Science of the Total Environment 598 (2017) 1065–1075

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Sensitivity of the LuminoTox tool to monitor contaminants of emerging concern in municipal secondary wastewater effluent

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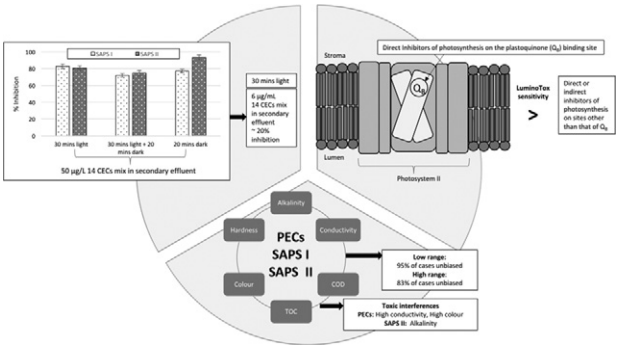
HIGHLIGHTS

- LuminoTox was sensitive to CECs in a range of concentration of 6 μg/L to 50 μg/L.
- Biosensors were applicable within the range of secondary wastewater characteristics.
- Best exposure method was exposing the biosensors for 30 min light or 20 min dark.
- Detection of CECs in wastewater required sample pre-concentration.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 1 March 2017
Received in revised form 13 April 2017
Accepted 15 April 2017
Available online 2 May 2017

Editor: D. Barcelo

Keywords:
Bioassay
CEC
Endocrine disruptor
Pesticide
Pharmaceutical
Toxicity detection tool

ABSTRACT

Contaminants of emerging concern (CECs) are generally poorly removed during conventional wastewater treatment. There is a need for rapid, sensitive and inexpensive methods to monitor the quality of treated wastewater effluent. The purpose of this study was to assess the applicability of the LuminoTox as a tool to monitor municipal secondary effluent (SE) and to determine its sensitivity to the presence of CECs. The effect of exposure method on a 14 CECs mix was explored; 20 min in the dark or 30 min under light were both recommended as they were sensitive to the detection of CECs in wastewater while providing a short run time. Stabilized aqueous photosynthetic systems (SAPS) detected the 14 CECs mix in the wastewater matrices when they were present at a concentration in the 6 μg/L to 50 μg/L range. Interference on the biosensors were examined in a range of wastewater characteristics commonly observed in SE and, for most cases, biosensors were not inhibited which suggests that, in most cases, wastewater characteristics would not cause toxic interferences. SAPS detected CECs in SE with different modes of action with the degree of sensitivity of individual CECs developed from experimental and literature values as follows: inhibitors of the plastoquinone binding site within photosystem II > direct or indirect inhibitors of photosynthesis acting on binding sites other than that of the Q_B. SAPS were assessed for their ability to detect residual CECs in SE without sample preparation, however, the effluent examined exhibited minimal inhibition for SAPS II (7 ± 1%) and no inhibition for SAPS I. These results highlight the need for the development of a sample pre-concentration method to increase the biosensor sensitivity towards native CECs. This would allow the LuminoTox to be an effective tool for monitoring wastewater quality with the intent of residual CECs detection.

Abbreviations: SAPS, stabilized aqueous photosynthetic systems; PECs, photosynthetic enzyme complexes; CECs, contaminants of emerging concern; SE, secondary effluent; SWW, synthetic wastewater; MQW, Milli Q water; MOAs, modes of action; ATZ, atrazine; MM, metsulfuron methyl; PCA, Principle Component Analysis; Q_B, plastoquinone; PS II, photosystem II.

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http://dx.doi.org/10.1016/j.scitotenv.2017.04.118
0048-9697/

1. Introduction

Wastewater treatment plants (WWTPs) were not traditionally designed to remove contaminants of emerging concern (CECs) (Henze et al., 2008) and, hence, many are poorly removed during conventional treatment (Rojas et al., 2013). WWTPs therefore contribute to the presence of CECs in the environment, as reported by various studies investigating different contaminants such as pesticides (Costanzo et al., 2007; Huber, 1993), antibiotics (Khan et al., 2013), prescription drugs (Bendz et al., 2005; Fang et al., 2012), and personal care products (Adolfsson-Erici et al., 2002). Ultimately, CECs end up in the environment in concentrations ranging from nanograms to micrograms per milliliter as reported by many studies (Hua et al., 2006; Luo et al., 2014; Metcalfe et al., 2010; Phillips et al., 2010; Rojas et al., 2013; Sengupta et al., 2014; Vidal-Dorsch et al., 2012). CECs are of concern as their long-term impacts on human and the environmental health are not fully understood (Daughton, 2004; Petrie et al., 2015).

There is a pressing need for new quality measurement methods for wastewater assessment which are more rapid, sensitive and less expensive than existing technologies (Bellemare et al., 2006; Connon et al., 2012; Krewski et al., 2010). Bioassays are attractive tools for quality assessment as they can account for the overall effect of complex mixtures, characterize a variety of wastewater samples and can offer toxicological information on diverse endpoints (Babić et al., 2017; Hemachandra & Pathiratne, 2017; Jarošová et al., 2014; Sun et al., 2017).

LuminoTox is a bioassay that uses stabilized aqueous photosynthetic systems (SAPS) which are green algae and photosynthetic enzyme complexes (PECs) which are spinach thylakoids to determine the photosynthetic inhibition of a sample. This bioassay measures the chlorophyll *a* fluorescence associated with photosystems I and II of the electron transport chain, which is then used to calculate the photosynthetic efficiency of a sample. More details about the instrument and bioassay can be found in Bellemare et al., 2006 and Dewez et al., 2007. It has been demonstrated that photosynthetic processes are sensitive to many different substances affecting either directly or indirectly the electron transport chain (Juneau et al., 2007). SAPS are less sensitive to metals due to the selectivity of the algae wall, and more sensitive to organic molecules compared to PECs (Bellemare et al., 2006).Organics known to act on extrinsic proteins at the end of the electron transport chain, such as ferredoxin and CF1 of ATPase, are disrupted during the PEC isolation process and, therefore, loose their sensitivity towards these compounds. On the other hand, PECs are more sensitive to metals as they have no cell wall for protection. LuminoTox could be an attractive instrument for the monitoring of municipal SE and could be used as a complementary tool to conventional chemical and microorganism monitoring. Similar to other *in vitro* bioassays, the LuminoTox is an integrative tool able to characterize the biological activity of water samples. However, unlike many others, this tool is rapid, user-friendly, inexpensive and portable.

Although LuminoTox has been tested and used for a wide variety of water samples, to our knowledge, there have been only three peer-reviewed articles published on municipal secondary effluent (SE) (De Luca et al., 2013; Gesuale et al., 2010; Souza et al., 2013). In all cases however, they have not explored the impact of specific SE parameters on measurements or considered the toxic responses associated with a mixture of CECs. Furthermore, only one biosensor was explored by each author: De Luca, Gesuale and Souza used SAPS I, PECs, and one of the SAPS biosensors (which was not specified), respectively. In this work, we assessed the sensitivity of the LuminoTox tool to monitor CECs in municipal SE using both types of biosensors. More specifically, we explored the impact of the method of exposure and type of matrix on the response and investigated if the LuminoTox could be used over a wide range of wastewater characteristics including: TOC, COD, conductivity, alkalinity, colour and hardness representative of SE characteristics. The sensitivity of the bioassay to CECs individually and in a mixture and with different contaminant modes of action (MOAs) was also examined.

