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EVALUATION OF WASTEWATER TREATMENT BY OZONATION FOR REDUCING THE TOXICITY OF CONTAMINANTS OF EMERGING CONCERN TO RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)

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Abstract:

While conventional wastewater treatment technologies are effective at removing many contaminants of emerging concern (CECs) from municipal wastewater, some contaminants are not removed efficiently. Ozonation may be a treatment option for reducing the concentrations of recalcitrant CECs in wastewater, but this process may generate toxic transformation-products. In the present study, we conducted semi-batch experiments to ozonate municipal wastewater effluent (WWE) spiked with 5 commonly detected CECs. The purpose of the study was to evaluate whether ozonation increased or decreased biological responses indicative of sublethal toxicity in juvenile rainbow trout (*Oncorhynchus mykiss*) injected intraperitoneally (i.p.) with extracts prepared from ozonated and non-ozonated WWE. Blood, liver and brain tissues were collected from the fish at 72 h post-injection for analysis of a battery of biomarkers. In fish i.p. injected with the extracts from non-ozonated WWE, significant induction of plasma vitellogenin (VTG) was observed, but ozonation of the MWWE spiked with CECs significantly reduced this estrogenic response. However, in fish injected with extracts from spiked MWWE after ozonation, the balance of hepatic glutathione in its oxidized (i.e. GSSG) form was altered, indicating oxidative stress. Levels of the neurotransmitter, serotonin, were significantly elevated in brain tissue from trout injected with the extracts from ozonated spiked MWWE; a biological response that has not been previously reported in fish. Other *in vivo* biomarkers showed no significant changes across treatments. These results indicate that ozonation reduces the estrogenicity of wastewater, but may increase other sublethal responses. The increase in biomarker responses after ozonation may be due to the formation of biologically active products of transformation of CECs, but further work is needed to confirm this conclusion. This article is protected by copyright. All rights reserved

Keywords: micropollutants, wastewater, ozonation, endocrine disruption, oxidative stress

INTRODUCTION

Contaminants of emerging concern (CECs), including pharmaceuticals and personal care products and endocrine disrupting compounds have been widely detected in surface waters close to urban areas [1,2]. These contaminants primarily enter the aquatic environment with discharges of industrial and domestic wastewater [3]. Conventional wastewater treatment technologies are effective at removing many of these contaminants during the wastewater treatment process, but some CECs are recalcitrant [4,5]. Many studies have demonstrated that these CECs can induce sublethal biological responses in fish at environmentally relevant concentrations [6,7]. In addition, pharmaceuticals and estrogenic contaminants may act additively [8,9] and some mixtures have been reported to act synergistically [10].

The environmental impacts of CECs discharged in wastewater have been demonstrated with recent studies in which gonadal inter-sex, endocrine disruption, oxidative stress, and metabolic enzyme induction has been observed in fish collected or caged at locations downstream of WWTPs [11-14]. Environmental regulations to protect aquatic life are now being developed for some CECs [15], but reductions in the use of these contaminants will prove difficult to enforce due to consumer demand for personal care products and the importance of pharmaceuticals for protecting human health and wellbeing.

CECs can be efficiently removed from wastewater using a range of advanced treatment technologies [16-19]. While chemical analysis of wastewater before and after treatment indicates whether the parent compounds are eliminated, these studies do not provide information on whether a treatment process reduces the toxicity of the final effluent [20, 21]. Advanced wastewater treatment may produce toxic by-products that increase the toxicity of wastewater [16, 22, 23]. Complementary *in vivo* toxicity testing with fish can be used to assess whether

wastewater treatment reduces toxicity to aquatic organisms [24]. In a recent study, we showed through *in vivo* exposures of early life stages of Japanese medaka (*Oryzias latipes*) to extracts prepared from municipal wastewater that biological effects increased with each step of sewage treatment, including delayed hatch in medaka exposed to extracts of wastewater after ozonation [25].

The purpose of this study was to assess whether ozonation of municipal wastewater effluent (WWE) spiked with five CECs reduces sublethal toxicity to juvenile rainbow trout (*Oncorhynchus mykiss*) that is associated with exposure to CECs and other micropollutants. Toxicity was also assessed in fish exposed to a CEC mixture composed of triclosan, diclofenac, carbamazepine, estrone and androstenedione. A bench scale semi-batch reactor was used to ozonate municipal WWE spiked with the five CECs. Ozonated and non-ozonated wastewater was extracted using solid phase extraction (SPE) techniques and concentrated for toxicity testing. The extracts were injected intraperitoneally (i.p.) into juvenile rainbow trout and 72 h post-injection, blood, liver and brain tissues were collected from the fish for analysis of biological responses, including biomarkers of oxidative stress, Phase I metabolism, endocrine disruption and alterations to a brain neurotransmitter.

MATERIALS AND METHODS

Test chemicals

High-performance liquid chromatography (HPLC) grade methanol was purchased from VWR International (Mississauga, ON, Canada). Reagent ACS grade ethyl ether was purchased from ACP Chemicals Inc. (Montreal, QC, Canada). Corn oil was purchased from a commercial retailer. Formic acid and dimethyl sulfoxide (DMSO) was purchased from Sigma-Aldrich

(Oakville, ON, Canada). The sources of the reagents and equipment used for the various biomarker protocols are described below for each method.

Triclosan (TCS), diclofenac (DCF), carbamazepine (CBZ) and estrone (E1) were purchased from Sigma-Aldrich, and androstenedione (ADD) was purchased from Toronto Research Chemicals (Toronto, ON, Canada). ADD is a natural androgen and E1 is a natural estrogen, and both compounds are produced through the steroid biosynthesis pathway. DFC is a non-steroidal anti-inflammatory (NSAID) drug and CBZ is a neuroactive drug originally developed as an anti-epileptic. TCS is a bacteriostatic compound added to many personal care products, soaps and surface cleaners.

Ozonation experiments

Semi-batch ozonation experiments were conducted using a bench scale ozonation system. The apparatus consisted of an ozone generator (OZONIA–TOGC2) fitted with an inlet online ozone gas monitor (WEDECO HC-400) and an outlet online ozone gas monitor (WEDECO MC-400), an Alicat digital gas flow meter, HOBO data acquisition system, ozone destruction unit for the off-gas, and a glass reaction vessel with a working volume of 700 mL. To spike the system with model CECs, 1.4 ml from a stock solution containing 50 µg/mL of target compounds in methanol was transferred into an empty reactor and the solvent was allowed to evaporate. Then, 700 mL of secondary wastewater effluent (WWE) collected on September 22nd, 2015 from the wastewater treatment plant serving the municipality of La Prairie (QC, Canada) was added to the reactor vessel and the solution was sonicated for 15 minutes to ensure that the CECs were dissolved in the wastewater matrix. The nominal concentrations of the model CECs in the final working solutions were 100 ng/mL. Following this, the reactor vessel was installed within the semi-batch ozonation system and the matrix was stirred for 30 min prior to ozonation.

