Environmental Science & Technology

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Environ. Sci. Technol., **Just Accepted Manuscript** • DOI: 10.1021/acs.est.6b05370 • Publication Date (Web): 27 Dec 2016

ACS Publications

Environmental Science & Technology is published by the American Chemical Society. 1155 Sixteenth Street N.W., Washington, DC 20036

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

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Abstract

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

1 Introduction

Feminization of male fish in association with municipal wastewater treatment plant (MWWTP) outfalls has been reported on a global scale.[1-4] Intersex (ova-testis) has been one of the most commonly reported effects observed in male fish downstream of MWWTPs.[5] This is concerning as severe cases of intersex have been associated with reduced reproductive success.[6-8] The feminization of male fish has been associated with compounds such as natural hormones (17ß-estradiol (E2) and estrone (E1)), synthetic estrogen (17α-ethynylestradiol (EE2)),[9] and industrialized products that mimic estrogens, including alkylphenolic chemicals,[10] which are routinely measureable in MWWTP effluents. Recent studies have suggested that additional compounds may also cause feminization of male fish, possibly through other pathways, including those with anti-androgenic activity.[11] EE2 added to a whole lake at an environmentally relevant concentration (~5 ng/L) resulted in near extirpation of a fish population[12] followed by changes to the whole ecosystem.[13] Changes in reproductive endpoints, including intersex in male fish, were observed in multiple species in this whole lake experiment.[12, 14] Thus, intersex is a prevalent and a biologically relevant marker of exposure to endocrine-disrupting compounds (EDCs) in MWWTP outfalls.

To protect aquatic species from potential deleterious effects of EDCs, it is necessary to reduce their exposure. The removal of compounds with estrogenic properties from MWWTPs depends on the plants' operational processes.[15-17] Enhanced wastewater treatment, including nitrifying activated sludge, has been shown to be effective at reducing the estrogenicity (natural and synthetic estrogens) of the final effluent and the associated endocrine disruption in fish exposed to it under laboratory conditions[18, 19] or in fish caged in the effluent outfalls.[20] However, it is difficult to extrapolate the findings from these studies to the recovery of free-living fish as these studies are typically short (do not cover the entire life cycle), and laboratory studies do not accurately reflect associated environmental conditions. Although numerous studies have documented endocrine responses in fish exposed to MWWTP outfalls, to our knowledge, no studies have documented the recovery of reproductive endpoints in free-living fish in receiving waters in response to MWWTP upgrades.

The Grand River watershed in southern Ontario, Canada, is the largest watershed that drains into Lake Erie. The area has a growing population of nearly 1 million people.[21] The rainbow darter (*Etheostoma caeruleum*), a native species of southern Ontario, has been studied extensively along a 60 km section of the central Grand River that includes two major MWWTP outfalls, Kitchener and Waterloo (Figure 1).[2, 6, 22-25] This sentinel species was selected for several reasons: they are highly abundant, are gonochoristic and sexually dimorphic, are short lived (5 years),[26] and are thought to have limited mobility.[2] The rainbow darter grow rapidly in their first summer and are sexually mature at 1 year of age.[46] They spawn asynchronously, with females laying multiple egg clutches each spring. Multiple studies indicated that the male rainbow darter in close proximity to the Waterloo and Kitchener MWWTPs were being feminized, with evidence of altered gene expression, vitellogenin induction,[23, 24] reduced steroid production,[2] and reduced gonad size.[2] The most consistent effect observed across multiple years and seasons was intersex in the male rainbow darter, with up to 100% incidences at sites below the Kitchener MWWTP.[2, 6, 22, 25, 27] High intersex severity scores were associated with the Kitchener effluent outfall, including many cases where the gonad had greater than 50% ovarian tissue,[6, 22] with instances of macroscopic intersex (e.g., Figure 2). Severe cases of intersex in the male rainbow darter were previously associated with altered gene expression[24] and reduced fertilization success.[6]

Major infrastructure upgrades were implemented at the Kitchener MWWTP to improve treatment efficiency and effluent quality. These included converting the plant from carbonaceous activated sludge (primarily the removal of biological oxygen demand (BOD)) to nitrifying activated sludge to enhance the removal of ammonia. Upgrades were initiated in mid-2012, and full nitrification was achieved by early 2013.[28] Although upgrades at the Waterloo MWWTP were planned during this period, construction delays at this MWWTP resulted in only minimal treatment changes and poor effluent quality over the course of the study period.[29]

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

2 MATERIALS AND METHODS

2.1 Description of sites

Nine sites in close proximity to the Kitchener and Waterloo MWWTP outfalls were sampled in multiple years between 2007 and 2015 in spring and/or fall seasons (Figure 1). Sites were selected to represent similar riffle/run habitats. Two upstream non-urban reference sites

(REF 1 and REF 2) and one urbanized reference site (REF 3) were included in addition to one near-field exposure site downstream of the Waterloo MWWTP (DSW 1) and two sites located farther downstream (INT 1 and INT 2), but upstream of the Kitchener outfall. Three sites were sampled downstream of the Kitchener MWWTP outfall (DSK 1, DSK 2, and DSK 3). The exact location (GPS coordinates) and distances between sites are provided in Table S1.