2. Materials and methods

2.1. Wastewater matrices and range of wastewater characteristics tested

SWW was used in some experiments because it provided a controlled environment and the possibility of adjusting wastewater composition, which is not possible using real SE. It also allow to eliminate the potential for toxicity from unknown compounds. SWW was used directly, or adjusted for certain characteristics. Table 1 outlines the six different characteristics examined at low and high conditions along with their respective methods of analysis. The conditions were defined based on the ranges observed in both literature values and experimental data from prior analysis of wastewater samples in our lab (data not shown). To obtain samples with characteristics typical of those found in SE, a synthetic wastewater recipe was adapted from other work (Klamerth, 2011) and used as a stock solution from which SWW with different composition was made. The base SWW recipe used in this work is as follows: 32 mg/L BBL Biosate peptone (pancreatic digest of casein 65%, yeast extract) and 22 mg/L BBL beef extract powder (BD Mississauga, Ontario); 6 mg/L 98% urea (Fisher Science Education, Fair Lawn, New Jersey); 28 mg/L 99+% potassium phosphate dibasic trihydrate, 4 mg/L 99% calcium chloride, 62 mg/L 99+% magnesium sulphate heptahydrate, 4 mg/L 99+% potassium chloride (Sigma Aldrich Canada, Oakville Ontario); 7 mg/L 95% sodium chloride (Fisher Scientific, Fair Lawn, New Jersey); 96 mg/L 99+% sodium carbonate and 60 mg/L 98+% calcium sulphate dehydrate (Acros Organics, Fair Lawn, New Jersey). Sample matrices were made by adjusting the base SWW either by diluting it for the low-range values or by adding a chemical to obtain the high-range values. Chemicals that were already in the base SWW recipe were selected to adjust the high-range values, whenever possible. If a new chemical was required for recipe adjustment, chemicals were selected on the basis that no specific inhibition to the biosensors was reported in the literature. Metals such as cadmium, mercury, lead, silver, and zinc were avoided as they were found to be toxic to biosensors (Boucher and Carpentier, 1999; Hiriart-Baer et al., 2006; Rai et al., 1981; Rai et al., 1991; Waldemar and Tadeusz, 1988; Winner et al., 1991). Furthermore, the US EPA produced an Environmental Technology Verification Joint Verification Statement, approving the LuminoTox for testing in drinking water and determined that the following metals caused interferences: aluminum, copper, iron, manganese, and zinc, and these were also avoided in the SWW recipe. Sodium, potassium, and magnesium however, were not specifically mentioned in the literature to be chemicals associated with toxicity; as such, chemicals containing these metals were preferentially selected for recipe adjustment. Humic acids have been reported to contribute to colour in wastewater (Czerpak et al., 2003; Zouboulis et al., 2004) justifying the selection of humic acid sodium salt (45%–75% technical as humic acid, Acros Organics Fair Lawn, New Jersey) to mimic the high colour range. The salt of this compound was selected instead of humic acid itself for improved solubility.

Secondary effluent (SE) was collected from a wastewater treatment plant to further assess the LuminoTox under real condition. The wastewater plant is serving a population of 95,000 inhabitants with a design capacity of 65,000 m^3/d and treats an average flow of 38,000 m^3/d. The influent to the plant is composed of 50% industrial and 50% domestic wastewater. Grab samples were collected at the effluent of secondary treatment (activated sludge) and immediately frozen and stored at −20 °C for further use.

2.2. CEC spiking of SWW and SE

CECs were added to synthetic and real wastewater matrices in order to operate in a range of concentrations where mid- to high-range inhibition is expected, with the exception of the experiment using unmodified wastewater samples without sample preparation (described in Section 3.7). A mix of 14 CECs (listed in Table 2 along with their

Table 1
Secondary effluent wastewater characteristics along with the methods used for measurement, dilution or chemical used to adjust the synthetic wastewater, typical values of the secondary wastewater effluent characteristics along with the references used for the determination of the range and the measured characteristics for the SWW prepared.

Characteristic	Method	Dilution[a]/chemical used to adjust synthetic wastewater	Condition	Measured characteristics of SWW prepared[b]	Typical secondary wastewater value[c]	References to determine the low and high values of the characteristics[d]
Colour (TCU, absorbance at 455 nm)	HACH 8025	Unadjusted Added humic acid sodium salt to obtain 14 mg/L	Low High	8 ± 14 234 ± 6	20 250	Paraskeva and Graham (2002), Singh (2012), WWTP A&C
Hardness (mg $CaCO_3$/L)	HACH 8030	1:2 dilution Adjusted recipe to 272 mg/L using magnesium sulphate heptahydrate	Low High	64 ± 8 453 ± 49	40 320	Kang et al. (2003), Kharajian et al. (1981), James et al. (2014), Metcalfe & Eddy Inc et al. (2002), WWTP A
Alkalinity (mg $CaCO_3$/L)	HACH drop count titration/sulfuric acid method (low- and high-range tests)	1:3 dilution Adjusted recipe to 314 mg/L using sodium bicarbonate	Low High	20 ± 5 225 ± 20	30 250	James et al. (2014), Kang et al. (2003), Kharajian et al. (1981), Singh (2012), WWTP A
Conductivity (μS/cm)	HI 98311 Instruction manual	1:4 dilution Adjusted recipe to 165 mg/L using potassium phosphate dibasic trihydrate	Low High	9 ± 80 1926 ± 80	1 1700	James et al. (2014), Mesut (2013), WWTP C
Chemical oxygen demand (mg COD/L)	HACH 8000	1:38 dilution Adjusted recipe to 144 mg/L using peptone	Low High	9 ± 0.1 94 ± 1	1 50	Kang et al. (2003), Mesut (2013), Metcalf & Eddy Inc. et al. (2002), Paraskeva and Graham (2002), WWTP B&C
Total organic carbon (mg TOC/L)	Adapted from 5310 B (Rice et al., 2012)	1:24 dilution Adjusted recipe to 134 mg/L using peptone	Low High	5 ± 3 193 ± 2	2 200	James et al. (2014), Kang et al. (2003), Metcalf & Eddy Inc. et al. (2002), Singh (2012), WWTP B&C

[a] The dilution is defined as SWW: MWQ.
[b] These are the measured characteristics of the SWW prepared for testing with the LuminoTox. The values are reported as an average of triplicate measurements and the standard deviation, with the exceptions of alkalinity and conductivity where the errors were reported as specified for the Low and High Test, and the error associated with the probe, respectively.
[c] The secondary effluent wastewater range was developed by using the lowest and highest measurement observed in the literature and in-house measurements.
[d] References consulted to determine the range of characteristics reported in literature for secondary effluent and results obtained for in-house characterization of samples collected at three different WWTPs nearby (WWTP A, B, C data not shown).

surrogates, purities, LOD, LOQs and suppliers) was defined to include frequently detected compounds and consider mixture effects. Single compound stock solutions were prepared in methanol with the exception of estrogens and their internal standards which were made with different ratios of dimethylsulfoxide (DMSO) to methanol from 30:70 to 100% DMSO. A stock solution containing 1000 mg/L of each of the 14 CECs (the CECs listed in Table 2 but excluding MM and diuron) and a stock solution 100 mg/L of each of the 14 CEC surrogates were made in methanol from the single CEC or surrogate stock solutions. Stock solutions were stored at −20 °C until required. Metsulfuron methyl (MM)

Table 2
CECs used for exploring the LuminoTox sensitivity to a 14 CECs mix and two compounds with different modes of action along with their internal standards, purities, LODs, LOQs and suppliers.

