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Behaviour (2019) DOI:10.1163/1568539X-00003576

Behaviour
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Proximity to wastewater effluent alters behaviour in bluegill sunfish (*Lepomis machrochirus*)

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Received 26 May 2019; initial decision 1 July 2019; revised 5 September 2019; accepted 9 September 2019

Abstract

Wastewater from municipal, agricultural and industrial sources is a pervasive contaminant of aquatic environments worldwide. Most studies that have investigated the negative impacts of wastewater on organisms have taken place in a laboratory. Here, we tested whether fish behaviour is altered by exposure to environmentally relevant concentrations of wastewater effluent in the field. We caged bluegill sunfish (*Lepomis macrochirus*) for 28 days at two sites downstream (adjacent to and 870 m) from a wastewater treatment plant and at a reference site without wastewater inputs. We found that exposed fish had a dampened response to simulated predation compared to unexposed fish, suggesting that fish may be at greater risk of predation after exposure to wastewater effluent. Fish held at the different sites did not differ in activity and exploration. Our results suggest that predator avoidance may be impaired in fish exposed to wastewater effluent, which could have detrimental implications for aquatic communities.

Keywords

stressors to aquatic ecosystem, fish behaviour, human-induced altered behaviour, contaminant exposure, wastewater treatment, effluent, risk-taking.

1. Introduction

Effluent released from wastewater treatment plants (WWTPs) has been globally recognized as a major source of aquatic contaminants (Luo et al., 2014).

DOI 10.1163/1568539X-00003576

These contaminants include persistent and biochemically active compounds, termed micropollutants, that can have profound impacts on the aquatic organisms living in environments receiving WWTP effluent. Micropollutants include pharmaceuticals and personal care products, pesticides, hydrocarbons, and many other natural and synthetic compounds, and they reach wastewater treatment plants from domestic, industrial, agricultural, and commercial sources (Mousel et al., 2017). Current WWTPs are not specifically designed to eliminate these micropollutants, which are thus released continuously into aquatic environments (Luo et al., 2014; Mousel et al., 2017). There are growing concerns regarding the negative impacts that wastewater contaminants may have on organisms residing in aquatic environments receiving treated effluent (Eggen et al., 2014). While our understanding of the impacts of contaminants on physiology, reproduction, and survival has been expanding, our knowledge about how wastewater effluent impacts behaviour is still in its infancy. Since behaviour is strongly linked to fitness and can often be a non-lethal indicator of disruption (Clotfelter et al., 2004), it is important to assess how wastewater effluent containing a complex mixture of micropollutants may affect key behaviours of aquatic organisms.

Only a handful of studies to date have attempted to address the impacts of treated wastewater on animal behaviour. The majority of these studies are controlled laboratory exposures focused largely on reproductive behaviours, and they have revealed mixed results about the impacts of wastewater. For example, male three-spined sticklebacks (*Gastrosteus aculeatus*) exposed to 50% and 100% treated wastewater for 21 days courted females less than unexposed control male fish (Sebire et al., 2011). In contrast, male mosquitofish (*Gambusia holbrooki*) collected from a wastewater exposed field site (chronic exposure) courted females more than fish collected from a clean reference site (Saaristo et al., 2014). Moreover, a study on male goldfish (*Carassius auratus*) found that exposure to 50% treated wastewater for 10 weeks did not influence courtship and spawning rates (Schoenfuss et al., 2002). Even fewer studies have explored the impacts of wastewater on non-reproductive behaviour, and these too have found varied results. For example, fathead minnows (*Pimphales promelas*) exposed to 100% wastewater effluent for 21 days were less aggressive than control fish, which could impact their ability to compete and to acquire and retain nests (Martinović et al., 2007; Garcia-Reyero et al., 2011), while round goby (*Neogobius melanostomus*) exhibited no change in aggression after exposure to wastewater effluent *in situ* (McCallum et al., 2017).

To the best of our knowledge, only two studies thus far have exposed fish (by caging) to WWTP effluent in the field (McCallum et al., 2017; Simmons et al., 2017). Male goldfish (*Carassius auratus*) caged downstream of a WWTP outfall were more active, more exploratory (crossed more unique grid cells) and took less time to resume motion after a startle than goldfish exposed to a reference site lacking WWTP inputs (Simmons et al., 2017). In contrast, round goby caged downstream of a WWTP exhibited no change in aggression or dispersal movement following exposure (McCallum et al., 2017). In general, the small number of studies conducted thus far, often using different species, different exposure durations and concentrations, and different effluent sources (all from different WWTPs), make it challenging to compare or generalize across studies.

Given that many behaviours can influence fitness, additional studies that explore the impacts of treated wastewater on behavioural endpoints are necessary and could provide tools for assessing sub-lethal effects of wastewater exposure. To assess if exposure to treated wastewater in environmentally realistic scenarios impacts the behaviour of fish in receiving waters, we caged bluegill sunfish (*Lepomis microchirus*) for 28 days at two distances from a WWTP outflow source, and we compared the behaviour of these fish to sunfish caged at a reference location that does not receive WWTP effluent. We used bluegill because it is a (i) well studied, (ii) widely distributed species found throughout freshwater ecosystems in North America, and (iii) is part of an economically important recreational fishery across most of its range (Werner & Hall, 1974; Gross & Charnov, 1980; Janssen, 1982). Bluegill are also an important species linking the trophic cascades of many freshwater aquatic communities (Mather et al., 1995; Carey & Wahl, 2010). To quantify behavioural changes resulting from exposure to treated wastewater, we targeted three key behaviours: (1) latency to exit a refuge as a measure of risk-taking in unfamiliar environments; (2) general movement in the absence of stimuli to represent overall baseline activity; and (3) response to a startle as a measure of risk-taking in familiar environments. These behaviours have been associated with growth, survival, and reproduction in other species (Dall et al., 2004; Bell, 2005; Smith & Blumstein, 2008; Belgrad & Griffen, 2016). We predicted that fish exposed to treated wastewater would (i) be less active and (ii) take longer to leave a refuge, due to the metabolic costs of wastewater exposure (Du et al., 2018; Mehdi et al., 2018), and would (iii) be less likely to respond to a startle, because exposure to treated wastewater can

