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TOXICITY OF EXTRACTS FROM MUNICIPAL WASTEWATER TO EARLY LIFE STAGES OF JAPANESE MEDAKA (*ORYZIAS LATIPES*) TO EVALUATE REMOVALS OF MICROPOLLUTANTS BY WASTEWATER TREATMENT

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Abstract: Treatment of municipal wastewater reduces the concentrations of some pharmaceuticals and personal care products, hormones and drugs of abuse. However, reduced concentrations of these micropollutants in wastewater may not correlate with reduced toxicity, as transformations of micropollutants and/or the formation of disinfection by-products may generate toxic compounds. In this study, we prepared extracts by solid phase extraction of samples collected from wastewater treatment plants (WWTPs) at various stages of treatment and tested these extracts for toxicity to early life stages of Japanese medaka (*Oryzias latipes*). Toxicity data for extracts prepared from a WWTP with secondary treatment showed that the numbers of exposed embryos (n=12 per treatment) that did not hatch increased from 1/12 for the treatment with untreated effluent to 5/12 for the treatment with final treated effluent. For extracts prepared from a WWTP with tertiary treatment, toxicity among exposed embryos (n=12 per treatment) also increased with each step of wastewater treatment, as shown by mortalities of 2/12 and 8/12 in treatments with extracts from untreated and final treated effluent, respectively, as well as an increase in the numbers of embryos that did not hatch from 2/12 to 9/12 in treatments with untreated and final treated effluent, respectively. Ozonation of treated wastewater collected from a third WWTP caused a high incidence of delayed hatch in exposed embryos (n=24 per treatment). However, hatching success and the numbers of developmental abnormalities in embryos from this ozonation treatment were not different from controls. This study demonstrates the value of including toxicity testing to assess the effectiveness of technologies for treatment of municipal wastewater.

Keywords: Japanese medaka, wastewater, developmental toxicity, mixture toxicity, ozonation

INTRODUCTION

Definitions of "micropollutant" vary widely, but there is a general consensus that the term refers to substances that originate from anthropogenic activities that are present in trace amounts in natural waters. These substances include pharmaceuticals and personal care products (PPCPs), drugs of abuse, hormones, stimulants and chemicals from household products are discharged from wastewater treatment plants (WWTPs) into the aquatic environment (Ratola et al., 2012; Luo et al., 2014; Petrie et al., 2015). Environmentally relevant concentrations of PPCPs in surface waters may induce biological responses in aquatic organisms, as reviewed previously (Santos et al., 2010; Brausch and Rand, 2011; Blair et al., 2013). Biological effects indicative of exposure to micropollutants have been observed in fish caged downstream of wastewater discharges, including oxidative stress in a freshwater fish species from South America (*Prochilodus lineatus*) caged for 96 h in the Colastine River, Argentina (Cazenave et al., 2014), and oxidative stress and induction of vitellogenin in fathead minnows (*Pimephales promelus*) caged for 4 weeks in the North Saskatchewan River, Canada (Jasinska et al., 2015). There is ample evidence that micropollutants released from WWTPs can cause endocrine disruption and gonadal inter-sex in wild fish exposed *in situ* (Sumpter and Johnson, 2005; Tetreault et al., 2011).

Removal of micropollutants by treatment of wastewater is an obvious solution to reducing the potential for adverse effects in the environment (Martín et al., 2012). There have been a number of studies investigating the removal of micropollutants in WWTPs with conventional activated sludge treatment, and these studies show that removals of these contaminants vary widely, depending on the physical and chemical properties of the compounds, as well as the characteristics of the WWTPs (Verlicchi et al., 2012; Luo et al., 2014; Blair et al., 2015). More efficient removals of micropollutants may be achieved using advanced oxidation technologies and various tertiary treatment options (Rosario-Ortiz et al., 2010; Reungoat et al, 2010; Lee et al., 2016; Sultana et al., in press). However, incomplete mineralization of contaminants using advanced oxidation can result in the formation of transformation products with the potential to induce greater levels of toxicity than the original parent compounds. Therefore, complementary toxicity testing is required to confirm that reduced levels of micropollutants correspond to reduced biological responses, and to ensure that greater toxicity does not occur through the production of toxic transformation products (Reungoat et al., 2010; Rizzo et al., 2011; Lee and von Gunten, 2016).

