# Chemical, Microbial and Toxicological Assessment of Wastewater Treatment Plant Effluents during Disinfection by Ozonation

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## Abstract

Municipal wastewater treatment plants (WWTP) effluents are primary sources of pathogenic microorganisms and contaminants of emerging concern (CECs) released into the aquatic environment. Main concerns regarding these pollutants include transmission of waterborne diseases to humans and toxic and endocrine disrupting effects on aquatic organisms. In the coming years, WWTPs are expected to invest billions of dollars in upgrades to meet new regulatory requirements for wastewater from Environment Canada. For this reason, we investigated the performance of ozone when the technology is used for disinfection to overcome multiple risk factors such as disinfection, CEC removal, endocrine activity and toxicity for real effluents collected from three WWTPs. Two secondary effluents required mean specific ozone doses for disinfection of 0.25 and 1.04 $gO_3$/gDOC whereas the advanced primary effluent required 1.52 $gO_3$/gDOC to achieve a total coliform target disinfection criteria of 1000 MPN/100 ml (equivalent to 200 MPN/100 ml *E. coli*). At ozone doses for disinfection, CECs with high reactivity with ozone

were removed at levels greater than the target CEC removal of 80% for all WWTP effluents. For the secondary effluents, ozone doses above $2.6 \pm 0.6$ $gO_3$/gDOC were required to satisfy the target removal for the recalcitrant CECs. Within the disinfection ozone dose range, estrogenic activity was reduced by more than 98% and androgenic activity was removed by more than 68%, while the anti-estrogenic activity remained unchanged. Lastly, based on the luminescence inhibition of *V. fischeri,* ozone doses for disinfection produced secondary effluents exhibiting less than 20% inhibition, thus falling under the hazard classification "no acute/chronic toxicity".

## 1. **Introduction**

Numerous studies have demonstrated that municipal wastewater is the primary source of pathogenic microorganisms in the aquatic ecosystem [1]. These pathogens are a risk factor for transmission of waterborne diseases to humans. Recent concerns about municipal wastewater effluents as potential sources for antibiotic resistant bacteria and genes in the environment have also increased attention to the significance of efficient wastewater effluent disinfection [2, 3]. In addition to the release of pathogenic microorganisms to the aquatic ecosystems, wastewater is one of the major sources of contaminants of emerging concern (CECs) released into the aquatic environment. The detection of CECs in the environment has raised concerns about the impact of these contaminants on both the environment and on public health. Studies published in the peer-reviewed literature have demonstrated that many CECs are not removed efficiently in conventional wastewater treatment plants [4, 5] and that significant amounts of these compounds are discharged into the environment [6, 7]. During a recent sampling campaign in a river system in Ontario that is impacted by WWTP discharges, we demonstrated that CECs discharged along with treated wastewater in receiving waters can make their way downstream into drinking water sources [8].

Another major concern is the occurrence of natural and synthetic hormones in the aquatic environment, leading to adverse effects on aquatic life by interfering with the endocrine system [9-11]. At environmentally relevant concentrations, the synthetic estrogen used in oral contraceptive pills (i.e. ethinylestradiol) affects fish and can lead to induction of inter-sex in males [12], reduced fertility [13, 14] and population failure in a dosed lake [15]. Much of the research on endocrine disrupting chemicals (EDC) has focused on the presence of estrogenic activity in wastewater effluents and its elimination via conventional and alternative treatment technologies [9, 16]; only recently the potential of WWTP effluents on disruption of other crucial hormonal pathways such as androgenic, anti-estrogenic and anti-androgenic responses received attention [17, 18].

Experiments performed in the last decade on ozonation of CECs clearly demonstrate that this technology is effective at removing several classes of contaminants from water [19-24]. Ozonation has been shown to inactivate viruses, bacteria and even more resistant protozoan pathogens (e.g. *Giardia*, *Cryptosporidium*) under conditions where disinfection with chlorine and chlorine dioxide fail [25]. Use of ozone for wastewater disinfection has been evaluated since 1970s [26]. Due to the economic inefficiencies of the first generation of technologies, ozonation has been viewed, especially in North America, as a less attractive alternative to chlorine and UV disinfection. However, in recent years, more advanced ozonation technologies have gained attention as an economical option for advanced wastewater treatment because of the high capacity for oxidation of emerging contaminants and demonstrated efficiency as a disinfectant. The combination of microbial disinfection and effective removal of micropollutants makes ozonation an attractive alternative for advanced wastewater treatment. In contrast to the numerous ozone disinfection studies in clean matrices only few studies investigated the disinfection efficiency of ozonation for

treatment of real WWTP effluents [27-31]. Significant variations in required ozone doses (2 to 30 mg/L) were reported in order to reach 1 to 3 log inactivation of indicator organisms, such as total coliforms or *E. coli*. Most importantly, the differences in these required ozone doses are attributed to differences in wastewater composition and secondly, due to the design of the ozone introduction systems. Additionally, information was generally missing on the toxicity and/or residual bioactivity of transformation products formed during ozonation of treatment plant effluents containing CECs at ozone doses used for disinfection.

The marine luminescent bacterium *Vibrio fischeri* has been commonly used in various biological testing protocols for investigation of acute toxic responses. Due to its experimental simplicity, the bioluminescent bacteria test according to EN ISO 11348, commercialized as Microtox by Modern Water, was applied to determine the acute toxicity of numerous. Short-term bioluminescence inhibition tests such as Microtox are useful tools for assessing the ecotoxicological impact of unspecific acting toxicants but their low sensitivity due to a short exposure time (5-30 min) and current test approach using cuvettes for standard luminometers limit their use. There are numerous reports on acute toxicity of CECs using *V. fischeri* but only a handful of reports investigated chronic effects using this test organism [32-37]. These studies demonstrated the applicability of a chronic toxicity test using *V. fischeri* but only looked at the contribution of specific contaminants, their mixtures or transformation products of single parent compounds in clean matrices. No literature currently exists on the application of a high-throughput chronic *V. fischeri* assay for assessment of the toxic potential of real wastewater samples. Consequently, no literature also exists about the effect of ozonation on the *V. fischeri* chronic toxicity in the treated WWTP effluents containing mixture of CECs.

In the present study, we evaluated under conditions representative of municipal wastewater treatment, the impact on effluent quality (residual CEC content, toxicity and agonistic and antagonistic androgenic and estrogenic endocrine activity) of using ozone as a disinfectant. Mechanisms of reaction between ozone and various CECs have been reported in literature in the context of ozonation being used as a tertiary treatment but to our knowledge, no prior study investigated the performance of ozone to simultaneously overcome these multiple risk factors related to WWTP effluents when the technology is used for disinfection only. In the coming years, many municipalities will invest money to upgrade treatment infrastructures in order to meet new regulations, including in Canada where WWTPs are expected to invest billions of dollars in upgrades to treatment infrastructure to meet new regulatory requirements for wastewater from Environment Canada or to respond to recommendations from the Canadian Council of Ministers of the Environment (CCME) aimed at improving wastewater quality [38]. The results presented here will ensure that investments in advanced treatment technologies for disinfection have the added benefit of removing CECs and reducing their biological and toxicological impact; thereby, protecting the environment and providing safe and secure sources of drinking water.

## 2. Materials and Methods

### *2.1. Chemicals*

16 CECs were chosen as target compounds to investigate the efficiency of ozone at removing CECs during the disinfection of three WWTP effluents. The compounds were chosen based on their prevalence in WWTP effluents and to represent various classes of compounds from beta-blockers to biocides and different reactivity towards ozone. The list of CECs and their second order reaction rate constants with ozone can be found in Supplementary Information Table S1. Based on their second order reaction rate constants ($k_{O3}$) they were categorized as low-moderate ($k_{O3} < 1000$

$M^{-1}s^{-1}$) and high reactivity with ozone ($k_{O3}$> 1000 $M^{-1}s^{-1}$). Based on this categorization benzotriazole, ibuprofen, isoproturon and mecoprop fall under the category of low-moderate reactivity, the rest of CECs fall under high reactivity with ozone. All target CECs (purity > 98%) were purchased from Sigma-Aldrich. Solvents used in preparation of stock solutions such as methanol (LC-MS grade), ethanol (LC-MS grade) and DMSO were purchased from Fisher-Scientific. The chemicals used in the culture medium for *V. fischeri* were purchased from Fisher Scientific: Sodium chloride, trypton, yeast extract; Sigma Aldrich: potassium chloride and magnesium chloride; and EMD Chemicals: glycerol. Positive controls required for yeast-based in vitro assays were flutamide (FLT) (purity > 99%) and β-Estradiol (E2) (purity 98%) purchased from Sigma-Aldrich, 4-Hydroxytamoxifen (4-HT) (> 97% pure) purchased from Abcam, and Dihydrotestosterone (DHT) (98% pure) purchased from Steraloids Inc. DHT and FLT were used as positive controls in the yeast androgen screen (YAS) assay and E2 and 4-HT were used as positive controls in the yeast estrogen screen (YES) assay.