Ozonation was completed using doses ranging from 0.41-0.56 mg of ozone/mg of chemical oxygen demand (COD) in wastewater. COD in wastewater used in the ozonation experiments was in the range of 25-30 mg/L. Extracts from ozonated and non-ozonated wastewater were prepared to assess the sublethal biological responses in trout. The treatments are described in Table 1, along with treatment codes.

Wastewater extract preparation and analysis

Aliquots of 7 x 100 mL from the treatments listed in Table 1 were extracted by SPE using both mixed-mode cation exchange and anion exchange cartridges. Extractions with Oasis® MAX (6 mL, 400 mg) and Oasis® MCX (6 mL, 150 mg) were conducted according to methods described by Metcalfe et al. [26] and by Yargeau et al. [27], respectively. 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 of 0.5 mL in acetone.

The model compounds in extracts from each treatment were analyzed by liquid chromatography coupled with high resolution mass spectrometry (LC-HRMS) using an Accela LC system coupled to a LTQ Orbitrap XL (Thermo Fisher Scientific, Waltham, MA, USA). Chromatographic separation of the target compounds was achieved using an Agilent Zorbax Eclipse plus C18 RRHD analytical column (2.1 x 5.0 mm, 1.8 μm) heated to a temperature of 30°C. The mobile phase was binary mixture of 2 mM aqueous ammonium formate (solvent A)

and methanol (solvent B), both containing 0.1% formic acid. The flow rate of the mobile phase was 0.3 mL/min. The percentage of organic phase (B) was changed as follows: 0 min (10%), 1 min (10%), 2 min (35%), 5 min (40%), 9 min (100%), 12 min (100%), 13 min (10%) and 15 min (10%). A heated electrospray ionization (HESI) source was used for ionization and the instrument was operated both in positive and negative modes. A linear calibration curve of seven points from 5 ng/mL to 150 ng/mL was used for quantification. The Limits of Detection for all model compounds were <1 ng/mL.

Treatments

Stock wastewater extracts for each treatment were prepared for toxicity testing by pooling together the seven replicate extracts from both the MAX cartridges (i.e. 700 mL) and MCX cartridges (i.e. 700 mL), then evaporating the acetone solvent from the pooled extracts to dryness using nitrogen gas, and dissolving in 5 mL of a carrier solvent. The carrier (i.e. CO) was corn oil with 8% DMSO added to reduce viscosity and aid in solvation of more polar spiked compounds. Fish were injected with extract volumes of 5 µL per g of body weight, which corresponds to a dose equivalent to 0.14 mL/g of the original volume of WWE collectively extracted by both the MAX and MCX cartridges (i.e. 1,400 mL). In addition, a CECs mixture was prepared by diluting the five model CEC compounds in CO for a nominal dose of 100 ng of each CEC compound per gram of fish body weight (n = 7 - 8 trout per treatment). A solvent control treatment (i.e. fish injected with CO) was used to assess baseline levels of biomarker responses (n = 5 - 6 trout per treatment).

Exposure experiments were conducted with juvenile hatchery-reared rainbow trout purchased from Linwood Acres Trout Farm, (Campbellcroft, ON, Canada), ranging from 15 - 20 cm in size. The trout were acclimated for two weeks in a 600 L tank containing partially

recirculating, filtered Otonabee River water with a 16:8 light:dark photoperiod. Fish were fed trout pellets daily at 1 - 2 percent of wet body weight. Water temperature (11-15 °C) was measured daily, and dissolved oxygen (80 – 100 % saturation), pH (6.5 - 7.5), nitrate, nitrite, and ammonia (< 0.5 ppm) were measured weekly. All protocols for experiments with rainbow trout were approved by the Animal Care Committee of Trent University, Peterborough, ON, Canada.

Fish were intraperitoneally (i.p.) injected with aliquots of the different stock solutions. Prior to injection, fish were placed for approximately 30 seconds in a 100 µg/mL solution of tricaine methanesulfonate (MS-222) purchased from Argent Chemical Laboratories (Redmond, WA, USA). Once sedated, the fish wet weight was recorded and injections were performed using a 26½ G tuberculin needle and 1 mL syringe in the mid-ventral section of the fish, directly behind the anal fin. Fish were injected with a volume of 5 µL per g of body weight, and the injection lesion was sealed with Gorilla Super Glue purchased from a commercial retailer to avoid leakage. The anal, adipose and/or dorsal fins were clipped to distinguish fish from the different treatments. Fish were allowed to recover in a bucket of well-aerated water and were later placed in a 600 L exposure tank with partially recirculating filtered Otonabee River water. At 72 h post-injection, fish were stunned with a blow to the head and blood was removed (1,000 µL ± 500 µL) from the caudal vein, using a 26½ G tuberculin needle and a 1 mL syringe, and the blood was placed into 1.5 mL Eppendorf tubes. Following this, fish were sacrificed by spinal severance and the liver, and brain tissue were quickly removed and immediately placed into cryogenic vials (Cole-Parmer Canada, Montreal, QC, Canada) and snap frozen in liquid nitrogen. To obtain plasma, the blood was stored on ice for 4 hours and then centrifuged at 5,000 g for 10 minutes at 4°C. Following centrifugation, blood plasma was removed, placed into cryogenic

vials and snap frozen in liquid nitrogen. Tissue and plasma samples were stored in an ultra-low temperature freezer at -80°C until removed for biomarker analysis.

Biomarkers

Total and oxidized glutathione. The concentrations of both total glutathione (i.e. tGSH) and oxidized glutathione (i.e. GSSG) were measured in homogenates prepared from liver tissue using protocols adapted from methods described previously [28, 29]. All reagents used in this assay were purchased from Sigma-Aldrich. These methods were previously described in detail by Martin et al. [30]. Briefly, the supernatant prepared from centrifuged homogenates of rainbow trout liver were added to wells of a 96-well microtiter plate. After adding glutathione reductase (GR), absorbance was read at λ 412 nm over 20 minutes using a SpectraMAX Plus 384 UV-Vis plate reader (Molecular Devices, Sunnyvale, CA, USA). The tGSH concentrations (nmol/g of tissue) were determined by comparing the sample absorbance values to a tGSH standard curve. To prepare the samples for GSSG analysis, the supernatant from homogenate was incubated in the dark for 90 minutes, and following incubation, the sample was added with reaction mixture and GR to the wells of a 96-well microtiter plate. Absorbance was read immediately after adding GR at λ 412 nm over 20 minutes using the SpectraMAX Plus 384 UV-Vis plate reader. GSSG concentrations (nmol/g of tissue) were determined by comparing the sample absorbance values to a GSSG standard curve.

