbon and nitrogen assimilated by darters in this system. Previous studies have demonstrated that increasing human inputs of nitrogen results in the increasing nitrogen δN ‰ values in aquatic organisms (Anderson and Cabana, 2005; Dube et al.,

2005; Loomer, 2008). Similar to Loomer (2008), δN^{15} values of fish in this study gradually increased downstream of the effluent plume of the Kitchener STP where δN^{15} values decreased significantly. Site similarities between δN^{15} values in fish muscle from the REF 2 and EXP 1 sites may have also been influenced by contributions from inputs from the Conestogo River tributary, which is a predominantly agricultural watershed that enters the Grand River upstream of the urban reference site REF 2. The Conestogo River is the only major tributary entering this stretch of the Grand River and it assimilates the sewage from the town of St. Jacobs (population of 2000), which is located approximately 7 km upstream of the confluence of the Conestogo and the Grand Rivers. Loomer (2008) also concluded that darters living within the Waterloo STP effluent plume were affected by the effluent and that nutrients from the STP were utilized by the darters within the plume. Alterations in the N-signatures are attributed to alterations in the speciation of the nitrogen available to primary consumers and the food web downstream of the STP. The existing MWWE treatment processes at the second treatment (Kitchener STP) plant result in high inputs of nitrogen, especially ammonia, into the river. The alteration in fractionation of nitrogen species within the treatment plant may alter the δN ‰ available to primary producers, resulting in much lighter signatures in the primary producers. This shift in nitrogen signature is manifested in a dramatic decrease in δN ‰ values in the EXP 2 exposed fish, which in turn are the prey items for higher trophic level predators. The impact of the alterations in nitrogen on the aquatic food web requires further investigation.

Failure to allocate energy resources toward reproductive development would indicate an alteration in energy allocation and reproductive impairment. Jobling et al. (2002a) was able to demonstrate that intersex in wild Roach was manifested in a wide variety of ways, including intersex, malformation of the gonads and/or reproductive ducts and altered gamete production. Intersex Roach had decreased milt production, sperm motility, and fertilization success compared to histologically normal male Roach. Spermiation in intersex male Roach was also affected as a lower proportion of

fish were found releasing sperm, and in those intersex fish that were spermiating, a reduced milt volume and a reduced sperm density were observed (Jobling et al., 2002b). Vajda et al. (2008) also documented significant reduction in sperm abundance among effluent exposed male White Suckers. All intersex fish had malformations of the reproductive duct(s), and in severely affected fish, ducts were occluded, thus preventing release of gametes. Fibrosis and inhibition of testicular development has also been observed in male wild White Sucker exposed to STP effluent (Woodling et al., 2006), and in male Fathead Minnow exposed to 17α-ethynylestradiol in a whole-lake exposure (Palace et al., 2002). Research investigating gamete quality of intersex darters in this system has merit and should be pursued in the future as well as duct formation, orientation of the gonad, genital pore, and duct blockage.

This study did not demonstrate any major alterations in female gonadal development as in wild female fish exposed to other STP effluents (Allen et al., 1999; Harshbarger et al., 2000; Minier et al., 2000; Viganò et al., 2001; Hecker et al., 2002; Jobling et al., 2002b; Sole et al., 2003; McMaster et al., 2005; Hinck et al., 2009). In Belgium, STP exposed Stoneloach did not affect female gonadal development, but Gudgeon from that same study demonstrated advanced oocyte development at one of the exposure sites relative to fish from the reference site (Douxfils et al., 2007). van Aerle et al. (2001) and Sole et al. (2002) also evaluated female gonad morphology but these results were compromised as fish were sampled at various times of the year preventing researchers from drawing conclusions. Woodling et al. (2006) observed asynchronous ovarian development in Colorado White Suckers exposed to STP effluents; however, the ovaries of only one female White Sucker contained malformed pre-vitellogenic oocytes. In a follow-up study, exposed female White Sucker displayed asynchronously developing ovaries with up to five oocyte stages when reference female were synchronous (Vajda et al., 2008). Female Smallmouth Bass exposed to STP effluent in the Potomac River displayed less advanced cellular developmental stages (Iwanowicz et al., 2009). Impacts on

ovarian development in darters in this study appear to be minor; however, male fish are experiencing major alterations in cellular development and organization suggesting that the compounds released in MWWE effluents have a tendency to act on the male reproductive system more so than female or that perhaps the male system is more susceptible to assault from anthropogenic compounds.

Wild darters collected immediately downstream of MWWE discharges in this study demonstrated a reduced ability to produce sex steroids in vitro. Examination of circulating sex steroids in these small-bodied fish was not possible due to low blood volume, however the in vitro production of sex steroids by gonadal tissue has been used successfully as a surrogate to circulating levels in fish (Gibbons et al., 1998; Tetreault et al., 2003; Chiang et al., 2010). Similar to results presented in this study, Longnose Sucker collected downstream of a STP discharge in north-western Alberta demonstrated a reduction in circulating E2 levels as well as a reduction in the ability of female ovarian tissue to produce E2 (McMaster et al., 2005). Female wild Roach collected downstream of a UK STP had approximately half the plasma E2 concentrations compared to reference females, while intersex male Roach had twice the circulating E2 levels than intersex fish sampled at the reference sites (Jobling et al., 2002b). Intersex males in the UK study demonstrated intermediate steroid profiles by having up to twice the amount of circulating T than males from the reference sites. A reduction in plasma E2 in wild Bream was also documented in the upper Elbe River downstream of the STP for the city of Dresden, Germany (Hecker et al., 2002). Douxfils et al. (2007) demonstrated lower plasma 11-ketotestosterone and testosterone in female Stoneloach and Gudgeon from STP exposed sites and male Gudgeon had higher than reference 11-ketotestosterone and 17β-estradiol levels. In an extensive study of the occurrence of intersex in Black Bass across the USA, plasma 11-ketotestosterone and the ratio between 17β-estradiol and 11-ketotestosterone did not differ regardless of the degree of intersex (Hinck et al., 2009). Given the high incidence of intersex in darters from this study it would be prudent to analyze the testicular tissue of male darters downstream of STP discharges in the Grand

River for the ability to produce E2 in vitro. In this study it was not possible to measure E2 as the measurement of T and 11KT used all of the incubation media. Future research should investigate the natural occurrence of intersex in these darters (*Ethoestoma sp.*), the potential for intersex in other species at these sites, and the capacity of testicular tissue in normal and intersex fish to produce E2 in vitro.

Municipal discharges have been identified as the major source of estrogenic compounds in the aquatic receiving environment (Purdom et al., 1994; Harries et al., 1996; Tyler et al., 1996; Servos et al., 2005, 2003). As the impacts of estrogenic compounds are of major concern in STP effluent contaminated waters, circulating plasma levels of vitellogenin (VTG), the egg yolk precursor, has been used extensively as a biomarker for exposure of aquatic biota to estrogenic contamination in males because the physiological production of vitellogenin is normally restricted to females (Purdom et al., 1994; Sumpter and Jobling, 1995; Jobling et al., 1998; Allen et al., 1999; Hecker et al., 2002; Diniz et al., 2005; Kavanagh et al., 2004; Vajda et al., 2008; Iwanowicz et al., 2009). Exposure to estrogens can trigger the expression of the vitellogenin gene in male fish. Vine et al. (2005) demonstrated no significant differences in the mean plasma vitellogenin concentrations of Pike sampled from sites upstream or downstream of sewage discharges, however the lack of a statistically significant difference was attributed to high variability in vitellogenin in reference male Pike. Reported plasma vitellogenin levels for intersex Pike in the study were similar to those of female Pike (Vine et al., 2005). Although many studies have measured elevated levels of vitellogenin in male fish and reported incidence of the intersex condition in male fish exposed to STP effluent, few studies have developed a strong correlation between vitellogenin concentration and presence of intersex. Substantive evidence has been documented to support the hypothesis of estrogenic compounds contributing to the cause of intersex in exposed fish, however estrogen exposure has correlated less with plasma vitellogenin in the same fish (Jobling et al., 2006). These relationships appear to be site- or species-specific. Analysis of

vitellogenin was consistent with the hypothesis that the intersex observed in White Perch was the result of exposure to estrogenic endocrine disrupting substances, however this study did not correlate vitellogenin concentration with degree of intersex as all exposed males displayed some measurable plasma vitellogenin (Kavanagh et al., 2004). Similarly, the incidence of intersex in White Sucker could not be consistently correlated with exposure to MWWE derived contaminants (Hinck et al., 2009). Currently, the vitellogenin protein is not yet commercially available for *Etheostoma sp.*, and we have been unsuccessful in finding cross-reactivity with other known vitellogenin genes. It is reasonable to postulate that due to the incidence of intersex in male darters presented in this study that male darters would have measurable levels of vitellogenin in their plasma. As it will not be possible to extract circulating vitellogenin from these small-bodied fish due to low blood volume, future research will attempt to isolate the vitellogenin gene from *Etheostoma sp.* hepatic tissue homogenates.

Long-term reproductive dysfunction in small-bodied forage fish may have a critical impact on the health of the ecosystem. Failure of these populations to sustain themselves would have significant impacts on the ecosystem as they are major constituents of the food chain for larger sport fisheries (walleye, bass, pike, and perch) (Scott and Crossman, 1998). Brown (2010) reported that the forage fish community in this region of the Grand River is often composed of 75-90% *Etheostoma sp.* making them a major food source for larger fish species. As these species are shorter lived (3-4 yrs), a reduction in reproductive output/success would manifest itself in population declines much more rapidly than would be seen with longer lived fish species (Gibbons et al., 1998a; Tetreault et al., 2003; Gray and Munkittrick, 2005; Kidd et al., 2007). In a whole lake exposure to the synthetic estrogen used in birth-control pills (17α-ethynylestradiol (EE2)), researchers demonstrated near extinction of the lake's population of Fathead Minnow (*Pimephales promelas*) as the result of reproductive failure (loss of recruitment, intersex in males, altered oogeneiss in females) (Kidd et al., 2007). This study demonstrated that continued inputs of natural and synthetic estrogens and estrogen mimics to the

aquatic environment in municipal wastewaters could decrease the reproductive success and sustainability of wild fish populations.

This study documents the first evidence of the presence of intersex in wild fish species observed in the Grand River. The increased incidence of intersex fish downstream of the second STP discharge is thought to be due to the cumulative impact of estrogenic compounds from both STP effluent discharges. It is not yet known the degree to which these intersex fish remain functionally male. Future research should also investigate E2 production in male fish (E2:T), aromatase activity (conversion of T to E2), and production of VTG in darter hepatic homogenates.

It is clear from this study that urban stretches of the Grand River are exposed to estrogenic compounds (man-made or otherwise), causing adverse biological effects (intersex in males) that might impair their ability to reproduce normally. It is not known if these fish populations are self-sustaining or if they rely in immigration from other stretches of the river less impacted by MWWE discharges to sustain their populations. It is important to establish this information prior to the projected human population growth in the watershed, which will require the aquatic environment to assimilate an increasing amount of municipal waste. In the future, investments in infrastructure will also be evaluated as both treatment plants in this study are scheduled for upgrades. This presents an opportunity to evaluate if facility modernization will alleviate the reproductive impacts currently seen in the receiving environment.

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Chapter 4

Reproductive and histopathological effects in wild fish inhabiting an effluent-dominated stream, Wascana Creek, SK, Canada.

4.1 Summary

During the winter low flow periods, Wascana Creek, Saskatchewan, Canada, can be 100% treated municipal wastewater downstream of the City of Regina's Wastewater Treatment Plant. The objective of this study was to determine if exposure to municipal effluent affects the health and reproductive development of fish in an effluent-dominated stream. Field studies were conducted on post-spawning (August 2006), spawning (June 2007), recrudescent (October 2007) and pre-spawning (May 2008) sentinel fish (Fathead Minnow *Pimephales promelas* and Brook Stickleback *Culaea inconstans*) to assess responses in terms of energy storage (condition factor and liver somatic index), reproduction (in vitro sex steroid biosynthetic capacity, gonadosomatic indices, and histology) and survival associated with exposure to the effluent. Sentinel species demonstrated varying responses depending on the season of field collections. While Stickleback collected downstream of the MWWE discharge were often longer, heavier and had greater condition, Fatheads from the same site were shorter and lighter. Exposed fish of both species exhibited delayed spawning and altered gonadal development depending on the season. Exposed male Fathead Minnows also had significantly lower scores of secondary sexual characteristics (fewer nuptial tubercles, little or no development of the dorsal pad, and the lack of presence of a dorsal fin dot). Histopathology of exposed Fathead Minnows revealed thickening of the gill lamellae and alterations in structure of the kidneys (inflammation of the proximal tubules and Bowman's capsule). It is not known if the effluents are affecting natural reproduction and recruitment into this population or if these impacted populations rely on immigration from upstream reaches to sustain the populations. Climate change and human population growth will further challenge the ability of this effluent-dominated stream to assimilate nutrients and contaminants which may further impair the performance of fish in this semi-arid environment.

Keywords: Reproductive impairment, fish responses, sewage, histopathology

4.2 Introduction

Municipal wastewater effluent (MWWE) is a mixture of liquid wastes and chemicals discharged from residential, institutional, commercial and industrial sources. The input of MWWE organic material into the aquatic receiving environment can result in eutrophication, elevated ammonia levels and lower dissolved oxygen content (Chambers et al., 1997; CanNorth, 2009; Waiser et al., 2011a). It is also a matrix containing environmental contaminants such as natural and synthetic hormones, pharmaceuticals and personal care products (Metcalfe et al., 2003, Servos et al., 2005, Waiser et al., 2011b), and industrial chemicals (Servos et al., 2003). Contaminants in MWWE are of particular concern in effluent-dominated streams where municipal and/or industrial wastewater effluents are discharged to these ephemeral or intermittent streams where they may comprise the majority of the stream flow (Brooks et al., 2006). Wascana Creek, Saskatchewan, is such a receiving environment as during the winter months treated MWWE from the City of Regina contributes 100% of the creek flow for 60 km downstream until confluence with the Qu'Appelle River (Waiser et al., 2011ab) (Figure 4.1). Given the continuous nature of effluent discharge and the low seasonal dilution of effluent, Wascana Creek is an ideal ecosystem for studying aquatic organism responses to pseudo-persistent emerging contaminants in MWWE. This creek does not receive any other major discharges downstream of the outfall.

Early studies evaluating the effects of sewage effluent on wild fish in the UK demonstrated an intersex condition (female oocytes in male testicular tissue) in the histopathology of male Roach (*Rutilus rutilus*) collected downstream of sewage treatment plants (Jobling et al., 1998). The presence of the intersex condition has been observed in other wild fish exposed to municipal wastewater effluent and has been strongly correlated with further reproductive impairments such as altered gonadal development and sex steroid levels (Hecker et al., 2002; Jobling et al., 2002a; Douxfils et al., 2007; Tetreault et al., 2011). Given the low seasonal dilution of municipal effluent in Wascana

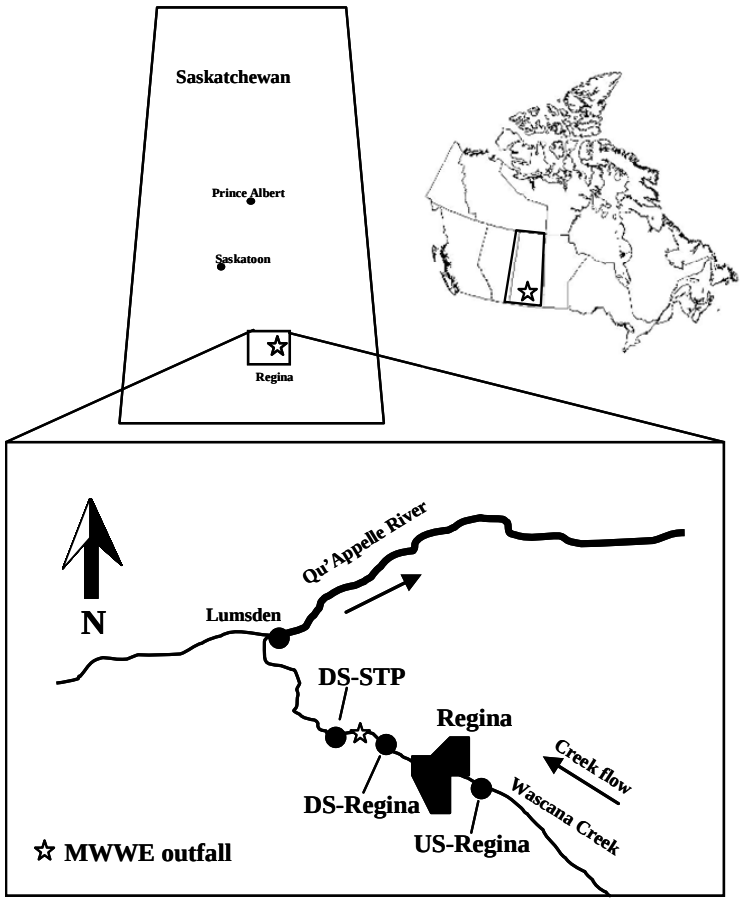


Figure 4.1. Map showing location of the Regina sewage treatment plant (star) and upstream reference (US-Regina, DS-Regina) and a downstream STP (DS-STP) exposure sites in Wascana Creek, Regina, Saskatchewan, Canada.

Creek there is potential that exposure to MWWE in this system can alter the reproductive performance in fish. MWWE effluent is a complex matrix whose composition may vary seasonally and temporally (Harries et al., 1996; Alvarez et al., 2009). As a result, the responses of fish exposed to municipal effluents also have the potential to vary (Jobling et al., 2002a; McMaster et al., 2005).

The overall objective of this research was to evaluate the effects of MWWE on wild fish populations in an effluent-dominated stream in Canada. The impact of municipal wastewater on fish performance, including detailed reproductive assays during several stages of the reproductive cycle and gonadal, respiratory and renal histopathology were evaluated to determine the influence of the effluent on small-bodied fish in a small aquatic receiving system.

4.3 Materials and methods

4.3.1 Study sites

The City of Regina is serviced by a tertiary treatment wastewater facility followed by ultraviolet disinfection (Waiser et al., 2011b). After primary treatment the effluent is transferred to a series of aerated facultative lagoons (90.82 hectares) using a combination of coarse and fine bubble tube aeration systems. Effluent retention time in the lagoon system is approximately 30 days. The secondary effluent then flows by gravity into clarifiers for tertiary treatment. Alum and an aionic polymer are then added for removal of phosphorus and algae. The tertiary effluent is then disinfected by ultraviolet light (US) for bacterial control. The characteristics of the treated effluent that discharges into Wascana Creek are described in Waiser et al. (2011a). Comparisons were made among fish collected upstream of the City of Regina (US-Regina), downstream of the city but upstream of the treatment facility (DS-Regina) (reference sites), and immediately downstream of the City of Regina's Wastewater Treatment Plant (DS-STP) (exposure site) (Figure 4.1). Wascana Creek field studies assessed sentinel fish species under post-spawning (August 2006), spawning (June 2007),

recrudescence (October 2007), and pre-spawning conditions (April 2008). The reproductive performance of resident fish was evaluated by in vitro sex steroid biosynthetic capacity, gonadosomatic indices, and histology. Condition factor and liver somatic index were also calculated to evaluate energy storage in exposed fish.

4.3.2 Field collections and fish health

Field studies conducted in August 2006 identified the small-bodied (<3.0 g body weight and <7.0cm in length) Brook Stickleback (*Culaea inconstans*) and Fathead Minnow (*Pimephales promelas*) as distributed throughout this reach of Wascana Creek making them desirable sentinel species for this study. Fish were collected using minnow traps deployed overnight for at least 12 h. In August 2006, fish were rendered unconscious by concussion, euthanized by spinal severance and preserved in 70% ethanol solution and shipped back to the laboratory for sampling at a later date. In the laboratory, 20 female and 20 male fish of each species were randomly selected to be sampled for the fish health assessment endpoints of condition factor [k=(body weight/length^3)*100], gonadosomatic index [GSI=(gonad weight/body weight)*100], and liver somatic index [LSI = (liver weight/body weight)*100]. Each fish was measured for length (± 1.0 mm) (total length for stickleback and fork length for minnows), and body weight (± 0.01 g). The internal organs were removed and the gonads (± 0.001 g) and liver (± 0.001 g) were weighed. For the remaining field collections, recrudescent (October 2007), spawning (June 2007) and pre-spawning (May 2008), the fish were removed from the traps and transported live to the on-site laboratory in aerated buckets, transferred to an aerated tank prior to sampling. When possible, 20 female and 20 male fish of each species were randomly selected to be sacrificed for the fish health assessment endpoints as previously described. A sample of gonad tissue was taken from 10 adults of each sex, weighed and placed in incubation media for analysis of in vitro steroid hormone production capacity (McMaster et al., 1995a). An additional

sample of gonad tissue was taken for histological analysis for ovarian and testicular development as described below.

Male Fathead Minnows were examined for secondary sex characteristics outlined in Parrott et al. (2003) to assess the secondary sex characteristics of that species. Male characteristics were subjectively evaluated visually, as banding strength, presence of nuptial tubercles, dorsal pad, and dorsal fin dot.

4.3.3 In vitro steroid production and vitellogenin

Due to the low volume of blood in small fish species, it is difficult to obtain enough plasma to determine circulating levels of sex steroids. As a surrogate to circulating levels of sex steroids, an in vitro bioassay to measure the production of sex steroids (testosterone (T) (both sexes), 17β-estradiol (E2) (females) and 11-ketotestosterone (11KT) (males)) by the gonadal tissue of fish was utilized as described in Tetreault et al. (2011). Concentrations of vitellogenin (VTG) were determined in liver homogenates of Fathead Minnow (June 2007) using an indirect competitive enzyme linked immunosorbant assay (ELISA) (Product no. V0108401, Fathead minnow vitellogenin ELISA kit, Bioscience Laboratories, Bergen Norway).

4.3.4 Histological analysis

Gonad histological analysis of fish of both species was conducted as described in Tetreault et al. (2011). Gonad tissues were collected from post-spawning (August 2006), spawning (June 2007), recrudescent (October 2007), and pre-spawning (May 2008) fish collected in Wascana Creek, SK. The gill and kidney tissues of Fathead Minnows were also dissected out and preserved in Davidson's solution. The gill histological assessment protocol used is described in Nero et al. (2006). Gill filaments were measured for secondary lamellar length (SLL - measured from 2^o filament tip to point of basal lamina contact) and width (SLW - measured at 2^o filament midpoint), interlamellar distance

(ID - measured from point of 2^o-basal lamina contact to another 2^o-basal lamina point of contact), and basal epithelial thickness (BET - measured from outer edge to opposite outer edge, width of branch). To evaluate histological alterations, namely the accumulation of inflammatory cells, of the kidney of Fathead Minnow a method was developed which allowed the fish kidney to be accurately assessed with quantitative data (Cheng, 2010).

4.3.5 Statistical Analyses

Analyses of fish data by species and by sex were conducted among sites. Differences in fish length and body weight were evaluated using analysis of variance (ANOVA). Condition factor, gonad size, and liver size were evaluated using analysis of covariance (ANCOVA). Data were subjected to a logarithmic transformation to ensure a normal distribution of the biological data as biological measures usually occur on an exponential scale (Environment Canada, 2002). Tukey's post-hoc test were used when $p<0.05$ to identify differences among sites. Non-parametric Kruskal-Wallis tests were used to compare in vitro steroid production and histological analyses. The error bars associated with the averages presented in all figures represent the standard error of the mean. All data analyses were conducted using SYSTAT 12.0 statistical software (SYSTAT, 2007).

4.4 Results

4.4.1 Fish health

Although both Fathead Minnow and Brook Stickleback are usually abundant species in Wascana Creek, in October (2007) and April (2008) only stickleback were collected in sufficient numbers for a complete adult fish health assessment. In August (2006), both exposed male and female Brook Sticklebacks were longer, heavier, and had greater condition than fish collected at the upstream US-Regina reference site ($p\leq0.001$), however there were no site differences with respect to relative

Table 4.1. Mean (± SE) (n) length, body weight, condition factor (k), liver somatic index (LSI), and gonadosomatic index (GSI) of post-spawning (August 2006), recrudescent (October 2007), spawning (June 2007) and pre-spawning (May 2008) Brook Stickleback collected in Wascana Creek, SK. Differences among sites ($p<0.05$) are denoted by different lowercase letters.

