LuminoTox as a tool to monitor contaminants of emerging concern in municipal secondary effluent and their removal during treatment by ozone

Meghan Marshall

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Doctor of Philosophy

Department of Chemical Engineering
McGill University
Montreal, Canada
August 2017

ABSTRACT

Conventional wastewater treatment plants were not designed to remove contaminants of emerging concern (CECs), and hence these chemicals have been shown to contribute to contamination into the environment, where CECs ultimately exist in the parts per trillion to parts per million concentration range. CECs have been shown to induce toxicity in aquatic life which has led to concern from researchers, governments and more recently the general public. Consequently, there is a pressing need for technologies to remove CECs and their associated toxicity, and for wastewater quality measurement methods to monitor these contaminants. Treatment of wastewater by ozone has been shown to reduce or remove many CECs; while many studies have demonstrated a decrease in the toxicity associated with their removal, some have reported a toxicity increase. This highlights the need to monitor the success of wastewater treatment by ozone using bioassays. The LuminoTox bioassay, which measures photosynthetic inhibition, was proposed as a tool for this application. While the LuminoTox has been used for different types of water analysis, there is limited research on its applicability for wastewater monitoring, and in particular, in municipal secondary effluents (SEs). In this PhD project, the LuminoTox was explored as a tool for the detection of CECs in wastewater and for monitoring the removal of CEC-associated toxicity during treatment by ozone. Two current LuminoTox biosensors were explored: Photosynthetic Enzyme Complexes (PECs), and Stabilized Aqueous Photosynthetic Systems I (SAPS I), as well as a new biosensor, SAPS II.

The results of this research are presented in three manuscripts. The first manuscript explored toxic interferences in the LuminoTox for ranges of characteristics typical of those found in SE. Furthermore, the limits of the LuminoTox sensitivity towards CECs in different water and wastewater matrices were explored by assessing the method of exposure, using different biosensors, and for different modes of action (MOAs). Results demonstrated that in most cases, the range of SE characteristics would not cause toxic interferences and demonstrate the potential for the LuminoTox to be used as a monitoring tool for SE. The most sensitive method of exposure was 20 minutes light or 30 minutes dark for a mixture of 14 CECs in various wastewater matrices using the biosensors SAPS I and SAPS II. In addition, the sensitivity of CECs with different MOAs was explored through experiments and the literature; the most sensitive toxicants were found to act on the plastoquinone (Q_B) binding site within photosystem II. Analysis of a mixture of 14 CECs

in SE revealed that the LuminoTox demonstrated limited sensitivity for detecting toxicity in the concentration range of native CECs in SE. This indicated that while the LuminoTox may be a promising tool for wastewater monitoring based on toxic interference results, if it is to be used for monitoring residual CECs, a pre-concentration method would be required.

Recently it was reported in another study that fines in freshwater sediments induce toxicity in the LuminoTox, however there has been no assessment of the effect of total suspended solids (TSS) on toxicity in wastewater. The second manuscript further addressed SE characteristics, and focused on the toxic interferences of a range of TSS characteristics typical of those found in SE. This work explored sensitivity to changes in atrazine concentration in a wastewater mixture containing constant total solids (TS), and also investigated a LuminoTox pre-concentration method for increased sensitivity towards CECs at their native SE concentrations. Results indicated that TSS does not contribute to toxicity over the range of concentrations typically exhibited by municipal SE, while the total dissolved solids (TDS) fraction does. Although the LuminoTox could detect 4 μg/L atrazine in the presence of TS, it could not distinguish between 4 μg/L and 6 μg/L atrazine; since native atrazine concentrations in SE are typically below these values, this further highlighted the need for sample pre-concentration. The pre-concentration method showed success; when applied to a mixture of 14 CECs in SE which were undetected without pre-concentration, the sensitivity of the LuminoTox was increased into the range of >70% photosynthetic inhibition. There were, however, limitations to this method involving the masking of toxicity indicating that optimization of the sample pre-concentration is still required.

Research reports conflicting evidence on the toxicity reduction associated with a decrease in CECs of wastewaters treated with ozone. The third manuscript explored the use of the LuminoTox as a monitoring tool, and ozone as a technology for CEC-associated toxicity removal. Chemical and LuminoTox analysis were performed on different wastewater mixtures containing atrazine or a mix of 14 CECs which had been exposed to different doses of ozone. Results indicated that LuminoTox was a sensitive tool for monitoring changes during ozone treatment; a decrease in CEC concentration corresponded to a decrease in toxicity. Furthermore, for equivalent ozone doses (achieved through varying ozone feed concentration and exposure time), the way in which ozone

is applied influenced the efficiency of toxicity removal; this removal also appeared to be specific to the wastewater mixture being treated.

In this PhD, the LuminoTox proved to be a good monitoring tool for toxicity of SEs and demonstrated the ability to detect and distinguish changes in CECs in wastewater mixtures. Furthermore, it proved to be excellent at monitoring wastewater during treatment by ozone. The LuminoTox, however, demonstrated limited CEC sensitivity at environmentally relevant concentrations. The LuminoTox pre-concentration method increased the sensitivity of the LuminoTox into the range applicable to native CECs in SE but further development of the sample preparation method is required prior to implementing the technology for wastewater monitoring.

RÉSUMÉ

Les stations de traitement des eaux usées utilisant des procédés conventionnels n'ont pas été conçues pour éliminer les contaminants d'intérêt émergent (CIEs). Ils contribuent donc à la contamination de l'environnement et les CIEs s'y retrouvent à des concentrations allant de parties par trillion jusqu'à des parties par million. Des recherches ont démontré que les CIEs peuvent avoir un impact négatif sur la vie aquatique, ce qui suscite des inquiétudes auprès des chercheurs, des gouvernements et plus récemment du grand public. Par conséquent, il y a un besoin pressent d'identifier des technologies pour éliminer les CIEs et leur toxicité, ainsi que des méthodes de mesure de la qualité des eaux usées afin de surveiller ces contaminants. Le traitement des eaux usées par ozonation permet de réduire ou d'éliminer de nombreux CIEs. De nombreuses études ont démontré une diminution de la toxicité associée à leur élimination, mais certaines ont signalé une augmentation. Cela démontre la nécessité de faire un suivi d'efficacité de traitement par ozonation en utilisant des biosenseurs. La technologie biologique LuminoTox, qui mesure l'inhibition photosynthétique, a été proposé comme outil pour cette application. Bien que le LuminoTox ait été utilisé pour différents types d'analyse d'eau, il existe peu d'information sur son applicabilité pour le suivi des eaux usées, en particulier pour les effluents secondaires municipaux. Dans ce projet de doctorat, le LuminoTox a été exploré comme un outil de détection des CIEs dans les eaux usées et pour surveiller l'élimination de la toxicité associée aux CIEs pendant le traitement par ozonation. Deux biocapteurs LuminoTox ont été explorés: les complexes enzymatiques photosynthétiques (CEPs) et les systèmes aqueux photosynthétiques stabilisés I (SAPS I), ainsi qu'un nouveau biocapteur, le SAPS II.

Les résultats de cette recherche sont présentés dans trois manuscrits. Les travaux présentés dans le premier manuscrit ont évalué les interférences toxiques affectant le LuminoTox pour des eaux usées ayant des caractéristiques typiques de celles des effluents secondaires. De plus, les limites de la sensibilité LuminoTox aux CIEs dans différentes matrices d'eau ont été explorées en évaluant la méthode d'exposition, en utilisant différents biocapteurs ainsi que différents modes d'action (MOA). Les résultats ont démontré que dans la plupart des cas, la gamme des caractéristiques des effluents secondaires ne provoquerait pas d'interférences toxiques et ont démontré le potentiel du LuminoTox comme outil de suivi ciblant ce type d'effluent. La méthode d'exposition la plus sensible était de 20 minutes d'exposition à la lumière ou de 30 minutes

d'obscurité pour un mélange de 14 CIEs dans diverses matrices d'eaux usées et pour les biocapteurs SAPS I et SAPS II. En outre, la sensibilité des CIEs avec différents MOA a été explorée par divers essais ainsi qu'une revue de la littérature; le constat étant que les biosenseurs sont plus sensibles aux substances toxiques agissant sur le site de liaison plastoquinone (Q_B) dans le photosystème II. L'analyse d'un mélange de 14 CIEs dans les effluents secondaires a démontré que le LuminoTox a une sensibilité limitée pour détecter la toxicité des CIEs présents. Cela indique que le LuminoTox peut être un outil prometteur pour la suivi de la qualité des eaux usées basée sur les résultats des interférences toxiques. Par contre, une méthode de pré-concentration serait nécessaire pour une détection à de faibles concentrations.

Récemment, une autre étude a rapporté que les particules fines dans les sédiments d'eau douce induisent une toxicité dans le LuminoTox. Cependant, il n'y a pas eu d'évaluation de l'effet des matières solides en suspension totales (MES) sur la toxicité dans les eaux usées. Les travaux présentés dans le deuxième manuscrit ont adressé ce point en étudiant les interférences toxiques d'une gamme de caractéristiques et concentrations de MES représentatives. Cette recherche a exploré la sensibilité aux changements de concentration d'atrazine dans l'eau usée en présence de solides totaux (ST) et a également étudié une méthode de pré-concentration de LuminoTox pour améliorer la sensibilité de détections des CIEs. Les résultats indiquent que les MES ne contribuent pas à la toxicité lorsque présents à concentrations représentatives des effluents secondaires municipaux, tandis que les matières totales dissoutes avaient un impact. Bien que le LuminoTox puisse détecter 4 µg / L d'atrazine en présence de ST, la distinction entre 4 µg / L et 6 µg / L d'atrazine n'était pas possible. Puisque les concentrations d'atrazine dans les effluents secondaires sont généralement inférieures à ces valeurs, ceci a mis en évidence la nécessité d'une pré-concentration de l'échantillon. Lorsqu'appliqué à un mélange de 14 CIEs dans l'eau usée, la méthode de pré-concentration a permis d'améliorer la sensiblité du Luminotox en augmentant l'inhibition photosynthétique de >70%, une toxicité sous la limite de détection sans pré-concentration. Cependant, un masquage de la toxicité par la matrice a été observée indiquant qu'une optimisation de la méthode de pré-concentration de l'échantillon est requise.

La litérature rapporte des preuves contradictoires sur la réduction de la toxicité associée à une diminution des CIEs dans les eaux usées traitées par ozonation. Le troisième manuscrit a donc

exploré l'utilisation du LuminoTox comme outil de suivi de l'élimination de la toxicité associée aux CIEs en cours de traitement par ozonation. Des analyses chimiques et par LuminoTox ont été effectuées sur différents mélanges d'eaux usées contenant de l'atrazine ou un mélange de 14 CIEs exposées à différentes doses d'ozone. Les résultats ont indiqué que le LuminoTox était un outil sensible pour suivre les changements pendant le traitement à l'ozone; une diminution de la concentration des CIEs ayant engendré une diminution measurable de la toxicité. En outre, pour des doses équivalentes d'ozone (obtenues grâce à une concentration variable de la charge d'ozone et à un temps d'exposition), les résultats ont également démontré que la concentration d'ozone dans le gaz utilisé pour le traitement influence l'élimination de la toxicité et que cet impact soit spécifique à la solution traitée.

Cette thèse de doctorat démontre que le LuminoTox s'avére être un bon outil de suivi de la toxicité des effluents secondaires ainsi que la capacité de détecter et de distinguer les changements dans les CIEs dans les mélanges d'eaux usées. En outre, il s'est avéré excellent pour surveiller les eaux usées pendant le traitement par ozonation. Cependant, le LuminoTox a démontré une sensibilité limitée aux CIEs à des concentrations normalement retrouvées dans les effluents secondaires, d'où la nécessité de poursuivre le développement d'une méthode de pré-concentration avant la mise en œuvre de la technologie pour le suivi de la qualité des eaux usées.

ACKNOWLEDGEMENTS

I would first like to thank my supervisor Prof. Viviane Yargeau for her constant support, guidance, encouragement, and advice throughout my PhD. Prof. Yargeau's drive for success, inspirational attitude, expert knowledge and constructive feedback were cornerstones in the completion of my PhD. I am grateful that she has provided me with the research equipment, supplies and lab personnel support to conduct my experiments and grow as a researcher. Finally, I would like to thank her for help with editing this thesis.

I would like to thank Marco Pineda for performing the chemical analyses for all three manuscripts integral to this PhD, and his commitment to sharing his deep knowledge and understanding of instruments and methods with students including myself. Thanks to Andrew Golsztajn and Ranjan Roy for their help with the particle size distribution analysis. I would also like to thank Deniz Nasuhoglu and Francois-Johan Chassaing for helping with the setup of my ozonation experiments, and at the WWTPs for their help with the sampling campaigns. I am grateful for having had support from the Yargeau 3Cs group members during the laborious sampling campaigns including Zeina, Liam, Sarah and Francois.

There is a community of people who have supported me throughout the journey of my PhD; their commitment to me was invaluable in the completion of this degree. I want to thank Zeina, my closest lab mate, for her continued moral support throughout the more challenging moments of my work, and also for being there to celebrate the milestones I achieved. Thanks to for daily support and encouragement from my other lab buddies, Linda, Petr, Marco, Karem, and Francois. Thanks to Isabelle, my life-long best friend who supported me at a distance, and to Evelyne for her constant encouragement and friendship throughout my PhD. Thanks to my partner David for making me laugh and taking me on adventures to inspire new perspectives. Thanks to my sister Rachael for giving me the strength, support, and inspiration through overcoming challenging moments in her own PhD. She really helped me see the path ahead in a positive light and aided me to envision completion. Thanks to my brother Duncan for his dry sense of humour that always keeps me laughing. Finally, last but not least, thanks to my parents for their continued belief in me and in my success; they have taught me that you can do anything if you really put your mind to it.

FUNDING

Funding for this PhD project was provided by the Natural Sciences and Engineering Research Council (NSERC) of Canada through the Discovery Grant Program (RGPIN/04635-2015). Thanks also to the Chemical Engineering Department at McGill University for awarding me with the McGill Engineering Doctoral Award (MEDA).

Table of Contents

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS AND UNITS

ATZ	Atrazine
ATP	Adenosine triphosphate
CEC	Contaminant of emerging concern
COD	Chemical oxygen demand
cm	Centimetre
CU	Colour units
DEET	N,N-Diethyl-3-methylbenzamide
EC	Electrical conductivity
F_o	Fluorescence reading representing the fully reduced state of plastoquinone
F_m	Fluorescence reading representing the fully oxidized state of plastoquinone
F_1	Fluorescence reading representing the fully reduced state of plastoquinone in the LuminoTox
F_2	Fluorescence reading representing the fully oxidized state of plastoquinone in the LuminoTox
k_d	Solids dissociation constant
k_{O3}	Second order rate constant for ozone
k_{OH}	Second order rate constant for the hydroxyl radical
K_{ow}	Octanol water coefficient
L	Litre
LC-HRMS	Liquid chromatography with high resolution mass spectrometry
LOD	Limit of detection
LOQ	Limit of quantification
M	Molar concentration
MCPA	4-Chloro-2-methylphenoxyacetic acid
µg	Microgram
mg	Milligram
MM	Metsulfuron methyl
MQW	Milli Q water
µS	MicroSiemen
NADPH	Nicotinamide adenine dinucleotide phosphate
ng	Nanogram
NOM	Natural Organic Matter
O_3	Ozone
OOM	Ozone/oxygen mixture
PCA	Principle component analysis
PCP	Personal Care Product
PEC	Photosynthetic Enzyme Complex
pH	Potential of hydrogen
PQ	Plastoquinone
PQH_2	Plastoquinol
PS II	Photosystem II

pK_a	Logarithmic acid dissociation constant
PSDA	Particle size distribution analysis
$QA^{-\cdot}$	Semiquinone radical
Q_A, Q_B	Plastoquinones of the reaction center
RuBisCO	Ribulose-1,5-bisphosphate carboxylase/oxygenase (enzyme)
SAPS I	Stabilized aqueous photosynthetic systems, type I (*chlorella vulgaris*)
SAPS II	Stabilized aqueous photosynthetic systems, type II (*chlamydomonas reinhardtii*)
SE	Secondary effluent
SW	Synthetic wastewater
TDS	Total dissolved solids
TOC	Total organic carbon
TS	Total solids
TSS	Total suspended solids
WWTP	Wastewater treatment plant
^oC	Degrees Celsius
Φ	Photosynthetic efficiency

CONTRIBUTIONS OF THE AUTHORS

MANUSCRIPT 1 - Sensitivity of the LuminoTox tool to monitor contaminants of emerging concern in municipal secondary wastewater effluent. Published in Science of the Total Environment, Volume 598, 15 November 2017, Pages 1065–1075.

