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Occurrence, environmental impacts and removal of legacy and emerging contaminants from two wastewater and one water treatment plant in Southern Ontario. Part II: environmental impacts

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Abstract

This is part two of a paper about the potential environmental impacts of treated effluent from a wastewater treatment plant (WWTP) discharging to the Detroit River in Windsor, Ontario, Canada. The WWTP uses conventional activated sludge with nitrification.

The assessment was conducted over six months using a variety of established tests, including *in vitro* cell-based screening assays, as well as acute, chronic and full-life cycle *in vivo* exposures. Effluent monitoring included pharmaceutically active compounds and endocrine disrupting compounds.

No tests reported significant toxicity. However, enhanced algal growth was observed in a *Pseudokerchneriella subcapitata* growth inhibition test. In full life-cycle fathead minnow exposure, liver-somatic index changes were noted in exposed fish – increases for males, decreases for females – and production of viable fry decreased. Neither alteration is thought biologically significant. Because the effluent is diluted substantially by the receiving water, the level of risk posed to aquatic receptors and the environment is probably negligible.

Key words: emerging contaminants, endocrine disrupting compounds (EDCs), pharmaceutically active compounds (PhACs), wastewater treatment, *in vitro* assay, fish life-cycle exposure

INTRODUCTION

This is the second of two papers on the occurrence, removal, and assessment of environmental impacts of many emerging and legacy contaminants, in water sources and drinking water. The study comprised two sets of experiments including occurrence and removal of endocrine disrupting compounds (EDCs) and pharmaceutically active compounds (PhACs) at two wastewater treatment plants (WWTPs) and one WTP in Windsor, Ontario, Canada (Part I), and the environmental impacts of the effluent from one of the WWTPs, referred to as WWTP-1 (this paper).

Although WWTPs are not designed to treat PhACs/EDCs, a portion of the substances is removed or transformed by conventional processes. However, a number of PhACs/EDCs persist through the treatment processes and are discharged with the final effluent. Even if the substances degrade or transform quickly, continuous discharge can result in pseudo-persistence in aquatic environments. Such discharge may harm the environment.

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The impacts of PhACs and EDCs in the environment have been widely noted. Examples include feminization of fish (Purdom *et al.* 1994), reproductive abnormalities in fish (Kidd *et al.* 2007), and stress on cyanobacteria and duckweed (Pomati *et al.* 2004).

Knowledge of the effects of PhACs/EDCs mixtures on ecological receptors is also limited. For example, it has been observed that PhACs mixed at individual 'no-effect' concentrations can have effects (Flaherty & Dodson 2005).

The toxicity of transformation by-products is also of concern (and often unknown). For example, UV photo-transformation by-products of diclofenac were shown to cause a 5- to 6-fold increase in toxicity to the green algae *Scenedesmus vacuolatus* (Schmitt-Jansen *et al.* 2007).

In this project, the potential environmental impacts of the target contaminants present in WWTP effluent were studied using a number of tests including fathead minnow full life-cycle exposure. Standard acute and chronic toxicity tests, *in vitro* yeast estrogenic screening (YES) and yeast androgenic screens (YAS), and thyroid disrupting screening (T4/hTTR) assays were carried out.

Long-term biological exposures combined with major effluent trace-chemistry have never been reported or published. Some controlled long-term exposures to WWTP effluent have been investigated by Environment Canada. However, many of the studies assessed the biological effects without detailed knowledge of the chemistry or composition of the effluent (J. Parrott, Environment Canada, unpublished data).

Fish life-cycle tests are the Organization for Economic Cooperation and Development's (OECD) definitive for assessing EDCs (Parrott *et al.* 2001). Exposing the fish for an entire life-cycle ensures that all critical (developmental) 'windows of exposure' are covered.

In this study, fathead minnows were exposed to samples of WWTP-1's treated effluent, collected before UV-disinfection, over a full life-cycle in a laboratory. The effluent was characterized for target pollutants to determine the types and concentrations of substances contained.

The standard acute and chronic toxicity tests were selected to provide information on the impacts of WWTP-1 effluent on various trophic levels of aquatic organisms. Recent studies (Kienle *et al.* 2011) indicate that such impacts depend on both the chemical composition of the effluent and the sensitivity of the receptors. Both short-term lethal (fish, invertebrates) and long-term sub-lethal (fish, invertebrates, aquatic plants/algae) assays were conducted. The impact of contaminants like EDCs that elicit sub-lethal responses may be estimated using a suite of longer-term, whole-effluent, toxicity tests that measure sensitive endpoints like reproduction or growth. The data from this series of experiments enabled assessment of the potential risks to aquatic life associated with exposure to the complex mixture of PhACs and other emerging pollutants in the WWTP effluent.

The potential effects of EDCs on the estrogen, androgen and thyroid hormone systems of aquatic organisms were studied using a two tier assay developed by USEPA (2009). Tier 1 screening assays (YES, YAS and thyroid receptor binding assays) are intended to identify the presence of EDCs, and Tier 2 full life-cycle assays (e.g. fathead minnow exposure) to confirm, characterize and quantify the EDCs' effects on the respective systems of aquatic organisms.

METHODOLOGY

WWTP-1 is a secondary sewage treatment plant incorporating conventional activated sludge with nitrification (CAS-N) and UV irradiation processes. The latter is used seasonally and was not operated during this study. The plant receives mainly residential wastewater.