impair sensory and cognitive systems important for predator avoidance (Pottinger et al., 2016; Heerema et al., 2018). We also tested if the behaviours were repeatable over time and if they were correlated, which would provide evidence of personality (Réale et al., 2007) and behavioural syndromes (Sih et al., 2004), respectively.

2. Methods

2.1. Fish collection and housing

Between 19 and 21 May 2016, we collected bluegill sunfish using a seine net in Opinicon Lake at Queen's University Biological Station (QUBS) near Kingston, Ontario, Canada. We collected fish from this site as it does not receive effluent from a WWTP facility. After capture, fish were placed in aerated coolers and transported to McMaster University where they were held in 500-litre recirculating holding tanks (L 1.2 $\times$ D 0.9 m) with charcoal filters in 19°C room temperatures and a 12:12 light/dark light cycle. Fish were fed four times a week with a combination of beef heart and squid purchased from a local grocery store. We housed all fish for fourteen days to ensure they were healthy and feeding regularly before we deployed them in the field. On average, fish deployed in cages were 8.99 $\pm$ 0.23 cm long (standard length) and 20.33 $\pm$ 1.73 g in body mass.

2.2. Caging exposure

We caged fish at three locations at varying distances from the outflow pipe of the Wastewater Treatment Plant located in the community of Dundas in Hamilton, Ontario, Canada (43°16′03.0″N, 79°56′37.2″W). This facility is a conventional activated sludge plant with tertiary sand filtration. The facility's treated effluent is released into the western-most end of the Desjardins Canal, and is the main source of water flowing into the canal, combined only with the minor input from a small drainage channel (Figure 1). Characterizing the impacts of this effluent in the canal is of special interest because the canal flows into Cootes Paradise Marsh, the largest coastal wetland on Lake Ontario that serves as important fish spawning and nursery habitat and a migratory stopover for birds (Hamilton Harbour Remedial Action Plan, 1992). Remediation of the wetland has been ongoing since 1993 following significant reductions in water quality due to combined sewer overflows and continued wastewater effluent release (Mayer et al., 2008; Thomasen &

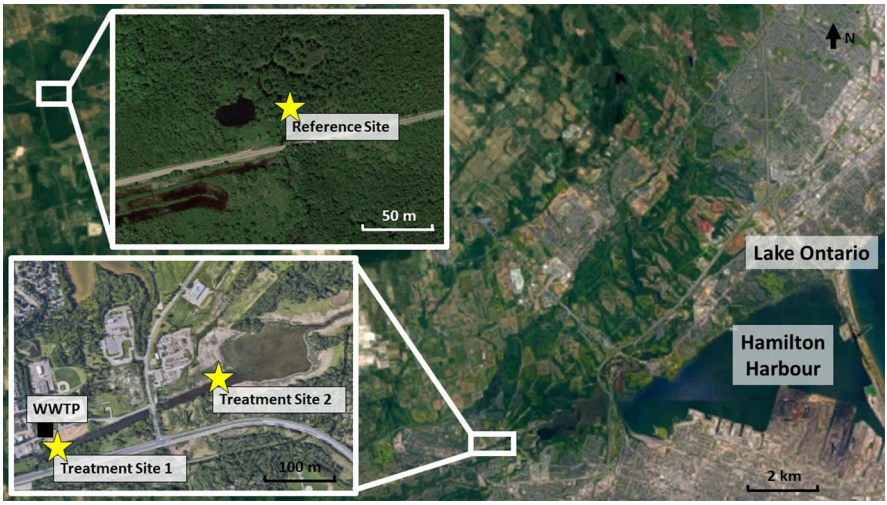

Figure 1. An aerial view of our caging locations (yellow stars), including our treatment sites near Cootes Paradise Marsh on the western end of the Hamilton Harbour in Lake Ontario and our reference site at Beverly Swamp in Flamborough, ON, Canada. The Wastewater Treatment Plant (WWTP) is indicated by the black square. Map data from Google (2018). Image adapted from McCallum et al. (2017).

Chow-Fraser, 2012). The first exposure site (hereafter referred to as Treatment site 1) was adjacent to the effluent outfall in the Desjardins Canal. The second site (hereafter referred to as Treatment site 2) was approximately 870 m downstream from the first site where the canal meets a pond known locally as West Pond (Figure 1). Our final caging site was located 17.4 km upstream from Treatment site 1 in Beverly Swamp in Flamborough, ON, Canada and served as our reference site. This site is part of the same watershed and does not receive effluent from any wastewater treatment facilities (Hamilton Conservation Authority, 2009).