Type	Subtype	Compound	Internal standard (surrogate)	Purity (%) or standard compound, surrogate	LOD, LOQ (μg/L)	Supplier: compound, surrogate
Pharmaceutical	Antibiotic	Sulfamethoxazole	Sulfamethoxazole-d4	VETRANAL, 98	1, 4	S, I
	Antibiotic	Trimethoprim	Trimethoprim-d9	VETRANAL, 99.9	1, 4	S, S
	Lipopenic	Gemfibrozil	Gemfibrozil-d4 (2,2-dimethyl-d6)	99.98, 99	1, 4	S, I
	Anti-epileptic	Carbamazepine	Carbamazepine-d10 (rings-d10)	98+, 98	1, 4	S, I
	Anti-depressant	D, L venlafaxine	(±)-Venlafaxine-d6 HCl (*N*,*N*-dimethyl-d6)	95, 99	1, 4	T, I
	Anti-inflammatory	Naproxen	(±)-Naproxen-d3 (α-methyl-d3)	98, 99	1, 3	T, I
	Anti-inflammatory	Ibuprofen	(±)-Ibuprofen-d3 (α-methyl-d3)	98, 99	1, 4	T, I
	Estrogen hormone	Estrone	Estrone 16, 16-d2	99+, 98	1, 4	S, I
	Estrogen hormone	17β-Estradiol	17β-Estradiol-2,4-d2	98+, 99	1, 4	S, I
	Estrogen hormone	17α-Ethinylestradiol	17α-Ethynylestradiol-2,4,16,16-d4	98, 98	1, 4	T, I
Pesticide	Herbicide	Atrazine	Atrazine-d5	98, 98	1, 4	T, T
	Herbicide	MCPA (4-chloro-2-methylphenoxyacetic acid)	4-Chloro-2-methylphenoxy-d3 acetic acid	99.8, 98	1, 3	S, I
	Herbicide	Diuron[a]	N/A	99.6	N/A	S
	Herbicide	Metsulfuron methyl[a]	N/A	99.5	N/A	C
	Insecticide	DEET (*N*,*N*-diethyl-3-methylbenzamide)	*N*,*N*-Diethyl-3-methyl-d3-benzamide-2,4,5,6-d4	99.5, 98	1, 4	S, I
Personal care product	Antibacterial/antifungal agent	Triclosan	Triclosan-d3	98, 98.1	1, 3	T, T

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; N/A: not applicable.
[a] Metsulfuron methyl and diuron were used to explore the LuminoTox sensitivity to CECs with different modes of action but were not included in the mix of 14 CECs.

and diuron, also listed in Table 2, were used in a separate experiment in order to explore the sensitivity of the LuminoTox to CECs with different MOA but were not included in the mix of CECs.

2.3. Quantification of CECs

Surrogates were added to each sample and then pre-concentration was performed by lyophilization using 800 mL Fast-Freeze Flasks and a FreeZone 4.5 Litre Benchtop Freeze Dry System (Labconco, Kansas City, MO). 3 mL of the sample was frozen in a disposable borosilicate glass tube (Fisher Scientific, Fair Lawn, New Jersey) and lyophilized. Each sample was reconstituted with 100 μL of a mix of 1:9 methanol to Milli Q water (MQW). Each sample was then centrifuged for 10 min at 3500 rpm using a Sorvall Legend X1R Centrifuge (Thermo Scientific, Waltham MA, USA). 90 μL was then decanted from each sample and again centrifuged for 10 min at 9000 rpm using a MicroCL 21 Centrifuge (Thermo Electron Corporation, Waltham MA, USA). 80 μL of each sample was then decanted and used for chemical analysis.

Analysis was performed on an Accela 600 LC System (Thermo Scientific, Waltham MA, USA) in tandem with an LTQ XL Orbitrap mass spectrometer. Both the LC and the MS systems were controlled by the Thermo Xcalibur 2.0 software (Thermo Scientific, San Jose CA, USA). A guard column (5 mm × 2.1 mm ID; 1.8 μm) was used prior to the analytical column (50 mm × 2.1 mm ID; 1.8 μm; C18 Zorbax Eclipse Plus) (Agilent Technologies, Santa Clara CA, USA). Separation of a 25 μL injection was conducted at 30 °C with a binary buffer system composed of 2 mM ammonium formate and 0.1% formic acid in MQW (Solvent A) and methanol 0.1% formic acid (Solvent B). A gradient elution at 0.25 mL/min of A:B was conducted as follows; initial 90:10 (0–1 min), 65:35 (1–2 min), ramp to 60:40 (2–5 min), 0:100 (5–9 min) and hold at 100% B (9–12 min).

Detection of analytes and surrogates was performed using an electrospray ionization source (ESI) in positive or negative mode. Target CECs run in negative mode include Triclosan, MCPA and their corresponding internal standards while the remainder were run in negative mode. Optimization of the instrument parameters for each type of mode was performed by direct infusion of standard solutions at 10 μL/min, while source optimization conditions were completed using infusion flow analysis. Nitrogen was used for all sheath, auxiliary and sweep gasses, while helium was used as the collision gas (Table 3). Analysis was done under data dependent acquisition mode with full scan at 30000 resolution for FT-MS (50 *m/z* to 700 *m/z*) while the ion

Table 3
Concentrations of the target CECs measured in SE and concentrations reported in the literature for municipal effluents.

