LuminoTox as a tool to optimize ozone doses for the removal of contaminants and their associated toxicity

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ABSTRACT

New treatment technologies and quality monitoring tools are needed for Contaminants of Emerging Concern (CECs) in wastewater. The purpose of this work was to assess the LuminoTox as a monitoring tool for CEC-associated toxicity in municipal wastewater during ozone treatment, and to evaluate the impact of different ozone feed concentrations at equivalent ozone doses for removing toxicity. The LuminoTox was sensitive at monitoring changes in toxicity of atrazine (ATZ) in synthetic wastewater (SWW) and in a 14 CECs mix in secondary effluent (SE) during ozone treatment. In both experiments, a decrease in toxicity was observed with increasing transferred ozone dose, which corresponded to a decrease in CEC concentration. For ATZ in SWW, a 5 ppm ozone feed showed better toxicity removal, up to 25% and 35% inhibition for LuminoTox algae biosensors SAPS I and SAPS II, respectively, for statistically equivalent ozone dose pairs of 43 mg (5 ppm ozone feed) and 36 mg (15 ppm ozone feed). The opposite was true for the 14 CECs in SE; the 15 ppm ozone feed showed better toxicity removal, up to 37% and 40% reduced for SAPS I and SAPS II inhibition, respectively, for statistically equivalent ozone dose pairs of 42 mg (5 ppm ozone feed) and 42 mg (15 ppm ozone feed). Different feed applications had an impact on the efficiency of toxicity removal for equivalent ozone doses; this efficiency appears to depend on the type of contaminants and/or wastewater matrix.

Key words: ozonation, ozone dose, contaminants of emerging concern, LuminoTox, *Chlamydomonas reinhardtii*, *chlorella vulgaris*

1 INTRODUCTION

Wastewater treatment plants (WWTPs) are not conventionally designed to remove contaminants of emerging concern (CECs), leading to their poor elimination during treatment (Henze et al., 2008; Rojas et al., 2013). CECs including endocrine disruptors, pharmaceuticals, antibiotics, herbicides, and insecticides ultimately end up in the environment where they exist in parts per billion to parts per trillion concentrations (Daughton, 2004; Diamond et al., 2011; Metcalfe et al., 2003; Snyder et al., 2006). CECs are of concern as the impact of their constant presence in the environment is not well understood (Bolong et al., 2009). There is an urgent need for new treatment methods to reduce or eliminate CECs, along with their transformation products (TPs) and associated toxicity.

Ozone is one promising technology for the advanced treatment of municipal wastewater as it has been shown to degrade most CECs for ozone doses in the range of about 3 to 20 mg O_3/L (Huber et al., 2005; Lassonde et al., 2015; Margot et al., 2013; Reungoat et al., 2010; Singh et al., 2015; Ternes et al., 2003; Yargeau & Danylo, 2015). CECs are oxidized via second order reactions through either direct attack by ozone, or indirectly by reaction with hydroxyl radicals with second order reaction rate constants range from approximately < 0.1 $M^{-1}s^{-1}$ to 7×10^9 $M^{-1}s^{-1}$ and from 10^9 $M^{-1}s^{-1}$ to 10^{10} $M^{-1}s^{-1}$ respectively (von Gunten, 2003). Ozone treatment of wastewaters has also demonstrated toxicity reduction or removal for many different organisms and endpoints such as an altered rate of rat fetal testicular development (Lassonde et al. 2015), immobilization of *Daphnia pulex* (Petala et al., 2006), and the inhibition bioluminescence of *Vibrio fischeri* (Reungoat et al., 2012). Different studies have confirmed a positive relationship between

increasing ozone dose and toxicity removal as for the inhibition of dehydrogenase activity (Uslu & Balcioglu, 2008), estrogenicity (Reungoat et al., 2012) and algal growth inhibition (Quero-Pastor et al., 2014) while others have reported an increased toxicity after ozone treatment such as for the inhibition of bioluminescence (Petala et al., 2006), mutagenicity (Petala et al., 2008), blocking of gap junction intracellular communication (Luster-Teasley et al., 2005) and developmental retardation, decreased body weight and length, and decreased vitellogenin levels in rainbow trout at various lifestages (Stalter et al., 2010). To our knowledge, there has been no assessment of the impact of applying the same ozone doses using different ozone feed conditions (thus different treatment times) on the efficiency of toxicity removal from wastewater.

The LuminoTox is a promising bioassay to monitor the quality of ozone-treated effluent as it was previously shown to detect a number of inorganic and organic molecules including CECs (Bellemare et al., 2006; Gesuale et al., 2010; Marshall & Yargeau, 2017; Souza et al., 2013) and to be applicable to secondary wastewater effluents (Marshall & Yargeau, 2017) and considering that photosynthesis inhibition is considered as one of the top 5 modes of action of environmental pollutants. Toxicants can bind specific sites within the thylakoid membrane which can interfere with the emission of chlorophyll a fluorescence associated with the photosystem I and II (PS I and II) reaction centres (Boucher & Carpentier, 1999; Maksymiec & Baszyński, 1988; Maxwell & Johnson, 2000; Tischer & Strotmann, 1977). The LuminoTox captures the change in fluorescence emission upon exposure to a contaminant which provides an indication of the impact on photosynthesis and is reported as photosynthetic inhibition. To our knowledge, there exists only one published article on monitoring the quality of secondary effluent (SE) during ozone treatment with the LuminoTox; Gesuale and colleagues reported a decrease in average

inhibition of photosynthetic enzyme complex (PEC) inhibition and in CECs including pharmaceuticals and nonylphenol ethoxylates with increasing ozone dose (Gesuale et al., 2010). However, since the average inhibition of their samples ranged ± 5% and error bars ranged ~ ± 3-7%, these results might be statistically equivalent to their blank (which was not shown) and to each other (t-tests were not reported), thus from their research, it is difficult to conclude if this trend was achieved.

In this study, LuminoTox was evaluated as a tool to monitor toxicity during ozone treatment of CECs in synthetic and real wastewater matrices. In addition, the tool was used to investigate the impact on removal of CEC-associated toxicity using equivalent doses of ozone applied using different application strategies (high and low ozone concentration in the gas phase).