Four cages were deployed at each of these sites before fish were added. The cages were 114-litre plastic totes (H 51 × W 81 × D 44.5 cm; Rubbermaid) with approximately 200 0.5 cm holes to allow water exchange. Each cage was equipped with a cylindrical piece of polyethylene foam (Water Noodle, Canadian Tire™, Hamilton Ontario) encircling the lid to keep the top of the cage (about 5 cm of the lid) above the waterline. The remainder of each cage was submerged. Cages were anchored in place using cement cinder blocks attached to each cage using galvanized chain. We staggered the deployment of fish over four weeks (one deployment per week) from 1

June to 22 July 2016 to allow time to conduct behavioural experiments on fish immediately following a four-week exposure. Each week, we placed 14 fish into a single empty cage at each site, creating four replicate cages per site. Fish were monitored weekly for health and survival, and were given supplementary food consisting of an approximately 100 g blend of fish meat from a local grocery store (labelled as 'salmon'). Fish were fed once weekly as a supplement because natural sources of food (e.g., zooplankton, aquatic insect larvae) could easily move in and out of the cages. The fish used in this study (and those described in another companion paper, Du et al. (2019) held in the same cages) had good body condition based on visual observation and were not malnourished when brought into the lab after four-week exposures with weekly supplementary feeding. Water temperature, pH, conductivity, total dissolved solids, salinity (Oakton Multi-Parameter Pocket Tester) and dissolved oxygen (WTW Oxi 3310 SET 2) were also measured weekly. To quantify pharmaceutical and personal care product (PPCP) concentrations at our sites, we deployed passive polar organic chemical integrative samplers (POCIS) at each site. Samplers were placed in empty cages identical to those holding fish and remained at each site for two weeks (from 21 June to 8 July 2016), on dates that overlapped with our fish deployment (POCIS-HLB, Environmental Sampling Technologies; Alvarez, 2010). After four weeks of being caged, fish were transported to McMaster University in aerated coolers. Fish were given 17–26 h in 40-litre tanks ($33 \times 51 \times 28$ cm) containing clean water (dechlorinated City of Hamilton tapwater) to recover from transport before behavioural trials commenced. In total 46 bluegill sunfish were subjected to behavioural testing, including 20 from the reference site, 15 from Treatment site 1, and 11 from Treatment site 2.

2.3. Water quality sampling

POCIS samplers (POCIS-HLB, Environmental Sampling Technologies, Alvarez, 2010) were collected after two weeks of field exposure and placed on ice for transport to McMaster University where they were stored at -20°C until they were analysed for PPCPs following the methods outlined in Metcalfe et al. (2014) and McCallum et al. (2017).

2.4. Quantifying behaviour

Each trial consisted of three experimental assays that were conducted in an indoor controlled lab environment. Sequentially, we measured (i) time to exit

a refuge, (ii) activity, and (iii) response to a marble being dropped into the centre of the tank for each fish. All trials were run between 0700 and 1900 (daylight hours) because bluegill sunfish are primarily diurnal (Reynolds & Casterlin, 1976). Fish were tested in a 92 × 45 × 42 cm (L × W × D) aquarium, lined with a thin layer of gravel, an aquarium filter, and an aeration stone and filled to water depth of 30 cm. A grid was drawn across the front and long side of the tank (Figure 2) with 10 × 10 cm cells which provided a simple way to score movement (see below).

Behavioural trials were performed under dim overhead lights to minimize stress (Jones, 1956; Owen et al., 2010) but still provide sufficient lighting to observe fish behaviour. Each trial began when an individual fish was gently guided from the holding tank with a hand-net into a 7.6 cm diameter by 18 cm long black acrylic (ABS) tube mounted on a 10 × 15 cm acrylic base that served as a refuge. One end of this refuge was blocked by a perforated black acrylic sheet. To ensure a degree of familiarity with such a refuge, ABS tubes of the same length and diameter were placed in each holding tank during the transport recovery period (see above). Once fish were in the refuge, the open end was closed off by a black acrylic sliding door. The refuge, with the fish inside, was transported to the test aquarium in a large container of water and gently placed on one end of the testing tank. Fish were then given 30 minutes to acclimate inside the refuge and the trial began when the sliding door was remotely lifted, leaving the entrance open. Fish behaviour was then recorded using video cameras (Canon Vixia HFS100 8.0 Megapixel).

For the first behavioural assay (Figure 2A), we measured the time (in s) taken for the focal fish to exit the refuge once the door was lifted. The test fish was considered to have exited once all of its body, including caudal fin, was outside of the refuge. If the test individual had not exited after 30 min (1800 s), it was assigned a time of 1800 s and the refuge was remotely lifted, gently forcing the fish to swim downwards and out to exit. The entire refuge was then remotely removed to prevent the fish from re-entering. Fish were given five minutes after the refuge was removed to recover before the second behavioural assay commenced.

In the second behavioural assay (Figure 2B), we determined the overall activity of individual fish by recording the number of times each fish crossed from one grid cell into another over a 600-s period. A fish was considered to have crossed into a grid cell when its operculum was observed to cross the