The toxicity tests conducted included:

Full life-cycle test:

- Fathead minnow (*Pimephales promelas)* life-cycle exposure test (5 months)

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Acute and chronic toxicity tests (standard Environment Canada biological test methods):

- Rainbow trout (Oncorhynchus mykiss) acute lethality (96-h)
- Water flea (Daphnia magna) acute lethality (48-h)
- Fathead minnow survival, growth (7-d)
- Water flea (Ceriodaphnia dubia) survival, reproduction (3-brood)
- Duckweed (Lemna minor) growth inhibition (7-d)
- Alga (Pseudokirchneriella subcapitata) growth inhibition (72-h)

In vitro endocrine disruption screening assays:

- YES assay
- YAS assay
- Thyroid transport receptor (T4/hTTR) binding assay

Fathead minnow life-cycle exposure test

The fathead minnow life-cycle test measures growth, development and reproduction throughout the fish's life-cycle. In this case, the fish were exposed to effluent from WWTP-1.

Each effluent sample was circulated through the fish aquaria for a week before being replaced with the next (fresh) batch. Two 1 liter samples were collected for chemical analysis every week: one from the fresh effluent batch (called 'new'), and the other from the same batch after circulation through the aquaria for a week ('old'). Concentrations of the trace contaminants in the new and old batches were compared for any variation – e.g., via degradation, volatilization, etc. – during the week-long experiments.

The control fish were exposed continuously to de-chlorinated municipal (Lake Ontario) tap water. The experimental fish were exposed to 100% effluent for the first 110 days and 70% effluent (diluted with tap water) for the remaining 28 days, because of unexplained fish deaths in one tank replicate. The concentration of WWTP-1 effluent was reduced, for fear of losing more fish and to keep the breeding phase of the experiment going.

Flow-through exposure was used (Parrott & Bennie 2009) providing 3 volume replacements daily. There were 8 replicate tanks of controls and 4 of 100%/70% effluent.

Exposure began at the egg stage and continued through hatching, maturation and breeding. Thirty newly-fertilized eggs were added in egg cups to each tank, and the larvae hatched in 5 days. Fish were observed during all life stages and reproduction. The endpoints examined included survival, sex ratio, secondary sex characteristics, egg production, fertilization and percentage hatch – see Figure 1.

The first generation of fish (F0) grew and was culled 42 days after hatching (dph) to 15 fish per tank. At 73 days, fish numbers were further reduced to 12 per tank. Fish thinning was random, except for the deliberate removal of a few small/stunted fish from all aquaria. Culled fish were killed, and their weights and lengths recorded. Stunted fish do not mature and their sex determination is difficult. Their presence would have skewed the fish sex ratio. Stunted fish occur similarly among both control and exposed fish. Care was taken to ensure that the number, weight, and length of stunted fish are not affected by the exposure. Exposure continued and the fish were sampled at 132 to 133 dph, when growth, health and reproductive status were assessed.

Fish maturation, breeding, and hatching

Secondary sex characteristics started to develop at 59 dph. By 73 dph, 58% of the fish could be sexed as male or female. Fish began breeding at about 80 to 81 dph (breeding groups comprised 3 males and 5 females, plus 2 immature fish, if needed).

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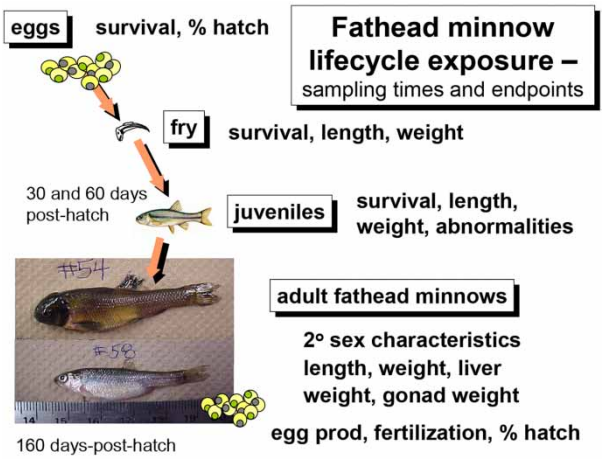

Figure 1 | Fathead minnow life-cycle exposure.

All eggs were counted, assessed for fertilization and rolled off tiles. A count of 100 eggs (or less, if the batch was smaller) was moved to hatching cups in clean, aerated water. Egg development in the batches was monitored daily, the un-eyed, dead eggs and mutants were counted, and the dead eggs removed. The second generation hatched was called F1. Hatching success in lab water was monitored, and the numbers of live and dead fry recorded. Larval deformities were also noted.

Fish sampling and sex characteristics

At 133 dph, the F0 fish were sampled, as per Parrott & Blunt (2005). Fish were killed by cervical severance, their weights and lengths recorded, and their livers and gonads dissected and weighed. Adult male and female fathead minnow can be distinguished by their body size and shape, and several secondary sexual characteristics – e.g., males have a black dot on their dorsal fin, etc., while females are smaller than males, and silvery and have an ovipositor.

The secondary sex characteristics of adult male fish were assessed and graded according to a pre-determined scale. Thus the presence or absence of a dorsal fin dot yielded 1 or 0 points, respectively. Dorsal fat pads were graded by size from 0 to 4. Fish body banding was also assessed, to give values from 0 to 3. The scores were summed to give the Male Index, which was between 5.3 and 10.9.