Author	**Contribution**
Meghan Marshall	Primary author who designed and executed fieldwork and experiments, performed, analyzed and interpreted results, and wrote manuscript
Marco Pineda	Second author who developed and helped write the analytical methods, performed chemical analysis of target CECs.
Viviane Yargeau	Third author who guided the first author on experimental design, helped with the interpretation of results and completed manuscript revision

MANUSCRIPT 2 - Impact of suspended solids on the use of LuminoTox to detect toxicity of micropollutants. Accepted in Archives of Environmental Chemistry and Toxicology, October 2017.

Author	**Contribution**
Meghan Marshall	Primary author who designed and executed fieldwork and experiments, performed, analyzed and interpreted results, and wrote manuscript
Viviane Yargeau	Second author who guided the first author on experimental design, helped with the interpretation of results and completed manuscript revision

MANUSCRIPT 3 - The optimization of ozone feed concentration to minimize LuminoTox toxicity in municipal secondary effluent. In review after submission of minor revisions in Chemosphere, October 2017.

Author	**Contribution**
Meghan Marshall	Primary author who designed and executed fieldwork and experiments, performed, analyzed and interpreted results, and wrote manuscript
Viviane Yargeau	Second author who guided the first author on experimental design, helped with the interpretation of results and completed manuscript revision

1. INTRODUCTION

As our society continues to create industrial and technological advances, new contaminants are produced, joining the existing chemicals that enter our wastewater, our environment, and contaminate our drinking water sources (Bettinetti et al., 2003; Langdon et al., 2014; Leusch et al., 2014c; Li et al., 2013; Uslu et al., 2013). These contaminants include personal care products, prescription drugs, antibiotics, endocrine disrupters and pesticides, all of which have been found in parts per trillion (ng/L) to parts per billion (μg/L) concentrations throughout the Great Lakes basin (Blair et al., 2013; Khairy et al., 2014; Metcalfe et al., 2010; Uslu et al., 2013), elsewhere in Canada (Gaultier et al., 2008; Jasinska et al., 2015b), and internationally (Jiang et al., 2013; Kiguchi et al., 2016; Ma et al., 2017; Petrovic et al., 2004).

Collectively, new contaminants whose risk to society is largely unknown have been labeled contaminants of emerging concern (CECs), micropollutants, or persistent organic pollutants, and have been shown to pose toxicity problems to the environment (Bharti & Banerjee, 2013; Celiz et al., 2009; Papa et al., 2013; Perkins et al., 2013). To date, it has been proven that many CECs have adverse effects on aquatic life. One of the most researched topics in this area is the estrogenic effects in fish, including adverse reproductive capabilities and altered gonadal development as a result of environmental or wastewater effluent exposure (Burkhardt-Holm et al., 2008; Harding et al., 2016; Kidd et al., 2007; Sellin et al., 2009). CECs have also been shown to cause or to have the potential to cause toxicity in bacteria, algae, rotifers, crustaceans, plants, and fish at environmentally relevant concentrations (Baldwin et al., 2016; Bellemare et al., 2006; Brun et al., 2006; Feito et al., 2013; Khan et al., 2013; Lonappan et al., 2016; Osorio et al., 2016; Villeneuve et al., 2017). In some cases, as little as parts per trillion concentrations are sufficient to induce a negative biological response (Uslu et al., 2013). Clearly, the release of CECs into the environment must be minimized to protect the environment.

As the chemical composition of wastewater becomes ever more complex and our understanding around toxicity increases, regulations surrounding discharge limits of CECs are being developed and put into force. In 2012, Switzerland became the first country to develop and enforce legislation regarding limits on CEC discharge from wastewater treatment plants (WWTPs) which required

the upgrade of approximately 100 Swiss facilities (Audenaert, 2014; Office Fédérale de l'Environnement Suisse, 2012). As of January 1, 2016, new Swiss legislation regarding CEC removal from municipal WWTPs requires that 12 target CECs are reduced by an average of 80% (Max & Nathalie, 2016). In 2014, the European Union was not far behind, with regulation and online monitoring for WWTPs in development (Audenaert, 2014). Although no CEC regulations for WWTPs currently exist in the US, Toxcast 21, a program developed by the National Institutes of Health, the US Environmental protection Agency (EPA), the US Food and Drug Administration (FDA), and the National Research Council (NRC), aims to advance computational toxicology, molecular toxicity, and systems biology, to improve shortcomings in existing chemical as well as *in vivo* and *in vitro* methods of toxicity testing for CECs (Krewski et al., 2010a; Thomas et al., 2013). While Canada currently has no regulations in place for CEC removal, it is expected they will soon be implemented. Globally, the shift towards implementing regulations for CEC removal has created an urgent need for better wastewater treatment technologies for CEC removal, and new quality methods for assessment of CECs. (Krewski et al., 2010a; Mitchell et al., 2013; Sipes et al., 2013; Tang et al., 2013b; Waters & Jackson, 2008).

In conventional municipal wastewater treatment, many CECs are poorly removed as evidenced by their presence in parts per trillion to parts per million concentrations in wastewater effluents (Arvai et al., 2014; Baalbaki et al., 2016; Baalbaki et al., 2017a; Baalbaki et al., 2017b; Baalbaki et al., 2017c; Leusch et al., 2014b; Margot et al., 2013; Phillips et al., 2010; Stalter et al., 2011). Municipal WWTPs, therefore, act as sources of contamination into the environment. Wastewater is a complex mixture containing compounds that react and degrade throughout the treatment process; as such its composition is poorly defined and difficult to characterize (Tang et al., 2013b). The degree to which CEC degradation occurs in wastewater is strongly influenced by the method of treatment, the persistence of the CEC, and the quality of the wastewater matrices in which it interacts (Celiz et al., 2009; Escher et al., 2006; Kasprzyk-Hordern et al., 2009; Larcher & Yargeau, 2013b). New wastewater treatment technologies for improved CEC removal are thus warranted to deal with the complex matrices.

The use of ozone for the advanced treatment of CECs in wastewater is currently of interest due to its strong oxidizing capabilities. The side reaction of ozone and wastewater also produces hydroxyl

radicals, which have rates of reaction higher than the ozone itself. The literature reports that ozonation has the potential to effectively degrade or mineralize many CECs at ozone doses from 3 mg/L O_3 to 20 mg/L O_3 (Benitez et al., 2004b; Chen et al., 2012; Huber et al., 2005b; Larcher et al., 2012; Larcher & Yargeau, 2013b; Macova et al., 2010; Magdeburg et al., 2014; Margot et al., 2013; Miik et al., 2011; Reungoat et al., 2010; Singh et al., 2015; Ternes et al., 2003). Ozone also has the ability to reduce different types of toxicity including non-specific (cytotoxicity), genotoxicity, endocrine activity, inhibition of photosynthesis and mutagenicity (Margot et al., 2013; Miik et al., 2011; Stalter et al., 2011). Research has confirmed the relationship between ozone dose and toxicity removal (Gesuale et al., 2010; Margot et al., 2013; Paraskeva et al., 1998; Petala et al., 2006; Quero-Pastor et al., 2014; Uslu & Balcioglu, 2008); the more ozone applied, the further labile and intermediate products are oxidized. In some cases, however, an increase in toxicity, which can be caused by transformation products (TPs) or disinfection byproducts (DBPs), was reported (Luster-Teasley et al., 2005; Magdeburg et al., 2014; Paraskeva et al., 1998; Petala et al., 2008; Stalter et al., 2010). The discrepancy in the literature regarding ozone treatment and toxicity removal underlines the importance of helping quantify the success of ozone treatment via toxicity testing.

Toxicity testing is being increasingly promoted as a strategy to better define the risks associated with WWTP effluent discharges, however, the complexity of the wastewater matrix poses challenges for its analysis (Escher et al., 2013; Jiang et al., 2013; Leusch et al., 2014c; Neale et al., 2012; Tang et al., 2013b). The assessment of wastewater toxicological properties through targeted chemical analysis poses a problem as this analysis focuses on selected contaminants, cannot account for the effect of the whole sample, nor can it predict potential interactive effects of a mixture, whether from addition, subtraction, antagonism or synergism (Altenburger et al., 2013; Boltes et al., 2012; Jonker et al., 2005; Pape-Lindstrom & Lydy, 1997; Sullivan & Spence, 2003; Tang et al., 2013b; Wilkinson et al., 2015). Although both *in vivo* and *in vitro* testing can account for the toxicological response of the overall sample, many *in vivo* and *in vitro* bioassays are lengthy, expensive and require advanced operator skill (Juneau et al., 2007; Stalter et al., 2010). Some *in vitro* bioassays, however, are a promising component to monitoring municipal wastewater effluents. They can be used to analyze a wide variety of water matrices including industrial, municipal, and agricultural effluents (Escher et al., 2013; Kontana et al., 2009; Langdon et al.,

2014; Schrank et al., 2009). They can characterize the overall toxic effect of a sample based on biological activity and can offer toxicological information for diverse endpoints. (Babić et al., 2017; Escher et al., 2013; Hemachandra & Pathiratne, 2017; Jarošová et al., 2014; Leusch et al., 2014c; Neale et al., 2012; Sun et al., 2017). These bioassays can account for effects of unknown compounds and the interactive effects of complex wastewater mixtures (Escher et al., 2013; Leusch et al., 2014c; Neale et al., 2012). Furthermore, some of these bioassays are rapid and cost effective compared to *in vivo* testing (Krewski et al., 2010a). These bioassays can be used as a complement to *in vivo* and chemical analysis to better define complex mixtures and unknown contaminants therein (Gesuale et al., 2010; Leusch et al., 2014a). They can also aid in better understanding and determining toxicity pathways and uncovering root causes of toxicity which are currently poorly defined (Krewski et al., 2010a).

One such promising bioassay is the LuminoTox, which reports the photosynthetic inhibition of green algae and spinach thylakoids for a sample of interest. CECs can bind specific sites within the thylakoid membrane which can interfere with the emission of chlorophyll a fluorescence. (Boucher & Carpentier, 1999a; Maksymiec & Baszyński, 1988; Maxwell & Johnson, 2000; Tischer & Strotmann, 1977). The LuminoTox measures the change in fluorescence emitted, compared to that of a blank (Mili Q water); the impact on photosynthesis can then be quantified through the computation of photosynthetic efficiency of photosystem II (PS II) and photosynthetic inhibition. This bioassay has been of recent interest for water testing and has been used to assess a wide variety of CECs in different water matrices (Angthararuk et al., 2015; Bellemare et al., 2006; Blaise et al., 2008; Burga-Perez et al., 2012; Chusaksri et al., 2010; Debenest et al., 2010; Dellamatrice et al., 2006; Dewez et al., 2007; Doussantousse, 2014; Manusadžianas et al., 2012; Souza et al., 2013). To date, there have been 17 peer-reviewed publications reporting results based on the LuminoTox (Angthararuk et al., 2015; Bellemare et al., 2006; Blaise et al., 2008; Burga Pérez et al., 2013; Chusaksri et al., 2010; De la Cruz et al., 2013; De Luca et al., 2013; Debenest et al., 2010; Dellamatrice et al., 2006; Dewez et al., 2007; Férard et al., 2015; Gesuale et al., 2010; Mamindy-Pajany et al., 2011; Manusadžianas et al., 2012; Perron et al., 2012; Souza et al., 2013; Toumi et al., 2013). Of these, three have explored municipal wastewater toxicity and only one of these three has addressed ozone treatment of municipal wastewater (De Luca et al., 2013; Gesuale et al., 2010; Souza et al., 2013). Further exploration of the LuminoTox as a tool to monitor

wastewater and CECs therein appears warranted to fill the knowledge gaps prior to implementation of the technology for this application.

This PhD thesis addressed the gaps identified in the aforementioned research and investigated the sensitivity of the LuminoTox in monitoring wastewater effluent for CECs, including certain prescription drugs, antibiotics, endocrine disrupters, pesticides and personal care products. In addition, the thesis assessed the applicability of the technology to evaluate the efficacy of ozone in reducing CEC-associated wastewater toxicity.

2. LITERATURE REVIEW

2.1 Specific CECs: Sources; routes into and presence in the environment; toxicity/ adverse effects

In this section, the sources of particular pharmaceuticals, pesticides, and personal care products targeted in this work are described, along with their routes into the environment. In each case, their environmental presence and known toxicity or adverse effects are described, justifying their selection for the project. This project focuses specifically on CECs in municipal secondary effluent (SE), which is the most common type effluent discharge in the environment. The concentrations of the target CECs in SEs are summarized in Table 1, and their structures, pKas and their CAS numbers are shown in Figure 1.

2.1.1 Pharmaceuticals

In 2016, in the USA alone, pharmaceutical sales were $333 billion or 1.9% of America's gross domestic product (International Trade Administration, 2016). The pharmaceuticals of interest in this work include antibiotics, lipopenics, epileptics, antidepressants, anti-inflammatories and synthetic hormones. These drugs are produced by manufacturers, and have been known to be released into the environment through the improper treatment of manufacturing effluents which are discharged into receiving surface and ground waters; the finished pharmaceuticals are purchased by the general public and hospitals (Benitez et al., 2013; Heberer, 2002; Larsson et al., 2007). Prescription/ non-prescription drugs are either ingested and excreted along with their metabolites, or disposed of, whether via household or hospital drains, toilets, or garbage (Bound & Voulvoulis, 2005; Guang et al., 2014; Kümmerer, 2003; Tong et al., 2011). Pharmaceuticals ultimately ending up in landfills have been shown to leach into the soils and the surrounding groundwater (Ahel & Jeličić, 2001; Heberer, 2002). Of those that pass through WWTPs, many are poorly removed and are ultimately discharged into receiving surface waters (Behera et al., 2011; Jelic et al., 2011; Luo et al., 2014). Moreover, wastewater sludge containing pharmaceuticals is often applied to farming crops and thus these drugs can be transported into the soil, groundwater and surface water (Liu, 2016; Prosser & Sibley, 2015). Information regarding medical uses and the occurrence of specific classes of pharmaceuticals are outlined in the following sections.

2.1.1.1 Antibiotics

Antibiotics are a class of pharmaceuticals used for the treatment and prevention of bacterial or parasitic protozoan infections by killing or preventing the growth of these microorganisms. Two of the target CECs selected for this project, sulfamethoxazole and trimethoprim. Trimethoprim and sulfamethoxazole are often used in combination to treat urinary tract infections or middle ear infections. Trimethoprim is also used to treat traveler's diarrhea, while sulfamethoxazole treats bronchitis and prostatitis. One of the main concerns with sulfonamides is that they will create bacterial resistance due to their environmental presence at concentrations which have been reported from 0.13 µg/L to 2.7 µg/L (Halling-Sørensen et al., 1998; Hirsch et al., 1999; Holm et al., 1995; Khan et al., 2013; Kolpin et al., 2002). In one study examining surface water in the vicinity of a hospital, sulfamethoxazole was found at 2.7 µg/L at while trimethoprim was found at 1.7 µg/L in the surface water along with six antibiotic resistant genes (Khan et al., 2013).

2.1.1.2 Lipopenics

Lipopenics are a class of pharmaceuticals that reduce the concentration of lipids in the blood for patients with hypercholesterolemia. Fibrates, a subclass of lipopenics, are amphipathic carboxylic acids. The fibrate Gemfibrozil, specifically lowers triglycerides, very low density lipoproteins (VLDLs), moderately decreases high density lipoproteins (HDLs) and modestly reduces low density lipoproteins (LDLs). Gemfibrozil was reported in groundwater up to a concentration of 6.86 µg/L (Fang et al., 2012) and at a median concentration of 0.015 µg/L (121 samples were analyzed) (Cabeza et al., 2012). Gemfibrozil was shown to cause growth inhibition of *chlorella vulgaris*, the inhibition of bioluminescence of *Vibrio fischeri* and the immobilization of *Daphnia magna* at concentrations of 60 µg/L, 40 µg/L, and 50 µg/L, respectively (Zurita et al., 2007).

2.1.1.3 Anti-epileptics

Anti-epileptic pharmaceuticals such as Carbamazepine are used traditionally for the treatment of epileptic seizures, but also for bipolar disorder, borderline personality disorder, and neuropathic pain. In an epileptic seizure, this pharmaceutical suppresses the excessive firing of neurons while in physiologic disorders, it acts as a mood stabilizer. Carbamazepine was reported in the

groundwater at a median concentration of 0.062 µg/L (121 samples were analyzed) (Cabeza et al., 2012). This CEC was also shown to cause growth stimulation of the algae *Parachlorella kessleri* at a concentration of 10 µg/L and growth inhibition of the algae *Neochloris pseudoalveolaris* at 100 µg/L (Haase et al., 2015).