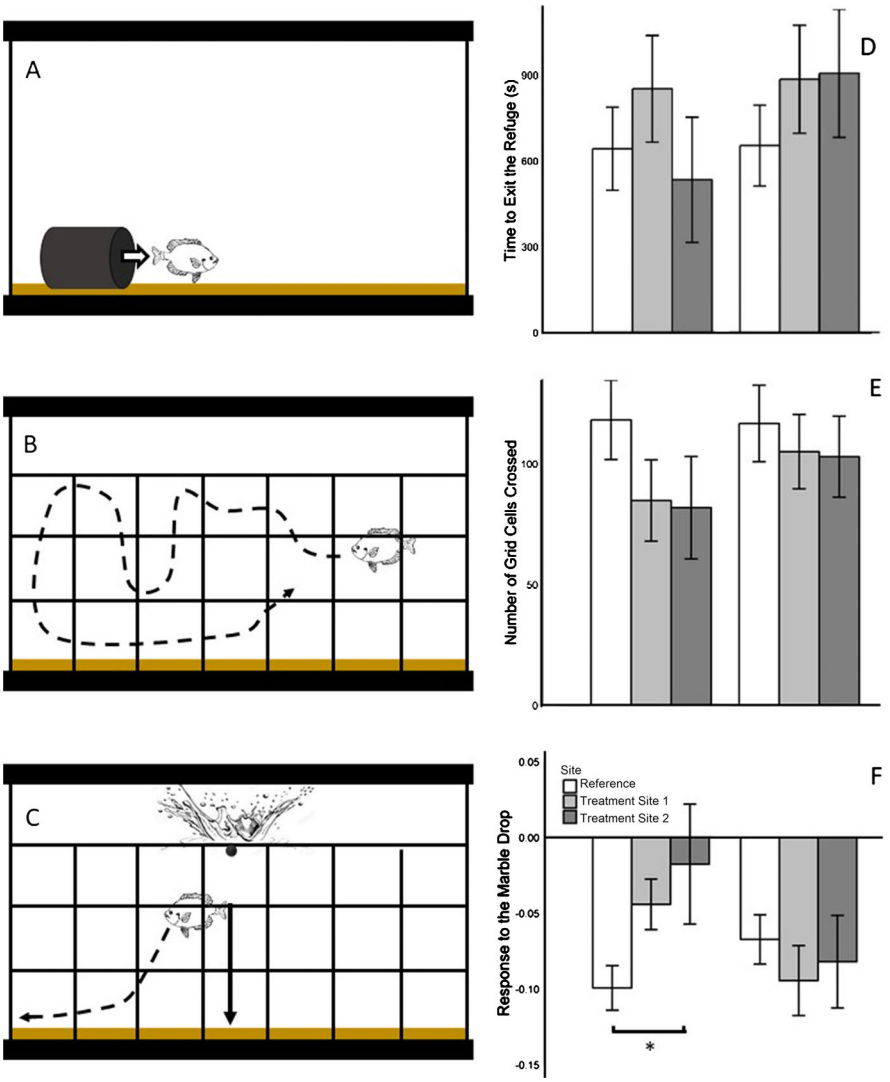

Figure 2. Schematics on the left illustrate the test arena and how (A) time taken to exit a refuge, (B) activity and (C) the response to something startling were measured. Figures on the right illustrate the average (D) time taken to exit the refuge in seconds, (E) number of grid cells crossed and (F) behavioural response to the marble drop, both one day (left) and one week (right) after exposure. The site fish were caged at is indicated by the legend. Error bars represent ± 1 standard error. An asterisk (*) denotes significant differences based on a LMM.

gridline. On average, fish were of a similar size to a single grid cell and were rarely stationary within a defined grid cell.

In the third behavioural assay (Figure 2C), we determined the response to simulated predation. We startled the fish by rolling a white opaque marble (1.25 cm diameter) into the centre of the aquarium from a PVC pipe fixed 40 cm above the centre of the aquarium. The marble drop causes a splash and concentric rings to form in the water much like the strike of a bird beak hitting the water surface. This method of simulated predation has been used in other studies and produces a standardized startle stimuli (Colson et al., 2015; Laubu et al., 2017; McCallum et al., 2017; Poisson et al., 2017). Following the marble drop, we quantified the change in activity of focal fish by subtracting the number of grid cells crossed per second for each individual during the 5 min that followed the marble drop from the grid cell crosses per second in the 10-min activity protocol. We chose this 5-min period for analysis because preliminary observations of several fish suggested that they return to pre-startle activity within this period of time. At the end of the third behavioural assay, the focal fish was removed from the aquarium and returned to its holding tank containing clean water. Videos were scored following the completion of all behavioural trials by an observer blind to the site where fish were caged.

Focal fish were retested using the same behavioural protocols described above six days after the initial tests. By retesting fish, we could quantify whether there were any changes in fish behaviour after being held in clean water in the laboratory for six days. However, three fish (two from the sites receiving wastewater effluent and one from the reference site) could not be retested because they died following the first set of experiments. Despite this, there were no obvious signs of injury, disease, or stress based on visual inspection of the fish. Once a second trial was complete, fish were euthanized and standard length (mm) and body mass (g) were measured. In most cases, fish were juveniles and could not be sexed (28/46 cases); however, fish were sexed whenever possible (1 female and 17 males).

2.5. Statistical analyses

We used univariate linear mixed models (LMMs) to test whether fish caged at sites receiving wastewater effluent differed in the time to exit a refuge, activity, and the response to startle as compared to individuals held at the reference site when tested one day after the four-week exposure and then

again after being held for one week in clean water. Individual values of time to exit the refuge, number of grid cells crossed (activity), or the change in the number of grid cells crossed per second following the marble drop (response to simulated predation attempt) was the dependent variable for each model, caging site (reference site, Treatment site 1 or Treatment site 2) and time since exposure (one day, or one week), were the independent treatment variables. An interaction term between caging site and time since exposure was also included to test if the relationship between caging site and behaviour differed when fish were held one day after exposure compared to one week. Body mass was included as a fixed covariate. Cage deployment date and individual identity were included as random effects. Post-hoc pairwise comparisons across sites and time since exposure were conducted using differences of least-squares means.