The early life stages of fish are a sensitive indicator of toxicity. Lethality and developmental toxicity resulting from exposure to PPCPs commonly detected in municipal wastewater have been observed in several studies using *in vivo* tests with fish in early life stages (Ferrari et al., 2003; Ishibashi et al., 2004; Sun et al., 2007; van den Brandhof and Monforts, 2010; Overturf et al., 2012). Biological responses have also been observed in early life stages of fish exposed directly to wastewater in *in vivo* bioassays (Zha and Wang, 2005; Cao et al., 2009). However, municipal wastewaters are a complex matrix containing a mixture of contaminants, as well as physical agents (e.g. suspended solids), chemical components (e.g. ammonia) and pathogens that could be harmful to fish. In the present study, we used solid phase extraction (SPE) to isolate and concentrate micropollutants and their transformation products from treated and un-treated municipal wastewater, and we tested these extracts for toxicity to early life stages of Japanese medaka (*Oryzias latipes*). The concentrations of selected PPCPs and drugs of abuse in extracts prepared from these wastewater samples were previously reported, and in all cases the levels of these compounds were reduced as a result of wastewater treatment (Baalbaki et al., 2016; Baalbaki et al., 2017; Maya et al., in press).

Toxicity testing with Japanese medaka was completed using a static, non-renewal assay over 19 days from egg fertilization to “swim up” of the fry (i.e. beginning of exogenous feeding). Endpoints of toxicity included lethality, hatching success, delayed time to hatch and developmental abnormalities. Japanese medaka are a convenient test model to study the effects of contaminants on early life stages of fish because they are a small aquarium fish, produce egg continuously if appropriate breeding conditions are maintained, and have a rapid rate of development of approximately 19 days from fertilized egg to exogenous feeding of the fry. Extracts were prepared from wastewater from the WWTPs serving three different communities located in Canada. Samples of treated and untreated wastewater were collected from two WWTPs with secondary and tertiary treatment, respectively. The removal of targeted PPCPs and drugs of abuse within these WWTPs has been reported previously (Baalbaki et al., 2016; Baalbaki et al., 2017), and samples collected at the same time were used in toxicity tests in the current study. Final effluent collected from another WWTP with secondary treatment was further treated by ozonation in a bench-scale experiment. The sublethal biological responses of juvenile rainbow trout (*Oncorhynchus mykiss*) to exposure to un-ozonated and ozonated wastewater from this WWTP was previously described by Maya et al. (in press). These three WWTPs were selected to evaluate changes in toxicity over a range of treatment technologies. The objective of the present study was to determine whether changes in the toxicity of extracts from wastewater collected throughout the treatment train were consistent with previously reported removals of micropollutants as a result of wastewater treatment.

METHODS AND MATERIALS

Chemicals

Triclosan, diclofenac, carbamazepine and estrone were purchased from Sigma-Aldrich (Oakville, ON, Canada), and androstenedione (ADD) was purchased from Toronto Research Chemicals (Toronto, ON, Canada). Calcium chloride was purchased from Caledon Laboratories (Georgetown, ON, Canada) and methylene blue was purchased from BDH Laboratory Supplies (Poole, Dorset, England). High-performance liquid chromatography (HPLC) grade methanol and acetone were purchased from VWR International (Mississauga, ON, Canada).

Wastewater

Municipal wastewater was collected from three different WWTPs at various stages of treatment using 24-h composite samplers at each location. The treatment parameters for each of the WWTP and the samples collected for toxicity testing are summarized in Table 1. WWTP 1 uses conventional activated sludge secondary treatment and WWTP 2 uses tertiary treatment. The treatment processes and schematics of the treatment trains are described in more detail for WWTP 1 by Baalbaki et al. (2017) and for WWTP 2 by Baalbaki et al. (2016). However, the basic treatment trains of these WWTPs are illustrated in Supplementary Information (Figures S1 and S2). The treated wastewater samples from WWTP 1 and WWTP 2 were collected before the disinfection stage, and there was no disinfection of treated wastewater at WWTP 3. All of the samples collected at WWTPs 1 and 2 were collected over 3 and 4 consecutive days, respectively and two 100 mL aliquots were removed each day for extraction.

WWTP 3 also uses secondary treatment, but the final effluent was further treated by ozonation using a bench-scale system to test the efficacy of this treatment process. Bench-scale ozonation was used because there are few wastewater treatment plants currently operating in

Canada that treat final effluents with ozone, although this technology will soon be installed at the WWTP serving the City of Montreal, QC, Canada (Ville de Montréal, 2016). The treated wastewater collected from WWTP 3 in a single sample collected with a 24-h composite sampler (3 L volume) was extracted as collected (i.e. WW treatment), and also extracted after spiking with 5 contaminants of emerging concern (CECs) that are commonly found in wastewater (i.e. WW + 5CEC treatment). The CECs include triclosan, diclofenac, carbamazepine, estrone and androstenedione, and each compound was spiked into the wastewater at a concentration of 100 µg/L, which is greater than the 0.1 to 1 µg/L levels of these compounds typically detected in treated wastewater (Luo et al., 2014). The CECs were added to the wastewater samples to ensure that biological responses would be observed in the *in vivo* bioassays. It was not known a priori whether the microcontaminant levels in the extracts would be sufficient to cause responses.