### *2.2. Collection, preservation and characterization of WWTP effluent samples*

24-hour composite samples of wastewater were collected from the effluents of treatment plants near Montreal, QC, Canada. Considering the variability of the composition of wastewater and the risk of obtaining site specific results if using only one source of wastewater, samples were collected from three different wastewater treatment plants. Description of the three treatment plants can be found in Table 1. WWTPs A and B are both using primary and activated sludge treatment whereas WWTP C is an advanced primary treatment facility (screening + de-gritting + coagulation/flocculation). Within four hours of collection, the effluent samples were characterized for chemical oxygen demand (COD), dissolved organic carbon (DOC), total nitrogen (TN), total suspended solids (TSS), conductivity, alkalinity and common anions (chloride, sulphate, nitrate

and nitrite) and metals (sodium, calcium, magnesium and potassium) using standard methods. Characteristics of the effluents can be found in Supplementary Information Table S2. Aliquots of the effluents were used within the same day of collection to determine the doses of ozone required to satisfy the target disinfection requirement of 200 MPN/100 ml (i.e. 2.3 log MPN/100 ml) of *E. coli* suggested by Ontario Ministry of Environment, Canada. The remaining effluent samples were frozen at -20°C until treatment by ozonation to determine CEC removal, endocrine activity and residual toxicity.

### *2.3. Preparation of working solutions and quantification of CECs in WW*

Frozen wastewater samples were thawed, brought to room temperature and an aliquot of 0.7L was spiked with CECs prior to treatment to obtain concentrations in the low µg/L (resulting measured concentrations – including native compounds – in the 20 – 100 µg/L range). The range of concentration was selected to obtain concentrations relevant to wastewater treatment while facilitating chemical analysis and detection of bioactivity associated with the CECs and their transformation products. CEC quantification and endocrine activity determination was performed on the same set of samples whereas chronic toxicity testing was performed on samples prepared and treated separately using slightly more elevated initial CEC concentrations (100 – 500 µg/L).

Spiking of CECs was performed by first transferring 1.4 ml of a stock solution containing a nominal concentration of 50 mg/L of target compounds in methanol into an empty reactor. The solvent was allowed to evaporate under a gentle flow of nitrogen considering that the presence of organic solvents is known to lead to enhanced consumption of ozone and scavenging of hydroxyl radicals. Following evaporation, 0.7 L of a WWTP effluent sample was added into the reactor and the mixture was sonicated for 15 minutes followed by stirring at 500 rpm for 30 minutes to allow homogenization. Concentrations of the target CECs were quantified using a Dionex ICS-5000

system coupled to a MSQ Surveyor mass spectrometer (Thermo Scientific). Samples were eluted on a C-18 Hypersil GOLD Column (2.1 x 50 mm, 1.9 μm particle size) maintained at 50 °C. The details of the chromatography, mass to charge ratios (m/z), ionization modes of respective molecular ions and limits of detection (LOD) of target CECs can be found in the Supplementary Information Table S3 and S4.

### *2.4. Ozonation of WWTP Effluent Samples*

The ozonation of WWTP effluents were carried out at room temperature in a bench-scale semi-batch reactor (working volume of 0.7 L), where ozone was introduced as a gaseous mixture of air and ozone. Ozone was generated using an ozone generator TRIOGEN TOGC2 and air as feed-stock. The inlet and outlet ozone concentrations were measured using two online ozone analyzers WEDECO HC-400 plus and WEDECO MC-400 plus, respectively. The flowrate of gas (~1 L/min) into the reactor was monitored using an ALICAT mass flow meter (M-5SLPM-D). The amount of ozone applied to the effluent was varied by increasing the treatment time. To account for the mass transfer limitations of small scale systems, the ozone concentrations in the inlet and outlet gas streams were used to determine the utilized ozone dose, which was used to report ozone doses (Calculations described in Supplementary Information). Prior to ozonation (t = 0) and then at different treatment times over a period of 30 minutes, 10 ml samples were collected from the reactor to quantify total coliforms, CEC concentrations, residual endocrine activity and chronic toxicity towards *V. fischeri*.

### 2.5. *Determination of Level of Disinfection*

The presence of total coliforms in the untreated and ozone treated WWTP effluent samples was quantified using a modified version of the USEPA approved Colilert® method developed by

IDEXX Laboratories. The method was adapted to a miniaturized microplate format to allow high-throughput analysis [39]. Two types of microplate configurations were used to quantify the total coliforms in the samples. Method 1 was used for samples expected to contain coliforms in the range of $8.9 \times 10^6 – 31$ MPN/ml (i.e. untreated samples and low ozone doses) and Method 2 was reserved for samples with total coliform concentrations in the range of 87 – 0.7 MPN/ml (i.e. treated with higher doses of ozone). First, the contents of a Colilert® snap-pack were transferred into a 100 ml of sterilized phosphate buffered saline solution (PBS). In Method 1, all the wells of a clear 96 well microplate were filled with 200 µl of this solution. 50 µl of sample was transferred into the first three wells in the first row and serially diluted 7 times (with a dilution factor of 5). This configuration allowed for analysis of 3 samples and 1 negative control (PBS solution only). In Method 2, all the wells of a 96 well microplate was filled with 150 µl of PBS containing the contents of the Colilert® snap-pack. No dilution was performed in this configuration as 100 µl of samples were added to all 4 wells in each row (total of 48 wells per sample). This arrangement allowed for analysis of 2 samples and 1 negative control (PBS only) in a plate. Once the microplates were loaded with the samples, they were sealed with transparent sealing tape and placed in an incubator at 35°C for 24 hours. Following the incubation period, positive wells (yellow) and negative wells (transparent) are recorded. An Excel based MPN calculator based on the work by Jarvis et al. (2009) [40] was used to enumerate the total coliforms.

### *2.6. Determination of endocrine activity using YES/YAS assays*

6 ml samples collected from the bench-scale ozonation system were dried to completion using a Thermo Scientific Speed Vac Concentrator (Savant SPD131DDA) coupled with a Refrigerated Vapor Trap (RVT4104) at 50 °C, 1 Torr at a ramp of 30 Torr/min. The dried samples were reconstituted in 0.6 ml of MeOH (preconcentration factor of 10) and vortexed vigorously.

### *2.6.1. Yeast Estrogen Screen Assay (YES) and Yeast Androgenic Screen Assay (YAS)*

Two independent recombinant yeast strain bioassays using the target species *Saccharomyces cerevisiae* were used to investigate the potential endocrine activity of wastewater extracts. These yeast-based assays had been stably transfected with the human estrogen receptor (hERα) and the human androgen receptor (hAR) and four different endpoints were examined (estrogenic, anti-estrogenic, androgenic, and anti-androgenic). The YES and YAS assays were perform as described in [41, 42] and in our previous works [43]. Briefly, the YES and YAS assays were conducted in 96-well plates that contained a triplicate 12 serial dilution of a sample of interest and a series of controls for validation (carrier solvent, yeast culture, and a positive control). The positive controls for the YES assay were E2 (estrogenic control) and 4-hydroxytamoxifan (anti-estrogenic control) and the positive controls for the YAS assay were DHT (androgenic control) and flutamide (anti-androgenic control). Details of data analysis and equivalency calculations can be found in the supporting information section.

## *2.7. Determination of chronic toxicity using V. fischeri*

The procedure used here for determination of chronic toxicity of samples using the marine luminescent bacterium *V. fischeri* is based on the method described in our previous study [44], which was modified to analyze aqueous samples. Briefly, the grown *V. fischeri* culture was diluted by nutrient supplemented seawater media (NSSWM) to yield an initial luminescence of 100 RLU. All the wells of an opaque 96-well microplate were then filled with 100 µL of this diluted *V. fischeri* solution. The nutrient concentration and the osmotic pressure in the samples were adjusted by diluting the samples with 10 times concentrated NSSWM (1 ml of 10X NSSWM to 9 ml of sample). 100 µL of the adjusted samples were then added into the microplate containing the diluted *V. fischeri* solution. The luminescence in each well was recorded over 24 hours every 15 minutes

by a Beckman-Coulter DTX 800 Multimode detector. Luminescence results were reported as an average of 8 recordings for each sample. The control in the plate was chosen to be NSSWM adjusted Milli-Q water (9 ml Milli-Q water + 1ml 10X NSSWM), providing the proper conditions for the bacteria to thrive. Due to the dynamic performance of the luminescence measurement over 24 hours, the integral approach was used to calculate the luminescence inhibition (%) rather than a specific time end-point, in accordance to our previous work [44].

## 3. Results and Discussion

### *3.1. Determination of ozone doses required for disinfection*

The evolution of total coliform concentration with respect to ozone dose is shown in Figure 1 (A, B and C) for effluents of the three WWTPs studied. The data collected over 4 separate sample collection and treatments per each WWTP were analyzed to identify global ozone doses which would satisfy the target disinfection requirement of 200 MPN/100 ml (i.e. 2.3 log MPN/100 ml) of *E. coli* for each plant. Considering that the method used was based on total coliforms and that literature reports that *E. coli* population represents about 10 – 20 % of total coliforms in WWTP effluents [45, 46], a range of target disinfection levels were determined to be between 2000 MPN/ 100 ml (Target 1) and 1000 MPN/100 ml (Target 2) of total coliforms (presented as red lines in Figure 1A, B and C).

The load of total coliforms present in the WWTP effluents prior to ozonation were on the same order of magnitude for WWTP A and WWTP B; however, the effluent from the WWTP C contained total coliforms at least two orders of magnitude larger than the other plants. The average initial total coliform loads of the effluents were 4.97 ± 0.53, 5.04 ± 0.76 and 7.10 ± 0.66 log MPN/100 ml for WWTP A, B and C, respectively. The values for the secondary effluents (WWTP A and B) were within the range of 3.3 – 6.5 log MPN/100 ml reported in literature [37, 46-48] and

the higher value observed at WWTP C can be explained by the different type of treatment, advanced primary, for which other authors have also reported levels of total coliforms up to 7.42 log MPN/100 ml [49-51].

