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Biological responses in fish exposed to municipal wastewater treatment plant effluent *in situ*

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ABSTRACT

Effluents from municipal wastewater treatment plants (MWTPs) are complex mixtures of chemicals including endocrine-disrupting compounds (EDCs) and 17α-ethynylestradiol (EE2). The objective of this study was to evaluate selected responses of two fish species, in two different years, exposed *in situ* to MWTP effluent. Biological markers of exposure (plasma vitellogenin (VTG) and antioxidant enzymes) were measured in two species of male fish, rainbow trout (*Oncorhynchus mykiss*) and fathead minnow (*Pimephales promelas*), caged at sites associated with wastewater outfall. The estrogenicity of the final effluent in 2010 was determined to be 17.0 + 0.4 ng/L estrogen equivalents (EEQ) and reduced to 7.5 + 2.9 ng/L EEQ after infrastructure upgrades. Pharmaceuticals and personal care products in the effluent and surface water in both years confirmed the exposures at each downstream site. Despite the presence of estrogenic compounds in the MWTP effluent, no effluent-caged male fish demonstrated plasma VTG induction. Minnows and trout that received an intraperitoneal injection of 5 mg/g EE2 showed VTG induction at both field sites. In 2012, the liver somatic index (LSI) of both species increased with exposure, as did changes in antioxidant enzymes, and reactive oxygen species (ROS) activity. Multiple biological mechanisms are modified by effluent exposure, and multiple endpoints are needed to assess risk.

Key words | estrogenicity, fathead minnow, rainbow trout, vitellogenin, wastewater

HIGHLIGHTS

- Effluent exposure resulted in induction of antioxidant enzymes and ROS activity in caged fish.
- Despite measurable estrogen equivalents in the effluent, downstream caged male fathead minnow did not demonstrate VTG induction.
- EE2 injected, control male fish exposed to wastewater or upstream reference conditions responded with elevated VTG concentrations.
- Several factors may have reduced the VTG response in caged fish.

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doi: 10.2166/wqrj.2021.031

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INTRODUCTION

Municipal wastewater treatment plants (MWTPs) have been identified as point sources of a broad suite of contaminants, including excess nutrients, metals, pharmaceuticals, and personal care products (Lishman *et al.* 2006; Metcalfe *et al.* 2010; Holeton *et al.* 2011). A wide variety of endocrine-disrupting substances (EDCs) have also been detected in both MWTP effluents and their receiving environments over the last two decades (Tyler *et al.* 1998; Ternes *et al.* 1999; Servos *et al.* 2003, 2005; Arlos *et al.* 2015). These EDCs may occur at concentrations high enough to elicit sub-lethal effects on aquatic organisms (Mills & Chichester 2005; Parrott & Blunt 2005). Endocrine related effects, especially intersex (testes-ova), have been documented in fish collected near wastewater treatment plant (WWTP) outfalls in many jurisdictions (Jobling *et al.* 1998; Blazer *et al.* 2007, 2012; Vajda *et al.* 2008; Tetreault *et al.* 2011; Bahamonde *et al.* 2013; Abdel-Moneim *et al.* 2015; Ekman *et al.* 2018). This is of concern because the feminization of male fish has been linked to reduced reproductive fitness (Jobling *et al.* 2002; Fuzzen *et al.* 2015), population collapse (Kidd *et al.* 2007), and possible changes in fish assemblages (Tetreault *et al.* 2013).

The Grand River in southern Ontario, Canada, was selected for the study based on numerous published reports of impacts on fish associated with wastewater effluents, including high incidence of feminized wild male fish (Tetreault *et al.* 2011; Hicks *et al.* 2017). Changes in physiology and energy allocation in wild fish have been associated with effluent outfalls (Ings *et al.* 2011; Tetreault *et al.* 2011, 2014) that have the potential to influence fish populations and communities (Tetreault *et al.* 2013). Alterations in endocrine function, including the expression of steroidogenic genes (Ings *et al.* 2011; Bahamonde *et al.* 2014; Marjan *et al.* 2017a), and regulation of sex steroids (Tetreault *et al.* 2011; Bahamonde *et al.* 2015) have been documented. The high occurrence of intersex (e.g., oocytes in testes) has also been observed in fish collected near wastewater outfalls in the Grand River (Tetreault *et al.* 2011; Tanna *et al.* 2013; Bahamonde *et al.* 2015; Fuzzen *et al.* 2015). These responses are consistent with predicted exposure to estrogens from municipal wastewater plants discharging into the Grand River (Arlos *et al.* 2018a), and the effluent studied herein has been demonstrated to be highly estrogenic (i.e., 16.99 ± 0.40 ng/L E2 equivalents – as estimated by the yeast estrogen screen (YES) assay in Tanna *et al.* (2013)). Smith (2013) conducted an effect-directed assay for estrogenic substances on effluent from the same MWTP using estrogen standards of estrone (E1), 17β-estradiol (E2), 17α-ethinylestradiol (EE2), bisphenol A (BPA), 4-nonylphenolethoxycarboxylic acids (NP) and 4-nonylphenolethoxylates (OP), and diethylstilbestrol (DES), and demonstrated that the major estrogenic constituents in the effluent were E1 and E2. Specific reproductive biomarkers, such as plasma vitellogenin (VTG) in male fish, have been suggested as key biomarkers for estrogenic chemical exposure downstream of wastewater outfalls (Sumpter & Jobling 1995) and Thorpe *et al.* (2009) reported that 2–5 ng/L E2eq (YES) was sufficient to increase plasma VTG by 9 fold, and 10–15 ng/L E2eq increased plasma VTG by 46–1,765 fold. However, despite the high effluent estrogenicity and the high prevalence of intersex in rainbow darters exposed to effluent in the Grand River demonstrate a marginal increase in heptic VTG mRNA (Bahamonde *et al.* 2014). Also, nonspecific biomarkers such as elevated reactive oxygen species (ROS) and detoxification enzymes have also demonstrated altered responses in fish exposed to municipal effluents (Oakes *et al.* 2004; Massarsky *et al.* 2013).

Studying biological responses of wild fish, while ideal and relevant, is often hampered by many confounding variables, particularly fish mobility affecting intermittent exposure and residency uncertainties. Although laboratory experiments provide more controlled conditions, they usually require the transport and holding of effluent that can confound results and do not reflect the natural conditions. Consequently, the use of an *in situ* design (e.g., caging study) to assess the responses of fish to MWTP effluent improves cause and effect linkages and realistically reflects an exposure of wild organisms (e.g., biological monitoring programs). The objectives of this study were to (1) investigate the change in effluent quality and estrogenicity following facility infrastructure upgrades, (2) compare responses of two species of fish in the receiving environment to those

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changes, and (3) evaluate the ability of those fish to physiologically respond to wastewater effluent when challenged with a known estrogen. In this study, fish were exposed to MWTP effluent in cages in two different years; prior to, and after, a major infrastructure upgrade. Selected metrics of altered homeostasis, including VTG induction and oxidative stress, in caged fish held at multiple sites below the effluent outfall were compared with upstream reference sites.