2 MATERIALS AND METHODS

2.1 Synthetic wastewater preparation and real wastewater collection and storage

Synthetic wastewater (SWW) was made with chemicals as previously described (Marshall & Yargeau, 2017). For the experiments performed using real wastewater, SE was collect at a WWTP serving a population of 95,000, having a design capacity of 65,000 m^3/d and receiving an average flow of 38,000 m^3/d. The influent consisted of approximately half industrial and half domestic wastewater and the facility consisted of an activated sludge secondary treatment train. Samples were collected from the SE and frozen at -20°C within 2 hours of collection. Samples were thawed before use. SWW was spiked with atrazine (ATZ) used as a model toxicant and SE was spiked with a mixture of CECs described in section 2.2.

2.2 Target CECs and internal standard stock solutions

The CECs were selected for this work because we have previously detected them in several secondary effluents (data not shown) and represent different classes of contamiants. Atrazine was added to the mix as it is the positive control recommended for the LuminoTox. Target CECs, their internal standards, suppliers, solvents for stock solutions, as well as LC-HRMS limits of detection (LODs) and limits of quantification (LOQs) are found in Table 1. Stock solutions (5 mg/L) were made for each individual CEC and surrogate. From the individual CEC and surrogate stock solutions, 1000 mg/L 14 CECs and a 100 mg/L 14 surrogate mixtures were both prepared in methanol.

Table 1: Target CECs with their internal standards, suppliers, solvents used for stock solutions, limits of detection and limits of quantification

Type	Subtype	Compound	Internal standard	Solvent for compound and surrogate	Surrogate (% purity or standard)	Supplier (compound, surrogate)	LOD, LOQ (µg/L)
Pharmaceutical	Antibiotic	Sulphamethoxazole	Sulfamethoxazole-d4	MeOH	VETRANAL, 98	S, I	1, 4
	Antibiotic	Trimethoprim	Trimethoprim-d9	MeOH	VETRANAL, 99.9	S, S	1, 4
	Lipopenic	Gemfibrozil	Gemfibrozil-d4 (2,2-dimethyl-d6)	MeOH	99.98, 99	S, I	1, 4
	Neurophathic/ epileptic	Carbamazepine	Carbamazepine-d10 (rings-d10)	MeOH	98+, 98	S, I	1, 4
	Antidepressant	D, L Venlafaxine	(±)-Venlafaxine-d6 HCl (N,N-dimethyl-d6)	MeOH	95, 99	T, I	1, 4
	Anti-inflammatory	Naproxen	(±)-Naproxen-d3 (α-methyl-d3)	MeOH	98, 99	T, I	1, 3
	Anti-inflammatory	Ibuprofen	(±)-Ibuprofen-d3 (α-methyl-d3)	MeOH	98, 99	T, I	1, 4
	Estrogen hormone	Estrone	Estrone 16, 16-d2	MeOH :DMSO, 1:1	99+, 98	S, I	1, 4
	Estrogen hormone	17β-estradiol	17β-estradiol-2, 4- d2	DMSO	98+, 99	S, I	1, 4
	Estrogen hormone	17α-ethinylestradiol	17α-ethynylestradiol -2,4,16,16-d4	MeOH :DMSO, 7:3	98, 98	T, I	1, 4
Pesticide	Herbicide	Atrazine	Atrazine-d5	MeOH;	98, 98	T, T	1, 4

				DMSO:MeOH, 1:9[1]			
	Herbicide	MCPA (4-Chloro-2-methylphenoxyacetic acid)	4-Chloro-2-methylphenoxy-d3 acetic Acid	MeOH	99.8, 98	S, I	1, 3
	Insecticide	DEET (N,N-Diethyl-3-methylbenzamide)	N,N-Diethyl-3-methyl-d3-benzamide-2,4,5,6-d4	MeOH	99.5, 98	S, I	1, 4
Antimicrobial agent	Antibacterial/ antifungal agent	Triclosan	Triclosan-d3	MeOH	98, 98.1	T, T	1, 3

T: Toronto Research Chemicals, Toronto Ontario; S: Sigma Aldrich Canada, Oakville Ontario; C: Chem Service, Wester Chester, Pennsylvania; I: CDN Isotopes, Point Claire, Quebec. LOD: Limit of Detection; LOQ: Limit of Quantification; MeOH: Methanol; DMSO: Dimethyl sulfoxide.

2.3 Ozone experiments

ATZ or the 14 CECs mix (which included ATZ) were added into the bottom of a 1L reactor; the solvent was left to evaporate and SWW or SE respectively was added to the reactor and stirred for 30 minutes. The concentration of CECs (200 µg/L ATZ or 50 µg/L of each CEC in the 14 CECs mix) in the samples before ozonation were selected with the intent of achieving a high inhibition prior to ozonation to ensure that potential changes in toxicity could be monitored during treatment. Ozone was generated by passing air or oxygen at 10 psi through a TOGC2 Compact Ozone Generator with a corona discharge (Triogen Ltd., East Kilbride, Scotland). Pure air and O_2 were fed to the O_3 generator in order to produce two different feed conditions in the inlet gas of the O_3 reactor: low concentration of ozone - 5 ppm O_3 and high concentration of ozone - 15 ppm O_3. The inlet and outlet of the semi-batch ozone reactor were monitored for O_3 concentration using Wedeco HC-400 plus and MC-400 plus ozone monitors (Xylem, Point Clair, Quebec), respectively. The inlet ozone/oxygen mixture (OOM) feed was maintained at 1L/min and continuous stirring using a stir bar was used to improve ozone contact. The reactor off gas was sent to a 10% w/v potassium iodide quenching solution (Fisher Chemical, Fair Lawn, New Jersey). An Alicat Scientific M Series Mass Flowmeter (Instrumart, Burlington, Vermont)

coupled to a HOBO UX 120-006M 4-Channel Analog Data Logger (Onset, Bourne, Massachusetts) was used for data collection (every second), and logged inlet and outlet ozone concentrations and OOM flowrate. The transferred ozone doses at each sampling time were computed by integrating equation 1 using the software program Graph, version 4.4 (Copyright: Ivan Johansen, 2012).

$$Transferred\ dose = \int_0^t \left(C_{in\ O_3} - C_{out\ O_3}\right) Q_{in\ O_3}\, dt \quad (1)$$

In equation 1, t is time, $C_{in\ O3}$ and $C_{out\ O3}$ are the concentrations of ozone entering and exiting the ozone treatment unit, and $Q_{in\ O3}$ is the flowrate. Samples were collected over the course of ozone treatment through a port in the top of the reactor. Samples were left to vent for 20 minutes and immediately frozen at -20°C.