Ozone doses to reach the two target disinfection levels were calculated from the first order decay fit and the corresponding 95% confidence intervals (C.I.) shown in Figure 1 and are presented here as "mean (95% C.I)". To reach the more conservative disinfection level (Target 2) of 1000 MPN/100 ml of total coliforms (3 log MPN/100 ml), utilized ozone doses were 7.9 (5.7 – 10.9), 1.7 (1.0 – 2.7) and 30.0 (26.3 – 35.8) mg $O_3$ /L for the effluents of WWTP A, B and C, respectively. The corresponding specific ozone doses ($gO_3$ /gDOC). specific ozone doses of 1.04 (0.75 – 1.43), 0.25 (0.15 – 0.40) and 1.52 (1.34 – 1.82) $gO_3$/gDOC were calculated for the effluents of WWTP A, B and C, respectively. At these conditions, approximately 2 log reduction was observed for the effluents of WWTP A and B and 4 log reduction for the effluent of WWTP C. Given the fact that only 10 - 20% of total coliform population is generally composed of *E.coli* and generally the log reduction for *E.coli* is greater than total coliforms at the same ozone dose [45, 52], it is safe to assume at the conditions mentioned above, the 200 MPN/100 ml *E.coli* target disinfection was satisfied. In a similar study, where secondary WWTP effluents were treated with ozone to achieve daily disinfection target of 235 cfu/100 ml of *E. coli*, between 1.5-2 log reduction was achieved at a transferred ozone dose of 6 mg/L and in order to achieve consistent compliance with effluent standards, due to variations in process conditions, an ozone dose in excess of 10 mg/L was suggested [53]. This result is consistent with the doses presented here except in the case of WWTP B, which required significantly less ozone to achieve the disinfection target. Except in the case of WWTP C (advanced primary effluent), the calculated specific ozone doses were within the economically feasible ozone doses of 0.5 to 1.0 $gO_3$ /gDOC [54].

The higher disinfection ozone dose required WWTP C can be explained by the higher total and dissolved organic load, the higher suspended solids and higher microbial load (Supplementary Information Table S2) of this advanced primary effluent. The higher concentration of suspended solids might have also contributed to the lower efficiency of inactivation considering that microorganisms can be shielded from ozone by the presence of activated sludge flocs [55], which can result in the release of microorganisms from the flocs at a later stage leading to positive responses during microbial enumeration [27]. When comparing the two secondary effluents (WWTP A and B), the higher disinfection ozone dose required for WWTP A might be explained by the higher alkalinity and soluble COD of this sample (Supplementary Information Table S2). In fact, the presence of carbonates and bicarbonates can lead to scavenging of hydroxyl radicals [55, 57] and when ozone decomposes into hydroxyl radicals in wastewater matrices (rather than reacting with COD), these hydroxyl radicals are more effective than ozone for the inactivation of *E.coli* [56].

For comparison of these bench scale experiments results with literature data, ozone doses required to reach several log reductions were calculated. Ozone doses ranging from 0.21 to 1.5 $gO_3$ /gDOC have been previously reported to achieve from 0.5 to 3 log reduction of *E. coli* at bench [45, 47, 54], pilot [37, 56, 57] and full scale [27] ozone treatment of secondary and tertiary effluents. Specific ozone doses of 1.09, 0.26 and 0.5 $gO_3$ /gDOC were required to reach a 2 log reduction for WWTP A, B and C, respectively, which are within the previously reported values. The values for WWTP A and B were also in the economically feasible ozone dose range of 0.5 – 1.0 $gO_3$ /gDOC [54], while WWTP C required a higher doses of ozone (up to 1.52 $gO_3$ /gDOC) to satisfy the disinfection target and reach up to 4 log total coliform reduction. Significantly higher *E. coli* inactivation of 5-6 logs at specific ozone doses of 0.5 and 1 $gO_3$ /gDOC was demonstrated by

Gamage et al. (2013) [46]; however, they performed their experiments with spiked *E. coli* in filtered secondary effluents thus the coliforms were more prone to attack by ozone since no shielding of bacteria from ozone would be possible. Additionally, it is reported that indigenous bacteria can have different inactivation efficiencies when compared to laboratory cultured bacteria as bacterial inactivation efficiency has been shown to be influenced by cell size, shape and membrane composition of the bacterial community [58].

### *3.2. CEC removal during disinfection*

The mean initial concentration of CECs and their standard deviations in the spiked WWTP effluents spiked with CECs are tabulated in Supplementary Information Table S5. The initial concentrations, including the native and spiked compounds, ranged from 20 to 90 µg/L. Considering the variability observed from one CEC to another and one wastewater sample to another, the measured concentrations were used in the calculations of removal as opposed to nominal concentration based on spiking. Removal levels of target CECs obtained at the specific ozone doses identified in the previous section as required for disinfection are presented in Figure 2 (removal at other ozone doses can be found in Supplementary Information Figure S1). The specific ozone doses leading to disinfection were 1.0 ± 0.3, 0.6 ± 0.2 and 1.4 ± 0.5 $gO_3$ /gDOC, respectively for WWTP A, B and C. In cases of concentrations <LODs, the removal was calculated assuming a residual concentration equal to the LOD, hence the reported values represent minimum removals. The target removal of CECs was set at 80% as per the guideline proposed in Switzerland [59, 60].

Results (Figure 2) indicate that for benzotriazole, ibuprofen, isoproturon and mecoprop, which are known to have low to moderate reactivity with ozone ($k_{O3}$<1000 $^{M-1}s^{-1}$), the 80% removal target was not satisfied in most cases. Other studies on the use of ozone as tertiary treatment have also

shown that higher doses of ozone were required to remove benzotriazole and mecoprop when compared to other CECs [20]. In a study where secondary effluents from six Swedish WWTPs were ozonated at bench scale, it was shown that in order to achieve more than 90% removal, CECs with high reactivity required only 0.4 – 1.33 $gO_3$ /gDOC while compounds with low reactivity required up to 3.87 $gO_3$ /gDOC [61]. Interestingly, for WWTP A, all the target CECs were removed by >80%, except for naproxen. Considering the high reactivity of naproxen with ozone ($k_{O3}>10^5$ $M^{-1}s^{-1}$), the lower estimated removal for naproxen might be biased by the fact that we are using the LOD value in our removal calculations whenever the concentration is <LODs. In this case, the initial concentration of naproxen being 21.8 ± 3.4 µg/L and the LOD being 7.67 µg/L, the maximum measurable removal is limited to 65%. Additionally, more than 95% removals of recalcitrant compounds were observed for the effluent of WWTP A, while the removal of these compounds were limited to around 50-75% for WWTP B and C. This significant difference might be associated to the higher conductivity, chloride and sulfate concentrations at WWTP A (Supplementary Information Table S2). The high conductivity suggests the presence of larger concentrations of cations and anions present in the wastewater and it has been previously shown that addition of NaCl enhanced the performance of ozonation [62] through generation of superoxide radicals. In addition, although sulfates can react with hydroxyl radicals and act as radical scavengers, it can also generate sulfate radicals [63], which may open other pathways of removal. Synergistic effects resulting from the presence of a variety oxidizing species such as ozone, hydroxyl radicals, sulfate radicals and superoxides might explain the higher removal of recalcitrant compounds during disinfection of the effluent of WWTP A.

### *3.3. Changes in endocrine activity during disinfection*

Figure 3 depicts the dose-response relationships for estrogenic (Figure 3 A-C), anti-estrogenic (Figure 3 D-F), androgenic (Figure 3 G-I) and anti-androgenic activity (Figure 3 J-L) of the extracts of WWTP effluents spiked with CECs and exposed to varying ozone doses. Figure S2 provided in the Supplementary information presents similar dose-response relationships for the unspiked wastewater effluents. Figure S2 indicates that no estrogenic, androgenic and anti-androgenic activity was in the extracts of unspiked wastewater, but strong anti-estrogenic response was observed for WWTP C, approximately 13.5 µg 4-HT/L and a moderate anti-estrogenic response was observed for WWTP A and B at a level of 1.3 µg 4-HT/L. For the spiked wastewater (Figure 3 J-L), no observable anti-androgenic activity was found in any of the untreated and treated samples of all WWTP effluents. The estrogenic, anti-estrogenic and androgenic activities of all samples were quantified by calculating the E2, 4-HT and DHT equivalencies, which are tabulated in Table 3.

The spiking of CECs resulted in variable initial bioactivities (No ozone, Table 3) which might be explained by differences in composition and nature of the compounds present in each wastewater effluent prior to spiking. The resulting initial E2-equivalencies were 0.75, 1.8 and 0.7 µg-E2/L for WWTP A, B and C, respectively, which is lower than the projected estrogenicity of about 200 µg-E2/L based on the amount of E1, E2 and EE2 spiked and the preconcentration of the samples. This reduced estrogenic activity of CEC spiked untreated effluent samples might be due to the native anti-estrogenicity of the wastewater matrix (Figure S2). Compounds such as diclofenac, ibuprofen and naproxen, which are also present in the mixture studied in this work, were shown to induce anti-estrogenic responses [64] and might have contributed to lowering the resulting initial estrogenic activity.

At close to specific ozone doses meeting disinfection requirements (0.3 – 1.1 $gO_3$ /gDOC) more than 99% estrogenic activity removal was observed for WWTP A and B and more than 98% for WWTP C (Table 3). However, anti-estrogenic activity was not removed for WWTP A (from 29.9 to 24.3 4-HT µg/L), increased for WWTP B (from 0.72 to 9.0 µg 4-HT/L) and remained not detectable WWTP C at all ozone doses studied. For WWTP A and B, the reduction in estrogenic activity could not be only attributed to ozone as presence of anti-estrogenicity could also mask the estrogenic response. It is probable that the observed persistent anti-estrogenic activity (for WWTP A and B) can be attributed to both the nature of transformation products, and/or effective oxidation of agonists than corresponding antagonists [65] or to the increased aqueous availability of compounds adsorbed to suspended solids upon ozonation.