MATERIALS AND METHODS

Experiment #1 – caging of fish in 2010

All animal use protocols were approved by the University of Waterloo's local Animal Care Committee (AUP# 07-16 and #10-17). The first fish caging study conducted on 28 September 2010 to 5 October 2010, utilized immature rainbow trout (RBT; *Oncorhynchus mykiss*: (~10 months, 50 g) purchased from Silver Creek Aquaculture Inc., Erin, ON, and 90 days post hatch (dph) fathead minnow (FHM; *Pimephales promelas*) purchased from Silhanek Baitfish, Bobcaygeon, ON). The sites selected for this study were within the urban central reach of the Grand River in the vicinity of the MWTP (43° 24′02.88″N; 80° 25′12.40″W) servicing the City of Kitchener (Figure 1). Five sites were used: two upstream reference sites (Sites 1–2) and three downstream effluent-exposed sites (Sites 3–5), within 500 m to 4 km of the facility (Kitchener MWTP) outfall (Figure 1; Supplementary Material, Table S1). Fish were exposed *in situ* (submerged in the river at depth of 40–70 cm) and were not fed for the duration of the experiments. Immature RBT cages (6 fish/cage) were deployed in triplicate, for a total of 18 fish/site. Thirty FHM were caged (15 fish/bucket) at the same sites in duplicate (30 FHM/site).

After the 7 days exposure period, fish were removed from cages and anesthetized with 0.1 g/L tricaine methanesulfonate (MS-222) prior to dissection on site in a field trailer. Blood was collected from the caudal vein of RBT using a 1½″ 28 G heparinized (1 mg/mL) syringe stored on ice while blood samples from FHM were collected by severing the caudal peduncle and collecting pooled blood with capillary tubes. All plasma samples were stored on ice for no more than 20 min prior to centrifugation (3,000 *g* for 5 min) to isolate plasma, which was flash-frozen in liquid nitrogen. Following bleeding, each fish was euthanized while still under MS-222 anesthesia by spinal severance. Fork length (±1.0 mm) and bodyweight (±0.001 g) were determined, and gonad and livers were removed, weighed (±0.001 g), and stored in liquid nitrogen prior to long-term storage at −80 °C. Morphometric characteristics were used to determine fish condition [$k = (\text{bodyweight}/\text{length}^3)*100$], gonadosomatic index [GSI = (gonad weight/bodyweight)*100], and liver somatic index [LSI = (liver weight/bodyweight)*100].

Experiment #2 – caging of fish in 2012

In 2012, the first of two studies were conducted with RBT caged from October 3 to 17 and FHM caged from October 4 to 18, at the same five sites as in 2010. In the first study, FHM and RBT were caged as described for the 2010 Experiment (Expt) #1, but with exposure time increased from 7 to 14 days. While the immature RBT (6 fish per container in triplicate; 18 fish/site) were identical to densities in Expt #1, adult male FHM were caged in lower densities (10 fish per container in triplicate; 30 fish/site) to reduce any density-dependent stress within each treatment container while still having sufficient replication. Due to the number of test organisms, distance between exposure sites, and to improve logistics, the deployment and retrieval dates were staggered to reduce stress to the test organisms and ensure QA/QC of the sampling data. Immature RBT were again purchased from Silver Creek Aquaculture Inc., Erin, ON, but this experiment utilized 90 dph male FHM purchased from Aquatic Resource Organisms (Hampton, NH, USA). Fish were held in the University of Waterloo Wet Laboratory for 14 days prior to deployment in the Grand River. A sampling of fish and storage of tissues was as described for Expt #1. Expt #2 also collected gill tissues from a subset of caged RBT and FHM which were dissected and preserved in Davidson's solution for histological assessment using a protocol described by Tetreault *et al.* (2012).

Experiment #3 – caging of injected fish

In conjunction with Expt #2, fish were injected intraperitoneally (*ip*) with either 5 μg/g 17α-ethynylestradiol (EE2) using corn oil as a vehicle or with corn oil alone (sham

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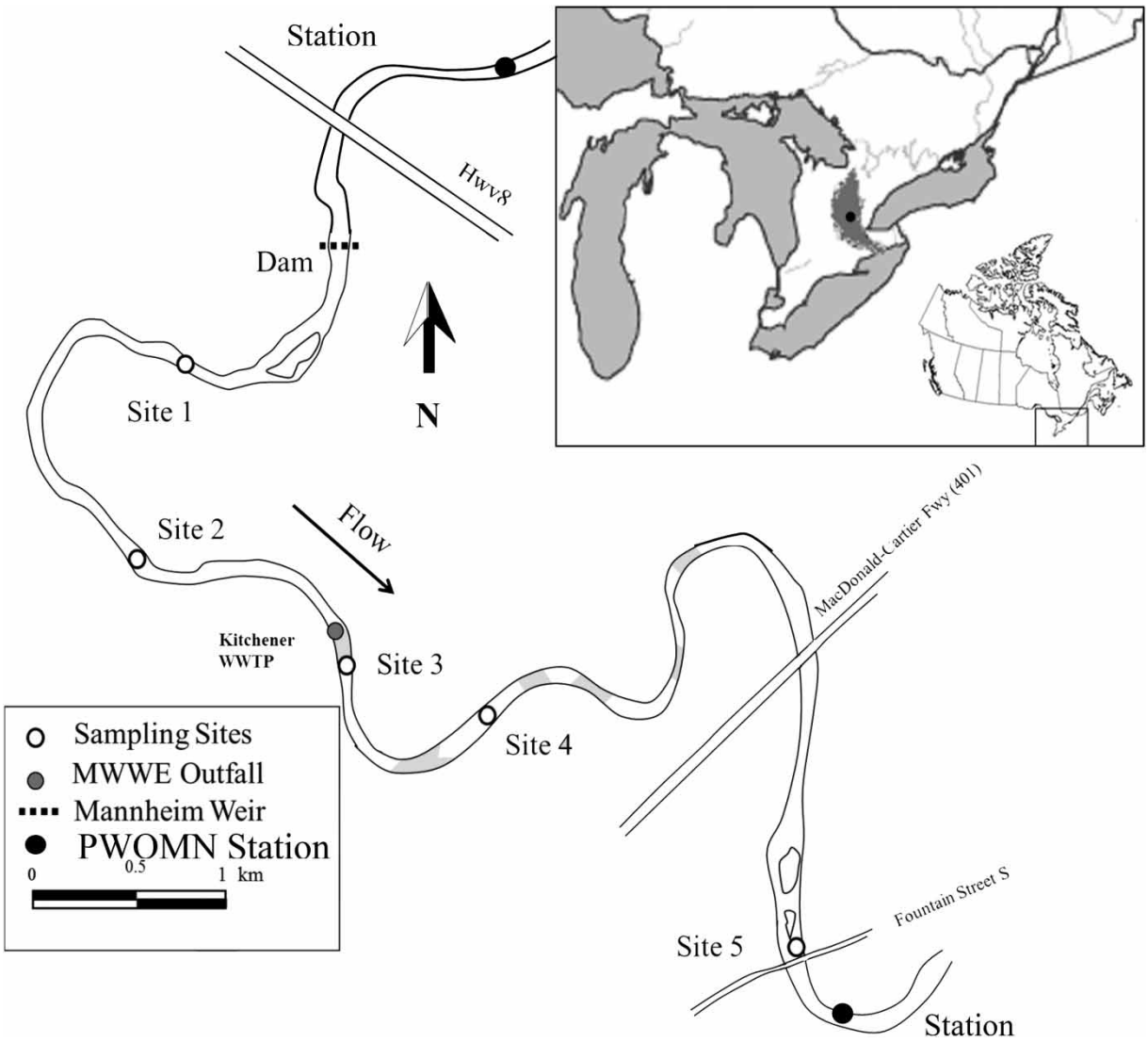

Figure 1 | The location of sites in the Grand River selected of caging experiments conducted near the outfall of the Kitchener wastewater treatment facility in October of 2010 and 2012.

treatment) to induce the VTG protein to ensure that the test organisms would elicit a biological response to an estrogen (Expt #3) (Vega-López *et al.* 2006). The EE2 and sham fish of each species were randomly distributed between the upstream control Site 2 and downstream MWTP-exposed Site 4 and were held for either 4 or 7 days, after which all fish were processed as described for Expt #1.