2.1.1.4 Antidepressants

Antidepressants are a group of pharmaceuticals used to treat many types of disorders or ailments some of which include major depressive disorder, anxiety disorders, eating disorders and pain. D, L Venlafaxine belongs to a class of antidepressants called serotonin-norepinephrine reuptake inhibitors and is mainly used to treat depression. D, L Venlafaxine was found at concentrations from 80 µg/L to 91 µg/L in fathead minnows that had been placed in the Grand River in Canada for a two week period (Metcalfe et al., 2010). D, L Venlafaxine was also found in creek water up to a concentration of 0.69 µg/L (Schultz et al., 2010). The fern *polystichum setiferum* was exposed to D, L Venlafaxine which induced phytotoxicity, and at 48 hours exposure, the lowest observed adverse effect level (LOAEL) for a reduction in mitochondrial activity was 10 µg/L; at one week's exposure, the LOAEL for DNA damage was 0.1 µg/L (Feito et al., 2013).

2.1.1.5 Anti-inflammatories

Anti-inflammatories are a group of pharmaceuticals used to relieve pain caused by excessive inflammation. Non-steroidal anti-inflammatory drugs (NSAIDs) alleviate pain by inhibiting the cyclooxygenase-2 enzyme. Ibuprofen and naproxen belong to the propionic acid subclass of NSAIDs; they can be used to treat migraines, menstrual cramps, and different types of arthritis. Ibuprofen and naproxen at concentrations between 0.006 µg/L to 0.032 µg/L and 0.016 µg/L to 0.034 µg/L respectively were found in the bile ducts of fish caught in surface water downstream of a wastewater treatment plant (Brozinski et al., 2013). Furthermore, out of five surface waters sampled downstream from a wastewater treatment plant, naproxen and ibuprofen were reported at mean concentrations up to 40 µg/L and 2.61 µg/L, respectively (Ascar et al., 2013). Both compounds were found to elicit growth inhibition in the algae *Selanstrum capricornutum,* concentrations of >32 µg/L produced a decrease in the number of offspring of the water flea *Ceriodaphnia dubia* (Brun et al., 2006).

2.1.1.6 Estrogen hormones

Estrogens can be either naturally derived and excreted by humans and livestock, or synthetically fabricated for medications. Synthetic estrogens are used for contraception, hormone replacement therapy and to treat prostate and breast cancers. Animal manure is arguably the largest source of estrogens in the environment (Adeel et al., 2017). The European Union estimates the annual livestock excretion of estrone and estradiol at 1315 kg/yr and 570 kg/yr respectively (Ray et al., 2013). The concentrations of estrogens in the environment vary greatly depending on their origin. For example, estrone, 17α-ethinylestradiol and 17ß-estradiol were found at 5.2 µg/L to 5.4 µg/L, 0.65 µg/L 0.68 µg/L, and 1.0 µg/L to 1.5 µg/L, respectively, in swine farm effluents (Francs, 2006) while these CECS were reported at concentrations up to 0.016 µg/L, 0.006 µg/L and 0.015 µg/L, respectively, in river water (Liu et al., 2017). Estrogens are one of the most studied classes of CECs, best known for their toxicity to fish in the environment; Kidd and colleagues conducted a 7-year study on fathead minnows in a lake dosed with estrogens. They determined that chronic exposure of synthetic 17α-ethinylestradiol as low as 0.005 µg/L to 0.006 µg/L lead to intersexed males and females with altered oogenesis, and over the study period leading to a near extinction of the species (Kidd et al., 2007). Altered sex development and reproductive capacities in fish is well known (Fenske et al., 2005; Razmi et al., 2011; Tabata et al., 2001). Chronic toxicity tests of the algae *Scenedusmus obliquus* exposed to 0.2 µg/L Biphenol A demonstrated an inhibition in population growth of 30% after 5 days (Wang et al., 2011). In *Chlamydomonas reinhardtii,* 1.9 µg /L, and 1.9 mg/L 17α-ethinylestradiol were shown to induce growth inhibition, and a 10% reduction in photosynthetic efficiency parameter F_v/F_m respectively (Polock, 2014).

2.1.2 Pesticides

Pesticides are a class of chemicals that kill unwanted plants, insects, or animals. Two subclasses of pesticides are herbicides and pesticides, which target specific plants and insects respectively. In 2007, herbicide and insecticide sales totaled approximately $17 million USD in the USA, and captured approximately 40% of the world market (US EPA, 2011). Herbicides enter the environment largely through land runoff into ground and surface waters after their application. In urban areas, however, the main pathway of entry into the environment begins with their use in

non-agricultural applications including grass management, industrial vegetating control, and non-agricultural crops such as horticulture and plant nurseries (Cahill et al., 2011). The primary pathway of the insect repellant DEET into the environment is through human consumption; DEET is adsorbed, rinsed off or excreted and ends up in WWTPs (Costanzo et al., 2007). Traditional wastewater treatment is not effective at the removal of pesticides such as atrazine or MCPA, nor insect repellants such as DEET (Aronson et al., 2012; Berthod et al., 2016; Hörsing et al., 2011; Köck-Schulmeyer et al., 2013), and thus WWTPs again act as a point source of environmental contamination. In a study which compiled data from Germany, France, the Netherlands and the USA comprising 4532 sites and 5,084 sampling occasions, MCPA and atrazine were reported in the list of the 59 most frequently detected herbicides (Schreiner et al., 2016). Atrazine was reported in flowing water at 90 µg/L and standing water at 2 µg/L (Huber, 1993). In a review by Costanzo and colleagues, they reported that DEET was present from 0.04 µg/L to 33.4 µg/L in aqueous samples (surface and ground water and treated effluents) in sites around the world (Campos et al., 2016; Costanzo et al., 2007). Atrazine has been shown to elicit LuminoTox photosynthetic inhibition in PECs and SAPS I at concentrations as low as 1.0 µg/L and 0.7 µg/L, respectively (Bellemare et al., 2006). MCPA induced toxicity (measured as a decrease in the number of healthy cells) in three species of diatoms when they were exposed at concentrations of 50 µg/L for 48hr (Wood et al., 2016). The information on the environmental toxicity of DEET is limited; DEET was reported to have an EC50 (measured by reduced O_2 flux) of the dinoflagellate *Gymnodinium instriatum* of 72,900 µg/L (Martinez et al., 2016).

Two of the pesticides in this PhD study, diuron and metsulfuron methyl, were used in a single experiment in this project to explore the sensitivity of the LuminoTox to different modes of action (MOAs) and were selected because diuron has the same MOA as atrazine, and metsulfuron methyl has a different MOA.

2.1.3 Personal care products

Personal care products (PCPs) comprise products including lotions, skin care creams, toothpaste, detergent, soap, shampoo and cosmetics. In 2010, direct and indirect sales of PCPs contributed $189 billion to the US economy (Personal Care Products Council, 2010). The main route of entry

of PCPs into the environment is through human use and consumption. PCPs are applied to skin and eventually washed off, or ingested and excreted. The routes of PCPs into the environment are the same as those of pharmaceuticals (Sui et al., 2015). Since the biological degradability of PCPs is variable, many are only partially removed during wastewater treatment and, ultimately, end up in the environment (Ternes et al., 2004). Triclosan is an antimicrobial and preservative agent found in personal care products, of which it typically comprises from 0.1% to 0.3% by weight (Montaseri & Forbes, 2016). In a study completed in the USA, triclosan was identified as one of the top seven CECs found in streams across 30 states, at concentrations up to 2.3 μg/L (Kolpin et al., 2002). Furthermore, triclosan was reported to elicit an IC50 (measured as survival or reproduction) of 4.7 μg/L in the algae *Selenastrum capricornutu*

Table 1 CECs concentrations in wastewater effluents reported in literature

Type	Subtype	Compound	Maximum concentration found in wastewater effluents (μg/L)	Type of municipal effluent
Pharmaceutical	Antibiotic	Sulfamethoxazole	0.871[a]	8 WWTPs including PE and SE
	Antibiotic	Trimethoprim	0.011[b], 0.344 ± 0.081[c]	L[b], SE[c]
	Lipopenic	Gemfibrozil	0.078 ± 0.028[c], 0.192 ± 0.020[d], 0.436e, 1.3[f]	SE[c, d]; 12 WWTPs including: SE and L[e]; 18 WWTP effluents including: SE, TE, PE and L[f]
	Anti-epileptic	Carbamazepine	0.135[b], 0.344 ± 0.005[c], 1.036 ± 0.279[d], 2.3[f]	L[b]; SE[c]; SE[d]; maximum value out of 18 WWTP effluents including: SE, TE, PE and L[f]
	Antidepressant	D, L Venlafaxine	1.8[g], 0.8[h]	SE[g], TE[h]
	Anti-inflammatory	Naproxen	0.599 ± 0.258[c], 0.180 ± 0.036[d], 1.189[e], 33.9[f]	SE[c, d]; 12 WWTPs including: SE and L[e]; 18 WWTP effluents including: SE, TE, PE, L[f]
	Anti-inflammatory	Ibuprofen	0.105 ± 0.041c, 0.444 ± 0.214d, 0.773e, 24.6f	SE[c, d]; 12 WWTPs including: SE and L[e]; 18 WWTP effluents including: SE, TE, PE and L[f]
	Estrogen hormone	Estrone	0.038[e], 0.1[i]	12 WWTPs including: SE and L[e]; 18 WWTPs including: PE, SE, TE and L[i]
	Estrogen hormone	17β-estradiol	0.1[j], 0.016[i]	4 WWTPs including SE and TE[j]; 18 WWTPs including: PE, SE, TE and L[i]
	Estrogen hormone	17α-ethinylestradiol	0.00763 ± 0.00301[k], 0.017[l]	TE[k]; 5 SE WWTPs[l]
Pesticide	Herbicide	Atrazine	0.055[b], 0.175[c]	L[b], SE [c]
	Herbicide	MCPA (4-Chloro-2-methylphenoxyacetic acid)	0.004 ± 0.003[d]	SE[d]
	Insecticide	DEET (N,N-Diethyl-3-methylbenzamide)	0.860[m]	TE[m]
Personal care product	Antibacterial/ antifungal agent	Triclosan	0.183 ± 0.005[n], 0.324[e]	SE[n]; 12 WWTPs including: SE and L[e]

a: (Okuda et al., 2009); b: (Radjenović et al., 2009); c: (Hörsing et al., 2011); d: (Berthod et al., 2016); e: (Martín et al., 2012); f: (Yan et al., 2014a); g: (Carballa et al., 2004); h: (Stasinakis et al., 2013); i: (Stevens-Garmon et al., 2011); j: (Zhu, 2014); k: (Lajeunesse et al., 2012).

References for municipal effluent concentrations: a: (Miao et al., 2004); b: (Carlson et al., 2013); c: (Hua et al., 2006b); d: (Kerr et al., 2008); e: (Lishman et al., 2006); f: (Metcalfe et al., 2003); g: (Lajeunesse et al., 2012); h: (Metcalfe et al., 2010); i: (Servos et al., 2005); j: (Metcalfe et al., 2013); k: (Cicek et al., 2007); l: (Fernandez et al., 2007); m: (Sengupta et al., 2014); n: (Buth et al., 2011).

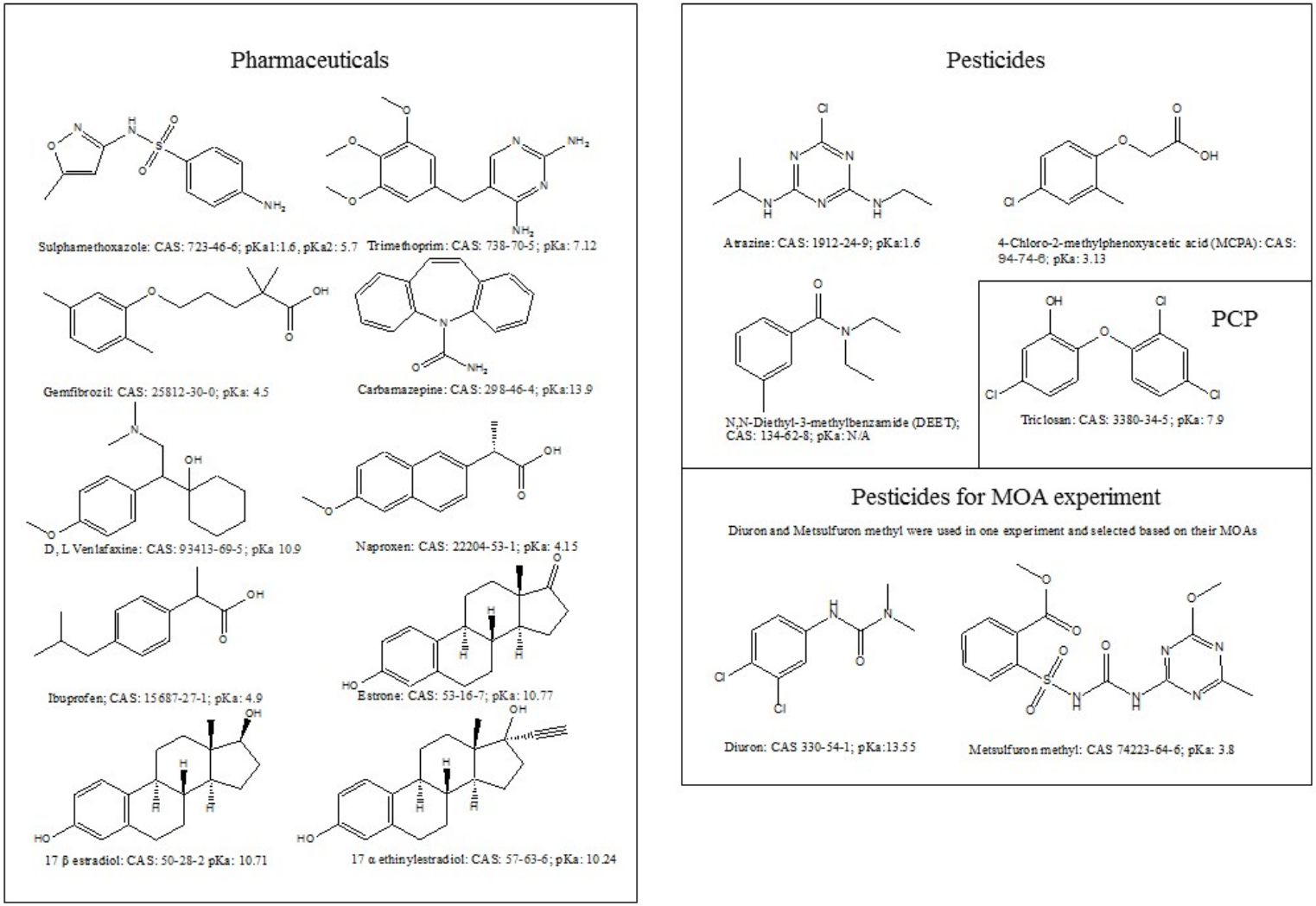


Figure 1 CECs including pharmaceuticals, pesticides, and one personal care product (PCP) as well as pesticides selected for MOA experiment

2.2 Secondary wastewater effluent

This project deals mainly with SE which was selected for analysis because it is currently the most common type of effluent that would be used for monitoring toxicity prior to discharge into the environment if wastewater quality regulations for toxicity were implemented at WWTPs. The composition of wastewater is poorly defined and poses a challenge to fully characterize. Wastewaters, including SEs, are a complex mixture of natural organic matter (NOM), soluble microbial products, and trace chemicals (Shon et al., 2006) some of which include CECs and their TPs, that react and degrade throughout the wastewater treatment process (Meng et al., 2010; Tang et al., 2013b) by biotic and abiotic mechanisms such as biodegradation (Larcher & Yargeau, 2013a), oxidation (Acero et al., 2000; Rodayan et al., 2013), hydrolysis (Andreozzi et al., 2004), and photolysis (Boreen et al., 2003; Rodayan et al., 2013; Yargeau & Leclair, 2008). In a wastewater or SE mixture, parent compounds, labile metabolites and TPs can all be present simultaneously, and will influence toxicological behaviour of the system (Celiz et al., 2009).

Traditional methods of characterization can be used as tools to help to define a wastewater sample. Typical SE characteristics and their ranges (outlined in Table 2) were selected for this project because they are well-established methods of wastewater quality testing for these characteristics, which are simple and produce results in minimal time. The wastewater characteristics of interest in this project are defined as follows:

- *Total Organic Carbon (TOC)* is defined as the total weight (in mg) of organic carbon per litre of sample. TOC is an indirect method of measuring the amount of organic carbon in a sample, achieved by measuring CO_2 produced from the complete oxidation of organic molecules.
- *Chemical Oxygen Demand (COD)* is the amount of oxygen needed to oxidize all of the organic carbon in a sample, reported as mass of oxygen consumed (in mg) per litre. The more an organic compound is able to be oxidized, the more COD will increase; it is therefore an indirect measurement of the organic carbon and is directly proportional to the TOC of a sample.