The number of nuptial tubercles was also counted and the result divided by 10, to obtain a Tubercle Index, which was between 0.4 and 3.9 in these tests.

For female fish, ovipositor length and width were measured under a dissecting microscope, and triangular ovipositor area was calculated as length $\times$ width/2.

Statistical analyses

The New and Old effluent compositions were analyzed statistically to look for variations in the effluent to which the fish were exposed during each week of the experiment. This was done using ProUCL v.5.0. A goodness of fit test was conducted to determine the best underlying distribution.

Average fish weight, length, condition factor (CF), liver-somatic index (LSI, ratio liver to body weight), and gonadosomatic index (GSI, ratio gonad [ovary or testis] to body weight), ovipositor area, and Tubercle and Male indexes were calculated for each replicate aquarium. SYSTAT (V. 11.0, Systat Software Inc., San Jose, CA, USA) was used for statistical analyses. Analysis of variance (ANOVA) enabled assessment for treatment differences in each endpoint.

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Acute and chronic toxicity tests

The biological effects of WWTP-1 effluent were assessed for acute and chronic effects using standard Environment Canada biological test methods (whole effluent toxicity tests).

Separate grab samples of WWTP-1 effluent were collected on the 45th, 90th, and 120th days of the fathead minnow life-cycle exposure test. This timing correlated with specific sensitive development stages in the fathead minnow life-cycle.

De-chlorinated municipal (Lake Huron) tap water was used for both control and dilution. Effect concentrations (LC_{50}, IC_{25}) were derived using CETIS v1.7.0, and a Mann–Kendall test for assessing control trends in the 72-h algal growth inhibition test was done using the '*Selenastrum* Trend Test 2' Excel program by B. Zadlijk (pers. comm.). ANOVA followed by Tukey's multiple comparison test was also used to assess potential treatment differences at each endpoint (significant ANOVA p-values <0.05), using Minitab 15. Further testing for differences from controls, using two sample t-tests to compare treatment means, was not required.

In vitro endocrine disruption screening assays

The assays are intended to detect known and unknown substances with endocrine disrupting potential. The *in vitro* assays were conducted at the Department of Environmental and Resource Studies, Trent University, Peterborough, Ontario, as per Metcalfe *et al.* (2013). These samples were collected along with the acute and chronic toxicity test samples, and frozen for shipping.

The YES and YAS assays indicate the presence of substances in an effluent that bind to a human estrogen (hERα) or human androgen receptor (α-hAR), respectively. The YES and YAS assays used recombinant yeast strains BJ3505 (Gaido *et al.* 1997, and modified according to Lorenzen *et al.* 2004), and YPH500 (Lorenzen *et al.* 2004), respectively. The positive binding controls for YES and YAS were estradiol (E2) and dihydro-testosterone (T) (Sohoni and Sumpter 1998), respectively.

The capacity to interfere with thyroxine (T4) binding to the human thyroid transport protein (i.e. T4/hTTR) was assessed (Ucan-Marin *et al.* 2009). Competition binding curves for tested compounds were made by plotting relative 125I-T4 protein binding against the concentration of the 'competitor', which is either T4 or the effluent extract.

YES and YAS assays

The *in vitro* YES and YAS assays were conducted at Agriculture and Agri-Food Canada, London, Ontario. All response data for the wastewater extracts were calculated as described by Lorenzen *et al.* (2004). The detection limits for estrogen and androgen equivalents were 0.003 ng/mL in the YES and 0.14 ng/mL in the YAS assays, respectively.

T4/hTTR assay

The wastewater extracts were tested at the National Wildlife Research Institute in Ottawa using methods described by Ucan-Marin *et al.* (2009).

Hazard quotient

A hazard quotient (HQ) is derived from a desktop screening level exercise and used to evaluate potential risk to aquatic receptors, defined as the ratio of the predicted environmental concentration of a substance to its corresponding predicted no-effect concentration (PNEC), based on toxicity data. HQ <1.0 indicates low (or acceptable) risk, while HQ ≥ 1.0 indicates a potential concern (e.g. further

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investigation may be warranted), but does not imply that adverse effects are certain. HQ screening was applied in relation to the PhACs, EDCs, and hormones occurring at the highest concentrations in WWTP-1 effluent.

RESULTS AND DISCUSSION

Comparison of Old and New sample chemistry

No statistically significant differences were observed between the New and Old effluent samples for any PhACs/EDCs, thus enabling the results to be pooled for subsequent analysis. Figure 2 shows comparisons of New and Old effluent samples for chlortetracycline and carbamazepine over the five months of the fathead minnow life-cycle test.

Fathead minnow full life-cycle exposure test

No differences were observed in egg hatching or larval growth in fish exposed to WWTP-1 effluent, compared to controls. Hatching for exposed and control fish occurred in 5 days, and larval survival was good for both treatments. Larval growth at 30 dph was similar for all treatments, as was survival through juvenile and adult stages. However, 7 of 12 fish died at 105 dph and all fish in one tank died at 130 dph. The 130 dph fish were sent for pathological analyses, and the cause of death was reported as bacterial infection (WWTP-1 effluent was not subject to UV disinfection). Similar tank deaths were not seen amongst the control fish. The fish growth and maturation characteristics are compared in Table 1.