Thiobarbituric acid reactive substances (TBARS). The concentrations of TBARS in juvenile rainbow trout liver tissue were measured with the assay adapted from the protocol described previously [28]. All reagents used in this assay were purchased from Sigma-Aldrich, with the exception of 2-thiobarbituric acid (TBA), which was purchased from Toronto Research Chemicals (Toronto, ON, Canada). These methods were described earlier by Diamond et al.

[14]. Briefly, homogenate prepared from rainbow trout liver was added to an Eppendorf tube with a reaction mixture containing TBA and buffer. This mixture was vortexed and immediately boiled for 15 minutes to initiate the reaction. Following boiling, samples were cooled on ice, vigorously vortexed with 750 µL of butanol, and centrifuged at 10,000 g for 5 minutes at room temperature. Following centrifugation, an aliquot of the organic phase from the homogenate or an analytical standard were added to the wells of a 96-well microtiter plate, and the absorbance was read at λ 532 nm using the SpectraMAX Plus 384 UV-Vis plate reader. Concentrations of TBARS (nmol/g of tissue) were determined by comparing the sample absorbance to a standard curve for solutions of malondialdehyde (MDA).

Vitellogenin (VTG). VTG in rainbow trout blood plasma was measured with a rainbow trout VTG enzyme-linked immunosorbent (ELISA) kit purchased from Biosense Laboratories (Bergen, Norway). All solutions and reagents required to complete the assay were provided in the kit. Plasma samples were thawed on ice, vigorously vortexed, and prepared at a minimum of two dilutions in buffer to ensure that sample absorbance was within the range of the standard curve. Dilutions of 25,000x, 50,000x, and 75,000x were used for plasma samples from the treatment with the CECs alone, and 25x and 50x dilutions were used for the remaining plasma samples. Two wells were used to calculate the non-specific binding of labeled VTG-specific antibody to the capture antibody in the absence of VTG (i.e. background noise) and all standards and sample dilutions were run in duplicate. Absorbance of 96-well plates was read at λ 405 nm, using a SpectraMAX Plus 384 UV-Vis plate reader. The VTG concentration (ng/mL of plasma) was determined by comparing the sample absorbance to a standard curve, constructed using VTG standard in dilution buffer, with concentrations ranging from 0.39 to 200 ng/mL.

17β-estradiol (E2) and testosterone (T). For the determination of plasma steroids in trout blood plasma, plasma samples were extracted from binding proteins using a previously described protocol [31]. Briefly, plasma volumes ranging from 50 to 200 µL were added to 16 x 150 mm glass test tubes, and the total volume in the tubes was adjusted to 1.0 mL with MilliQ water. Five mL of ethyl ether was then added to each test tube and the solution was vigorously vortexed for 20 seconds. The mixture was allowed to sit and then the organic phase was removed and placed into a clean test tube. The extraction was completed in triplicate for each plasma sample and the extracts were pooled and evaporated to dryness in a warm water bath (40°C ± 5°C) under a slow stream of nitrogen. Steroid extracts were then reconstituted in enzyme immunoassay buffer provided in the enzyme immunoassay kits.

Concentrations of E2 and T extracted from blood plasma were measured using an estradiol enzyme immunoassays (EIA) Kit® and a testosterone EIA Kit®, respectively, purchased from Cayman Chemical (Ann Arbor, MI, USA). All solutions and reagents required for the assays were provided in the kits. Plasma extracts were prepared at two dilutions to ensure that sample absorbance values were within the standard curve and all dilutions and standards were run in duplicate. Dilutions were prepared as follows: a) 5x and 10x dilutions for all E2 analyses, b) 10x and 12x dilution for T analyses from the treatment with the CECs alone, and c) 4x and 6x dilutions for T analyses from all other treatments. Two non-specific binding wells, and two maximum binding wells were run with each plate to account for non-immunological binding of the tracer to the well and maximum binding of tracer to the antibody, respectively. Absorbance of the 96-well plate commercial kits was read at λ 412 nm using a SpectraMAX Plus 384 UV-Vis plate reader. Steroid concentrations (pg/mL of plasma) were determined by comparing sample absorbance values to standard curves constructed from E2 EIA standard in

buffer at concentrations ranging from 6.6 to 4000 pg/mL and from T EIA standard in EIA buffer at concentrations ranging from 3.9 to 500 pg/mL.

Serotonin. Stock solutions of serotonin HCl purchased from Sigma Aldrich and serotonin-d4 creatine purchased from CDN Isotopes were prepared by dissolving in a solvent of MeOH-Milli Q water (90:10, v/v). Serotonin was extracted from brain tissue of rainbow trout using a formic acid extraction methodology adapted from a method developed for extraction of rodent brain tissue [32]. Briefly, rainbow trout brain tissue (134 ± 40 mg) was weighed and placed in a 1.5 mL Eppendorf tube and was spiked with 10 µL of a 25 µg/mL solution of serotonin-d4 (i.e. internal standard) and then mixed with 990 µL of ice-cold 0.1 M formic acid in MilliQ water. Tissues were homogenized and then centrifuged at 13,300 g for 20 minutes at 4°C. The top aqueous layer was removed and placed into a clean 1.5 mL Eppendorf tube and samples were analyzed on the same day of preparation.

Serotonin was analyzed by LC-HRMS using an Orbitrap Q Exactive instrument (Thermo Fisher, Bremen, Germany) equipped with an electrospray ionization source (ESI). The analytes were separated chromatographically using a Dionex Ultimate 3000 HPLC operated with a reverse phase column, which was a Genesis C18 column (150 mm x 2.1 mm ID; 4 mm particle size) purchased from Chromatographic Specialties (Brockville, ON, Canada), coupled with a guard column with the same packing material (4 mm x 2.0 mm) purchased from Phenomenex (Torrance, CA, USA). The mobile phase for chromatographic separation was a binary mixture of [A] 0.1 % formic acid in MilliQ water, and [B] methanol (100%). Separation by HPLC was performed using a flow rate of 0.2 mL/min with the following gradient: [B] 5% over 0.5 minutes, increasing to [B] 95% over 6.5 minutes, held at [B] 95% for 5 minutes, decreased to [B] 5% over 1 minute, held at [B] 5% for 5 minutes for a total run time of 18 minutes.

Protonated molecular ions and product ions for the serotonin and serotonin-d4 were detected in positive polarity mode using targeted selected ion monitoring (t-SIM) in data dependent MS^2 (i.e. t-SIM/ddMS^2) mode. The t-SIM analysis was conducted with a mass resolution of 70,000 and ddMS^2 was conducted with a mass resolution of 35,000. The electrospray needle voltage was 4 kV, the flow of ultrapure nitrogen sheath gas was 30L/min, the heated metal capillary temperature was 250°C, and the scan range was 0 to 200 m/z. Serotonin concentrations (ng/mg of brain tissue) were determined by comparing the sample peak area to a standard curve constructed using the ratio of the peak area of the serotonin-d4 internal standard, to the peak area of the calibration standard, with concentrations ranging from 0.25 to 150 ng/mL.