- *Colour* (also called true colour) is the colour (measured in colour units or CU) of a sample after being filtered through a 0.45 μm filter. The unit of colour is equivalent to the absorbance at 455nm or 465 nm of 1 mg/L platinum (in the form of a chloroplatinate ion).
- *Hardness* is defined as the sum of concentrations of calcium and magnesium ions in a water sample, expressed in mg of $CaCO_2$ per L of sample.
- *Alkalinity* is the ability of an aqueous solution to neutralize a strong acid. Alkalinity can be determined by measuring the amount of acid required to neutralize a sample, and is reported in mg/ L $CaCO_2$ equivalents.
- *Electrical Conductivity (EC)* is the ability of a solution to conduct electricity, measured in S/m. EC was used as an indirect indicator of TDS.
- *Total Solids (TS)* is the total amount of solids (the addition of TSS and TDS) in a sample, measured in mg TS per litre.
- *Total Suspended Solids (TSS)* is defined as the amount of suspended solids with diameter greater than 0.45 μm, measured as mg of TSS per litre
- *Total Dissolved Solids (TDS)* is the weight (in mg) of all of the dissolved solids per litre of water. It can be measured by drying a water sample with the TSS portion removed. In this project, EC was used as an indication of TDS due to the simplicity of the EC measurement. If a sample is made of constituents that are mostly conductive, EC is directly proportional to TDS (Hem, 1985; Lloyd, 1985; Wood, 1976). A relationship to relate EC to TDS seen in equation 1 was developed for industrial wastewater effluent (milk production) where the main sources that contribute to EC are Ca^+ and Na^+ (Ali et al., 2012).

$$TDS\ (ppm)\ = K \cdot EC(\mu S/cm)\,,\ K = 0.64 \pm 0.25, range: 1189 \pm 242\ \mu S/cm) \qquad (1)$$

Where K is a proportionality constant.

If a high proportion of substituents exist in the water that are not conductive (as could be the case for municipal wastewater), a more complicated relationship can arise. For example, a model relating EC and TDS has been developed by Atequana and colleagues for an aquifer contaminated with hydrocarbons (Atekwana et al., 2004). Dissolved organics such as hydrocarbons can contribute to the TDS but can reduce the specific conductance due to their high resistivity (Atekwana et al., 2004). It is also possible that organics can

increase specific conductance due to polar organic species such as organic acids and biosurfactants produced during degradation (Cassidy et al., 2002). To our knowledge, there exists no relationship between EC and TDS developed in the literature for municipal wastewater.

Table 2 Typical secondary effluent wastewater characteristics

Characteristic	Colour (TCU, absorbance at 455 nm)	Hardness (mg $CaCO_3$/L)	Alkalinity (mg $CaCO_3$/L)	COD (mg COD/L)	TOC (mg TOC/L)	TS (mg TS/L)	TSS (mg TSS/L)	EC (µS/cm)
Secondary wastewater range	20-100[a, b]	40-320[c, d, f]	60-240[b, c, d, e]	28-50[a, d, f, g, h, i]	18-38[b, c, d, f, i]	270-810[h, i, j]	4-230[a, d, g, h, i, j, k, l]	470-1490[c, g, m, n]

COD: Chemical Oxygen Demand; TOC: Total Organic Carbon; TS: Total Solids; TSS: Total Suspended Solids; EC: Electrical Conductivity

a: (Panagiota Paraskeva, 2002); b: (Singh, 2012); c: (James et al., 2014); d: (Kang et al., 2003); e: (Khararjian et al., 1981); f: (Metcalfe & Eddy Inc. et al., 2002); g: (Mesut & 2013); h: (Acero et al., 2010a); i: (Harris et al., 1987); j: (Karthikeyan & Meyer, 2006); k:(Ragush et al., 2015); l: (Rusten et al., 1998); m: (Acero et al., 2010b); n:(Ghayenia et al., 1998)

2.3 Ozone treatment for removal of CECs from wastewater

Traditionally, ozone treatment of wastewater was used primarily for disinfection, and for removing colour and odour (Paraskeva et al., 1998), but in the past two decades this technology has been of increasing interest for removal of CECs (Gomes et al., 2017; Hua et al., 2006a; Margot et al., 2013; Paraskeva et al., 1998; Rodayan et al., 2014; Singh et al., 2015; Sundaram et al., 2014; Ternes et al., 2002). The following sections outline ozone reaction, kinetics and its reactivity with CECs, detail the main parameters affecting the efficacy of ozone treatment, and review ozone's ability to remove CECs and their associated toxicities.

2.3.1 Ozonation reaction, kinetics and reactivity with CEC moieties

Ozone oxidizes wastewater constituents via two reactions- directly, through ozone degradation, and indirectly, through side reaction by hydroxyl radicals (Nöthe et al., 2009; Paraskeva & Graham, 2002). In a series of complex reaction mechanisms, ozone decomposes into hydroxyl radicals; both ozone and the hydroxyl radical react via second order rates of reaction with CECs,

as seen in equation 2 (von Gunten, 2003):

$$\frac{-dS}{dt} = k_{O_3}[O_3][S] + k_{OH\cdot}[OH\cdot][S] \quad (2)$$

where S is the concentration of a CEC, O_3 is the concentration of dissolved ozone, $OH^{\cdot}$ is the concentration of dissolved hydroxyl radical, and $k_{O3,}$ k_{OH} are the second order reaction rate constants for ozone and the hydroxyl radical, respectively. Ozone reacts selectively with compounds that contain electron-rich moieties such as activated aromatic systems, carbon–carbon double bonds, and non-protonated primary, secondary and tertiary amines; (Huber et al., 2003a; Margot et al., 2013; Ternes et al., 2003). The hydroxyl radical however, reacts non-selectively with electron-rich moieties within a compound (Gomes et al., 2017). The hydroxyl radical is a stronger oxidant compared to ozone; their oxidation potentials are 2.80 eV and 2.08 eV respectively (Sharma et al., 2011). Consequently, hydroxyl radical rates of reaction with CECs are typically 3 to 10 orders of magnitude higher and approach diffusion control for compounds such as aromatic hydrocarbons, aliphatic alcohols, unsaturated compounds and formic acid (Hoigné & Bader, 1976). The half-life of the hydroxyl radical, however, is on the microsecond scale, thus its concentration never reaches above 10^{-12} M (Glaze & Kang, 1988).

Ozone dose can be calculated using three different approaches, seen in equations 3-5. The applied dose is the amount of ozone (mg) applied to the wastewater in the ozonation treatment unit where the ozone and wastewater come into contact. The transferred dose is the difference between the dose entering (applied) and leaving the wastewater in the ozone treatment unit. The consumed dose is the actual amount of ozone being consumed by the wastewater, and is an indication of material in the water reacting with ozone, measured by taking the difference between the transferred dose of the wastewater and a high purity water control. In addition, the consumed dose can be used to understand the efficiency of transfer of ozone into the wastewater. In this project, the transferred ozone dose is reported.

$$Applied\ dose = \int_0^t C_{in\ O_3} Q_{in\ O_3}\, dt \quad (3)$$

$$Transferred\ dose = \int_0^t \left(C_{in\ O_3} - C_{out\ O_3}\right) Q_{in\ O_3}\, dt \quad (4)$$

$$Consumed\ dose = \ Transferred\ dose_{wastewater} - Transferred\ dose_{pure\ water} \quad (5)$$

Where 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.

Substances with high ozone reactivity such as carbamazepine and 17ß estradiol (Ikehata et al., 2008) can be degraded rapidly even at low ozone doses; for example, twelve CECs with high ozone reactivity were removed to over 90% at an ozone dose of 2.3 mg/L (Margot et al., 2013). Removal of CECs with lower reactivity towards ozone were reported to be dependent on ozone dose and wastewater quality (specifically pH, hydroxyl radical and ozone demand; for more information see section 2.3.3) (Hollender et al., 2009; Margot et al., 2013). Substances with low ozone reactivity but high hydroxyl radical reactivity, such as atrazine, can be removed at low to moderate rates (Hollender et al., 2009; Margot et al., 2013). Second order reaction rate constants for ozone and the hydroxyl radical with the CECs in this project along with their degradability rank are shown in Table 3.

Table 3 Ozone and hydroxyl radical second order rate constants of CECs in wastewater, their degradability classifications and rates of removal of CECs using ozone

Compound	k_{03} ($M^{-1}s^{-1}$)	k_{0H} ($M^{-1}s^{-1}$)	Degradability rank (ozone; hydroxyl radical)	Maximum removal of CECs using ozone treatment (% removed)
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	96[a]; 100[b]; 93[c]; >92[d]; 99[e]
Trimethoprim	2.7 x 10^{5}[g, n]	6.9 ± 0.2 x 10^{9}[g]	Rapid; rapid	96[f]; 99[c]
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	>90[a]; 94[c]; 100[f]
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	60[a]; >98[d]; 97[c]
Venlafaxine	Not found in literature	8.46 x 10^{9}[r]; 8.15 ± 0.37 x 10^{9}[s]	N/A; rapid	75[c]
Naproxen	~2 x 10^{5}[c, n]	9.6 x 10^{9}[d]	Rapid; rapid	100[a]; 100[f]; 90[c]
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	100[a]; 100[f]
estrone	9.4 ± 2.7 x 10^{5}[u]	1.6 ± 0.88 x 10^{10}[u]	Rapid; rapid	100[a]; 93[f]
17β-estradiol	10^{6}[h]	*1.41 x 10^{10}[v]	Rapid; *rapid	100[a]
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	97[g] **
Atrazine	6[a, n]	3 x 10^{9}[a]	Slow; rapid	62[h]; 34[c]
MCPA (4-Chloro-2-methylphenoxyacetic acid)	4.4 ± 0.2 x 10^{5}[p]	*6.6 x 10^{9}[q]	Rapid; *rapid	100[i]; 75[h]
DEET (N,N-Diethyl-3-methylbenzamide)	0.126 ± 0.006[k]; <10[n]	4.95± 1.8 x 10^{9}[l]	Slow; rapid	79[a]; 62[h]
Triclosan	3.8 x 10^{7}[e, n]	*5.4 ± 0.3 x 10^{9}[f]; 9.6 x 10^{9}[m]	Rapid; rapid	100[a];100[j]

*Experiment was not conducted at pH 7; ** experiment was conducted in drinking water; 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.

References for second order rate constants: a: (Acero et al., 2000) pH 7, T = 20°C; b: (Wert et al., 2009) pH 7, T = 20°C; c: (Huber et al., 2005b); 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., 2003a) 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

References for maximum CEC removals using ozone: a: (EPA 2010); b: (Singh et al., 2015); c: (Margot et al., 2013); d: (Ternes et al., 2003); e: (Yargeau & Leclair, 2007); f: (Singh et al., 2015); g: (Huber et al., 2003b); h: (Hollender et al., 2009); i: (Gimeno et al., 2014); j: (Chen et al., 2012)

2.3.2 Effects of ozonation operating parameters on efficacy of treatment

The main parameters affecting the efficacy of ozone treatment are ozone solubility, pH, temperature, reactor design and the quality of the wastewater itself. The following discussion describes the main effects of these parameters on the ozone treatment of wastewater.

Ozone solubility and mass transfer: Ozone is a gas at ambient conditions and minimally soluble in water; at 20^{o}C, its solubility in water at 100% is 570 mg/L (Kinman & Rempel, 1975). Ozone concentrations used for applications in wastewater are typically below 14% by weight, which limits the mass transfer of ozone into water (EPA, 1999). Consequently, typical ozone concentrations in wastewater range from <0.1 mg/L to 1 mg/L. For ozone systems that have not been optimized for mass transfer, an increase in solubility will cause an increase in mass transfer and increase the efficacy of ozone treatment; major parameters affecting ozone solubility are pH and temperature.

pH: The concentration of both ozone and hydroxyl radicals is highly dependent on the pH of the wastewater. Under acidic conditions, ozone oxidation dominates, while under basic conditions, the production of the hydroxyl radical will dominate and therefore be the predominant oxidant (Hoigné & Bader, 1976). The removal rate of CECs whose pKa value is close to that of the wastewater will be particularly susceptible to changes in pH. For example, a pH change from 6.3 to 8 of a sample containing fluoroquinone antibiotics such as norfloxacin, ofloxacin and ciprofloxacin can increase the susceptibility of these compounds towards ozone attack by 1 to 2 orders of magnitude (Dodd, Buffle, & von Gunten, 2006).

Temperature: The solubility of ozone increases as the temperature of water is decreased; rates of reaction of ozone and the hydroxyl radical with CECs increase with temperature, thus these two effects work against each other (Nöthe et al., 2009; Yargeau & Leclair, 2007, 2008).

Formation of the hydroxyl radical: The formation of the hydroxyl radical during ozone treatment is largely due to the reaction of ozone with NOM or bicarbonate (measured as alkalinity) and, hence, will vary with wastewater composition (Margot et al., 2013). The effluent

composition can therefore be varied to influence hydroxyl radical formation, for example, basic pH adjustment, the application of ultraviolet (UV) light, or the additional of hydrogen peroxide (Buffle et al., 2006).

2.3.3 Ozone demand

Ozone and the hydroxyl radical reacts with various constituents within the wastewater; the amount of oxidizable material within the wastewater is called the ozone demand. The following wastewater constituents effect the ozone demand.

NOM: The oxidation of NOM leads to the formation of organic acids, aldehydes, aldoacids and ketoacids (EPA, 1999). NOM can be measured as dissolved organic carbon (DOC). NOM and can greatly contribute to the ozone demand by directly reacting with ozone or by scavenging of hydroxyl radicals, depending on their concentration and the wastewater pH (Staehelin & Hoigne, 1985; von Gunten, 2003).

Bicarbonate or carbonate ions: Measured as alkalinity, these ions scavenge hydroxyl radicals to form carbonate radicals (Glaze & Kang, 1988). Alkalinity can greatly contribute to the ozone demand depending on the concentration of bicarbonate and carbonate ions in wastewater (Staehelin & Hoigne, 1985).

Organic oxidation byproducts: Organic oxidation byproducts are typically biodegradable and can be measured as biodegradable dissolved organic carbon (BDOC).

Bromide ion: Oxidization of this ion produces brominated organics, bromamines, hypobromous acid, hypobromite and the bromate ion (EPA, 1999; Gomes et al., 2017)

CECs or other synthetic organic contaminants: Oxidation of these contaminants can lead to smaller, more polar and reactive species and ultimately to mineralization (Margot et al., 2013).

Other ions: This category includes ions such as nitrate, nitrite, chloride (Gomes et al., 2017)

2.3.4 Ozone treatment of wastewater and trends in CEC concentration and toxicity

As previously shown in our labs (Larcher & Yargeau, 2013b; Lassonde et al., 2015; Rodayan et al., 2014; Yargeau & Danylo, 2015) and by others (Huber et al., 2005b; Margot et al., 2013; Reungoat et al., 2010; Singh et al., 2015; Ternes et al., 2003), it is well known that when wastewater is treated with ozone, a reduction in, or mineralization of most CECs occurs. Furthermore, a reduction in toxicity for many different organisms and endpoints has been reported for wastewater treated with ozone (Macova et al., 2010; Petala et al., 2006; Quero-Pastor et al., 2014; Reungoat et al., 2012; Reungoat et al., 2010; Uslu & Balcioglu, 2008). However, the research is conflicting regarding toxicity removal, which is it is important to use bioassays to gauge the success of ozone treatment for the removal of toxicity. Many studies have confirmed that increasing the ozone dose decreases toxicity (Paraskeva et al., 1998; Petala et al., 2006; Quero-Pastor et al., 2014; Uslu & Balcioglu, 2008). Other studies, however, have reported an increase in toxicity (Luster-Teasley et al., 2005; Paraskeva et al., 1998; Petala et al., 2008; Stalter et al., 2010). In the literature, a decrease in toxicity corresponding to a decrease in CECs has also been reported for diverse toxicity tests (Gunnarsson et al., 2009; Quero-Pastor et al., 2014; Uslu & Balcioglu, 2008). The following discussion provides an overview of the literature connecting the trends in toxicity and CEC concentration with ozone treatment, outlines the occurrence and toxicity of TPs and DBPs in ozonated wastewater, and analyzes the findings in the literature.