The spiked wastewater was extracted after ozonation (i.e. WW + 5CECs + O3 treatment) in experiments using a bench scale ozonation system. The semi-batch ozonation experiment using ozone gas introduced into the reaction vessel was completed as described previously by Maya et al. (in press). The ozone dose used for each experiment was calculated based on the chemical oxygen demand (COD) of the wastewater or the spiked reverse osmosis (RO) water used in the bench scale system. Ozone doses used for the wastewater experiments ranged from 0.41 to 0.56 mg of ozone per mg of COD in wastewater, which is within the usual range for disinfection of municipal wastewater (Rodayan et al., 2014).

Extraction

Extracts were prepared from wastewater collected as 24-h composite samples from various points in the WWTPs. The wastewater samples were pre-filtered through 0.45 µm glass fibre filters and then 100 mL aliquots were extracted by SPE using Oasis® MAX (6 mL, 400 mg) and Oasis® MCX (6mL, 400 mg) ion exchange cartridges. Extractions with MAX and MCX cartridges were conducted according to methods described by Metcalfe et al. (2014) and by Yargeau et al. (2014), respectively. Aliquots of wastewater samples collected from WWTPs 1 and 2 were extracted only with the anion exchange (i.e. MAX) cartridges, which are specific for acidic and neutral compounds, while wastewater samples collected from WWTP3 were extracted with both MAX and MCX cartridges. The latter cation exchange cartridge extracts basic and neutral compounds. By combining extracts from both types of cartridges, our objective was to expose the developing Japanese medaka embryos to a range of acidic, basic and neutral micropollutants extracted from the wastewater.

Briefly, the pH of the aliquots of wastewater were adjusted to 8 or to 2.5 for extraction using MAX and MCX cartridges, respectively, and the samples were extracted with pre-conditioned cartridges using a loading rate of 1 mL/min. Cartridges were then washed with 2 mL of MilliQ water adjusted to either pH 8 or pH 2.5, and allowed to dry. The MAX cartridges were eluted using: a) 2 mL of methanol and b) 3x3 mL of 2% formic acid in methanol. The MCX cartridges were eluted using 3x3 mL of 5% ammonium hydroxide in methanol. The eluate was evaporated just to dryness and made up to a final volume in acetone (WWTP 2, WWTP 3) or in methanol (WWTP 1). The earliest tests were conducted with methanol as the carrier solvent, but acetone was used in later tests because of concerns about background toxicity induced by exposure to methanol. The number of aliquots of each wastewater sample that were extracted and pooled for toxicity testing and the final volumes of the extracts are described in Table 1. All solvent controls were prepared by going through the same blank extraction procedures as those used for extraction of wastewater samples.

Toxicity testing

In this study, we evaluated the toxicological and developmental responses of the early life stages of Japanese medaka exposed to micropollutants extracted from wastewater collected at various stages of treatment. All test protocols were reviewed by the Animal Care Committee of Trent University. Eggs were collected from mature female Japanese medaka from a brood stock that has been maintained by Trent University for 3 decades. This breeding stock was maintained at a 16h:8h photoperiod and a temperature of 23°C. Eggs were collected after fertilization by males, which occurred approximately one hour after daily feeding. Eggs were removed from the abdomen of the females, and the chorionic fibers were removed by gently rolling the eggs between the fingers. Once collected, eggs were placed in a petri dish containing rearing solution for a 24 h period to confirm viability. The embryo rearing solution used to sustain eggs prior to and during toxicity testing (Paterson et al, 2011) was prepared by dissolving 10 g NaCl, 0.3 g KCl, 0.4 g $CaCl_2$•2 H_2O and 1.63 g $MgSO_4$ in 200 mL of MilliQ water. Twenty mL of this nutrient mixture were added to a clean 1L volumetric flask and made up to a final volume of 1 L with MilliQ water (pH□7.4). Two drops of the antifungal agent, methylene blue were then added and the mixture was thoroughly mixed.