When considering the androgenic response, the untreated samples of all WWTP effluents spiked with CECs showed a moderate androgenic activity at calculated DHT equivalents of 1.5, 1.8 and 2.3 µg-DHT/L for WWTP A, B and C, respectively (Table 3). Since no-anti androgenic activity was observed for background wastewater matrices (Supplementary Information Figure S2) and in spiked samples (Table 3); it is safe to assume that the observed reduction in androgenic activity with increased ozone dose is not due to any masking effects. More than 68% androgenic activity removal was observed for WWTP A at an ozone dose of 0.5 $gO_3$ /gDOC; at an elevated ozone dose of 2.2 no detectable androgenic activity was present for this effluent. In the cases of WWTP B and C, at ozone doses of 0.6 and 0.8 $gO_3$ /gDOC respectively, more than 90% androgenic activity removal was possible. Similarly, the effluents of WWTP B and C showed no detectable androgenic activity at elevated ozone doses of 2.6 and 3.3 $gO_3$ /gDOC, respectively.

In line with previously published works, the efficacy of ozone in altering the endocrine disrupting potential of WWTP effluents is also clearly demonstrated here, especially for reduction in

estrogenic activity. Such changes in endocrine activity might be explained by the transformation of compounds initially present in the wastewater into less bioactive compounds. Similar to the values obtained in this study, at ozone doses of 0.6 – 1.1 $gO_3$ /gDOC, more than 90% removal in estrogenic activity of WWTP effluents were reported previously [52, 65-67]. However, it is necessary to investigate further if the reduction in agonistic activity is in fact due to transformation products or due to antagonistic activity becoming more pronounced following ozonation which in turn masks the agonistic response. Even though some previously published works reported removals of 65-87% of anti-estrogenic activity at approximately 0.5 $gO_3$ /gDOC [18, 68], others have also reported an increase in antagonistic activity during ozonation [65]. Our results indicate that at ozone doses for disinfection provide a significant reduction in estrogenic and androgenic activity of wastewaters however anti-estrogenicity is not eliminated even at elevated ozone doses.

### 3.4. *Changes in chronic Toxicity towards V. fischeri during disinfection*

Figure 4 shows the evolution of bioluminescence of *V. fischeri* over 24 hours for the effluents of the three WWTPs with (Figure 4 A-C, graphs on the left) and without CECs (Figure 4 D-F, graphs on the right) at varying utilized ozone doses. Sensitivity experiments in Milli-Q water (Supplementary Information Table S6) showed that a luminescence inhibition of 50% and 20% was detectable at average CEC concentrations of 169.0 µg/L and 72.2 µg/L, respectively. This shows that if CECs in real WWTP effluent samples can be concentrated up to around 100 µg/L range, it will be likely to see a response by this chronic *V. fischeri* assay and allow assessing if same extracts induce acute or chronic responses in *V. fischeri* allowing the differentiation of specific and unspecific acting contaminants in effluents, simultaneously.

The untreated samples of all CEC spiked WWTP effluents induced a strong inhibitory response (~100%) due to the presence of CECs (Figure 4 A-C). As ozone dose was increased, the inhibitory

effects of the CECs and other contaminants found in the wastewaters diminished since the luminescence responses of treated effluents start approaching that of the control, suggesting that the transformation products formed have lower toxic responses than the parent compounds and that some mineralization might be occurring. In all ozone treated cases, the lag phase of the treated samples did not differ significantly than that of the control; however, in the case of CEC spiked effluent of WWTP A (Figure 4A) at ozone doses of 1.1±0.4 and 2.3±0.6 $gO_3$ /gDOC, the initial rate of luminescence was higher than that of the control. This trend was also observed in the effluent of WWTP A not spiked with CECs even when no ozone was introduced (Figure 4D). The presence of already available dissolved organic carbon in the wastewater matrix could have led to an increase in the food source for *V. fischeri* and hence affected the initial luminescence kinetics in a positive manner. As the ozone dose was increased in both systems, the breakdown products of CECs and the conversion of suspended organic material to dissolved organic carbon could have resulted in a further increase in the food source for *V. fischeri* and hence improvements in luminescence kinetics were observed. The lower level of luminescence measured at 20-24 hours in both spiked and not spiked cases, even for the highest ozone concentration studied, could be due to the combined synergistic contribution of chemicals of unknown origin already present in the wastewater, formation of transformation products with toxic nature and to accumulation of bacterial metabolites which induce product inhibition. For WWTP B (Figure 4B), the rate of initial luminescence approached that of the control with increasing ozone dose, in contrast to WWTP A, no improvement in this rate over the control with increasing ozone dose was observed suggesting that the material of unknown origin in the effluent of WWTP B either was not degraded by ozone or that this system was cleaner and increased ozonation did not lead to more readily degradable material. Similar trend was also observed for this effluent not spiked with CECs (Figure 4E). At

an ozone dose of 0.3±0.2 $gO_3$ /gDOC there was still residual toxicity for WWTP B effluent spiked with CECs as indicated by the reduced luminescence value at 20-24 hours, at higher ozone doses this the luminescence response was improved and approached that of the control.

Effluent of the WWTP C (Figure 4C and F), showed a distinctly different trend when compared to the effluents of other treatment plants. At an ozone dose of 0.4±0.2 $gO_3$ /gDOC for both spiked and not spiked systems as well as the untreated case of the not spiked system, the lag phase was unchanged, initial rate luminescence was slightly reduced but maximum luminescence was significantly lower than the control and the stationary phase is extremely short. This delayed toxic response trend was also observed previously in the presence of 30 mg/L streptomycin sulfate [69] and can be an indication of presence of similar acting compounds naturally present in the wastewater and not completely eliminated with ozone. At an ozone dose of 1.4±0.2 $gO_3$ /gDOC for the effluent spiked with CECs and at ozone doses larger than 1.2±0.2 $gO_3$ /gDOC for the effluent not spiked with CECs, the rate of initial luminescence was similar to that of the control, but the stationary phase luminescence value was significantly higher than that of the control. Similarly to the case of WWTP A, presence of naturally occurring organic carbon, conversion of suspended carbon to dissolved carbon upon ozonation and transformation products of CECs could collectively contribute to an increase in substrate concentration for *V. fischeri*, hence this stimulatory response could lead to over population at later stages and exhaustion of the food source at this elevated microbial load.

Figure 5 summarizes the luminescence inhibition percentages calculated using the integral approach applied at three levels of ozone doses for the three WWTPs either spiked (Figure 5A) or not spiked with CECs (Figure 5B). The target inhibition percentage of 20% (represented as a red horizontal line on the graphs) is based on the hazard classification system suggesting “no

acute/chronic toxicity" below that percentage of inhibition [70]. Control experiments performed using varying amounts of suspended matter up to a concentration of 1,000 mg/L (simulated by using the suspended material obtained after freeze drying influent of WWTP A) indicate that suspended material did not interfere with the readings (results not shown). The wastewater matrix itself induced approximately 60% and 72% luminescence inhibition, for WWTP A and C, respectively, whereas wastewater matrix of WWTP B showed a response <20% even before treatment (Figure 5A, 0 ozone dose). The spiking of the effluent with CECs elevated the initial inhibitions to almost 100% for all WWTPs. Toxicity of the unspiked effluents was reduced to below the target inhibition value of 20% at ozone doses of 0.3 ± 0.2 and 1.2 ± 0.2 $gO_3$ /gDOC for WWTP A and C, respectively (Figure 5A), while higher doses of 1.1 ± 0.4, 1.3 ± 0.5 and 1.4 ± 0.2 $gO_3$ /gDOC were required to reach the "no acute/chronic toxicity" level for CEC spiked effluents of WWTP A, B and C, respectively (Figure 5B). Except for the effluent of WWTP B spiked with CECs, ozone doses required to satisfy the target inhibition value of < 20% was below or very close to the dose required to reach disinfection. Similar decrease in toxicity and/or no increase in toxicity upon treatment of mixtures of CECs and secondary effluents with ozone was also shown in other works employing short-term bioassays with algae, *Daphnia magna* and *V. fischeri* [66, 71-73]. However, other studies on ozonation of secondary effluents reported increase in acute toxicity towards *V. fischeri* [63, 74] and decrease in hatching rates and severe developmental effects in fish were also reported [75, 76] suggesting that effluent characteristics might be impacting changes in toxicity during ozonation.

## 4. Conclusion

Secondary effluents of WWTP A and B were shown to require ozone doses within the previously reported economically feasible ozone dose range of 0.5 – 1 $gO_3$/gDOC, whereas the advanced

primary effluent of WWTP C required slightly larger ozone dose to satisfy the disinfection target. At close to disinfection doses determined for each type of effluent, 80% target CEC removal was satisfied for all CECs with high reactivity with ozone, however only for the effluent from WWTP A, the more recalcitrant CECs were also sufficiently removed (>95%) at these ozone doses. It is speculated that this enhanced removal could be due to the synergistic effects of generation of other oxidizing species such as sulfate, superoxide and hydroxyl radicals. On the other hand, ozone dose of more than $2.6 \pm 0.6$ $gO_3$/gDOC was required to remove more than 80% of the recalcitrant CECs in the effluents of WWTP B and C, which high demand might be explained by a high industrial component at WWTP B and a primary effluent at WWTP C. For samples spiked with CECs, more than 98% removal of estrogenic activity and 68-90% removal of androgenic activity was achieved for all WWTP effluents at ozone doses in the range satisfying disinfection criteria. However, anti-estrogenic activity of CEC spiked effluent samples did not seem to be influenced by the dose of ozone. Hence, the reduction in estrogenic activity could not be only attributed to ozone as presence of anti-estrogenicity could also mask the estrogenic response. Even though it is desirable to achieve significant reduction in estrogenic activity of WWTP effluents, the effect of anti-estrogenicity towards other aquatic species needs further evaluation. Further investigation regarding chronic toxicity towards *V. fischeri* revealed that in order to reduce the effluent toxicity to less than the target inhibition of 20%, ozone doses in the rage of $0.7 – 1.8$ $gO_3$/gDOC were required for all three WWTP effluents, which were slightly larger than the ozone doses required for disinfection. Considering the data presented here, the benefit of using ozonation as a wastewater effluent disinfection step was demonstrated via its capacity to simultaneously overcome multiple risk factors. Hence, regulatory framework for wastewater effluent should include bioactivity or toxicity

in addition to disinfection criteria in order to get the full benefits associated with optimization of treatment technologies using ozone.