Ozone, between doses of 3 mg O_3/L and 20 mg O_3/L, has been shown to degrade most CECs (Huber et al., 2005b; Margot et al., 2013; Reungoat et al., 2010; Singh et al., 2015; Ternes et al., 2003). The dose required for CEC removal is highly dependent on CEC reactivity with ozone and hydroxyl radicals (section 2.3.1) (Reungoat et al., 2012), and on the ozone demand (section 2.3.3) (Wert et al., 2011). At the beginning of ozone treatment, ozone and hydroxyl radicals are not readily available for the oxidation of CECs due to their scavenging by NOM, alkalinity and other compounds outlined in section 2.3.3. Scavenging can be overcome by increasing the ozone dose (Zwiener & Frimmel, 2000). In 2010, the EPA published a literature review data base for removal of CECs by different technologies from wastewater (EPA, 2010). They analyzed 22 lab-scale, 32 pilot scale, and 15 full scale ozone units in the USA, and concluded that ozone was an excellent technology for the removal of CECs. In 2014, Switzerland introduced the first full-scale

wastewater treatment plant in the world specifically designed for CEC removal using ozone treatment (Max & Nathalie, 2016). The plant exceeds the requirements of the legislation for CEC removal (an average of 80% of the 12 target CECs); it achieves an average removal of 84% of the target CECs which include: Amisulpride; Carbamazepine; Citalopram; Clarythromycin; Diclofenac; Hydrochlorothiazide; Metoprolol; Venlafaxine; Benzotriazole; Candesartan; Irbesartan; and Methylbenzotriazole. Table 3 outlines the maximal rates of removal of the target CECs in this project as reported in the literature. Clearly, there is widespread agreement that ozone is a good tool for the reduction of many CECs.

To further describe the relationship between toxicity, CEC concentration, and ozone-treated wastewater, some key studies examining bioassay-based toxicity are reported below. In 2013, Margot and colleagues completed a study at the Lausanne municipal WWTP in Switzerland where a pilot-scale ozone reactor was placed after secondary treatment. Out of 70 CECs analyzed after treatment by ozone doses from 2.3 mg O_3/L and 9 mg O_3/L, on average, 80% of the CECs were removed, some of which included: carbamazepine (90%); gemfibrozil (76%); naproxen (82%); ibuprofen (83%); trimethoprim (94%); venlafaxine (46%); 17β estradiol (>61%); estrone (>92%); atrazine (74%); DEET (66%). Although the production of bromate was confirmed, a decrease in toxicity was reported in all bioassays tested on the effluent, including the Combined Algae Test (*Pseudokirchneriella subcapitata* growth inhibition: 75%, photosynthesis inhibition: 82%), Early Life Fish Toxicity Test (mortality, swim-up, malformations, and abnormal behaviour: all significantly improved post ozonation), YES (estrogenic effects: 89%).

At the South Caboolture Water Reclamation Plant in Queensland, Australia, Reungoat and colleagues studied a treatment train consisting of six stages which included denitrification, pre-ozonation (at a dose of 0.1 mg O_3/mg DOC), coagulation/ flocculation/ dissolved air flotation and filtration, main ozonation (at a dose of 0.5 mg O_3/mg DOC), activated carbon filtration, and ozonation (at a dose of 0.3 mg O_3/mg DOC) for disinfection (Reungoat et al., 2010). After the overall treatment, 50 of the 54 micropollutants quantified in the influent were removed to below their limits of detection, indicating an average overall 90% concentration reduction. Bioassays revealed an overall decrease in toxicity: Microtox demonstrated a decrease in baseline toxicity of 78%, E-SCREEN presented a decrease in estrogenicity of 99%, CAFLUX revealed a decrease in

aryl hydrocarbon receptor response of 62%, acetylcholinesterase inhibition decreased by >90%, (neurotoxicity), the I-PAM Assay presented a decrease in photosynthetic inhibition of 75%, and umuC Assay revealed a decrease in genotoxicity of >93%. In this study, bioassays provided additional information that was not captured through chemical analysis alone. For example, although estrogenic chemicals were not detected in the secondary treatment effluent, an estrogenic response was detected through the E-SCREEN assay. In a subsequent study by Reungoat at three full-scale water reclamation plants in Australia, ozone at a range of 0.2 mg O_3/mg DOC to 0.8 O_3/mg DOC, followed by biologically activated carbon filtration, was implemented at the end of the secondary treatment (Reungoat et al., 2012). The 21 quantified CECs were removed by varying degrees, which authors reported was highly dependent on the structure of the CEC and the ozone dose applied. Post-ozonation bioassays were applied to observe toxicity removal: Microtox revealed that non-specific toxicity (cytotoxicity) was reduced by 31% to 39% while E-SCREEN indicated an estrogen reduction of 87%.

At the Neuss-South municipal WWTP in Germany, a pilot ozonation system was added after secondary treatment (Magdeburg et al., 2014). The chemical analysis revealed that approximately half of the compounds analyzed were removed by >90% at an ozone dose of 0.7 g O_3/g DOC. A >90% decrease in mutagenic effects was observed post-ozonation however, the Ames test for mutagenicity revealed a dose-dependent response with mutagenicity post-ozonation. Further, an increase in mortality of the Early Life Fish Toxicity Test was reported. Finally, the Comet Assay reported *in vivo* genotoxicity of fish erythrocytes.

At the El Ejido municipal WWTP in Spain, a pilot ozonation system was added after secondary wastewater treatment (Prieto-Rodríguez, 2013). At an ozone dose level of 3.4 mg O_3/L, 90% of the sum of the CEC concentration were removed, while at an ozone dose of 9.5 mg O_3/L, 98% had been removed. In addition, after a dose of 9.5 mg O_3/L, the ozone showed 0% inhibition in both the Biofix Lumi-10 Assay (*Vibrio fischeri*), and the Respirometry Assay (activated sludge).

In all of the above studies, CECs were removed during ozone treatment, while four of the five studies reported a corresponding decrease in toxicity for many different endpoints. Magdelburg reported an increase in mutagenicity, mortality, and genotoxicity. This difference in effluent

quality highlights the need to optimize ozone treatment for toxicity removal.

2.3.5 Formation of transformation products and disinfection byproducts in wastewater by ozone

It is well known that the oxidation of CECs produces TPs, while the oxidation of NOM (with or without bromide ions) produces DBPs. It is important to consider both ozone TPs and DBPs as they both have the potential to contribute to the toxicity of the overall wastewater mixture (Celiz et al., 2009). The following section will discuss occurrence, toxicity, and related issues surrounding TPs and DBPs.

TPs can be formed in wastewater through oxidation agents such as ozone (Rodayan et al., 2013), and some have been shown to influence toxicity (Rosal et al., 2009). In addition, some TPs are more recalcitrant than their parent CECs, and thus recalcitrance and toxicity are both important factors to consider for toxicity removal during ozone treatment (Margot et al., 2013). While many TPs have yet to be identified and their toxicities are largely unknown (Celiz et al., 2009), for some CECs such as sulfamethoxazole and atrazine, both TP occurrence and removal rates have been studied in WWTPs (Acero et al., 2000; Göbel et al., 2007). For example, it is well known that the ozonation of atrazine produces the primary TPs deethylatrazine (DEA), deisopropylatrazine (DIA), 4-acetamido-2-chloro-6-isopropylamino- s-triazine (CDIT) and 2-chloro-4-ethylimino-6-isopropylamino-s-triazine (ATRA-imine) (Acero et al., 2000; von Gunten, 2003). Furthermore, Acero and colleagues showed that when atrazine is exposed to ozone alone, the reaction pathway produces 4% DEA, 5% DIA, 24% CDIT, and 67% ATRA-imine, while when exposed to hydroxyl radicals alone, the reaction pathway produces 8% DEA, 25% DIA, 17% CDIT, and 50% ATRA-imine. In their batch experiment with initial ozone concentration of 10 mg/L, Acero and colleagues showed that over a reaction period of 120 mins these TPs are stable intermediates, indicating recalcitrance towards ozone. In the case of atrazine TPs, it was reported for different algae species that the photosynthetic EC50s for DEA and DIA were one order of magnitude smaller than that of ATZ thus, in this case, the TPs were less toxic compared to their parent compounds (Belfroid et al., 1998; Stratton, 1984). However, Rosal and colleagues demonstrated an increase toxicity associated with TP formation; toxicity in *Vibrio fischeri* and *Daphnia magna* was shown to increase when the CEC chlorofibric acid was exposed to ozone (Rosal et al., 2009). This was

attributed to a ring-opening reaction which produced TPs, including 4-chlorophenol, hydroquinone, 4-chlorocatechol, 2-hydroxylisobutyric acid, and three nono-aromatic compounds. In another example, Margot and colleagues demonstrated that TPs can be recalcitrant to ozone degradation compared to their parent CEC; while the CECs carbamazepine and sulfamethoxazole were both removed by >90%, their TPs 10, 11-dihydro-10, 11-dihydroxy carbamazepine and N-acetyl sulfamethoxazole were removed only by 47% and 50% respectively (Margot et al., 2013). Hydroxyl or acetyl groups on the reactive species change the electron density within the structure, slowing down the rate of reaction between the metabolite and ozone (Huber et al., 2005a). Although Margot and colleagues reported an overall decrease in toxicity for the effluent from the WWTP, no specific analysis of toxicity related to TPs was performed (Margot et al., 2013). Clearly the issue of toxicity and recalcitrance of TPs is complex and warrants further study.

Although DBPs will not specifically be explored in this PhD project, it is important to understand they can potentially contribute to the toxicity of wastewater during ozone treatment (Yan et al., 2014b). When ozone reacts with NOM, it forms a variety of organic and inorganic DBPs; well-known DBPs include aldehydes (formaldehyde, acetaldehyde, glyoxal, and methyl glyoxal), acids (oxalic, succinic, formic and acetic), and others such as pyruvic acid and hydrogen peroxide. If the bromide ion is present in the mixture with NOM, halogenated DBPs are known to be formed (Pan et al., 2017). The well-known brominated DBPs include the bromate ion, bromoform, brominated acetic acids, bromopicrin, and brominated acetonitriles (EPA, 1999). Nitrosamines are DBPs known to be formed during chlorination and chloramination of NOMs (Kristiana et al., 2013), but more recently, ozone-induced formation of nitrosamines in wastewater has been reported (Gerrity et al., 2015). Several DBPs have been shown to exhibit toxicity in ozonated wastewater effluents. For example, when Japanese medaka (*Oryzias latipes*) embryos were exposed to SEs treated with increasing doses of ozone (from 0.26 to 0.96 mg O_3/ mg DOC_o), total aldehyde was formed from 41.5 µg/L to 114.7 µg/L, which resulted in an increase in deformed larvae, from 2.2% to 4.1% (Yan et al., 2014b). More recently it has been shown that 13 new polar phenolic chlorinated and brominated DBPs are of concern because they have been shown to induce higher toxicity compared to well-known aliphatic DBPs (Pan et al., 2017); for example, higher developmental toxicity in the marine polychaete *Platyneris dumerilii* (Yang & Zhang, 2013). In addition, these DBPs can act as precursors for the formation of the EPA regulated DPBs (Pan et al., 2017).

2.4 Bioassays for monitoring the toxicity of municipal secondary effluent

A bioassay is an analysis that measures change in biological activity of a biological host exposed to a toxicant. *In vivo* bioassays use whole living organisms as the host while *in vitro* bioassays use plant or animal tissues, cells, or other biological derivatives. Bioassays are used to study adverse physiological effects on organisms, including endocrine disruption, photosynthetic inhibition, reproduction, or oxidative stress; these effects are referred to as MOAs. Toxicants that act non-specifically (non-specific MOAs) lead to narcosis, while those that act specifically (specific MOAs) at a specific target site produce a specific action. For example, cytotoxicity is classified as a non- specific MOA in that it does not target a specific metabolic pathway, but rather the whole cell, while estrogen disruption targets a specific pathway. Within a bioassay that targets a MOA, an endpoint is simply the point at which the researcher choses to measure within the MOA. For example, within cytotoxicity, the EC20 or EC50 endpoints, which in this case are the effective concentrations at which the population is decreased by 20% or 50%, respectively, can be measured. Endpoints can also be acute or chronic; for example, rate of growth has a chronic endpoint, because it can be measured over a period of time, while androgen disruption has an acute endpoint because it can be measured at one specific time point. The degree to which a toxicant will impact the organism is dependent on a number of factors, some of which include the toxicant concentration and potency, time of exposure of the toxicant to the host, the type, age, and health of the host, and the environmental conditions in which the host is exposed to the toxicant.

There is a pressing need for more rapid and sensitive tools for wastewater quality monitoring (Bellemare et al., 2006; Connon et al., 2012; Krewski et al., 2010b; Maruya et al., 2016); in the past decade, bioassays have become of increasing interest for this application (Escher et al., 2013; Magdeburg et al., 2014; Maruya et al., 2016; Sun et al., 2017). The focus of this PhD project will be *in vitro* analyses which are of growing importance as tools for wastewater monitoring (Macova et al., 2010; Mehinto Alvine et al., 2016; Mendonça et al., 2009; Rizzo, 2011). The following are examples of *in vitro* bioassays:

AREc32: The Antioxidant Response Element Cell line 32 bioassay assesses the induction of oxidative stress responses.

MTT: The tetrazolium dye bioassay assesses cytotoxicity.

YES: The Yeast Estrogenic Screening bioassay assesses estrogen disruption.

YAS: The Yeast Androgenic Screening bioassay assesses androgenic disruption.

Quiagen Cignal Finder 10-nuclear receptor reporter assay: This bioassay can quantify biological changes in the following nuclear receptors: Estrogen (ER), Androgen (AR), Perioxisome Proliferation-Activation (PPAR), Retinoic Acid (RAR), Vitamin D (VDR), Glucocorticoid (GR), Progesterone (PR), Retinoid X (RXR), Liver X (LXR), Hepatocyte Nuclear Factor 4 (HNF4A).

Microtox: Microtox is one of the most common bioassays for measuring non-specific aquatic cytotoxicity.

PEA: the plant efficiency analyzer bioassay, for measuring photosynthetic inhibition and other parameters (see section 2.4.3.4)

Combined Algae Test: This bioassay measures photosynthetic inhibition and other parameters (see section 2.4.3.4)

LuminoTox: The LuinoTox measures the photosynthetic inhibition; for more information, see section 2.4.3.

The following sections will describe the advantages of bioassays for municipal wastewater monitoring, the impact of mixture effects on toxicity and bioassays, and review the LuminoTox as a tool for this purpose.

2.4.1 Advantages of bioassays for wastewater quality monitoring

Bioassays have the following advantages; they can:

- Account for the overall effect of a complex mixture (Jia et al., 2015) rather than probe for only specific compounds as achieved by chemical analysis
- Characterize toxicity based on biological activity rather than chemical structure as achieved by more traditional methods of analysis (Couling et al., 2006; Russom et al., 1997)

- Be sensitive to the detection of chemicals at environmentally relevant concentrations (Leusch et al., 2010; Roberts et al., 2015)
- Analyze a wide variety of water matrices some of which include industrial, municipal and agricultural effluents (Christou et al., 2017; Čučak et al., 2017)
- Offer toxicological information for diverse endpoints (Babić et al., 2017; Maruya et al., 2016)
- Be used as a complement to *in vivo*, chemical or traditional wastewater quality markers for regulated municipal wastewater analysis (Gesuale et al., 2010; Leusch et al., 2014a)
- Lead to a better understanding of toxicity pathways and root causes of toxicity which are currently poorly understood (Krewski et al., 2010a)
- Be rapid compared to *in vivo* analysis: for example, the Fish Early Life Test for rainbow trout takes 40 days to complete (Stalter et al., 2010). Furthermore, bioassays take less time than it would take to define the composition of wastewater samples via chemical analysis (Jia et al., 2015)
- Be cost effective compared to chemical (Jia et al., 2015) and *in vivo* (Leusch & Snyder, 2015) analysis

Each bioassay has its particular strength, however, typically, one bioassay does not offer all of the advantages outlined above. The LuminoTox was selected for this project because it is easy to use, has a short time to result, and a relatively low cost, making it attractive for potential use at municipal WWTPs. For more detail, see section 2.4.3.

2.4.2 Impact of mixture effects on toxicity and bioassays

Mixture effects of CECs and their TPs in wastewater are largely unknown (Chen et al., 2013). In many cases, the toxicity of a mixture that consists of compounds with similar MOAs can be predicted using a concentration addition model (Altenburger et al., 2000; Backhaus et al., 2000), while mixtures of compounds with dissimilar MOAs exhibit independent action models of toxicity (Tang 2013). For traditional environmental pollutants, with the exception of herbicides that have a specific MOA (section 2.4.3.6), compounds in a mixture often elicit a baseline non-specific toxicity (narcosis) (Di Toro et al., 2000; Dyer et al., 2000; Escher et al., 2005) whereby individual

compounds contribute minimally towards toxicity via accumulation in the cellular membrane (Wezel & Opperhuizen, 1995).