Exposure characterization and pharmaceutical analysis

River flow data were obtained from the Government of Canada water quality monitoring network (station 16018404102). Mean streamflow for September 2010 and 2012 was 14.5 and 16.5 m^3/s, respectively. In 2012, at each of the caging exposure sites, conductivity and water temperature loggers (ONSET Hobo U24–001) were deployed for the duration of the experiment. Surface water grab samples were collected (in triplicate) near the caged fish during the exposures on 1, 8, and 16 days to characterize the exposure. Water quality parameters were analyzed by the Laboratory Services Branch of the Ontario Ministry of Environment, Etobicoke, Ontario. The selected pharmaceuticals (carbamazepine, ibuprofen, naproxen, and venlafaxine) have been measured as previously described

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in Tanna *et al.* (2013). Total estrogenicity was determined on the whole effluent grab samples using the YES assay (Smith 2013; Hicks *et al.* 2017).

Polar organic contaminants integrated samplers

Polar organic contaminants integrated samplers (POCIS) for monitoring polar (water-soluble) compounds were deployed alongside the fish cages and retrieved after 2 weeks. The description of the deployment, analytical specification, and analysis for POCIS were previously described in Gillis *et al.* (2014). Both *in situ* experiments conducted in 2010 and 2012 followed the same protocol for deployment, retrieval, and analysis of the POCIS units. The compounds monitored included pharmaceuticals (carbamazepine, ibuprofen, and gemfibrozil), two antibiotics (sulfamethoxazole and trimethoprim), an estrogen (estrone), an anti-bacterial compound (triclosan), and two synthetic musks (Galaxolide® and Tonalide®). In 2012, the selection of target analytes also included an androgen (androstenedione) and an artificial sweetener (sucralose).

Plasma VTG

In 2010, the primary antiserum for the VTG ELISA was produced using European white rabbits (4E1 and 3A2) and the inoculum consisted of 300 μg RBT VTG per mL saline solution. The methods for inoculation to induce the primary antisera were carried out in accordance with University of Waterloo Animal Care Committee UWSOP457 (Use of Freund's Complete Adjuvant in Rabbits) and UWSOP452 (Subcutaneous Injection in the Rabbit) (Smith 2013). In 2012, the detection of plasma VTG concentrations collected from males (FHM and RBT) was conducted separately between years and followed a Competitive Enzyme-Linked Immunosorbent Assay (ELISA) protocol developed at the University of Waterloo is described in the Supplementary Material (Smith 2013).

Oxidative stress

In both Expt #1 and #2, a subsample of tissues (liver and gill) from FHM (both years) and RBT (2012) were assessed for oxidative stress endpoints (catalase (CAT); glutathione S-transferase (GST); glutathione peroxidase (GPx); glutathione reductase (GR); and superoxide dismutase (SOD)). GST catalyzes the conjugation of the reduced form of glutathione (GSH) to xenobiotic substrates for detoxification. GPx reduces lipid hydroperoxides to alcohols and reduces hydrogen peroxide to water. GR reduces glutathione disulfide (GSSG) to GSH which is an important antioxidant. CAT is a common enzyme found in nearly all living organisms exposed to oxygen and catalyzes the decomposition of hydrogen peroxide to water and oxygen. It is an important enzyme in protecting the cell from oxidative damage by ROS. Lastly, SOD is another antioxidant which catalyzes the dismutation of superoxide anion (O_2-) into oxygen and hydrogen peroxide. Each of these endpoints was analyzed using the methods outlined in detail by Massarsky *et al.* (2013).

Statistical analyses

Data were tested for normality using Kolmogorov–Smirnow Normality tests. In some cases, data were not normally distributed or Levene's test of equal variance failed, and therefore analysis of variance (ANOVA) was performed on ranks instead. Fish performance indicators including condition (k), GSI, and LSI were analyzed by sex and species and among sites within each experiment using analysis of covariance (ANCOVA) with the 'site' as the categorical variable so that sites sampled within a given year were compared within experiment. Condition factor was analyzed using bodyweight in response to body length as a covariate, and relative gonad and liver weight were analyzed using bodyweight as the covariate. The Holm–Sidak multiple comparison test was used to identify differences that were statistically significant. Differences in VTG induction and oxidative stress endpoints, as well as fish length and weight were analyzed individually by site by species within each experiment separately using a one-way ANOVA. Non-detects in VTG analysis were substituted with detection limit for statistical analyses. For Exp #3, VTG levels in fish were analyzed individually by species with treatment (sham or EE2) and period (4 and 7 days) as categorical independent variables using a two-way ANOVA (Wilkinson 1990). Correlations between oxidative stress endpoints and VTG were calculated using the Pearson correlation and

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Kendal tau concordance methods. All data analyses were conducted using SigmaPlot® 12.0 statistical software.

RESULTS AND DISCUSSION

Exposure characterization and pharmaceutical analysis

Water quality parameters from 2010 (Expt #1) obtained from Provincial Water Quality Monitoring Network (PWQMN) stations upstream (#16018404102; Lat.: 43.6304701388° Long.: −80.4766093575°; approximately 2.5 km upstream of Site 1 collection site) and downstream (#16018401202; Lat.: 43.4220137636° Long.: −80.4104843575°; approximately 1.2 km downstream of Site 5) of the exposure reach were summarized in Supplementary Material, Table S2. Surface water ammonia, water temperature, and conductivity were always highest at the downstream near-field Site 3 and remained higher than the upstream reference sites at the far-field Site 5 (4 km downstream) in both years (Supplementary Material, Tables S2 and S3). The conductivity of the Kitchener effluent in 2010 and 2012 was 2,680 and 2,330 μS/cm, respectively, which was reflected in an increase in conductivity at the downstream exposure Sites 3, 4, and 5 (691, 667, and 597 μS/cm, respectively). Based on conductivity, the effluents per year exposure in 2010/2012 was estimated as 6.7%/13.2%, 5.8%/5.8%, and 3.2%/5.0% at Sites 3, 4, and 5. In the summer of 2012, the treatment plant had initiated part of a planned major upgrade that came online in the fall of 2012. This resulted in a major change in the effluent quality with partial nitrification in the new system resulting in a decrease in ammonia (and corresponding increase in nitrate) (Supplementary Material, Table S3). Although the measured ammonia in the Kitchener final effluent in the summer of 2012 was potentially lethal to aquatic organisms (~24 mg/L), the concentration in the effluent in the fall of 2012 was significantly reduced (~12 mg/L) as a result of treatment upgrades coming online (i.e., total ammonia at Site 3 was <4 mg/L in the river). Despite this dramatic change in effluent, most water quality parameters were higher at the near-field exposure site (Site 3) than reference sites at all time periods (Supplementary Material, Table S3).