2.2 Effluent characterization and river water quality

Data for traditional effluent quality parameters including monthly total ammonia, nitrate, total Kjeldahl nitrogen (TKN), BOD, total phosphorous (TP), and total suspended solids (TSS) were provided by the Region of Waterloo for the Waterloo and Kitchener MWWTPs for the duration of the study (2007–2015). Effluent quality was also assessed on the basis of the removal of select pharmaceuticals and total estrogenicity at the Waterloo MWWTP (2010–2015) and the Kitchener MWWTP before (2010 to July 2012), during (August 2012 to January 2013), and after the upgrades (2013–2015). Effluent samples from both the Kitchener and Waterloo MWWTPs were collected, preserved, extracted, and analyzed for three pharmaceuticals (ibuprofen, naproxen, and carbamazepine) following the protocols outlined in Arlos et al.[31] The yeast estrogen screen (YES) assay was used to assess total estrogenicity quantified in estradiol equivalence (E2eq) following the method described by Arlos et al.[32] Dissolved oxygen (DO) concentrations in river water below the Kitchener MWWTP were provided by the Grand River Conservation Authority.

2.1 Fish collection and processing

Historical data on rainbow darter in proximity to the Kitchener and Waterloo MWWTPs before the Kitchener upgrades (collected between 2007 and 2012) were included in several earlier studies.[2, 22, 23, 25, 26] Fuzzen et al.[6] reported on the first post-upgrade data set; these data were collected in spring 2013, 9 months after the Kitchener MWWTP upgrades. Rainbow darter were sampled again in the fall of 2013, 2014, and 2015 and in the spring of 2015 at the same sites and in the same manner. Briefly, rainbow darter were collected in riffle/run habitats at the selected sites by backpack electrofishing (Smith Root LR-24). A target sample size of 20 males and 20 females was established to collect fish to analyze all sampling endpoints, including intersex, somatic indices, and additional endpoints for other research studies. For each sampling event, rainbow darter were held in well-aerated buckets until they were sampled on site in a portable laboratory. Fish were rendered unconscious by concussion and then euthanized by spinal severance. Fish total length (± 0.1 cm), weight (± 0.01 g), gonad weight (± 0.001 g) and liver weight (± 0.001 g) were recorded. A single testis lobe from a subset of the male rainbow darter was transferred to Davidson's solution for 48 hours and stored in 70% ethanol before being processed for histology. All fish were handled in accordance with the approved University of Waterloo animal care protocols (AUPP# 10-17 and 14-15).

2.2 Histology

Gonad tissues were dehydrated and embedded in paraffin wax. Embedded samples were microtomed at a thickness of 5 µm, put on slides with slide mount, and stained with hematoxylin and eosin. A minimum of 40 sections per fish were scanned for intersex at 100x magnification using a Leica DM100 light microscope. Two parameters were calculated for each site at each sample date. The first parameter was intersex incidence, which is the percentage of male rainbow darter with intersex (based on presence or absence of oocytes). The second parameter was intersex severity, which was based on the scoring index adopted from Bahamonde et al.[22] using the number and development stage of oocytes in addition to the proportion of ovarian tissue to testicular tissue. From 2007 to 2015, an average of 18 male rainbow darter were sampled each year for intersex incidence and severity but this number ranged from 5 to 68 depending on the availability of archived samples (Table S2).

2.3 Data analysis and statistics

A BACI (before-after control-impact) design was used to test for differences in intersex incidence and severity between upstream and downstream sites before and after the upgrades [two-way ANOVA with factors site (upstream vs. downstream) and period (pre-upgrade vs. post-upgrade) with year as the replicate]. This was completed separately for each combination of downstream site and a single reference site (REF 3), where there were at least 3 years pre-upgrade and 3 years post-upgrade. The third reference site (REF 3) was selected because it is the only reference in the urbanized region above both the Waterloo and Kitchener MWWTPs. This resulted in four two-way ANOVAs, one for intersex incidence and one for severity for each of the following pairs (fall data only): DSK 1 versus REF 3 and DSK 2 versus REF 3. For the remaining sites in the fall and for all of the spring collections, differences in intersex incidence and severity among years within sites were tested. Differences in intersex incidence were tested with the Fisher's exact test, while differences in intersex severity were tested with the Kruskal–Wallis test with Dunn's pairwise comparison (with individual fish as replicates). The relationship between intersex incidence and severity across all seasons and years was assessed with linear regression. Fish body weight, total length, liver weight, and gonad weight were used to calculate condition factor (k = body weight/length^3 x 100), gonadosomatic index (GSI = gonad weight/body weight x 100), and liver somatic index (LSI = liver weight/body weight x 100). These data are provided to support interpretation of the intersex and were not compared statistically (Figure S1). Annual changes in effluent nutrient concentrations (ammonia and nitrate) at both MWWTPs (Kitchener and Waterloo) as well as river DO concentrations were assessed with a Kruskal–Wallis test with Dunn's pairwise comparison. Changes in pharmaceuticals and E2eq across different time points were also assessed for both the Waterloo and Kitchener effluent using one-way ANOVAs with Tukey's pairwise comparisons. Pharmaceutical and E2eq data from the Kitchener WWTP were pooled into three categories: pre-upgrade, during upgrades, and post-upgrade. All data were plotted and tested with SigmaPlot version 13 using $\alpha < 0.05$.