The concentration addition and independent action models do not always predict the toxicity of a mixture, as more complex patterns such as synergism, or antagonism, which can be dose level, or dose ratio specific can arise for certain mixtures due to specific chemical interaction between species (Altenburger et al., 2013; Boltes et al., 2012; Jonker et al., 2005; Pape-Lindstrom & Lydy, 1997; Tang et al., 2013b). For example, Pape-Lindstrom and colleagues demonstrated that atrazine combined with binary and ternary combinations of organochloride insecticide, methoxychlor, and organophosphate insecticide methyl-parathion, and other organophosphates exhibited less than additive to marginally synergistic toxicity (measured as acute toxicity of *Chironomus tentans*) with certain combinations of these compounds (Pape-Lindstrom & Lydy, 1997). On the other hand, sometimes mixtures do exhibit concentration addition effects such as atrazine, dialuron and isoproturon (herbicides) and terbutyrn (algaecide) (Brust et al., 2001; Knauert et al., 2010; Nyström et al., 2002; Pape-Lindstrom & Lydy, 1997). Yang and colleagues assessed the mixture effects of different antibacterial agents on the growth inhibition of *Pseudokirchneriella subcapitata*; while some binary mixtures which included sulfamethoxazole or triclosan plus another antibacterial agent have an additive mixture effect, other combinations, such as sulfamethoxazole and trimethoprim exhibit synergistic effects (Yang et al., 2008). In a final example illustrating the potential complexity of mixture effects, a mixture of carbamazepine and chlofibrinic acid was shown to act alone by a non-specific MOA (non-polar narcosis); combined in the *Daphnia* acute immobilization test (using *Daphnia magna*) they exhibited a concentration addition effect, while combined in the algal growth inhibition test (using *Desmodesmus subspicatus*), they exhibited the independent action effect (Cleuvers, 2003).

There are several cautionary issues to consider when applying bioassays to complex mixtures in an ozonation process. There may exist a subtle effect created by the formation of new TPs, which could be shielded by the bulk of the mixture (Macova et al., 2010). Moreover, the bioassay may not always detect all effects from specific chemicals; this highlights the importance of combined chemical and bioanalytical analysis. For example, aldehydes and other electrophiles form beta blockers during ozonation (Benner & Ternes, 2009; Macova et al., 2010). The toxic

effect of these compounds may be insignificant compared to the bulk of the mixture and therefore their specific toxicity may not necessarily be captured by the suite of bioanalytical tools, but may require targeted chemical analysis.

2.4.3 LuminoTox

The LuminoTox was developed in 1999 in Quebec, Canada by Francois Bellemare and his two colleagues Lucie Lorrain and Nathalie Boucher (Doussantousse, 2014). This bioassay, which measures the photosynthetic inhibition of a sample, is of interest for monitoring of municipal SEs as it possesses all of the advantages of bioassays outlined in section 2.4.1, while being cost effective, rapid (3 hours maximum including setup, activation, exposure and cleanup), and sensitive to a variety of organic and inorganic pollutants (Bellemare et al., 2006; Gesuale et al., 2010; Mamindy-Pajany et al., 2011; Souza et al., 2013). Furthermore, given that it has been shown that CECs affect aquatic life (Baldwin et al., 2016; Bellemare et al., 2006; Brun et al., 2006; Feito et al., 2013; Khan et al., 2013; Lonappan et al., 2016; Osorio et al., 2016; Villeneuve et al., 2017), the LuminoTox is appropriate for monitoring the effect of CECs since it measures aquatic, non-specific toxicity with the MOA being photosynthetic inhibition, which gives a global measure of toxicity of a plant cell.

To date, there have been 17 peer-reviewed journal articles published on LuminoTox toxicity testing on a wide variety of water samples including sediments, certified reference material sediments spiked with a nanoparticle, mining wastewater, different types of filtered laboratory water or municipal wastewater spiked with one pollutant, and municipal wastewater (Angthararuk et al., 2015; Bellemare et al., 2006; Blaise et al., 2008; Burga Pérez et al., 2013; Chusaksri et al., 2010; De la Cruz et al., 2013; De Luca et al., 2013; Debenest et al., 2010; Dellamatrice et al., 2006; Dewez et al., 2007; Férard et al., 2015; Gesuale et al., 2010; Mamindy-Pajany et al., 2011; Manusadžianas et al., 2012; Perron et al., 2012; Souza et al., 2013; Toumi et al., 2013). Of these articles, there have only been three published on municipal SE (De Luca et al., 2013; Gesuale et al., 2010; Souza et al., 2013), one of which also includes monitoring of ozone treatment (Gesuale et al., 2010). The following section summarizes the theory of photosynthesis and details the light-dependent reactions; outlines the theory of chlorophyll a fluorescence including its traditional

bioassays and measurements compared to those of the LuminoTox; and discusses thylakoid and algal biosensors. In addition, the LuminoTox and its use as a tool to monitor advanced oxidation processes, including ozone treatment, are subsequently described. While there is no published literature on toxic interferences of SE characteristics in the LuminoTox , the information provided by the manufacturer for water samples will be described.

2.4.3.1 Photosynthesis as a mean to measure toxicity

Photosynthesis is essentially a series of reactions that enables photosynthetic organisms such as plant cells and algae to convert light energy, carbon dioxide and water into chemical energy stored as carbohydrate molecules, which can later be released to fuel the organisms' metabolism (with oxygen gas as a major byproduct). Photosynthesis can be divided into two phases: light-dependent and light-independent reactions. Light-dependent reactions happen within the thylakoid membrane of a plant cell. In this process, photons initiate a series of redox reactions which create a proton gradient across the thylakoid membrane that drives the production of adenosine triphosphate (ATP), and ultimately produces nicotinamide adenine dinucleotide phosphate (NADPH). The light-independent pathways occur in the stroma of the chloroplast (outside the thylakoid membrane). The enzyme Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) uses atmospheric carbon dioxide along with the nicotinamide adenine dinucleotide (NADH) to form three carbon sugars that are subsequently converted into sugar and starch molecules and stored in the plant cell. The metabolism of a plant cell is affected by the presence or absence of light (Pfannschmidt et al., 2009). Furthermore, it has been reported in numerous studies that in the presence of an inhibiting compound, toxicity pathways are affected differently in the light compared to in the dark (Krupa, 1999; Veeranjaneyulu et al., 1992). Thus in this project, it was important to explore toxicity when exposure is occurring under both conditions. Section 2.4.3.2 will focus on the light-depended reactions and particularly the reactions within PS II as they are relevant to sites of inhibition of organic and inorganic molecules and pertinent to the function of chlorophyll a fluorescence measurements within the LuminoTox.

2.4.3.2 Photosystem II and the electron transport chain of the light-dependent reactions

In the light-dependent reactions, an electron transport chain is mainly composed of a series of protein complexes in the following order: PS II; cytochrome b6f; PS I, ATP synthase, as well as different electron transporters throughout. PS I and PS II contain antenna complexes that are composed of a variety of pigments including chlorophyll. In the first step of the light-dependent reactions, photons are absorbed by the PS II antenna pigments and transferred to chlorophyll a molecules within the reaction centre. Through photo-induced charge separation, an electron is liberated from a chlorophyll molecule and through a series of steps, creates the radical pair: Antenna complex $P680^{+\cdot}$ and semiquinone radical ($Q_A^{-\cdot}$) (Renger & Renger, 2008). $P680^{+\cdot}$ provides the driving force for the oxidation of water, producing H^+ and oxygen gas while $Q_A^{-\cdot}$ is the reductant in a series of steps that, along with Q_A, coverts plastoquinone (PQ) to plastoquinol (PQH_2) (Renger, 2011). The latter two steps take place in the water-splitting complex at the Mn_4Ca^{2+} cluster, and at the Q_B binding site within the D1 protein of PS II respectively (Barber, 2012). Subsequently, PQ is released from the Q_B binding site and passes electrons to the cytochrome b6f and a series of proteins which ultimately end at the reaction centre of PS I. PS I absorbs light within its antenna pigments which further excites the electron. The electron is then passed to subsequent electron acceptors; catalyzed by ferredoxin $NADP^+$ reductase, ferridoxin (the final electron acceptor) converts $NADP^+$ to NADPH. Finally, via ATP synthase driven by passive diffusion of the H+ from the thylakoid lumen to the stroma, ADP is reduced to ATP.

2.4.3.3 Theory of chlorophyll a fluorescence and some of the conventional measurements

Chlorophyll a fluorescence is a well-known traditional indicator of plant cell health (Cao & Govindjee, 1990; Fai et al., 2007; Maxwell & Johnson, 2000; Zushi & Matsuzoe, 2017). In the light-dependent reactions, photons of light are absorbed by chlorophyll molecules which then transfer energy in one of three ways: the energy can be converted into photochemical energy which drives photosynthesis, it can be dissipated as heat, or it can be re-emitted as chlorophyll a fluorescence (Maxwell & Johnson, 2000). During photosynthesis, these three energy fluxes compete, thus, a decrease in efficiency of one results in the increase in yield of the others or vice versa.

When a thylakoid is transferred from dark to light, there is an increase in fluorescence which occurs due to a change in electron acceptors available for reaction; specifically Q_A cannot accept an electron until it has passed one onto Q_B; in this state, the reaction centre is said to be "closed" as Q_A is reduced. An increase in the efficiency of photochemical reactions or heat dissipation is called photochemical and non-photochemical quenching respectively, processes which take approximately 15 to 20 mins to achieve steady state (Maxwell & Johnson, 2000). Different light application techniques can be used to isolate photochemical and non-photochemical parameters to provide specific information about these energy fluxes within the plant cell (Maxwell & Johnson, 2000; Perron et al., 2012; Perron & Juneau, 2011); in this section, only photochemical parameters will be described as information regarding non-photochemical quenching is beyond the scope of this work.

Chlorophyll a fluorescence is sensitive to changes in temperature and NaCl content, so care should be taken to keep these parameters constant (Conrad et al., 1993; Morton et al., 2014; Murata et al., 2007; Neale & Melis, 1989). At room temperature, the chlorophyll a fluorescence emission spectra peaks just below 700 nm for PS II while PS I peaks just below 750 nm (Morton et al., 2014; Vilfan et al., 2016). Fluorescence emission spectra of chlorophyll a fluorescence technologies are typically measured at > 700 nm which is the case for the LuminoTox. (Bellemare et al., 2006; Conrad et al., 1993; Dewez et al., 2007).

In a photosynthetic organism in the absence of actinic light, reaction centres are in their oxidized states and are said to be "open". The traditional fluorescence measurement of the open reaction centre, called minimal or ground fluorescence (F_o), is measured by applying low intensity photons for a short period of time to a dark-adapted thylakoid. Upon exposure to a saturating light beam for a short time, the reaction centre becomes completely oxidized and is said to be "closed"; the fluorescence produced is traditionally called maximum fluorescence (F_m). Due to the short period of light exposure for all of the aforementioned fluorescence measurements (in the μsec range), there is no change in photochemical efficiency (Maxwell & Johnson, 2000). All of these measurements can be performed in the presence and absence of an inhibitor (Dewez et al., 2007; Perron et al., 2012). Section 2.4.3.4 discusses specific conventional bioassays that use these fluorescence measurements.

2.4.3.4 Conventional methods of analysis and comparison to the LuminoTox

Conventional, well-accepted technologies for evaluating photosynthetic performance include the Pulse Amplitude Modulated (PAM) fluorometry, used alone or in the Combined Algae Test (including name variations such as the Algae Inhibition, Combined Algae Assay, or Phytotoxicity Tests) and the Plant Efficiency Analyzer (PEA) (Conrad et al., 1993; Dewez et al., 2007; Escher et al., 2008b; Escher et al., 2011; Escher et al., 2006; Fai et al., 2007; Leusch et al., 2014b; Li et al., 2013; Macova et al., 2010; Margot et al., 2013; Mestankova et al., 2011; Neale et al., 2012; Sarafi et al., 2017; Tang et al., 2013a).

The Combined Algae Test, quantifies two endpoints: growth rate and photosynthetic inhibition (Dewez et al., 2007; Escher et al., 2008a; Mestankova et al., 2011). The photosynthetic inhibition test was the inspiration for the creation of the LuminoTox (Doussantousse, 2014). In the photosynthetic inhibition test, algae are exposed to contaminates for 24 hours and over the course of exposure, the quantum yield of PS II, the proportion of open reaction centres in PS II, and the maximum quantum yield of PS II are typically determined (see equations 6 - 8) at one or more time points by PAM fluorometry (Escher et al., 2008a; Mestankova et al., 2011). PAM fluorometry measures F_o, and F_m as described in section 2.4.3.3; the transient fluorescence (F_t) is also measured after a period of 15 min 20 mins of exposure to pulses of un-saturating light. Immediately after the measurement of F_t , F_m' and F_o' are then both measured by applying a saturating light and removing the actinic light respectively. A decrease in quantum yield indicates a toxic effect.

$$\varphi_{PS\,II} = (F_m' - F_t)/F_m' \quad Quantum\ yield\ of\ PS\ II \tag{6}$$

$$qP = (F_m' - F_t)/(F_m' - F_o') \quad Proportion\ of\ open\ reaction\ centres\ in\ PS\ II \tag{7}$$

$$F_v/F_m = (F_m - F_o)/(F_m) \quad Maximum\ quantum\ yield\ of\ PS\ II \tag{8}$$

The PEA provides information on the location of photosynthetic inhibition (mode of toxic action) within PS II and its kinetics (Perron et al., 2012; Strasserf & Srivastava, 1995). The PEA measures the F_o as described in section 2.4.3.3, but then a constant saturating light is applied until the

photosynthetic organism reaches F_m (over a period of under 1s). The instrument measures the fluorescence over the course of this light exposure and at regular intervals, and produces a graph that reflects the transport of electrons from the water to the plastoquinones. If the graph is plotted on a log scale, transitions O, J, I and P can be seen, which represent different reduction states of Q_A and Q_B. Different parameters such as the absorption flux (ABS), trapping flux (TP), electron transport flux (ET) and dissipation flux can be calculated. For definitions and formulas see: (Perron, 2011). A change in slope or amplitude of this graph indicates a toxic effect.

The main advantage that the LuminoTox has over the PAM and PEA technologies is its superior sensitivity (Dewez et al., 2007). The LuminoTox had the smallest EC50 value (greatest sensitivity) for atrazine by up to 141X and 137X compared to PAM fluorometry and the PEA respectively, and for copper up to 70X (for both the PEA and PAM fluorometry). A similar result was demonstrated by Perron and colleagues who reported that the LuminoTox had a higher inhibitory response compared to that of the PEA (Perron et al., 2012). Furthermore, the LuminoTox is a significantly shorter test compared to that of the Combined Algae Test. Although the traditional methods of analysis provide more information on photosynthetic performance, for municipal wastewater treatment, this may not be necessary or practical.

2.4.3.5 LuminoTox analyzer function and measurements

The LuminoTox analyzer comprises an excitation light at 470 nm and a photodiode protected by a HB700 filter (Lab Bell, 2006). The baseline fluorescence is monitored. A sample is subsequently subjected to a low intensity light of 20 µmol photons $m^{-2}s^{-1}$, triggered for 2 secs and the fluorescence is measured >700 nm (Bellemare et al., 2006), thus capturing the chlorophyll a fluorescence emitted by PS I and PS II. Following the low intensity light exposure, the sample is immediately subjected to a high intensity exposure of 500 µmol photons $m^{-2}s^{-1}$ triggered for 0.7 secs and the fluorescence is again measured over the same emission spectra. Within the instrument, the baseline fluorescence is subtracted from the fluorescence measurements. These readings are reported on the LuminoTox screen as F_1 (from the low intensity light exposure) and F_2 (from the high intensity light exposure) and are similar to the traditional F_o and F_m measurements respectively, as they represent the photosynthetic reaction centres in their fully reduced and oxidized states respectively (Bellemare et al., 2006). The photosynthetic yield and the % inhibition

can then be computed from the F_1 and F_2 readings and are seen in equations 6 - 8:

$$\varphi_{PS\ II\ sample} = \left(F_{2\ sample} - F_{1\ sample}\right)/F_{2\ average\ blank}\ \ Quantum\ yeild\ of\ PS\ II \quad (6)$$

$$\varphi_{PS\ II\ blank} = \left(F_{2\ blank} - F_{1\ blank}\right)/F_{2\ average\ blank}\ \ Quantum\ yeild\ of\ PS\ II \quad (7)$$

$$\%\ inhibition = \left(1 - {}^{\varphi_{PS\ II\ sample}}/_{\varphi_{PS\ II\ blank}}\right) \times 100\%\ \ Photosynthetic\ inhibition \quad (8)$$

2.4.3.6 Algae and thylakoids as biosensors

Many chlorophyll a fluorescence technologies use thylakoids and algae biosensors as they are more sensitive and have better reproducibility than whole plant biosensors (Bellemare et al., 2006; Clijsters & Van Assche, 1985; Dewez et al., 2007). The LuminoTox uses Stabilized Aqueous Photosynthetic Systems (SAPS) which are green algae and Photosynthetic Enzyme Complexes (PECs) which are spinach thylakoids. More specifically, SAPS I is the algae *chlamydomonas reinhardtii* and SAPS II is *chlorella vulgaris*. This section describes the MOAs of different pollutants on these biosensors and also outlines how the LuminoTox biosensors have traditionally been used.