Ethoxyresorufin-O-deethylase (EROD). The activity of this enzyme from the cytochrome P4501A (CYP1A) monooxygenase family of metabolic enzymes was measured in liver tissue from juvenile rainbow trout using an EROD assay with hepatic S-9 preparation, adapted from previously described protocols [33, 34]. All reagents used in this assay were purchased from Sigma-Aldrich, with the exception of the Bio-Rad protein assay reagent, which was purchased from Bio-Rad Laboratories Canada (Mississauga, ON, Canada). The EROD analysis method was previously described in detail by Diamond et al. [14]. Briefly, homogenate of rainbow trout liver tissue was prepared in buffer and then centrifuged at 9,000 g for 20 minutes at 4°C to isolate the S-9 fraction. Aliquots of the S-9 preparation and a solution of ethoxyresorufin (ER) in HEPES buffer were added to wells of a 96-well microtiter plate. Following a 10-minute incubation period in the dark, NADPH was quickly added to each well to initiate the conversion of 7-ER to resorufin. The reaction was read every 30 seconds for 12 minutes at λ_{exc} 530 nm and λ_{em} 586 nm, using a Gemini EM fluorescence plate reader (Molecular Devices, Sunnyvale, CA, USA). The rate of resorufin production in each S9 fraction was compared to a resorufin standard

curve. The protein content in each microsomal fraction was determined using Bio-Rad reagent added to wells of a 96-well microtiter plate. Following a 5-minute incubation period in the dark, absorbance levels were measured at λ 600 nm using a SpectraMAX Plus 384 UV-Vis plate reader. Protein concentrations were determined by comparing sample absorbance values to a standard curve prepared with bovine serum albumin in MilliQ water. The specific EROD activity was measured as pmol/mg/min of protein by normalizing the EROD activity to the protein content in the S9 fraction.

Statistical analyses

The assumptions of normality and homogeneity of variance for biomarker responses within each treatment were tested using a Kolmogorov-Smirnov test ($p<0.05$) and a Levene's test ($p<0.05$), respectively. A one-way analysis of variance (ANOVA) was used to determine if there were statistically significant differences in biomarker responses between treatment groups. When the assumptions of normality and/or homogeneity of variance were not met, data on biomarker responses were either log-transformed or transformed with a reciprocal transformation in order to meet both assumptions. When a significant difference in biomarker responses was observed between treatments ($p<0.05$), a Tukey's *post hoc* test was used to determine differences between paired treatments.

The assumptions of normality and homogeneity of variance for model CEC concentrations in the WW+5CECs and WW+5CECS+O3 extract treatments were tested using a Kolmogorov-Smirnov test ($p<0.05$) and an F-test ($p<0.05$), respectively. A one-tailed independent samples t-test ($p<0.05$) was used to determine if there were statistically significant differences in CEC concentrations between the two CEC-spiked extract treatments. When the assumptions of normality and/or homogeneity of variance were not met, data on CEC

concentrations were log-transformed to meet both assumptions. Finally, a Welch t-test ($p<0.05$) was used to determine statistically significant differences in CEC concentrations between treatment groups when the assumption of homogeneity of variance could not be met. 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

CECs in wastewater extracts

All of the model CECs were detected at low concentrations in pooled extracts from unspiked wastewater (i.e. WW), with the exception of androstenedione (ADD), which was below the limit of detection (Table 2). The toxicity testing conducted using extracts pooled from both types of SPE cartridges excluded other elements of the wastewater matrix which could have influenced toxicity (e.g. ammonia). However, the use of extracts for dosing fish may lead to unrealistically high exposures of fish to CECs, and it also makes it difficult to relate dosimetry by i.p. injection back to exposures to the CECs in the original volumes of WWE.

As shown in Table 2, all model CEC compounds spiked into WWE were present at µg/mL concentrations in the pooled extract from the non-ozonated treatment (i.e. WW+5CECs), but the levels of these model CECs were significantly reduced to ng/mL concentrations in extracts from the ozonation treatment (i.e. WW+5CECs+O3), which were comparable to levels present in unspiked wastewater (i.e. WW). Therefore, ozonation greatly reduced the concentrations of all model CECs in the MWWE extracts to concentrations close to or below those detected in extracts prepared from non-spiked WWE (Table 2). The volumes of the wastewater extracts injected into fish were 5 µL/g, so from the concentrations listed in Table 2, the doses of CECs in the extracts can be calculated. The doses of the CECs in extracts from the

WW+5CECs treatment (i.e. non-ozonated) varied from approximately 17 ng/g for E1 to 175 ng/g for CBZ. The doses of CECs in extracts from the WW+5CECs+O3 treatment were all <0.4 ng/g.

Oxidative Stress

One of the treatments with rainbow trout was injection of a mixture prepared by diluting the five model CEC compounds in corn oil solvent for a dose of 100 ng of each CEC compound per gram body weight. As illustrated in Figure 1, the levels of hepatic tGSH in fish injected with this model CEC mixture (i.e. 5CECs) were significantly higher than the levels of hepatic tGSH in fish injected with the extracts prepared from the WW+5CECs treatment ($p \leq 0.04$). However, these levels were not significantly greater than the hepatic tGSH levels observed in fish injected with the CO solvent or the WW extract ($p > 0.05$). The mean concentration of tGSH was significantly reduced in the treatment with WW+5CECs+O_3 relative to all other treatments (Figure 1).

As shown in Figure 2, there was a significant increase in the ratio of oxidized glutathione-to-total glutathione (GSSG-to-tGSH) observed in fish from the WW+5CECs+O3 treatment relative to fish from all other treatments, including the CO treatment (log-transformed data; $p > 0.05$). The decrease in tGSH (Figure 1) and increase in the GSSG-to-tGSH ratio (Figure 2) in fish injected with the ozonated wastewater extract indicates that exposure to extracts prepared from ozonated wastewater resulted in a decrease in antioxidant defenses (i.e. tGSH) and an increase in the levels of the oxidized form of glutathione; both of which are indicators of oxidative stress.

Severe oxidative stress can inhibit the production of GSH, as described by Zhang et al. [35] or increase the rate of oxidation of GSH into GSSG, as described by Hellou et al. [36]. The stress response in fish exposed to the extracts from the ozonation treatment was most likely due

to exposure to toxic transformation products formed during ozonation. The significant reduction in tGSH may have been caused by exposure to halides and/or aromatic moieties formed during ozonation, as both classes of compounds have been detected as toxic by-products resulting from the ozonation of wastewater [22]. The reaction of ozone with phenols and polycyclic aromatics can also produce various redox-active compounds, including phenoxy radicals, superoxide radicals and quinones [23].