Transferred ozone doses for pilot and full-scale ozonation of municipal SE are typically between 0.5 mg/L and 30 mg/L (Hollender et al., 2009; Huber et al., 2005; Xu et al., 2002; Zimmermann et al., 2011). It may be advantageous for observing toxicity removal to look at doses greater than these as there may be significant additional reduction in toxicity for doses higher than typically applied. Toxicity and CECs removals were thus investigated at ozone doses up to 55 mg/L. Furthermore, the CODs of SWW and SE were determined using HACH method 8000 in order to interpret ozone demand without the addition of CECs.

2.4 LuminoTox

2.4.1 Justification of use and theory

The intention of Aquacion Inc., the company that produces LuminoTox, was to eventually use LuminoTox as an online monitoring tool at WWTPs with goal of monitoring CECs. As such, in

this work, the LuminoTox was explored for its ability to monitor wastewaters containing CECs for use in combination with a battery of bioassays to monitor wastewater quality. The LuminoTox measures photosynthetic inhibition of a sample of interest by subjecting it first to a high intensity photon emission at 420 nm and measuring the emitted fluorescence > 700 nm; this is called F2 reading. The F1 reading is then measured using a similar procedure but instead, using a low intensity photon emission. F2 and F1 represent the reduced and oxidized states of plastoquinone (Q_B) (an electron carrier found within PS II) respectively and are used to compute the photosynthetic efficiency (Φ, equation 2) and % inhibition (equation 3) (Dellamatrice et al., 2006).

$$\Phi_x = [F2_x\text{-}F1_x]/F2_{zero}\,,\ x = zero\ or\ sample,\ zero = sample\ blank \quad (2)$$

$$\%\ inhibition = 100 \cdot [\Phi_{zero}\text{-}\Phi_{sample}]/\Phi_{zero} \quad (3)$$

2.4.2 Protocol of use of the LuminoTox

ATZ standards and biosensors including SAPS I (prod # LBLP15AA-L) and SAPS II (prod # LBLP16AA) were obtained from Aquacion Inc. (Montreal, Canada). These two biosensors were selected for experimental analysis to compare their sensitivities during ozone treatment of wastewaters containing CECs. Biosensors were activated for 90 minutes prior to testing using a BAZZ lighting system (DC 12 V, 1.2 W, model # MK-B01-3528-0.25M). 2 mL of each sample was added to a disposable borosilicate glass tube (Fisher Scientific, Fair Lawn, New Jersey). 100 µL of SAPS I or SAPS II was then added to each sample every 30 seconds. Biosensors were left exposed in the light on the lab bench for 30 minutes (Marshall & Yargeau, 2017). One at a time, each sample was poured into a Fisherbrand disposable cuvette (Fisher Scientific, Fair Lawn,

New Jersey) and read using the pesticide toxicity setting (for SAPS) in the LuminoTox Analyzer (Model LBLX01AA). F1 and F2 readings were recorded for each sample. An ATZ control and a Milli-Q water (MQW) blank were run with each experiment and passed manufacturer's specifications which were as follows: the average 10 µg/L ATZ control inhibition was from 35% to 45% and the blank F2 replicates were all be above 500 000.

2.5 CEC chemical analysis

Samples were pre-concentrated using 800 mL Fast-Freeze Flasks and a FreeZone 4.5 Litre Benchtop Freeze Dry System (Labconco, Kansas City, MO) and reconstituted in a mixture of 1:8 methanol to water as described previously (Marshall & Yargeau, 2017). Although it has limitations, yophilisation was selected to evaluate the use a simple and cheap method prior to LuminoTox rather than the usual solid-phase extraction method. Analysis was conducted with an Accela 600 LC System (Thermo Scientific, Waltham MA, USA) coupled with an LTQ XL Orbitrap mass spectrometer. LC and MS systems were controlled using Thermo Xcalibur 2.0 software (Thermo Scientific, San Jose CA, USA). LC separation was executed as described previously (Marshall & Yargeau, 2017). ATZ recoveries were in the range of 38% to 59%, which was sufficient to obtain concentrations above the LOQ. CECs from the 14 CEC mix were reported as relative removal.

2.6 TP chemical analysis

TPs have the potential to contribute to toxicity, thus, chemical analysis was performed on TPs of ATZ in SWW. ATZ TPs were selected for analysis because (1) the response of their parent compound in the LuminoTox is well known (2) these TPs are well known and have previously

been detected using LC-MS (Acero et al., 2000) and (3) Analysis of the mixture of one CEC and its TPs was less complex compared to that the 14 CECs mix in SE thus there is better potential to relate changes in toxicity and TP concentration. The four major TPs analyzed include: deethylatrazine (DEA), deisopropylatrazine (DIA), 4-acetamido-2-chloro-6-isopropylamino- s-triazine (CDIT) and 2-chloro-4-ethylimino-6-isopropylamino-s-triazine (ATRA-imine). TP chemical analysis was performed as in section 2.5. Due to the lack of analytical standards, ATZ TPs were reported as counts, which were used only to determine relative removal.

2.7 Statistical analysis

Ozonation experiments were conducted in triplicates. For each experiment, chemical analysis was performed and LuminoTox measurements were run in triplicate (resulting in 9 replicates per conditions tested for toxicity assessment). Average and standard deviations are reported for CEC concentration and % inhibition while only the average ozone dose is reported to improve the readability of the graphs and tables. T tests: Two-Sample Assuming Unequal Variances were performed in Excel using a two-tailed distribution and $p<0.05$.