Wastewater extracts were either used as is, or diluted (1:1) prior to exposure to Japanese medaka in their early life stages. Dilutions were necessary or extracts from WWTP 1 because of mortalities close to 100% in all treatments with undiluted extracts. There were three control treatments for each test, including a positive control (i.e. triclosan), a solvent control and a negative (no solvent) control. Each treatment consisted of either 12 or 24 embryos, each placed in an individual well of a 24 well microtitre plate. Treatments with 24 embryos were necessary in tests with extracts from WWTP 3 in order to generate statistically significant results. Each well contained 2 mL of rearing solution and 6 µl of test material; either wastewater extract, positive control solution or solvent. The positive control consisted of 6 µl of a 0.5 ppm solution of triclosan dissolved in methanol (WWTP 1 treatments) or acetone (WWTP 2 and 3 treatments) solvent. Only one concentration of the positive control was tested in treatments with extracts from WWTP 1 and WWTP 2. After addition of the triclosan solution to the wells of microtiter plates with the rearing medium, the final concentration of triclosan in the wells was 1.0 µg/mL. For treatments with extracts from WWTP 3, five positive control treatments were conducted over a range of triclosan concentrations from 0.1 to 5.0 µg/mL in the wells of the microtiter plates (Table 2). The positive control treatments were run simultaneously with the other treatments to ensure that the Japanese medaka were responding in an expected manner to a personal care product known to affect early life stage development of fish (Ishibashi et al., 2004).

Early life stages of medaka were reared in a 16h:8h light/dark photoperiod and a temperature of 23±1°C. Daily observations were made using a dissecting microscope to record developmental changes. Each day, the medaka were observed for mortalities, hatching success, day of hatch and developmental abnormalities, including tail, fin and eye deformities, tube heart and edema of the pericardial region and/or yolk sac. Medaka were observed from 1 day post-fertilization to the “swim up” stage, which is the point at which the fry complete sorption of the yolk sac, for a total observation period lasting 19 days. Fry were euthanized with MS-222 (100 mg/L in water buffered with sodium carbonate) if they survived to the end of the test period.

For treatments with extracts from WWTP 1 and WWTP 2, toxicity was evaluated (n=12 per treatment) on the basis of mortalities and hatching success only, because these are definitive endpoints of toxicity that can be easily evaluated. For the treatments with extracts from WWTP 3, toxicity was evaluated (n=24 per treatment) on the basis of mortalities and hatching success, plus data on the numbers of embryos showing delayed hatch and numbers that were moribund after hatch (i.e. not showing a swimming response to physical stimulus). “Delayed hatch” was judged to occur in embryos that hatched after Day 13, which in our experience over the past 30 years with this colony of Japanese medaka originally from the Carolina Biological strain is the usual time to hatch at the rearing temperature of 23ºC. The mean time to hatch of the medaka in each treatment was also calculated, although these data could not be analyzed statistically. “Total toxicity” was calculated as the total number of animals showing lethal (i.e. mortality) or sublethal (i.e. no hatch, delayed hatch, moribund) responses in each treatment. The experiments were not repeated and each embryo exposed in a well of the microtiter plate (i.e., n=12 for WWTP 1 and 2; n=24 for WWTP 3) was considered a replicate for statistical purposes.

Toxicity data for all treatments stages were not normally distributed and transformations of the data did not result in a normal distribution. Therefore, a Chi-square test ($p<0.05$) was used to determine if there were statistically significant differences in toxicity endpoints between the solvent control and various treatments with wastewater extracts. Chi-square analysis was only used for statistical analysis of “total toxicity” data as simultaneous testing of multiple endpoints introduces family-wise error which reduces the statistical power of the Chi-square test. Statistical analysis was conducted in R (version 3.1.2), using the open-source integrated development environment, R studio (version 0.98.1091).

RESULTS AND DISCUSSION

Control treatments

No significant differences in biological effects were observed between the negative and solvent control treatments for tests with extracts from WWTP 2 and 3, indicating that the carrier solvent (i.e. acetone) did not induce any grossly observable biological responses in Japanese medaka. However, mortalities were elevated in the solvent control treatment for tests with extracts from WWTP 1, so the methanol solvent was discontinued and acetone was used as a carrier in all other tests with wastewater extracts. Methanol is the final solvent typically used for the SPE extractions. Biological responses in all wastewater treatments were compared to the solvent control treatment.