Year	Month	Species	Site	Sex	Length (cm)	Weight (g)	*k* [a]	LSI[b]	GSI[c]
2006	August	Brook Stickleback	US-Regina	Female	4.54 ± 0.01 (19) a	0.68 ± 0.04 (19) a	0.72 ± 0.02 (19) a	5.64 ± 0.38 (19) a	1.77 ± 0.16 (19) a
			DS-STP		5.36 ± 0.12 (20) b	1.25 ± 0.08 (20) b	0.80 ± 0.02 (20) b	7.47 ± 0.57 (20) s	1.92 ± 0.26 (20) a
			US-Regina	Male	4.90 ± 0.07 (21) a	0.98 ± 0.04 (21) a	0.75 ± 0.03 (21) a	4.91 ± 0.30 (21) a	0.53 ± 0.08 (21) a
			DS-STP		5.44 ± 0.07 (25) b	1.60 ± 0.06 (25) b	0.90 ± 0.04 (25) b	6.10 ± 0.41 (25) a	0.76 ± 0.07 (25) b
2007	June	Brook Stickleback	US-Regina	Female	5.38 ± 0.06 (23) a	1.06 ± 0.03 (23) a	0.68 ± 0.02 (23) a	9.14 ± 0.65 (23) a	5.59 ± 1.15 (23) a
			DS-STP		5.44 ± 0.09 (20) a	1.11 ± 0.04 (20) a	0.71 ± 0.03 (20) a	9.73 ± 0.82 (20) a	20.81 ± 2.46 (20) b
			US-Regina	Male	5.32 ± 0.09 (20) a	1.20 ± 0.06 (20) a	0.80 ± 0.03 (20) a	4.89 ± 0.40 (20) a	0.39 ± 0.06 (20) a
			DS-STP		5.36 ± 0.12 (14) a	1.23 ± 0.07 (14) a	0.81 ± 0.03 (14) a	3.38 ± 0.22 (14) b	0.40 ± 0.08 (13) a
2007	October	Brook Stickleback	US-Regina	Female	5.32 ± 0.08 (20) a	0.91 ± 0.07 (20) a	0.59 ± 0.03 (20) a	6.23 ± 0.43 (20) a	3.86 ± 0.11 (19) a
			DS-Regina		5.77 ± 0.12 (19) b	1.28 ± 0.08 (19) a	0.66 ± 0.01 (19) b	6.58 ± 0.33 (19) a	3.77 ± 0.14 (19) a
			DS-STP		5.14 ± 0.07 (23) a	0.96 ± 0.04 (23) a	0.71 ± 0.01 (23) b	6.48 ± 0.37 (23) a	3.41 ± 0.14 (21) b
			US-Regina	Male	5.41 ± 0.11 (20) a	1.03 ± 0.07 (20) a	0.64 ± 0.02 (20) a	6.17 ± 0.44 (20) ab	1.33 ± 0.10 (20) a
			DS-Regina		5.48 ± 0.08 (20) a	1.13 ± 0.05 (20) ab	0.68 ± 0.01 (20) ab	5.35 ± 0.27 (20) a	1.52 ± 0.16 (20) a
			DS-STP		5.59 ± 0.09 (20) a	1.23 ± 0.06 (20) b	0.70 ± 0.02 (20) b	6.97 ± 0.40 (20) b	1.33 ± 0.08 (20) a

[a] *k*, Condition factor = weight/length^3)×100

[b] LSI, Liver somatic index = (liver weight/body weight)×100.

[c] GSI, Gonadosomatic index = (gonad weight/body weight)×100.

Table 4.1 cont.

Year	Month	Species	Site	Sex	Length (cm)	Weight (g)	k [a]	LSI[b]	GSI[c]
2008	May	Brook Stickleback	US-Regina	Female	5.52 ± 0.12 (19) a	1.03 ± 0.12 (19) a	0.60 ± 0.02 (19) a	8.70 ± 0.76 (19) a	11.04 ± 0.93 (19) a
			DS-Regina		5.43 ± 0.09 (20) a	0.94 ± 0.05 (20) a	0.58 ± 0.01 (20) a	7.18 ± 0.52 (20) a	7.91 ± 0.51 (20) b
			DS-STP		5.61 ± 0.15 (18) a	1.08 ± 0.09 (18) a	0.59 ± 0.01 (18) a	6.67 ± 0.45 (18) a	7.74 ± 0.56 (18) b
			US-Regina	Male	5.61 ± 0.09 (22) a	1.25 ± 0.06 (22) a	0.70 ± 0.02 (22) a	5.87 ± 0.44 (22) a	0.62 ± 0.03 (21) a
			DS-Regina		5.67 ± 0.18 (20) a	1.28 ± 0.08 (20) a	0.69 ± 0.01 (20) a	4.74 ± 0.28 (20) ab	0.72 ± 0.04 (20) a
			DS-STP		5.53 ± 0.08 (21) a	1.08 ± 0.06 (21) a	0.63 ± 0.01 (21) a	4.24 ± 0.24 (21) b	0.66 ± 0.05 (20) a

liver size in either sex ($p \geq 0.211$) (Table 4.1). Exposed male fish had larger gonads than reference fish ($p=0.030$), however there were no site differences for female stickleback ($p=0.442$) (Table 4.1). In June 2007 (spawning period), there were no significant differences among sites with respect to length, weight or condition for either sex ($p \geq 0.284$), or for relative liver size in female stickleback ($p=0.481$). Exposed male stickleback had significantly smaller relative liver size when compared to reference fish ($p=0.048$). Exposed spawning female stickleback had significantly larger gonads (>3 fold) than reference fish ($p<0.001$), whereas there were no significant site differences in male gonadosomatic index ($p=0.804$) (Table 4.1). Field collections of recrudescent stickleback in the fall of 2007 demonstrated that exposed female fish did not differ significantly from upstream (US-Regina) fish in terms of length or weight ($p \geq 0.298$), but had greater condition ($p=0.007$). Immediately downstream of the STP discharge (DS-STP) female fish were shorter and lighter than fish from the DS-Regina reference site ($p=0.004$) but there was no difference in terms of condition ($p=0.500$). There were no significant site differences in the length of male stickleback ($p>0.358$), however DS-STP males were significantly heavier and had greater condition than US-Regina fish ($p \leq 0.038$) (Table 4.1). There were no site differences in relative liver size of female stickleback ($p=0.709$), however exposed male stickleback had significantly larger liver size when compared to DS-Regina fish ($p=0.031$). In terms of gonadal development, exposed female stickleback had significantly smaller relative gonad size when compared to fish from both reference sites ($p \leq 0.033$). There were no significant site differences in relative gonad size of recrudescent male stickleback ($p \geq 0.451$) (Table 4.1).

Pre-spawning stickleback (May 2008) demonstrated no significant differences in length or weight of stickleback of either sex or in the condition of female fish ($p \geq 0.078$) among sites. Exposed male fish had significantly lower condition than fish from either reference site ($p \leq 0.013$) (Table 4.1). Exposed male fish also had significantly smaller liver size when compared to fish from the US-Regina site ($p=0.025$), but did not differ in liver size from the DS-Regina site ($p \geq 0.135$) (Table 4.1). Female

stickleback from the DS-STP site had significantly reduced gonadal development when compared to fish from US-Regina site ($p=0.013$), but did not differ when GSI was compared to fish downstream of the City of Regina ($p=0.818$) (Table 4.1).

In August of 2006 (post-spawning), female Fathead Minnow demonstrated no significant site differences with respect to length, weight or condition ($p \geq 0.464$), however exposed female fish had larger livers and gonads when compared to organ sizes of US-Regina fish ($p \leq 0.020$) (Table 4.2). Exposed male minnows were smaller (shorter and lighted) when compared to US-Regina fish ($p<0.001$) but did not differ statistically in condition ($p=0.059$). Male fish collected downstream of the STP discharge had larger livers when compared to organ sizes of US-Regina fish ($p=0.008$), but did not differ in relative gonad size ($p=0.158$) (Table 4.2). Effluent exposed post-spawning male minnows (August 2006) demonstrated a significant reduction in the number of nuptial tubercles (Table 4.3).

Exposed Fathead Minnows collected in June 2007 (spawning) demonstrated that both exposed male and female minnows were shorter and lighter when compared to reference fish collections ($p \leq 0.006$), however they did not differ significantly in condition ($p \geq 0.368$). Exposed Fathead Minnows of both sexes also had significantly larger livers relative to body weight than non-exposed fish ($p \leq 0.035$) and larger relative gonad size however only male minnows were statistically significant ($p<0.001$) (Table 4.2). Although the relative gonad size of exposed female minnows was 2x larger than that of reference fish it was not statistically significantly different. This was potentially due to the fact that the high variability in the gonad size of these exposed multiple-spawners precluded the detection of a significant site difference ($p=0.087$). Female minnows collected downstream of the sewage discharge in June (2007) demonstrated a significant reduction in the size of the ovipositor (Table 4.3).

Table 4.2. Mean (± SE) (n) length, body weight, condition factor (k), liver somatic index (LSI), and gonadosomatic index (GSI) of post-spawning (August 2006), recrudescent (October 2007), and spawning (June 2007) Fathead Minnow collected in Wascana Creek, SK. Differences among sites ($p<0.05$) are denoted by different lowercase letters.

Year	Month	Species	Site	Sex	Length (cm)	Weight (g)	*k* [a]	LSI[b]	GSI[c]
2006	August	Fathead Minnow	US - Regina	Female	4.74 ± 0.17 (11) a	1.27 ± 0.15 (11) a	1.14 ± 0.05 (11) a	2.43 ± 0.22 (11) a	4.01 ± 0.37 (11) a
			DS - STP		4.74 ± 0.05 (22) a	1.18 ± 0.04 (22) a	1.10 ± 0.02 (22) a	3.12 ± 0.21 (20) b	4.93 ± 0.37 (22) b
			US - Regina	Male	5.78 ± 0.11 (20) a	2.25 ± 0.12 (20) a	1.16 ± 0.03 (20) a	0.60 ± 0.11 (20) a	3.50 ± 0.22 (20) a
			DS - STP		4.62 ± 0.10 (20) b	1.11 ± 0.07 (20) b	1.10 ± 0.03 (20) a	0.85 ± 0.16 (20) b	5.54 ± 0.31 (20) a
2007	June	Fathead Minnow	US - Regina	Female	5.32 ± 0.13 (20) a	1.50 ± 0.11 (20) a	0.97 ± 0.04 (20) a	3.28 ± 0.24 (20) a	4.47 ± 0.95 (19) a
			DS - STP		4.87 ± 0.08 (20) b	1.19 ± 0.07 (20) b	1.02 ± 0.07 (20) a	3.98 ± 0.31 (20) b	10.05 ± 1.82 (19) b
			US - Regina	Male	6.08 ± 0.08 (20) a	2.62 ± 0.13 (20) a	1.15 ± 0.03 (20) a	2.59 ± 0.16 (20) a	1.54 ± 0.19 (20) a
			DS - STP		5.62 ± 0.08 (20) b	1.97 ± 0.09 (20) b	1.02 ± 0.02 (20) a	3.70 ± 0.18 (20) b	2.38 ± 0.22 (20) b
	October	Fathead Minnow	US - Regina	Female	5.67 ± 0.12 (19) a	1.79 ± 0.10 (19) a	0.97 ± 0.04 (19) a	5.71 ± 0.42 (19) a	5.60 ± 0.63 (19) a
			DS-Regina		5.66 ± 0.16 (24) a	1.89 ± 0.15 (24) a	1.00 ± 0.02 (24) a	6.66 ± 0.35 (24) a	6.12 ± 0.27 (24) a
			DS - STP		4.80 ± 0.07 (5) b	1.05 ± 0.05 (5) b	0.94 ± 0.02 (5) a	7.24 ± 0.68 (5) a	4.18 ± 1.81 (5) a
			US - Regina	Male	6.70 ± 0.13 (21) a	3.05 ± 0.18 (21) a	0.99 ± 0.02 (21) a	4.90 ± 0.24 (20) a	1.02 ± 0.08 (21) a
			DS Regina		5.88 ± 0.20 (16) b	2.26 ± 0.23 (16) b	1.04 ± 0.02 (16) a	6.09 ± 0.31 (16) a	1.00 ± 0.11 (16) a
			DS - STP		6.20 ± 0.00 (1) ab	2.30 ± 0.00 (1) ab	0.97 ± 0.00 (1)	5.21 ± 0.00 (1)	0.78 ± 0.00 (1)

[a] *k*, Condition factor = weight/length^3)×100

[b] LSI, Liver somatic index = (liver weight/body weight)×100.

[c] GSI, Gonadosomatic index = (gonad weight/body weight)×100.

Table 4.3. Secondary sex characteristics of post-spawning (August 2006) and spawning (June 2007) male Fathead Minnows collected upstream of the City of Regina and downstream of the sewage treatment plant. Also included is the ovipositor size of spawning female Fathead Minnows (June 2007). Differences between sites ($p<0.05$) are denoted by "*".

Year	Month	Site	Reproductive status	Tubercles (# / fish)	Fin dot (%)	Dorsal pad (%)	Bands (%)	Ovipositor (μm^2)
2006	August	US-Regina	Post-spawning	5.26 $\pm$ 1.13	40	65	50	NA
		DS-STP		1.48 $\pm$ 0.41 *	85	95	70	NA
2007	June	US-Regina	Spawning	17.05 $\pm$ 3.63	70	65	60	133.90 $\pm$ 18.20
		DS-STP		18.10 $\pm$ 2.76	85	50	30	55.29 $\pm$ 9.85 *

Similar to June results, female Fathead Minnows collected downstream of the MWWE discharge in October 2007 (recrudescent) were shorter and lighter when compared to reference fish ($p \leq 0.037$), however they did not differ significantly in body condition or relative liver size ($p \geq 0.074$) (Table 4.2). Gonad size in exposed fish was similar regardless of the weight of the fish, however this result may be an artifact resulting from the sample size of fish collected downstream of the STP discharge being very small. Fall collections of Fathead Minnow downstream of the STP discharge yielded only a single male minnow; this prevented inter-site comparisons.

4.4.2 In vitro steroid production and vitellogenin

In June 2007 samples were taken to measure sex steroid production capacity from Brook Stickleback and Fathead Minnows during the spawning period. Female stickleback collected downstream of the STP outfall had significantly lower basal and forskolin-stimulated T production ($p<0.001$) and had significantly higher production of E2 when the tissue was treated with forskolin ($p<0.001$) (Table 4.4). Male steroid production was highly variable during the spawning period resulting in no significant site differences in the production of either androgen ($p \geq 0.096$) (Table 4.5). There were no significant differences among sites in the capacity of Fathead Minnows of either sex for any steroid in either treatment ($p \geq 0.260$) in June (Table 4.6). Although the mean VTG of female Fathead Minnows collected downstream of the discharge were half of those measured in reference fish the differences were not significant (Figure 4.2). Male Fathead Minnows collected downstream of the STP discharge in June demonstrated significantly higher levels (5x) of VTG when compared to levels from male fish collected upstream ($p=0.020$) (Figure 4.2).

There were no significant site differences in post-spawning stickleback steroid production capacity of any steroids in either sex collected in October 2007 (Tables 4.4 and 4.5). Vitellogenin in

Table 4.4. Mean (±SE) basal and forskolin-stimulated in vitro production (pg/mg) of testosterone and 17β-estradiol by spawning (June 2007), recrudescent (October 2007), and pre-spawning (May 2008) ovarian tissue from female Brook Stickleback collected from reference and exposed sites in Wascana Creek, SK. Differences among sites ($p<0.05$) are denoted by different lowercase letters.

Year	Month	Species	Site	Testosterone		17β-Estradiol	
				basal	forskolin	basal	forskolin
2007	June	Brook Stickleback	US - Regina	20.87 ± 2.03 a	27.41 ± 3.12 a	3.28 ± 0.41 a	2.51 ± 0.28 a
			DS - STP	9.16 ± 1.25 b	11.29 ± 1.51 b	4.68 ± 0.47 a	5.88 ± 0.33 b
	October	Brook Stickleback	US - Regina	3.09 ± 1.02 a	9.72 ± 0.73 a	3.64 ± 0.31 a	21.21 ± 2.38 a
			DS Regina Up STP	2.81 ± 0.39 a	9.19 ± 1.12 a	3.47 ± 0.49 a	23.32 ± 2.14 a
			DS - STP	6.77 ± 1.19 a	9.91 ± 1.19 a	4.31 ± 0.82 a	21.06 ± 2.27 a
2008	May	Brook Stickleback	US - Regina	0.71 ± 0.16 a	0.71 ± 0.16 a	0.49 ± 0.09 a	0.98 + 0.21 a
			DS Regina Up STP	0.38 ± 0.13 b	0.38 ± 0.13 b	0.44 ± 0.11 b	0.82 + 0.24 b
			DS - STP	0.38 ± 0.14 b	0.38 ± 0.14 b	0.44 ± 0.12 b	0.82 + 0.25 b

Table 4.5. Mean (±SE) basal and forskolin-stimulated in vitro production (pg/mg) of testosterone and 11-ketotestosterone by spawning (June 2007), recrudescent (October 2007), and pre-spawning (May 2008) testicular tissue from male Brook Stickleback collected from reference and exposed sites in Wascana Creek, SK. Differences among sites ($p<0.05$) are denoted by different lowercase letters.

Year	Month	Species	Site	Testosterone	11-Ketotestosterone
2007	June	Brook Stickleback	US - Regina	19.40 ± 4.70 a	79.59 ± 18.47 a
			DS - STP	40.18 ± 8.77 a	136.61 ± 34.55 a
	October	Brook Stickleback	US - Regina	15.67 ± 2.32 a	9.72 ± 0.73 a
			DS Regina Up STP	11.52 ± 1.78 a	9.19 ± 1.12 a
			DS - STP	16.74 ± 2.45 a	9.91 ± 1.19 a
2008	May	Brook Stickleback	US - Regina	71.38 ± 6.59 a	ND
			DS Regina Up STP	34.32 ± 5.38 ab	ND
			DS - STP	56.01 ± 10.99 ab	ND

ND indicates non-detect = sample concentration below detection limit.

Table 4.6. Mean (±SE) basal and forskolin-stimulated in vitro production (pg/mg) of sex steroids by spawning (June 2007) gonadal tissue from female and male Fathead Minnow collected from reference and MWWE exposed sites in Wascana Creek, SK. Differences among sites ($p<0.05$) are denoted by different lowercase letters.

Year	Month	Species	Sex	Site	Testosterone		17β-Estradiol	
					basal	forskolin	basal	forskolin
2007	June	Fathead Minnow	Female	US - Regina	0.70 ± 0.05 a	1.49 ± 0.29 a	ND[1]	1.75 ± 0.20[2]
				DS - STP	1.03 ± 0.21 a	3.20 ± 0.73 a	1.59 ± 0.15	3.50 ± 0.59

Year	Month	Species	Sex	Site	Testosterone		11-Ketotestosterone	
					basal	forskolin	basal	forskolin
2007	June	Fathead Minnow	Male	US - Regina	0.97 ± 0.26 a	5.61 ± 1.0 a	5.09 ± 0.94 a	20.79± 3.93 a
				DS - STP	2.38 ± 1.38 a	6.00 ± 1.20 a	11.86 ± 6.03 a	15.78 ± 1.94 a

1. ND = Below detection limit of the assay

2. Not enough detectible replicates for statistical analysis

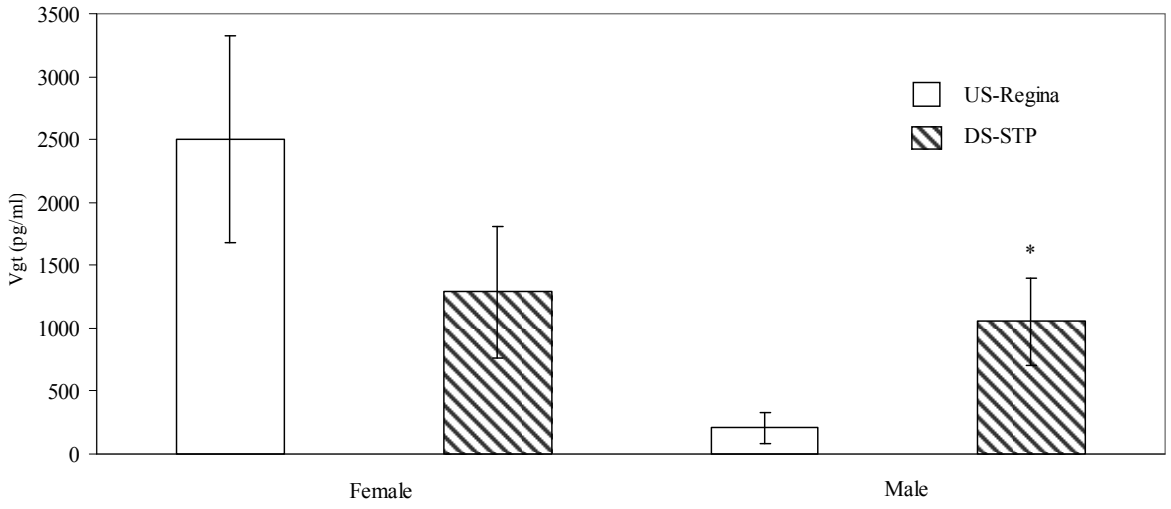


Figure 4.2. Mean (±SE) concentration of vitellogenin measured from liver homogenates of spawning female and male Fathead Minnow collected at an upstream reference (US-Regina) and a downstream STP (DS-STP) exposure site in Wascana Creek, SK in June 2007. "*" denotes statistically significant difference.

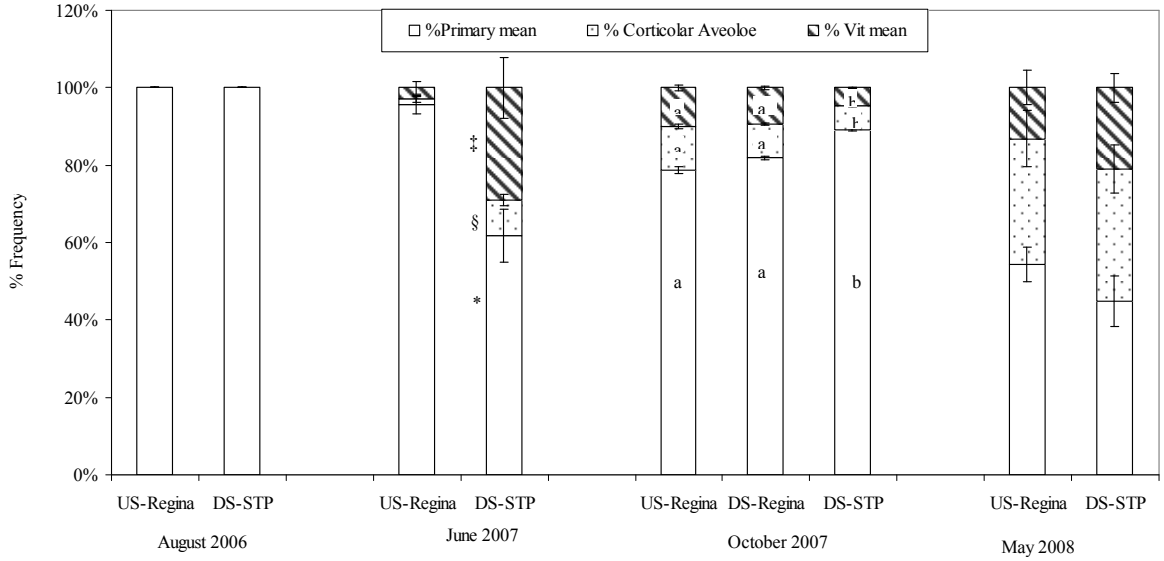
liver homogenates from Fathead Minnows collected in October was not detectable at any of the sites (data not shown). Due to insufficient numbers of Fathead Minnows collected in October of 2007, gonad tissue was not analyzed for sex steroid production capacity. Spring steroid production capacity of female Brook Stickleback was significantly decreased at both sites downstream of the City of Regina for both basal and stimulated T and E2 when compared to US-Regina levels ($p<0.001$) (Table 4.4). Testosterone production by exposed male stickleback did not differ statistically from either upstream site ($p\geq0.071$) and 11KT production was below the detection limit of the assay at all sites (Table 4.5).

4.4.3 Histopathology

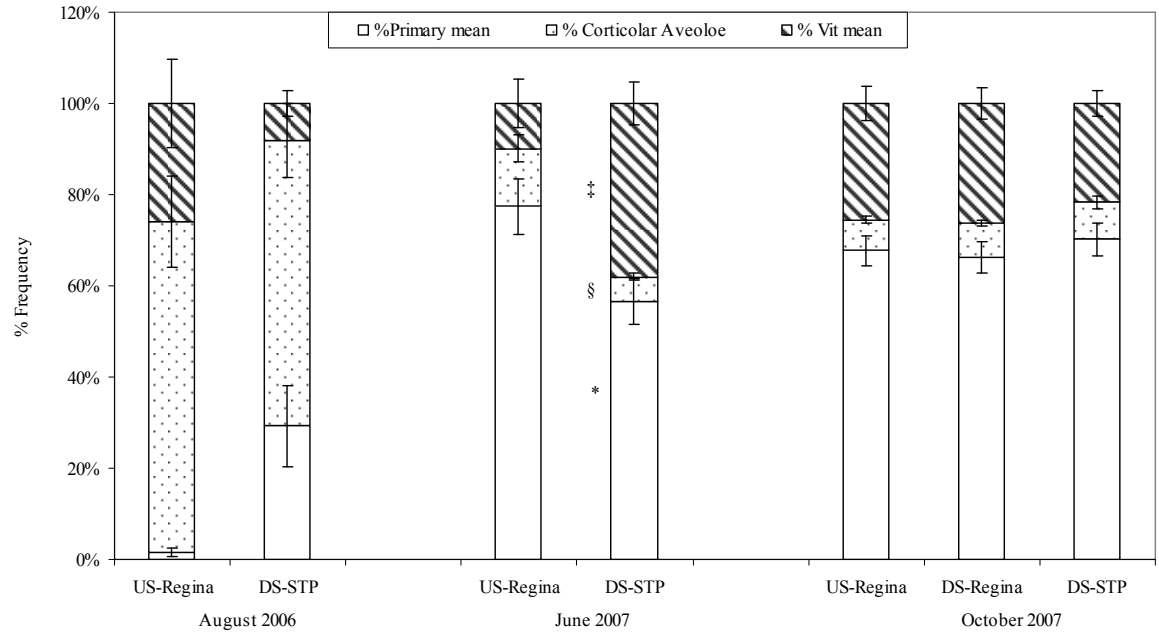
4.4.3.1. Female gonad histology

There was no difference in ovarian development of female stickleback in August 2006 as only primary follicles were observed in samples from both sites (Figure 4.3a). In ovaries collected from Fathead Minnow in August of 2006, oocyte development from exposed female Fathead Minnow appears to be delayed relative to the gonad development observed in reference fish as exposed fish had higher levels of primary and cortical alveolar cell types and fewer vitellogenic cells (Figure 4.3b). There was no significant site difference in the size of the vitellogenic follicles ($p=0.588$) (Figure 4.3d). Field collections of spawning fish in June 2007 demonstrated a delay in the spawning activity of fish collected downstream of the MWWE discharge for the City of Regina (Figures 4.3ab and 4.4ab). Both exposed female stickleback and minnows had significantly greater frequency of both cortical alveolar and vitellogenic follicles ($p\leq0.045$) then those found in reference females which had 96% and 78% primarily follicles, respectively, suggesting that reference fish have already successfully spawned.