Type	Compound	Experimental	Literature		
		Concentration in SE (μg/L)	Maximum concentration reported in municipal effluents examined (μg/L)	Municipal effluents (number and type)	References
Pharmaceuticals	Sulfamethoxazole	<LOD	0.871	8 WWTPs including PE and SE	Miao et al. (2004)
	Trimethoprim	<LOD	0.011	L	Carlson et al. (2013); Hua et al. (2006)
			0.344 ± 0.081	SE	
	Gemfibrozil	2.7 ± 2.2	0.078 ± 0.028	SE	Hua et al. (2006); Kerr et al. (2008); Lishman et al. (2006); Metcalfe et al. (2003)
			0.192 ± 0.020	SE	
			0.436	12 WWTPs including: SE and L	
			1.3	18 WWTP effluents including: SE, TE, PE and L	
	Carbamazepine	<LOQ	0.135	L	Carlson et al. (2013); Hua et al. (2006); Kerr et al. (2008); Metcalfe et al. (2003)
			0.344 ± 0.005	SE	
			1.036 ± 0.279	SE	
			2.3	Maximum value out of 18 WWTP effluents including SE, TE, PE and L	
	D, L venlafaxine	0.7 ± 0.3	1.8	SE	Lajeunesse et al. (2012); Metcalfe et al. (2010)
			0.8	TE	
	Naproxen	2.3 ± 1.9	0.599 ± 0.258	SE	Hua et al. (2006); Kerr et al. (2008); Lishman et al. (2006); Metcalfe et al. (2003)
			0.180 ± 0.036	SE	
			1.189	12 WWTPs including SE and L	
			33.9	18 WWTP effluents including SE, TE, PE and L	
	Ibuprofen	2.1 ± 0.6	0.105 ± 0.041	SE	Hua et al. (2006); Kerr et al. (2008); Lishman et al. (2006); Metcalfe et al. (2003)
			0.444 ± 0.214	SE	
			0.773	12 WWTPs including: SE and L	
			24.6	18 WWTP effluents including SE, TE, PE and L	
	Estrone	2.4 ± 1.0	0.038	12 WWTPs including SE and L	Lishman et al. (2006); Servos et al. (2005)
			0.1	18 WWTPs including PE, SE, TE and L	
	17β-Estradiol	3.0 ± 0.9	0.1	4 WWTPs including SE and TE	Metcalfe et al. (2013); Servos et al. (2005)
			0.016	18 WWTPs including PE, SE, TE and L	
	17α-Ethinylestradiol	11.0 ± 6.2	0.00763 ± 0.00301	TE	Cicek et al. (2007); Fernandez et al. (2007)
			0.017	5 SE WWTPs	
Pesticides	Atrazine	<LOQ	0.055	L	Carlson et al. (2013); Hua et al. (2006)
			0.175	SE	
	MCPA (4-chloro-2-methylphenoxyacetic acid)	<LOD	0.004 ± 0.003	SE	Kerr et al. (2008)
	DEET (*N*,*N*-diethyl-meta-toluamide)	10.3 ± 0.9	0.860	TE	Sengupta et al. (2014)
Antimicrobial agent	Triclosan	<LOD	0.183 ± 0.005	SE	Buth et al. (2011); Lishman et al. (2006)
			0.324	12 WWTPs including SE and L	

WWTP: wastewater treatment plant; PE: primary effluent; SE: secondary effluent; TE: tertiary effluent; L: lagoon; for studies including multiple WWTPs, only the maximum concentrations are reported.

trap was used to generate the MS2 identification fragments. Internal standard recoveries were calculated in SE and applied to adjust all concentrations. The average recovery for the 14 CECs in secondary effluent was 37%, with a low recovery for ibuprofen 8% and all other recoveries between 19% to 90%. Despite the low recovery obtained for ibuprofen, the concentrations were still above the LOQ.

2.4. LuminoTox

PECs (prod # LBLP13AA-L), reaction buffer (prod # LBLP1321), SAPS I (prod # LBLP15AA-L), SAPS II (prod # LBLP16AA-L) and atrazine standard were obtained from Aquacion Inc. (Montreal Canada). SAPS were activated for 90 min prior to testing using a BAZZ lighting system (DC 12 V, 1.2 W, model # MK-B01-3528-0.25 M) as per the manufacturer's instructions. 2 mL of each sample of interest was pipetted in triplicate into disposable borosilicate glass tubes (Fisher Scientific, Fair Lawn, New Jersey). 100 μL of SAPS was then added to each sample (separated by 30 s intervals) and exposed to either 30 min light, 30 min light followed by 20 min dark or 20 min dark. After each exposure (including in between 30 min light followed by 20 min dark), one sample at a time was poured into a Fisherbrand plastic disposable cuvette (Fisher Scientific, Fair Lawn, New Jersey) and inserted into the LuminoTox analyzer and F1 and F2 readings were taken. Lyophilized PECs were combined with reaction buffer in a darkroom with a green light, vigorously mixed, and placed on ice for 30 min prior to testing. Subsequent sample testing was the same as for that of the SAPS but with an exposure of 20 min in the dark. Blanks (Milli Q water) and controls (10 μg/L atrazine) were run for each set of samples analyzed and passed the manufacturers specifications, which are 10 μg/L atrazine control inhibition should be within 35% to 45% and each triplicate F2 reading of the sample blank should be above 500,000. Results of the controls were not always presented to improve readability of the graphs. Photosynthetic efficiency and inhibition were computed for all samples in triplicate and the average and standard deviation were reported.

2.5. Principle Component Analysis & statistical analysis

Principle Component Analysis (PCA) was completed using XLSTAT version 2016.5 for Mac iOS to investigate similarities in behaviour of (1) the inhibition response of wastewater characteristics including TOC, COD, colour, alkalinity conductivity and hardness and (2) those of the biosensors when exposed to these conditions. *t*-Tests (Paired Two Sample for Means) were performed in Excel Version 15.27 using a two-tailed distribution with $p < 0.05$ and applied to data comparing the inhibition of CECs in MQW to the inhibition if CECs in wastewater as well as for the comparison of the sensitivity at different exposure methods. Paired *t*-tests were also used to determine if any of the SWW samples exhibiting different characteristics typical of those found in SE had inhibition significantly different than MQW blanks.

3. Results and discussion

3.1. Effect of matrices on inhibition

Fig. 1 presents the inhibition of SAPS I and SAPS II exposed to a 14 CECs mix in SE-A, SWW (base recipe) or MQW using different methods of exposure: 30 min of light, 30 min of light followed by 20 min in the dark or 20 min in the dark. PECs were not considered in this analysis as spinach thylakoids are sensitive to light; they experience structural changes which ultimately damage their structure and function (Ashikawa et al., 1986; Siefermann-Harms, 1978). No effect of the matrix was observed on the inhibition of atrazine (Paired *t*-tests). However, under certain conditions, the SWW and SE matrices exhibited a slight protective effect when both biosensors were exposed to the 14 CECs as indicated by the lower inhibition observed in these matrices compared to that of the 14 CECs present in MQW. More specifically, for SAPS I, both wastewater matrices were statistically lower (as confirmed by Paired *t*-tests) compared to that of the MQW for all exposure methods, while for SAPS II, the protective effects (lower inhibitions) were significant for an exposure of 30 min light. Furthermore, SAPS II exhibited a statistically lower inhibition for the SWW exposed for 30 min light followed by 20 min dark and for SE exposed to 20 min dark when comparing both to their respective Milli Q matrices for the same treatment. The different behaviours observed may be explained by different MOAs of atrazine compared to the CECs mix (further discussed in Section 3.6). Another possible explanation for the different responses observed in the atrazine control compared to that of the 14 CECs mix is that CEC mixtures can have synergistic, antagonistic or several other effects (Jonker et al., 2005; Pape-Lindstrom and Lydy, 1997), that could behave differently for diverse methods of exposure. The observed protective effects of the wastewater matrices when exposed to the CEC mix might be explained by the presence of organic matter (OM) in the synthetic and real wastewater. Other studies have indeed reported that metal cations (Brown and Markich, 2000) and organic contaminants (including CECs) (Landrum et al., 1987) can partition or form complexes with the OM, making them less bioavailable to the biosensors. It has been demonstrated that the amount of dissolved organics is inversely proportional to the biological uptake rate due to decrease in freely available organic xenobiotics (Landrum et al., 1985). Another possible explanation for protective effects of the matrix has to do with the hardness of the samples considering that the EC_{50} value of pentachlorophenol (a biocide) towards *Selenastrum capricornutum* was reported to be more than doubled in the presence of hard water compared to soft water although the reason was not elucidated (Smith et al., 1987). Despite the slight protective effects observed in wastewater matrices, the LuminoTox was able to capture the toxicity of CECs in the high-range of inhibition at a concentration of 50 μg/L.