Exposure to triclosan in the positive control treatments induced a variety of lethal and sublethal responses, including mortalities, reduced hatching success and delayed hatch (Table 2). The developmental abnormalities observed included yolk sac edema and vascular hemorrhaging. Total toxicity in the 1.0 and 5.0 μg/mL treatments was 100% due to mortalities before hatch for all embryos. Mortalities were not observed in the 0.1 and 0.5 μg/mL treatments, but there was a trend towards elevated numbers of embryos experiencing delayed or no hatch in the 0.5 μg/mL treatment (Table 2). However, total toxicity for the treatments with the lowest concentrations of triclosan was not significantly different from the solvent control treatment. The high incidence of mortalities observed in the triclosan positive control treatment in the experiments with extracts from WWTP 1 and WWTP 2 (Table 3) was consistent with the toxicity of the two highest nominal concentrations of triclosan in the experiment with extracts from WWTP 3 (Table 2). Ishibashi et al. (2004) reported 100% mortalities in Japanese medaka embryos exposed to nominal concentrations of triclosan between 0.62 and 2.5 μg/mL, and a significant reduction in hatching success and increase in time to hatch in treatments with nominal concentrations of 0.313 μg/mL.

The statistical significance of differences in hatch time between treatments could not be tested, since the assumptions of parametric and non-parametric statistical tests could not be met. However, mean hatch times in the 0.1 and 0.5 μg/mL treatments were 12.3 and 12.6 days, respectively, which is very similar to the mean hatch times in the solvent control treatment of 12.9 days; indicating no effects of triclosan on hatch time. Overall, these data indicate that early life stages of Japanese medaka responded in a concentration dependent manner to exposure to triclosan; a compound commonly found in municipal wastewater as a result of its use as an antimicrobial agent in personal care products.

Wastewater extracts

a) WWTP 1 and WWTP 2

In the treatments with wastewater collected from WWTP 1, there were high mortalities in treatments with extracts from all points in the plant (Table 3). Therefore, the tests were repeated with extracts diluted by 50% in the carrier solvent. Elevated mortalities and embryos that did not hatch were observed in treatments with the diluted extracts from WWTP 1 and the extracts from WWTP 2. Surprisingly, the lowest numbers of mortalities and numbers of embryos that did not hatch were observed in the treatments with extracts prepared from the untreated effluents from WWTP 1 and WWTP 2 (Table 3). It is possible that more hydrophobic micropollutants in the untreated wastewater were associated with suspended particulates or dissolved organic constituents (Artola-Garicano et al., 2003). Since SPE extraction of the wastewater was preceded by a filtration step, this may have removed a significant portion of the micropollutants.

In the tests with diluted extracts from WWTP 1, mortalities were greater (i.e. 5/12) and numbers of embryos that did not hatch (i.e. 3/12) in the treatment with pre-aeration wastewater (i.e. after primary clarifier) relative to the untreated wastewater (Table 3). This was also the case for WWTP 2, where mortalities (i.e. 6/12) and numbers of embryos that did not hatch (i.e. 7/12) were higher than in the treatment with untreated effluent (Table 3). In our previously published studies of the removals of targeted micropollutants (e.g. PPCPs, drugs of abuse) in these WWTPs (Baalbacki et al., 2017; Baalbacki et al., 2016), removal was relatively poor (i.e. <20%) in the primary clarifier, and there were negative removals (i.e. higher concentrations) after the primary clarification step for some compounds. However, it is instructive to note that there was relatively efficient removal of triclosan (i.e. >80%) in the primary clarifiers for both WWTP 1 and WWTP 2 (Balbaaki et al., 2016; Baalbaki et al., 2017).

Other studies have shown that removals of micropollutants in the primary clarifiers of WWTPs are a function of the balance between the pKa of the compound and the pH of the wastewater, as well as the octanol-water partition coefficient (log K_{ow}), which influences their partitioning to sludge (Behera et al., 2011; Stamatis and Konstantinou, 2013). For the toxicity assessment of wastewater from these two WWTPs, the extracts were prepared by SPE with MAX cartridges to concentrate acidic and neutral compounds. Therefore, changes in pH between the untreated wastewater and the pre-aeration (i.e. after primary clarifier) wastewater may have influenced the proportion of some acidic compounds in the neutral (i.e. more hydrophobic) and ionized (i.e. more hydrophilic) forms, and therefore, the efficiency with which these compounds were extracted from wastewater prior to toxicity testing. Of course, the bioassays were conducted in buffered rearing medium at a consistent pH (□7.4) and under controlled conditions of temperature, etc.