At maturity, there were few growth differences between fish exposed to WWTP-1 effluent and the controls (Table 1), although the exposed males were slightly longer than control males ($p = 0.035$). Both exposed males and females had changes in LSI, exposed males showing increased LSIs ($p = 0.036$) and females reduced LSIs ($p = 0.034$), compared to control males/females. These variations are not thought to be biologically significant related to toxicant exposure, compared to those seen in other lifecycle fathead minnow exposures using pulp mill effluents (Parrott *et al.* 2004). Generally, the growth and external sex characteristics (female ovipositor area, and tubercle and male indexes in males) of exposed fish were very similar to those of the control fish (Table 1).

The similarity between the exposed and control fish was evident from their general breeding success. Cumulative egg production was similar in the groups – see Figure 3. The quality of the eggs from effluent-exposed fish was high, with over 98% fertilization success, and, as shown in Table 2,

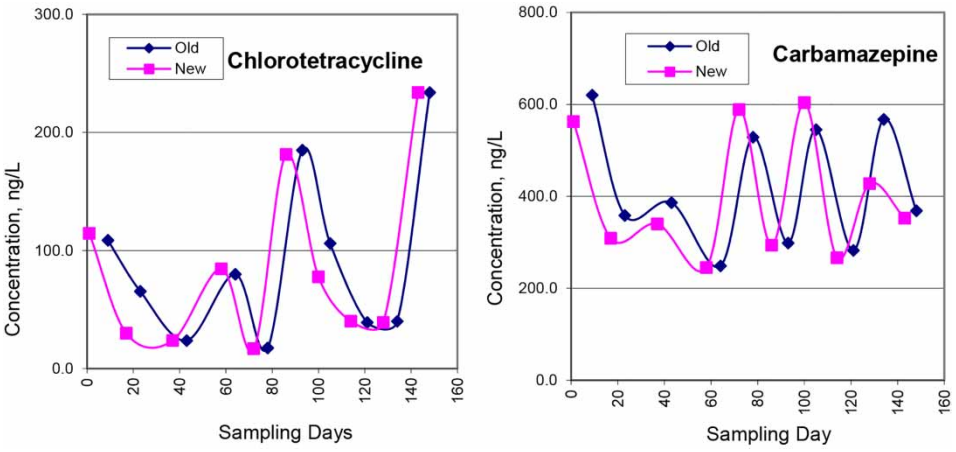

Figure 2 | 'New' and 'Old' effluent samples determinations with respect to chlortetracycline and carbamazepine concentrations.

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Table 1 | Growth and maturation characteristics of male and female fathead minnows exposed for a life-cycle to WWTP-1 effluent or lab water (controls). Bold text shows statistically significant differences

	Male		Female	
	Control	100% then 70% effluent	Control	100% then 70% effluent
No. of tanks	8	3	8	3
L ± sd, mm	60 ± 3	63 ± 5	47 ± 3	48 ± 4
W± sd, g	3.0 ± 0.4	3.3 ± 0.7	1.2 ± 0.2	1.3 ± 0.3
CF ± sd	1.4 ± 0.1	1.3 ± 0.1	1.1 ± 0.1	1.2 ± 0.1
LSI ± sd	**3.0 ± 0.8**	**4.5 ± 0.5**	4.2 ± 1.1	3.2 ± 0.4
GSI ± sd	1.5 ± 0.3	2.3 ± 0.5	13 ± 5	16 ± 5
O ± sd mm^2	0.2 ± 0.2	0.2 ± 0.2	1.0 ± 0.5	1.2 ± 0.2
TI ± sd	2.2 ± 0.7	2.3 ± 0.5	na	na
MI ± sd	8 ± 1	8 ± 1	na	na

L: length; W: weight; CF: condition factor; LSI: liver-somatic index; GSI: gonadosomatic index; O: ovipositor area; TI: tubercle index; MI: male index.
Note: Effluent exposures were to 100% concentration for the first 105 days, and 70% concentration for the remaining 28 days.

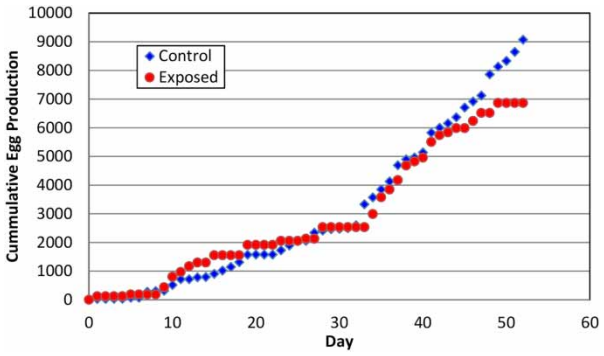

Figure 3 | Plot of cumulative egg production over time for control and WWTP-1 exposed fathead minnows.

Table 2 | Assessment of egg quality from control and exposed fish

	Eggs from Control Fish		Eggs from exposed Fish	
	Based on each batch of eggs	Proportion of affected eggs or fry	Based on each batch of eggs	Proportion of affected eggs or fry
Total no. of eggs	18,173		6,865	
No. of batches of eggs	130		44	
% fertilized	98.7 ± 0.3	98.8	98.5 ± 0.9	98.1
% good eggs	96.4 ± 0.4	96.5	96.7 ± 0.9	97.0
Number of eggs assessed for hatch	3,769		1,921	
Number of batches assessed for hatch	52		25	
% hatch of all eggs assessed	69 ± 5	73	55 ± 8	61
% deformed fry	8 ± 4	3	2 ± 1	1
% good live fry of all eggs assessed	58 ± 5	60	42 ± 7	47

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more than 96% of the eggs were of good quality for both exposed and control groups. The excellent fertilization success suggests that the synthetic estrogen EE2 was not present at concentrations above 0.3 ng/L in WWTP-1 effluent (although the LoD for EE2 is 5 ng/L), as EE2 is a potent disrupter of fertilization in fathead minnows (Parrott & Blunt 2005). The potential lack of estrogenic compounds in WWTP-1 effluent was also supported by undetectable responses for WWTP-1 in the YES assay.