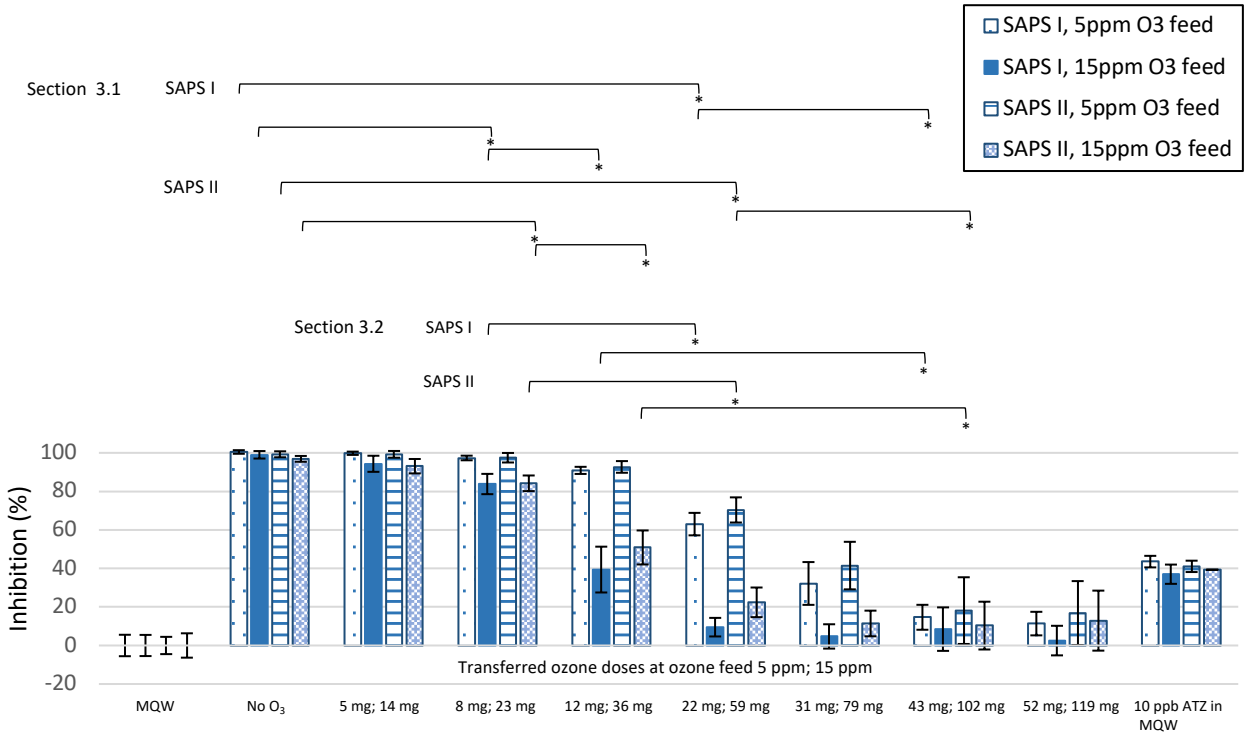
3 RESULTS AND DISCUSSION

3.1 Relationship between toxicity, CECs, and ozone dose

Figure 1 and Figure 2 indicate that the LuminoTox was able to detect the toxic effect of ATZ and CECs, as the toxicity of spiked waters were higher than the toxicity of un-spiked SWW and SE (toxicity data of un-spiked matrices are reported in the notes below Figures 1 and 2). For both ATZ in SWW (Figure 1) and CECs in SE (Figure 2), the toxicity did not change significantly at the low doses (in Figure 1, an average of 8 mg for the 5 ppm feed, and an average of 15 mg for

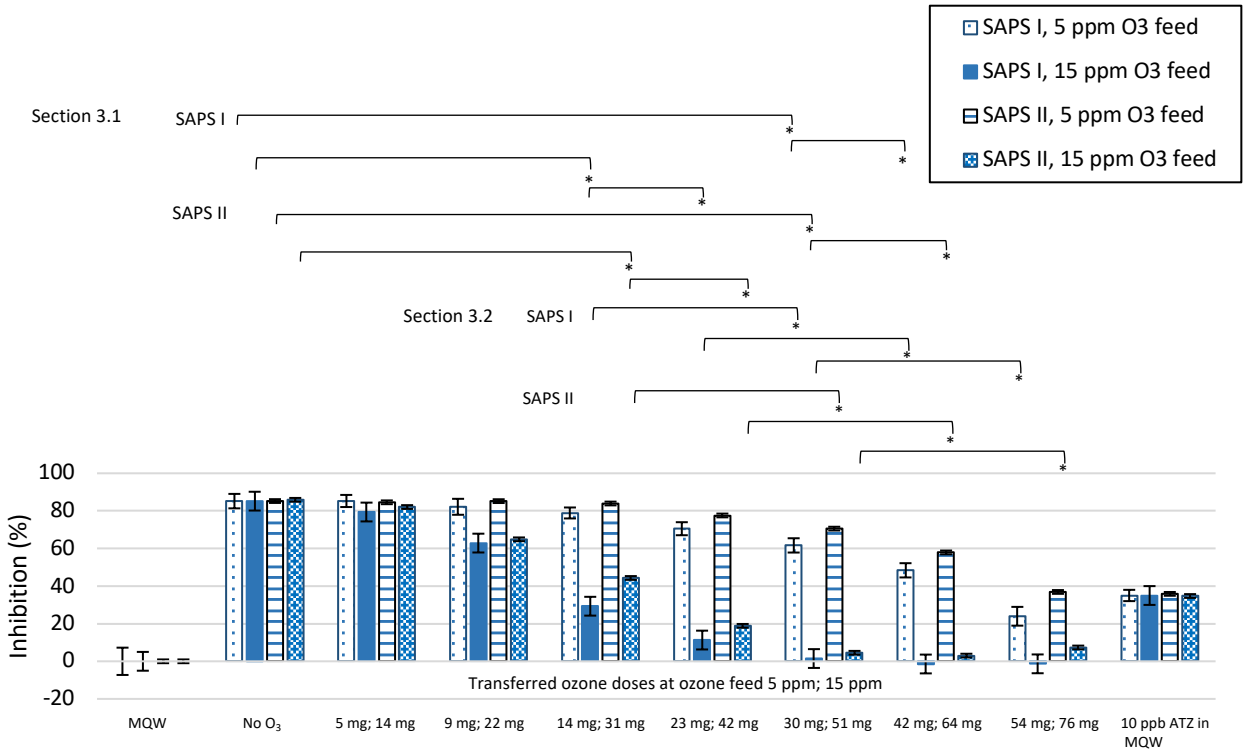
the 15 ppm feed for ozone applied to SWW containing ATZ; in Figure 2, an average of 14 mg for the 5 ppm feed, and an average of 14 mg for the 15 ppm feed for ozone applied to SE containing CECs). The minimal reduction in toxicity observed is likely due the initial ozone demand (COD 76 mg COD/L and 24 mg COD/L for SWW and SE respectively) caused by the preferential electrophilic attack of moieties such as poly-phenols and amines present in NOM as reported by others (Saroj et al., 2005; Wang et al., 2007; Yavich et al., 2004), which is limiting the removal of more toxic constituents. Furthermore, it has been demonstrated that limited hydroxyl radicals are available for CEC destruction for exposure to low doses of ozone due to their scavenging by the wastewater matrix (Wert et al., 2009). CEC hydroxyl radical destruction is important for compounds such as ATZ, DEET and ibuprofen whose reactivity with ozone is low (see the k_{O3}s summarized in Table 2). These observations can explain why the LuminoTox did not detect changes in toxicity for low ozone doses.