We then estimated repeatability for each behavioural measure across all individuals, regardless of treatment site, using the intraclass correlation coefficient, $r = \sigma_a^2/(\sigma_a^2 + \sigma_w^2)$, where r is the intraclass correlation coefficient, σ_a^2 is the variance component estimated among individuals and σ_w^2 is the variance component estimated within individuals (residual variance). For each behavioural measure, the variance components were estimated using mixed effects models in the ICC package in R (Wolak, 2016). In each univariate analysis, the response variable was a behavioural measure and the predictor variable was individual identity, which was modeled as a random effect. Intraclass correlation coefficients were considered statistically significant if their 95% confidence intervals did not encompass zero (Wolak, 2016). We also tested if the time taken to exit the refuge was correlated with activity and response to the startle using two univariate LMMs where time taken to exit the refuge was the response variable and either activity or the response to the startle was the predictor variable. Time since exposure and mass were included as fixed covariates. Cage deployment date and individual identity were included as random effects. We did not test if activity and response to the startle were correlated because the response to the startle was quantified using the activity measure and thus the two measures are not independent.

In all analyses, time to exit the refuge was base-10 log-transformed, the number of grid cells crossed was square-root transformed, and the response to the marble being dropped was not transformed to meet assumptions of normality. We used the Satterthwaite approximation to estimate parameter specific p-values using the R package lmerTest (Kuznetsova et al., 2017).

Differences between water quality parameters measured weekly across sites were assessed using multivariate analysis of variance (MANOVA) where pH, salinity, conductivity, dissolved oxygen, total dissolved solids and water temperature ($N = 8$ for each parameter at each site) were the response variables and the site was the predictor variable. The effect of each water quality parameter was then evaluated using analysis of variance (ANOVA). Additionally, differences in survival measured weekly between sites were assessed using a generalized linear mixed model assuming a binomial distribution with cage and experimental week set as random effects and caging site as a fixed effect. We used likelihood ratio tests to test for main effects of our fixed predictor variable (caging site).

All statistical analyses were conducted in R version 3.1.0 (R Core Team, 2017). Figures were plotted using the package gpplot2 (Wickham, 2016) and LMMs were run using the package lme4 (Bates et al., 2014).

2.6. Ethical note

All methods for collecting and handling bluegill were approved by McMaster University's Animal Research Ethics Board (Animal Utilization Protocol 13-12-51) and adhere to the standards of the Canadian Council on Animal Care.

3. Results

3.1. Water quality

Water quality parameters differed significantly between the sites (MANOVA: $F_{2,21} = 3.65$, $p = 0.001$). Specifically, salinity (ANOVA: $F_{2,21} = 104.8$, $p < 0.0001$), conductivity (ANOVA: $F_{2,21} = 95.07$, $p < 0.0001$), total dissolved solids (ANOVA: $F_{2,21} = 95.37$, $p < 0.0001$), and water temperature (ANOVA: $F_{2,21} = 7.45$, $p = 0.004$) were higher at the sites receiving wastewater effluent (Table 1). Dissolved oxygen (ANOVA: $F_{2,21} = 2.44$, $p = 0.11$) and pH (ANOVA: $F_{2,21} = 0.41$, $p = 0.67$) were similar between these sites (Table 1).

Eighteen of the 24 targeted PPCP compounds were detected using the POCIS samplers (see Table A1 in the Appendix; note that these data are partially reproduced in Du et al., 2019). Sixteen of the 18 compounds were found at Treatment site 1, and all eighteen were detected at Treatment site 2 while only two compounds were found at the reference site (sucralose,

Table 1.
Average water quality measured at each site during weekly point sampling (± SE).

	Reference	Treatment site 1	Treatment site 2
pH	8.16 (± 0.15)	7.91 (± 0.17)	7.97 (± 0.27)
Salinity (ppm)	368.75* (± 7.56)	567.25* (± 12.91)	568.88* (± 13.32)
Conductivity (S/m)	765.38* (± 15.00)	1150.75* (± 24.39)	1148.75* (± 27.10)
Dissolved oxygen (mg/l)	6.63 (± 0.21)	11.90 (± 3.16)	11.87 (± 1.82)
Total dissolved solids (ppm)	542.38* (± 11.14)	815.38* (± 17.31)	815.75* (± 18.94)
Water Temperature (°C)	17.35* (± 1.21)	20.81* (± 0.85)	22.63* (± 0.83)

Asterisks indicate water quality parameters that differed significantly between sites.

an artificial sweetener, and gemfibrozil, an oral drug used to lower lipid levels). Interestingly, Treatment site 1 had lower concentrations of certain compounds (e.g. food additives, beta blockers) compared to Treatment site 2; however, these levels were still much higher than at the reference site (Figure 3). Treatment site 2 had some of the highest concentrations of certain pharmaceuticals (e.g. carbamazepine, food additives) despite it being further away from the WWTP outflow site than Treatment site 1, suggesting these compounds degraded little over the 870 m distance from the outfall pipe.

3.2. Survival

Exposure to wastewater and, more specifically, the distance from the WWTP outflow, did not significantly reduce bluegill survival (Binomial GLMM: LRT_{site}: $\chi^2 = 1.56$, $N_{\text{cages}} = 12$; $p = 0.46$), but survival was highest on average in fish from the reference site and lowest in fish caged at Treatment site 2 (Figure 4; note that these data are partially reproduced in Du et al., 2019).