Various studies have established that estrone (E1), 17β-estradiol (E2) and 17α-ethinyl estradiol (EE2) are among the steroidal estrogens found in WWTP effluent that may be responsible for estrogenic effects in fish in receiving waters (Kunz *et al.* 2015). In WWTP-1, E1 was present in all influent samples at concentrations between 66 and 618 ng/L and was removed at 93 to 99% efficiency. E2 was also detected in all influent samples at 34.4 to 42.4 ng/L. Its removal efficiency was sporadic, ranging from 1 to 94%, and it was detected in two of four effluent samples at 31.9 and 34 ng/L. EE2 was only detected in one influent sample, at 14.8 ng/L, and was removed at 9% efficiency with an effluent concentration of 13.4 ng/L.

The total numbers of influent and effluent samples analyzed were 4 and 25, respectively. Across the full set of effluent samples – see Table 5 – the maximum concentrations of E1, E2, and EE2 in the effluent were 140 ng/L, 174 ng/L, and 107 ng/L, respectively. These concentrations exceed reported reproductive NOEC values based on laboratory studies (Caldwell *et al.* 2012), however, no adverse effects were observed following exposure to WWTP-1 effluent. Effluents are a complex mixture of known and unknown substances, and even in instances where impairments to fish reproduction have been observed, this may not have been caused by estrogenic compounds. (Cavallin *et al.* 2016).

In Table 2, the quality of eggs from control and exposed fish is compared. All eggs were hatched in de-chlorinated municipal (Lake Ontario) tap water. Each batch of eggs was assessed separately, and the mean and standard error calculated. The cumulative totals of affected eggs or fry were assessed as a proportion of the entire number of eggs or fry. These parallel methods verify the trends and show the general impact of breeding success for both the control and WWTP-1 exposed fish.

As shown in Table 2, despite the good fertilization, some reduction in hatching success was observed in eggs from exposed parents. Hatching success from exposed F1 fish was 55% compared to 69% for control parents. Percentages of lethal deformities in hatched fry were extremely variable, while the percentage of deformed fry was higher in control (8%) than exposed offspring (2%). Hatched fry survival was similar for both control and exposed groups. Taking all the factors into account, the percentage of good live fry produced from eggs from exposed fish was 42% compared to 58% from controls. It is difficult to know whether this drop would be environmentally significant, as, in one season, fathead minnows produce several hundred fry, most of which do not survive to adulthood.

Acute and chronic eco-toxicity test results

The results of standard acute and chronic toxicity tests are shown in Table 3. No toxicity was observed in any of the tests from the three sampling events. However, the algal test (*P. subcapitata*, 72 hour IC25, growth, cell count) results indicated some growth stimulation at higher effluent concentrations across all sampling events, corresponding to days 45, 90, and 120 of the fathead minnow life-cycle test – see Figure 4.

The growth stimulation followed a eutrophic concentration-response relationship, meaning that higher growth was observed at higher nutrient concentrations. The concentrations of total phosphorus were 0.16 mg/L, 0.07 mg/L and 0.07 mg/L, and of nitrogen (nitrate plus nitrite) 9.37 mg/L, 12.1 mg/L and 7.81 mg/L, on days 45, 90 and 120, respectively. Larval fathead minnows in the 7-day survival and growth test with the day 90 effluent sample were also significantly larger (mean dry weight in 6.25%, 12.5% and 100% effluent was 0.834 mg, 0.842 mg, and 0.895 mg, respectively) than the controls (mean dry weight of 0.700 mg) (one-way ANOVA, $F(7,16) = 3.41$, $p = 0.020$).

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Table 3 | Summary effect concentrations calculated for the suite of standard (Environment Canada) acute and chronic toxicity tests conducted with WWTP-1 effluent

	Effect Concentrations[†] (% volume effluent)		
Biological Test*	**Day 45**	**Day 90**	**Day 120**
Rainbow trout (O. mykiss) 96 hour LC50 (survival)	> 100	Pass[a]	Pass[a]
Water flea (D. magna) 48 hour LC50 (survival)	> 100	Pass[a]	Pass[a]
Fathead minnow (P. promelas) 7 day LC50 (survival)	> 100	> 100	> 100
Fathead minnow (P. promelas) 7 day IC25 (growth)	> 100	> 100	> 100
Water flea (C. dubia) 3-brood LC50 (survival)	> 100	> 100	> 100
Water flea (C. dubia) 3-brood IC25 (reproduction)	> 100	> 100	> 100
Algae (P. subcapitata) 72 hour IC25 (growth, cell count)	> 90.91[b] (stimulatory effect)[c,f]	> 90.91[b] (stimulatory effect)[d,f]	> 90.91[b] (stimulatory effect)[e,f]
Duckweed (L. minor) 7d IC25 (growth, frond number)	> 97[b]	> 97[b]	> 97[b]
Duckweed (L. minor) 7d IC25 (growth, dry weight)	> 97[b]	> 97[b]	> 97[b]