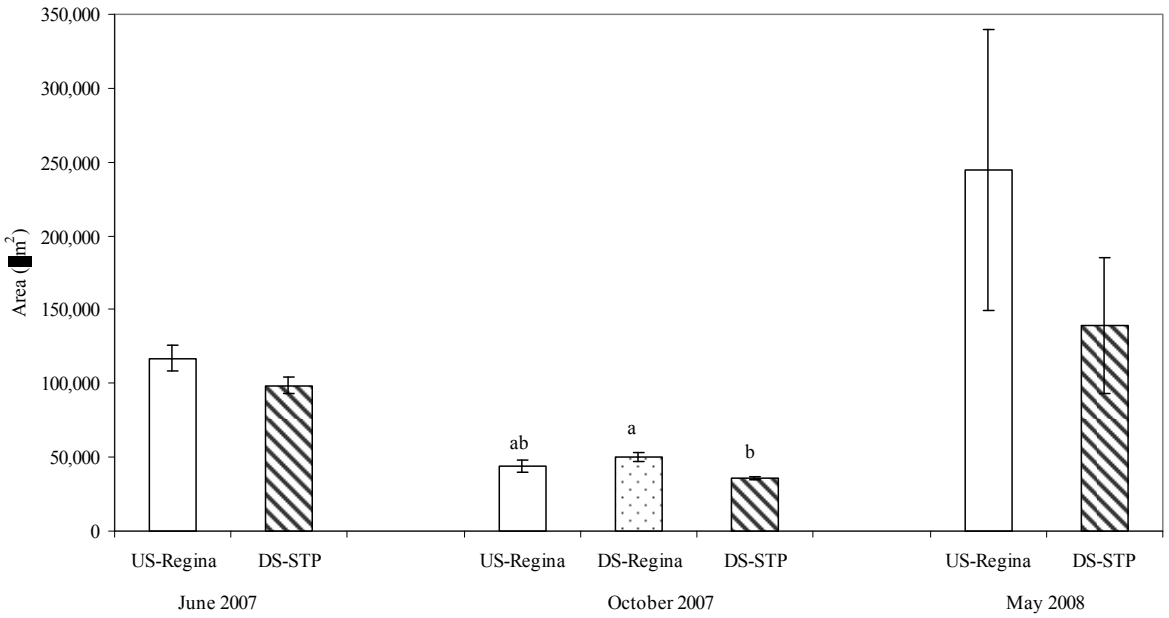
a)


%Primary mean
% Corticolar Aveoloe
% Vit mean
% Frequency
120%
100%
80%
60%
40%
20%
0%
US-Regina
DS-STP
August 2006
June 2007
October 2007
DS-Regina
May 2008
a
b
‡
§
*

b)


%Primary mean
% Corticolar Aveoloe
% Vit mean
% Frequency
120%
100%
80%
60%
40%
20%
0%
US-Regina
DS-STP
August 2006
June 2007
DS-Regina
October 2007
‡
§
*

c)


350,000
300,000
250,000
200,000
150,000
100,000
50,000
0
Area (µm²)
ab
a
b
US-Regina
DS-STP
US-Regina
DS-Regina
DS-STP
US-Regina
DS-STP
June 2007
October 2007
May 2008

d)

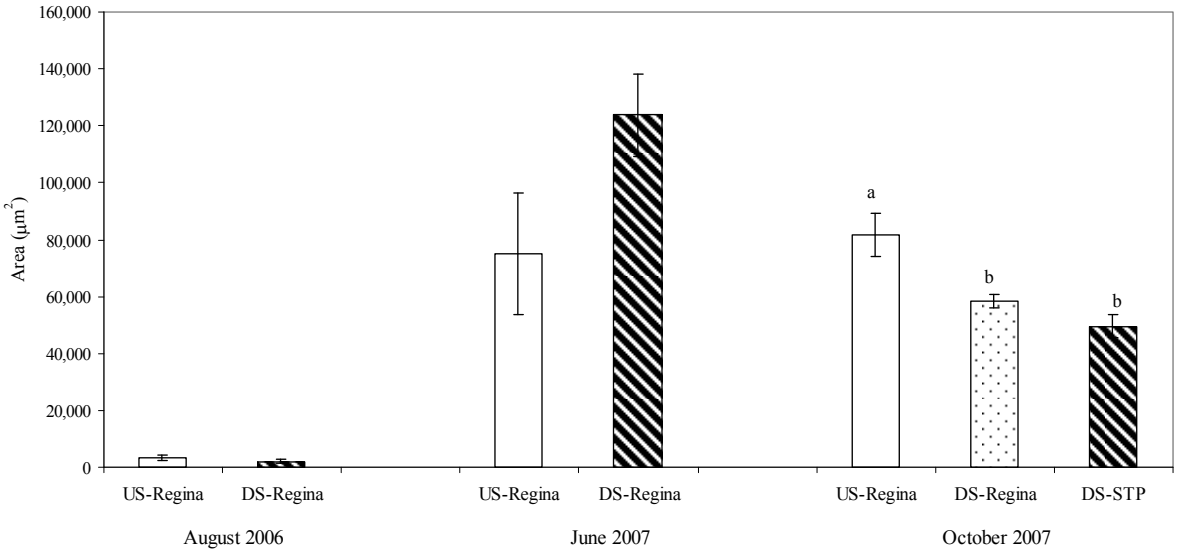

160,000
140,000
120,000
100,000
80,000
60,000
40,000
20,000
0
Area (µm²)
a
b
b
US-Regina
DS-Regina
US-Regina
DS-Regina
US-Regina
DS-Regina
DS-STP
August 2006
June 2007
October 2007

Figure 4.3. Histopathology of female a) Brook Stickleback and b) Fathead Minnow, and mean area (μm^2) ($\pm$SE) of vitellogenic follicles of c) Brook Stickleback and d) Fathead Minnow collected at reference (US-Regina; DS-Regina) and a MWWE exposed sites (DS-STP) during post-spawning (August 2006), spawning (June 2007), recrudescence (October 2007) and pre-spawning (May 2008) in Wascana Creek, SK. Different letters and symbols denote statistically significant differences among sites within each sampling event.

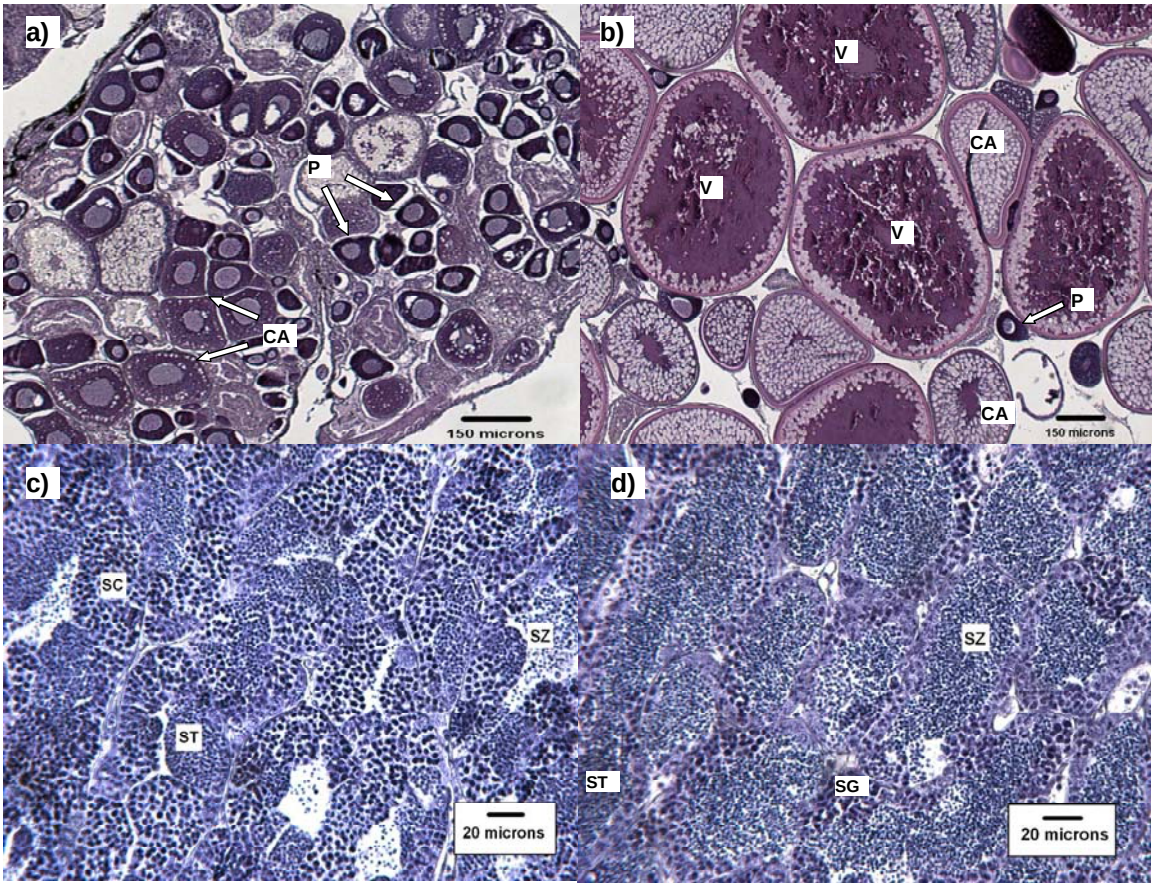


Figure 4.4. Gonadal tissue sections from female and male Fathead Minnow sampled in June, 2007. Ovarian tissue from female minnows collected from (a) the US-Regina reference site with clusters of primary (P) and (b) DS-STP exposed site which retain vitellogenic (V) and cortical alveolar (CA) follicles. Testicular tissue from male minnow collected from (c) the US-Regina reference site and (d) DS-STP exposed site containing spermatogonia (SG), Spermatocytes (SC), spermatids (ST) and spermatozoa (SZ).

There was no significant site difference in the size of exposed stickleback and minnow vitellogenic follicles when compared to US-Regina reference follicles ($p=0.091$) (Figure 4.3cd). Vitellogenic follicles from exposed female stickleback were significantly smaller when compared to those from females collected from the DS-Regina site ($p<0.001$). Female stickleback collected downstream of the STP discharge continued to demonstrate alterations in gonad development relative to reference condition in October 2007. Ovaries from exposed female stickleback had a greater frequency of primary follicles and significantly fewer follicles in the cortical alveolar and vitellogenic stages ($p\leq0.028$) (Figure 4.3a). There was no significant difference in stage of ovarian development of female minnows in October 2007 ($p\geq0.712$) (Figure 4.3b), however vitellogenic follicles in STP exposed fatheads were significantly smaller than those collected from US-Regina females ($p<0.001$) (Figure 4.3d). Although the size of vitellogenic follicles of exposed pre-spawning female stickleback was smaller relative to those in reference females, this difference was not statistically significant ($p\geq0.221$) (Figure 4.3c).

4.4.3.2 Male gonad histology

A visual scan of histological slides of testicular tissue of both sentinel species in this study did not detect the intersex condition. Male Brook Stickleback collected downstream of the MWWE discharge for the City of Regina in August of 2006 had altered histopathology when compared to gonad development from male fish collected from the US-Regina reference site (Figure 4.4c,d & 4.5). Exposed male fish had significantly fewer spermatocytes, spermatozoa and more spermatids when compared to reference males (Figure 4.5a) ($p\leq0.033$), whereas exposed male Fathead Minnows had relatively more spermatocytes and significantly fewer spermatogonia when compared to fish from the reference site ($p\leq0.033$) (Figure 4.5b). Testicular tissue of spawning male stickleback (June 2007) from both sites was miniscule ($\geq$ 0.40% GSI) with weak structural integrity, and prevented any

samples from being collected for histological analysis. Male Fathead Minnow collected downstream of the wastewater discharge during the spawning season (June 2007) had significantly greater proportion of the later stage spermatids and spermatozoa, and significantly less spermatogonia than male fish collected from the reference site (Figure 4.5c). Similar to the histopathology of female fish these results suggest a delay in the spawning activity of fish collected downstream of the STP discharge for the City of Regina.

4.4.3.3. Gill histology

Brook Stickleback and Fathead Minnows collected downstream of the Regina STP discharge in August of 2006 had significantly shorter and thicker secondary lamellae compared to fish from the reference site ($p<0.001$) (Figures 4.6a and 4.7). Analysis of the distance between lamellae of stickleback in 2006 was not possible because the secondary lamellae were too close together for measurement, however the interlamelar distance was significantly shorter in exposed stickleback in 2007 (Figure 4.6b). Due to problems with the fixation of the stickleback gills from US-Regina from 2007 there is no analysis from this site. The gills of exposed Fathead Minnow collected in June 2007 had significantly thinner primary (BET) and secondary lamellae (SLW) resulting in the 2^{o} lamellae significantly further from each other (lower ID) ($p\leq0.005$) (Figure 4.6b).

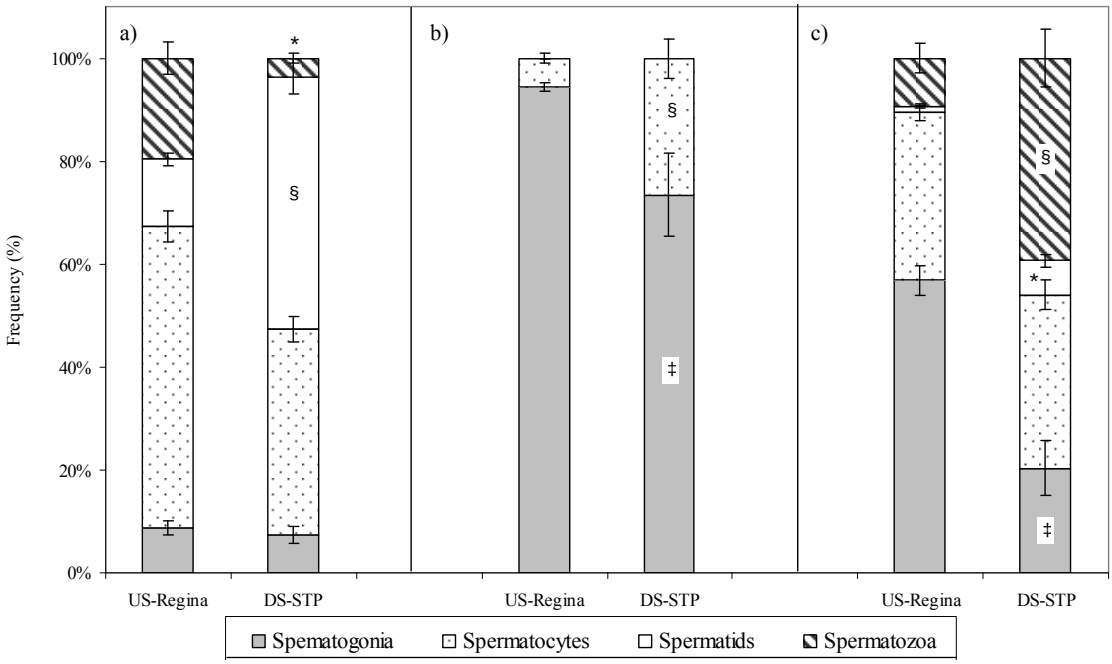


Figure 4.5. Histopathology of post-spawning male (August 2006) a) Brook Stickleback and b) Fathead Minnow, and c) spawning Fathead Minnow (June 2007) collected at reference (US-Regina) and a STP exposed sites (DS-STP) in Wascana Creek, SK. Different symbols denote statistically significant differences within cell type between sites.

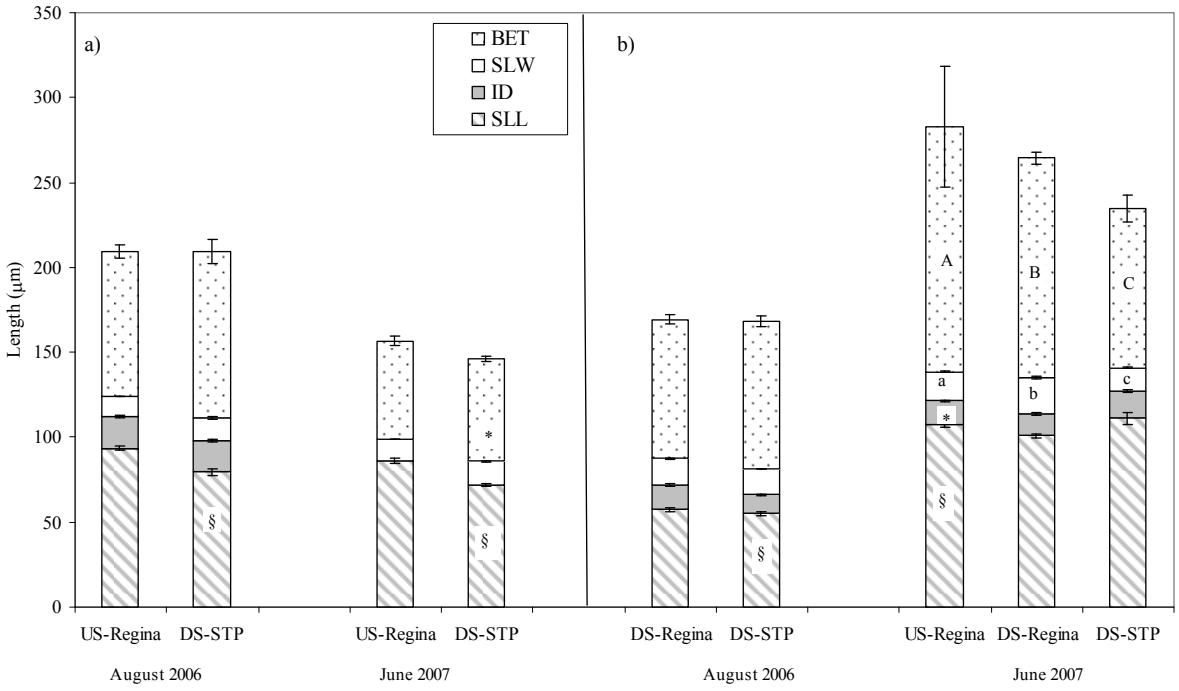


Figure 4.6. Gill histopathology of post-spawning (August 2006) and spawning (June 2007) a) Brook Stickleback and b) Fathead Minnow, collected at reference (US-Regina, DS-Regina) and a STP exposed sites (DS-STP) in Wascana Creek. Full variable names are listed in Materials and Methods. Different symbols denote statistically significant differences within structures. The error bars represent the standard error of the mean.

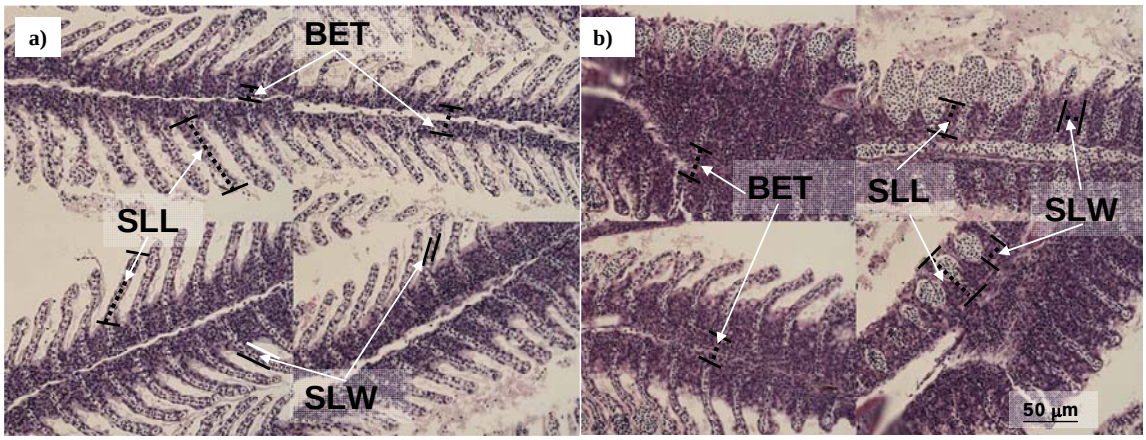
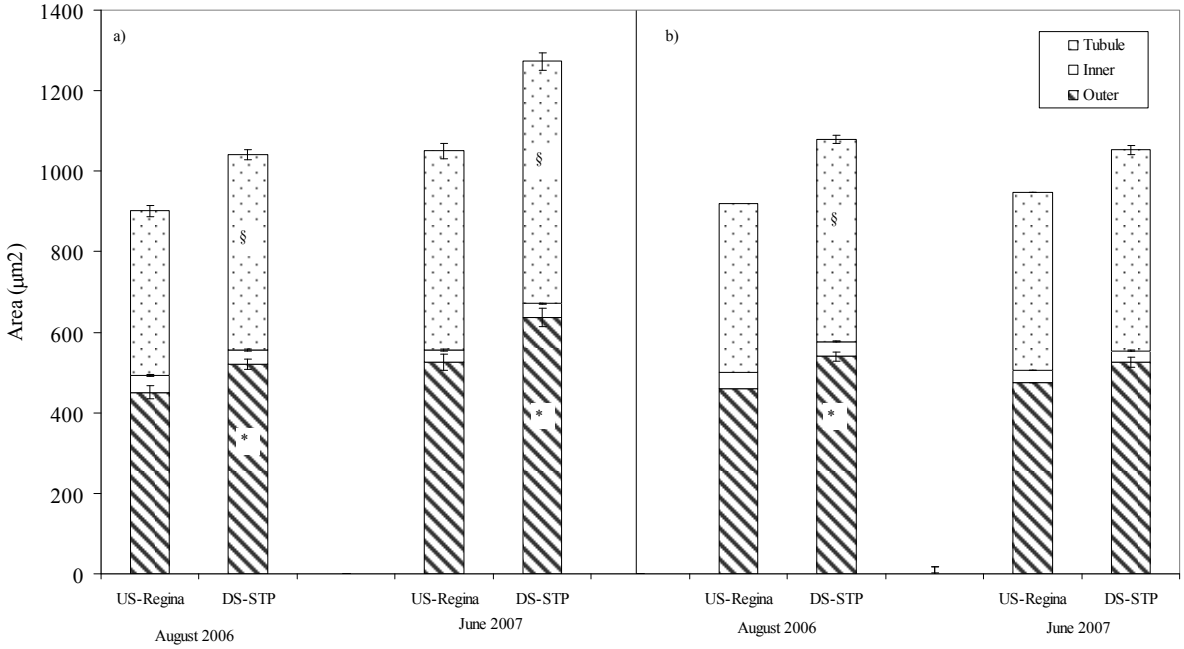


Figure 4.7. Gill histopathology of Fathead Minnow collected at (a) reference (US-Regina) and (b) downstream of a STP discharge (DS-STP) in Wascana Creek.

4.4.3.4. Kidney histology

The proximal tubules in kidneys of male and female Fathead Minnows collected downstream of the STP discharge were inflamed when compared to kidneys in fish from the reference site ($p \leq 0.002$) (Figure 4.8ab). Inflammation is described as the overall size of the proximal tubule of exposed fish being significantly larger while the size of the inner tubule did not change (Figure 4.9). The size of the Bowman's capsule of exposed fish demonstrated sex differences as the size of the structure in exposed male fish are smaller although not statistically significant (Figure 4.8c & 4.9), while the structure in exposed female Fathead Minnows was significantly larger when compared to the Bowman's capsule in reference kidneys in June 2007 ($p \leq 0.063$) (Figure 4.8d).


a)
b)
Tubule
Inner
Outer
Area (µm2)
1400
1200
1000
800
600
400
200
0
§
*
US-Regina
DS-STP
August 2006
June 2007

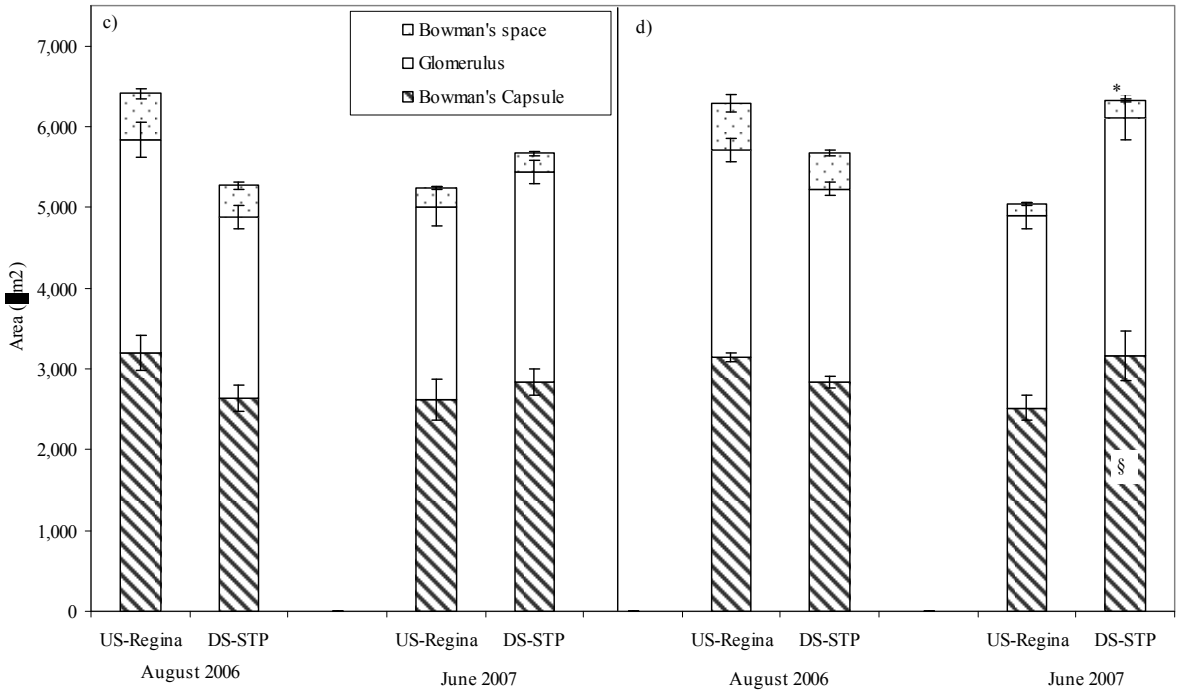

c)
d)
Bowman's space
Glomerulus
Bowman's Capsule
Area (µm2)
7,000
6,000
5,000
4,000
3,000
2,000
1,000
0
*
§
US-Regina
DS-STP
August 2006
June 2007

Figure 4.8. Mean size (±SE) of the inner, outer and total kidney proximal tubule of a) male and b) female Fathead Minnow, and mean size of the kidney Bowman's capsule, the glomerulus and the Bowman's space between the two structures of c) male and d) female Fathead Minnow collected at reference (US-Regina) and a STP exposed sites (DS-STP) in Wascana Creek, SK. Different symbols denote statistically significant differences within structures.