Figure 1: SAPS I and SAPS II toxicity of atrazine in synthetic wastewater exposed to different transferred ozone doses using 5 ppm and 15 ppm ozone feed concentrations



X axis: Transferred ozone doses for the following ozone feed concentrations: 5 ppm (first two bars); 15 ppm (last two bars). Samples were run in triplicate. The error bars represent one standard deviation. * $p < 0.05$. Synthetic wastewater (SWW) was run in a separate experiment and achieved toxicities of -2% ± 0%, inhibition (SAPS I), and -1% ± 0%, inhibition (SAPS II). Equivalent ozone dose pairs (see Section 3.2) for the 5 ppm and the 15 ppm ozone feeds were confirmed by paired t test ($p < 0.05$).

Figure 2: SAPS I and SAPS II toxicity of a mixture of 14 CECs in in secondary effluent exposed to different transferred ozone doses using 5 ppm and 15 ppm ozone feed concentrations



X axis: Transferred ozone doses for the following ozone feed concentrations: 5 ppm (first two bars); 15 ppm (last two bars). Samples were run in triplicate. The error bars represent one standard deviation. * $p < 0.05$. Secondary effluent (SE) was run in a separate experiment and achieved toxicities of 9% ± 2%, inhibition (SAPS I), and 12% ± 1%, inhibition (SAPS II). Equivalent ozone dose pairs (see Section 3.2) for the 5 ppm and the 15 ppm ozone feeds were confirmed by paired t test ($p < 0.05$).

At higher doses of ozone, a decrease in toxicity of SWW containing ATZ, and in SE containing CECs was observed, and significant reductions of toxicity were identified (seen in Figures 1 and 2, respectively; statistical difference confirmed by paired t tests). In Figure 1, SAPS I and SAPS II toxicity was reduced by an average of 85% and 81%, respectively, at an average ozone dose of 43 mg (5 ppm ozone feed) and by an average of 60% and 46% respectively at an average ozone dose of 36 mg (15 ppm ozone feed). A reduction in toxicity was also observed in Figure 2; SAPS I and SAPS II toxicity was reduced by an average of 37% and 27%, respectively, at an average ozone dose of 42 mg (5 ppm ozone feed) and by an average of 74% and 67% respectively at an average ozone dose of 42 mg (15 mg/L ozone feed). Thus, the LuminoTox demonstrated

sensitivity to changes in SAPS toxicity, and was able to monitor the overall reduction in toxicity during ozone treatment in different wastewater matrices containing single or a mixture of CECs.

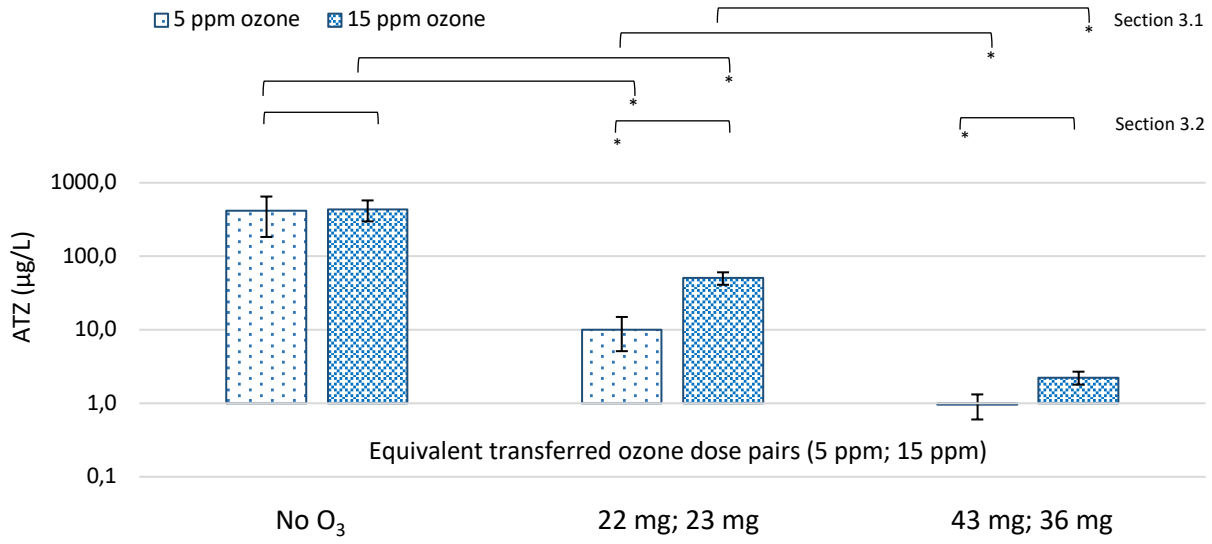
In all cases, results demonstrated a change in toxicity which corresponded to a decrease in CECs. The change in toxicity observed in Figure 1 corresponded to a decrease in ATZ, as confirmed by paired t tests. The decreasing toxicity trend with ozone doses also observed for CECs (Figure 3) was similarly associated with removal of these compounds, as summarized in Table 3. This trend has been reported in other work for different CECs in wastewaters for diverse endpoints such as estrogenicity, differences in male fish gene expression, differences in rat fetal testicular development, bacterial inhibition of dehydrogenase activity, and non-specific toxicity (Microtox) (Gunnarsson et al., 2009; Lassonde et al., 2015; Reungoat et al., 2012; Uslu & Balcioglu, 2008). In the 14 CECs mix in SE, ATZ, to which the biosensors are sensitive because of the mode of action (MOA) of this contaminant, was removed by up to 69 % and 96 % for average ozone doses of 54 mg (5 ppm ozone feed) and 51 mg (15 ppm ozone feed), respectively. Sulfamethoxazole, carbamazepine, naproxen and estrone had the greatest rates of removal, which ranged from 98% to 100% for the 5 ppm ozone feed (average ozone dose of 54 mg), and the 15 ppm ozone feed (average ozone dose of 51 mg), respectively. By contrast, ibuprofen, 17ß-estradiol and 17-α-ethinylestradiol were removed at less than 26% for both feed conditions at the same highest dose of ozone tested. Overall, the LuminoTox was able to detect changes in toxicity of ATZ and of the mixture of 14 CECs in synthetic and real wastewater matrices during ozone treatment which corresponded to a decrease in CECs.