Notes:
LC: Lethal concentration, IC: inhibition concentration.
*De-chlorinated municipal (Lake Huron) tap water was used for both control and dilution.
[†]Effect concentration using CETIS v1.7.0.
[a]Tests conducted on days 90 and 120 used 0% and 100% effluent only (pass/fail test. Pass means 100% survival in 100% effluent).
[b]Highest concentration tested, based on test method.
[c]Algal growth stimulation was 49%, 246% and 337% (compared to average for effluent concentrations of 10.1%, 30.3% and 90.9%, respectively).
[d]Algal growth stimulation was 57%, 287% and 273% (compared to average for effluent concentrations of 10.1%, 30.3% and 90.9%, respectively.
[e]Algal growth stimulation was 15%, 28%, 46%, 234% and 293% for effluent concentrations of 0.125%, 3.37%, 10.1%, 30.3% and 90.91%, respectively).
[f]S (%) = (T-C)/C * 100, where S (%) = percent stimulation, T = average cell yield at test end in test solutions, and C = average cell yield in the controls.

The observation of 'no adverse effect' at 100% effluent concentration indicates that the WWTP treatment currently employed is sufficient to avoid causing acute and chronic toxicity to standard test organisms. This, in turn, suggests that impacts to aquatic organisms in the receiving environment, where substantial effluent dilution takes place, are unlikely.

YES and YAS assays

The results of the *in vitro* testing of extracts prepared from the effluent samples are summarized in Table 4, and indicate no response. This suggests that the concentrations of compounds with estrogenic or androgenic activity, or with the capacity to bind with the hTTR thyroid hormone transporter protein, were below the LoDs of those compounds set by this test. The lack of estrogenic responses in the YES assay for the effluent supports the finding of no effect on fertilization success in fathead minnow exposed to the same effluent for a full life-cycle.

Review of effluent chemistry

Table 5 provides monitoring data for the ten commonest PhACs/EDCs and four hormones measured in WWTP-1 effluent. HQ exceeded 1.0 for nine substances (indicated by bold font).

Table 6 is a summary of the effects of these substances on aquatic organisms, showing both typical apical and non-traditional endpoints, as well as the respective concentrations at which these effects were observed.

For the two antibiotics (sulfamethoxazole and ciprofloxacin), the lowest reported effect concentrations were for cyanobacteria (blue–green algae), which are known to be significantly more sensitive than green algae (EMEA 2006). The suite of standard toxicity tests utilized in this study did not include a blue–green algal species.

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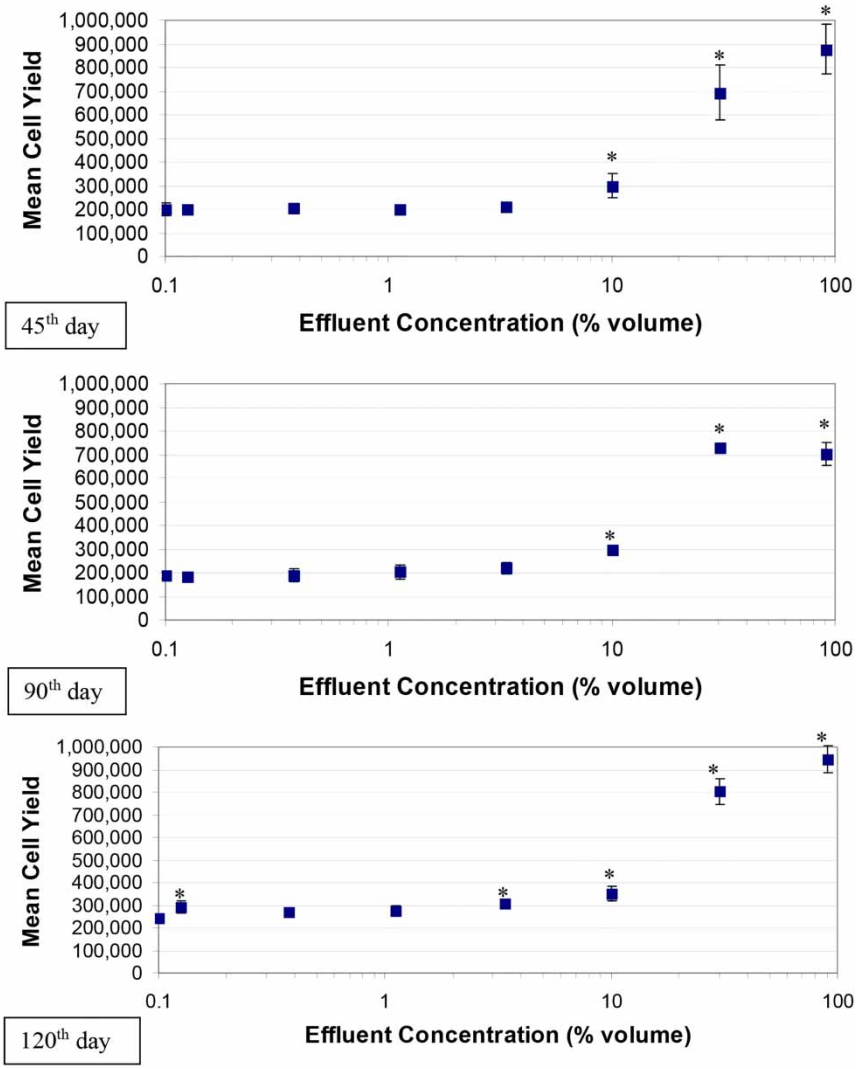

Figure 4 | Mean cell yield (with standard deviation) following a *Pseudokerchneriella subcapitata* 72-hour growth inhibition test exposed to the WWTP-1 effluent. Asterisks (*) indicate where growth stimulation was observed. Note: this test was developed to assess growth inhibition not growth stimulation.