3.3. Behaviour

Exposure to wastewater effluent altered the behaviour of fish when tested one day after the four-week exposure period for one of the behaviours quantified. Fish held at the reference site did not differ in the time required to exit the refuge compared to fish held at Treatment site 1 ($\beta = 0.51$, SE $= 0.27$, $t = 1.92$, $p = 0.06$; Figure 2D) and Treatment site 2 ($\beta = 0.12$, SE $= 0.24$, $t = 0.50$, $p = 0.62$). Nor did they differ in their activity, as measured by grid crosses, compared to fish held at Treatment site 1 ($\beta = 1.98$, SE $= 1.31$, $t = 1.51$, $p = 0.19$; Figure 2E) and Treatment site 2 ($\beta = 1.54$, SE $= 1.17$, $t = 1.31$, $p = 0.14$). However, the change in activity after the marble was

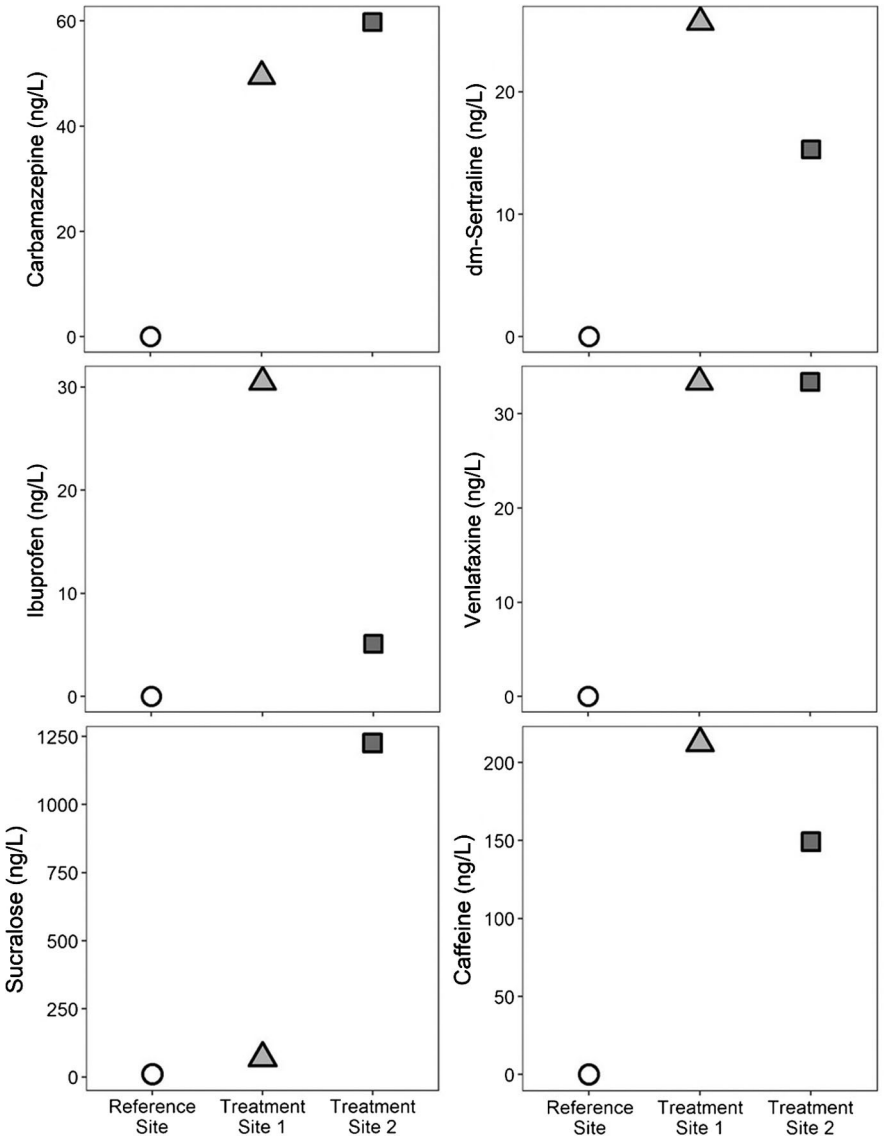

Figure 3. The concentration of the six most abundant PPCP substances found at the three study sites. Treatment site 2 and Reference Site data have been previously published in Du et al. (2019).

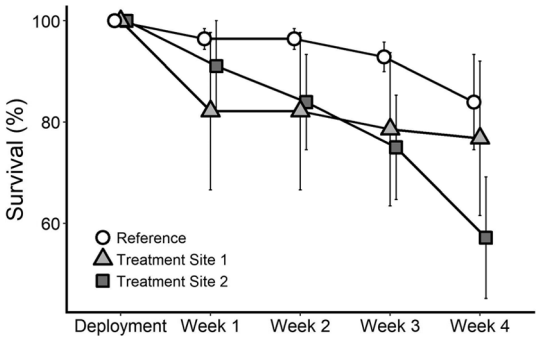

Figure 4. Average cumulative percentage survival of Bluegill Sunfish plotted by exposure week and exposure site. Error bars represent ±1 standard error. Note that these data are partially reproduced in Du et al. (2019).

dropped varied across treatment groups, where fish caged at the reference site showed a stronger decrease in activity after the marble was dropped compared to fish held at Treatment site 2, located 870 m from the outflow from the WWTP ($\beta = -0.08$, SE $= 0.03$, $t = 2.31$, $p = 0.02$; Figure 2F). However, fish at the reference site did not differ from fish held at Treatment site 1, adjacent to the outflow location ($\beta = -0.04$, SE $= 0.03$, $t = -1.52$, $p = 0.13$) and fish held at Treatment site 1 did not did not differ compared to fish held at Treatment site 2 ($\beta = -0.03$, SE $= 0.04$, $t = -0.91$, $p = 0.37$).