3.2. Sensitivity associated to different methods of exposure

Levels of inhibitions obtained using an exposure of 30 min light resulted in a higher inhibition of SAPS I; this exposure method resulted in a greater inhibition of the 14 CECs mix compared to the others in all matrices as confirmed by Paired *t*-tests. For SAPS II, an exposure of 20 min in the dark was the most sensitive for the SWW and SE matrices, while 30 min light was the most sensitive for the MQW matrix, again confirmed using Paired *t*-tests. It is therefore, recommended to either use an exposure of 30 min light or 20 min dark and both methods were more sensitive and limit the exposure time compared to a combination of 30 min of light followed by 20 min in the dark.

3.3. Variability of results

It was observed, for all biosensors, in different matrices and for different toxicants, that as the inhibition of a sample decreases, the standard deviation tends to increase, which was also reported in other work (Burga Pérez et al., 2013; Gesuale et al., 2010). This trend was further investigated using dilutions of an atrazine solution and results are presented in Fig. 2. Results indicate that samples in the mid- to high-range of inhibition (~30% to ~100%) exhibit errors between 1% to 3% inhibition, while those that elicit ~0% to ~15% of inhibition have errors between 7% to 11%. As such, inhibitions values <15% are less reliable compared to those in the mid- to high-range of inhibition and should be interpreted with care.

3.4. Interference or toxic responses of biosensors to samples with different SE characteristics

The potential for toxic interferences of wastewater matrix characteristics were explored by varying the SWW recipe. Fig. 3 shows the inhibition of PECs, SAPS I and SAPS II exposed (30 min light) to samples prepared to mimic the low and high-range wastewater characteristics

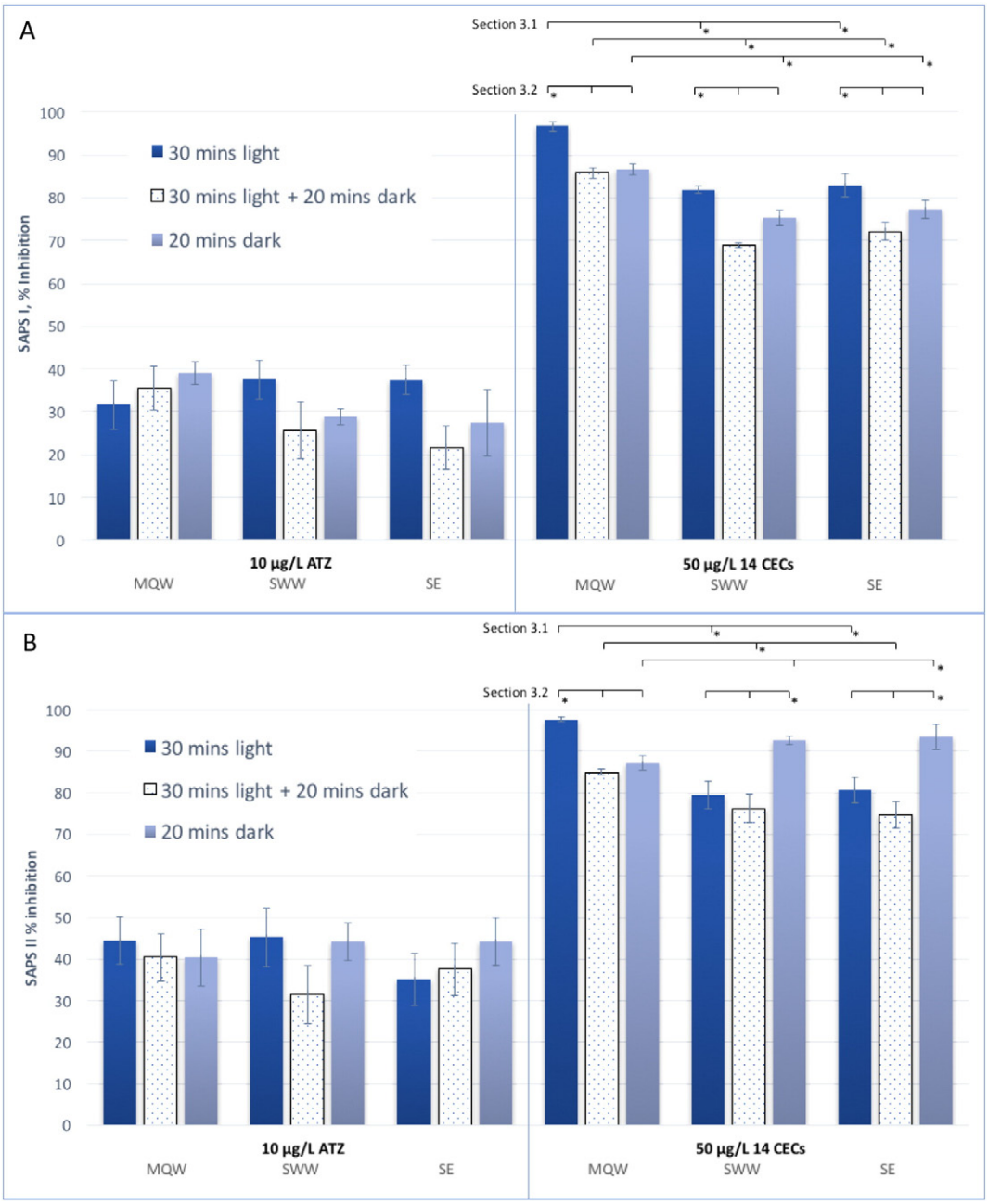

Fig. 1. Measurements of inhibition in SE using different methods of exposure for different matrices and biosensors. A) SAPS I; B) SAPS II. The error bars represent one standard deviation, n = 3. *p < 0.05.

representative of municipal secondary. All low-range values were statistically equivalent to their blanks as confirmed by Paired *t*-tests, with the exception of alkalinity (20 ± 5 mg $CaCO_3$/L) which was greater for SAPS II (8 ± 4% inhibition); thus, in most cases, no inhibition was observed for the low-range values. In some cases, samples that elicited an inhibition statistically equivalent to their blank, had one or more replicate exhibiting a stimulation (negative inhibition), which is a hormetic effect that was also observed in other LuminoTox studies (Gesuale et al., 2010; Mamindy-Pajany et al., 2011). Hormetic effects have also been reported for other assays and are known to be independent of species, mode of action and toxicant (Calabrese & Baldwin, 2002; De Nicola et al., 2007; Lippert et al., 2000; Mamindy-Pajany et al., 2011). In only 3 of high-range conditions tested resulted in significantly higher inhibitions compared to their blanks, while the remainder were statistically equivalent as confirmed by Paired *t*-tests. For the high-range values, PECs exhibited inhibitions of 28 ± 12% and 36 ± 5% for colour (234 ± 6 TCU) and conductivity (1926 ± 80 µS/cm) respectively, while the SAPS II exhibited an inhibition 19 ± 4% for alkalinity (225 ± 20 mg $CaCO_3$/L). It has been demonstrated that metal cations are toxic to the biosensors at concentrations as low as parts per billion (Maksymiec and Baszyński, 1988) and as mentioned previously, PECs are more sensitive to metal cations compared to SAPS (Bellemare et al., 2006). However, also mentioned previously, metals in the presence of humic acid will form complexes and consequently decrease metal bioavailability (Brown and Markich, 2000). As such, any free Na^+ could be contributing to the observed toxicity. More likely, however, the humic acid is contributing to the toxicity. It has been demonstrated that humic acids can interfere with the electron transport in thylakoids; the authors suggested that humic acid quinoid structures act as electron scavengers which thereby inhibit the photosynthetic production of oxygen (Pflugmacher et al., 2006). The reason that SAPS do not show inhibition towards humic acid sodium salt is likely because they have a cell