Table 4 | *In vitro* assay results for extracts prepared from wastewater

Sampling date[a]	EEQ (YES) ng/mL	TsEQ (YAS) ng/mL	hTTR binding % of control
Day 45	ND	ND	98
Day 90	ND	ND	101
Positive control	0.093	2.182	56
Day 120	ND	ND	102
Day 120	ND	ND	100

[a]Corresponds with day 45, 90, and 120 of the 5-month fathead minnow test, correlating with specific sensitive development stages in the fathead minnow life-cycle.
ND: not detected.

The most sensitive endpoint reported for diclofenac is a histological effect in the kidney and gills. Although histological effects are not assessed as apical endpoints in standard toxicity tests, they were considered relevant for derivation of a PNEC value for aquatic life protection, because such changes may impact populations by affecting reproduction or growth (EC 2010).

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Table 5 | The ten commonest PhACs/EDCs (ng/L) and four hormones (ng/L) measured in WWTP-1 effluent, with their associated PNECs and log K_{ow} values. An HQ (maximum measured concentration in effluent/PNEC concentration) has been computed for full strength (100%) effluent

Substance	Limit of Detection (LoD)	Detection frequency	Min[1] concentration measured in effluent	Max concentration measured in effluent	Median	Mean (± Standard Error)	% Removal Efficiency	PNEC	HQ (Max/ PNEC)	Log K_{ow}
Trimethoprim	1	25/25	134	791	448	460 ± 40	Moderate[iii]	60,000 [a]	0.01	0.73
Carbamazepine	1	23/25	ND/196	838	409	440 ± 30	No/-ve removal[i]	10,000 [b]	0.08	2.25
Sulfamethoxazole(antibiotic)	2	25/25	ND/194	841	451	425 ± 25	Moderate[iii]	600 [a]	**1.4**	0.48
Diclofenac (anti-inflammatory)	1	23/25	116	749	403	420 ± 30	No/-ve removal[i]	50 [a]	**15**	0.57
Bisphenol A (endocrine disruptor)	2	23/25	ND/10	782	318	350 ± 50	High[iv]	175 [c]	**4.5**	3.32, 3.64
Ciprofloxacin (antibiotic)	0.5	25/25	80	343	158	170 ± 20	Moderate[iii]	89 [a]	**3.9**	0.01
Norfloxacin	10	25/25	70	235	161	150 ± 10	Inconclusive[vi]	15,000 [d]	0.02	−0.31
Bezafibrate	0.5	23/25	ND/49	258	140	150 ± 10	Moderate[iii]	460 [a]	0.6	**4.25**
Clofibric acid (lipid regulator)	1	21/25	ND/3	360	134	130 ± 20	Low[ii]	100 [e]	**36**	2.57
Enrofloxacin	5	23/25	ND/85	143	115	115 ± 5	No/-ve removal[i]	795,000 [f]	0.00	3.1
17-α-Ethynyl Estradiol (EE2) (hormone)	5	17/25	ND/11	107	5	19 ± 5	Inconclusive[vi]	0.5[g]	**214**	3.67–4.2
17-β-Estradiol (E2) (hormone)	2	6/25	ND/3	174	20.5	31 ± 8	Moderate[iii]	0.4 [a]	**435**	**4.01**
Estrone (E1) (hormone)	2	21/25	ND/5	140	2	11 ± 5	Excellent[v]	3.6 [a]	**39**	**3.13**
Estriol (E2) (hormone)	5	16/25	ND/6	136	5	23 ± 17	Excellent[v]	60 [h]	**2.3**	2.45–2.81

[1]If one or more values were non-detect both ND and the minimum detected values are reported.
[i]No/negative removal (≤0%, substances showed higher or similar concentrations in the effluent compared to the influent).
[ii]Low (removal efficiencies greater than 0% and up to 50%).
[iii]Moderate (removal efficiencies greater than 50% and up to 75%).
[iv]High (removal efficiencies greater than 75% and up to 95%).
[v]Excellent (removal efficiencies greater than 95%).
[vi]Inconclusive (removal efficiencies with standard errors spread over three or more categories).
[a]Swiss Ecotox 2014 (http://www.oekotoxzentrum.ch/expertenservice/qualitaetskriterien/vorschlaege/index_EN).
[b] CCME (2015).
[c]Cited in EC (2010).
[d]Verlicchi *et al.* (2012).
[e]Hull *et al.* (2015).
[f]Boxall *et al.* (2006).
[g]Nagpal & Meays (2009).
[h]Caldwell *et al.* (2012).