For extracts prepared from both WWTP 1 and WWTP 2, toxicity increased in treatments with extracts prepared from samples collected post-aeration (i.e. after biological treatment by activated sludge) relative to the extracts prepared from samples collected pre-aeration (Table 3). This was not expected, as our previous studies showed that the measured concentrations of most targeted PPCPs and drugs of abuse declined significantly to low ng/L concentrations after activated sludge treatment in both of these WWTPs (Balbaaki et al., 2016; Balbaaki et al., 2017). Negative removals were observed for some pharmaceuticals, including ephedrine, tramadol and carbamazepine, after activated sludge treatment in both WWTPs. This may be due to the de-conjugation of metabolites to the parent compound during the activated sludge treatment, as has been suggested by other authors (Ternes et al., 1998; Clara et al., 2005; Brorström-Lunden, 2008; Jelic et al., 2011). It is possible that the elevated toxicity observed in extracts prepared from samples collected after activated sludge treatment (i.e. post-aeration) in WWTP 1 and WWTP 2 was due to de-conjugation and subsequent release of parent compounds from sludge. Another explanation may be formation of transformation products as a result of microbial activity during activated sludge treatment. Reduced hatching success of fish has been previously observed in studies where early life stages of Japanese medaka were exposed directly to secondary wastewater effluents from egg fertilization to fry swim-up (Zha and Wang, 2005; Cao et al., 2009).

In tests with extracts of the final treated effluents from WWTP 1 that were diluted by 50%, the numbers of mortalities (i.e. 5/12) and the numbers of embryos that did not hatch (i.e. 4/12) were reduced relative to the post-aeration treatments (Table 3). This is consistent with the efficient removals of several PPCPs and drugs of abuse observed in our previous study of the final treated effluents from this WWTP, although there were several recalcitrant compounds (i.e.

carbamazepine, sucralose, tramadol, codeine) that were not removed efficiently by the treatment process (Baalbacki et al., 2017).

In tests with extracts from WWTP 2, surprisingly, there was not a reduction in toxicity in the tests conducted with extracts prepared from the treated effluent (Table 3). This is not consistent with data from our previous study that showed significant reductions (i.e. >75%) in the measured concentrations of several PPCPs and drugs of abuse in the treated wastewater from WWTP 2 (Baalbaki et al., 2016). This WWTP has a tertiary treatment step with rotating biological contactors and sand filtration. It is possible that these treatment technologies are releasing toxic parent compounds through de-conjugation, and this is contributing to the elevated toxicity. Our previous studies have shown that sand filtration is relatively efficient at removing hydrophobic micropollutants, but is less efficient at removing more water-soluble compounds (Sultana et al., in press). In any event, further work is required to determine whether similar observations of elevated toxicity occur in other WWTPs that have tertiary treatment stages.

b) WWTP 3

Table 4 summarizes the data on biological responses of early life stages of Japanese medaka exposed to extracts prepared from treated wastewater collected at WWTP 3. These extracts were prepared from treated wastewater (WW), treated wastewater spiked with 5 model CECs (WW + 5CECs) and spiked wastewater after ozonation (WW + 5CECs + O3). Total toxicity was significantly greater in the WW relative to the solvent control. Total toxicity in the WW+5CECs treatment was also elevated relative to that observed in the solvent control treatment (Table 4).

The number of embryos that did not hatch and the number of mortalities observed in the WW treatment was greater than the incidence of these endpoints observed in the solvent control treatment (Table 4). The observations of no hatch were similar to the reduced hatching success observed in the treatments with treated wastewater from WWTP 2 and WWTP 1 (Table 3), although the severity of the response was lower in the WW treatment from WWTP 3.

In addition, embryos in the WW treatment experienced delays in time to hatch relative to the solvent control treatment. The mean hatch time of 14.6 days observed for embryos in the WW treatment was approximately one day longer than the 13.5 days observed in the solvent control treatment. However, as assumptions of parametric and non-parametric statistical tests could not be met, statistical differences in hatch time between treatments could not be tested. Moribund embryos upon hatch and developmental abnormalities (haemorrhage, pericardial edema) were also observed in two fish from the WW treatment (Table 4).

It was expected that toxicity would have been higher in the spiked wastewater (i.e. WW+5CECs) when compared to the WW extract. However, similar levels of total toxicity were observed between these two treatments (Table 4). It is possible that the toxic effects of the spiked CECs were masked by the other constituents in the wastewater extracts through antagonistic effects. Among the embryos in the WW+5CECs treatment, four individuals were observed with the developmental abnormality of yolk sac edema, but there was the same hatching success in this treatment relative to the solvent control, with a mean hatch time of 13.8 days.

In Japanese medaka from the WW+5CECs+O3 treatment, low incidences of mortalities, no hatch, moribund embryos upon hatch and developmental abnormalities were observed (Table 4). The only developmental abnormality observed in this treatment was one embryo with a subcutaneous hemorrhage. Although most of the embryos hatched in the WW+5CECs+O3 treatment, the time to hatch for these embryos was delayed, with embryos in the ozonated treatment having a mean hatch time of 15.5 days compared to 13.5 days in the solvent control treatment. However, all but one embryo hatched in this treatment.