Many different classes of pollutants have been shown to have an effect on photosynthesis. It is well known that herbicides such as tirazines, are direct photosynthetic inhibitors, specifically of the Q_B binding site within PS II (Fuerst & Norman, 1991; Muller et al., 2008; van Rensen, 1982). Specific to the LuminoTox, a study on fifteen pesticides with diverse MOAs revealed that PECs were more sensitive to herbicides than insecticides and suggested that this was related to the specific site of action within the photosynthetic structures (Chusaksri et al., 2010). The study noted that the herbicides atrazine, diuron, and ametryne were the most toxic compounds to the PECs. It is well accepted that Cd^{2+}, Hg^{2+}, Cu^{2+}, Pb^{2+} and Zn^{2+} affect the stability of the Mn_4Ca^{2+} cluster within the water splitting complex (Bernier & Carpentier, 1995; Bernier et al., 1993; Boucher & Carpentier, 1999b; Maksymiec & Baszyński, 1988; Rashid et al., 1994; Rashid & Popovic, 1990; Sabat, 1996). These metals were shown to compete or displace one or more of the native Ca^{2+}, Cl^- and Mn^{2+} ions which causes the release of extrinsic polypeptide chains and ultimately inhibit the oxidation of water and thus photosynthesis. More recently, it was demonstrated using the Plant Efficiency Analyzer (PEA) that endocrine disrupters 4-nonylphenol (5 mg/L), 4-octylphenol (5

mg/L) and 17ß-estradiol (4 mg/L) significantly disrupt the PS II energy fluxes in *chlamydomonas reinhardtii* and *Pseudokirchneriella subcapitata* (Perron & Juneau, 2011). Table 4 shows the sensitivity- the photosynthetic effect observed and the concentration at which the effect was elicited- of different biosensors (from the LuminoTox and other technologies) to selected organic and inorganic pollutants.

SAPS and PECs have often been used as complementary tools because they are sensitive to different types of contaminants. SAPS are used because they are more sensitive to organics such as phenolic compounds which are known to act at the end of the electron transport chain (Bellemare et al., 2006). However, when PECs are isolated, extrinsic proteins, ferredoxin, and CF_1 of ATP synthase are disrupted and therefore lose their sensitivity to these types of compounds (Bellemare et al., 2006; Environment Canada, 2005). PECs, on the other hand, can be used to detect metals after 10 mins which are not all immediately detected in SAPs at this exposure level (Bellemare et al., 2006; Macfie & Welbourn, 2000). Algae cells which possess intact cell walls are thought to have selective metal binding capabilities and can bind metal ions in negatively charged sites; metals, therefore, will not always alter the electron transport chain and affect the fluorescence emission (Macfie & Welbourn, 2000).

SAPS are sensitive to inorganic ammonia/ ammonium detection compared to PECs which are relatively insensitive (Bellemare et al., 2006). At a pH of 7, the ammonium ion is favoured while at a pH of 11, ammonia is the predominant species. While PECs only tolerate pH ranges between 6.5 to 7.8, SAPS can tolerate high pHs such as pH 11. The use of PECs and SAPS as complementary tools to separate non-ammonia and ammonia toxicity is illustrated in an experiment with influent and effluent wastewater samples (LBI Innovations Inc, 2014). In the influent, PECs detected toxicity at a pH of 7 while SAPs I did not, indicating that there are contaminants in the water to which PECs are sensitive. At a pH of 11, there was toxicity in the influent indicating the presence of ammonia. In the effluent, SAPs I again indicated toxicity at pH 11 while both SAPS and PECs at pH 7 elicited none. This indicates that the treatment failed to remove ammonia toxicity but more importantly, this combination of LuminoTox biosensors was able to differentiate between two different types of toxicity.

Table 4 Sensitivity of LuminoTox biosensors to different chemicals tested in literature

Pollutant	Pollutant Class	Biosensor	Effect on photosynthesis	Concentration (µg/L) at which effect was observed
Atrazine	Pesticide (Herbicide)	PECs (15 mins), SAPS I (10 mins)	Threshold effect (8-10% inhibition)	1.0, 0.7[a]
Diuron	Pesticide (Herbicide)	PECs (15 mins), SAPS I (10 mins)	Threshold effect (8-10% inhibition)	0.6, 0.5[a]
DEET	Pesticide (Insecticide)	*Chlorella protothecoides*[1]	EC50 (oxygen flux)	72,900[b]
Tetrabromobisphenol A	Flame retardant	SAPS I (15 mins)	IC50	17,300[c]
Carbamazepine	Pharmaceutical	SAPS I (15 mins)	Threshold effect (8-10% inhibition)	1000[d]
Sulfamethoxazole	Pharmaceutical	SAPS II (15 mins)	Threshold effect (8-10% inhibition)	10[a]
ß estradiol	Estrogen	*Chlamydomonas reinhardtii* SAPS I	Significant difference in the following ratios in the PEA test compared to those of the control: TR_0/ABS, ET_0/TR_0, DI_0/RC; Threshold effect (8-10% inhibition)	4000[e] 10[a]
4-nonylphenol	Endocrine disruptor	*Chlamydomonas reinhardtii*	Significant difference in the following ratios in the PEA test compared to those of the control: TR_0/ABS, ET_0/RC, ABS/RC, ET_0//RC, ET_0/TR_0, MO, DI_0/RC	5000[e]
Cu^{2+}	Heavy metal	PECs (15 mins), SAPS I (10 mins)	Threshold effect (8-10% inhibition)	20, >2,500[a]
Pb^{2+}	Heavy metal	PECs (15 mins), SAPS I (10 mins)	Threshold effect (8-10% inhibition)	0.6, 0.5[a]

1: Could not find LuminoTox biosensor therefore, another algae species was used. PECs: Photosynthetic enzyme complexes; SAPS: Stabilized Aqueous Photosynthetic Systems; SAPS are algae while PECs are spinach thylakoids; TR_0/ABS: Trapping probability or the maximum efficiency of PS II photochemistry; ET_0/RC: Electron transport rate in an active reaction center; ABS/RC: Effective antenna size of an active RC; ET_0//RC: Electron transport per active reaction centre; ET_0/TR_0: Electron transport probability; MO: Net rate of PS II closure ; DI_0/RC: Effective dissipation of an active reaction centre; a:(Bellemare et al., 2006); b:(Martinez et al., 2016); c:(Debenest et al., 2010); d:(Aquacion Inc., 2014); e:(Perron & Juneau, 2011)

2.4.3.7 Possible interferences of secondary effluent characteristics in the LumimnoTox

In all of the literature on the LuminoTox, there has been no exploration of toxic interferences related to specific municipal SE characteristics such as those found in section 2.2. Aquacion Inc. has established ranges of parameters for water samples including conductivity (0 μS to 3000 μS), colour (0 CU to 450 CU), hardness (4 mg/L $CaCO_3$ to 320 mg/L $CaCO_3$), turbidity (0 NTU to 350 NTU), TSS (0 mg/L to 500 mg/L), temperature (20 ^oC to 25 ^oC), and pH (6.8 to 7.8), however, no data or explanations are available to explain how these parameters were set (LBI Innovations Inc, 2014). Further, these ranges do not cover the expected values for some SE parameters and important SE characteristics such as TOC and COD have not been investigated.

2.4.3.8 Possible interferences due to suspended solids and fines in the LuminoTox

The presence of fines in natural freshwater sediment samples inducing toxicity has been demonstrated in several ecotoxicity tests (Péry et al., 2006) including the Microtox (Ringwood et al., 1997; Tay et al., 1998). The results from these studies prompted Environment Canada to establish guidelines for interpretation of sediment toxicity data in the MicroTox (Environment Canada, 2002). The LuminoTox Solid Phase Assay (Lum-SPA) and the MicroTox Solid Phase Assay (Mic-SPA) were also shown to be correlated with each other (Dellamatrice et al., 2006; Férard et al., 2015). Four peer-reviewed journal articles have dealt with LuminoTox toxicity testing of natural freshwater sediment samples containing fines (Burga Pérez et al., 2013; Dellamatrice et al., 2006; Férard et al., 2015; Mamindy-Pajany et al., 2011), three of which have demonstrated the toxic interference of fines in natural freshwater sediment samples (Burga Pérez et al., 2013; Dellamatrice et al., 2006; Férard et al., 2015).

Fines were defined as particles < 74 μm, < 45 μm (max filter pore size), and < 63 μm by Burga Pérez, Dellematrice, and Férard respectively (Burga Pérez et al., 2013; Dellamatrice et al., 2006; Férard et al., 2015). In the literature five different varriants on the LuminoTox were used for sediment sample testing:

- *Lum-SPA* (Burga Pérez et al., 2013; Dellamatrice et al., 2006; Férard et al., 2015): Natural freshwater sediment samples are mixed in a vertical rotator for 4 hours at 20 ^oC to

maximize the solubilization of hydrophobic contaminants and lower molecular weight hydrophobic chemicals. Subsequently, PECs and the samples are added together and exposed for 15 mins. The sample is then filtered using a column filter (pore size range of 15 to 45 μm) and the PECs and filtrate are read in the LuminoTox analyzer.

- *LuminoTox Leachate Phase Assay (Lum-LPA)* (Burga Pérez et al., 2013): Natural freshwater sediment samples are initially mixed using the same method as that of the Lum-SPA. The samples are then filtered using the same column filter as the Lum-SPA and the filtrate (which contains fines) and the PECs are then added togeather, exposed for 15 mins and read in the LuminoTox.
- *LuminoTox Centrifugate Leachate Phase Assay (Lum-cLPA)* (Burga Pérez et al., 2013): This assay is run in the same way as the Lum-LPA except that there is an extra centrifugation step (run at 3000 rpm, 15 mins) after the filtration and before the addition of PECs (which removes the fines).
- *LuminoTox Direct contact Assay (Lum-DCA)* (Burga Pérez et al., 2013): This assay is run in the same way as the SPA, except without the filtration step (the whole natural freshwater sediment samples and the PECs are measured in the LuminoTox).
- *LuminoTox Elurate Assay (Lum-ELU)* (Dellamatrice et al., 2006; Férard et al., 2015): Natural freshwater sediment samples are mixed in the same way as in that of the Lum-SPA, but then the samples are centrifuged (3000 rpm, 10 mins) and the eluate and the PECs are then added together, exposed for 15 mins and then read in the LuminoTox. This assay should be roughly equivalent to that of the Lum-cLPA (different centrifugation times were used).

While the Lum-SPA, Lum-DCA and Lum-LPA toxicity are dependent on grain size, the Lum-cLPA and the Lum-ELU are not dependent on the presence of fines which have been removed by centrifugation.

Dellematrice first showed a sharp decrease in the IC20 value in the LuminoTox Solid Phase Assay (Lum-SPA) with mixtures of kaolin (particle diameter > 4 μm) and silica sand (particle diameters between 125 μm and 250 μm) when the kaolin content was ≥ 20% (an inverse correlation was reported) (Dellamatrice, 2006). Subsequently, a significant inverse correlation was demonstrated

between IC50s and fines content (Burga Pérez et al., 2013; Férard et al., 2015), as well as between IC50s and organic matter content (Burga Pérez et al., 2013) in natural freshwater sediment samples using the Lum-SPA. Burga Perez and colleagues observed that the Lum- LPA always reported a higher toxicity than that of the Lum-SPA which was thought to be due to the presence of fines in the Lum-LPA (Burga Pérez et al., 2013). They demonstrated that in the natural freshwater sediment samples, the IC20s produced in the Lum-LPA compared to those of the Lum-cLPA was always greater, and the only difference between these two assays is the removal of fines by centrifugation in the Lum-cLPA. It was thought that the difference in IC20s was due to the characteristics of the fines which could also have bound pollutants. Earlier studies had shown that both kaolin and bentonite clays have the ability to adsorb ions such as copper (Veli & Alyüz, 2007; Yavuz et al., 2003). Subsequently, the Lum-LPA, Lum-SPA and Lum-DCA were run on samples containing kaolin or bentonite (Burga Pérez et al., 2013); it was determined that the inhibition of the Lum-DCA was significantly more than for the other two assays (when all of the fines remained in the sample). Furthermore, the Lum-DCA showed bentonite having greater inhibition compared to kaolin while the opposite was true in the Lum-SPA which was thought to be a difference in absorption of the two clays. Using the filtrates of the Lum-SPA tests, Burga Pérez and colleagues confirmed that PECs produced 2.5X more chlorophyll a fluorescence in the kaolin sample compared to that of the bentonite. This result was attributed to a difference in the adsorption of the clays, and could also have been due to a different composition, sedimentation rate and capacity to inhibit electron transport of the clays. Thus the main factors influencing toxicity of natural fresh water sediment samples are the characteristics of the clay particles themselves, and any bound pollutants. Recently, Férard and colleagues used both the Lum-SPA and the Lum-ELU as complementary bioassays for the assessment of toxicity due to the difference in dependency of fines (Férard et al., 2015).

To date, no studies have investigated the effect of fines and TSS present in wastewater on the use of LuminoTox. Since concentrations of TSS typically range from 4 mg/L to 230 mg/L in SE (see Table 2) and will include particles of the sizes that others have defined as fines (Burga Pérez et al., 2013; Dellamatrice et al., 2006; Ferard Jean et al., 2015), exploring the toxicity of particulate matter in wastewater is of interest.

2.4.4 The applicability of the LuminoTox to monitoring wastewater treatment technologies

There have only been three peer-reviewed articles published on municipal SE all of which focus on LuminoTox as a monitoring tool for different doses of oxidative treatments (De Luca et al., 2013; Gesuale et al., 2010; Souza et al., 2013). Of these articles, only one biosensor was explored by each author: De Luca - SAPS, Gersuale - PECs and Souza - SAPS biosensor (De Luca et al., 2013; Gesuale et al., 2010; Souza et al., 2013).

Souza and colleagues evaluated the toxicity removal of UV/H_2O_2 used for the disinfection of municipal SEs. They revealed that toxicity was significantly decreased, from over 90% to under 20%, with 50 mins of UV/H_2O_2 exposure. The data they presented however, may not be a completely accurate representation of the LuminoTox toxicity of the sample, as the alkalinity was 507 ± 24 mg/L HCO_3, and the pH was 7.9, both of which are above the acceptable range for LuminoTox. Exceeding the ranges can cause a falsely large inhibition.

De Luca and colleagues used LuminoTox to examine the toxicity removal of SEs spiked with atrazine after being exposed to UV-A and UV-C photo-Fenton treatments (De Luca et al, 2013). When the atrazine solution was exposed to UV-A for the first 20 mins, an initial decrease in toxicity was observed. From 20 mins to 45 mins, there was an increase in toxicity, after which the toxicity decreased until it reached an inhibition of 20% at 120 mins of exposure. Refinements in the LuminoTox data presented, indicating error bars, and a statistical analysis would be useful in determining if this trend (toxicity decrease followed by increase followed by decrease again) could be attributed to the sensitivity of LuminoTox as a monitoring tool. In addition, in order to optimize the photo-Fenton procedure, the pH of the wastewater sample DeLuca and colleagues used was adjusted to 2.8 and there was no indication in the methods section of pH was neutralized for the LuminoTox analysis. If the pH was not adjusted, there will be a significantly higher inhibition observed than if the LuminoTox protocol was followed. The trend identified by DeLuca and colleagues was exhibited in an ozone treatment train with the PSII inhibition I-PAM assay (Reungoat et al., 2010) and with Early Life Fish Toxicity Test (Magdeburg et al., 2014). If this is

what is occurring in De Luca and colleagues' research, the LuminoTox does appear to be a sensitive tool for monitoring toxicity changes in wastewater effluents.