Fish had recovered from the above effects of exposure to wastewater effluent when fish were retested after one week of exposure to clean water. We found no differences between fish held at the refuge site compared to those held at treatment sites 1 and 2 in the time required to exit the refuge ($\beta = -0.29$, SE $= 0.30$, $t = -0.96$, $p = 0.34$ and $\beta = -0.13$, SE $= 0.24$, $t = -0.54$, $p = 0.59$, respectively; Figure 2D), the number of grid cells crossed ($\beta = 1.05$, SE $= 1.52$, $t = 0.69$, $p = 0.49$ and $\beta = 0.51$, SE $= 1.21$, $t = 0.42$, $p = 0.68$, respectively; Figure 2E), or the response to the marble being dropped ($\beta = -0.01$, SE $= 0.04$, $t = -0.22$, $p = 0.83$ and $\beta = 0.02$, SE $= 0.03$, $t = 0.73$, $p = 0.44$, respectively; Figure 2F).

Estimates of repeatability suggested that individual fish were consistent in their time to exit a refuge (r $= 0.52$, $CI_{95} = 0.26$–0.70) and activity ($r = 0.35$, $CI_{95} = 0.05$–0.58) between tests run one day and one week after exposure. Not surprisingly, given the recovery of startle behaviour after one week of recovery, there was poor intra-individual repeatability in the response to marble drop between tests run one day and one week after exposure ($r =$

0.03, $CI_{95} = -0.30$–0.32). Additionally, fish that exited the refuge more quickly were also more active ($\beta = -1.84$, SE = 1.54, $t = -3.59$, $p = 0.0006$), but time to exit the refuge was not correlated with response to the startle ($\beta = -0.83$, SE = 0.81, -1.02, $p = 0.31$).

4. Discussion

Our study revealed two main findings. First, that bluegill caged in natural areas receiving WWTP effluent showed a dampened response to a simulated predation event. Second, that this behavioural disruption disappeared after fish recovered in clean water in the lab for one week. This result is consistent with one of our predictions. One interpretation of this result is that bluegill sunfish at sites receiving wastewater effluent may not respond appropriately to predation attempts, as they showed little to no change in activity after a startle. No other behavioural traits changed with exposures. We also found that bluegill sunfish were consistent in their time to exit a refuge and their general activity between the first and second trials and that fish that exited a refuge more quickly were more active, consistent with earlier studies demonstrating personality in these traits in bluegill (Wilson & Godin, 2009; Wilson et al., 2011). However, bluegill were not consistent in their response to the startle between the first and second trials and this response was not correlated with time to exit a refuge. This is likely because individuals caged at the WWTP exposure sites showed little to no response to the startle response test within 24 hours of being tested, but had recovered in the follow-up trial one week later after being held in clean water.

Our finding that animals caged at sites receiving treated wastewater differ in response to predation cues is consistent with a few recent studies. Frog tadpoles (*Rana pipiens*) exposed to treated wastewater did not show avoidance behaviour in response to a predator cue compared to tadpoles held in control conditions that exhibited normal avoidance behaviour (Heerema et al., 2018). McGee et al. (2009) found evidence that larval fathead minnows have reduced predator response when exposed to concentrations of estrogens found in treated wastewater. Weinberger & Klaper (2014) found that adult fathead minnows exposed to fluoxetine — a serotonin reuptake inhibitor that is found in treated wastewater — did not react to a mock predator. Mosquitofish exposed to fluoxetine had reduced freezing behaviour following a simulated predator strike and they entered a predator 'strike zone' more rapidly than

those that were not exposed (Martin et al., 2017). Male goldfish caged downstream of a WWTP outfall took less time to resume motion after a startle compared to fish from a reference site lacking WWTP inputs (Simmons et al., 2017). Therefore, there appear to be pervasive effects of exposure on startle behaviour in many species, that can be detected using relatively small numbers of individuals (sample sizes generally range from 10-20 individuals in our study and in these other studies). Fish exposed to WWTP effluent are known to accumulate pharmaceuticals in the brain, which is one of the organs with the highest burdens of some contaminants (McCallum et al. 2017), suggesting that neural disruption may be ultimately responsible for changes in behaviour. Nevertheless, although this research suggests that organisms living in aquatic environments receiving WWTP effluent might be at higher risk of predation, there is evidence suggesting that predators that also live in these environments may be worse at capturing prey (Bisesi et al., 2014). Future research should focus on how these impacts of chronic contaminant mixtures on fish behaviour might alter the function of aquatic communities.

The impacts of exposure to WWTP effluent and the pharmaceuticals therein on fish exploration and activity are less clear. Our study found that WWTP effluent had little effect on these traits, but that has not been the case in some previous studies. For example, Simmons et al. (2017) found that goldfish caged downstream of a WWTP outfall were more active and more exploratory than those at a reference site. In contrast, *Daphnia pulex* exposed to treated wastewater decreased swimming distance compared to those held in control water (Zein et al., 2015). Interestingly, in a study exposing tadpoles to concentrations of triclosan, caffeine, and acetaminophen (all of which are found in treated wastewater), only those tadpoles exposed to triclosan had lower activity and reduced startle response, while tadpoles exposed to caffeine had highest activity and increased startle response (Fraker & Smith, 2004). This last result suggests that exposure to a mixture of contaminants could lead to synergistic, additive, or antagonistic effects, and could explain why changes in activity were not observed in our study.