Both the river grab samples and POCIS indicate that the downstream environment where the fish were caged was exposed to a variety of pharmaceuticals. Numerous emerging contaminants were present in the Kitchener final effluent and river water grab samples during the caging exposures in 2012 (Supplementary Material, Tables S4 and S5). Concentrations increased immediately downstream of the Kitchener outfall, and while they were still detectable further downstream, some chemicals such as ibuprofen and naproxen declined, while others (gemfibrozil, sucralose, sulfamethoxazole, androstenedione) were more persistent. The concentrations of four selected pharmaceuticals (carbamazepine, ibuprofen, naproxen, and venlafaxine) in the Kitchener effluent are presented for 2010 (Figure 2(a)) and 2012 (Figure 3(a)). The concentrations of many of the contaminants (except venlafaxine) in effluent were reduced in 2012. The concentrations of

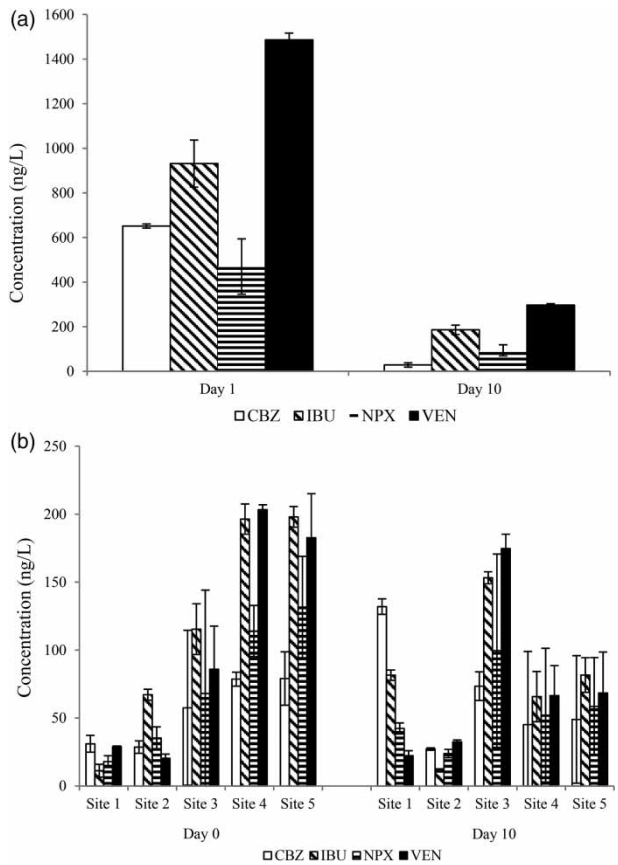

Figure 2 | The concentration of selected emerging contaminants (CBZ, Carbamazepine; IBU, Ibuprofen; NPX, Naproxen; VEN, Venlafaxine) (a) in the Kitchener effluent, and (b) at the reference sites upstream (Sites 1 and 2) and the exposure downstream (Sites 3, 4, and 5) during an *in situ* study (day 1 and 10) in October of 2010.

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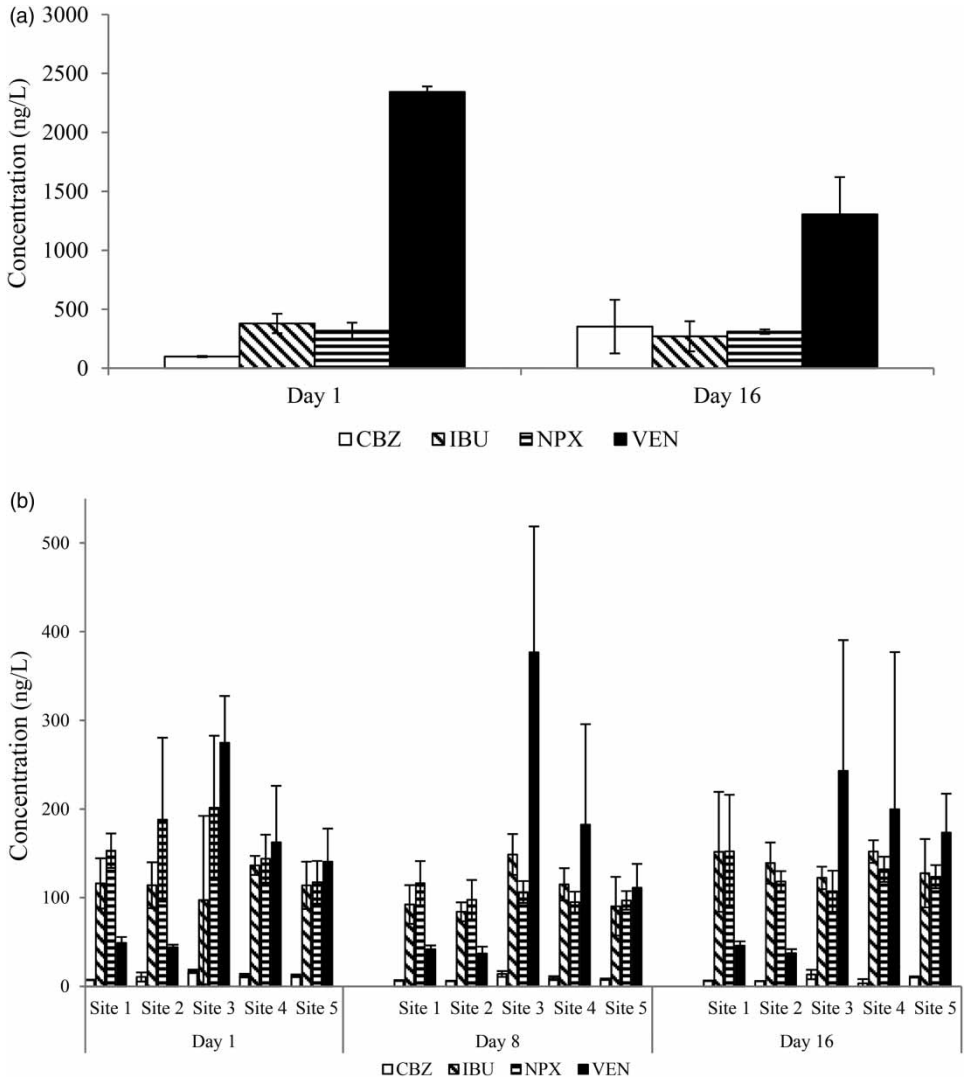

Figure 3 | The concentration of selected emerging contaminants (CBZ, Carbamazepine; IBU, Ibuprofen; NPX, Naproxen; VEN, Venlafaxine) (a) in the Kitchener effluent (day 1 and day 16), and (b) at upstream (Sites 1 and 2) and the exposure sites (Sites 3, 4, and 5) during the fish caging study (1, 8, and 16 days) in October of 2012.

these contaminants at the exposure sites in the river were generally elevated compared with upstream reference sites in either 2010 or 2012 (Figures 2(b) and 3(b), respectively). For example, ibuprofen concentrations in the 2012 effluent were at least 10 times lower than 2 years previously in 2010. The detected contaminants at the upstream sites are likely originating from the upstream Waterloo MWTP. The chemicals found in high concentrations in the Kitchener effluents, such as ibuprofen and venlafaxine, were found in much higher concentrations downstream of the Kitchener outfall compared with upstream.

POCIS accumulated each of the target pharmaceutical analytes at all sites, demonstrating consistency between the active and passive sampling approaches and both support that fish deployed in cages downstream of the effluent outfall were exposed to elevated concentrations of a variety of contaminants. Concentrations estimated by POCIS were lower than those measured by grab samples (SPE extraction) possibly due to river temperatures and flows that are known to affect estimates (Li *et al.* 2010). POCIS measured additional emerging compounds including an estrogen (estrone) and an androgen (androstenedione) that were detected in low concentrations at the downstream locations

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but not at the two upstream (reference) sites (Supplementary Material, Table S5). Ibuprofen in POCIS samples was consistent with surface water samples throughout the study area but carbamazepine, naproxen, and venlafaxine in POCIS were slightly elevated immediately downstream of the MWTP outfall (Site 3) compared with upstream reference sites; however, these differences were not statistically significant. Both antibiotics (sulfamethoxazole and trimethoprim), as well as sucralose, remained elevated at the far-field exposure site (Site 5) suggesting potential for persistence in the system. Arlos *et al.* (2014) modeled the fate of several pharmaceuticals in this branch of the Grand River and suggested that dilution as well as chemical-specific degradation was important in river fate. In both years, many pharmaceuticals were detected in the upstream surface waters, likely due to the influence of the Waterloo MWTP outfall; approximately 20 km upstream (Arlos *et al.* 2018b).