The lack of a change in the GSSG-to-tGSH ratio observed in fish treated with the model CEC mixture, despite the significant change in levels of tGSH may indicate that the fish were under mild oxidative stress, but exhibited an adaptive response by increasing the synthesis of GSH, as described by Zhang et al. [35]. Li et al. [37] observed a similar "adaptive stage" in rainbow trout exposed to carbamazepine, whereby fish adapted to the production of reactive oxygen species (ROS) through an increase in their antioxidant defenses within the first 7 days of exposure. Similarly, diclofenac exposure has also been demonstrated to cause an increase in the concentrations of antioxidant enzymes in fish [38, 39].

Apart from GSH being an important non-enzymatic co-factor involved in balancing the cellular redox potential of cells, it is also used in phase II conjugation reactions in the presence of glutathione-S-transferase (GST). In this biotransformation reaction, GSH is conjugated to toxic biogenic molecules by GST, creating polar products that can be more easily removed from cells [36]. Cazenave et al. [12] and Jasinska et al. [13] reported significant elevations in GST in fish caged downstream of WWTPs, demonstrating that wastewater contains pollutants that undergo detoxification via phase II biotransformation. Therefore, the decrease in hepatic tGSH in fish exposed to the wastewater extracts may have been due to an increase in exposure to contaminants subject to phase II biotransformation via the conjugation of GSH, thereby,

reducing intracellular GSH levels. It is likely that the CECs present in the unspiked wastewater or a combination of these contaminants and the spiked model CECs contributed to the biomarker responses.

Although a significant reduction in antioxidant defenses (i.e. lower tGSH) and a significant increase in oxidative stress (i.e. higher GSSG ratio) were observed in fish injected with the extract from ozonated wastewater (i.e. WW+5CECs+O3), no differences in hepatic TBARS were observed between treatments ($p=0.37$), as shown in Figure 3. No elevation in TBARS indicates that there was no damage to cellular lipids observed in the fish, and this indicates that cellular antioxidant systems were able to successfully scavenge reactive compounds. The intracellular removal of ROS is mediated through antioxidant defenses, including vitamins, proteins and enzymes that scavenge these reactive species and reduce cellular damage [36, 40]. Elevated TBARS has been observed in fish exposed to high concentrations of carbamazepine and diclofenac [37-39]. The lack of a TBARS response in the present study could be attributed to the short exposure period and the low dose used.

Gagné et al. [41] observed an increase in lipid peroxidation in gill tissue of freshwater mussels exposed to diluted ozonated wastewater. This response was attributed to the formation of toxic transformation products, including carboxylic acids, aldehydes and ketones [41]. The lack of lipid damage observed in trout from the present study exposed to extracts from ozonated wastewater may be due to a lower production of toxic transformation products by the treatment system, species-specific differences in antioxidant defenses, the presence of different micropollutants and/or the use of extracts for exposures in the present study, rather than whole effluents to which mussels were exposed. The results observed for fish injected with the two non-ozonated wastewater extracts were very similar to those reported by Gagné et al. [42] in an

in vitro study with rainbow trout hepatocytes exposed to 1% wastewater, where the hepatocytes showed evidence of oxidative stress, but no elevation of TBARS.

Endocrine disruption

As illustrated in Figure 4, the concentrations of plasma VTG were significantly elevated in fish injected with the model CECs mixture when compared to all other treatments (log-transformed data; $p<0.05$). The induction of VTG observed in fish from the 5CECs treatment can probably be attributed to the high doses of estrone (E1), androstenedione (ADD) and triclosan (TCS) in this treatment. Although ADD is androgenic, this steroid compound can be converted to estrogens by aromatase enzyme [43]. In addition, the estrogenic activity has been observed previously in fish exposed to TCS [44]. Therefore, the estrogenic response observed in fish injected with the model CEC mixture could have been due to the combined exposure to E1, ADD and TCS.

Mean plasma VTG levels were slightly elevated relative to controls in trout from the treatments with unspiked wastewater (i.e. WW) and spiked wastewater (i.e. WW+5CECs), as illustrated in Figure 4. Mean plasma VTG was significantly lower in fish injected with the extracts from ozonated wastewater when compared to fish injected with extracts from non-ozonated wastewater (log-transformed data; $p\leq0.004$). The levels of VTG in these fish did not differ significantly from fish from the CO treatment (log-transformed data; $p>0.05$), as shown in Figure 4.

The reduction in this estrogenic response observed in fish injected with the extract from ozonated wastewater (i.e. WW+5CECs+O3) is consistent with the low concentrations of E1, ADD and TCS measured in this extract (Table 2). No significant difference in plasma VTG was observed in fish exposed to extracts from the WW and WW+5CECs treatments (Figure 4),

despite the large difference in the concentrations of E1, ADD and TCS in the two extracts. Estrogenic chemicals have been shown to act additively [9], so all of the estrogenic compounds extracted from wastewater in the WW treatment may have contributed to the induction of VTG. The estrogenic effects were reduced in trout injected with extracts from the WW+5CECs+O3 treatment. The ER-binding affinity of steroidal and non-steroidal estrogenic compounds is dependent on the presence of a phenolic functional group. Because ozone reacts with phenols and activated aromatic compounds [23], it is likely that oxidation reduced the levels of estrogenic contaminants that bind to the ER.

The reduction in estrogenicity observed in fish following ozonation was very similar to findings reported previously [18, 24], where reductions in VTG were observed in juvenile rainbow trout following exposure to ozonated wastewater. The reduction in estrogenicity observed in the present investigation contributes to the literature showing that ozonation of wastewater reduces estrogenic effects in fish.

Plasma E2 levels in fish injected with the model CEC mixture were significantly greater than E2 levels in fish from all other treatments (log-transformed data; $p<0.05$), as shown in Table 3. Similarly, plasma T levels in fish injected with the model CEC mixture were significantly greater when compared to fish injected with the wastewater extracts (reciprocal transformed data, $p\leq0.03$). However, no differences in plasma T levels were observed in fish injected with the model CEC mixture when compared to fish injected with CO (reciprocal transformed data, $p>0.05$), as shown in Table 3.

Androstenedione (ADD) can promote aromatase activity in fish, resulting in enhanced production of T and E1 through steroidogenic pathways [45]. Furthermore, exposure to E1 can ultimately lead to the production of estradiol (E2) via the activity of 17β-hydroxysteroid

dehydrogenase [46]. Therefore, the significant elevation in plasma E2 and T observed in fish injected with the model CEC mixture can be attributed to the high dose of E1 and ADD administered to fish in these treatments. No statistically significant differences in mean plasma steroid levels were observed between fish injected with the wastewater extracts and CO solvent (log-transformed data; $p>0.05$). This finding suggests that either the concentrations of ADD and E1 present in the spiked MWWE extracts were not high enough to elicit any significant changes in plasma steroids, or that contaminants present in the wastewater extracts may have acted as inhibitors within the hypothalomo-pituitary gonadal (HPG) axis, counteracting the stimulatory effects of ADD and E1. For example, wastewater contaminants may have altered the function and expression of aromatase and other enzymes in the HPG-axis, modulating the endogenous production of estrogens [43].