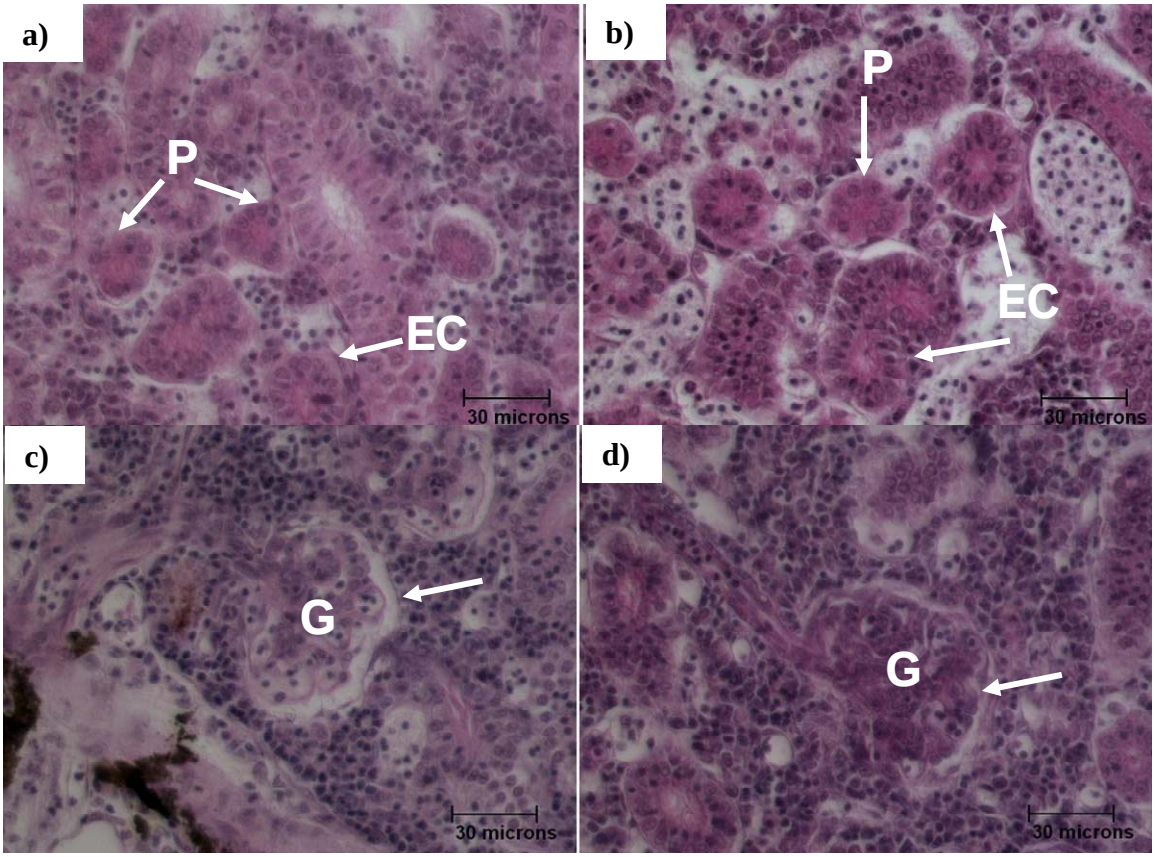


Figure 4.9. Histopathology of kidney of male Fathead Minnows from a reference (a, c) and effluent exposed (b, d) sites in Wascana Creek (2006) displaying proximal (P), the glomerulus (G), and cuboidal epithelial cells (EC) in the distal tubules.

4.5 Discussion

Sublethal effects were detected in two forage fish species exposed to municipal wastewater discharges in Wascana Creek, Saskatchewan. Brook Stickleback tended to be longer and heavier when collected at downstream exposed sites compared to fish collected from reference sites. However fish responses demonstrated seasonal site differences with respect to fish condition between the spring, summer and fall collections. Stickleback of both sexes collected downstream of the STP discharge in the summer of 2006 and the fall of 2007 had greater condition relative to reference fish, while site differences in condition and liver somatic indices were not observed in spring spawning stickleback. Although Fathead Minnows exposed to MWWE discharge were consistently smaller than reference fish, they had higher liver somatic indices indicating increased energy storage regardless of sex. These results are similar to previous studies that have measured health parameters of fish exposed to STP effluents. In a study by Tetreault et al. (2011), responses to nutrient enrichment were observed as increased energy storage in Rainbow Darter (*Etheostoma caeruleum*) and Greenside Darter (*E. blennioides*) which tended to be longer, heavier, and have greater condition and liver somatic indices at sites downstream of STP discharges compared to reference sites. Similar to the stickleback results from this study, spring spawning darters collected downstream of the STPs in the fall had the potential to have greater growth (longer and heavier) (Tetreault et al., 2011). Municipal wastewater effluent is a complex matrix whose composition may vary seasonally and temporally (Harries et al., 1996; Alvarez et al., 2009). As demonstrated in this study, the responses of fish exposed to municipal effluent also have the potential to vary. This study also demonstrated seasonal site differences with respect to fish condition between the spring and fall collections

Although McMaster et al. (2005) observed increased condition and relative liver size (LSI) in Longnose Sucker (*Catostomus catostomus*) downstream of a STP discharge in Alberta, Canada, there is increasing evidence that exposure to STP effluent does not always manifest in increased fish

condition. In various studies, wild fish including Roach (Jobling et al., 1998), Flounder, *Platichthys flesus* (Allen et al., 1999), Gudgeon, *Gobio gobio* (van Aerle et al., 2001), and White Sucker, *Catostomus commersoni* (Hinck et al., 2009), collected downstream of STPs tended to invest energy into body mass in a similar way as reference fish. Slimy sculpin (*Cottus cognatus*) exposed to MWWE effluents did however show increased condition (Arciszewski et al., 2011). A number of studies have reported both increased enzymatic activity and increased liver size in fish exposed to industrial effluents (Gibbons et al., 1998ab; Arcand-Hoy and Metcalfe, 1999; McMaster et al., 2005). Seasonal differences in energy storage (condition and LSI) in exposed stickleback were observed in this study. Further research is required to investigate the relationship between these higher energy stores and liver enzymatic activity and size.

Sentinel species in this study demonstrated seasonal-dependant responses in gonadal development and histopathology as well as significant alterations in steroid production. During recrudescent and pre-spawning periods (October and May, respectively) exposed female stickleback have smaller relative gonad size, while in fish collected during spawning season and soon afterwards (June and August, respectively) the relative size of the ovaries are significantly larger (2 to 4 fold) when compared to reference females. In August, female fish of both species collected downstream of the City of Regina municipal discharge had larger gonads retaining significantly more larger vitellogenic follicles, while reference fish had smaller gonads with very few vitellogenic follicles. Male fish exposed to MWWE discharge also had altered testicular development relative to that of reference males during spawning and post-spawning periods. If spawning is delayed or does not occur at the downstream site, that could explain larger gonad sizes and vitellogenic follicles in exposed female fish observed in August as fish are potentially reabsorbing gonadal material that was not released during the spawning period. This indicates that environmental conditions in the MWWE

effluent receiving environment are capable of producing larger fish, however these fish are demonstrating alterations in reproductive development, particularly the females.

Reduction in gonadal development in MWWE effluent exposed fish has been reported in Roach (Jobling et al., 1998), Flounder (Allen et al., 1999), Bream (*Abramis brama L*) (Hecker et al., 2002), White Sucker (Vajda et al., 2008), and Rainbow and Greenside Darters (Tetreault et al., 2011). Gonadal development of Bass exposed to treated sewage in the Conococheague Creek, USA, was reported to be 33-50% reduced when compared to reference populations (Iwanowicz et al., 2009). Pre-spawning female Bream collected downstream of major urban areas on the Elbe River, Germany, demonstrated lower GSI than control site fish which was attributed to an inhibition of maturation (Hecker et al., 2002).

This study demonstrated major alterations in the histopathology of female gonadal development of spawning Fathead Minnow and Brook Stickleback, as well as recrudescent stickleback as described in wild female fish exposed to other STP effluents (Allen et al., 1999; Hecker et al., 2002; Jobling et al., 2002; Viganò et al., 2001; Sole et al., 2003; McMaster et al., 2005; Hinck et al., 2009). In Belgium, STP exposed Stoneloach (*Barbatula barbatula*) displayed no impacts on female gonadal development, but Gudgeon from that same study demonstrated advanced oocyte development at one of the exposure sites relative to reference fish (Douxfils et al., 2007).

Alterations in gonadal development observed in this study could possibly be due to the exposure of sentinel species to estrogenic compounds in the STP effluent. Municipal discharges have been identified as a major source of estrogenic compounds in aquatic receiving environments (Purdom et al., 1994; Tyler et al., 1996; Servos et al., 2005). Circulating plasma levels of vitellogenin, the egg yolk precursor, has been used extensively as a bioindicator of exposure of aquatic biota to estrogenic compounds in males because the physiological production of vitellogenin is normally restricted to females (Purdom et al., 1994; Jobling et al., 1998; Allen et al., 1999; Sumpter and Jobling, 1995;

Hecker et al., 2002; Vajda et al., 2008). In the current study male Fathead Minnows collected downstream of the MWWE discharge demonstrated a significant induction of vitellogenin to similar levels of induction of vitellogenin as that of female fish from the same site. Similar to this study, plasma vitellogenin levels for intersex Pike were similar to those of female pike (Vine et al., 2005). Although many studies have measured elevated levels of vitellogenin in male fish and reported increased incidence of the intersex condition in fish exposed to MWWE, a strong correlation between vitellogenin concentration and presence of intersex has not been established. Exposure to other contaminants, such as those with anti-androgenic properties may contribute to the expression of intersex in fish (Jobling et al., 2009).

One of the objectives of this study was to investigate the potential for the intersex condition (female oocytes in male testicular tissue), which has been previously reported in wild fish exposed to MWWE. Although neither sentinel species demonstrated intersex, failure to allocate energy resources towards reproductive development, as demonstrated in this study, resulted in reproductive impairment. Exposure to MWWE has been correlated with deformity of the gonads and/or reproductive ducts and altered gamete production in Roach (Jobling et al., 2002a), as well as malformations of the reproductive duct(s) preventing release of gametes (Vajda et al., 2008). Fibrosis and inhibition of testicular development of male White Sucker exposed to STP effluent has also been reported (Woodling et al., 2006). Male Fathead Minnow exposed to 17α-ethynylestradiol (EE2) in a whole lake exposure also showed similar malformations (Palace et al., 2002). However, fibrosis or other malformations of testicular tissue were not observed in the fish collected from Wascana Creek in the current study.

There also is the potential for indirect effects of estrogen exposure. Palace et al. (2006) documented that tubules of the kidney tissue in Pearl Dace (*Margariscus margarita*) exposed to EE2 were enlarged, had intertubular edema and hyaline degeneration in the proximal tubules relative to

reference fish. This was attributed to the filtration by kidneys of a high concentration of the large vitellogenin molecule leading to inflammation and leakage across the glomeruli resulting in malfunction of the kidneys and eventual renal failure (Länge et al., 2001, Zaroogian et al., 2001, Palace et al., 2006). The kidney tissue of exposed Fathead Minnows in the effluent-dominated Wascana Creek also demonstrated enlargement of the proximal tubules and a reduction of the Bowman's space within the glomeruli suggesting inflammation of the kidney which could cause impaired functioning of this organ. We were unable to detect hyaline droplet degeneration or vacuolar alterations in the first segment of the proximal tubule, which would have indicated necrosis of the renal tubules (Bucher and Hofer, 1993; Palace et al., 2006). This may be an artifact of the tissue embedding technique in this study, which utilized paraffin rather than methacrylate which allows thinner sectioning for greater resolution.

This study demonstrated a reduction in the ability of sentinel species collected in an effluent-dominated stream to produce sex steroids in vitro. Although the examination of circulating sex steroids in these small-bodied fish was not possible due to the low blood volume, the production of sex steroid by gonadal tissue was used successfully as a surrogate to circulating levels in fish (Van Der Kraak et al. 1992; McMaster et al. 1995; Tetreault et al., 2011). Pre-spawning female stickleback gonad tissue collected downstream of the MWWE discharge in this study had significantly reduced ability to produce testosterone and 17β-estradiol. While reduction of 50% in testosterone production was also observed in exposed spawning female stickleback, 17β-estradiol production by exposed fish increased 2-fold of reference levels and exposed male steroid production was also significantly higher than reference levels. Although in October (2007), the production of testosterone by ovarian tissue by MWWE exposed females was double that of levels in fish from the reference site there were no significant alterations in steroid production by stickleback undergoing gonadal recrudescence. The reduction in the ability of female ovarian tissue to produce 17β-estradiol in fish undergoing

recrudescence collected downstream of the MWWE is consistent with observations in MWWE exposed Longnose Sucker (McMaster et al., 2005), and Greenside Darters (Tetreault et al., 2011), as well as pre-spawning female and male Rainbow Darters (Tetreault et al., 2011), Roach (Jobling et al., 2002a) and Bream (Hecker et al., 2002).

A seasonal water survey in Wascana Creek revealed that downstream of the STP discharge for the City of Regina, PPCPs were always present up to mg/L levels, and ammonia frequently exceeded the guidelines for both drinking water and for the protection of sensitive aquatic species (<2.0 mg/L) (Waiser et al. 2011ab). In a freshwater environment ammonia, a metabolic cell waste product, is eliminated from fish's blood via passive branchial ammonia excretion or through the conversion of ammonia to urea in the kidneys (Randall and Wright, 1987). In an environment receiving MWWE waste, elevated environmental ammonia levels can result in a net uptake of ammonia by the fish. The accumulation of ammonia in tissue is deleterious to fish. Several studies have documented sublethal effects of ammonia on aquatic organisms (Daoust and Ferguson, 1984, Soderberg et al,. 1984, Bucher and Hofer, 1993, Person-Le Ruyet et al., 1997). Significant reduction in growth of larval Fathead Minnow (Fairchild et al., 2005), reduced growth rate and irregular gill ventilation and morphology in Turbot (*Scophthalmus maximus*) (Person-Le Ruyet et al., 1997), and hyperplasia of the gill epithelium and fused lamellae in Channel Catfish (*Ictalurus punctatus*) (Soderberg et al., 1984) have all be demonstrated in organisms chronically exposed to environmentally relevant concentrations of ammonia. In this study we demonstrated that fish collected downstream of the MMWE discharge in waters containing elevated concentrations of ammonia displayed thicker, shorter and fused secondary gill lamellae and the restriction of the primary gill lamellae. This appears to be the result of loss of structural and cellular integrity. The level of cellular structural disorganization observed in the exposed fish in Wascana Creek would significantly reduce the surface area by which respiration and excretion of waste products could occur. Daoust and Ferguson (1984) also demonstrated that Rainbow

Trout (*Oncorhrynchus mykiss*) fingerlings exposed to high concentrations of un-ionized ammonia in water had acidophilic droplets in epithelial cells of their renal proximal convoluted tubules. Hence, environmental exposure of sentinel species in this study to ammonia may have contributed to the observed kidney abnormalities.

Sentinel fish species exposed to concentrated municipal effluent caused adverse biological effects such as delays in spawning, induction of vitellogenin in males, alterations in gill and kidney morphology, all of which could impair their ability to survive and reproduce normally. Sampling efforts at far-field downstream reaches demonstrated a complete lack of fish, therefore, it is not known if the populations in Wascana Creek downstream of the City of Regina outfall are self-sustaining or if they rely on immigration from upstream stretches of the river. Future changes in water quantity and quality resulting from climate change, human population growth and altered land use may further challenge the assimilative capacity of the creek and further impair its ecological integrity. Future studies should investigate if the planned upgrades in effluent treatment at the Regina facility will help improve the health of the aquatic life in Wascana Creek.

4.6 Acknowledgements

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Chapter 5

Fish community responses to multiple municipal wastewater inputs in a watershed

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This paper incorporates fish community data from Brown (2010).

5.1 Summary

Municipalities utilize aquatic environments to assimilate their domestic and industrial effluent, which can result in eutrophication and anoxia in the receiving environment and toxicity and endocrine disruption in resident aquatic biota. The objective of this study was to assess the impacts of MWWE discharges on the fish community downstream of two MWWE discharges in the Grand River. The fish communities downstream of the MWWE outfalls demonstrated differences in the abundance and diversity, species and family richness, % tolerance and % vulnerability when compared to the fish community upstream or further downstream of the effluent discharges. In both years studied (2007 & 2008), the fish community exposed to MWWE in riffle-run habitats demonstrated reductions in the proportion of the most prominent fish (Rainbow Darter, *Etheostoma caeruleum*) found in the reference sites, and a significant increase in the proportion of large, mobile, tolerant, omnivourous fish species such as suckers and sunfish relative to the reference populations. There was less variability in the responses of the fish community to MWWE in the same season between years than between seasons within the same year. This study demonstrates the effects of multiple outfalls of treated MWWE on fish populations and communities in urban areas. Municipalities, through MWWE, are a major source of nutrients and PPCPs to aquatic systems and careful management of their effluent is critical to minimize the impacts on the aquatic environment, especially considering strong growth trends in urban populations.

Keywords: Municipal wastewater, fish community, principal component analysis, watershed management

5.2 Introduction

MWWE is one of the largest anthropogenic waste discharges by volume in Canada and there is concern over the potential impacts it may have on aquatic ecosystems (Environment Canada, 2001). The input of organic material and nutrients from MWWE into the aquatic receiving environment can result in eutrophication and lower dissolved oxygen content (Chambers et al., 1997; Cooke, 2006). MWWE also contain a variety of toxic contaminants such ammonia, chloride, inorganic chloramines (Environment Canada, 2001), natural and synthetic hormones, pharmaceuticals and personal care products (Ternes et al., 1998; Ternes et al., 1999; Servos et al., 2005; Lishman et al., 2006; Metcalfe et al., 2009), and industrial chemicals such as alkyphenol polyethoxylates (Servos et al., 1999). Over the last decade, studies have been conducted that demonstrate that the endocrine function of individual fish collected near treatment plant effluents is impaired (Purdom et al., 1994; Harris et al., 2001; Jobling et al., 2002; Blazer et al., 2007; Tyler and Jobling, 2008; Vajda et al., 2008; Tetreault et al., 2011). MWWE may also cause effects at the population level as demonstrated by the collapse of a fish population when a whole lake was exposed to low levels of an endocrine disrupting substance (17α-ethynylestradiol) (Kidd et al., 2007) commonly found in municipal effluents (Ternes et al., 1999).

The Grand River watershed in southern Ontario, Canada, is highly influenced by urban development and MWWE discharges. The river assimilates the effluent from 30 wastewater treatment plants as well as urban stormwater, and run-off from numerous non-point pollution sources such as agriculture (crop and livestock operations), and aggregate extraction. This watershed is expected to have a 38% increase in population by 2036 which may further threaten water quality (Environmental Commissioner of Ontario, 2010). Historically the watershed was impacted significantly from MWWE inputs but major infrastructure investment has greatly improved water quality (Cooke, 2006; Environmental Commissioner of Ontario, 2010). However, several sections of the river, especially

those flowing through heavily urbanized areas in the middle of the watershed, continue to have degraded water quality. Fish collected downstream of MWWE discharges in this watershed have been shown to have altered gonadal development, impaired capacity to produce sex steroids, and high rates of intersex (Tetreault et al., 2011). How these MWWE inputs and changes in fish performance translate into changes in fish populations and communities is currently unknown. In addition, the capacity of the river system to assimilate increasing amounts of MWWE and runoff due to rapidly growing populations and changing land use, exacerbated by the impacts of climate change, remains very uncertain and presents a management challenge that is shared by many watersheds worldwide. Managers need a thorough understanding of how key environmental stressors impact biological components of the aquatic system such as fish populations and communities to implement adaptive management of the watershed.

Fish community surveys (FCSs) have been used as an assessment tool worldwide to detect impact on aquatic environments. They typically involve determination of species composition, relative abundance, and diversity in specific stream reaches that are compared with other locations with comparable natural environmental attributes (Wickert, 1995; Wichert and Rapport, 1998; Taylor et al., 2001; Argent and Carline, 2004). These studies usually focus on evaluating the changes in species composition along a gradient of the river and associate these changes with changes in river characteristics and stressors. In this study, the impacts of MWWE on fish communities along the urbanized middle reach of the Grand River were assessed in the same locations near two major MWWE outfalls where physiological effects have been previously observed in individuals of a sentinel fish species (e.g., gonadosomatic indices, changes in gonadosomatic indices, suppressed steroid production, and intersex).

5.3 Materials and methods

5.3.1 Treatment plant and site selection

The Grand River watershed is located in southern Ontario and is the largest drainage basin on the northern shore of Lake Erie (Figure 5.1). Throughout the watershed nutrient levels are consistently elevated and oxygen levels frequently fall below the Ontario Provincial Water Quality Objective of 4 mg/L (dissolved oxygen at night during the summer season) especially downstream of the Kitchener MWWE discharge (Cooke, 2006). The municipality of Kitchener-Waterloo (>280,000 people) is serviced by two secondary-conventional activated sludge treatment plants with differing volumes and operational conditions that result in effluent of differing quality (Table 5.1). The distribution of sites in this study represents a 50 km stretch of the river along a gradient from a mainly agricultural landscape to an urbanized central section of the watershed (from West Montrose to Cambridge) (Figure 5.1). In the stretch of the river between the Waterloo and Kitchener MWWE discharges there is a low head dam (Mannheim Weir) equipped with two fishways. Sites were chosen along the 50 km reach based on proximity to the MWWE discharges, wadeability, habitat similarity (riffle/run and substrate size), distance from adjacent sites, and accessibility. All attempts were made to minimize variability in the fish community due to differences in river habitat by selecting riffle/runs with similar characteristics. In September of 2007, nine sites were sampled on the Grand River from the village of West Montrose upstream of Waterloo to downstream to the hamlet of Blair just west of the city of Cambridge. Habitat assessments of these sites in 2008 confirmed that there were minimal differences among sites (Brown, 2010). Sites in the study area exhibited similar physical characteristics; rubble and gravel bottom, little shade and a mean water depth of 10 to 60 cm. Sites were categorized and numbered sequentially downstream as being upstream (US) or downstream (DS) of a MWWE discharge, or intermediate (INT) between the two MWWE discharges to be consistent with a previous

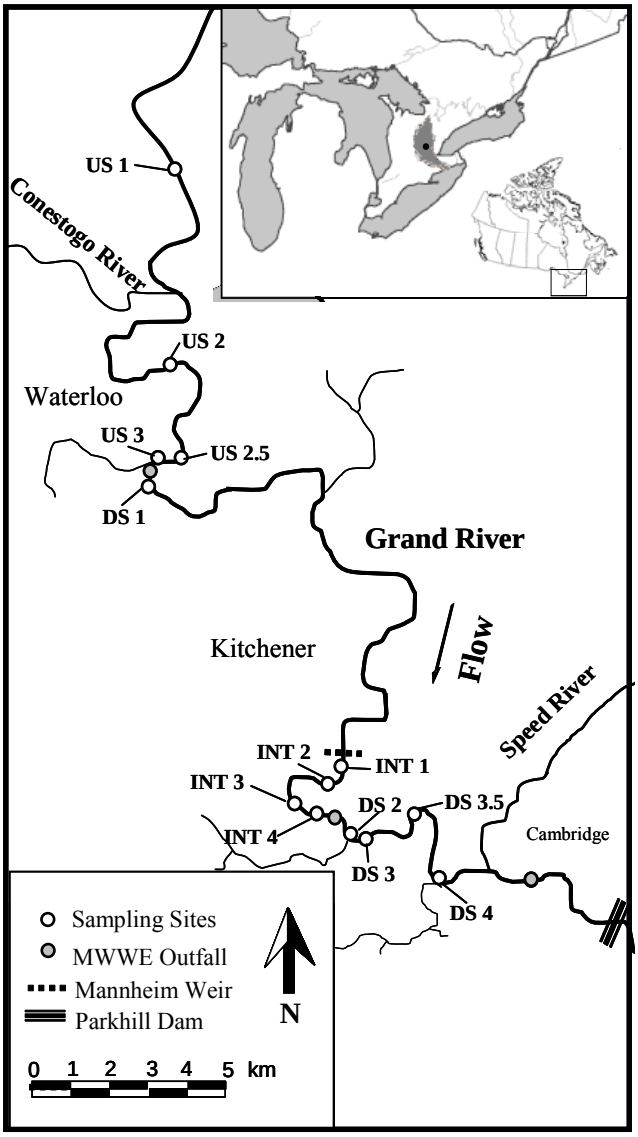

Figure 5.1. Map of Grand River and its major tributaries (Conestogo and Speed Rivers) through Waterloo and Kitchener, Ontario, Canada. Sampling sites for 2007 and 2008 access the river intermittently along a 50 km reach of the Grand River. All other sites are presented in order downstream as listed in Table 5.2.