VTG analysis

Despite the detection of estrogenic activity in the final effluent at Kitchener, caged FHM did not show elevated levels of plasma VTG after 7 days of exposure in 2010 (Figures 4 and 5). Analysis of plasma VTG of male FHM from the 2010 caging study did not demonstrate induction with only 11% of the downstream fish having levels above the (0.0586 μg/mL) detection limit (Figure 4). Similarly, in 2012, neither FHM nor RBT demonstrated induction of VTG after 14 days of caging at sites downstream of the Kitchener MWTP outfall. In the 2012 exposure, no differences in VTG were observed across the sites in male FHM or RBT (Figure 5). FHM males at the upstream sites had only 33% and the downstream sites only 31% of individuals with detectable levels of VTG (detection limit 0.429 ± 0.03 ng/mL) (Figure 5). Forty-two percent of the caged male RBT had VTG levels below the detection limit (0.478 ± 0.08 ng/mL). VTG was generally less than 1 μg/mL, and there were no statistically significant differences in VTG among sites (Figure 5). The observed lack of VTG induction was not due to the inability to produce VTG since FHM and RBT injected with EE2 responded with significant induction after 4 days and a heightened induction after 7 days of exposure. FHM and RBT injected with EE2 had elevated plasma VTG levels after 4 days, and further elevated levels after 7 days of caging (Figure 6). Male FHM and RBT injected *ip* with EE2 expressed VTG concentrations of up to 20,000 μg/mL (Figure 6). While sham injected FHM also had detectable levels of VTG, these levels were lower than EE2 injected males and did not increase between 4 and 7 days (Figure 6). Unfortunately, the plasma samples from the RBT sham injected fish were compromised and were not analyzed. In general, injected FHM males and juvenile RBT exposed downstream of the sewage treatment plant had lower concentrations of VTG than those that were exposed at the upstream site after both 4 and 7 days of exposure. However, due to the variability in this small sample size, this decrease in plasma VTG at the downstream site was not statistically significant (Figure 6; $p > 0.05$).

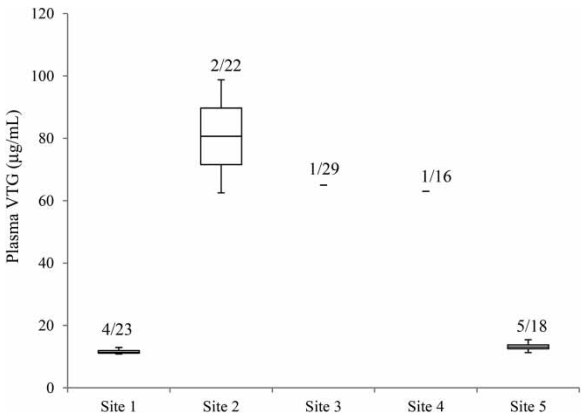

Figure 4 | Plasma VTG levels of male FHM caged for 7 days at upstream reference (Sites 1 and 2) and the exposure sites (Sites 3, 4, and 5) near the Kitchener Treatment Plant outfall in October of 2010. Numbers indicate the number of fish with measurable levels/total number of fish sampled. The detection limit of the ELISA for the VTG concentration in fathead minnows was 0.0586 μg/mL.

Considering that wild male fish at this site show evidence of feminization, such as intersex (Tetreault *et al.* 2011; Tanna *et al.* 2013), delayed spermatogenesis (Tetreault *et al.* 2011; Fuzzen *et al.* 2016), and inhibition of androgen production (Bahamonde *et al.* 2015; Fuzzen *et al.* 2015) associated with effluent exposure, the lack of VTG induction in caged fish seems inconsistent. There are several possible explanations for these observations. The lack of VTG induction in MWTP effluent-exposed fish may be due to low levels of estrogens in the effluent and river sites; however in 2010, the total estrogenicity of the effluent was 17.0 ± 0.4 ng/L estrogen equivalents (EEQ), which would be high enough

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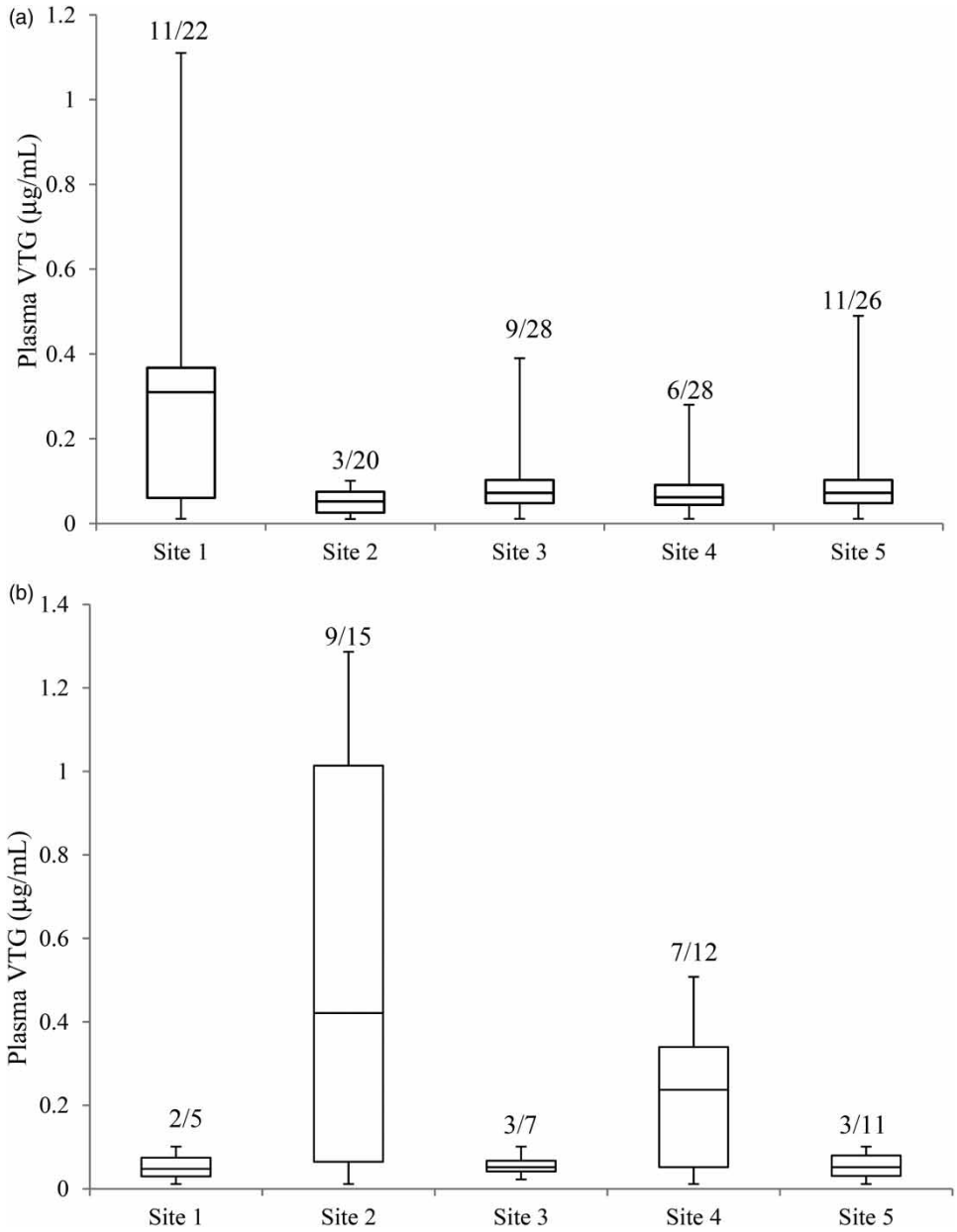