3 Results and discussion

3.1 Effluent characterization before and after MWWTP upgrades

Before the upgrades (2007–2012), the Kitchener MWWTP lacked nitrification primarily because of inefficient aeration and short solids retention time (SRT; < 2 d).[29] The upgrades, which included more efficient aeration and higher SRT (> 5 d), significantly improved the removal of ammonia, resulting in a decrease in the median annual ammonia concentration from 25 mg/L to 2–6 mg/L in post-upgrade years (Figure 3). In contrast, ammonia concentrations in the Waterloo MWWTP increased over the course of the study, with concentrations in 2013 reaching levels similar to those in the Kitchener MWWTP before the upgrades (Figure 3). A partial upgrade at Waterloo was implemented in 2014 to treat the centrate (elevated in ammonia), derived from the centrifugation of the biosolids;[29] however, full nitrification was not achieved and ammonia concentrations remained high in the final effluent (> 20 mg/L; Figure 3). The 2014 upgrade also resulted in a decrease in both BOD and TSS (Table S3). Nitrate at both MWWTPs was inversely related to ammonia concentrations and was a good indicator of the degree of nitrification.

Additional effluent characterizations included the measurement of select pharmaceuticals (indicators of treatment quality) and total estrogenicity (in E2eq) (Figure 4). Before the upgrades, when nitrification was lacking at the Kitchener MWWTP, both ibuprofen (IBU) and naproxen (NPX) concentrations were significantly higher than after the implementation of nitrification (IBU: one-way ANOVA, F = 20.2, df = 32, $p < 0.001$; NPX: one-way ANOVA, F = 10.5, df = 32, $p < 0.001$). This was not surprising, as these compounds have high biotransformation potential.[33] IBU concentrations were up to 135 fold higher and NPX concentrations were up to 20 fold higher before the upgrades. In contrast, compounds that have low biotransformation potential and low sorption rates onto solids typically have slow removal rates, and more advanced treatment is needed to achieve removal.[33] Therefore, it is not surprising that carbamazepine was more persistent and not affected by the upgrades (Figure 4; one-way ANOVA, F = 3.0, df = 32, $p = 0.08$). The pattern of pharmaceuticals at the Waterloo MWWTP was variable and reflected the lack of nitrification over the years (Figure 4). This study is consistent with previous studies that have demonstrated that nitrification and extended SRT are associated with greater removal of pharmaceuticals.[33, 34]

Total effluent estrogenicity (E2eq) was also assessed at both MWWTPs. At the Kitchener MWWTP, there was a significant reduction from 18 ng/L E2eq before the upgrades to < 2 ng/L E2eq (one-way ANOVA, F = 17.6, df = 20, $p = < 0.001$) in post-upgrade years. These values are similar to those in other studies that have quantified E2eq in secondary treated effluent.[19, 35, 36] Although the reduced estrogenicity is probably associated with the changes in effluent treatment, influent was not measured during the study so a change in the source cannot be ruled out. The population was increasing over the years of the study so MWWTP inputs were probably increasing (Table S3). The E2eq at Waterloo was usually lower than at Kitchener in pre-upgrade years (Figure 4).

Natural and synthetic estrogens could have contributed to E2eq in the effluents.[9] An attempt was made to quantify E1, E2, and EE2 in this study; however, matrix effects resulted in the data failing quality assurance, probably because of low selectivity (unit resolution) in the LS-MS/MS method. Nitrifying activated sludge have been shown to be associated with the removal of estrogenic compounds (including E1, E2, and EE2) with 90–99% efficiency.[15, 16, 37] This has mainly been attributed to biodegradation processes,[38] which are favourable under nitrifying conditions.[33] It has also been shown that the longer the solids retention time, the greater the removal of estrogenic compounds: an SRT of > 5 d is typically associated with enhanced removal.[17, 39] The higher aeration and SRT (going from < 2 d to > 5 d) at the Kitchener MWWTP, which resulted in nitrifying conditions after the upgrades, is probably also contributing to a more diverse biological community in the treatment system and therefore a reduction in many contaminants as well as in total estrogenicity in the final effluent.

3.2 Intersex before and after MWWTP upgrades

The main objective of this study was to evaluate whether the high occurrence (70–100%) of observed intersex in the wild male rainbow darter downstream of MWWTPs would be reduced following major infrastructure upgrades to improve effluent quality. The implementation of nitrification at the Kitchener MWWTP corresponded with a distinct decrease in the incidence and severity of intersex in wild rainbow darter (Figure 5). At the second downstream site (DSK 2), intersex incidence had already decreased from 100% (in fall 2012) to 29% (a 71% reduction) in the first fall season (2013) after the upgrades. In contrast, the decrease at the first site immediately downstream of the Kitchener MWWTP (DSK 1) was more gradual from 2013 to

2015. By the third fall season after the upgrades (2015), intersex incidence had decreased to 9% (DSK 1) and 14% (DSK 2). Similarly, intersex severity scores also decreased gradually in post-upgrade years downstream of the Kitchener MWWTP. The mean intersex score at DSK 1 and DSK 2 before the upgrades ranged from two to three, with maximum scores of six (including visible eggs). By fall 2015 (3 years post-upgrade), the mean intersex scores were less than one, the lowest mean score recorded at these sites below the Kitchener MWWTP outfall since these studies began in 2007. The decrease in intersex in post-upgrade years in the fall was also supported by spring data collections, where intersex incidence and severity was at its lowest in spring 2015 at all three sites below the Kitchener MWWTP (Figure S2). Supporting statistics for comparing years within sites for both intersex incidence and severity are provided in Tables S5 (fall) and S6 (spring).