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Tables and Figures

Table 1. Summary of the description of three WWTPS studied

| **Characteristics** | **WWTP A** | **WWTP B** | **WWTP C** |
|---|---|---|---|
| Capacity ($m^3$/day) | 65,000 | 55,000 | 2,500,000 |
| Residential/Industrial (%) Load Distribution | 50/50 | 30/70 | 70/30 |
| Sewer (Separate/Combined, %) | 0/100 | 10/90 | 40/60 |
| Population served | 92,000 (2012) | 55 657 (2015) | 1,886,000 (2011) |
| SRT (days) | 5-6 | 7 | N/A |
| Treatment train | Conventional Activated Sludge: Screening + Aeration basin + Secondary clarifier | Conventional Activated Sludge: Screening + Aeration basin + Secondary clarifier | Advanced Primary: Screen + Degritting + Coagulation + Flocculation |

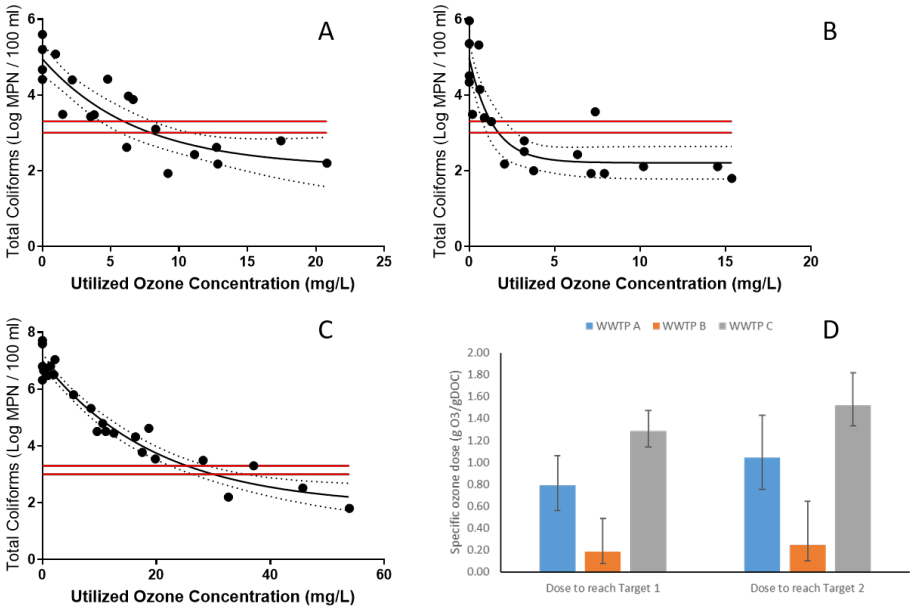


Figure 1. Variation of total coliform concentration (Log MPN/100ml) as a function of ozone dose for bench scale treatment of wastewater collected from A) WWTP A B) WWTP B C) WWTP C. The red lines indicate target total coliform concentrations of 2000 (Target 1) and 1000 (Target 2) MPN/100 ml. Dashed lines represent 95% Confidence Intervals on the fit. D) Specific ozone doses (g $O_3$/ g DOC) to achieve disinfection. Error bars represent 95% Confidence Intervals.

Table 2. Ozone doses to achieve log reduction of total coliforms during ozonation of effluents from three WWTPs

| Log Reduction | Specific Ozone dose (g O3 / g DOC) Mean (95% Confidence Interval) | | |
|---|---|---|---|
| | WWTP A | WWTP B | WWTP C |
| 1 | 0.39 (0.32 – 0.45) | 0.09 (0.08 – 0.1) | 0.22 (0.20- 0.24) |
| 2 | 1.09 (0.97 – 1.26) | 0.26 (0.25 – 0.27) | 0.5 (0.49 – 0.51) |
| 3 | N/A | N/A | 0.89 (0.88 – 0.90) |
| 4 | N/A | N/A | 1.52 (1.50 – 1.54) |

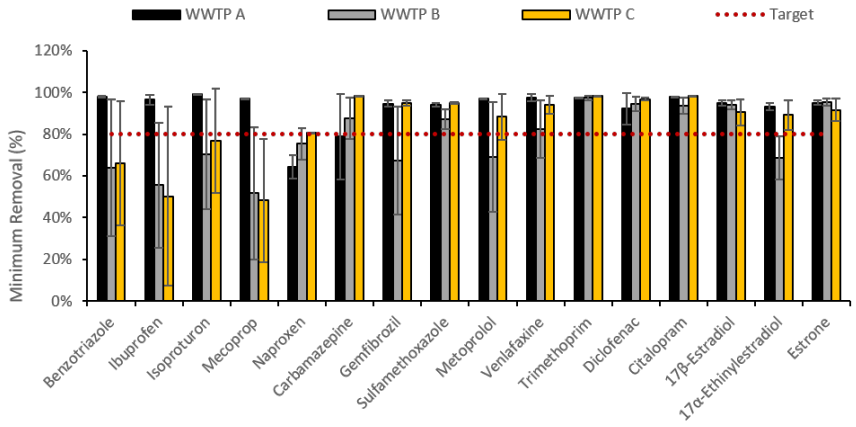


Figure 2. Minimum removals (%) of CECs at close to disinfection conditions for A) WWTP A (1.0 ± 0.3 g $O_3$/g DOC), B) WWTP B (0.6 ± 0.2 g $O_3$ / g DOC) and C) WWTP C (1.4 ± 0.5 g $O_3$/ g DOC). Error bars represent standard deviation (n=3)

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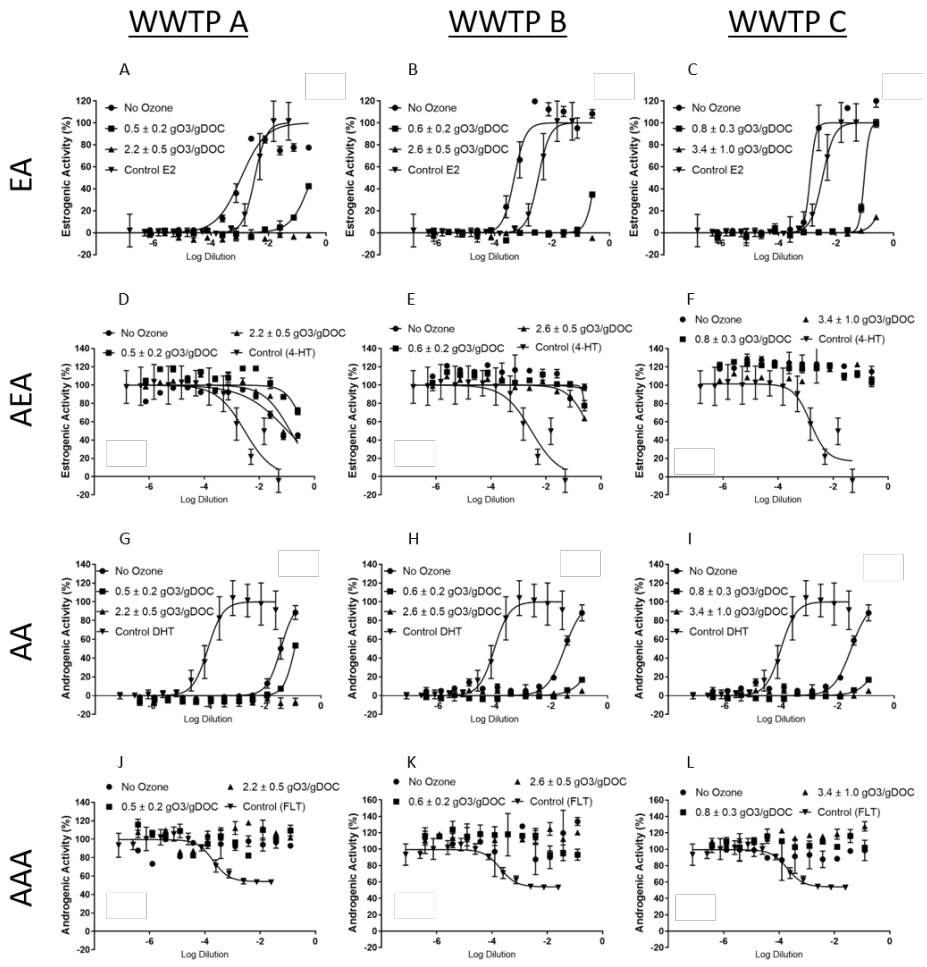