Figure 5 | VTG induction in plasma of (a) male FHM and (b) RBT caged at upstream reference (Sites 1 and 2) and the exposure sites (Sites 3, 4, and 5) in the Grand River for 14 days near the Kitchener wastewater treatment outfall in October of 2012.

to elicit an estrogenic response in laboratory FHM (Länge *et al*. 2001; Parrott & Blunt 2005; Caldwell *et al*. 2008). Although the analysis of estrogenicity indicated that a number of estrogenic compounds remained present (i.e., bisphenol A, estrone, 17β-estradiol, EE2, and nonylphenol) (Arlos *et al*. 2018a), the total estrogenicity measured in the effluent was only 7.2 ± 2.9 ng/L EEQ during the 2012 exposure period in this study. This would result in an exposure of less than 1 ng/L EEQ to the fish at Site #3 downstream cage site. The caged fish closest to the MWTP outfall experienced only 6.7% effluent (based on water conductivity levels) which means that the estrogen exposure would have been approximately 1 ng/L EEQ at the most exposed site. Also, the 2010 exposure lasted for only

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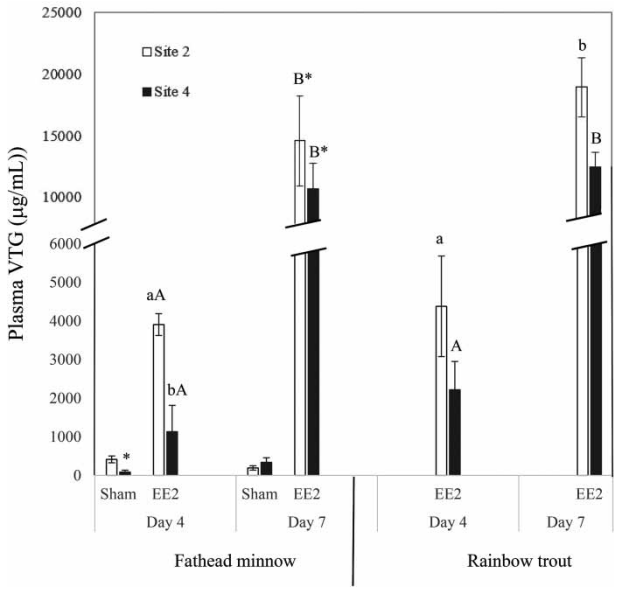

Figure 6 | Induction of circulating plasma VTG in mature male FHM and in immature RBT exposed upstream reference (Site 2) and downstream exposure site (Site 4) of the Kitchener treatment plant outfall in the Grand River for 4 and 7 days after injection with EE2 or corn oil sham in October of 2012. Lower case letters indicate significant different VTG levels within a treatment and species on each day. Upper case letters indicate significant difference of VTG of EE2 injected fish between days within species within sites. '*' indicates statistical difference between sham and EE2 injections within species and days.

7 days which may not have been sufficient to allow the activation of the cellular pathways; hence, in 2012, the exposure period was extended to 14 days and the lower flows resulted in a higher effluent exposure as well (13.1% MWTP effluent) (Vega-López *et al.* 2006). Male FHM and RBT in the study were capable of expressing a VTG response upon exposure to a synthetic estrogen as demonstrated when they were injected with 5 µg/g bodyweight EE2. Males of both species responded with a large, rapid increase in VTG protein production (Figure 6). Pawlowski *et al.* (2003) measured hepatic VTG expression (226 ± 38 to 3,373 ± 1,958 pg/µg total RNA) in male RBT caged in an effluent for 14 days and Ekman *et al.* (2018) demonstrated significant VTG mRNA induction in male FHM after only 5 days of wastewater effluent exposure *in situ*.

Another factor is that the treatment plant had initiated an upgrade just prior to the exposures that resulted in at least partial nitrification of the effluent which increased nitrate in the effluent (Bicudo *et al.* 2016), reduced the ammonia, and unexpectedly reduced the effluent estrogenicity (Hicks *et al.* 2017). The total estrogenicity measured in the Kitchener effluent using the YES assay declined from 17.0 ± 0.4 ng/L in 2010 to 7.5 ± 2.9 ng/L in 2012 and continued to decline after additional upgrades (Hicks *et al.* 2017). Numerous other studies have documented the decline of total estrogenicity in effluents with increased treatment (Servos *et al.* 2005). The profile of estrogenic compounds observed using an effects-directed assessment (EDA) of the Kitchener effluent showed that at least part of the estrogenicity in the effluent was associated with 17β-estradiol, estrone, EE2, and alkyphenols, although additional unidentified estrogens may be present in the effluent (Arlos *et al.* 2018a). Desbrow *et al.* (2008) using a similar EDA on WWTP effluent in the UK demonstrated a similar profile of estrogenicity. Decreases in ammonia levels resulting from increased aeration/nitrification upgrades at this facility have also been associated with declines in estrogenicity of the effluent and a reduction in the incidence and severity of intersex in wild fish (Hicks *et al.* 2017). However, making a direct causal link between specific chemicals and the impacts on wild fish remains difficult to establish as the effluent contains a wide diversity of endocrine active chemicals. For example, the androgen, androstenedione, was detected by POCIS, and antiandrogens such as triclosan have been reported in the effluent and river (Arlos *et al.* 2014). It is possible that mRNA synthesis or VTG production was disrupted in fish exposed to the effluent as has been suggested in other studies (Miller *et al.* 1999). In this study, caged fish exposed to the effluent showed a tendency to have reduced induction compared with the upstream reference fish when injected with the synthetic estrogen EE2. It is possible that androgenic activity or anti-estrogenic chemicals present in the effluent and surface water may have partially suppressed the estrogenic effects in caged fish.

Previous studies in the Grand River have found increased incidence and severity of the intersex condition at sites downstream of the Kitchener MWTP in four different species of fish (rainbow darter, greenside darter (*E. blennioides*), johnny darter (*E. nigrum*), and longnose dace (*Rhinicthys cataractae*)) (Tetreault *et al.* 2011; Tanna *et al.* 2013). Additionally, this elevation of intersex incidence and severity at the site downstream of the Kitchener MWTP was consistent in rainbow darter when examined across 4 years of field collections (Fuzzen *et al.* 2016). Although an

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association between reproductive impairment, intersex incidence/severity, and VTG induction has been documented in some studies (Jobling *et al.* 2002), caution should be taken when assessing these endpoints as they may occur through different mechanisms of action. The possible suppression of the VTG response of EE2 injected fish at the downstream sites suggests other compounds may be interfering (Miller *et al.* 1999). Hultman *et al.* (2017) provided evidence that other compounds are capable of exerting anti-estrogenic effects through potential suppression of estrogen receptor (ER)-mediated responses, but not necessarily directly associated with the ER-mechanism of action. This certainly confounds efforts to isolate and identify specific compounds or classes of compounds responsible for the expression of VTG in fish exposed to whole effluent. As a result, the linkages of exposure to estrogenic compounds in wild fish leading to induction of VTG in males or the development of intersex in wild fish is still not clear.