A BACI analysis was used to assess whether sites below the Kitchener MWWTP (DSK 1 and DSK 2) returned to reference conditions after the upgrades. The analyses revealed significant interaction between factors (upstream vs. downstream x pre-upgrade vs. post-upgrade). For the test between DSK 2 (second site downstream of the Kitchener MWWTP) and REF 3, pairwise comparisons for the interactions revealed significant differences in intersex incidence before the upgrades ($p < 0.001$) but not after ($p = 0.226$). The finding was similar for intersex severity, indicating that both intersex incidence and severity at DSK 2 are returning to reference conditions. The test for DSK 1 (first site below the Kitchener MWWTP) and REF 3 also revealed a significant difference in intersex severity before the upgrades ($p < 0.001$) but not after ($p = 0.129$), thus also indicating that this site is returning to reference conditions. Interestingly, there was a difference in intersex incidence between DSK 1 and REF 3 both before ($p < 0.001$) and after the upgrades ($p < 0.034$), possibly indicating that intersex incidence at DSK 1 is taking longer to recover than intersex severity. Intersex incidence was lower at DSK 1 in post-upgrade years than in pre-upgrade years (p = 0.001); however, it might be that intersex incidence was taking longer to recover than intersex severity. This would not be surprising, since with decreasing exposure, severity could be decreasing more rapidly than incidence. It is interesting to note that across all sites, years, and seasons, intersex incidence was positively correlated with severity (r^2 = 0.88; df = 82, $p < 0.001$). Additional supporting statistics for the two-way ANOVAs (BACI analysis) are provided in Table S7–S10.

Mean severity scores at the furthest downstream site (DSK 3) were highly variable among the years, but by fall 2015, it was at its lowest ever reported, with a maximum score of two (Figure 5). This site is approximately 5 km downstream from the Kitchener MWWTP outfall, where the effluent would be more evenly distributed and diluted across the river.[40] Although intersex incidence was slightly elevated at this site relative to the immediate upstream reference site (INT 2), intersex severity was similar to that at the sites below Waterloo (DSW 1, INT 1) and never as severe as at the sites immediately below the Kitchener outfall.

Intersex below the Waterloo MWWTP occurred less frequently and was less severe than at the sites below the Kitchener MWWTP throughout the study period (Figure 5). Intersex incidence ranged from 7 to 40% with no significant differences among years in either the spring (Fisher's exact, p = 0.237) or fall (Fisher's exact, p = 0.204). Similarly, intersex severity did not differ among years in either the spring (Kruskal–Wallis, H = 3.203, p = 0.202) or fall (Kruskal–Wallis, H = 5.596, p = 0.347), where the mean scores were consistently less than one every year. The maximum severity score reported at this site was four; however, this score was infrequently observed. An additional site located 12 km downstream of the Waterloo outfall (INT 1) that was sampled less frequently beginning in 2013 had similar trends to DSW 1, with intersex incidence ranging from 12 to 33%. The Waterloo MWWTP services 100,000 fewer people and produces 40% less volume of effluent (Table S3) than the Kitchener MWWTP, and the receiving environments of the Waterloo and Kitchener effluent outfalls have similar river flows.[21] This lower loading is a possible explanation for the reduced impacts at this site compared with the sites below Kitchener before the upgrades.

Intersex was infrequent at the reference sites (REF 1, REF 2, and REF 3). Incidence averaged 7.3 ± 1.2% (mean ± SE), ranging from 0 to 20% over the study period, and severity scores were low at these sites. There were no differences between years within reference sites for either intersex incidence or severity (Table S5 and S6). It is unknown whether intersex at these sites was due to anthropogenic stressors or a natural phenomenon. It is not unusual to find intersex at reference sites, especially when the sites are not free of anthropogenic influences. A review on intersex in teleost fish by Bahamonde et al.[5] noted that other studies reported 0.5-55% intersex incidence at reference sites.

The site immediately above the Kitchener MWWTP outfall (INT 2) had highly variable rates of intersex incidence, ranging between 0 and 55%, with a maximum severity score of six, which was normally only ever observed below the Kitchener MWWTP. This site is 19 km downstream of the Waterloo MWWTP outfall, which may have contributed to the intersex observed at this site. However, a more plausible explanation may be that the rainbow darter are moving between sites as there are no physical barriers in this section of the river and INT 2 is a short distance (1 km upstream) from the Kitchener MWWTP outfall. It is interesting to note that intersex incidence and severity also significantly decreased at this site after fall 2013, mirroring the period of the upgrades. Hicks et al.[29] previously showed site-specific stable isotope signatures ($\delta^{15}N$ and $\delta^{13}C$) in rainbow darter at the same sites as the current study, suggesting that although most fish have high site fidelity some fish may move across larger spatial scales (among closely situated sites). More knowledge on the movement patterns of rainbow darter is needed to better interpret these data.