The delayed development observed in embryos exposed to the ozonated wastewater extract followed a similar pattern of delayed or incomplete hatch in medaka exposed over early life stage development to high (i.e. 1-10 µg/mL) concentrations of the pharmaceutical, diclofenac (Yamagami, 1981). In the case of exposure to diclofenac, it was suggested that the mechanism for delayed or incomplete hatch was inhibition of the secretion of hatching enzymes and/or enzymatic choriolysis (Yamagami, 1981). Although we are not suggesting that diclofenac was the causative agent for delayed hatch in the present study, exposure to micropollutants that induced this biological effect through a similar mechanism may have caused the delayed hatch of medaka exposed to extracts from ozonated wastewater. Other microcontaminants could have affected the time to hatch through other mechanisms. For instance, it has been shown that exposure of early life stages of salmonids to steroidal estrogens can alter time to hatch (Marlatt et al., 2014; Schubert et al., 2014).

Our previous studies with extracts prepared from wastewater collected from WWTP 3 showed that ozonation significantly reduced the levels of the 5 model CECs, but did not reduce oxidative stress (i.e. changes in ratios of reduced and oxidized glutathione) in juvenile trout exposed by intraperitoneal injection over 72 h to the same extracts (Maya et al., in press). Treatment of wastewater by ozonation can result in the formation of transformation products which are more toxic than the original parent compounds (Lee and von Gunten, 2016). Multiple studies have reported the formation of toxic transformation products following wastewater treatment by ozonation, including the formation of aldehydes, carboxylic acids, brominated organic compounds, and N-nitrosodimethylamine (Huang et al., 2005; Sgroi et al., 2014; Gerrity et al., 2015). Cao et al. (2009) observed significant disruptions in the hatching of Japanese medaka embryos exposed to ozonated wastewater. Yan et al. (2014) demonstrated a clear dose-response relationship between total aldehyde concentrations produced during ozonation and the frequency of adverse effects on developmental endpoints observed in early life stages of Japanese medaka exposed to ozonated wastewater. Aldehydes are very reactive with proteins, as the toxicity of both aldehydes and ketones to Japanese medaka has been shown to be related to reactivity with thiol-groups on proteins (Furuhama et al., 2012). Overall, it is likely that the toxicity observed in the WW+5CECs+O3 treatment, and specifically increased times to hatch was caused by exposure of the Japanese medaka embryos to toxic transformation products. Future work should include more extensive analysis to identify the compounds that may be responsible for these biological effects.

CONCLUSIONS

Removals of micropollutants within WWTPs vary widely, but treatment of municipal wastewater has been shown to reduce the measured concentrations of PPCPs, drugs of abuse and steroid hormones in the treated wastewater relative to the untreated wastewater (Verlicchi et al., 2012; Luo et al., 2014; Blair et al., 2015; Baalbaki et al., 2016; Baalbacki et al., 2017). However, the toxicity data presented in this study with early life stages of Japanese medaka indicate that declines in the measured concentrations of micropollutants in wastewater extracts may not correlate with reductions in toxicity. Biological transformations of conjugated metabolites back to the parent compounds, or formation of toxic transformation products may generate compounds in treated wastewater that are toxic to early life stages of fish and other aquatic organisms. The early life stages of medaka were exposed to extracts prepared from wastewater. This has the advantage of excluding toxic macropollutants (e.g. ammonia) from the bioassay matrix and focusing only on micropollutants isolated by the solid phase extraction technique. However, the extraction conditions and the methods (e.g. MAX vs MCX extraction cartridges) may alter the proportions and composition of these compounds, so care must be taken in extrapolating these results to exposure to whole effluents. More studies are needed to confirm the results from the present study that indicates that biological effects increase with level of wastewater treatment.

This study demonstrates the value of toxicity testing along with measurements of removals of micropollutants from wastewater to assess the effectiveness of treatment, since biological effects increased with the stages of wastewater treatment. *In vitro* bioassays are useful tools for rapid screening of large numbers of wastewater samples (Reungoat et al., 2010; Rizzo et al., 2011; Lee and von Gunten, 2016). Magdeberg et al. (2012) evaluated the toxicity of wastewater using *in vivo* bioassays with algae and invertebrates. However, early life stages of fish, and in particular, Japanese medaka are sensitive to the toxic effects of micropollutants in wastewater because of the high rates of metabolism and rapid changes at various levels of biological organization (e.g. gene expression, enzyme function, organogenesis) that occur during the development of embryos and larvae (Zha and Wang, 2005; Cao et al., 2009). The present study has contributed to the literature that demonstrates that early life stages of medaka can be used as a sensitive and relatively rapid test system for evaluating the toxicity of municipal wastewater.