The use of VTG as a biomarker of estrogen exposure is frequently documented in the literature in fish exposed to estrogenic compounds, as in a whole lake experiment with EE2 (~5 ng/L) conducted at the Experimental Lakes Area. Palace *et al.* (2002) and Kidd *et al.* (2007) observed induction of VTG in both genders of wild FHM after 1 year of exposure. It is important to distinguish between single compound (estrogen) and mixture exposures. In complex mixtures, such as in MWTP effluent, the response of fish becomes less predictable, such as in this study. RBT have also demonstrated the ability to respond (induction of VTG) to wastewater effluent exposure in a variety of caging experiments (Purdom *et al.* 1994; Harres *et al.* 1997; Ings *et al.* 2011) as have FHM (Jasinska *et al.* 2015). Tetreault *et al.* (2012) observed VTG in wild male FHM from Wascana Creek, downstream of a secondary treated effluent outfall in Saskatchewan. In these studies, VTG induction was influenced by both dose (degree of exposure) as well as duration of the exposure. FHM exposed *in situ* to WWTP effluent in the South Saskatchewan River had a 4-fold induction of VTG mRNA after a few days (Jasinska *et al.* 2015).

VTG has been utilized as a tool to assess the efficacy of upgrades to effluent treatment. The induction of RBT hepatic VTG mRNA exposed to pilot plant, conventional effluent *in situ* was expressed up to 50-fold, the relative abundance of VTG mRNA relative to controls, or effluent undergoing additional advanced treatment (Gunnersson *et al.* 2009). Ings *et al.* (2011) reported altered VTG mRNA in RBT exposed to a tertiary treated effluent (Guelph, ON). Prior to facility upgrades at the well-studied Boulder MWTP (Colorado, USA), the estrogenicity of the effluent (approximately EEQ 26 ng/L) corresponded with the presence of endocrine-disrupting chemicals and observed reproductive effects in effluent-exposed free-living white suckers (*Catostomus commersoni*) (Vajda *et al.* 2011) and FHM exposed *in vivo* (Barber *et al.* 2007). Upgrades at this facility corresponded with improved removal efficiency of EDCs from the effluent, reduction of the estrogenicity of the effluent below detectable levels, and absence of biological responses (i.e., VTG induction, reduction in nuptial tubercle abundance) in male FHM (Barber *et al.* 2012). This suggests that these management actions could lead to the potential recovery of wild fish exposed to the upgraded MWTP effluent (Ekman *et al.* 2018) including intersex (Hicks *et al.* 2017).

Gill pathology and oxidative stress

There were no differences in gill histopathology of fish caged in association with the Kitchener wastewater effluent after 14 days of exposure (Supplementary Material, Table S9). Also, as these short-term (4, 7, and 14 days) exposures were not expected to alter fish length, weight, condition, LSI, nor GSI, the results of the whole fish parameters are summarized in Supplementary Material, Tables S6–S8. FHM caged in 2010, indicated no consistent site differences in oxidative stress downstream of the MWTP discharge in either liver or gill tissue (Figure 7(a)–7(d)). However, some differences in activity between tissues were observed. For instance, GPx and CAT activity was found to be greater in liver tissue (Figure 7(a)), while GR activity was greater in gill tissue (Figure 8(b)–8(d)). In 2012, site differences in enzyme activity were observed, but these alterations were generally small in magnitude (Figure 8(e)). SOD activity in 2012 was modestly elevated at the near-field exposure site (Site 3) compared with the far-field exposure sites (Sites 4 and 5) and not different compared with reference sites suggesting another stressor was responsible for the higher SOD. An increase in activity for GST (Figure 8(a)) was detected in gills of exposed RBT and FHM at the near-

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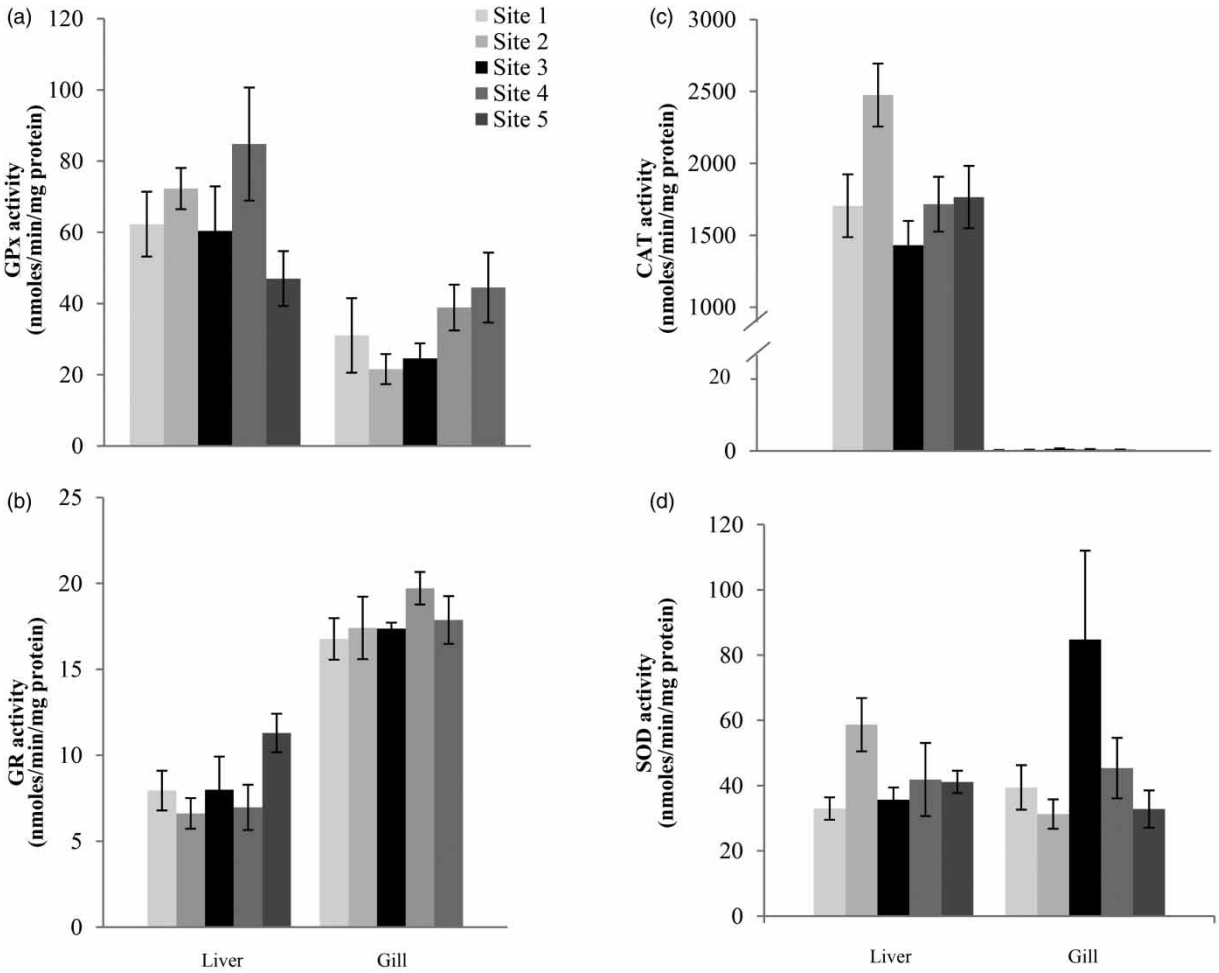

Figure 7 | Detection of antioxidant enzymes and ROS activity of (a) GPx, (b) GR, (c) CAT, and (d) SOD in liver and gills of male FHM (*Pimephales promelas*) caged upstream reference (Sites 1 and 2) and exposure sites (Sites 3, 4, and 5) downstream of the discharge of the Kitchener municipal effluent the Grand River in October of 2010 for 7 days. There were no significant differences detected across sites.