Figure 3. Dose-response relationship of treated and untreated WWTP (spiked with CECs) showing the evolution of estrogenic activity, anti-estrogenic activity, androgenic activity, and anti-androgenic activity for: WWTP A (A, D, G, and J), WWTP B (B, E, H, and K), and WWTP C (C, F, I, and L), respectively. EA-Estrogenic activity, AEA-Antiestrogenic activitiy, AA-Androgenic activiy, AAA-Antiandrogenic activity

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Table 3. Equivalencies of endocrine activities in the extracted samples

| Treatment plant | Sample | Estrogenic activity E2-EQ (ng/L)* | Anti-estrogenic activity 4HT-EQ (µg/L)** | Androgenic activity DHT-EQ (µg/L)*** |
|---|---|---|---|---|
| | | Mean (Min – Max) | | |
| WWTP A | Background**** | Not detected | 1.3 (0.3 – 2.3) | Not detected |
| | No Ozone | 746 (494-987) | 29.9 (26.4 – 33.5) | 1.5 (1.4 – 1.9) |
| | 0.5±0.2 $gO_3$/gDOC | 2.60 (2.55 – 2.63) | 6.8 (6.2 – 7.4) | 0.47 (0.45 – 0.50) |
| | 2.2±0.5 $gO_3$/gDOC | Not detected | 24.3 (24.0 – 24.6) | Not detected |
| WWTP B | Background | N/A | 1.3 (0 – 4.0) | Not detected |
| | No Ozone | 1781(1127 – 2466) | 0.72 (0.45 – 0.87) | 1.8 (1.5 – 2.1) |
| | 0.6±0.2 $gO_3$/gDOC | 2.8 (2.5 – 3.1) | 9.0 (8.1 – 9.8) | 0.12 (0.11 – 0.13) |
| | 2.6±0.6 $gO_3$/gDOC | N/A | 7.6 (7.3 – 7.9) | Not detected |
| WWTP C | Background | N/A | 13.5 (12 – 15.3) | Not detected |
| | No ozone | 691 (372 – 1009) | Not detected | 2.3 (1.7 – 3.0) |
| | 0.8±0.3 $gO_3$/gDOC | 9.3 (7.8 – 10.3) | Not detected | 0.10 (0.07 – 0.14) |
| | 3.3±1.1 $gO_3$/gDOC | 1.2 (0.8 – 1.6) | Not detected | Not detected |

* Ethinylestradiol equivalency
** 4hydroxytamoxifen equivalency
*** DHT equivalency
**** Wastewater background activity (wastewater not spiked with CECs)
No Anti-androgenic activity was present in any of the samples therefore calculation of FLT equivalency was not possible

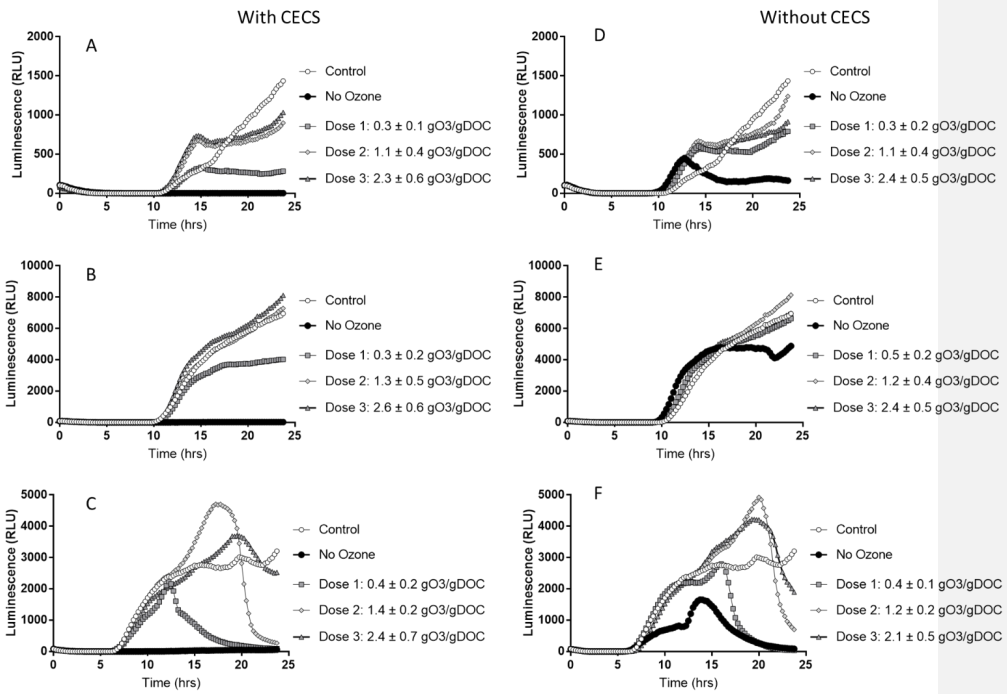


Figure 4. Evolution of *V. fischeri* luminescence with respect to time at different concentrations of utilized ozone dose for WWTP effluents with (graphs on the left) and without CECs spiked (graphs on the right): WWTP A (A & D), WWTP B (B & E), and WWTP C (C & F). Control refers to NSSWM + *V. fischeri*, No Ozone refers to the sample prior to ozonation (dose of 0 mg/L $O_3$).

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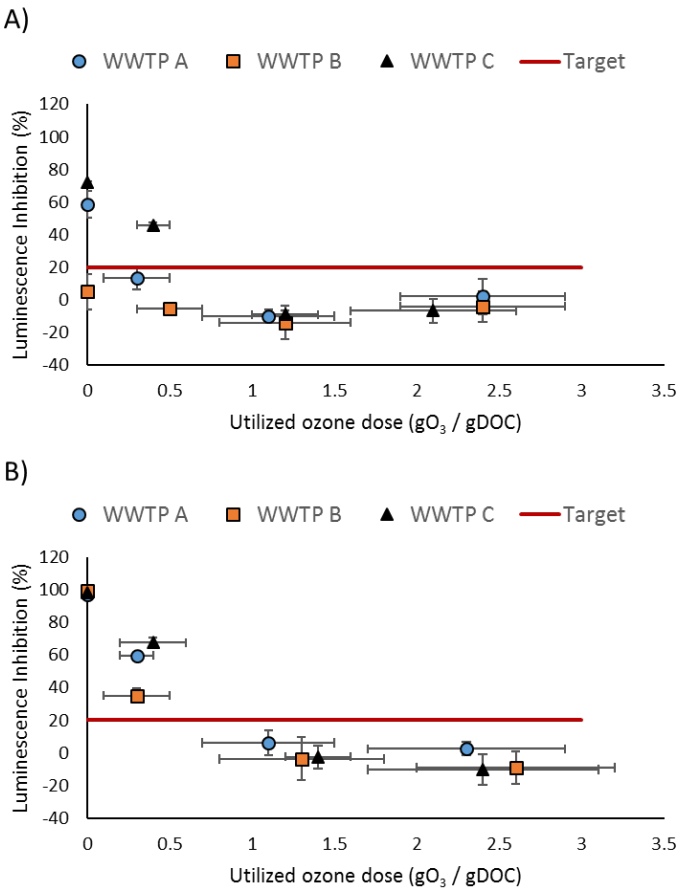


Figure 5. Comparison of integral luminescence inhibition calculated over 24 hours for WWTP effluents: A) without CECs (B) with spiked CECs

**Supplementary Information**

*Utilized ozone calculation*

The inlet and outlet ozone concentrations were measured using two online ozone analyzers WEDECO HC-400 plus and WEDECO MC-400 plus, respectively. The flowrate of gas into the reactor was monitored using an ALICAT mass flow meter (M-5SLPM-D). The amount of ozone utilized was varied by increasing the treatment time. Generally, in large scale applications the amount of ozone leaving the system without reacting is close to zero, thus normalized ozone doses (O3:DOC) are based on an applied ozone dose. It is highly likely when using small bench scale reactors that efficient mass transfer of ozone can be limited, hence in order to compare the results obtained at lab scale with other pilot or large-scale applications it is beneficial to use the utilized ozone dose instead. Using applied ozone doses in lab scale studies can lead to an overestimation of required ozone doses to reach target criteria. The amount of ozone utilized ($O_{3,utilized}$) was calculated by taking the difference between the amount of ozone transferred into wastewater ($O_{3,WW}$) and the amount of ozone transferred into MilliQ water (($O_{3,MQ}$) only as follows:

$$O_{3\,WW}\left(\frac{mg}{L}\right) = \int_0^t \frac{Q}{V}\left(C_{g,in} - C_{g,out}\right)dt \quad (1)$$

Where, $C_{g,in}$ and $C_{g,out}$ are the inlet and outlet gaseous ozone concentrations converted to mg/L, $t$ is time of treatment (min), $Q$ is the gaseous flowrate (0.9 – 1 L/min) and $V$ is the volume of reactor (0.7 L). By plotting $(Q/V)(C_{g,in} - C_{g,out})$ vs $t$ and taking the area under the curve up to a determined time the $O_{3,\,WW}$ was calculated.

$$O_{3_{MQ}}\left(\frac{mg}{L}\right) = \int_0^t \frac{Q}{V}\left(C_{g,in} - C_{g,out}\right)dt \quad (2)$$

Similarly to Eq.1, the $O_{3,MQ}$ was calculated by Eq.2. And by using Eq.3 $O_{3,utilized}$ was calculated.

$$O_{3_{utilized}}\left(\frac{mg}{L}\right) = O_{3\,WW}\left(\frac{mg}{L}\right) - O_{3_{RO}}\left(\frac{mg}{L}\right)$$

*Data analysis for agonistic and antagonistic endocrine disruption*

To account for the absorbance associated with yeast turbidity the corrected absorbance for the YES and YAS assays were obtained using Equation 1 and Equation 2, respectively:

$$A_{S,Corr.} = (A_{s,540\,nm} - A_{Y.B.,540\,nm}) - (A_{s,630\,nm} - A_{S.B.,630\,nm}) \quad (1)$$

Where, $A_{s,corr.}$ is the corrected absorbance of the sample, $A_{s,540nm}$ is the absorbance measured at 540 nm of the sample, $A_{Y.B.,540nm}$ is the absorbance measured at 540 nm of the yeast blank, $A_{s,630nm}$ is the absorbance measured at 630 nm of the sample and $A_{S.B.,630nm}$ is the absorbance measured at 630 nm of the solvent blank.

$$A_{S,Corr.} = (A_{s,415\,nm} - A_{Y.B.,415\,nm}) - (A_{s,595\,nm} - A_{S.B.,595\,nm}) \quad (2)$$

Where, $A_{s,corr.}$ is the corrected absorbance of the sample, $A_{s,415nm}$ is the absorbance measured at 415 nm of the sample, $A_{Y.B.,415nm}$ is the absorbance measured at 415 nm of the yeast blank, $A_{s,595nm}$ is the absorbance measured at 595 nm of the sample and $A_{S.B.,595nm}$ is the absorbance measured at 595 nm of the solvent blank.