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Table 6 | Summary of PhACs/EDCs in 100% effluent with HQ ≥1.0 and associated effects on aquatic organisms at low concentrations. The lowest effect concentrations (LOEC) reported in the literature are listed (unless otherwise specified)

Compound	Endpoint	Lowest Concentration Reported in Literature	Organism	Reference
Sulfamethoxazole	Growth inhibition	LOEC 5,900 ng/L EC(10) 11,000 ng/L	Cyanobacteria (blue–green algae) plant (duckweed)	Brain *et al.* (2004)
Diclofenac	Adverse histological effects, necrosis in kidney and gills	NOEC 1,000 ng/L	*Oncorhynchus mykiss*	Mehinto *et al.* (2010)
Bisphenol A	reduced semen quality and delayed ovulation	LOEC 1,750 ng/L	*Salmo trutta f. fario*	Lahnsteiner *et al.* (2005)
Ciprofloxacin	Growth inhibition (cell density)	EC(50) 5,000 ng/L	*Microcystis aeruginosa* (blue–green algae)	Halling-Sorensen *et al.* (2000)
Clofibric acid	growth, reproduction	NOEC 10,000 ng/L	*Daphnia magna*	Flaherty & Dodson (2005)
17-α-Ethynyl Estradiol (EE2)	Reproduction, egg production	LOEC 1.0 ng/L	*Pimephales promelas* (fathead minnow)	Parrott & Blunt (2005)
17-β-Estradiol (E2)	Vitellogenin induction *in vivo*, adult stage (150–171 dph)	NOEC 5 ng/L	*Gabiocypris rarus* (Chinese rare minnow)	Caldwell *et al.* (2012)
Estrone	Vitellogenin induction *in vivo*	LOEC 484 ng/L	*Oryzias javanicus* (Java medaka)	Imai *et al.* (2007)
Estriol	*in vivo* observation of a testis-ova in at least one medaka	NOEL 75 ng/L LOEL 750 ng/L	*Oryzias latipes* (Japanese medaka)	Metcalfe *et al.* (2001)

With respect to bisphenol A, reduced semen quality and delayed ovulation – another non-standard endpoint – were found to be the most sensitive and relevant to population effects. Standard toxicity tests do not monitor for this endpoint.

It is noted that many non-standard endpoints were evaluated in the fathead minnow full life-cycle exposure tests, but that none showed any significant impacts compared to the controls. Adverse reproduction impacts in fish – shown experimentally to be the most sensitive endpoint following exposure to constant low levels of estrogenic compounds – occur after longer-term exposures (e.g. several weeks to months, as employed in this study, or over multiple generations) (Caldwell *et al.* 2012). This study did not investigate multi-generational impacts.

In future, consideration may be given to employing a battery of *in vitro* bioassays with increased sensitivity, each linked to identifying a specific mode of action (in addition to YES, YAS, hTTR employed in this study), in order to be able to improve selection of whole organism tests and associated endpoints in the evaluation of impacts of trace contaminants in treated municipal effluent (EC 2014).

It is noted that WWTP-1 effluent is diluted by the primary receiver (Little River) and substantially by the final receiver (Detroit River), so the level of risk posed to aquatic receptors and the environment is probably negligible.

The WWTP-1 effluent samples (collected on three separate occasions) were not toxic to aquatic life when tested using Environment Canada's standard acute and chronic bioassays. Acute toxicity to rainbow trout was probably not evident as WWTP-1 is a nitrifying plant, where un-ionized ammonia is converted to nitrate. In addition, UV disinfection eliminates the potentially toxic effects of chlorine residuals that can be present at a chlorinating plant. There was no evidence of chronic toxicity of WWTP-1 effluent.

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SUMMARY AND CONCLUSIONS

The potential impacts of WWTP effluent on aquatic life were assessed in Windsor, Ontario, Canada, through extensive tests over approximately six months. The work included life-cycle tests, acute and chronic toxicity tests, and *in vitro* biological assays. A desk top screening approach was also applied to assess the potential risks from the effluent's constituents to aquatic organisms.

No statistically significant difference was found in the compositions of the New and Old water samples. The aim was to determine whether the effluent's composition changed for any reason during a week's storage. No such change was found.

In the fathead minnow life-cycle exposure tests, no statistically significant differences were observed between the control fish and those exposed to WWTP effluent, in terms of survival, growth, or sexual development. Egg production was excellent in exposed fish, and fertilization success was high for eggs from the exposed parents. Egg-hatching success was reduced for eggs from exposed fish compared to controls, but it is difficult to assess whether this would be environmentally significant. Generally, fathead minnow growth and reproduction were very good, considering that the fish were exposed to high effluent concentrations for an entire life-cycle.

The acute and chronic toxicity tests showed no statistically significant difference between organisms exposed to WWTP effluent and the controls, except for algal growth stimulation. The latter probably arose from increased nutrient concentrations in the effluent and, in any case, could not be determined reliably in the test used.

The HQ assessment indicated potential for further investigation into nine substances when present in 100% effluent, i.e. before dilution. The substances were sulfamethoxazole, diclofenac, bisphenol A, ciprofloxacin, and clofibric acid, and four hormones (E1, E2, EE2, estriol). However following dilution in the Detroit River, exposure is considered to be negligible.

In vitro YES, YAS, and T4/hTTR assays indicated, in all cases, that the concentrations of potentially hazardous compounds were below detection limits. No samples produced a positive response in any biological assay.

In conclusion, WWTP-1 effluent is expected to have no or negligible impact on aquatic life after discharge to the surface water. However, it is noted that the tests selected may not have been sensitive enough to detect non-traditional endpoints that may be more valid for PhACs/EDCs/hormones.

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