Supplemental Data—The Supplemental Data are available on the Wiley Online Library at DOI: 10.1002/etc.xxxx.

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Data availability—Readers should contact Chris Metcalfe at Trent University (cmetcalfe@trentu.ca). The data are not yet available in a repository because a co-funder of the project is an industrial partner, Air Liquide Canada.

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Table1: Treatment parameters for the three WWTPs from which wastewater was collected for extraction and toxicity testing, and information on the samples collected from the WWTPs and the number of aliquots (100 mL) of the samples extracted for toxicity testing. UV = ultraviolet; CECs = Contaminants of Emerging Concern; WW = Wastewater; O3 = Ozonated; MAX and MCX = Oasis anion exchange and cation exchange extraction cartridges, respectively.

Parameter	**WWTP 1**	**WWTP 2**	**WWTP 3**
Population served	~79,000	~135,000	~92,000
Design capacity (m^3/day)	68,200	64,000	65,000
Treatment	Secondary (activated sludge); UV disinfection	Activated sludge plus tertiary (rotating biological contactors and sand filtration); Chlorine disinfection & de-chlorination	Secondary (activated sludge); No disinfection
Samples collected	Untreated, Pre-aeration, Post-aeration, Treated; 3 consecutive days	Untreated, Pre-aeration, Post-aeration, Treated; 4 consecutive days	Treated (WW); Samples spiked with CECs (WW + 5CECs); Ozonated (WW + 5CECs + O3)
Aliquots extracted and pooled	3 x 100 mL; MAX extractions	4 x 100 mL; MAX extractions	7 x 100 mL; MAX and MCX

			extractions
Final solvent	Methanol (0.5 mL)	Acetone (0.5 mL)	Acetone (1.5 mL)

Table 2: The numbers of early life stages of Japanese medaka (n=12 per treatment) showing mortality, delayed hatch, no hatch, moribund embryos after hatch, developmental abnormalities and total toxicity in the solvent (acetone) control treatment and in the positive control treatments with triclosan. Treatments with total toxicity that were significantly different than the solvent control treatment ($p<0.05$) are marked by asterisks (*). NA = Not applicable because of 100% mortality in the treatments.

Nominal concentration (µg/mL)	Mortality	Delayed hatch	No hatch	Moribund	Developmental abnormalities	Total toxicity
Solvent control	0	0	0	0	1	1
Triclosan						
0.1	0	0	0	0	5	5
0.5	0	1	2	0	2	5
1.0	12	NA	NA	NA	NA	12*
5.0	12	NA	NA	NA	NA	12*

Table 3: The number of early life stages of Japanese medaka (n=12) showing mortalities and no hatch in the positive control (triclosan) and solvent control treatments and in the treatments with extracts from WWTP1 and WWTP 2. For treatments with extracts from WWTP 1, data from toxicity tests conducted with 50% dilution of the extracts are shown in brackets. Treatments with total toxicity that were significantly different than the solvent control treatments ($p<0.05$) are marked with an asterisk (*).

Treatment	Mortality	No Hatch	Total Toxicity
Triclosan (1 µg/mL)	9	1	10*
WWTP 1			
Solvent control	2 [2]	0 [0]	2 [2]
Untreated	11 [3]	1 [2]	12* [5*]
Pre-aeration	12 [5]	[3]	12* [8*]
Post-aeration	12 [8]	[8]	12* [16*]
Treated	7 [5]	5 [4]	12* [9*]
WWTP 2			
Solvent control	1	0	1
Untreated	2	2	4*
Pre-aeration	6	7	13*
Post-aeration	6	8	14*
Treated	8	9	17*

Table 4: The number of early life stages of Japanese medaka (n=24) showing mortalities, delayed hatch, no hatch, moribund embryos after hatch, developmental abnormalities and total toxicity in the solvent control treatment and in the treatments with extracts from WWTP 3. Treatments with total toxicity that were significantly different than the solvent control treatment ($p<0.05$) are marked by an asterisk (*).

Treatment	Mortality (%)	Delayed hatch (%)	No hatch (%)	Moribund (%)	Developmental abnormalities (%)	Total toxicity (%)
Solvent control	1	1	3	0	0	5
WW	2	3	5	2	2	14*
WW+5CECs	1	1	3	2	4	13*
WW+5CECs+O3	1	13	1	1	1	14*