Table 2: Ozone and hydroxyl radical second order rate constants of CECs in wastewater taken from the literature and their degradability classifications

Compound	Literature values		Degradability rank (ozone; hydroxyl radical)
	k_{03} ($M^{-1}s^{-1}$)	k_{0H} ($M^{-1}s^{-1}$)	
Sulfamethoxazole	~2.5 x $10^{6}_{b, c, h}$; 5.55 x 10^{5}_{g} ; 5.7 x 10^{5}_{n}	5.5 x $10^{9}_{b, c, h}$; 5.5 ± 0.7 x 10^{9}_{g}	Rapid; rapid
Trimethoprim	2.7 x $10^{5}_{g, n}$	6.9 ± 0.2 x 10^{9}_{g}	Rapid; rapid
Gemfibrozil	6.82 ± 0.38 x 10^{4}_{i}; ~5 x 10^{4}_{n}	13.1 ± 1.8 x 10^{9}_{i} : ~10 x 10^{9}_{o}	Medium; rapid
Carbamazepine	~3 x $10^{5}_{b, c, h, n}$	8.8 x 10^{9}_{b}; 8.8 ± 1.2 x 10^{9}_{h}	Rapid; rapid
Venlafaxine	Not found in literature	8.46 x 10^{9}_{r} ; 8.15 ± 0.37 x 10^{9}_{s}	N/A; rapid
Naproxen	~2 x $10^{5}_{c, n}$	9.6 x 10^{9}_{d}	Rapid; rapid
Ibuprofen	9.1 ± 1_{c}; 9.6 ± 1_{h}; 9.6_{n}	7.4 x 10^{9}_{b}; 7.4 ± 1.2 x $10^{9}_{c, h}$	Slow; rapid
Estrone	9.4 ± 2.7 x 10^{5}_{u}	1.6 ± 0.88 x 10^{10}_{u}	Rapid; rapid
17β-estradiol	10^{6}_{h}	*1.41 x 10^{10}_{v}	Rapid; *rapid
17α-ethinylestradiol	~3 x 10^{6}_{c}; ~7 x 10^{9}_{h}	9.8 ± 1.8 x 10^{9}_{c}; 9.8 ± 1.2 x 10^{9}_{h}	Rapid; rapid
Atrazine	$6_{a, n}$	3 x 10^{9}_{a}	Slow; rapid
MCPA (4-Chloro-2-methylphenoxyacetic acid)	4.4 ± 0.2 x 10^{5}_{p}	*6.6 x 10^{9}_{q}	Rapid; *rapid
DEET (N,N-Diethyl-3-methylbenzamide)	0.126 ± 0.006_{k}; $<10_{n}$	4.95± 1.8 x 10^{9}_{l}	Slow; rapid
Triclosan	3.8 x $10^{7}_{e, n}$	*5.4 ± 0.3 x 10^{9}_{f}; 9.6 x 10^{9}_{m}	Rapid; rapid

k_{03}: ozone second order rate constant; k_{0H}: hydroxyl radical second order rate constants. *Experiment was not conducted at pH 7; Slow: second order rate constant ≤10 $M^{-1}s^{-1}$; Medium: second order rate constant >10 $M^{-1}s^{-1}$ < 1 x 10^{5} $M^{-1}s^{-1;}$ Rapid: ≥1 x 10^{5} $M^{-1}s^{-1}$; N/A: Not available. a: (Acero et al., 2000) pH 7, T = 20°C; b: (Wert et al., 2009) pH 7, T = 20°C; c: (Huber et al., 2005); d: (Packer et al., 2003); e: (Suarez et al., 2007) pH 7; f: (Latch et al., 2005) pH 3.5, T = 22°C; g: (Dodd et al., 2006) pH 7, T = 20°C for k_{O3} and T = 25°C for k_{OH}; h: (Huber et al., 2003) pH 7, T = 20°C; i: (Uslu et al., 2015) pH 7, T = 20°C; j: (MacBean, 2008-2010); k: (Latch et al., 2005) pH 7; l: (Song et al., 2009) pH 7, room temperature; m: (Lee & von Gunten, 2012) pH 7; n: (Lee et al., 2013) pH 7; o: (Razavi et al., 2009) pH 7, room temperature; p: (Solís et al., 2015); q:(Benitez et al., 2004) pH 9, T = 20°; r: (Abdelmelek et al., 2011) pH 7, room temperature; s: (Santoke et al., 2012); t: (Toxnet, 2016); u: (Nakonechny et al., 2008) pH 7, room temperature; v: (Rosenfeldt & Linden, 2004) pH 6.8; w: (Lewis & Archer, 1979); x: (Jones et al., 2002); y: (Ryu et al., 2014)

Figure 3: Chemical analysis of atrazine in samples containing atrazine in synthetic wastewater exposed to different equivalent transferred ozone dose pairs for 5 ppm and 15 ppm ozone feed



The error bars represent one standard deviation. * $p < 0.05$. Equivalent ozone dose pairs for the 5 ppm and the 15 ppm ozone feeds were confirmed by paired t test ($p < 0.05$).

Table 3: Range of removals for select equivalent transferred ozone dose pairs for the 14 CECs mix in secondary effluent

CEC	Range of removals[1]	
	5 ppm ozone feed (%)	15 ppm ozone feed (%)
Sulfamethoxazole	98 - 100	100
Carbamazepine	100	100
Naproxen	99 - 100	100
Ibuprofen	3 - 14	7 - 37
Estrone	98 - 99	99 - 100
17ß-estradiol	0.0 - 21	1 - 31
17α-ethinylestradiol	18 - 26	11 - 65
Atrazine	31 - 69	43 - 96
DEET	56-84	67-93

1: The 5 ppm and 15 ppm ozone feed dose pairs examined for CEC removals were (30 mg; 31 mg), (42 mg; 42 mg) and (54 mg; 51 mg). All compounds were detected in the 14 CECs mix in SE before ozone was applied (data not shown).

3.2 Ozone efficiency of toxicity removal for different ozone feed applications

To evaluate the potential impact of using different ozone feed concentrations on the removal of toxicity, results presented in Figure 1 and Figure 2 that had statistically equivalent transferred dose pairs (confirmed by paired t tests) were compiled for comparison, as presented in Table 4.

It was observed that, equivalent mid-range transferred ozone dose pairs of the 5 ppm and the 15 ppm ozone feed experiments elicited different removal efficiencies. In the ATZ and SWW ozone experiment (Table 4), the 5 ppm ozone feed was more efficient at toxicity removal compared to that of the 15 ppm; the maximum differences observed were 25% (SAPS I) and 35% (SAPS II) for the highest level of equivalent ozone dose pairs. Differences in removal by the two feed concentrations for equivalent dose pairs can be attributed to the better removal of ATZ (Figure 3) by the 5 ppm ozone feed as confirmed by paired t tests.