Using the corrected absorbance obtained by Equations 1 and 2 the estrogenic activity (%) and androgenic activity (%) was then calculated using Equation 3 and Equation 4, respectively:

$$Estrogenic\ Activity\ (\%) = (A_{S,Corr.}/A_{E2,Corr.})\times 100 \quad (3)$$

$$Androgenic\ Activity\ (\%) = (A_{S,Corr.}/A_{DHT,Corr.})\times 100 \quad (4)$$

The $EC_{50}$ and $IC_{50}$ of extracts of treated and untreated WWTP effluent samples were evaluated based on the dilution factor. The dilution factor was defined as volume of sample added to a well divided by the total well volume of 200 µl.

The effective concentration showing a 50% effect, $EC_{50}$ for agonistic and $IC_{50}$ for antagonistic effects, were calculated from the log-logistic fit of the concentration-effect curve (Eq.5) of activity (%) against log dilution (or log concentration (µg/L) for reference compounds) using Prism GraphPad Software:

$$Endocrine\ Activity\ (\%) = Bottom + \frac{(Top-Bottom)}{1+10^{(LogEC_{50}-LogC)\times Hill\ Slope}} \quad (5)$$

where, Bottom is the minimum activity (set to 0%), Top is the maximum activity (set to 100%), C is sample dilution factor or the concentration of reference compound and $EC_{50}$ is the effective dilution or concentration at 50% effect.

When possible, estrogenic, androgenic, anti-estrogenic, anti-androgenic activity of samples were quantified by calculating E2, DHT,4-HTAM and FLT equivalencies, respectively as follows:

$$EQ\ (\mu g/L) = EC_{50,ref}(\mu g/L) \times \frac{EC_{50,sample,d}}{EC_{50,ref,d}} \quad (6)$$

Where, $EQ\ (\mu g/L)$ is the equivalency with respect to the reference compound, $EC_{50,ref}\ (\mu g/L)$ is the concentration at which a 50% effect is observed for the reference compound, $EC_{50,sample,d}$ is the dilution factor where a 50% effect is observed for the sample and $EC_{50,sample,d}$ is the dilution factor where a 50% effect is observed for the reference compound. For the antagonistic activities such as anti-estrogenicity and anti-androgenicity $IC_{50}$ is used instead of $EC_{50}$.

Table S1. Class and Second order reaction rate constants of target CECs

| Compound | Second order reaction rate constant, $k_{O3}$ ($M^{-1}s^{-1}$) | Class | References |
|---|---|---|---|
| Benzotriazole | $2.3 \times 10^2$ | Chemical/drug precursor | (Hollender et al. 2009) |
| Ibuprofen | 9.1 | NSAID | (Huber et al. 2005) |
| Isoproturon | 141 | Herbicide | (De Laat et al. 1996) |
| Mecoprop | < 350 | Herbicide | (Margot et al. 2013) |
| Naproxen | $2 \times 10^5$ | NSAID | (Huber et al. 2005) |
| Carbamazepine | $3 \times 10^5$ | Anti-epileptic | (Huber et al. 2005) |
| Gemfibrozil | $4.9 \times 10^5$ | Lipid regulator | (Jin et al. 2012) |
| Sulfamethoxazole | $2.5 \times 10^6$ | Antibiotic | (Huber et al. 2005) |
| Metoprolol | 2000 | Beta-blocker | (Benner et al. 2008) |
| Venlafaxine | $8.5 \times 10^3$ | Antidepressant | (Lee et al. 2014) |
| Trimethoprim | $2.7 \times 10^5$ | Antibiotic | (Wert et al. 2009) |
| Diclofenac | $1 \times 10^6$ | NSAID | (Huber et al. 2005) |
| Citalopram | N/A | Antidepressant | N/A |
| 17β-Estradiol | $2.21\times 10^5 – 3.7\times 10^9$ | Natural | (Broséus et al. 2009) |
| 17α-Ethinylestradiol | $3 \times 10^6$ | Synthetic estrogen | (Huber et al. 2005) |
| Estrone | $6.2 \times 10^3 – 2.1\times 10^7$ | Natural estrogen | (Broséus et al. 2009) |

Table S2. Characterization of effluents from 3 WWTPs (n=3)

| **Wastewater characteristics** | **WWTP A** | **WWTP B** | **WWTP C** |
|---|---|---|---|
| | Mean ± Std Dev | Mean ± Std Dev | Mean ± Std Dev |
| Total Chemical Oxygen Demand, TCOD (mg/L) | 32.0 ± 1.4 | 29.5 ± 4.9 | 52.5 ± 0.7 |
| Soluble Chemical Oxygen Demand, SCOD (mg/L) | 25.5 ± 6.4 | 15.5 ± 4.9 | 40.5 ± 2.1 |
| Dissolved organic carbon, DOC (mg/L) | 7.9 ± 0.4 | 6.9 ± 1.6 | 19.7 ± 1.0 |
| Total Nitrogen, TN (mg/L) | 13.2 ± 0.5 | 11.4 ± 1.7 | 16.4 ± 0.1 |
| Total suspend solids, TSS (mg/L) | 11.5 ± 2.1 | 11.3 ± 0.7 | 15.4 ± 0.8 |
| Conductivity | 1011.5 ± 123.7 | 577.5 ± 53 | 798.5 ± 31.8 |
| Alkalinity (as CaCO3 mg/L) | 155.0 ± 28.3 | 102.5 ± 3.5 | 170.0 ± 7.1 |
| pH | 7.2 ± 0.5 | 6.7 ± 0.4 | 7.6 ± 0.3 |
| UVA at 254 nm ($cm^{-1}$) | 0.14 ± 0.04 | 0.10 ± 0.01 | 0.19 ± 0.01 |
| SUVA 254 nm (L $mg^{-1}$ $m^{-1}$) | 1.7 ± 0.4 | 1.5 ± 0.4 | 0.9 ± 0.1 |
| Chloride (mg/L) | 140.5 ± 40.9 | 84.3 ± 9.1 | 128.0 ± 1.4 |
| Sulphate (mg/L) | 125.1 ± 29.1 | 48.5 ± 0.6 | 65.5 ± 6.4 |
| Nitrate (mg/L) | 8.7 ± 2.6 | 7.9 ± 1.8 | 0.7 ± 1.0 |
| Nitrite (mg/L) | 1.0 ± 0.4 | 1.4 ± 1.3 | 0.5 ± 0.7 |
| Sodium (mg/L) | 68.4 ± 5.2 | 39.6 ± 4.7 | 47.6 ± 2.0 |
| Calcium (mg/L) | 34.5 ± 1.3 | 17.7 ± 1.6 | 23.9 ± 5.5 |
| Magnesium (mg/L) | 12.5 ± 2.3 | 3.0 ± 0.4 | 7.4 ± 0.1 |
| Potassium (mg/L) | 8.5 ± 1.8 | 3.6 ± 0.4 | 5.0 ± 0.2 |
| Copper (mg/L) | 0.2 ± 0.1 | 0.1 ± 0.01 | 0.3 ± 0.2 |

Table S3. LC-MS methods used for quantification of CECs in spiked treated and untreated WWTP effluents. Method 2 was used for quantification of hormones (i.e. estrogens) and Method 1 was for the other target contaminants.

| **Specifications** | **Method 1** | **Method 2** |
|---|---|---|
| Column type | C-18 | C-18 |
| Eluant A | $H_2O$ + 0.1% Acetic acid | $H_2O$ (1 mM $NH_4OH$) |
| Eluant B | ACN + 0.1% Acetic acid | ACN (1 mM $NH_4OH$) |
| Gradient (%B) | 10.5% – 85 % in 9 minutes | Isocratic (32% B) in 10 minutes |
| Flow Rate | 0.3 ml/min | 0.3 ml/min |
| Injection Volume | 25 µl | 25 µl |
| Ionization | ESI | ESI |
| Monitoring | SIM | SIM |
| Probe Temperature | 475 °C | 500 °C |