Table 5.1. The Municipality of Kitchener-Waterloo and associated population, wastewater treatment plant processes, and the composition in the final effluent of the two sewage treatment plants in September of 2007 and 2008 and October-November 2008 (2007 and 2008 Ontario Clean Water Agency Performance Assessment Report - Wastewater Treatment Plant; Waterloo and Kitchner (K. Chow personal communication)). * Data from the Kitchener plant were not reported for November 2008.

Municipality	Waterloo			Kitchener		
Treatment plant characteristics	Secondary- conventional activated sludge			Secondary- conventional activated sludge		
Population served	105,100	120,055		185,000	210,999	
Year	2007	2008	2008	2007	2008	2008
Month	Sept	Sept	Nov	Sept	Sept	Oct*
Avg. Suspended Solids (mg/L)	5.03	11.20	8.30	10.13	7.70	7.30
Avg. Biological Oxygen Demand (mg/L)	<2.00	17.80	15.60	8.75	3.00	5.30
Avg. Phosphorus Load (mg/L)	0.51	0.48	0.31	0.84	0.68	0.40
Avg. NH3 + NH4 (mg/L)	4.92	9.48	5.85	26.28	16.88	16.50
Avg. Nitrate Load (mg/L)	17.40	7.47	9.01	1.31	1.71	2.04
Avg. Nitrite Load (mg/L)	0.02	1.16	0.86	0.64	1.55	1.73

study (Tetreault et al., 2011). The upper site (US 1) was approximately 15 km downstream of a large reservoir (Shand Dam), a distance far enough for the surface water temperatures not to be influenced by the summer hypolimnetic release from the dam (Figure 5.1). The furthest downstream site (DS 4) was approximately 10 km upstream of the Parkhill Dam in Cambridge and not directly affected by the reservoir. In 2008, the US 3 and INT 1 sites were omitted and replaced by sites US 2.5 and INT 3, respectively to increase the distances between adjacent reference and exposure sites. Also in 2008, two additional sites were included in the survey (INT 4 and DS 3.5) to increase the representative sample of reference, intermediate and exposed fish community sampling sites in this section of the study reach.

The summer of 2007 was very dry with water levels below the 10^{th} percentile on record for the Grand River (Station No. 02GA003) near the furthest downstream site (DS 4), while 2008 was a very wet year, with water levels above the 90^{th} percentile (Environment Canada, 2009). The water temperature at this site in was 20.6^{o}C and 17.8^{o}C September 2007 and 2008, respectively. The mean monthly discharge recorded at this location in 2007 was 29.2 m^3/s. The discharge rate for September at the time of the field study was 14.1 m^3/s. In 2008, the mean monthly discharge was 3-fold higher (63.7 m^3/s) with a rate of 48.4 and 63.2 m^3/s in September and November, respectively.

5.3.2 Fish community

The fish community assessments in the Grand River watershed were conducted in the months of September (2007 and 2008) and November (2008). At each sampling site, a 100 m wadeable riffle/run site was divided into ten equal subsections extending 10 m from shore. Six randomly selected sub-sites from the ten subsections were sampled for 300 seconds each using a backpack electrofisher (Halltech HT-2000; Guelph). Starting downstream, the operator and two netters moved upstream in a zig-zag pattern catching as many fish as possible. Voltage was not corrected for

conductivity to take into account the influence of effluent quality on fishing effort. Sampling occurred in the morning (between 8:00 a.m. and 12:00 p.m.) to minimize confounding factors such as diurnal fluctuations in water temperature and sunlight/shading influences on fish behaviour and distribution. Fish from each subsection were transported live to the on-site laboratory in aerated buckets and identified to species before being returned to the river. All fish were handled according to the University of Waterloo's and Environment Canada's Animal Care Committee Protocols (AUP 02-24, 08-08 and AUP-810; respectively).

5.3.3 Analysis

The number of individual species, families, and mean total catch per unit effort (CPUE) were compared among sites, CPUE was calculated by the mean abundance of sub-sites divided by shocking time (i.e., CPUE - #individuals/300s). Tukey's post-hoc tests were used to identify differences among sites when $p \leq 0.05$. The composition of the fish community was compared among sites using the Shannon-Wiener diversity index, species evenness, species and family richness, and trophic status (invertivores, omnivores and carnivores).

Characteristics such as tolerance (ability to adapt to disturbance and/or stress (S=Sensitive, I=Intermediate and T=Tolerant)), resilience (ability to withstand exploitation, expressed as population doubling time = period of time for the population to double based on fecundity, age to maturation and longevity (L=Low, I=Intermediate, H=High)), and vulnerability (ability to avoid predation/collection) were derived from the literature for each species caught and compared among sites (Froese, 2010; Eakins, 2011). Diet classification (invertivore, omnivore, carnivore) of each species was also determined from the literature (Scott and Crossman, 1998; Froese, 2010; Eakins, 2011) and percent site composition of each diet class were compared among sites. Classification of fish characteristics is listed in supporting information (Table S1).

Principal Components Analysis (PCA) and ordination plots using SYSTAT 12 (SYSTAT, 2007) incorporated the following parameters: CPUE, diversity, evenness, species and family richness, % Percidae, % Catostomidae, % Cyprinid, and % Centrarchid. The biological data was log-transformed and standardized using SYSTAT to a mean of zero and the generated variables represent the mean $\pm$ SD for analysis. This multivariate technique (Principal Component Analysis) was utilized to generate an ordination diagram to identify and quantify which sites would group together based on all of the biological data collected. A measure of Bray-Curtis dissimilarity among sites and cluster analysis was analyzed using the statistical software PAST version 2.03 (Hammer, 2001).

5.4 Results

5.4.1 CPUE

CPUE in 2007 was significantly lower at US 3, INT 1 and DS 4 when compared to all other sites ($p \leq 0.009$) (Table 5.2). CPUE was highest at DS 1, DS 3 and at US 1, while US 2, INT 2 and DS 2 demonstrated intermediate CPUE. In September of 2008, CPUE was significantly higher at INT 2, INT 3 and INT 4 when compared to US 1 ($p \leq 0.009$) (Table 5.2). CPUE was highest in the middle stretch of the sampling area (downstream of the first discharge at DS 1, to INT 4), while sites US 2, US 2.5, DS 2, DS 3.5 and DS 4 demonstrated similar CPUE. In November of 2008 CPUE was lower than in September collections in either year and was highest upstream of the first MWWE discharge (US 2.5) and immediately downstream of the second (DS 2). CPUE was lowest further downstream of the second discharge (DS 3, DS 3.5 and DS 4) (Table 5.2).

Table 5.2. Mean catch-per-unit-effort (CPUE), diversity, richness, evenness, species and family richness collected along an urban gradient (km) in the Grand River in September (2007 and 2008) and November (2008). Differences in CPUE among sites ($p<0.050$) are denoted by different lowercase letters.

Year	Month	Site	km	CPUE	Shannon-Weiner Diversity	Evenness	Species Richness	Family Richness
2007	Sept	US 1	0	31.00 a	0.78	0.75	11	5
		US 2	17	18.20 b	0.69	0.72	9	4
		US 3	21	7.20 c	0.68	0.88	6	3
		DS 1	23	30.50 ab	0.70	0.67	11	5
		INT 1	37	7.50 c	0.72	0.85	7	4
		INT 2	39	18.00 ab	0.52	0.54	9	4
		DS 2	43	18.70 ab	0.81	0.71	14	5
		DS 3	44	36.70 a	0.75	0.60	18	3
		DS 4	50	8.50 c	0.72	0.80	8	3
2008	Sept	US 1	0	16.00 a	0.69	0.73	9	4
		US 2	17	23.80 ab	0.30	0.33	9	4
		US 2.5	20.5	22.00 ab	0.67	0.70	9	4
		DS 1	23	30.30 ab	0.50	0.46	12	4
		INT 2	39	30.80 b	0.52	0.67	6	3
		INT 3	40	31.20 b	0.59	0.62	9	5
		INT 4	41	34.20 b	0.67	0.60	13	5
		DS 2	43	24.00 ab	0.45	0.43	11	5
		DS 3	44	18.70 ab	0.45	0.54	6	3
		DS 3.5	46	23.70 ab	0.93	0.76	17	4
		DS 4	50	23.50 ab	0.79	0.69	14	4
2008	Nov	US 1	0	8.03 ab	0.68	0.61	8	5
		US 2	17	9.80 a	0.20	0.19	14	5
		US 2.5	20.5	9.63 a	0.86	0.73	8	4
		DS 1	23	7.47 ab	0.66	0.58	9	4
		INT 3	40	7.70 ab	0.77	0.74	12	4
		INT 4	41	3.90 b	0.77	0.77	5	3
		DS 2	43	8.10 a	0.58	0.52	9	5
		DS 3	44	0.93 c	0.61	0.79	13	5
		DS 3.5	46	3.23 bc	0.85	0.74	11	5
		DS 4	50	3.17 bc	0.85	0.76	7	3

5.4.2 Fish community

The fish community assessments conducted in the Grand River watershed demonstrated temporal variability in several parameters. Sampling from the nine sites in 2007 resulted in a total of 1,058 individuals collected comprising 30 different species of fishes representing seven families. Eighteen of the 30 species represented < 1% of the total fish captured across all sites. Thirteen of these 18 species are classified as unique as five or less individuals were collected across all sites. Ten of the 30 species were collected at only one site. Both an increased incidence of unique species and site-specific collections of certain species generally occurred at sites downstream of MWWE discharges (DS 1, DS 2 and DS 4). As described in subsection 5.3.2, in 2008 sites US 3 and INT 1 were replaced by US 2.5 and INT 3, and two additional sites, INT 4 and DS 3.5 were added resulting in a total of 11 sites assessed in 2008. Site INT 2 was omitted from the study design for the November 2008 collections. These additional sites were similar in habitat to all other sites as described in Brown (2011).

Sampling from the 11 sites in September and November 2008 resulted in a total of 1,670 and 1,541 individuals, respectively, collected comprising 22 different species of fishes representing five families. In September, 12 of these 22 species represented <1% of the total abundance of fish across all sites, and 13 of the 22 species in November. In September 2008, eight of these species are classified at unique as five or fewer individuals were collected across all sites, while in November collections unique species accounted for nine of the 22 species. In order to allow comparisons among all sites, the remaining results presented address the data from the four dominants families, Percidae, Catostomidae, Cyprinidae, and Centrarchidae.

The fish community assessment in both years demonstrated that the most abundant family across all collections at all sites was Percidae with the exception of the far-field sites in 2007 (DS 4), where no members of this family were observed (Table 5.3). The abundances of Percidae downstream

Table 5.3. Proportion (%) of family represented as well as characteristics and trophic strategies of fish collected in the Grand River in September (2007 and 2008) and November (2008).

Year	Month	Site	Family				Characteristic				Trophic status		
			Percidae	Catostomidae	Cyprinidae	Centrarchidae	Tolerant	Sensitive	Vulnerable	Resilient	Invertivore	Omnivore	Carnivore
2007	Sept	US 1	66.67	2.15	13.98	16.67	3.76	43.55	2.15	43.55	69.35	29.57	1.04
		US 2	86.24	0	2.75	10.09	2.75	49.54	0	49.54	83.49	12.84	0
		US 3	58.14	20.93	0	20.93	23.26	0	20.93	0	51.16	41.86	0
		DS 1	46.2	35.33	10.87	7.07	42.93	27.72	35.32	27.72	55.43	44.02	0
		INT 1	31.11	2.22	35.56	31.11	37.78	2.22	2.22	2.22	57.78	33.33	8.89
		INT 2	81.48	2.78	0.93	14.81	3.7	14.81	2.78	14.81	80.56	17.59	0
		DS 2	53.57	34.82	5.36	5.36	11.61	3.57	36.61	3.57	77.68	20.54	0
		DS 3	66.36	19.09	9.55	3.64	17.73	51.36	19.09	52.05	76.36	23.64	0
		DS 4	0	29.41	47.06	23.53	33.33	0	31.37	0	43.14	56.86	0
2008	Sept	US 1	65.63	2.08	29.17	3.13	31.25	40.63	2.08	40.63	93.75	5.21	1.08
		US 2	95.8	2.1	0.7	0	1.4	14.69	2.1	83.22	97.2	2.8	3.67
		US 2.5	87.12	3.03	0.76	9.09	3.03	49.24	2.27	49.24	87.88	12.12	6.89
		DS 1	80.22	3.85	14.84	1.1	6.59	73.63	3.3	72.53	92.31	6.59	0
		INT 2	93.51	0	1.08	5.41	1.08	62.7	0	62.7	94.59	5.41	0
		INT 3	85.03	0.53	12.3	1.07	10.16	56.15	0.53	56.15	97.33	2.67	0
		INT 4	87.8	0.98	7.32	2.93	1.95	52.68	0.49	52.68	90.73	5.85	3.14
		DS 2	84.03	3.47	10.42	1.39	4.17	75	3.47	75	93.06	6.94	0.89
		DS 3	78.57	3.57	17.86	0	3.57	69.64	3.57	69.64	96.43	3.57	0
		DS 3.5	65.03	5.59	18.88	10.49	8.39	23.78	4.9	42.55	83.1	14.08	1.41
		DS 4	58.16	19.15	9.22	12.77	23.4	42.55	18.44	22.54	66.67	32.62	0
2008	Nov	US 1	23.64	34.55	20	13.64	2.73	49.09	37.27	14.55	74.55	25.45	0
		US 2	58.57	19.29	9.29	12.86	23.4	42.55	18.44	42.55	66.67	33.33	0
		US 2.5	95.8	2.1	0.7	0	1.4	83.22	2.1	83.22	97.2	2.8	0
		DS 1	88.46	3.08	0.77	7.69	3.03	49.24	2.27	49.24	87.88	12.12	0
		INT 3	80.65	3.76	14.52	1.08	7.69	72.53	3.3	72.53	92.31	7.69	0
		INT 4	93.51	0	1.08	5.41	1.08	62.7	0	62.7	94.59	5.41	0
		DS 2	85.03	0.53	12.3	1.07	10.16	56.15	0.53	56.15	97.33	2.67	0
		DS 3	87.8	0.98	7.32	2.93	1.95	52.68	0.49	52.68	90.73	5.85	3.41
		DS 3.5	84.62	3.5	10.49	1.4	4.17	75	3.47	75	93.06	6.94	0
		DS 4	78.57	3.57	17.86	0	3.57	69.64	3.57	69.64	96.43	3.57	0

of MWWE discharges were lower when compared to sites upstream of the individual outfalls, however returned to upstream levels at the intermediate sites (≥85%) before gradually decreasing again downstream of the second MWWE discharge. In both years, the relative abundance of Catostomidae in September increased in the vicinity of the MWWE discharges and was rarely present at sites in the upper reach or at the intermediate sites. In November (2008), the abundance of Catostomidae was highest at the upper most sites (US 1 and US 2). Across all sites, the relative abundance of Cyprinidae and Centrarchidae were in general opposite to those of Percidae and accounted for 70% of the fish collected at the DS 4 site in 2007 where there was a complete absence of Percidae (Table 5.3). In 2008, Percidae accounted for 58% and 79% (September and November, respectively) of the fish community at this site due mainly to the presence of darter species.

5.4.3 Species diversity

In September 2007, the Shannon-Weiner Index of species diversity was highest (≥0.72) at the upstream US 1 site and downstream of the second MWWE discharge (DS 2, DS 3 and DS 4) while in September 2008, species diversity was highest at a distance downstream of the first discharge (INT 2 (0.52) to INT 4 (0.67)) with abrupt decreases downstream of both discharges (0.50 and 0.45; respectively) (Table 5.2). In both months of 2008 (September and November), species diversity was highest downstream of the second discharge (DS 3.5 (0.93, 0.85) and DS 4 (0.79, 0.85)). Species diversity was lowest among all sites between years and seasons at US 2. In 2007, sites located in the middle reach of the sampling region (DS 1 to INT 2, and DS 3) had the lowest score of evenness (≤0.60) with the exception of INT 1, which had a high evenness score (0.85) (Table 5.2). Sites at the upper reach prior to these MWWE inputs had higher evenness scores (0.72-0.88). INT 1, DS 4 and US 3 demonstrated the greatest evenness (≥0.80). Species evenness in 2008 was similar to that of species diversity as evenness decreased immediately downstream of both MWWE discharges (0.46-0.58 and 0.43-0.52 for DS 1 and DS 2 in September and November; respectively) and gradually increased at a distance downstream of each discharge (≥ 0.60) (Table 5.2). The low indices of

diversity and evenness at US 2 in 2008 is a reflection of the fact that darters were the primary species collected at this site. Species richness was elevated downstream of both MWWE discharges and highest downstream of the second MWWE discharge for both years and in each season (Table 5.2). No trends in family richness were observed among sites (Table 5.2).

5.4.4 Trophic status and classification

Fish collected in the community survey were categorized into three major fish trophic classes, invertivores, omnivores, and carnivores (Table 5.3). Fish communities sampled in 2007 were predominately invertivores with the exception of US 3, DS 1 and INT 1, which had the lowest percentage of invertivores (51, 55 and 58%, respectively) and US 3 and DS 1, which had the highest incidence of omnivores (42, and 44 %, respectively). Incidences of invertivores and omnivores at all other sites in 2007 were 69 to 83% and 13 to 30%, respectively. The altered incidence of invertivores and omnivores at these sites corresponded to the reduced prominence of darter species and increased presence of sucker species at these sites. In both seasons of 2008, the fish community was comprised primarily of invertivores ($\geq$ 67%) due to the prominence of darter species. Omnivorous fish were the second most common feeding type at all sites in 2008 and their distribution across sites was uniform (3 to 12%) with the exception of the two furthest downstream sites from the second MWWE discharge in September (14% and 33%, respectively) and the two furthest upstream sites in November (25% and 33%, for US 1 and US 2, respectively). The increase in the proportion of omnivorous fish in September is a reflection of the increased occurrence of Rock Bass (Centrarchidae) and White Sucker (Catostomidae) at DS 4 site (Tables 5.3) and of the increased incidence of Rock Bass and Golden Redhorse at US 1 and US 2 in November. Across all upstream and intermediate sites the incidence of carnivores was low (2 to 9%) in 2007, and only present at US 1 and INT 1. In September 2008, carnivores were absent from DS 1, INT 2, INT 3, DS 3 and DS 4 and only at low levels (1-7%) at the

rest of the sites and at a single site (DS 3) in November 2008 (Table 5.3). The proportion of carnivores in the fish communities did not appear to be correlated with MWWE discharges.

Fish communities sampled were also categorized by the proportion of tolerant, resilient and vulnerable species present (Table 5.3). In 2007, 44 to 50% of fish communities collected upstream of the first MWWE discharge were comprised of sensitive fish species and only 0-3.8% of the remaining community were classified as tolerant (Table 5.3). The proportion of tolerant species increased downstream of the first MWWE discharge (43% at DS 1), and the contribution of sensitive fishes was greatly reduced (28%) when compared to upstream sites, except US 3. The number of tolerant species gradually decreased downstream of the first MWWE discharge and returned to pre-discharge levels at INT 2 before gradually increasing again downstream of the second outfall (12, 18, and 33% at DS 2, DS 3 and DS 4; respectively). In 2007, sensitive fish decreased downstream of DS 2 and were completely absent at the far field exposure site (DS 4). The presence of sensitive species in September 2008 increased downstream of the first MWWE discharge (74%) relative to sites upstream of this location (15-49%), and then gradually decreased in the middle reach of thie study area (63, 56 and 53% for INT 2, INT 3 and INT 4, respectively) then increased immediately downstream of the second discharge (75 and 70% for sites DS 2 and DS 3, respectively) (Table 5.3). In November 2008, the proportion of sensitive species was lower downstream of the first MWWE discharge (49%) relative to the intermediate sites and then decreased again downstream of the second discharge (56 and 53% for DS 2 and DS 3, respectively), and then increased at further downstream site DS 3.5 and DS 4 (75 and 70%, respectively) (Table 5.3).

In 2007, the distribution of fish vulnerable to predation/collection among sites mirrored that of the proportion of tolerant fish. Immediately downstream of the first MWWE discharge 35% of the fish were classified as vulnerable, whereas only 2% of the population were vulnerable upstream of the first outfall. The distributon of vulnerable fish varied little among sites in 2008 with <5% of the

community sampled characterized as vulnerable with the exception of the far-field exposure site downstream of the second MWWE discharge in September (DS 4) (18%) and the upper most reference sites in November (US 1 and US 2) ($\geq$ 18%) of 2008 (Table 5.3).

In 2007, the fish communities sampled downstream of the MWWE discharges had lower proportions of resilient species when compared to fish communities upstream with exception of the community at DS 3. In September 2008, the proportion of resilient fishes increased immediately downstream of MWWE discharges (73 to 75%) and then gradually decreased approaching pre-discharge levels (<50%) at a distances further from the point source (DS 4). In November of 2008 the proportion of resilient fish among sites mirrored that of sensitive fish across all sites (Table 5.3).

5.4.5 Principal component analysis

Principal component analysis (PCA) was conducted among sites within each year for all 16 of the fish community parameters listed in the methods section. An absolute component loading (PC) value of $\geq$0.800 was considered to be significantly correlated with the site (Table 5.4). The PCA of the first principal component (PC1) explained 44%, 44%, and 48% of the total variance observed in September of 2007 and 2008 and November 2008, respectively. In both seasons and years, PC1 had strong positive correlations to the proportion of omnivores (Figure 5.2bdf; Table 5.4). In September 2008, PC1 also had strong positive correlations with diversity, and in November 2008 with the presence of Catostomidae (Figure 5.2df; Table 5.4). In both seasons and years, PC1 had a strong negative relationship with the proportion of invertivores (Figure 5.2bdf and Table 5.4). In 2007 PC1 also had strong negative relationships with CPUE, and the proportion of sensitive, resilient fish, and in September 2008 PC1 was additionally negatively correlated with the proportion of resilient fish and of Percidae. The PCA of the second principal component (PC2) explained 23%, 16%, and 17% of the total variance observed in September of 2007 and September 2008 and November 2008, respectively.

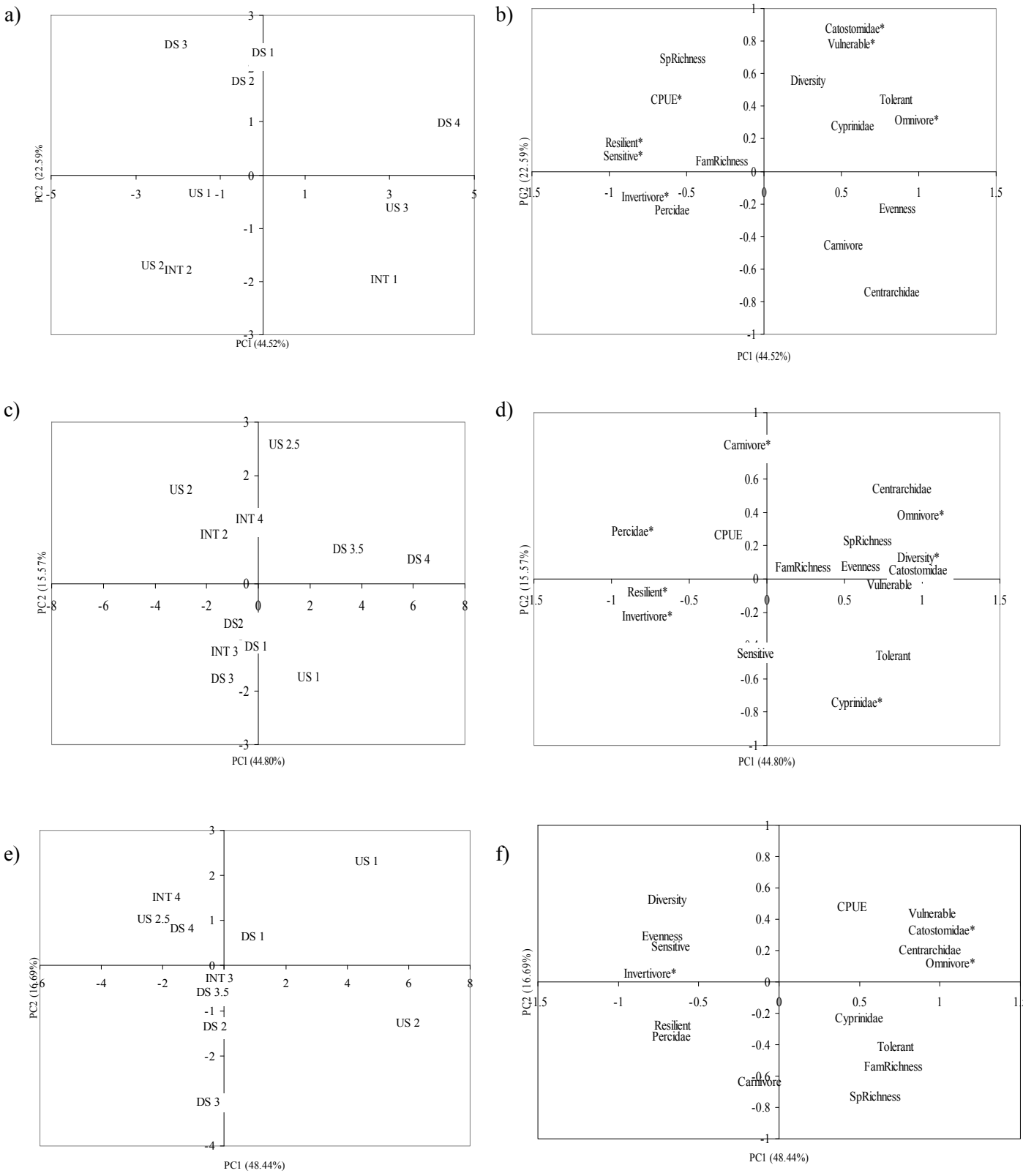
a)
DS 3
DS 1
DS 2
DS 4
US 1
US 3
US 2
INT 2
INT 1
PC2 (22.59%)
PC1 (44.52%)
b)
Catostomidae*
Vulnerable*
SpRichness
Diversity
CPUE*
Tolerant
Cyprinidae
Omnivore*
Resilient*
Sensitive*
FamRichness
Invertivore*
Percidae
Evenness
Carnivore
Centrarchidae
PG2 (22.59%)
PC1 (44.52%)
c)
US 2.5
US 2
INT 4
INT 2
DS 3.5
DS 4
DS2
INT 3
DS 1
DS 3
US 1
PC2 (15.57%)
PC1 (44.80%)
d)
Carnivore*
Centrarchidae
Omnivore*
Percidae*
CPUE
SpRichness
Diversity*
FamRichness
Evenness
Catostomidae
Vulnerable
Resilient*
Invertivore*
Sensitive
Tolerant
Cyprinidae*
PC2 (15.57%)
PC1 (44.80%)
e)
US 1
INT 4
US 2.5
DS 4
DS 1
INT 3
DS 3.5
DS 2
US 2
DS 3
PC2 (16.69%)
PC1 (48.44%)
f)
Diversity
CPUE
Vulnerable
Catostomidae*
Evenness
Sensitive
Centrarchidae
Omnivore*
Invertivore*
Cyprinidae
Resilient
Percidae
Tolerant
FamRichness
Carnivore
SpRichness
PC2 (16.69%)
PC1 (48.44%)

Figure 5.2. Biplots of the Principal Component Analysis (PCA) of sites from the Grand River sampled in September of a) 2007, c) 2008 and e) November 2008 and the eigenvector values for the fish community variables from September b) 2007, d) 2008 and f) November 2008 derived from the 16 standardized fish community parameters. Full variable names are listed in Materials and methods. Asterisk "*" indicate significant correlation of the parameter to the principal component.