Table 4: Differences in removal of SAPS I and SAPS II toxicity for equivalent ozone doses using different ozone feed treatments

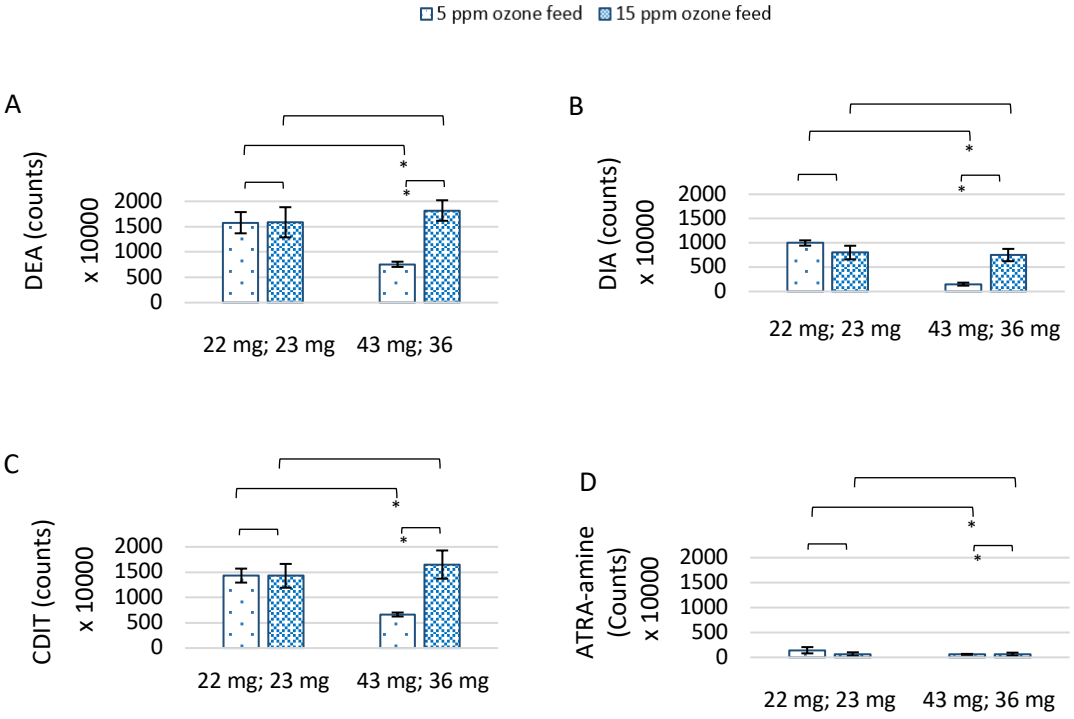
Statistically equivalent transferred ozone doses (dose in mg at 5 ppm feed; dose in mg at 15 ppm feed)	**Difference in average toxicity reduction between the two ozone feed treatments (5 ppm relative to 15 ppm)**	
	Based on SAPS I %	Based on SAPS II %
Atrazine in SWW (Figure 1)		
22; 23	22%	16%
43; 36	25%	35%
14 CECs mix in SE (Figure 2)		
30; 31	- 33%	- 28%
42; 42	- 37%	- 40%
54; 51	- 23%	- 33%

Equivalent ozone dose pairs for the 5 ppm and the 15 ppm ozone feeds were confirmed by paired t test ($p < 0.05$).

To further investigate the differences in toxicity removal at different ozone feeds, TPs of ATZ at two levels of equivalent ozone dose pairs were analyzed and results are reported in Figure 4. The presence of the major TPs: DEA, DIA, CDIT and ATRA-imine were confirmed at the lowest level of ozone dose pairs analyzed. For the 5 ppm ozone feed, a decrease was observed for all TPs from the first to the second level of ozone dose pairs for both feed concentrations, as confirmed by paired t tests. However, the 15 ppm feed produced stable intermediates, as indicated by the lack of statistical difference observed for both ozone dose levels, also confirmed

by paired t tests. The formation and near plateau of the same ATZ TPs was also reported over time in a batch ozone experiment with an initial ozone concentration of 10 ppm (Acero et al., 2000). Despite the presence of TPs for both 5 ppm and 15 ppm ozone feed concentrations, in other work, it was reported for different algae species that the photosynthetic EC50s of four ATZ TPs were one order of magnitude (for DEA and DIA) to three orders of magnitude (other TPs not addressed in this work) smaller than that of ATZ (Belfroid et al., 1998; Stratton, 1984). Thus, although differences in TP removal were observed for different feed concentrations at an equivalent ozone dose, their overall effect on the reported toxicity may be less dominant than that of their parent compound.

Figure 4: Chemical analysis of four atrazine transformation products in samples containing atrazine in synthetic wastewater exposed to different transferred ozone doses



A: DEA; B: DIA; C: CDIT; D: ATRA-imine. Statistically equivalent transferred ozone doses (confirmed by paired t test, $p < 0.05$) are presented along the X- axis as follows: 5 ppm ozone feed; 15 ppm ozone feed. The error bars represent one standard deviation. * $p < 0.05$.

Table 4 showed a difference in the efficiency of toxicity removal for the 14 CECs mix in SE for the two feed treatments; unlike ATZ in SWW, the CECs in SE show that the 15 ppm ozone feed was more efficient compared to that of the 5 ppm feed. For the three levels of ozone dose explored, the differences in toxicity removal for SAPS I and SAPS II by the 15 ppm ozone feed compared to that of the 5 ppm were: 33% and 28%; 37% and 40%; and 23% and 33%, respectively. Table 5 presents the difference in CEC removals for equivalent ozone dose pairs (15 ppm feed compared to the 5 ppm feed) for select CECs from the 14 CECs mix. ATZ, DEET, and 17α-ethinylestradiol exhibited the greatest differences in removals between the 15 ppm and the 5 ppm ozone feeds; for the three levels of ozone dose pairs, differences in ATZ removal were 15%, 24% and 48%; for DEET, 15%, 9%, and 19%; and for 17α-ethinylestradiol, 2%, 5%, and 46%. At the lowest ozone dose pair in Table 5, while only 44% of the CECs showed a better removal efficiency for the 15 ppm ozone feed, at the highest dose pair, this percentage increased to 78%. Overall, results demonstrate that as the ozone dose is increased for the 15 ppm ozone feed, many CEC removals become larger compared to an equivalent ozone dose at the 5 ppm feed. Thus, it appears that the ozone feed concentration can greatly influence the efficiency of toxicity removal and this appears to be specific to the matrix and/or the CECs being removed although more studies would need to be conducted to confirm this idea.