Neurotransmitters

Mean levels of serotonin (5-HT) in the brain tissue of fish injected with the extracts from the WW and WW+5CECs treatments, as well as the 5CECs treatment were not significantly different from the levels of this neurotransmitter in fish from the control treatment (log-transformed data; $p>0.05$), as shown in Figure 5.

These results with the WW, WW+5CECs and 5CECs treatments are similar to previous studies assessing the effects of exposure to naphthalene on brain monoamine neurotransmitters in rainbow trout, where no changes in the concentrations of hypothalamic and pituitary serotonin were observed following an exposure period of 3 days or less [47, 48]. However, significant changes in brain serotonin were observed in the hypothalamus at 5 days post exposure [47].

Therefore, it is possible that the acute exposure period conducted in the present study was not long enough to induce changes in the concentrations of brain serotonin.

In contrast, mean serotonin levels in fish brain tissue were significantly greater in fish injected with the extract from the WW+5CECs+O3 treatment when compared to fish from the CO treatment (log-transformed; $p \leq 0.02$), as shown in Figure 5. These observations indicate that exposure to extract prepared from ozonated wastewater induced a serotonergic response. It is likely that this serotonergic response was caused by exposure to transformation products of ozonation. However, the exact mechanism(s) by which these by-products exerted their serotonergic effect remains to be evaluated.

To our knowledge, this is the first study demonstrating neuroendocrine effects in fish due to exposure to a complex mixture of compounds present in municipal wastewater, although serotogenic responses were previously reported in freshwater mussels exposed to municipal effluents [49]. In addition, serotonin and other brain monoamines were modulated in rainbow trout exposed to venlafaxine, an antidepressant that is widely detected in wastewater [50]. In fish, changes to the levels of neurotransmitters can act as important modulators of the endocrine cascade that controls reproduction. For example, serotonin can alter steroidogenesis by interacting with gonadotropin-releasing hormone (GnRH), resulting in gonadotropin secretion and the induction of steroidogenesis [51]. However, in the present study, the serotogenic response in fish from the WW+5CECs+O3 treatment was not sufficient to alter steroidogenesis, as indicated by the lack of changes to circulating levels of E2 and T (Table 4). Endogenous hormones can also act as regulators of neurotransmitters by up-regulating or down-regulating hypothalamic monoaminergic systems [47, 48]. However, it cannot be discounted that long-term

alterations to brain serotonin levels in fish exposed to ozonated wastewater could affect reproduction.

EROD

As shown in Figure 6, no significant differences in hepatic EROD activity were observed between the various treatments (log-transformed data; $p>0.05$). Many organic contaminants that have been detected in wastewater can induce CYP1A enzyme activity, and the induction of CYP1A in fish has been observed in fish exposed to wastewater [39]. However, pharmaceuticals and personal care products commonly found in WWE have been observed to both induce and inhibit CYP1A-mediated metabolism. For example, *in vitro* hepatic CYP1 inhibition has been observed in zebrafish exposed to fluoxetine and erythromycin [52], and E2 and octylphenol inhibited *in vitro* EROD activity in rainbow trout hepatocytes [53]. In contrast, bezafibrate, ibuprofen, naproxen, and antidepressants from the selective serotonin reuptake inhibitor (SSRI) class induced CYP1A in PLHC-1 cell lines from topminnow, *Poeciliops lucida* [54]. In an *in vitro* study with a rainbow trout gonadal cell line, exposure to a mixture of pharmaceuticals and fragrances resulted in the synergistic induction of EROD, while exposure to other mixtures of wastewater-derived chemicals did not induce EROD activity [10]. The results from the present study for fish injected with the model CEC mixture alone were consistent with this study by Fernández et al. [10], where EROD induction was not observed in the rainbow trout cell line exposed to a mixture of pharmaceuticals and endocrine disruptors.

CONCLUSIONS

The variety of *in vivo* responses observed in juvenile rainbow trout exposed to extracts from the various treatments reinforces the importance of measuring a range of biological responses when assessing whether wastewater treatment increases or decreases toxicity [55]. The

approach used in this study to expose rainbow trout to extracts prepared from WWE has the advantage of isolating and concentrating the CECs that are present in the wastewater. However, this approach may also elevate CECs to unrealistically high doses that do not reflect direct exposures to WW effluent.

Biological responses were observed in fish injected with the extracts from ozonated wastewater . These fish showed a reduction in plasma VTG when compared to fish injected with extracts from non-ozonated wastewater, but fish injected with extracts from ozonated wastewater experienced an increase in oxidative stress, as well as elevation of brain serotonin. These responses could be caused by exposure to oxidative transformation products of the chemicals present and/or spiked into the wastewater. Previous studies have shown that many redox-active products can be formed by ozonation [23].

There are wastewater treatment options to mitigate the production of toxic transformation products by ozonation. The use of biofiltration following ozonation may be a beneficial addition to treatment systems in order to reduce toxicity [56]. Catalytic ozonation could also be tested as an alternative wastewater treatment technology as reaction pathways with hydroxy free-radicals could improve micropollutant removal and reduce formation of toxic by-products [57]. In addition, future studies should further assess the *in vivo* biological responses induced by CEC mixtures, and in particular the serotogenic effects of the by-products of ozonation.

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Figure 1: Mean tGSH + S.E. (nmol/g) in livers of juvenile rainbow trout (n = 5 - 8 per treatment) i.p. injected with CO, wastewater extracts, or model CEC mixture. Wastewater extracts injected into fish at doses of 5 µL per gram were equivalent to 0.14 mL/g of the original wastewater volume extracted by MAX and MCX cartridges, and the nominal doses of each compound in the 5 CECs treatment injected into the fish were 100 ng/g. Statistical differences between treatment groups are denoted by different letter codes ($p<0.05$). Note that standard errors are shown for reference only, as the data were log-transformed for statistical analysis.

Figure 2: GSSG as a percent of tGSH + S.E. (nmol/g) in livers of juvenile rainbow trout (n = 5 - 8 per treatment) i.p. injected with CO, wastewater extracts or a model CEC mixture. Wastewater extracts injected into fish at doses of 5 µL per gram were equivalent to 0.14 mL/g of the original wastewater volume extracted by MAX and MCX cartridges, and the nominal doses of each compound in the 5 CECs treatment injected into the fish were 100 ng/g. Statistical differences between treatment groups are denoted by different letter codes ($p<0.05$). Note that standard errors are shown for reference only, as the data were log-transformed for statistical analysis.