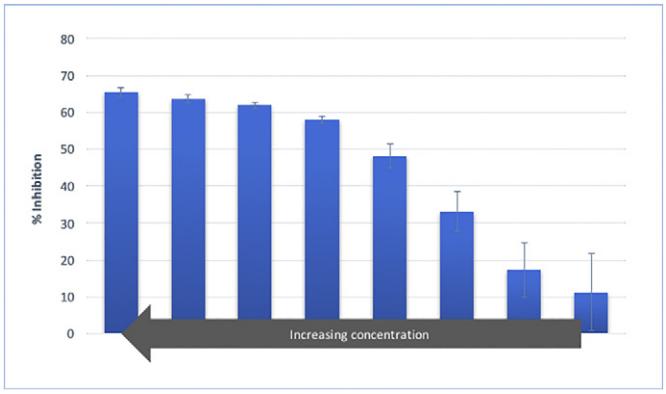

Fig. 2. Increasing variability of the measured inhibition observed in the low range of inhibition for PECs. Error bars represent on standard deviation, n = 3.

wall which protects their thylakoids from disruption. As alkalinity and conductivity are increased, inherently, so are metal cations, thus, the selection in the present study of sodium bicarbonate and potassium hydrogen phosphate to simulate these characteristics may have better mimicked what would happen with real wastewater. The toxicity of samples with low or high alkalinity, high conductivity or high colour should be taken into account as they could contribute to an observed toxicity.

3.5. PCA on samples with different characteristics and biosensors

PCA was performed on the high-range inhibition values in Fig. 3 in order to explore possible relationships between the high-range characteristics or the biosensors. Two cases were explored: first, characteristics were set as variables while the biosensors were their observations and secondly, the opposite case was investigated. To assess the appropriateness of the dataset for both cases, Barette's Sphericity Test and the Kaiser-Meyer-Olkin measure of sample adequacy were performed. The biosensors as variables passed both tests while the high-range characteristics inhibitions as variables failed due to the multicollinearity that existed between variables and was not further considered.

Fig. 4 displays a scatter plot of the biosensor's principle component loadings: PC1 and PC2. The more a variable is correlated to a specific PC axis, the more strongly a conclusion can be drawn with regards to how they relate to other variables that are strongly correlated to the same axis. Furthermore, the closer the variables (highly correlated to a specific axis) are on the scatter plot, the more similar their behaviour. In Fig. 4, the PC1 and PC2 correlations were as follows respectively: 0.81, −0.59 (PECs); 0.96; 0.16 (SAPS I); 0.91, 0.35 (SAPS II). In all cases, PC1 captured most of the variance for all biosensors, thus if the biosensors are projected onto this axis, Fig. 4 can be pictured as a line graph. In this case, SAPS I and SAPS II are more similar in their behaviour compared to the PECs. This might be explained by the fact that while all biosensors have photosynthetic machinery, the SAPS are alive and have a cell wall, while the PECs are non-living and comprise only the photosynthetic machinery of the spinach thylakoid. SAPS thus produced more similar inhibition data over the range of characteristics examined compared to results obtained with the PECs.

Since PECs appeared as responding differently to toxicants based on the PCA analysis, this suggests the potential to use them as a complementary tool to the SAPS. This is in alignment with a study of Bellemare et al. (2006) who suggested that PECs and SAPS can be used as complementary biosensors for testing metals in different water matrices because of their differences in sensitivity. Furthermore, both types of biosensors were suggested as complementary for the detection of NH_3; while PECs are insensitive to inorganic amines as they do not contain the machinery for NH_3/NH^{4+} assimilation, SAPS I is increasingly sensitive to NH_3 with increasing pH (the pH was confirmed to be independent of toxicity up to pH 11) (Bellemare et al., 2006). They also demonstrated, that, generally, PECs are significantly less sensitive to organic compounds which was suggested to be a consequence of the thylakoid isolation process. PECs will require further investigation, to determine if they can be used as a complementary tool for monitoring CECs in wastewater but this was beyond the scope of this work.

3.6. Sensitivity of the LuminoTox towards CECs with different modes of toxic action

The sensitivity of LuminoTox towards compounds with different MOAs in SE was assessed and results are presented in Fig. 5. CECs selected for this test included those with the same (Diuron, a urea), different (MM, a sulfonylurea) and mixed (14 CECs mix) MOAs compared to that of atrazine, which directly inhibits photosystem (PS) II by competing with the plastoquinone (Q_B) to bind the D1 protein (Q_B) binding site (Muller et al., 2008). MM indirectly inhibits photosynthesis through the inhibition of acetolactase synthase (Reboud, 2002), a protein

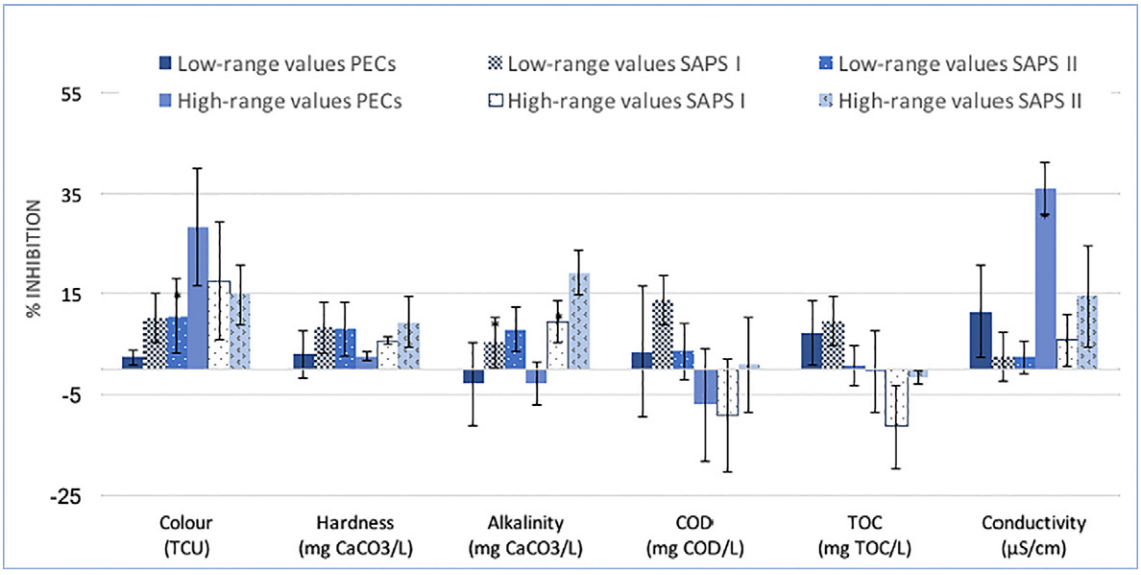

Fig. 3. PECs, SAPS I and SAPS II inhibition of SWW samples having different secondary effluent characteristics. COD: chemical oxygen demand; TOC: total organic carbon. Sample exposure: 30 min light. Error bars represent one standard deviation, n = 3. *p < 0.05, compared to blanks not shown.