Table 5.4. Eigenvalues from the first (PC1) and second (PC2) component analysis from the standardized (mean of zero ± SD) 16 fish community parameters measured in the Grand River in September of 2007 and September and November of 2008. PC loading value of ≥0.800 was considered to be positively or negatively influencing the fish community (in **bold**).

Parameter	2007 September		2008 September		2008 November	
	PC1	PC2	PC1	PC2	PC1	PC2
Omnivore	**0.824**	0.335	**0.882**	0.327	**0.922**	0.089
Evenness	0.748	-0.214	0.629	0.036	-0.784	0.247
Tolerant	0.746	0.453	0.733	-0.512	0.583	-0.437
Centrarchidae	0.635	-0.701	0.745	0.533	0.765	0.201
Cyprinidae	0.420	0.303	0.426	**-0.814**	0.373	-0.275
Vulnerable	0.414	**0.837**	0.709	-0.043	0.799	0.443
Catostomidae	0.410	**0.838**	0.752	0.048	**0.832**	0.358
Carnivore	0.367	-0.442	-0.204	**0.816**	-0.146	-0.667
SW diversity	0.157	0.508	**0.803**	0.064	-0.759	0.414
Family Richness	-0.435	0.007	0.028	-0.005	0.528	-0.541
Species Richness	-0.630	0.698	0.609	0.165	0.427	-0.765
Percidae	-0.738	-0.205	**-0.931**	0.301	-0.760	-0.372
CPUE	**-0.815**	0.486	-0.317	0.252	0.394	0.456
Resilient	**-0.895**	0.109	**-0.908**	-0.119	-0.752	-0.279
Sensitive	**-0.895**	0.108	-0.083	-0.486	-0.762	0.229
Invertivore	**-0.923**	-0.150	**-0.891**	-0.264	**-0.976**	0.006

In 2007, PC2 had strong positive correlations with the proportion of vulnerable species and the proportion of Catostomidae (Figure 5.2b; Table 5.4).

In September 2008 there was a strong positive relationship between PC2 and the proportion of carnivorous fish and a strong negative correlation with the proportion of Cyprinidae (Figure 5.2d; Table 5.4). There were no significant positive or negative correlations of the PC2 observed in November 2008. In both years and seasons, fish parameters characteristic of larger-bodied fish such as Catostomidae and Centrarchidae (omnivore, tolerant, and vulnerable) tend to plot to the right along the first principal component and characteristic of smaller-bodied Percidae (invertivores, resilient, sensitive) tend to plot to the left of the bi-plot (Figure 5.2bdf).

Bi-dimensional spatial distribution of the September PCA for both years oriented DS 4 to the right along the x-axis indicating that this site had more omnivores (Figure 5.2ac). In 2007, this section also included the sites INT 1 and US 3, while in 2008 this also included DS 3.5. In 2007, the sites US 1, US 2, INT 2 and DS 3 were associated with the parameters descriptive of members of the Percidae family (to the left of the y-axis), while the sites DS 1, DS 2 and DS-3 (above the x-axis) had more vulnerable large-bodied fish. Bi-dimensional spatial distribution of the September 2008 PCA oriented the sites DS 3.5 and DS 4 far to the right of the y-axis as these sites were influenced by greater species diversity, and more omnivorous Catostomidae and Centrarchidae fish (Figure 5.2d). Similar to the September 2007 distribution, the sites US 2, INT 2, INT 3, DS 1, DS 2 and DS 3 were associated with the parameters descriptive of members of the Percidae family (to the left of the y-axis), while the US 1 and DS 3 sites (below the x-axis) had more Cyprinidae. Spatial distribution of the November 2008 data demonstrated a significant change in the fish community among sites as US 1 and US 2 were oriented to the right along the x-axis indicating that these sites had more omnivores and Catostomidae fish (Figure 5.2ef), and US 2.5, INT 4 and DS 4 were associated with invertivore fish (to the left of the y-axis). If the sites US 1 and US 2 are removed from the November 2008 analysis, the PCA

demonstrates that the sites US 2.5, INT 3, DS 3.5 and DS 4 are strongly positively correlated to higher species diversity, and higher proportions of the smaller-bodied sensitive fish that are resilient to capture (data not shown).

5.4.6 Bray-Curtis analysis

Indications of site dissimilarity were conducted using the Bray-Curtis Index, which represents the similarity between sites using cluster analysis dendrograms (Figure 5.3). Cophenetic correlation coefficients for the September 2007, September 2008 and November 2008 Bray-Curtis clusters are 0.7847, 0.7456, and 0.9039; respectively. Analysis conducted on the 2007 data demonstrated two distinct clusters of sites (Figure 5.3a). The three near-field exposure sites (DS 1, DS 2 and DS 3) were grouped together (93% similarity) and the reference and intermediate sites were grouped together (US 1, US 2 and INT 2) (94% similarity). These observations are directly related to the observed changes in the fish communities downstream of the MWWE discharges. Based on the parameters measured, the Bray-Curtis analysis grouped the sites DS 4, US 3 and INT 1 furthest away from all other sites (Figure 5.3a).

The September 2008 Bray-Curtis Index analysis demonstrated four distinct site clusters (Figure 5.3b**).** As in the 2007, the three near-field exposure sites (DS 1, DS 2 and DS 3) were grouped together (96% similarity) (Figure 5.3b). The reference and intermediate sites were grouped together in two separate clusters (US 2, US 2.5 and INT 2 (92% similarity); and INT 3 and INT 4 (95% similarity); respectively). In September 2008, the site US 1 clustered much differently when compared to September 2007 and was not clustered with the other upstream sites. The furthest downstream sites DS 3.5 and DS 4 were strongly grouped furthest away from these other sites indicating that the far-field fish communities were more unique when sampled in September 2008 (Figure 5.3b). In November 2008 three clusters were evident; the two furthest upstream (US 1 and US 2) sites, which were 93% similar, sites in the middle reach of the river (US 2.5, DS 1 and INT 4), which were ~95%

similar to each other and the third cluster was INT 3, DS

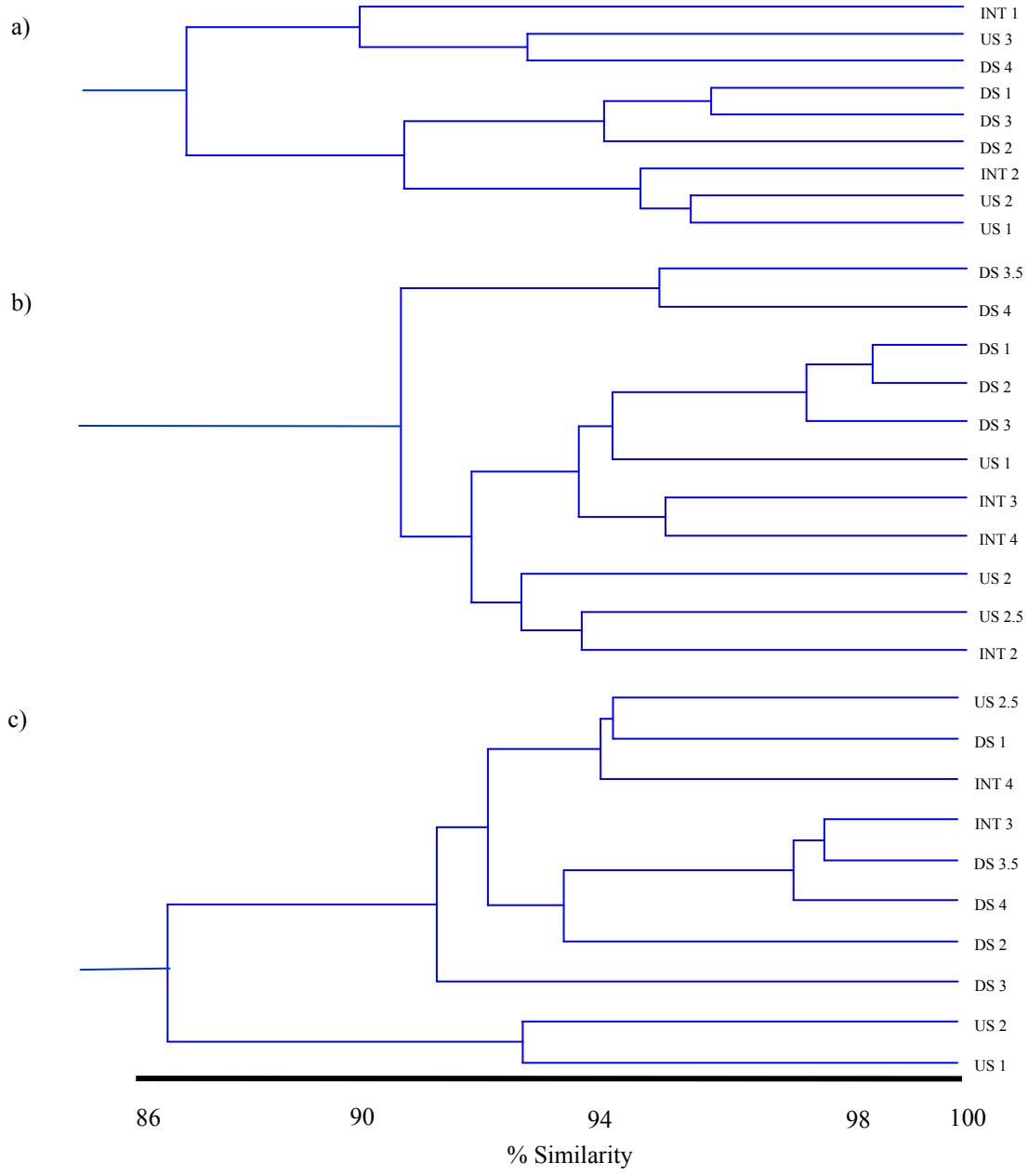

Figure 5.3. Bray-Curtis similarity analysis of fish community data among the sampling sites collected from the Grand River in a) September 2007, b) September 2008, and c) November of 2008.

3.5 and DS 4 which were 97% similar to each other (Figure 5.3c). The sites DS 2 and DS 3 were more distinct and were more separated from all other sites.

5.5 Discussion

The biological responses observed in the middle reach of the Grand River (which included two large MWWE discharges) demonstrate evidence of impacts at the level of the fish community. The fish communities downstream of the MWWE outfalls studied here demonstrate differences in the abundance and diversity of fish species, the type of dominant fish families and proportion of fish classified as tolerant, resilient and vulnerable when compared to fish communities upstream or at a distance from these MMWE outfalls. Regardless of the extremes in water flows between years, the fish community exposed to MWWE in this reach of the river in both years demonstrated reductions in the proportion of the most prominent fish (Rainbow Darter) found in the upstream sites and increases in the proportion of sucker species. Near MWWE outfalls, there were also increases in the proportion of tolerant fish species, as well as the proportion of vulnerable (catchable) and omnivourous fish, which is indicative of the increase in the number of larger, more mobile sucker species collected downstream of the MWWE outfalls. This study demonstrated seasonal variability in the fish community in this reach of the Grand River as the fish community observed in the late summer (September) was significantly different from that observed in November. In November small-bodied fish were more evenly distributed throughout the urban study area and the community in the upstream rural areas which were comprised of more sucker species.

The close proximity of the MWWE exposed sites in the spatial distribution of the ordination diagrams and similarity plots support the conclusions that site differences were due to changes in the fish community downstream of wastewater discharges. MWWE exposed sites were grouped based on increased proportions of omnivorous, tolerant and vulernable species, which is indicative of large-

bodied fish and reflected the shift from the prevalence of darter species in the upstream and intermediate reference sites to the increased presence of sucker species at MWWE exposed site in September. The clustering of sites demonstrated by the PCA and the Bray-Curtis analyses can be generalized into reference (upstream), exposure (downstream), and recovery zones (intermediate) (Tsai, 1975). In the exposure zone where the MWWE enters the river, fish abundance increases relative to upstream as fish are able to take advantage of the increase in nutrients. River habitat analysis at all study sites in 2008 revealed an increase in macrophyte production at the furthest downstream sites, likely from the accumulation of nutrients provided by the two MWWE outfalls (Brown, 2010). Further downstream of the Kitchener discharge, BOD increases and nocturnal respiration of abundant aquatic vegetation (i.e., macrophyte growth) consumes O_2 resulting in decreased DO (Rosamond et al., 2011). In September (both years), macrophyte growth downstream of the MWWE outfalls may have obscured the vision of the sampling crew thus reducing the number and types of fish collected at sites downstream of MWWE discharges. However, collections at the same downstream sites in November, when the plants had died back, also demonstrated low abundance, which indicates that resilience to capture of fish in September did not likely influence the results, however the macrophyte growth could have influenced the change in community by providing additional habitat.

As demonstrated by the September collections in both years in this study, fish communities downstream of the MWWE outfalls were altered compared to upstream sites and dominated by omnivorous species such as suckers, which are more tolerant of low oxygenated environments. More sensitive and less mobile fish such as darters avoid or suffer in these MWWE exposed zones due to lack of DO. The recovery zone occurs at a distance to the MWWE outfall where DO levels start to increase, which leads to more habitable conditions for most fish species. The intermediate sites in this study demonstrate gradual increases in fish diversity, and the relative presence of more sensitive, less

resilient fish such as *Etheostoma nigrum*, *E. blennioides* and *Rhinichthys cataractae* relative to exposure zones. Whether the shift in the proportion of darter species upstream of MWWE outfalls to sucker species in the fish communities downstream of these outfalls is due to the observed reproductive impairement of darters at these dowstream sites or due to their inability to tolerate low oxygen in some reaches is still to be determined (Tetreault et al., 2011).

In the early fall (November 2008), there appeared to be less of a distinction between exposure and recovery zones as the fish communities at the intermediate sites were more similar to the MWWE exposed locations than in the summer field collections (September 2007 and 2008). However, it should be noted that water flows in November 2008 were four times greater than September 2007 flows, and an increase in proportion of darter species was captured at the far field exposure zone in the fall (November 2008) relative to September 2007 collections. This may suggest that increased water flow in early fall can overcome some of the water quality issues observed at the far field sites in the summer months. Reduced macrophyte growth in early fall could also contribute to improved DO levels due to the reduced levels of nocturnal respiration. Contrary to what was observed in the late summer collections (September 2008), fish communities sampled in November immediately upstream of the first discharge (US 2.5) and further downstream of the second discharge (DS 3.5, DS 4) had higher species diversity. These sites also had higher proportions of the smaller-bodied sensitive fish resulting in an overall even distribution of these types of fish among all sites in early fall. As the quality of the effluent did not change dramatically between September and November, further studies are required to evaluate if the changes in the fish community within sites between seasons are due to changes in overall water quality of the river, in the river's ability to assimilate waste material or due to migration of fish to overwintering areas.

Human interference can alter the fish community by a simple elimination of some species which is often accompanied by a corresponding increase in the remaining species (Hynes, 1970).

Changes in fish communities downstream of MWWE outfalls have previously been documented (Porter and Janz, 2003; Ra et al., 2007; Yoem et al., 2007; Brown, 2010). Porter and Janz (2003) observed the fish community downstream of a MWWE outfall to be dominated by the very tolerant cyprinid Red Shiner (*Cyprinella lutrensis*), have few top predators and comprised of >50% omnivores at the exposure site relative to the reference location. These researchers concluded that downstream habitat was not a limiting factor so changes in the fish community were attributed to the impaired water quality at the MMWE discharge and although secondary treatment was removing many pollutants from the effluent, further treatment may be necessary to provide protection for aquatic ecosystems. Ra et al. (2007) demonstrated a cumulative effect of municipal wastewater in fish communities relative to control sites in South Korea. Similar to the impacted fish community in this study, fish communities influenced by MWWE in South Korea were comprised of a greater proportion of tolerant and omnivorous fish which resulted in the mean Index of Biotic Integrity (IBI) score of MWWE exposed sites to be half of the score of control sites. Impacts on the species composition and trophic guilds observed in this study suggest degradation of the stream ecosystem health of the Grand River.

In a previous study, reproductive development of two sentinel species (Rainbow Darter, *Etheostoma caeruleum* and Greenside Darter, *E. blennioides*) collected downstream of these two MWWE outfalls were studied (Tetreault et al., 2011). Exposed fish had greater growth (longer and heavier) when compared to reference fish (fish collected at the upstream sites) regardless of sex. However, these fish did not assimilate additional nutrient resources into energy storage as measured by fish condition or liver somatic index. Impacts on ovarian development appeared to be minor with no differences in gonad size or cellular development. Male fish exposed to MWWE were more impacted with an impaired capacity to produce sex steroids in vitro and altered testicular development. Impacts were identified downstream of the first MWWE discharge (DS 1) relative to background levels, then

appeared to recover somewhat at the intermediate site (INT 2), and then decreased again downstream of the second MWWE discharge (DS 2 and DS 4) (Tetreault et al., 2011). Male darters collected in the upstream agricultural region demonstrated no evidence of intersex whereas fish from the urban reference sites had incidence of intersex up to 20%. Rates of intersex were elevated downstream of both MWWE discharges studied (33% and ≥ 60%, respectively) and up to 100% at the far-field location (Tetreault et al., 2011). These results suggest that fish populations have the potential to recover from exposure to the fish MWWE effluent outfall (Waterloo) prior to exposure to the second MWWE effluent outfall (Kitchener). Feminization of male fish has been linked to reduced breeding success in wild (Harris et al., 2011) and laboratory cultured fish (Vajda et al., 2011) exposed to MWWE. Although investment in advanced secondary treatment processes to improve effluent quality has become more frequent over the last two decades, even secondary- and tertiary-treated effluents have been shown to have impacts on fish reproductive responses (Yoem et al., 2007; Vajda et al., 2008; Tetreault et al., 2011) and cause stress in fish (Ings et al., 2010) as treated MWWE still contains natural and synthetic hormones (Ternes et al., 1999; Servos et al., 2005) and other bioactive chemicals (Servos et al., 1999; Lishman et al., 2006; Metcalfe et al., 2009). This study provides evidence that at locations downstream of MWWE outfalls in the Grand River where reproductive impairment of a sentinel species (darters) have been observed, there are also subtle but detectable alterations in the fish community. It is not clear if the changes downstream of MWWE outfalls reflect differences in the water quality or subtle changes in habitat, or a result of the combination of both stressors.

Elevated levels of nutrients have been previously correlated with increases in growth and reduced population size of the Longear Sunfish (*Lepomis megalotis*) collected downstream of a MWWE discharge in Oklahoma, USA (Porter and Janz, 2003), and the Pale Chub (*Zacco platypus*) in Korea (Yoem et al., 2007). Increases in condition factor and liver somatic index of female chub or sunfish did not translate to increased reproductive success and young fish were absent from the

downstream fish population. Researchers concluded that size-selective mortality was due to ammonia toxicity and recruitment failure was the result of habitat degradation. They also attributed reproduction impairment to the cumulative impact of industrial and treated sewage effluents which lead to a >50% decrease in the abundance of individuals in the treated sewage exposed fish community. This is a community that was composed of a greater proportion of tolerant fish species (Yoem et al., 2007). The system in that study has a number of parallels to the Grand River watershed, which also assimilates large volumes of ammonia and organic/nutrient enriched effluent.

The amount of dissolved oxygen in a river is essential to its ability to sustain aquatic life and thus it is an important indicator of water quality. The Grand River downstream of the second sewage outfall experiences low DO levels (<4.0 mg/L) in the early morning hours particularly in late summer and early fall when the water flow is the lowest. Also, the impact of urban development on the Grand River is reflected by the significant increase of total ammonia which peaks at DS 4 (5.56 mg/L), and exceeds the Provincial Water Quality Objective (PWQO) of 0.0165 mg/L for unionized ammonia 47% of the time (Cooke, 2006). The concentrations of phosphorus, chloride and nitrate increases gradually downstream in the river as expected throughout the watershed from nonpoint sources carried by tributaries to the main stem of the Grand River. However, median total nitrite concentrations tend to be above the federal guideline of 0.06 mg/L in the Grand River at the lower reach where this study took place and may be the result of high levels of ammonia in the effluent of the second MWWE discharge denitrifying as it travels downstream (Tsai, 1975; Cooke, 2006). The increased levels of nutrients from the MWWE discharges coupled with low DO in the reach of the river downstream of the second sewage outfall are two primary factors that create conditions that promote denitrification; elevated nitrates can also contribute to significant impacts on aquatic fauna in this section of the river.

This study demonstrated some temporal variability (annual and seasonal) in the fish community as opposite trends were observed between and within years in abundance, diversity of fish,

and the proportion of certain fish families collected downstream of MWWE discharges in this reach of the Grand River. A significant difference between years was the presence of darter species at the DS 4 site in both seasons in 2008, a site that had no darter species in 2007. In the summer of 2007, recorded water levels were below the 10th percentile for the Grand River while water levels in the summer of 2008 were above the 90th percentile, resulting in a 3-fold increased in flow rate between years (Environment Canada, 2009). The lower flows in the summer of 2007 often occurred when water temperatures peaked; this contributed to critically low DO levels and resulted in an impaired ability to sustain aquatic life in this reach of the river. Increased water temperature, decreases in DO and low water flow at the most impacted site in this reach (DS 4) corresponds with our observations of changes to the fish commmunity downstream of these MWWE outfalls. However, the fish communities downstream of MWWE outfalls were altered in both years regardless of the discharge rate of the river.

The Region of Waterloo is currently undertaking significant infrastructure upgrades at the two wastewater treatment facilities in this study. Investment in secondary treatment of wastewater and improvement in management of operations has resulted in the re-colonization of sensitive fish species in locations downstream of MWWE outfalls in other similar systems where they had been previously excluded (Wichert, 1995). Upgrades in treatment at both major facilities on the Grand River have the potential to mitigate the effects currently documented at all levels of biological organization in this river including alterations in the fish responses (Ings et al., 2010; Tetreault et al., 2011), fish populations (Tetreault et al., 2011), food webs (Loomer, 2008), and fish communities (Wichert, 1995; Brown et al. 2011).

In this study we have successfully demonstrated the potential for MWWE discharges in the Grand River to impact fish communities. Future research should investigate the relationships between identified stressors and biological responses at the population and community level for the purpose of developing relationships between levels of biological organizations that may aid in predicting the

impacts of MWWE. This approach would aid in forecasting the impacts of further urban and industrial development, changes in land use practices, and investments in infrastructure upgrades on aquatic biological responses in watersheds (Colombo et al., 2007).

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Chapter 6

General Conclusions

The research presented in this thesis documented the impacts of MWWE on wild fish in the Canadian environment. This was accomplished through a series of field studies in the vicinity of MWWE discharges looking at the health and reproductive status of sentinel fish species across multiple levels of biological organization (Figure 6.1). Fish exposed to MWWE demonstrated changes in sex steroid production, reproductive development (gonadosomatic index, histopathology, intersex), and energy storage (condition, liver somatic index). The responses to MWWE were also observed at the fish community level. This research contributes to our understanding of the linkages among levels of biological organization which could aid in the design of monitoring programs.