Figure 3: Mean TBARS + S.E. (nmol/g) in livers of juvenile rainbow trout (n = 5 - 8 per treatment) i.p. injected with CO, wastewater extracts or a model CEC mixture. Wastewater extracts injected into fish at doses of 5 µL per gram were equivalent to 0.14 mL/g of the original wastewater volume extracted by MAX and MCX cartridges, and the nominal doses of each compound in the 5 CECs treatment injected into the fish were 100 ng/g. Note that standard errors are shown for reference only, as the data were log-transformed for statistical analysis.

Figure 4: Mean plasma vitellogenin + S.E. (ng/mL plasma) in juvenile rainbow trout (n = 5 - 7 per treatment) i.p. injected with CO, wastewater extracts or a model CEC mixture. Wastewater extracts injected into fish at doses of 5 µL per gram were equivalent to 0.14 mL/g of the original wastewater volume extracted by MAX and MCX cartridges, and the nominal doses of each compound in the 5 CECs treatment injected into the fish were 100 ng/g. Statistical differences between treatment groups are denoted by different letter codes ($p<0.05$). Note that the y-axis in this figure is on a log scale. Note that standard errors are shown for reference only, as the data were log-transformed for statistical analysis.

Figure 5: Mean brain serotonin (5-HT) + S.E. (ng/mg tissue) in juvenile rainbow trout (n = 5 - 7 per treatment) i.p. injected with CO, wastewater extracts or a model CEC mixture. Wastewater extracts injected into fish at doses of 5 µL per gram were equivalent to 0.14 mL/g of the original wastewater volume extracted by MAX and MCX cartridges, and the nominal doses of each compound in the 5 CECs treatment injected into the fish were 100 ng/g. Statistical differences between treatment groups are denoted by different letter codes ($p<0.05$). Note that standard errors are shown for reference only, as the data were log-transformed for statistical analysis.

Figure 6: Mean EROD activity + S.E. (pmol/mg/min) in livers of juvenile rainbow trout (n = 5 - 8 per treatment) I.P. injected with CO, wastewater extracts or a model CEC mixture. Wastewater

extracts injected into fish at doses of 5 µL per gram were equivalent to 0.14 mL/g of the original wastewater volume extracted by MAX and MCX cartridges, and the nominal doses of each compound in the 5 CECs treatment injected into the fish were 100 ng/g. Note that standard errors are shown for reference only, as the data were log-transformed for statistical analysis.

TABLES

Table 1: Description of the experimental treatments for generating extracts for *in vivo* assays with juvenile rainbow trout.

Wastewater extract	Description
CO	Carrier alone (i.e. 8% DMSO in corn oil)
WW	Extract prepared from non-ozonated wastewater
WW+5CECs	Extract prepared from non-ozonated wastewater previously spiked with five model CECs
WW+5CECs+O3	Extract prepared from ozonated wastewater previously spiked with five model CECs
5CECs	Carrier spiked with five model CECs

Table 2: Mean (±SD) measured concentrations of model CECs (ng/mL) detected in extracts prepared from the different wastewater treatments. Values are means of extract replicates (n = 14 per extract treatment). The concentrations of all target analytes were <LODs in the CO treatment. The concentrations of the target analytes were not measured in the 5CEC treatment.

	Concentration (ng/mL)		
Compound	WW	WW+5CECs	WW+5CECs+O3
ADD	<LOD[1]	12,832 ± 5,250	45 ± 23
CBZ	17 ± 6.2	35,438 ± 14,834	33 ± 21
DCF	411 ± 384	25,511 ± 8,682	42 ± 21
E1	195 ± 241	3,371 ± 1,496	75 ± 42
TCS	82 ± 33	12,969 ± 5,473	53 ± 26

1) Below level of detection (i.e. <0.5 ng/mL)

Table 3: Plasma 17β-estradiol and testosterone concentrations (pg/mL plasma) in juvenile rainbow trout i.p. injected with CO, wastewater extracts or a model CEC mixture. Values are means ± S.D. (n = 5-7 per treatment). Wastewater extracts injected into fish at doses of 5 µL per gram were equivalent to 0.14 mL/g of the original wastewater volume extracted by MAX and MCX cartridges, and the nominal doses of each compound in the 5 CECs treatment injected into the fish were 100 ng/g. Statistical differences between treatment groups are denoted by different letter codes ($p<0.05$).

Treatments	17β-estradiol (pg/mL plasma)	Testosterone (pg/mL plasma)
CO	959 ± 368 (a)	851 ± 389 (ab)
WW	747 ± 268 (a)	517 ± 138 (a)
WW+5CECs	648 ± 263 (a)	736 ± 195 (a)
WW+5CECs+ O3	596 ± 139 (a)	664 ± 132 (a)
5CECs	7179 ± 4343 (b)	2917 ± 2005 (b)