3.3 Potential causative agents of intersex

The implementation of nitrification at the Kitchener MWWTP dramatically improved the plant's overall effluent quality in terms of observed concentrations of nutrients, concentrations of pharmaceuticals, and total estrogenicity. This corresponded to a reduction in the occurrence and severity of intersex at sites below the Kitchener MWWTP. The exact cause of intersex in this study is still not known, although strong evidence in the literature suggests that these types of responses are related to natural (E1/E2) and synthetic (EE2) estrogens[9] as well as to some industrial contaminants such as bisphenol A[41] and alkylphenols.[42] Recent studies have also suggested that chemicals such as metformin (an anti-diabetic) detected in MWWTP effluents can cause intersex in fathead minnows (*Pimephales promelas*).[43] Jobling, et al.[11] have also suggested that chemicals acting as anti-androgens may be contributing to some intersex found downstream of MWWTP outfalls in England. Two anti-androgens, the microbial agents triclosan and chlorophene, have been measured in both the Kitchener and Waterloo MWWTP effluents.[31] Hypoxia has also been suggested as a mechanism for endocrine disruption (i.e., oxygen levels reduced below 1.0 mg/L).[44] The excessive nutrients released into the Grand River have historically caused severe oxygen sags downstream of the Kitchener outfall, where mean summer daily DO levels were well below the recommended objective of 4 mg/L and were as low as 1.2 mg/L in the early morning before the upgrades.[21] After the upgrades, daily summer DO never dropped below 6 mg/L, and median values were the highest in post-upgrade years (Figure

S3). Multiple possible chemicals or conditions might have worked through various pathways or mechanisms to cause the intersex observed in this study.

Advanced treatment technologies (e.g., granular activated carbon (GAC), chlorine dioxide (ClO_2), and ozonation) have been demonstrated to reduce effluent estrogenicity and associated endocrine disruption in laboratory-exposed fish compared with conventional activated sludge.[18, 19] Baynes et al.[18] found that nitrifying activated sludge processes (e.g., nitrification) were less effective at removing estrogenic compounds and reducing associated intersex and vitellogenin induction in laboratory-exposed roach. More advanced treatment (GAC) was required to completely remove intersex. A study by Barber et al.[20] demonstrated that an upgrade from a trickling filter to nitrifying activated sludge was sufficient to reduce total effluent estrogenicity and associated endocrine disruption (as measured by its effects on vitellogenin induction, sperm abundance, gonad size, and secondary sexual characteristics) in caged fish. The current study further supports that nitrifying activated sludge can be an effective and perhaps sufficient upgrade for removing many estrogenic compounds and reducing their associated biological effects such as intersex.

3.4 Manifestation of intersex in the rainbow darter

The timing and duration of exposure to EDCs and the resulting manifestation of intersex in fish is still poorly understood.[45] The recovery of the rainbow darter population from intersex after the MWWTP upgrades suggests that adult rainbow darter can recover quickly from past exposure to EDCs. This is demonstrated by the decrease in intersex incidence (up to 71% reduction) in the first year post-upgrade, which eventually declined to levels similar to those observed at reference sites. If exposure during early life stages (e.g., gonad differentiation) caused intersex to be manifested during the darters' entire lifetime, a rapid decrease in intersex in older fish (with life expectancy of about 5 years[26]) would not be expected. The largest (i.e., oldest) fish did not show a tendency to retain high intersex in the years after the upgrades (Figure S4). Unfortunately, rainbow darter were not aged for this study, but a consistent range in lengths was always sampled from the population and the majority of the fish sampled (Figure S4) were probably 2 or more years old based on studies on rainbow darter growth conducted by Crichton[46] in the Grand River. Other studies support the hypothesis that fish can recover from exposure to EDCs. For example, zebrafish (*Danio rerio*) (including adults) exposed to environmentally relevant concentrations of EE2 have been observed to recover from endocrine-disrupting effects at multiple levels of biological organization including gene expression, protein production (vitellogenin induction), proportion of gonad cell types, gonad size, growth, and sex ratios.[49-51] The recovery of the wild rainbow darter from intersex in the Grand River and zebrafish in the laboratory is in contrast to the findings of Liney et al.[47], who suggested that intersex induced by municipal wastewater effluent in early life stage roach (*Rutilus rutilus*) was permanent. However, the manifestation of intersex in roach was based on the presence of an ovarian cavity in male fish and not ova-testis as in this study. Similarly, Schwindt et al.[48] suggested that fathead minnow populations may not recover from exposure to EE2, including potential transgenerational effects. Therefore, there are studies that document cases where exposure to EDCs may either be irreversible or reversible, and this may depend on species sensitivities, the duration (exposure and recovery) and type of exposure (compound specific versus whole effluents), and the manifestation of the effect in question. Most studies on the recovery from exposure to EDCs are laboratory based, and field observations may involve many confounding factors. Additional studies are needed to further understand how different chemicals, effluents, and species of fish may respond to altered EDC exposure.