The seasonal variability of key endpoints in sentinel species must be understood to interpret effects and to design effective monitoring programs. Energy storage (condition factor, liver somatic index), energy utilization (gonadosomatic index) and sex steroid production were shown to change across the annual reproductive cycle of the Greenside Darter, Brook Stickleback and Fathead Minnow. The period prior to spawning in the early spring or during recrudescence in the late fall represented the optimal time to sample Greenside Darters in the Grand River as they demonstrated temporal stability and minimum variability in key parameters of energetic storage, utilization, as well as, sex steroid production (Chapter 2). The optimal time to conduct biomonitoring studies on Brook Stickleback and Fathead Minnows in Wascana Creek was determined to be late spring-early summer (Chapter 4). Selection of the fish collection period needs to be specific to the annual reproductive cycles of the sentinel species selected as it will minimize confounding factors that can result in

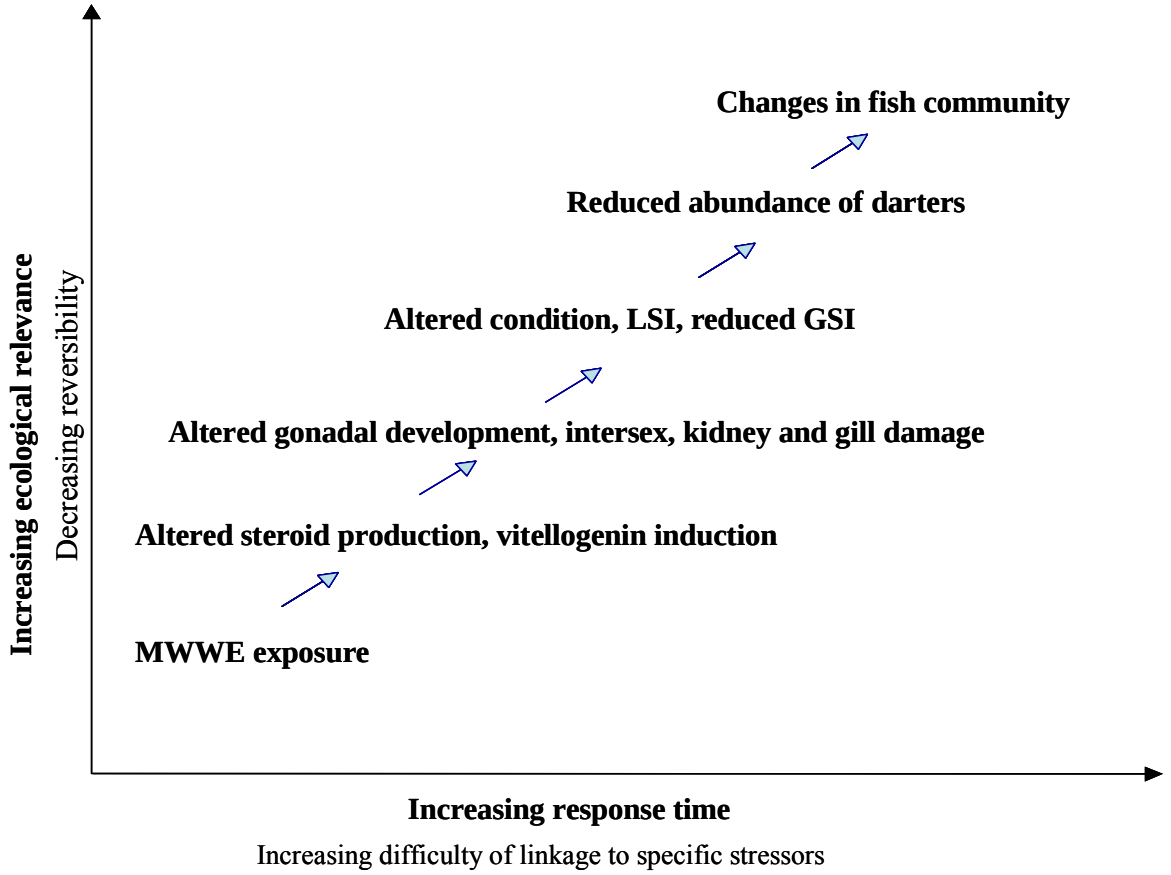

Figure 6.1. Levels of biological responses in fish exposed to MWWE (stressor). Exposed fish demonstrate impaired steroid production (physiological change) which contributes to the alterations in gonadal development (tissue response), which resulted in reductions in gonad size (whole organism response). Impairment in recruitment will lead to subsequent changes in the abundance of the fish species (population response) and eventually to its relative abundance in community assemblages (community response).

underestimation of the magnitude of impacts in the receiving environment. Studies where biomonitoring was not conducted during the appropriate life history periods will have a reduced ability to detect responses of fish to wastewater due to high variability and reduced statistical power, and/or minimal organ size (i.e., gonads) that may confound the interpretation (Chapters 3 and 4). The knowledge generated in this thesis can direct future research and biological monitoring using similar small-bodied fish as sentinel species.

MWWEs in the Grand River have the potential to impair gonadosomatic growth, steroid production and histopathology (e.g., intersex). The sentinel species, Rainbow Darter and Greenside Darter showed effects at multiple levels of biological organization that are involved in reproduction (physiological, cellular, organ and whole organism) (Chapter 3). In a receiving environment dominated by effluent, fish (Brook Stickleback, Fathead Minnow) exposed to MWWE in Wascana Creek demonstrated reproductive impairments, including altered gonadal development, delayed spawning, altered sex steroid production capacity, vitellogenin induction in male fish, and reduced expression of male sex characteristics (Chapter 4). These impacts on reproduction may lead to changes in recruitment and it is possible that these populations are sustained by immigration from upstream. Interestingly, unlike the Grand River, the high exposure to effluent in Wascana Creek did not lead to intersex in male fish. Exposure to MWWE in Wascana Creek also induced adverse effects on the respiratory and excretory organs in wild fish as demonstrated by the loss of cellular integrity in both kidney and gill tissues (Chapter 4). Effects on the gills are likely due to the high ammonia in the receiving environment and potential hypoxic conditions which may also explain the absence of fish further downstream. This requires further study as facility upgrades may lead to future changes in water quality. These findings emphasize the need to define not only the sublethal endocrine effects of emerging contaminants (PPCPs, estrogens) in MWWE, but also of the conventional toxicity of ammonia and impacts of nutrient enrichment when examining the impact of MWWE on the aquatic

environment. Only by measuring effects across multiple biological processes will monitoring generate sufficient knowledge for managers to understand the nature of the impacts on fish and formulate appropriate remedial actions.

Analysis of fish communities downstream of MWWE discharges (Chapter 5) demonstrated reductions in the proportion of the most prominent fish group (*Ethoestoma sp.*) which were also shown to have reproductive impairments (Chapter 3). There was a significant increase in the proportion of large, mobile, tolerant fish species such as suckers and sunfishes downstream of the second MWWE discharge. However, assigning causality of community level effects is difficult as there are many factors that control fish assemblages (Chapter 5). Although fish community assessments have considerable ecological relevance, fish communities respond slowly and it is difficult to make linkages to specific environmental stressors. Changes in fish assemblages can be the cumulative effect from various influences over a longer period of time (years) and recovery at this level may take several generations (Figure 6.1). Biomonitoring should therefore include measurement endpoints at several levels of biological organization that are mechanistically linked and provide the supporting information necessary to complete comprehensive assessments and make predictions at appropriate time scales.

Whole organism responses and fish community surveys have been proposed by Environment Canada to monitor compliance of the Environmental Quality Objectives for MWWE. Although Environment Canada's EEM Program has been successful in assessing the biological effects of pulp and paper and metal mining activities, it has not been tested for it applicability to municipal wastewaters. The studies presented in this thesis demonstrate that the effects-based approach used in the EEM is capable of detecting changes in fish over time and space. This approach provides a wide variety of population-level responses, ranging from changes in recruitment to individual-level measurements such as indicators of survival, reproduction and energy storage. Poor design of a

monitoring program such as selection of unsuitable endpoints or conducting studies at inappropriate periods during the fish's reproductive cycle will introduce confounding influences which limit the ability of the studies to identify effects or determine the magnitude of the impacts. Environmental monitoring programs optimized using a knowledge of the life history of the sentinel species can be a powerful tool to evaluate the impact of municipal wastewater effluent on the aquatic environment.

Whole organism effects (condition and organ size) most consistently demonstrate responses to wastewater and should be incorporated into monitoring programs for municipal wastewater effluents. Physiological endpoints can complement biological monitoring to aid in directing more detailed mechanistic studies to identify causative agents for the reproductive alterations observed. Future research should be directed at establishing the thresholds which elicit subsequent responses at the next level of biological organization. This would facilitate a better understanding of the linkages among the measurement endpoints, allow better assessments and increase our ability to predict how fish and fish populations may respond to remedial actions. In addition to MWWEs, numerous natural (habitat, flow, etc.) and anthropogenic (storm water, agricultural runoff, eutrophication, etc.) stressors can also impact fish populations in these watersheds. MWWEs are very complex mixtures of chemicals that can have low concentrations of endocrine disrupting substances as well as traditional toxicants such as ammonia and nutrients that may contribute to seasonal hypoxia. Many municipalities across Canada are making significant investments in wastewater infrastructure with the intention of improving effluent quality to meet more stringent effluent quality standards. The knowledge generated in this thesis will contribute to our ability to evaluate the effectiveness of these investments and the potential recovery of these receiving environments.

Further research is needed to assess the potential mechanisms responsible for intersex (estrogens and/or hypoxia) in the sentinel species studied herein by further incorporating laboratory research on the HPG axis to identify the pathway responsible for intersex in fish. New research using reproductive

endpoints should also take into account factors such as photoperiod and water temperature as these are important environmental cues for the timing and duration of gonadal recrudescence. It is imperative to address the significance of intersex in relation to reproductive success and how these impacts translate into effects at the population level. Lastly, it is crucial to evaluate if planned infrastructure investments at Waterloo, Kitchener and Regina will result in improvements in the receiving environment. In order to develop our understanding of the impact of MMWE on higher levels of organization, it is important to determine if the observed effects of wastewater on fish communities can be separated from changes in habitat, land use, and other factors.

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References

Allen, Y., Scott, A.P., Matthiessen, P., Haworth, S., Thain, J.E., Feist, S. 1999. Survey of estrogenic activity in United Kingdom estuarine and coastal waters and its effects on gonadal development of the Flounder (*Platichthys flesus*). Environ. Toxicol. Chem. 18, 1791-1800.

Alvarez, D.A., Cranor, W.L., Perkins, S.D., Schroeder, V.L., Iwanowicz, L.R., Clark, R.C., Guy, C.P., Pinkney, A.E., Blazer, V.S., Mullican, J.E. 2009. Reproductive health of bass in the Potomac, USA, Drainage: Part 2: Seasonal occurrence of persistent and emerging organic contaminants. Environ. Toxicol. Chem. 28(5), 1084-1095.

Anderson, C., Cabana, G., 2005. Delta15N riverine food webs:effects of N inputs from agricultural watersheds. Can. J. Fish Aqua. Sci. 62, 333-340.

Ankley, G.T., Bencic, D.C., Breen, M.S., Collette, T.W., Conolly, R.B., Denslow, N.D., Edwards, S.W. Ekman, D.R., Gracia-Reyero, N., Jensen, K.M., Lazorchak, J.M., Martinovic, D., Miller, D.H., Perkins, E.J., Orlando, E.F., Villeneuve, D.L., Wang, R.L., Watanabe. 2009. Endocrine disrupting chemicals in fish: developing exposure indicators and predictive models of effects based on mechanism of action. Aquat. Toxicol. 92, 168-179.

Ankley, G.T., Bennett, R.S., Erickson, R.J., Hoff, D.J., Hornung, M.W., Johnson, R.D., Mount, D.R., Nichols, J.W., Russom, C.L., Schmieder, P.K., Serrano, J.A., Tietge, J.E., Villeneuve, D.L. 2010. Adverse outcome pathways: A conceptual framework to support ecotoxicology research and risk assessment. Environ. Toxicol. Chem. 29, 730-741.

Arcand-Hoy, L.D., Metcalfe, C.D., 1999. Biomarkers of exposure of Brown Bullheads (*Ameirus nebulosus*) to contaminants in the lower Great Lakes, North America Environ. Toxicol. Chem. 18(4), 740-749.

Arciszewski, T.J., Kidd, K.A., Munkittrick, K.R. 2011. Comparing responses in the performance of sentinel populations of stoneflies (Plecoptera) and Slimy Sculpin (*Cottus cognatus*) exposed to enriching effluents. Ecotoxicol. Environ. Saf. 74(7), 1844-1854.

Argent, D.G., Carline, RF. 2004. Fish assemblage changes in relation to watershed landuse disturbance. Aqua. Ecosys. Health & Manage. 7(1), 101–114.

Barrett, T.J., Munkittrick, K.R. 2010. Seasonal reproductive patterns and recommended sampling times for sentinel fish species used in environmental effects monitoring programs in Canada. Environ. Rev. 18, 115-135.

Barrett, T.J., Lowell, R.B., Tingley M.A., Munkittrick, K.R. 2010. Effects of pulp and paper mill effluent on fish: influence of sentinel species on variability in responses among sampling cycles. Environ. Toxicol. Chem. 29, 440-452.

Beneteau, C.L., Mandrak, N.E., Heath, D.D. 2009. The effects of river barriers and range expansion of the population genetic structure and stability in Greenside Darter (*Etheostoma blennioides*) populations. Cons. Gen. 10(2), 477-487.

Bennie, D. 1999. Review of the environmental occurrence of alkylphenols and alkylphenol ethoxylates. Water Qual. Res. J. Can. 1, 79-122.

Bjerregaard, L.B., Korsgaard, B., Bjerregaard, P. 2006. Intersex in wild Roach (*Rutilus rutilus*) from Danish sewage effluent-receiving streams. Ecotox. Environ. Saf. 64(3), 321-328.

Blazer, V.S., Iwanowicz, L.R., Iwanowicz, D.D., Smith, D.R., Young, J.A., Hedrick, J.D., 2007. Intersex (testicular oocytes) in Smallmouth Bass from the Potomac River and selected nearby drainages. J. Aqua. Animal Health 19, 242-253.

Brasfield, S.M. 2007. Investigating and interpreting reduced reproductive performance in fish inhabiting streams adjacent to agricultural operations. Ph.D. Thesis in Biology, University of New Brunswick, Saint John, New Brunswick, Canada.

Brooks, B.W., Riley, T.M., Taylor, R.D. 2006. Water quality of effluent-dominated ecosystems: ecotoxicoloical, hydrological, and management considerations. Hydrobio. 556, 365-379.

Brown, C.J.M. 2010. The effects of municipal wastewater effluent on fish communities in the Grand River watershed. M.Sc. Thesis in Biology, University of Waterloo, Waterloo, Ontario, Canada.

Brown, C.J.M., Knight, B.W., McMaster, M.E., Munkittrick, K.R., Oakes, K.D., Tetreault, G.R., Servos, M.R. 2011. The effects of tertiary treated municipal wastewater on fish communities of a small river tributary in Southern Ontario, Canada. Envion. Pollut. 159, 1923-1931.

Bunt, C.M., Cooke, S.J., McKinley, R.S. 1998. Creation and maintenance of habitat downstream from a weir for the Greenside Darter, Etheostoma blennioides - a rare fish in Canada. Environ. Bio. Fish. 51(3), 297-308.

Bucher, F., Hofer, R. 1993. The effects of treated domestic sewage on three organs (gills, kidney, liver) of Brown Trout (*Salmo trutta*). Wat. Res. 27(2), 255-261.

CanNorth. 2009. Biological and limnological assessment of Wascana Creek and the Qu'Appelle River systems. Final Report Project No. 1123. Prepared by Canada North Environmental Services, Saskatoon, Saskatchewan, pp. 62.

CEPA. 1999. Canadian Environmental Protection Act c. 33, Schedule. 1; SOR/2000-109; SOR/2001-1, 147; Canada Gazette Part II, err.(F) 135. pp. 382.

Chambers, P.A., Allard, M., Walker, S.L., Marsalek, J., Lawrence, J., Servos, M., Busnarda, J., Munger, K.S., Adare, K., Jefferson, C., Kent, R.A., Wong, M.P. 1997. Impacts of municipal wastewater effluents on Canadian waters: a Review. Water Qual. Res. J. Can. 32 (4), 659-713.

Cheng, C.S. 2010. Kidney histology assessment methodology development. Report for Environment Canada. pp. 21.

Chiang, G., Munkittrick, K.R., Saavedra, M.F., Tucca, F., McMaster, M.E., Urrutia, R., Tetreault, G., Gavilan, J.F., Barra, R. 2010. Seasonal changes in *Trichomycterus areolatus* and *Percilia gillissi*, and their suitability for environmental monitoring. Acc. Stud. Neotrop. Fauna Environ. 46(3), 185-196.

Colombo, S.J., McKenney, D.W., Lawrence, K.M., Gray, P.A. 2007. Climate change projections for Ontario: practical information for policymakers and planners. Climate change research report; CCRR-05. Queen's Printer for Ontario, Ontario Ministry of Natural Resources, Sault Ste. Marie, ON.

Cooke, S. 2006. Water quality in the Grand River: A summary of current conditions (2000-2004) and long term trends. Cambridge: Grand River Conservation Authority. pp. 88.

COSEWIC. 2006. COSEWIC assessment and update status report on the Greenside Darter Etheostoma blennioides in Canada. Committee on the Status of Endangered Wildlife in Canada. Report. pp. 34.

Craig, J.F. 2008. A short review of pike ecology. Hydrobiologia 601, 5-16.

Daoust, P.-Y., Ferguson, H.W. 1984. The pathology of chronic ammonia toxicity in Rainbow Trout, *Salmo gairdneri* Richarson. J. Fish Diseases 7, 199-205.

Daughton, C.G., Ternes. T.A. 1999. Pharmaceuticals and Personal Care Products in the Environment: Agents of Subtle Change? Environ. Health Perspect. 107(S6), 907-938.

Dextrase, A. 2005. Update COSEWIC status report on Greenside Darter (*Etheostoma blennioides*). Report. pp. 42.

Douxfils, J., Robert, M., Frederic, S., Arnaud, B., Delphine, L., Thome, J.-P., Patrick, K. 2007. Do sewage treatment plant discharges substantially impair fish reproduction in polluted rivers? Sci. Total Environ. 372, 497-514.

Drimmie, R. J., Heemskerk, A. R. 2005. Leco Combustion of Organics. In Technical Procedure 9.0, Revision 02, p. 8: Environmental Isotope Laboratory, Department of Earth Sciences, University of Waterloo, Ontario, Canada.

Dube, M.G., MacLatchy, D.L. 2001. Identification and treatment of a waste stream at a bleached kraft pulp mill that depresses a sex steroid in the Mummichog (*Fundulus heteroclitus*). Environ. Toxicol. Chem. 20(5), 985-995.

Dube, M.G., Benoy, G.A., Blenkinsopp, S., Ferone, J.-M., Brua, R.B., Wassenaar, L.I. 2005. Application of multi-stable isotope (13C, 15N, 34S, 37Cl) assays to assess spatial separation of fish (Longnose Sucker *Catostomus catostomus*) in an area receiving complex effluents. Water Qual. Res. J. Can. 40(3), 275-287.

Dyer, S.D., Wang, X.H. 2002. A comparison of stream biological responses to discharge from wastewater treatment plants in high and low population density areas. Environ. Toxicol. Chem. 21, 1065-1075.

Eakins, R.J. 2011. Ontario Freshwater Fishes Life History Database. Version 3.92. Online database. Accessed February 2009. Available from: www.fishdb.ca.

Edeline, E., Le Rouzic, A., Winfield, I.J., Fletcher, J.M., Ben James, J., Stenseth, N.C., Vollestad, L.A. 2009. Harvest-induced disruptive selection increases variance in fitness-related traits. Proceedings of the Royal Society B-Biological Sciences 276(1676), 4163-4171.

Empix Imaging. 2006. Northern Eclipse 7.0. Mississauga, On, Canada. p. Imaging software.

Environment Canada. 2002. Guidance for data assessment. National EEM Office, Environment Canada, Ottawa, Ontario, pp. 37.

Environment Canada. 2009. The Green Lane. Water Level and Streamflow Statistics. Cited February, 2011. Available from: http://www.wsc.ec.gc.ca/staflo/index_e.cfm?cname=flow_monthly.cfm&first=1913&last=2009.

Environment Canada. 2011. Metal mining environmental effects monitoring (EEM) technical guidance document. Chapter 3: effects on fish and fisheries resources. National EEM Office, Environment Canada, Ottawa, Ontario, pp. 36.

Environmental Commissioner of Ontario. 2010. Sewage Treatment: Not Good Enough. Redefining Conservation, ECO Annual Report, 2009/10. Toronto: The Queen's Printer for Ontario.

Fahy, W.E. 1954. The life history of the northern Greenside Darter, *Etheostoma blennioides* Rafinesque. J. Elisha Mitchell Sci. Soc. 139-205.

Fairchild, J.F., Allert, A.L., Sappington, L.C., Wadell, B. 2005. Chronic toxicity of un-ionized ammonia to early life-stages of endangered Colorado Pikeminnow (*Ptychocheilus lucius*) and Razorback Sucker (*Xyrauchen texanus*) compared to the surrogate Fathead Minnow (*Pimephales promelas*). Arc. Environ. Cont. Tox. 49, 378-384.

Fent, K., Escher, C., Caminada, D. 2006. Estrogenic activity of pharmaceuticals and pharmaceutical mixtures in a yeast reporter gene system. Reprod. Toxicol. 22, 175-185.

Fish Survey Expert Working Group. 1997. Recommendations from Cycle 1 review. EEM/1997/6. Environmental Effects Monitoring Program. Environment Canada, Ottawa, ON, Canada.

Fitzgerald, D.G., Lanno, R.P. Dixon, D.G. 1999. A comparison of a sentinel species evaluation using Creek Chub (*Semotilus atromaculatus* Mitchill) to a fish community evaluation for the initial identication of environmental stressors in small streams. Ecotoxicology 8, 33-48.

Froese, R. 2010. FishBase World Wide Web electronic publication. Version (11/2010) Online database. Accessed February 2009. Available from: www. fisbase.org.

Galloway, B.J., Munkittrick, K.R. 2006. Influence of seasonal changes in relative liver size, condition, relative gonad size and variability in ovarian development in multiple spawning fish species used in environmental monitoring programs. J. Fish Bio. 69(6), 1788-1806.

Gibbons, W.N., Munkittrick, K.R., Taylor, W.D. 1998a. Monitoring aquatic environments receiving industrial effluents using small fish species 1: Response of Spoonhead Sculpin (*Cottus ricei*) downstream of a bleached-kraft pulp mill. Environ. Toxicol. Chem. 17(11), 2227-2237.

Gibbons, W.N., Munkittrick, K.R., McMaster, M.E., Taylor, W.D. 1998b. Monitoring aquatic environmental receiving industrial effluents using small fish species 2: Comparison between responses of Trout-Perch (*Percopsis omiscomaycus*) and White Sucker (*Catostomus commersoni*) downstream of a pulp mill. Environ. Toxicol. Chem. 17(11), 2238-2245.

Gray, M.A., Curry, A.R., Munkittrick, K.R. 2002. Non-lethal sampling methods for assessing environmental impacts using a small-bodied sentinel fish species. Wat. Qual. Res. J. Can. 37(1), 195-211.

Gray, M.A., Cunjak, R.A., Munkittrick, K.R. 2004. Site fidelity of Slimy Sculpin (*Cottus cognatus*): insights from stable carbon and nitrogen analysis. Can. J. Fish. Aquat. Sci. 61(9), 1717-1722.

Gray, M.A., Munkittrick, K.R. 2005. An effects-based assessment of Slimy Sculpin (*Cottus cognatus*) populations in agricultural regions of northwestern New Brunswick. Wat. Qual. Res. J. Canada 40, 16-27.

Gross, B., Montgomery-Brown, J., Naumann, A., Reinhard, M. 2004. Occurrence and fate of pharmaceuticals and alkylphenol ethoxylate metabolites in an effluent-dominated river and wetland. Environ. Toxicol. Chem. 23(9), 2074-2083.

Guillette, L.J., Edwards, T.M. 2005. Is nitrate an ecologically relevant endocrine disruptor in vertebrates? Integr. Comp. Biol. 45, 19-27

Hammer, O., Harper, D.A.T., Ryan, P.D. 2001. PAST: Paleontological Statistics software package for education and data analysis. Palaeo. Elect. 4(1).

Harries, J.E., Sheahan, D.A., Jobling, S., Matthiessen, P., Neall, P., Routledge, E.J., Rycroft, R., Sumpter, J.P., Tylor, T. 1996. Survey of estrogenic activity in United Kingdom inland waters. Environ. Toxicol. Chem. 15(11), 1993-2002.

Harries, J.E., Sheahan, D.A., Jobling, S., Matthiessen, P., Neall, P., Sumpter, J.P., Tylor, T., Zaman, N. 1997. Estrogenic activity in five United Kingdom rivers detected by measurement of vitellogenesis in caged male trout. Environ. Toxicol. Chem. 16(3), 534-542.

Harris, C.A., Hamilton, P.B., Runnalls, T.J., Vinciotti, V., Henshaw, A., Hodgson, D., Coe, T.S., Jobling, S., Tyler, C.R., Sumpter, J.P. 2011. The consequences of feminization in breeding groups of wild fish. Environ. Health Perspect. 119(3), 306-311.

Harshbarger, J.C., Coffey, M.J., Young, M.Y. 2000. Intersexes in Mississippi River shovelnose sturgeon sampled below Saint Louis, Missouri, USA. Mari. Environ. Res. 50, 247-250.

Hecker, M., Tyler, C.R., Hoffmann, M., Maddix, S., Karbe, L. 2002. Plasma biomarkers in fish provide evidence for endocrine modulation in the Elbe River, Germany. Environ. Sci. Tech. 36(11), 2311-2321.

Hinck, J.E., Blazer, V.S., Schmitt, C.J., Papoulias, D.M., Tillitt, D.E. 2009. Widespread occurrence of intersex in black basses (*Micropterus spp.*) from US rivers, 1995-2004. Aquat. Toxicol. 95(1), 60-70.

Holeton, C., Chambers, P.A., Grace L. 2011. Wastewater release and its impacts on Canadian Waters. Can. J. Fish. Aquat. Sci. 68, 1836–1859.

Hynes, H.B.N. 1970. The ecology of flowing waters in relation to management. Water Poll. Control Fed. 42 (3), 418-424.