Figure 1

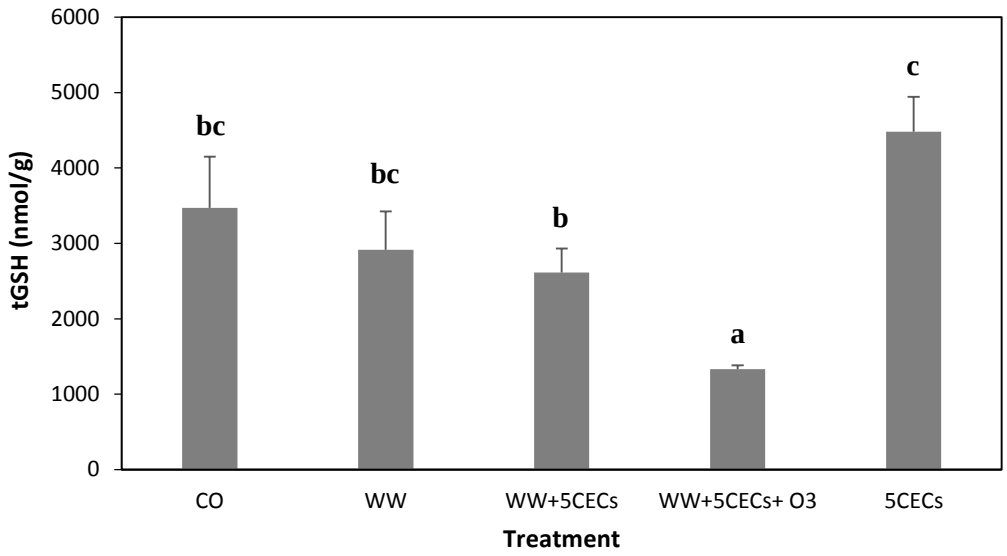

Figure 2

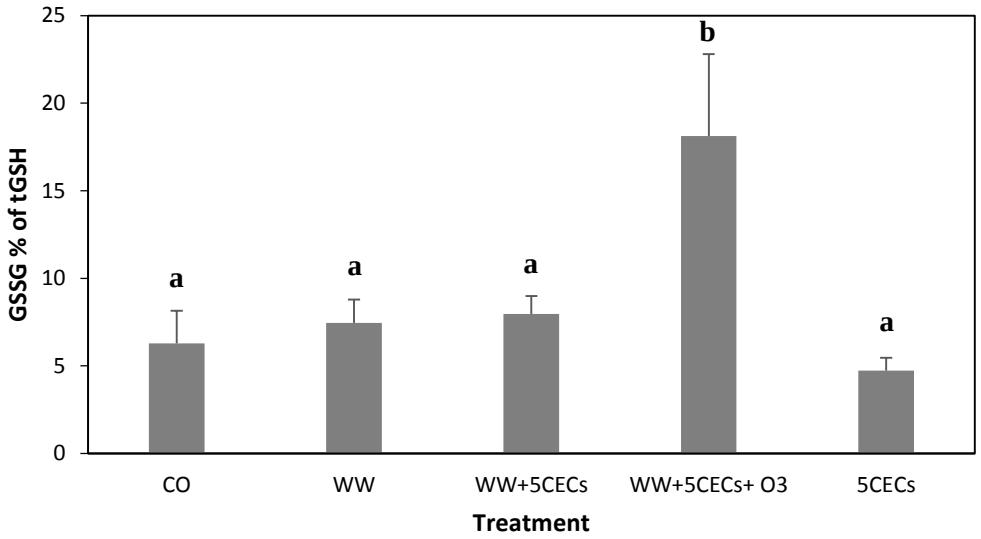

Figure 3

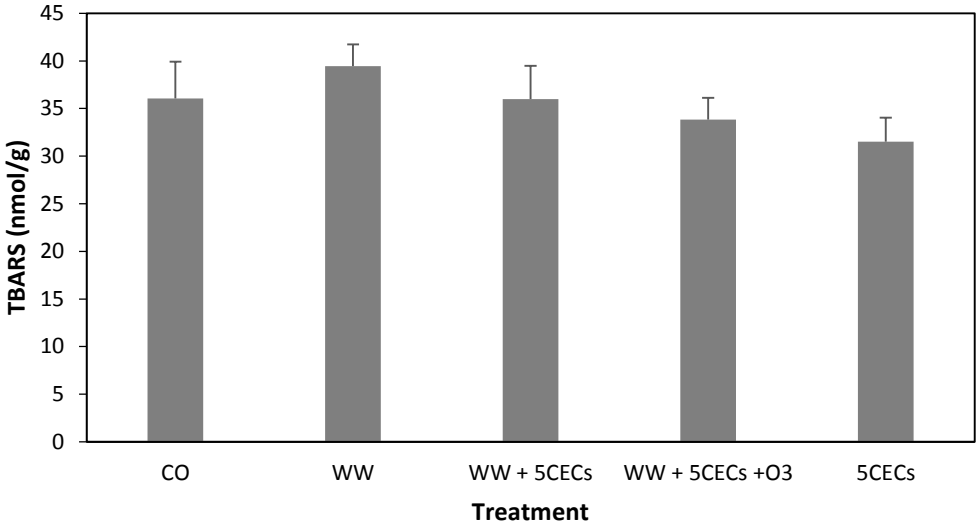

Figure 4

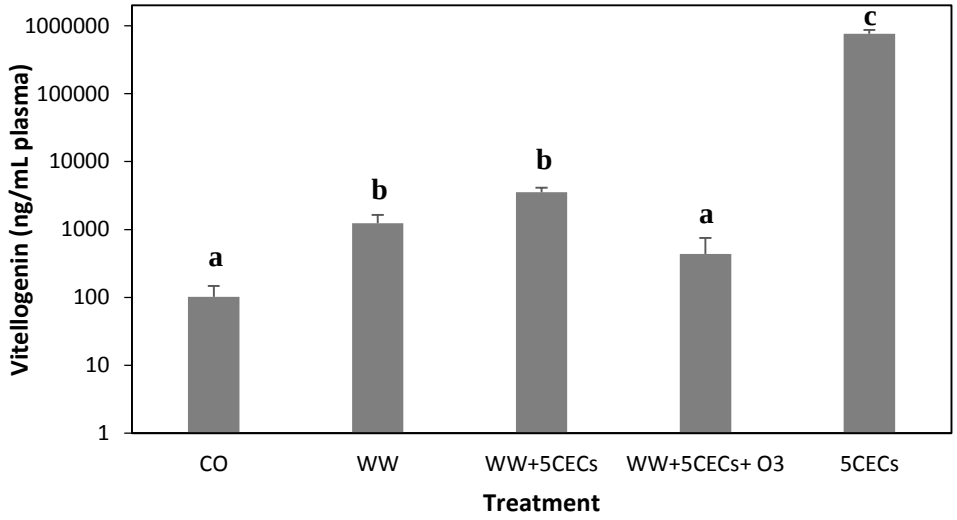

Figure 5

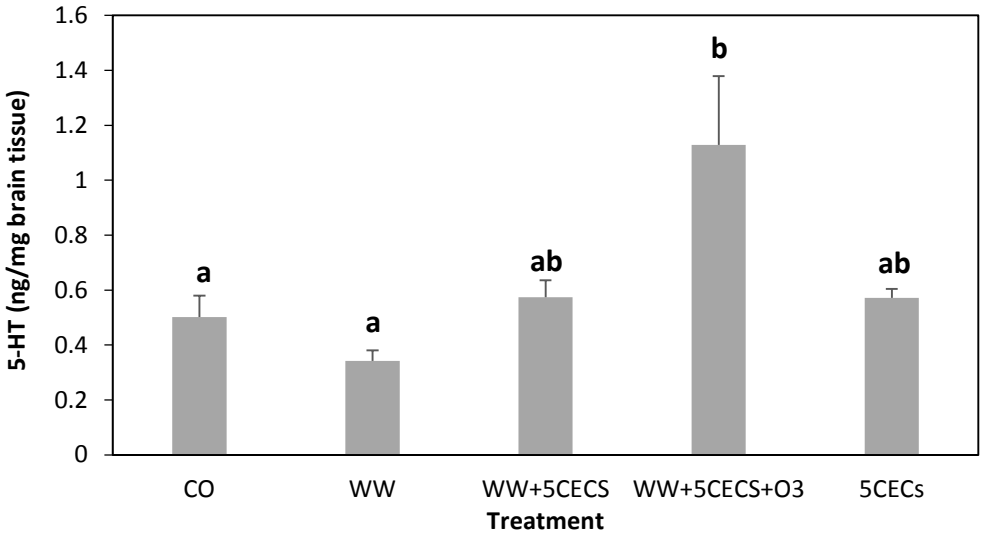

Figure 6