field exposure site (Site 3). Catalyze activity in gills of FHM, but not RBT, was reduced at the exposed sites (Figure 8(d)). The GST activity in both RBT and FHM caged at Site 3 was significantly elevated relative to upstream of the Kitchener outfall or further downstream. These results are consistent with those from the literature where moderate induction of some antioxidant enzyme activity in fish adjacent to MWTP outfalls was observed (Isamah *et al.* 2000; Oakes *et al.* 2004; Almroth *et al.* 2008). Cavenave *et al.* (2014) showed an increase in oxidative stress (i.e., GST, GR) in caged fish (*Prochilodus lineatus*) exposed to wastewater effluents. In studies with FHM caged for 21 days, elevated GST and GR as well as decreased SOD activity downstream of a wastewater outfall were shown (Jasinska *et al.* 2015). Freshwater mussels demonstrated responses in lipid peroxidation and GST activity, when held downstream of the Kitchener outfall for 4 weeks in 2010 (Gillis *et al.* 2014). Biomarkers of oxidative stress and immuno-modulation or biomarkers of cellular communication may be general indicators of exposure to wastewater; however, they are not specific to contaminant exposure (Oakes *et al.* 2004; Lacaze *et al.* 2019) and responses to contaminants inducing oxidative stress may be species-dependent (Oakes *et al.* 2005). In this study, Pearson correlations of oxidative stress biomarkers and fish health parameters K, GSI, and LSI were negatively correlated ($p \leq -0.800$) with CAT, and GR and SOD had the same relationship with GSI (Supplementary Material, Table S1) suggesting possible links between

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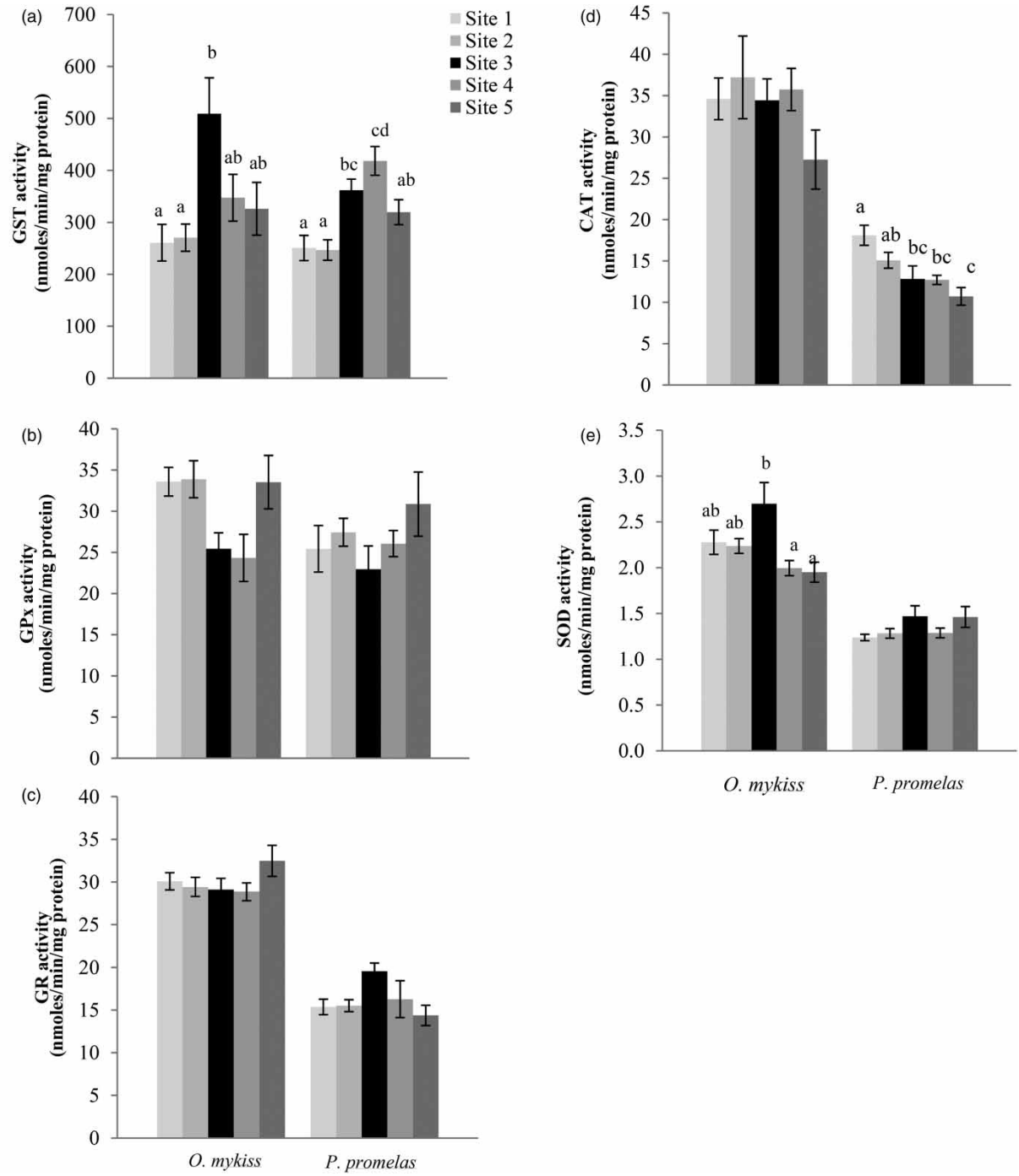

Figure 8 | Detection of antioxidant enzymes and ROS activity of (a) GST, (b) GPx, (c) GR, (d) CAT, and (e) SOD in the gills of juvenile RBT (*Oncorhynchus mykiss*) and male FHM (*Pimephales promelas*) caged in upstream reference (Sites 1 and 2) and exposure sites (Sites 3, 4, and 5) downstream of the discharge of the Kitchener municipal effluent in the Grand River in October of 2012 for 14 days.

these general indicators to health outcomes. As well Kendal tau analysis indicate some positive concordance ($p \geq 0.800$) between SOD and conductivity and $NO_2 + NO_3$ and GPx and pH, while a negative concordance was observed between VTG and alkalinity (Supplementary Material, Table S11). New genomic techniques such as metabolomics,

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transcriptomic, and pathway analysis may provide further insight to the potential effects in fish exposed to municipal effluents (Marjan *et al.* 2017b; Ekman *et al.* 2018; Lacaze *et al.* 2019).

CONCLUSION

MWTPs are point sources of a broad suite of substances including contaminants of emerging concern. Elevated estrogenic content of the effluents has been associated with changes in reproductive function, including severe intersex in wild fish in the Grand River (Tanna *et al.* 2013; Fuzzen *et al.* 2015, 2016). Although fish exposed to effluent *in situ* downstream of the Kitchener outfall were capable of responding (e.g., EE2 injections), they showed only minor responses to effluent exposure, including VTG which is often used as a biomarker of estrogen exposure. This may be due to the short duration of the cage exposure, dilution of effluent in the river water, low exposure to specific chemicals (e.g., estrogens), or other factors that altered the response (e.g., antiestrogens, water quality). Although *in situ* experiments can be a useful tool for assessing effects of wastewater, the results must be carefully interpreted as there are many factors that alter exposure and expression of effects in fish in the receiving environment. The inclusion of different approaches and endpoints is critical in building a weight of evidence to support future remedial actions if needed.

ACKNOWLEDGEMENTS

This project was funded through grants from Standards Development Branch, Ontario Ministry of the Environment and Climate Change (OMECC), the Canadian Water Network, Natural Sciences and Engineering Research Council, and Canada Research Chairs to MRS. Technical and laboratory support was provided through OMECC Laboratory Services Branch, Trent University (MDM), University of Ottawa (TWM), and Environment and Climate Change Canada (MEM).

DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

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First received 23 September 2020; accepted in revised form 9 April 2021. Available online 16 April 2021

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