A decrease in toxicity may not be directly associated with a decrease in CECs, due to the complexity of the wastewater samples. It is well known that CECs in environmental samples have the potential to elicit mixture effects such as additive, synergistic, or antagonistic (Altenburger et al., 2013; Boltes et al., 2012; Jonker et al., 2005; Pape-Lindstrom & Lydy, 1997; Tang et al., 2013). In addition, the CECs themselves may have different potencies. For example,

ATZ belongs to a specific class of herbicides which inhibit the plastoquinone (Q_B) binding site of PS II which we suggested and observed in other work to be the most toxic target site of action for SAPS (Chusaksri et al., 2010; Marshall & Yargeau, 2017). The insect repellent DEET exhibited an EC50 in the green algae *Chlorella protothecoides* of 388 mg/L (Martinez et al., 2016) which was 5,400 x less potent than that reported for ATZ in the same species (Al Qasmi, 2013). Thus, due to the influence of mixture effects and different CEC potencies, it is difficult to conclude for certain the contribution of CEC removals on the difference in toxicity removal observed, nonetheless, for a given ozone dose pair, the 15 ppm feed was better at removing many CECs during ozone treatment.

Table 5: Difference in removals for select equivalent transferred ozone dose pairs for the 14 CECs mix in secondary effluent

CEC		**Difference in removals of equivalent doses of the 15 ppm ozone feed compared to that of the 5 ppm[1]**		
	5 ppm feed	30 mg	42 mg	54 mg
	15 ppm feed	31 mg	42 mg	51 mg
Sulfamethoxazole		1%	0%	2%
Carbamazepine		0%	0%	0%
Naproxen		0%	0%	0%
Ibuprofen		-4%	-1%	34%
Estrone		2%	0%	1%
17ß-estradiol		0%	-3%	9%
17α-ethinylestradiol		2%	5%	46%
Atrazine		15%	24%	48%
DEET		15%	9%	19%

1: Reported as: Dose of the 5 ppm ozone feed; dose of the 15 ppm ozone feed and both doses are statistically equivalent as confirmed by paired t test ($p > 0.05$). All compounds were detected in the 14 CECs mix in SE before ozone was applied (data not shown).

To our knowledge, there have been no articles published specifically addressing the efficiency of toxicity removal using different feed concentrations that compare equivalent ozone doses. However, some studies report toxicity removal for various treatment conditions, which can be reanalyzed to determine the potential relationship between feed concentration and toxicity

removal similar to what was observed in the present study. In a semi-batch lab-scale ozone experiment, Zhang and colleagues used different ozone feeds (40 ppm and 80 ppm) to treat water over given treatment times, i.e. leading to different ozone doses (Zhang et al., 2008). We reanalyzed their data to obtain toxicity removals for comparable ozone doses obtained with the two feed concentrations (see Table 6 for details). For similar applied ozone dose pairs of 320 mg (20 ppm feed) and 340 mg (85 ppm feed), the 20 ppm ozone feed reduced estrogenicity by 13% more, which again suggests differences in toxicity removal associated with feed concentration. Similarly, we calculated equivalent ozone doses (see Table 6) using data published by Petala and colleagues from their semi-batch lab-scale ozone experiment (Petala et al., 2006). The 2.5 ppm ozone feed achieved greater toxicity removal by 10% to 12% for % immobilization of *Thamnoephalus platyurus* compared to ozone feeds of 5 ppm, 6.5 ppm, and 7.3 ppm for similar or equivalent average applied ozone doses. Furthermore, the 2.5 ppm ozone feed achieved a 15% greater reduction in toxicity for % immobilization of *Daphnia pulex* compared to that of the 6.5 ppm feed for similar average applied ozone doses of 38 mg and 39 mg, respectively. This interpretation of literature data supports our findings, which highlighted that the concentration of ozone in the feed gas can impact the efficiency of toxicity removal in wastewater for equivalent ozone doses.

Table 6: Difference in toxicity removal for similar or equivalent average applied ozone doses for two different ozone feed concentrations calculated from literature sources

Ozone feed concentrations compared (feed 1 in ppm; feed 2 in ppm)	Equivalent (or similar) average applied ozone dose pairs calculated[1] (Dose in mg of ozone feed concentration 1; dose in mg of ozone feed concentration 2)	Type of toxicity	Difference in toxicity removal of ozone feed 1 compared to ozone feed 2 for equivalent (or similar) average applied ozone dose pairs (%)	Figure used in reference	Time used from Figure (for ozone feed concentration 1 in min; for ozone feed concentration 2 in min)	Feed Flowrate (L/min)	Reference
40; 85	320; 340	Estrogenicity (ng EEQC/L)	13	Figure S3	4; 2	2	Zhang 2008
2.5; 5	225; 225	Crustacean test	12	Figure 3	30; 15	3	Petala

		using *Thamnoephalus platyurus* (% immobility)					2006
2.5; 6.5	37; 39	Crustacean test using *Thamnoephalus platyurus* (% immobility)	10	Figure 3	5; 2	3	Petala 2006
2.5; 7.3	113; 110	Crustacean test using *Thamnoephalus platyurus* (% immobility)	12	Figure 3	15;5	3	Petala 2006
2.5; 6.5	37; 39	Crustacean test using *Daphnia pulex* (% immobility)	15	Figure 3	5; 2	3	Petala 2006

1: Applied ozone doses were calculated by multiplying the ozone feed concentration by the feed flowrate by the time

4 CONCLUSIONS

Results show that the LuminoTox was a sensitive tool for monitoring changes in toxicity of different mixtures of CECs in wastewaters during ozone treatment which corresponded to a decrease in CEC concentration. For ATZ in SWW samples exposed to a 5 ppm ozone feed compared to a 15 ppm feed, a maximum difference in toxicity of 25% (SAPS I toxicity) and 35% (SAPS II toxicity) was observed for equivalent ozone doses. For CECs in SE, the 15 ppm feed was more efficient at toxicity removal, with a maximum difference in toxicity of 37% (SAPS I toxicity), and 40% (SAPS II toxicity). Thus, it was demonstrated that different ozone feed concentrations had an effect on the efficiency of toxicity removal for an equivalent transferred ozone dose, which was further confirmed by our new interpretation of literature data, and appears to be specific to the wastewater matrix and/ or CECs being removed.

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