The time of the year in which adult fish are exposed to EDCs may also be important in determining the manifestation of intersex. In the first spring (2013) immediately following the upgrades, intersex incidence and severity remained high at DSK 1 and DSK 2 (Figure S2). This was probably because the Kitchener MWWTP had still had poor effluent quality in the previous summer (June –July 2012), before the initial upgrade in August 2012. The summer is the post-spawning period of the rainbow darter, when they build their gonads (recrudescence) for the next spring. The following post-spawning period (summer 2013) would have been the first full period of recrudescence in post-upgrade effluent, and this coincided with reduced intersex in the fall of 2013. This suggests that the manifestation of intersex may be related to the exposure to EDCs during a critical window of each year, such as the post-spawning period when germ cell proliferation is occurring in the gonads.[52] It has been suggested that there is a window of sensitivity during which exposure to EDCs can induce intersex in the early life stages of fathead minnows.[53] Liney et al.[47] were also able to induce intersex in roach when exposure occurred during the critical window of germ cell proliferation in early life stages. Intersex has also been induced in post-spawning adult roach exposed to MWWTP efflents,[18] but not in adult roach where the testes were fully mature,[54] further supporting the theory of a window of sensitivity. For the rainbow darter, further studies are needed to validate whether intersex can be induced in post-spawning adults.

This is a unique study with an important finding that investments in treatment infrastructure at MWWTPs can improve ecosystem health. The results of this study suggest that the relatively conventional treatment plant upgrades at the Kitchener MWWTP reduced exposure to contaminants or conditions that had previously induced the severe intersex condition in fish. The recovery of the rainbow darter from high intersex incidence and severity below the

Kitchener MWWTP outfall suggests that wild fish can recover from previous exposure to EDCs. This study complements work in the laboratory as well as the whole lake exposures conducted at the Experimental Lakes Area[12] that predict that chemicals typically found in MWWTP effluents can cause histological responses in fish. Fortunately this study also demonstrates that improved treatment (targeted at conventional parameters) can greatly reduce the effects in the environment. This study has implications for wastewater management at other sites around the globe in that it confirms that treatment upgrades can reduce biologically relevant indicators of EDC responses in wild fish in a relatively short period of time.

SUPPORTIVE INFORMATION

Supportive information contains data on GSI, LSI, and condition factor for rainbow darter; spring intersex incidence and severity; river DO; Kitchener and Waterloo effluent flows, BOD, TSS, TP, and TKN; and summary statistics.

ACKNOWLEDGMENTS

The authors are grateful for all the assistance received from the University of Waterloo and Environment and Climate Change Canada crews who assisted in sample collection and processing. The treatment plant effluent data were supplied under an agreement with the Regional Municipality of Waterloo. The authors also acknowledge Mark Anderson and Sandra Cooke from the Grand River Conservation Authority (GRCA) for providing access to Grand River water quality data. These data were provided under a licence with the GRCA. This manuscript was copy-edited by Jennifer Thomas. This work was funded by the Natural Sciences and Engineering Research Council of Canada (NSERC), the Canadian Water Network, the

Ontario Ministry of the Environment and Climate Change (OMECC), and the Canada Research Chairs Program.

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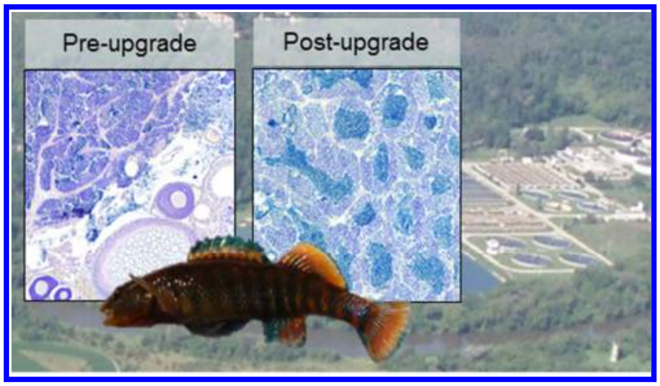

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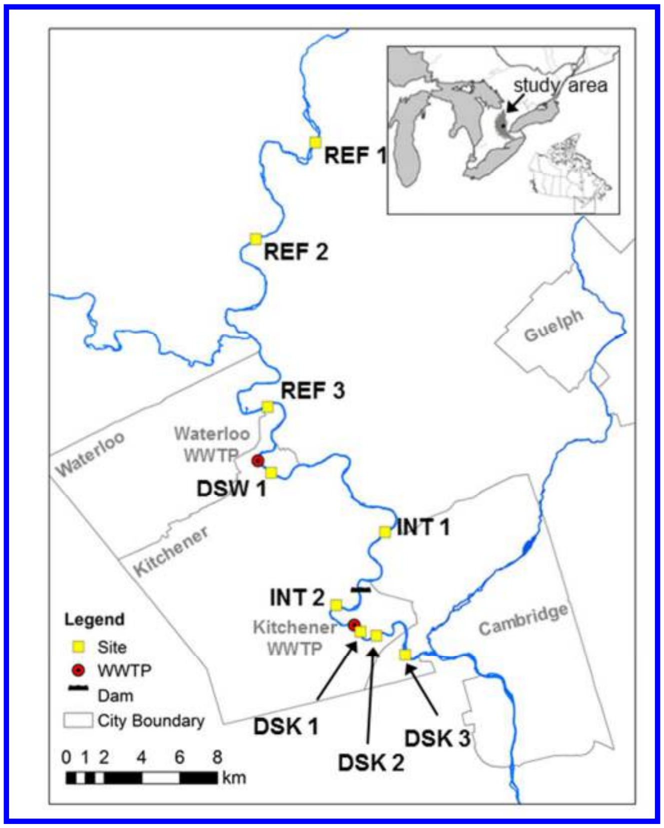

Figure 1. Map of sampling sites along the Grand River, Ontario, where fish were collected in multiple years during the fall and spring periods of 2007–2015. There were three upstream reference sites (REF), four downstream sites (DSW, DSK), and two sites located between the Kitchener and Waterloo wastewater treatment plant (WWTP) outfalls (INT). A full description of the sites is provided in the materials and methods section.