Ings, J, Servos, M, Vijayan, M. 2010. Hepatic transcriptomics and protein responses to in situ municipal wastewater effluent exposure in Rainbow Trout. Environ. Sci. Technol. 44(6), 2176– 2182.

Iwanowicz, L.R., Blazer, V.S., Guy, C.P., Pinkney, A.E., Mullican, J.E., Alvarez, D.A. 2009. Reproductive health of bass in the Potomac, USA, Drainage: Part 1. Exploring the effects of proximity to wastewater treatment plant discharge. Environ. Toxicol. Chem. 28(5), 1072-1083.

Jefferies, K.M., Nelson, E.R., Jackson, L.J., Habibi, H.R. 2008. Basin-wide impacts of compounds with estrogen-like activity on Longnose Dace (*Rhinichthys cataractae*) in two prairie river of Alberat, Canada. Environ. Toxicol. Chem. 27(10), 2042-2052.

Jobling S., Tyler C.R. 2006. Introduction: The ecological relevance of chemically induced endocrine disruption in wildlife. Environ. Health Persp. 114, 7-8.

Jobling, S., Nolan, M., Tyler, C.R., Brighty, G., Sumpter, J.P. 1998. Widespread sexual disruption in wild fish. Environ. Sci. Tech. 32, 2496-2506.

Jobling, S., Beresford, M., Nolan, M., Rodgers-Gray, T., Brighty, G., Sumpter, J.P., Tyler, C.R. 2002a. Altered sexual maturation and gamete production in wild Roach (*Rutilus rutilus*) living in rivers that receive treated sewage effluents. Bio. Reprod. 66, 272-281.

Jobling, S., Coey, S., Whitmore, J.G., Kime, D.E., Van Look, K.J.W., McAllister, B.G., Beresford, N., Henshaw, A.C., Brighty, G., Tyler, C.R., Sumpter, J.P. 2002b. Wild intersex Roach (*Rutilus rutilus*) have reduced fertility. Bio. Reprod. 67, 515-524.

Jobling, S., Williams, R., Johnson, A., Taylor, A., Gross-Sorokin, M., Nolan, M., Tyler, C.R., van Aerle, R., Santos, E., Brighty, G. 2006. Predicted exposures to steroid estrogens in UK rivers correlate with widespread sexual disruption in wild fish populations. Environ. Health Persp. 114, 32-39.

Jobling, S., Burn, R.W., Thorpe, K., Williams, R., Tyler, C.R. 2009. Statistical modeling suggests that antiandrogens in effluents from wastewater treatment works contribute to widespread sexual disruption in fish living in English Rivers. Environ. Health Perspect. 117(5), 797-802.

Kavanagh, R.J., Balch, G.C., Kisparissis, Y., Niimi, A.J., Sherry, J., Tinson, C., Metcalfe, C.D. 2004. Endocrine disruption and altered gonadal development in White Perch (*Morone americana*) from the Lower Great Lakes Region. Environ. Health Persp. 112(8), 898-902.

Kavlock, R.J., Daston, G.P., DeRosa, C., Fenner-Crisp, P., Gray, L.E., Kaattari, S, et al. 1996. Research needs for the risk assessment of health and environmental effects of endocrine disruptors: a report of the U.S. EPA-sponsored workshop. Environ. Health Perspect. 104(Suppl. 4), 715-740.

Kidd, K.A., Blanchfield, P.J., Millls, K.H., Palace, V.P., Evans, R.E., Lazorchak, J.M., Flick, R.W. 2007. Collapse of a fish population after exposure to a synthetic estrogen. Proc. Natl. Acad. Sci. 104(21), 8897-8901.

Kilgore, B.W., Munkittrick, K.R., Portt, C.B., Hedley, K., Culp, J., Dixit, S., Pastershank, G. 2005. Biological criteria for municipal wastewater effluent monitoring programs. Wat. Qual. Res. J. Can. 40 (3), 374-387.

Kocovsky, P.M., Stapanian, M.A., Knight, C.T. 2010. Night sampling improves indices used for management of yellow perch in Lake Erie. Fish. Manag. Eco. 17(1), 10-18.

Kolpin, D.W., Furlong, E.T., Meyer, M.T.Thurman, E.M, Zaugg, S.D., Barber, L.B., Buxton, H.T. 2002. Pharmaceuticals, hormones, and other organic wastewater contaminants in U.S. streams, 1999-2000: A national reconnaissance. Environ. Sci. Tech. 36, 1202-1211.

Kramer, V.J., Etteron, M.A., Hecker, M., Murphy, C.A., Roesijadi, G., Spade, D.J., Spromberg, J.A., Wang, M., Ankley, G.T. 2011. Adverse outcome pathways and ecological risk assessment: bridging to population-level effects. Environ. Toxicol. Chem. 30(1), 64-76.

Länge, R., Hutchinson, T.H., Croudace, C.P., Siegmund, F., Schweinfurth, H., Hampe, P., Panter, G.H., Sumpter, J.P. 2001. Effects of the synthetic estrogen 17α-ethinylestrdiol on the life-cycle of the Fathead Minnow (*Pimephales promelas*). Environ. Toxicol. Chem. 20(6), 1216-1227.

Lange, A., Paull, G.C., Hamiton, P.B., Iguschi, T., Tyler, C.R. 2011. Implications of persistent exposure to treated wastewater effluent for breeding in wild Roach (*Rutilis rutilis*) populations. Environ. Sci. Tech. 45, 1673-1679.

Lee, H.-B., Peart. T.E. 1995. Determination of 4-nonylphenol in effluent and sludge from sewage treatment plants. Anal. Chem. 67, 1976-1980.

Lee, H.B., T.E. Peart, Chan, J., Gris, G. 2004. Occurrence of endocrine-disrupting chemicals in sewage and sludge samples in Toronto, Canada. Water Qual. Res. J. Can. 39(1), 57-63.

Lee, H.B., T.E. Peart and M.L. Svoboda. 2005. Determination of endocrine-disrupting phenols, acidic pharmaceuticals, and personal-care products in sewage by solid-phase extraction and gas chromatography-mass spectrometry. J. Chromatogr. A. 1094, 122-129.

Lee, H.-B., Sarafin, K., Peart. T.E. 2007. Determination of β-blockers and β_2-agonist in sewage by solid-phase extraction and liquid chromatography-tandem mass spectrometry. J. Chromatogr. A. 1148, 158-167.

Lishman, L., Smyth, S.A., Sarafin, K., Kleywegt, S., Toito, J., Peart, T., Lee, B., Servos, M., Beland, M., Seto, P. 2006. Occurrence and reductions of pharmaceuticals and personal care products and estrogens by municipal wastewater treatment plants in Ontario, Canada. Sci. Total Environ. 367, 544-558.

Loomer, H.A. 2008. The dynamics of carbon and nitrogen stable isotope signatures of aquatic food webs in the Grand River watershed. M.Sc. Thesis in Biology. University of Waterloo, Waterloo, Ontario, Canada.

Marentette, J.R., Gooderham, K.L. McMaster, M.E., Ng, T. Parrott, J.L., Wilson, J.Y., Wood, C.M., Balshine, S. 2010. Signatures of contamination in invasive Round Gobies (*Neogobius melanostomus*): A double strike for ecosystem health? Ecotox. Environ. Saf. 73, 1755-1764.

McMaster, M.E., Munkittrick, K.R., Jardine, J.J., Robinson, R.D., Van Der Kraak, G.J. 1995a. Protocol for measuring in vitro steroid production by fish gonadal tissue. Can. Tech. Rep. Fish. Aquat. Sci. 1961. pp. 78.

McMaster, M.E., VanDerKraak, G.J., Munkittrick, K.R. 1995b. Exposure to bleached kraft pulp mill effluent reduces the steroid biosynthetic capacity of White Sucker ovarian follicles. Comp. Bio. Phys. C- Pharm. Tox. Endocrin. 112(2), 169-178.

McMaster, M.E., VanDerKraak, G.J., Munkittrick, K.R. 1996. An epidemiological evaluation of the biochemical basis for steroid hormonal depressions in fish exposed to industrial wastes. J. Great Lakes Res. 22(2), 153-171.

McMaster, M.E., Frank, M., Munkittrick, K., Riffon, R., Wood, C. 2002. Follow-up studies addressing questions identified during cycle 1 of the adult fish survey of the pulp and paper EEM program. Wat. Qual. Res. J. Can. 37, 133-153.

McMaster, M.E., Hewitt, L.M., Tetreault, G.R., Janoscik, T., Boyco, C., Peters, L., Parrott, J.L., Van der Kraak, G.J., Portt, C.B., Kroll, K.J., Denslow, N.D. 2005. Detailed endocrine assessments of wild fish in the Northern River Basins, Alberta, in comparison to EEM monitored endpoints. Wat. Qual. Res. J. Can. 40(3), 299-314.

Metcalf & Eddy Inc., Tchobanoglous, G., Burton, F., Stensel, H.D. 2004. Wastewater engineering treatment and reuse. Fourth Edition. Published by McGraw-Hill Science Engineering pp. 1,819.

Metcalfe, C.D., Miao, X.S., Koenig, B.G., Struger, J. 2003. Distribution of acidic and neutral drugs in surface waters near sewage treatment plants in the lower Great Lakes, Canada. Environ. Toxicol. Chem. 22(12), 2881-2889.

Metcalfe, C.D., Chu, S., Judt, C., Li, H., Oakes, K., Servos, M., Andrews, D. 2009. Antidepressants and their metabolites in municipal wastewater, and downstream exposure in an urban watershed. Environ. Toxicol. Chem. 29, 79-89.

Mikaelian, I., De Lafontaine, Y., Harshbarger, J.C., Lee, L.J., Martineau, D. 2002. Health of Lake Whitefish (*Coregonus clupeaformis*) with elevated tissue levels of environmental contaminants. Environ. Toxicol. Chem. 21(3), 532-541.

Miller, R.V. 1968. A Systematic Study of the Greenside Darter, *Etheostoma blennioides* Rafinesque (Pisces: Percidae). Copeia 1968(1), 1-40.

Minier, C., Caltot, G., Leboulanger, F., Hill, E.M. 2000. An investigation of the incidence of intersex fish in Seine-Maritime and Sussex regions. Analusis 28(9), 801-806.

Mobrand, L.E., Lichatowich, J.A., Lestelle, L.C., Vogel, T.S. 1997. An approach to describing ecosystem performance "through the eyes of salmon". Environ. Toxicol. Chem. 54(12), 2964-2973.

Mohapatra, D.P., Brar, S.K., Tyagi, R.D., Surampalli, R.Y. 2011. Occurrence of bisphenol-A in wastewater and wastewater sludge of CUQ treatment plant. J. Xenobiotics 1, 9-16.

Munkittrick, K.R., Portt, C.B., Van Der Kraak, G.J., Smith, I.R., Rokosh, D.A. 1991. Impact of Bleached Kraft Mill Effluent on population characteristics, liver MFO activity, and serum steroid-Levels of a Lake Superior White Sucker (*Catostomus commersoni*) population. Can. J. Fish. Aquat. Sci. 48, 1371-1380.

Munkittrick, K.R., Van Der Kraak, G.J., McMaster, M.E., Portt, C.B. 1992. Response of hepatic MFO activity and plasma sex steroids to secondary-treatment of bleached kraft pulp-mill effluent and mill shutdown. Environ. Toxicol. Chem. 11, 1427-1439.

Munkittrick, K.R., McMaster, M.E., Van Der Kraak, G.J., Portt, C.B., Gibbons, W.N., Farwell A., Gray, M. 2000. Development of methods for effects-driven cumulative effects assessment using fish populations: Moose River Project. SETAC Press Ed. By C.G. Ingersoll. ISBN 1-880611-41-4. pp. 236.

Nero, V., Farwell, A., Lee, L.E.J., Van Meer, T., MacKinnon, M.D., Dixon, G.D. 2006. The effects of salinity on naphthenic acid toxicity to Yellow Perch: Gill and liver histopathology. Ecotoxicol. Environ. Saf. 65, 252-264.

Palace, V.P., Evans, R.E., Wautier, K., Baron, C., Vandenbyllardt, L., Vandersteen, W., Kidd, K. 2002. Induction of vitellogenin and histological effects in wild Fathead Minnows from a lake experimentally treated with the synthetic estrogen, ethynylestradiol. Wat. Qual. Res. J. Can. 37(3), 637-650.

Palace, V.P., Wautier, K., Evans, R.E., Blanchfield, P.J., Mills, K.H., Chalanchuk, S.M., Godard, D., McMaster, M.E., Tetreault, G.R., Peters, L.E., Vandenbyllardt, L., Kidd, K. 2006. Biochemical and histopathological effects in Pearl Dace (*Margariscus margarita*) chronically exposed to a synthetic estrogen in a whole lake experiment. Environ. Toxicol. Chem. 25(4), 1114-1125.

Parrott, J.L., Wood, C.S., Boutout, P., Dunn, S. 2003. Changes in growth and secondary sex characteristics of Fathead Minnows exposed to bleached sulfite mill effluent. Environ. Toxicol. Chem. 22(12), 2908-2915.

Person-Le Ruyet, J., Galland, R., Le Roux, A., Chartois, H. 1997. Chronic ammonia toxicity in juvenile Turbot (*Scophthalmus maximus*). Aquaculture 154, 155-171.

Porter, C.M., Janz, D.M. 2003. Treated municipal sewage discharge affects multiple levels of biological organization in fish. Ecotoxicol. Environ. Saf. 54, 199-206.

Purdom, C., Hardiman, P., Bye, V., Eno, N., Tyler, C., Sumpter, J. 1994. Estrogenic effects of effluents from sewage treatment works. Chem. Ecol. 8, 275–285.

Ra, J.S., Kim, S.D., Chang, N.I., An, K.G. 2007. Ecological health assessments based on whole effluent toxicity tests and the index of biological integrity in temperate streams influenced by wastewater treatment plant effluents. Environ. Toxicol. Chem. 26, 2010-2018.

Randall, D.J., Wright, P.A. 1987. Ammonia distribution and excretion in fish. Fish Phys. Biochem. 3(3), 107-120.

Rosamond, M.S., Thuss, S.J., Schiff, S.L., Elgood, R.J. 2011. Coupled cycles of dissolved oxygen and nitrous oxide in rivers along a trophic gradient in southern Ontario, Canada. J. Environ. Qual. 40, 256-270.

Shang, E., Yu, R.M.K, Wu, R.S.S., 2006. Hypoxia affects sex differentiation and development, leading to a male-dominated population in Zebrafish (*Danio rerio*). Environ. Sci. Technol. 40, 3118-3122.

Scott, W.B., Crossman, E.J. 1998. Freshwater fishes of Canada. Oakville: Galt House Publications Ltd. pp. 966.

Servos, M.R. 1999. Review of the aquatic toxicity, estrogenic responses and bioaccumulation of alkylphenol and alkylphenol polyethoxylates. Wat. Qual. Res. J. Canada 34, 123-177.

Servos, M.R., Maguire, R.J., Bennie, D.T., Lee, H.B., Cureton, P.M., Davidson, N., Sutcliffe, R., Rawn, D.F.K. 2003. An ecological risk assessment of nonylphenol and its ethoxylates in the aquatic environment. Human Ecol. Risk Ass. 9(2), 569-587.

Servos, M.R., Bennie, D.T., Burnison, B.K., Jurkovic, A., McInnis, R., Neheli, T., Schnell, A., Seto, P., Smyth, S.A., Ternes, T.A. 2005. Distribution of estrogens, 17β-estradiol and estrone, in Canadian municipal wastewater treatment plants. Sci. Tot. Environ. 336(1-3), 155-170.

Soderberg, R.W., McGee, M.W., Boyd, C.E. 1984. Histology of cultured Channel Catfish, *Ictalurus punctatus* (Rafinesque). J. Fish Bio. 24, 683-690.

Sole, M., Barcelo, D., Porte, C. 2002. Seasonal variation of plasmatic and hepatic vitellogenin and EROD activity in Carp, *Cyprinus carpio*, in relation to sewage treatment plants. Aqua. Toxicol. 60, 233-248.

Sole, M., Raldua, D., Piferrer, F., Barcelo, D., Porte, C. 2003. Feminization of wild Carp, *Cyprinus carpio*, in a polluted environment: plasma steroid hormones, gonadal morphology and xenobiotic metabolizing system. Comp. Bio. Phys. C- Pharm. Tox. Endocrin. 136, 145-156.

Sumpter, J.P., Jobling, S. 1995. Vitellogenesis as a biomarker for estrogenic contamination of the aquatic environment. Environ Health Persp. 103(Suppl.7), 173-178.

Sumpter, J.P., Johnson, A.C. 2005. Lessons from endocrine disruption and their application to other issues concerning trace organics in the aquatic environment. Environ. Sci. Technol. 39(12), 4321-4332.

SYSTAT, I. 2007. SYSTAT 12.0: The System for Statistics. Evanson, IL, USA.

Tanna, R.N. 2012. Association of intersex in wild fish with wastewater effluent in the Grand River, Ontario. M.Sc. Thesis. University of Waterloo, Waterloo, Ontario, Canada.

Taylor, C.A., Knouft, J.H., Hiland, T.M. 2001. Consequences of stream impoundment on fish communities in a small North American drainage. Regul. Rivers Res. Manage. 17, 687-698.

Ternes, T.A. 1998. Occurrence of drugs in German sewage treatment plants and rivers. Wat. Res. 32(11), 3245-3260.

Ternes, T.A., Stumpf, M., Mueller, J., Haberer, K., Wilken, R.-D., Servos, M. 1999. Behavior and occurrence of estrogens in municipal sewage treatment plants -I. Investigations in Germany, Canada and Brazil. Sci. Total Environ. 225, 81-90.

Tetreault, G.R. 2003. Monitoring the aquatic environment in the Athabasca Oil Sands using reproductive responses in small-bodied fish. M.Sc. Thesis. University of Waterloo, Waterloo, Ontario, Canada.

Tetreault, G.R., McMaster, M.E., Dixon, D.G., Parrott, J.L. 2003. Using reproductive endpoints in small forage fish species to evaluate the effects of Athabasca Oil Sands activities. Envinron. Toxicol. Chem. 22, 2775-2782.

Tetreault, G.R., Bennett, C.J., Shires, K., Knight, B., Servos, M.R., McMaster, M.E. 2011. Intersex and reproductive impairment of two species wild fish exposed to multiple municipal wastewater discharges. Aquat. Tox. 104, 278-290.

Thomas, P., Rahman, S., Khan, I.A., Kummer, J.A. 2007. Widespread endocrine disruption and reproductive impairment in an estuarine fish population exposed to seasonal hypoxia. Proc. R. Soc. B 274, 2693-2701.

Tsai, C.-f. 1975. Effects of sewage treatment plant effluents on fish: a review of literature, Smithsonian Institution and Virginia Institute of Marine Science, Chesapeake Research Consortium Incorporated. pp. 238.

Tyler, C.R., Eerden, v.d., Jobling, S., Panter, G., Sumpter, J.P. 1996. Measurement of vitellogenin, a biomarker for exposure to oestrogenic chemicals, in a wide variety of cyprinid fish. J. Comp Phys. B 166(7), 418-426.

Vajda, A.M., Barber, L.B., Gray, J.L., Lopez, E.M., Woodling, J.D., Norris, D.O. 2008. Reproductive disruption in fish downstream from an estrogenic wastewater effluent. Environm Sci. Tech. 42(9), 3407-3414.

Vajda, A.M., Barber, L.B., Gray, J.L., Lopez, E.M., Bolden, A.M., Schoenfuss, H.L., Norris, D.O. 2011. Demasculinization of male fish by wastewater treatment plant effluent. Aquat. Tox. 103(3-4), 213-221.

van Aerle, R., Nolan, M., Jobling, S., Christiansen, L.B., Sumpter, J.P., Tyler, C.R. 2001. Sexual disruption in a second species of wild cyprinid fish (the Gudgeion, *Gobio gobio*) in United Kingdom freshwaters. Envinron. Toxicol. Chem. 20(12), 2841-2847.

Van Der Kraak, G.J., Munkittrick, K.R., McMaster, M.E., Portt, C.B., Chang, J.P. 1992. Exposure to bleached kraft pulp-mill effluent disrupts the pituitary-gonadal axis of White Sucker at multiple sites. Tox. Appl. Pharma. 115(2), 224-233.

Vethaak, A.D., Larh, J., Kuiper, R.V., Grinwis, G.C.M., Rankouhi, T.R., Giesy, J.P., Gerritsen, A. 2002. Estrogenic effects in fish in The Netherlands: some preliminary results. Toxicol. 181-182, 147-150.

Viganò, L., Arillo, A.B., S., Massari, A., Mandich, A. 2001. First observation of intersex cyprinids in the Po River (Italy). Sci. Tot. Environ. 269, 189-194.

Vine, E., Shears, J., Van Aerle, R., Tyler, C.R., Sumpter, J.P. 2005. Endocrine (sexual) disruption is not a prominent feature in the pike (*Esox lucius*), a top predator, living in English waters. Envinron. Toxicol. Chem. 24(6), 1436-1443.

Waiser, M.J., Tumber, V., Holm, J. 2011a. Effluent-dominated streams. Part 1: presence and effects of excess nitrogen and phosphorus in Wascana Creek, Saskatchewan, Canada. Envinron. Toxicol. Chem. 30(2), 496–507.

Waiser, M.J., Humphries, D., Tumber, V., Holm, J. 2011b. Effluent-dominated streams. Part 2: presence and possible effects of pharmaceuticals and personal care products in Wascana Creek, Saskatchewan, Canada. Envinron. Toxicol. Chem. 30(2), 508-519.

Walker, C.H., Hopkin, S.P., Sibly, R.M., Peakall, D.B. 2001. Principals of ecotoxicology. Second ed. Taylor & Francis, pp. 309.

Wichert, G.A. 1995. Effects of sewage effluent remediation and urbanization on fish associations of Toronto streams. North Amer. J. Fish. Manag. 15, 440-456.

Wichert, G.A., Rapport, D.J. 1998. Fish community structure as a measure of degradation and rehabilitation of riparian systems in an agricultural drainage basin. Environ. Manage. 22(3), 425-443.

Woodling, J.D., Lopez, E.M., Maldonado, T.A., Norris, D.O., Vajda, A.M. 2006. Intersex and other reproductive disruption of fish in wastewater effluent dominated Colorado streams. Comp. Bio. Phys. C- Pharm.Tox. End. 144, 10-15.

Yeom, D.H., Lee, S.A., Kang, G.S., Seo, J., Lee, S.K. 2007. Stressor identification and health assessment of fish exposed to wastewater effluents in Miho Stream, South Korea. Chemosphere 67, 2282-2292.

Zarrogain, G., Gardner, G., Borsay Horowitz, D., Gutjahr-Gobell, R., Haebler, R., Mills, L. 2001. Effect of 17β-estradiol, o,p'-DDT, octylphenol and p,p'-DDE on gonadal development and liver and kidney pathology in juvenile male summer Flounder (*Paralichthys dentatus*). Aquat. Toxicol. 54, 101-112.

Appendix A

Supporting Information for Chapter 5

Table S1. Fish fauna Tolerance, Resiliency, Vulnerability, of fish collected along an urban gradient in the Grand River in the September of 2007 and 2008, and November of 2008. Tolerance (S = sensitive, I = Intermediate, T = Tolerant) is the ability of a species to adapt to disturbance and stress; Resiliency is the ability to withstand exploitation (doubling time), period of time of the population to double based on fecundity, age to maturation and longevity; Vulnerability is predisposition to predation/collection.

Family	Common name	Species	Tolerance	Resiliency	Vulnerability
Percidae	Blackside Darter	*Percina maculata*	I	I	L
	Yellow Perch	*Perca flavescens*	T	L	H
	Johnny Darter	*Etheostoma nigrum*	I	I	L
	Fantail Darter	*Etheostoma flabellare*	I	I	I
	Greenside Darter	*Etheostoma blennioides*	I	I	I
	Rainbow Darter	*Etheostoma caeruleum*	S	H	L
Catostomidae	White Sucker	*Catostomus commersonii*	T	L	H
	Northern Hog Sucker	*Hypentelium nigricans*	S	H	L
	Greater Redhorse	*Moxostoma valenciennesi*	S	L	H
	Golden Redhorse	*Moxostoma erythrurum*	I	L	H
Centrarchidae	Bluegill	*Lepomis macrochirus*	I	I	I
	Pumpkinseed	*Lepomis gibbosus*	I	I	I
	Rock Bass	*Ambloplites rupestris*	I	I	I
	Smallmouth Bass	*Micropterus dolomieu*	I	I	I
	Largemouth Bass	*Micropterus salmoides*	T	L	I
Cyprinidae	Blacknose Shiner	*Notropis heterolepis*	S	H	L
	Rosyface Shiner	*Notropis rubellus*	S	H	L
	Silver Shiner	*Notropis photogenis*	S	H	L
	Common Shiner	*Luxilus cornutus frontalis*	I	I	L
	Spottail Shiner	*Notropis hudsonius*	I	I	L
	Longnose Dace	*Rhinichthys cataractae*	I	I	I
	Hornyhead Chub	*Nocomis biguttatus*	I	I	I
	Blacknose Dace	*Rhinichthys obtusus*	I	H	L
	Striped Shiner	*Luxilux chrysocephalus*	T	I	L
	Brassy Minnow	*Hybognathus hankinsoni*	T	I	L
	Bluntnose Minnow	*Pimephales notatus*	T	I	I
	Creek Chub	*Semotilus atromaculatus*	T	I	I
	Common Carp	*Cyprinus carpio*	T	I	H
Cottidae	Mottled Sculpin	*Cottus bairdii*	I	L	I
Ictaluridae	Stonecat	*Noturus flavus*	I	I	I
Escocidae	Northern Pike	*Esox lucius*	I	L	H
Umbridae	Central Mudminnow	*Umbra limi*	T	I	L