Table S4. Exact mass, ionization mode, mass to charge ratios (m/z) and limit of detection (LOD) of target CECs determined as signal-to-noise ratio of 3

| **Compound** | **Exact mass (Da)** | **Ionization mode (+/-)** | **m/z** | LOD (µg/L) |
|---|---|---|---|---|
| Benzotriazole | 119.05 | + | 120.05 | 0.96 |
| Ibuprofen | 206.13 | - | 205.13 | 1.90 |
| Isoproturon | 206.14 | + | 207.14 | 0.32 |
| Mecoprop | 214.04 | + | 215.04 | 1.03 |
| Naproxen | 230.09 | - | 229.09 | 7.67 |
| Carbamazepine | 236.10 | + | 237.10 | 0.58 |
| Gemfibrozil | 250.16 | - | 249.16 | 1.83 |
| Sulfamethoxazole | 253.05 | + | 254.05 | 1.74 |
| Metoprolol | 267.18 | + | 268.18 | 0.91 |
| Venlafaxine | 277.20 | + | 278.20 | 1.14 |
| Trimethoprim | 290.14 | + | 291.14 | 0.70 |
| Diclofenac | 295.02 | + | 296.02 | 2.21 |
| Citalopram | 324.16 | + | 325.16 | 0.63 |
| 17β-Estradiol | 272.18 | - | 271.18 | 1.53 |
| 17α-Ethinylestradiol | 296.18 | - | 295.18 | 1.83 |
| Estrone | 270.16 | - | 269.16 | 1.50 |

Table S5. Initial CEC concentrations in the spiked wastewater samples used for CEC removal experiments (n=3) – includes native and spiked compounds

| Compounds | Concentrations (µg/L) | | |
|---|---|---|---|
| | WWTP A | WWTP B | WWTP C |
| | Mean ± Std Dev | Mean ± Std Dev | Mean ± Std Dev |
| Benzotriazole | 49.9 ± 9.1 | 81.8 ± 40.6 | 53.8 ± 5.9 |
| Ibuprofen | 35.5 ± 3.5 | 56.7 ± 24.7 | 43.6 ± 0.2 |
| Isoproturon | 34.9 ± 0.5 | 56.9 ± 27.9 | 37.6 ± 8.6 |
| Mecoprop | 35.1 ± 1.2 | 61.3 ± 27.7 | 40.6 ± 2.2 |
| Naproxen | 21.8 ± 3.4 | 56.6 ± 25.2 | 39.4 ± 0.2 |
| Carbamazepine | 41.0 ± 17.0 | 41.8 ± 19.1 | 33.7 ± 1.0 |
| Gemfibrozil | 35.9 ± 9.5 | 52.0 ± 23.8 | 37.4 ± 8.3 |
| Sulfamethoxazole | 29.3 ± 4.4 | 38.2 ± 25.8 | 34.7 ± 2.2 |
| Metoprolol | 29.4 ± 1.1 | 57.0 ± 28.0 | 39.6 ± 8.7 |
| Venlafaxine | 29.4 ± 1.1 | 54.7 ± 24.9 | 37.1 ± 2.6 |
| Trimethoprim | 27.9 ± 0.9 | 61.1 ± 31.1 | 41.0 ± 0.4 |
| Diclofenac | 91.4 ± 1.9 | 72.8 ± 47.0 | 69.6 ± 11.8 |
| Citalopram | 32.0 ± 1.7 | 40.9 ± 18.8 | 36.6 ± 1.4 |
| 17β-Estradiol | 31.7 ± 8.3 | 28.9 ± 12.7 | 20.7 ± 11.0 |
| 17α-Ethinylestradiol | 27.9 ± 6.5 | 28.3 ± 14.5 | 21.9 ± 11.7 |
| Estrone | 31.5 ± 6.6 | 35.3 ± 15.9 | 23.2 ± 12.4 |


Figure S1. CEC removal data at varying specific ozone doses ($gO_3/gDOC$) for the three effluents: A) WWTP A, B) WWTP B and C) WWTP C. Error bars represent standard deviation (n=3)

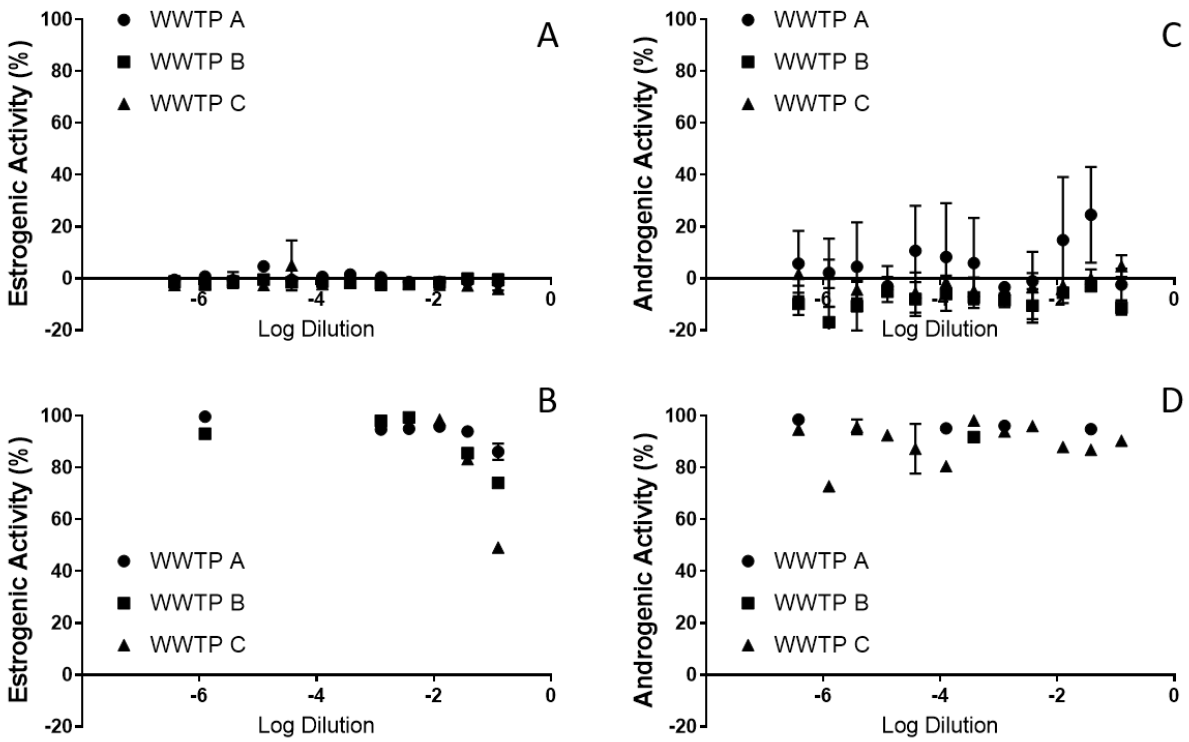


Figure S2. Dose-response relationship showing A) Estrogenic, B) anti-estrogenic, C) androgenic and D) anti-androgenic behaviour of extracts of WWTP effluents without the addition of CECs (i.e. contribution of the wastewater matrix). Error bars represent the standard deviation (n=3)

Table S6. 24-h Luminescence inhibition at various concentrations of CECs to determine the sensitivity of the Chronic *V. fischeri* assay

| Target CECs | CEC concentrations (µg/L) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | **MIX 8** | **MIX 7** | **MIX 6** | **MIX 5** | **MIX 4** | **MIX 3** | **MIX 2** | **MIX 1** |
| Benzotriazole | 7.0 | 54.0 | 126.0 | 265.0 | 590.0 | 496.0 | 1183 | 956 |
| Ibuprofen | 2.0 | 20.0 | 57.0 | 165.0 | 442.0 | 541.0 | 1026 | 1322 |
| Isoproturon | 2.0 | 25.0 | 68.0 | 171.0 | 459.0 | 591.0 | 1111 | 1455 |
| Mecoprop | 3.0 | 31.0 | 80.0 | 179.0 | 485.0 | 597.0 | 1127 | 1399 |
| Naproxen | 8.0 | 36.0 | 83.0 | 166.0 | 433.0 | 513.0 | 1001 | 1237 |
| Carbamazepine | 4.0 | 19.0 | 50.0 | 123.0 | 346.0 | 436.0 | 844 | 1123 |
| Gemfibrozil | 1.0 | 19.0 | 65.0 | 181.0 | 468.0 | 597.0 | 1008 | 1294 |
| Sulfamethoxazole | 2.0 | 22.0 | 60.0 | 155.0 | 401.0 | 509.0 | 987 | 1303 |
| Metoprolol | 2.0 | 20.0 | 44.0 | 146.0 | 433.0 | 408.0 | 1055 | 1006 |
| Venlafaxine | 2.0 | 13.0 | 39.0 | 111.0 | 300.0 | 379.0 | 746 | 952 |
| Trimethoprim | 3.0 | 41.0 | 109.0 | 238.0 | 664.0 | 735.0 | 1564 | 1687 |
| Diclofenac | 2.0 | 18.0 | 57.0 | 149.0 | 436.0 | 540.0 | 1084 | 1364 |
| Citalopram | 1.0 | 10.0 | 28.0 | 78.0 | 179.0 | 246.0 | 422 | 490 |
| 17β-Estradiol | 6.0 | 28.0 | 56.0 | 112.0 | 223.0 | 279.0 | 446 | 558 |
| 17α-Ethinylestradiol | 18.0 | 88.0 | 176.0 | 352.0 | 704.0 | 881.0 | 1409 | 1761 |
| Estrone | 6.0 | 28.0 | 57.0 | 113.0 | 226.0 | 283.0 | 453 | 566 |
| **Average concentration** | **4.3** | **29.5** | **72.2** | **169.0** | **424.3** | **501.9** | **966.6** | **1154.6** |
| **Luminescence Inhibition (%)** | **-7.4** | **11.2** | **20.2** | **50.3** | **99.1** | **99.4** | **98.8** | **97.1** |