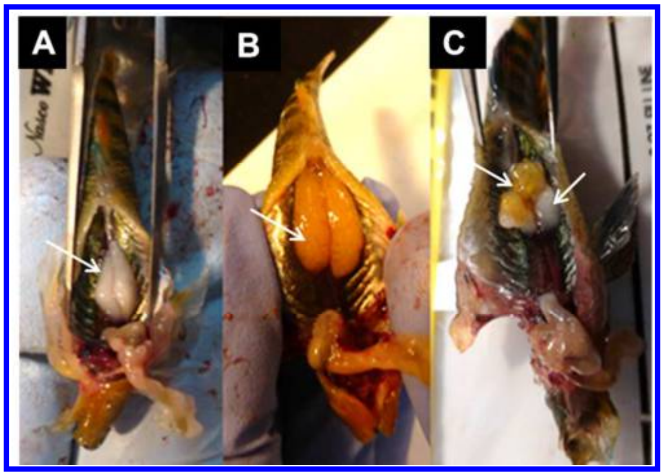

Figure 2. (A) Normal male, (B) normal female, and (C) a severely intersexed male rainbow darter. Arrows point to gonad tissue (testis or ovaries). The intersex male caught in close proximity to the Kitchener MWWTP outfall reveals macroscopic intersex (presence of both testicular and ovarian tissues).

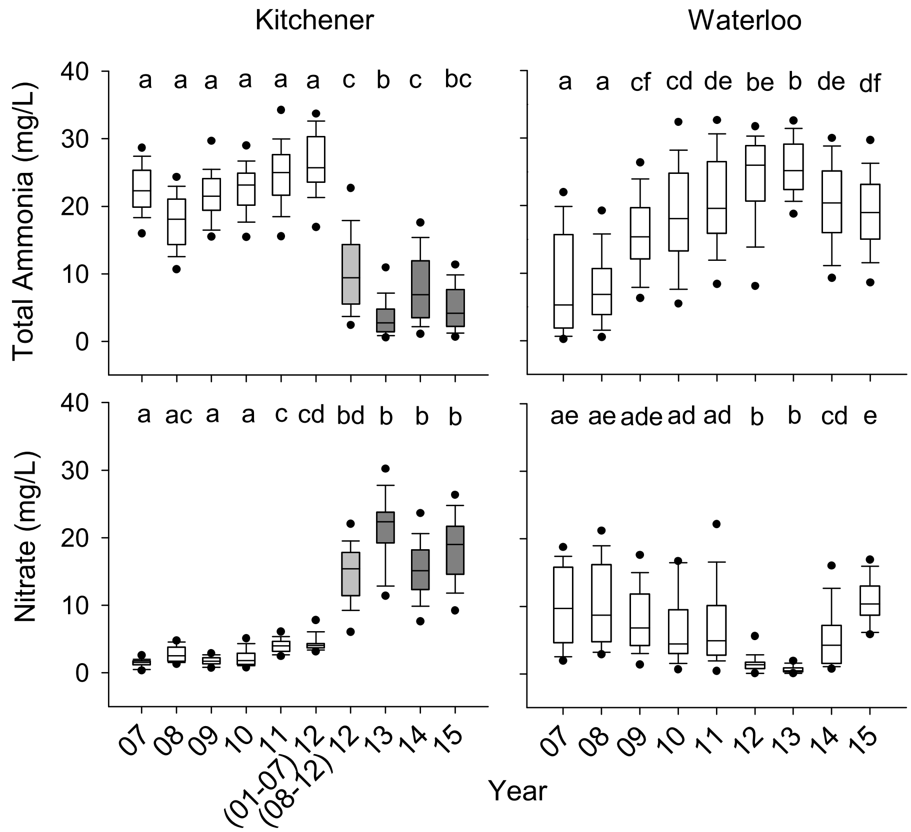

Figure 3. Total ammonia (top panel) and nitrate (bottom panel) for both the Kitchener (left) and Waterloo (right) MWWTPs from 2007 to 2015. For Kitchener only, the white boxes indicate pre-upgrade years (up until July 2012), light grey indicates the period during the upgrades (Aug–Dec 2012), and dark grey indicates post-upgrade years (2013–2015). Black dots represent the upper 95% and lower 5%. Boxplots that do not share a letter in common are significantly different at $p < 0.05$. Boxplots are represented by weekly measurements ($n = 52$) with the exception of Kitchener from 2013 to 2015 and Waterloo from 2014 to 2015 where the frequency of measurements was increased ($n = 153–158$).