We assayed behaviour in the lab in clean water so we could compare between exposure treatments in common conditions. The advantage of this approach is that it excludes any acute behavioural changes associated with being in contaminated water, and focusses on the persistent behavioural disruption that results from chronic exposure. However, our results suggest that

fish behaviour can recover after one week in clean water, and it is possible that there was some more modest recovery of behaviour during the first 24 hours in clean water before our first test period. Despite this, we still observed that fish held at the sites receiving treated wastewater differed in behaviour from those held in the reference site that did not receive treated wastewater. It will be useful in future studies to distinguish whether the testing environment affects behavioural outcomes, thus distinguishing the acute versus persistent effects of being in contaminated water, and to more finely discern how long the impacts of WWTP exposure last in a clean environment, which has implications for recovery potential of contaminated systems.

The levels of contaminants and the behavioural changes that we observed varied between the two WWTP exposure sites. Interestingly, the treatment site located 870 m downstream of the WWTP outfall had higher levels of some contaminants compared to the treatment site located immediately adjacent to the outfall pipe. In previous caging experiments conducted at these sites in 2015 (see McCallum et al., 2017), contaminant concentrations were highest at the treatment site closest to the outfall, as expected. This difference between our study and the study conducted by McCallum et al. (2017) may be explained by small differences in the caging location of the treatment site closest to the WWTP outfall between the studies. The major inputs for these sites are the WWTP outfall and a small surface water drainage ditch that inputs upstream of the outfall pipe. In our study, the cages placed at the treatment site nearest the outfall was nearer to the drainage ditch and adjacent to the outfall pipe, while the cages used in the McCallum et al. (2017) study were placed downstream of the outfall pipe and further from the drainage ditch. Thus, the caging location at the outfall treatment site used in our study may have been affected by inputs of cleaner water from the drainage channel, as the relative flow of receiving waters affects the dilution of effluent and the resultant exposure concentrations. Despite this, there were still signs of behavioural disruption at both treatment sites, as fish startle response differed between sites (based on the significant main effect) and the average startle response was similar between treatment sites.

It is possible that some of the water quality differences between our sites might have affected our results. Our experimental design standardized many factors that were within our control, but carrying out exposures at different sites in the field makes it impossible to control all variables other than the

presence or absence of micropollutants. For example, slight site differences in temperature (Kinouchi et al., 2007), nutrient concentrations (Carey & Migliaccio, 2009), and dissolved oxygen levels (Smith et al., 1999), including pronounced oxygen level fluctuations in eutrophic systems (Domenici et al., 2007), could alter fish physiology and behaviour, and interact with the effects of effluent exposure. Whereas field exposures to WWTP effluent are critical to understanding impacts in a real-world setting, controlled lab exposures will be critical for understanding the interactive impacts of exposure to contaminants and other stressors on aquatic organisms.

The discharge of WWTP effluent remains one of the largest sources of contaminants to aquatic environments, and can severely degrade water and habitat quality for fish and other wildlife. Our study adds to the growing recognition that these complex mixtures are altering the behaviour of aquatic species living in receiving waters. Only by recognizing how exposures might generate behavioural deficits and pinpointing how these deficits might be linked to fitness in wild populations can we begin to make suggestions about regulatory practices for wastewater treatment that will mitigate real-world impacts on aquatic animals.

Acknowledgements

The authors would like to thank Naylor Lobban, Jennifer Bowman, Tys Theysmeyer, Andrea Court, Kyle Mataya, Theresa Warriner, Noah Houpt, Lianne Ralph and Nathasha Leadbetter for lab and field assistance. We would also like to thank Pat Chow-Fraser, Marten Koops, Mark Bainbridge and Bert Posedowski for their support. This work was financially supported by a Natural Sciences and Engineering Research Council of Canada (NSERC) Discovery Grant to S. Balshine, a Royal Bank of Canada Bluewater grant to S. Balshine and G.R. Scott, a NSERC Engage Plus Grant to G.R. Scott, and support from the City of Hamilton. G.R. Scott is supported by the Canada Research Chairs Program.

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Appendix

Table A1.
Summary of average PPCPs using POCIS samplers ($N = 3$ replicates per site).

Compound	Class	Estimated time-weighted concentration (ng/l)		
		Treatment site 1	Treatment site 2	Reference site
Caffeine	food	212.6	149.2	ND
Sucralose	food	70.2	1226.4	10.24
Trimethoprim	anti-biotic	ND	ND	ND
Sulfamethoxazole	anti-biotic	ND	ND	ND
Carbamazepine	anti-seizure	49.5	59.8	ND
Acetaminophen	analgesic	63.9	11.9	ND
Ibuprofen	anti-inflammatory	30.5	5.1	ND
Gemfibrozil	lipid regulator	0.7	0.9	0.3
Naproxen	anti-inflammatory	ND	ND	ND
Triclosan	antibacterial	ND	ND	ND
Estrone (E1)	hormone	ND	ND	ND
Estradiol (E2)	hormone	ND	ND	ND
Androstenedione	hormone	0.08	0.05	ND
Testosterone	hormone	0.2	0.3	ND
Venlafaxine	antidepressant	33.4	33.4	ND
O-dm-venlafaxine	metabolite	3.6	3.4	ND
N-dm-venlafaxine	metabolite	8.9	9.5	ND
Sertraline	antidepressant	5.5	8.0	ND
dm-sertrailne	metabolite	25.7	15.3	ND
Citalopram	antidepressant	1.7	0.5	ND
Fluoxetine	antidepressant	0.1	0.03	ND
Atenolol	beta-blocker	2.7	3.3	ND
Metoprolol	beta-blocker	ND	4.3	ND
Propanolol	beta-blocker	ND	20.9	ND

Estimated time-weighted PPCP concentrations from the POCIS samplers were derived from sampling rates previously reported in the literature. ND indicates 'not detected'. Data from Treatment site 2 and Reference Site have been previously published in Du et al. (2019).