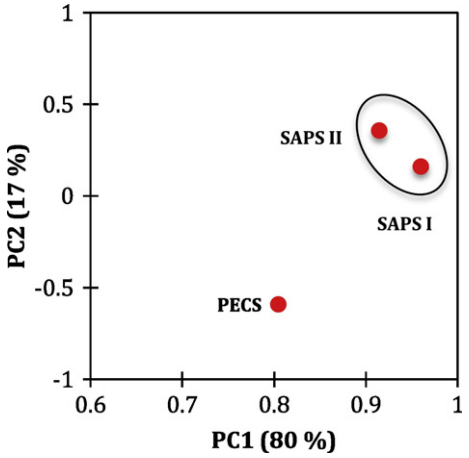

Fig. 4. Scatter plot of the biosensor's principle component loading (PC1 and PC2). The variables that have been circled behave similarly. Variables are the biosensors, and observations are the high-range characteristics. PC1 and PC2 principle components comprise 97% of the variance.

biocatalyst involved in branched-chain amino acid synthesis. The 14 CECs mix comprises many MOAs. For example, triclosan has been shown to inhibit photosynthesis in cyanobacteria (Huang et al., 2016); it has been demonstrated that different algae species possess different sensitivities to triclosan and thus, it is thought that the CEC targets multiple sites of action (Franz et al., 2008). Carbamazepine has been shown to reduce the photosynthetic activity of *Neochloris pseudoalveolarisin* in a dose dependent manner; the proposed MOA was the disruption of sugar and protein biosynthesis (Haase et al., 2015). Sulfamethoxazole has been shown to inhibit folate synthesis in plant cells by targeting hihydropteroate synthase by behaving as a structural analog (Brain et al., 2008). Estrogen disrupting compounds have been shown to cause antioxidant stress to *Chlorella* sp. (Wang et al., 2013) and growth inhibition and stimulation in SAPS I (Czerpak et al., 2003) although, to our knowledge, no specific MOA has been determined for green algae.

The toxicities observed for the individual compounds which was as follows: diuron < atrazine < MM correspond well with their EC_{50} values for the same biosensors: *Chlorella vulgaris* in the Reboud study (equivalent to SAPS I); diuron (0.0043 mg/L) < atrazine (0.4132 mg/L) (Ma et al., 2002) (MM not available) and *Chlamydomonas reinhardtii* in the Reboud study (equivalent to SAPS II); diuron (0.09 mg/L) < atrazine (0.15 mg/L) < MM (185 mg/L) (Reboud, 2002). SAPS were slightly less sensitive to the 14 CECs mix than to atrazine but much more sensitive to the 14 CECs mix than to MM. LuminoTox appears to be more sensitive to toxicants that act directly on the Q_B binding site within PS II. Moreover, the lack of sensitivity of MM compared to the other CECs tested is likely related to its indirect MOA on photosynthesis. As such, for the CECs investigated, the sensitivity of the LuminoTox to CECs with diverse MOA is as follows: direct inhibitors of the Q_B binding site within PS II > multiple MOA > indirect photosynthetic inhibition. A comparison of 40 and 29 herbicide EC_{50}s of the same algae species as SAPS I and SAPS II respectively, revealed that, for the most part, compounds that directly inhibited the Q_B binding site within PS II had EC_{50}s below 0.5 mg/L, while those that did so through other MOAs, had EC_{50}s between 1 mg/L to 963 mg/L (Ma et al., 2002; Reboud, 2002). Furthermore, a study on IC_{20}s of fifteen pesticides with diverse MOAs revealed that PECs were more sensitive to herbicides compared to insecticides (the IC_{20}s spanned four orders of magnitude) and the authors suggested that the variety of sensitivities was related to the specific site of action within the photosynthetic machinery (Chusaksri et al., 2010). They also noted that within the herbicides, atrazine, diuron, and ametryne were the most toxic group of compounds but did not further elaborate. These three compounds all act on the Q_B binding site of PS II which further highlights that the LuminoTox is the most sensitive to toxicants acting on the Q_B binding site within this photosystem. Furthermore, it can be seen in this paper that paraquat, a PS I inhibitor, has an IC20 three orders of magnitude more compared to the PS II inhibitors; even though it still acts directly on photosynthesis, it appears that the LuminoTox is significantly less sensitive to PS I compared to PS II inhibitors. Thus, for the analysis of individual compounds, based on experimental and literature data, the LuminoTox sensitivity is as follows: direct photosynthetic inhibitors that act on the Q_B binding site within PS II > direct photosynthetic inhibitors on sites other than those of the Q_B binding site within PS II and indirect photosynthetic inhibitors.

The MOA of pesticides in plants and, more specifically, green algae are more studied and elucidated in the literature compared to that of CEC of other classes. However, the following comparison between atrazine and protanol (a β-blocker pharmaceutical) supports the idea that toxicants with different sensitivities due to their specific MOAs on photosynthesis (as previously outlined) extends beyond pesticides. In the LuminoTox, atrazine produced an inhibition of ~95% at a concentration of 0.1 mg/L (Souza et al., 2013) while that of propranolol, which has a specific MOA on green algae that has not yet been determined but was confirmed to be different from that of PS II inhibition, was ~35% at a concentration of 50 mg/L (De la Cruz et al., 2013; Escher et al., 2005; Neuwoehner and Escher, 2011). Furthermore, Aquacion Inc.

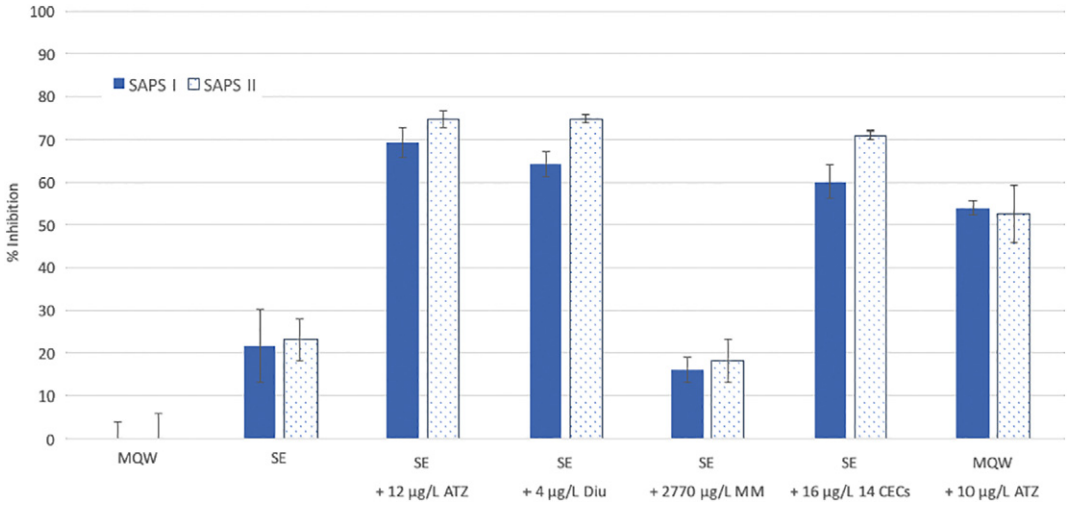

Fig. 5. Sensitivity of the LuminoTox to different CECs with diverse modes of action (MOAs) present in SE. Exposure method: 30 min light. MM: metsulfuron methyl; ATZ: Atrazine. Error bars represent one standard deviation, n = 3.