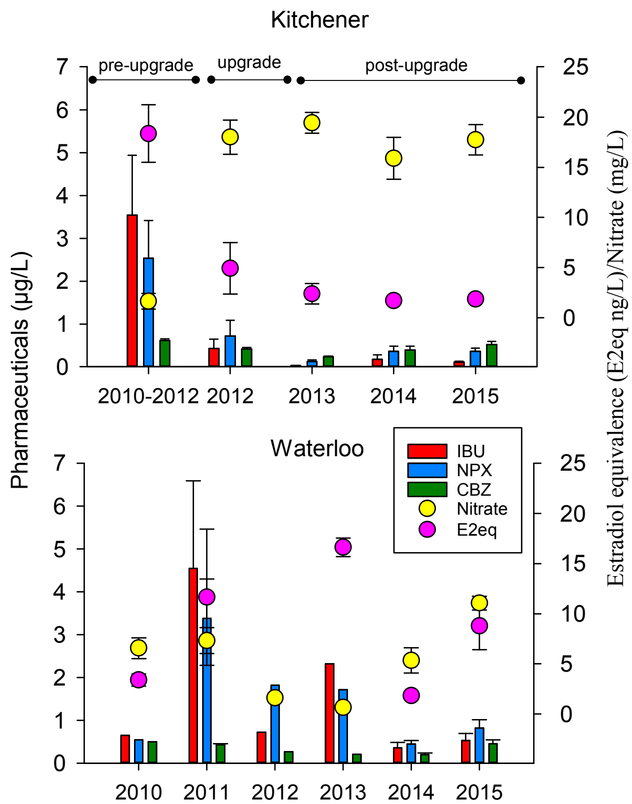

Figure 4. Effluent characterization for the Kitchener (top) and Waterloo (bottom) MWWTPs between 2010 and 2015. For Kitchener only, the pre-upgrade years are 2010 to July 2012; the period during the upgrades is August to December 2012, and the post-upgrade years are 2013 to 2015. The bars represent three pharmaceuticals (ibuprofen (IBU), naproxen (NPX), and carbamazepine (CBZ)),the pink filled circles represent estradiol equivalence (E2eq), and the yellow filled circles represent nitrate. All parameters are represented by the means (±SE) of multiple sample points (days) with the exception of pharmaceuticals in 2010, 2012, and 2013 at Waterloo, where only one sample point (one day) was available. Otherwise, the sample sizes range from 2 to 9, where each replicate represents one event (day) sampled in triplicate. Sample sizes are provided in Table S4.

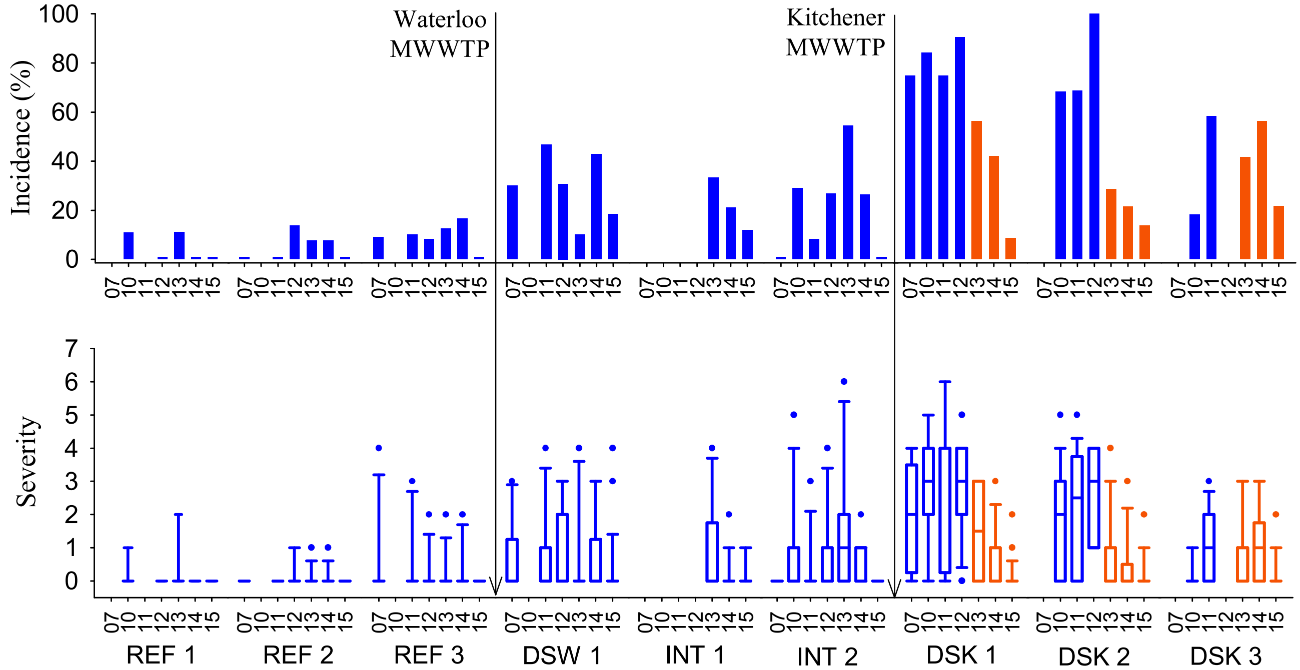

Figure 5. Intersex incidence (top panel) and severity (bottom panel) for fish collected in the fall in 2007 and 2010–2015. Sites are arranged from upstream (REF 1) to downstream (DSK 3), with the black arrows indicating the inputs of MWWTP effluents. Orange bars and orange box plots indicate post-upgrade years (2013–2015) below the Kitchener MWWTP. Sample sizes are provided in Table S2.