determined that carbamazepine, and sulfamethoxazole have a minimum detection level (defined as 8% to 10% inhibition) of 1 mg/L and 0.01 mg/L respectively, while that of atrazine and diuron are 0.001 mg/L and 0.0005 mg/L respectively. Again, this highlights that biosensors are more sensitive to toxicants acting on the Q_B binding site within PS II compared to those acting indirectly on photosynthesis. Furthermore, herbicides are typically designed to act directly on photosynthesis while other CEC classes will tend to act indirectly, making the LuminoTox to be likely less sensitive to CECs classes other than that of herbicides.

3.7. Sensitivity of LuminoTox to the detection of residual CECs in wastewater effluents

The sensitivity of LuminoTox towards CECs present at the low concentrations observed in secondary wastewater effluents was investigated in order to determine if the tool was able to detect the effect of CECs in SE without sample preparation. Results indicated that it was not possible to observe residual CEC toxicity in SE: SAPS I experienced no toxicity compared to its blank as confirmed by Paired *t*-tests, while SAPS II elicited an inhibition of 7 ± 1% which was statistically larger compared to its blank (data not shown) but below the threshold (about 15%) identified earlier to obtain reliable results. The low inhibition of SE obtained compare well to other LuminoTox results reported for SEs, where minimal to no inhibition was observed (Environment Canada, 2005; Gesuale et al., 2010; Souza et al., 2013). Chemical analysis of the SA sample was performed to determine the concentration of the 14 target CECs and results are reported in Table 3. Eight of the target CECs were found in the SE and, out of these, 17α-ethinylestradiol and DEET had the highest concentrations (11.0 ± 6.2 μg/L and 10.3 ± 0.9 μg/L respectively). Six of the target CECs, however, were either below the chemical limit of quantification (LOQ) or limit of detection (LOD) reported for each compound in Table 2. The values obtained were comparable to data in the literature (Table 3).

An additional experiment was then conducted using different concentrations of the 14 CECs mix in SWW to establish the lowest concentration at which the measured inhibition was in a range of acceptable variability (~20% inhibition). SAPS I and SAPS II exhibited inhibitions of 21 ± 6% and 25 ± 4% respectively when CECs were present at 6 μg/L (data not shown). Comparison to the chemical analysis results for SE reported in Table 3 indicated that 17α-ethinylestradiol and DEET were present at concentration ~2× higher than this threshold, while the remainder of the target CECs that were detected above their LOQ, were below this concentration. Based on the literature compiled, only ibuprofen and naproxen were present above that threshold value 6 μg/L (~5× and ~4× respectively). These results suggest that the contribution CECs to toxicity measured by LuminoTox is likely minimal at their native concentrations and that sample preparation is required for the LuminoTox to be an effective tool for wastewater effluent monitoring with the intent of detecting residual CECs.

If a slight inhibition is observed in sample, it may be difficult to distinguish if the observed toxicity is due to CECs or some other characteristic within the wastewater matrix. For example, the alkalinity of SE which measured to be 140 mg $CaCO_3$/L and could have contributed to the slight SAPS II inhibition observed. As previously outlined, low- and high-range alkalinity in the absence of CECs elicited some toxicity in SAPS II (Fig. 3). The low SAPS II inhibition observed in SE further underlines the need for the development of a sample preparation method for the LuminoTox to distinguish the toxicity of residual CECs from other potential sources of toxicity within the matrix.

4. Conclusion

Three exposure methods were examined for SAPS; although, slight protective effects were observed for the 14 CECs mix in the SE and SWW matrices, the LuminoTox was able to detect the mix when it was present at a concentration range between 6 μg/L to 50 μg/L. The recommended SAPS exposure methods are an exposure time of 30 min light or 20 min dark as both methods exhibited sensitivity to samples while providing minimal time of analysis. For all biosensors, as inhibition decreases the standard deviation of the sample tends to increase. As such, samples exhibiting <15% inhibition should be interpreted with care. SAPS I, SAPS II and PECs were shown to be suitable for monitoring samples with wastewater characteristics representative of secondary effluent, including TOC, COD, conductivity, alkalinity, colour and hardness. However, some limitations were observed for SAPS II that were more sensitive to high alkalinity (225 ± 20 mg $CaCO_3$/L leading to an inhibition of 19 ± 4%) and for PECS when exposed to samples with high conductivity (1926 ± 80 μS/cm) or colour (234 ± 6 TCU), which exhibited inhibitions of 28 ± 12% and 36 ± 5%, respectively. The toxicity of samples with these characteristics should therefore, be taken in consideration as interferences from the matrix may hide the contribution to toxicity of CECs. The SAPS were able to detect CECs with different MOAs with the degree of sensitivity as follows: direct inhibition of the Q_B binding site within PS II > multiple MOAs > indirect photosynthetic inhibition. Combining this result with those found in the literature, the LuminoTox was shown to be most sensitive to direct photosynthetic inhibitors acting on the Q_B binding site within PS II and less sensitive to other inhibitors acting either directly on photosynthesis on sites other than that of Q_B or indirectly on photosynthesis. The ability of the LuminoTox to detect native CECs in SE was explored, but no SAPS II inhibition was observed and SAPS I exhibited an inhibition of 7 ± 1%, which is lower than the threshold of 15% for reliable measurements. Chemical analysis confirmed that most native CECs were present at concentrations below the concentrations observed to induce a response above 20% of inhibition in the LuminoTox. It was thus impossible to distinguish the inhibition caused by the CECs from that of other potential sources of toxicity within the matrix. The development of a sample preparation protocol for testing on the LuminoTox is thus required for this technology to be used as an effective tool for wastewater monitoring with the intent of detecting residual CECs.

5. Acknowledgements

The authors thank the WWTP staff for their tour of the treatment facility, showing the authors locations for sampling, and for their general commitment to collaborate. Thanks to Émilie Doussantousse and Aquacion for training on the use of the LuminoTox tool and biosensors.

Funding

This work was supported by the Natural Sciences and Engineering Research Council (NSERC) of Canada through the Discovery Grant Program (RGPIN/04635-2015) and McGill University for the McGill Engineering Doctoral Award to Meghan Marshall. Funding sources did not in any form help write this article or participate in research design, execution or analysis.

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