Gersuale and colleagues exposed municipal SE to ozone, and reported a decrease in average LuminoTox inhibition and CECs, including pharmaceuticals and nonylphenol ethoxylates, with increasing ozone dose (Gesuale et al., 2010). In their paper however, it is difficult to conclude for certain if there is, in fact, a decrease in LuminoTox toxicity because the inhibition of their samples ranged from ± 5% and error bars ranged ~ ± 3-7%. As such, toxicities reported at different ozone doses could be statistically equivalent (t-tests were not reported) and could also be statistically equivalent to the blanks due to the low inhibition reported (blanks with error bars and related statistics were not shown). Clearly, more investigation needs to be conducted on the LuminoTox as a tool for monitoring wastewater treatment technologies such as ozone.

3. OBJECTIVES

The current limit of knowledge described in the literature review clearly demonstrates the need for further research on rapid and effective bioassays to monitor the elimination of CEC-related wastewater toxicity. As such, this PhD thesis work was designed to reach the following objectives:

1 To evaluate the applicability of the LuminoTox for use in the range of characteristics typical of those found in SE, including: TOC; COD; alkalinity; conductivity; hardness; colour; and TSS.
2 To evaluate a new algae biosensor called SAPS II (*chlamydomonas reinhardtii*) for use in wastewater applications.
3 To evaluate the sensitivity of the LuminoTox as a tool for the detection of CECs in wastewater matrices.
4 To investigate the possibility of using the LuminoTox for the monitoring of the removal of CECs and their associated toxicity during treatment of wastewater by ozonation.
5 To evaluate ozone for its capacity to reduce CEC-associated toxicity as measured by the LuminoTox.

4. MANUSCRIPT 1: SENSITIVITY OF THE LUMINOTOX TOOL TO MONITOR CONTAMINANTS OF EMERGING CONCERN IN MUNICIPAL SECONDARY WASTEWATER EFFLUENT

4.1 Preface

As countries around the world begin to develop regulations for limiting the discharge of CECs, there is an urgent need for wastewater quality monitoring tools such as bioassays; the LuminoTox has shown potential for this application. While the LuminoTox has been used for many different types of water analysis, there is limited information on its applicability for its use in wastewater, and in particular, in municipal secondary effluents. Further, the sensitivity of the LuminoTox using different biosensors and modes of exposure has not been broadly explored for CECs in wastewater. The work presented in this manuscript further addresses these issues and objectives 1 and 2 of this PhD project. The range of water characteristics published for the LuminoTox by LBI Innovations Inc is expanded. The characteristics typical of those found in SE have been studied, along with the sensitivity of the LuminoTox towards CECs in water, and in a variety of wastewater matrices.

Samples were collected from the SE of a WWTP and SWW was made in the lab. These samples were spiked with either a single CEC (atrazine), or a mixture of 14 CECs, which was used for studying the sensitivity of method of exposure for SAPS I and SAPS II. From the literature, and in-house analyses of wastewater from three different WWTPs, ranges of characteristics typical of those found in SE were developed, including TOC, COD, alkalinity, EC, hardness, and colour. A number of samples were made by adjusting SWW for different SE characteristics; these samples were used to explore toxic interferences in the LuminoTox for SAPS I, SAPS II and PECs. Finally, using literature values and the toxicity results from SE samples spiked with different CECs, the sensitivity of different MOAs was explored in the LuminoTox using SAPS II.

It was determined that either 30 mins light or 20 mins dark induced the most sensitive response for SAPS I and SAPS II, and the literature showed that these biosensors were more sensitive to organic compounds compared to the PECs. Furthermore, in most cases, the SE wastewater characteristics explored would not induce a toxic interference. The characteristics that did induce

toxicity should therefore, be taken in consideration when analyzing samples showing these characteristics, since interference from the matrix may hide the CECs' contribution to toxicity. This work expands the existing knowledge about sensitivity of LuminoTox to different MOAs; it was determined that CECs that act directly on photosynthesis by inhibiting the Q_B binding site within PS II are more sensitive than CECs that act in different locations within PS II or those that act indirectly on photosynthesis. Finally, chemical analysis of the SE confirmed that the presence of most native CECs in the effluent were below concentrations which induced a 20% inhibition response in the LuminoTox. The development of a sample pre-concentration method for increasing the sensitivity of the LuminoTox towards residual CECs in wastewater was recommended.

This manuscript was published in **Science of the Total Environment, Volume 598, 15 November 2017, Pages 1065–1075**.

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

Meghan Marshall, Marco Pineda, Viviane Yargeau[1]

Department of Chemical Engineering, McGill University, Montreal, Quebec, Canada H3A 0C5

Email addresses: meghan.marshall2@mail.mcgill.ca, marco.pinedacastro@mcgill.ca, vivane.yargeau@mcgill.ca

4.2 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

[1] Viviane Yargeau: corresponding author. Email address: vivane.yargeau@mcgill.ca. Tel.:+1 514 398 2273; fax: +1 514 398 6678.
Mailing address: 3610 University St. Department of Chemical Engineering, McGill University, Montreal, Quebec, Canada H3A 0C5

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; Principle Component Analysis: PCA; Q_B: Plastoquinone; PS II: Photosystem II

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 of 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.

Key words: bioassay, CEC**,** endocrine disruptor, pesticide, pharmaceutical, toxicity detection tool

4.3 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., 2006b; 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., 2010b). 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; 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, Gersuale 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.

4.4 Materials and methods

4.4.1 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 5 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 & Carpentier, 1999a; Hiriart-Baer et al., 2006; Rai et al., 1981; Rai et al., 1991; Waldemar & Tadeusz, 1988; Winner & Owen, 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 conditions. 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.

Table 5 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 455nm)	HACH 8025	Unadjusted	Low	8 ± 14	20	(Panagiota Paraskeva, 2002; Singh, 2012), WWTP A&C
		Added humic acid sodium salt to obtain 14 mg/L	High	234 ± 6	250	
Hardness (mg $CaCO_3$/L)	HACH 8030	1:2 dilution	Low	64 ± 8	40	(Kang et al., 2003; Kharajian et al., 1981) (James et al., 2014; Metcalfe & Eddy Inc. et al., 2002), WWTP A
		Adjusted recipe to 272 mg/L using magnesium sulphate heptahydrate	High	453 ± 49	320	
Alkalinity (mg $CaCO_3$/L)	HACH Drop Count Titration/Sulfuric Acid Method (Low- and High-range Tests)	1:3 dilution	Low	20 ± 5	30	(James et al., 2014; Kang et al., 2003; Kharajian et al., 1981; Singh, 2012), WWTP A
		Adjusted recipe to 314 mg/L using sodium bicarbonate	High	225 ± 20	250	

Table 5.. continued

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]
Conductivity (µS/cm)	HI 98311 Instruction manual	1:4 dilution	Low	9 ± 80	1	(James et al., 2014; Mesut & 2013), WWTP C
		Adjusted recipe to 165 mg/L using potassium phosphate dibasic trihydrate	High	1926 ± 80	1700	
Chemical oxygen demand (mg COD/L)	HACH 8000	1:38 dilution	Low	9 ± 0.1	1	(Kang et al., 2003; Mesut & 2013; Metcalfe & Eddy Inc. et al., 2002; Panagiota Paraskeva, 2002), WWTP B&C
		Adjusted recipe to 144 mg/L using peptone	High	94 ± 1	50	
Total organic carbon (mg TOC/L)	Adapted from 5310 B (Rice et al., 2012)	1:24 dilution	Low	5 ± 3	2	(James et al., 2014; Kang et al., 2003; Metcalfe & Eddy Inc. et al., 2002; Singh, 2012), WWTP B&C
		Adjusted recipe to 134 mg/L using peptone	High	193 ± 2	200	

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 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).

4.4.2 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 4.4.1). A mix of 14 CECs (listed in Table 6 along with their 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 1,000 mg/L of each of the 14 CECs (the CECs listed in Table 6 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) and diuron, also listed in Table 6, 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.

Table 6 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-ethinylestradiol-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

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.

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.

4.4.3 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 3,500 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 9,000 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 x 2.1 mm ID; 1.8 μm) was used prior to the analytical column (50 mm x 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 30ºC 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 positive 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 7). 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 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.

Table 7 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., 2006b)
			0.344 ± 0.081	SE	
	Gemfibrozil	2.7 ± 2.2	0.078 ± 0.028	SE	(Hua et al., 2006b; 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., 2006b; 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., 2006b; 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., 2006b; 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	

Table 7 continued

Type	Compound	Experimental	Literature		
		Concentration in SE (µg/L)	**Maximum concentration reported in municipal effluents examined (µg/L)**	**Municipal effluents (number and type)**	**References**
	Estrone	**2.4 ± 1.0**	**0.038** **0.1**	**12 WWTPs including SE and L** **18 WWTPs including PE, SE, TE and L**	**(Lishman et al., 2006; Servos et al., 2005)**
	17β-estradiol	3.0 ± 0.9	0.1 0.016	4 WWTPs including SE and TE 18 WWTPs including PE, SE, TE and L	(Metcalfe et al., 2013; Servos et al., 2005)
	17α -ethinylestradiol	11.0 ± 6.2	0.00763 ± 0.00301 0.017	TE 5 SE WWTPs	(Cicek et al., 2007; Fernandez et al., 2007)
Pesticides	Atrazine	< LOQ	0.055 0.175	L SE	(Carlson et al., 2013; Hua et al., 2006b)
	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 0.324	SE 12 WWTPs including SE and L	(Buth et al., 2011; Lishman et al., 2006)

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.

4.4.4 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-B01-3528-0.25M) 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 sec 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.

4.4.5 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

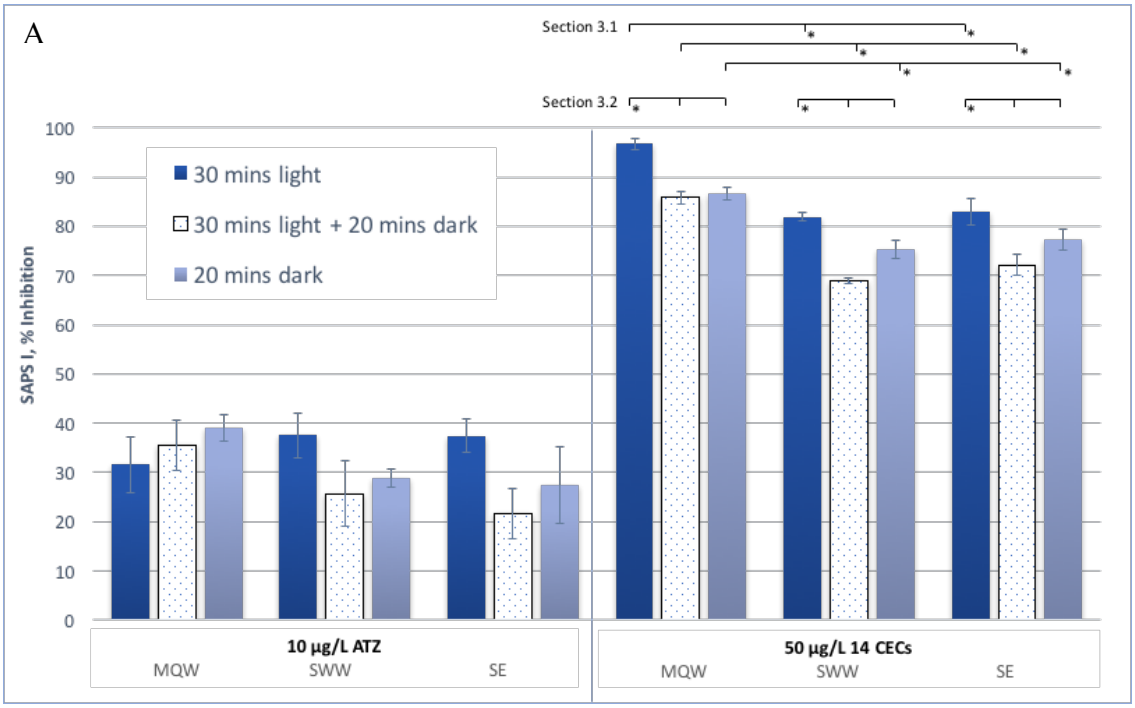
of the SWW samples exhibiting different characteristics typical of those found in SE had inhibition significantly different than MQW blanks.

4.5 Results and discussion

4.5.1 Effect of matrices on inhibition

Figure 2 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 4.5.6). 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 & 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 natural organic matter (NOM) in the synthetic and real wastewater. Other studies have indeed reported that metal cations (Brown & Markich, 2000) and organic contaminants (including CECs) (Landrum et al., 1987) can partition or form complexes with the NOM, 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.



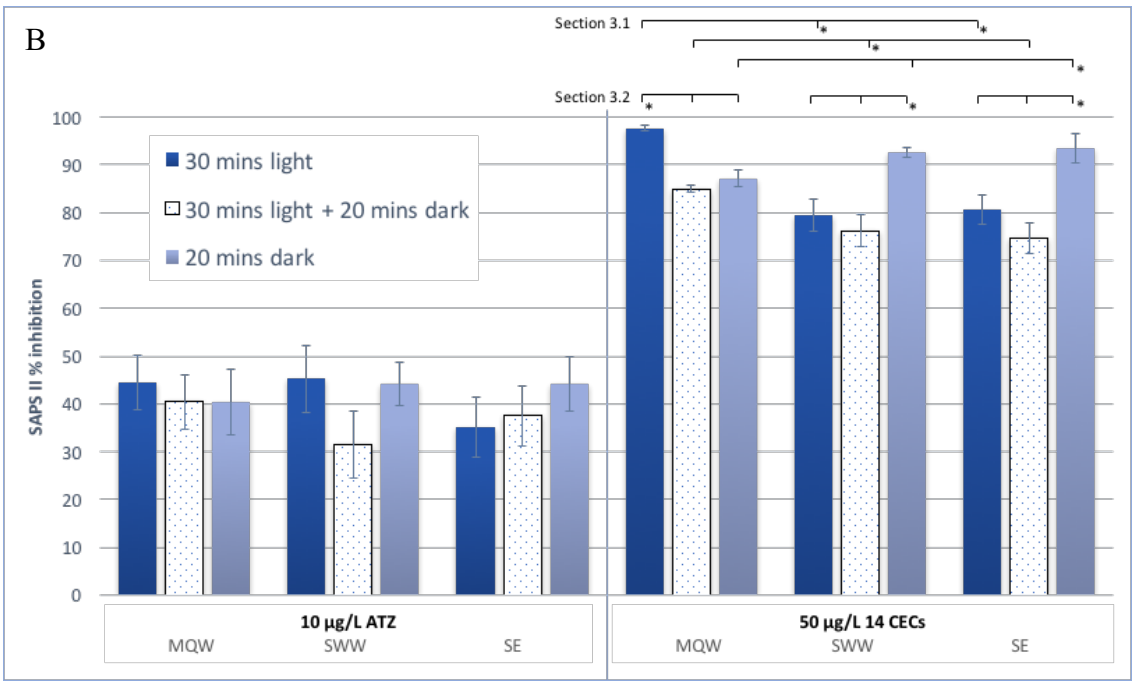


Figure 2 Measurements of inhibition in SE using different methods of exposure for different matrices and biosensors. A) SAPS I; B) SAPS II. The error bars represent one standard deviation, n=3. * $p < 0.05$.

4.5.2 Sensitivity associated with 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 minutes 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.

4.5.3 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 Figure 3. 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.

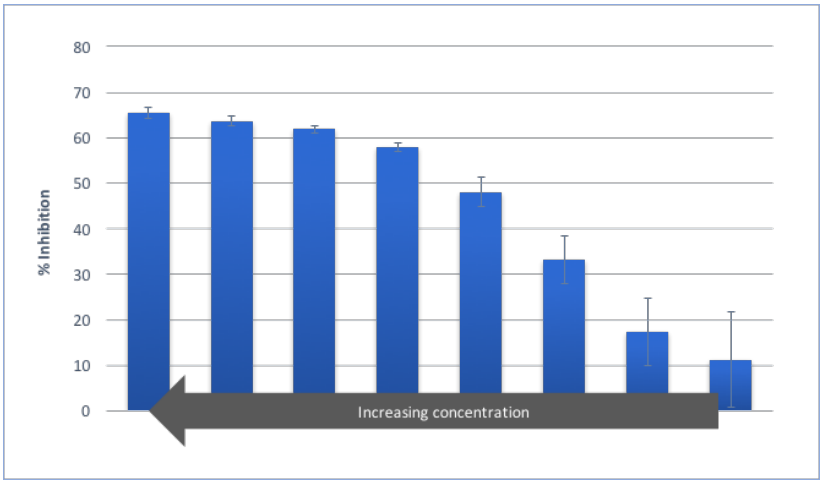


Figure 3 Increasing variability of the measured inhibition observed in the low range of inhibition for PECs. Error bars represent on standard deviation, n=3.

4.5.4 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. Figure 4 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 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 & 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 & 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 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.

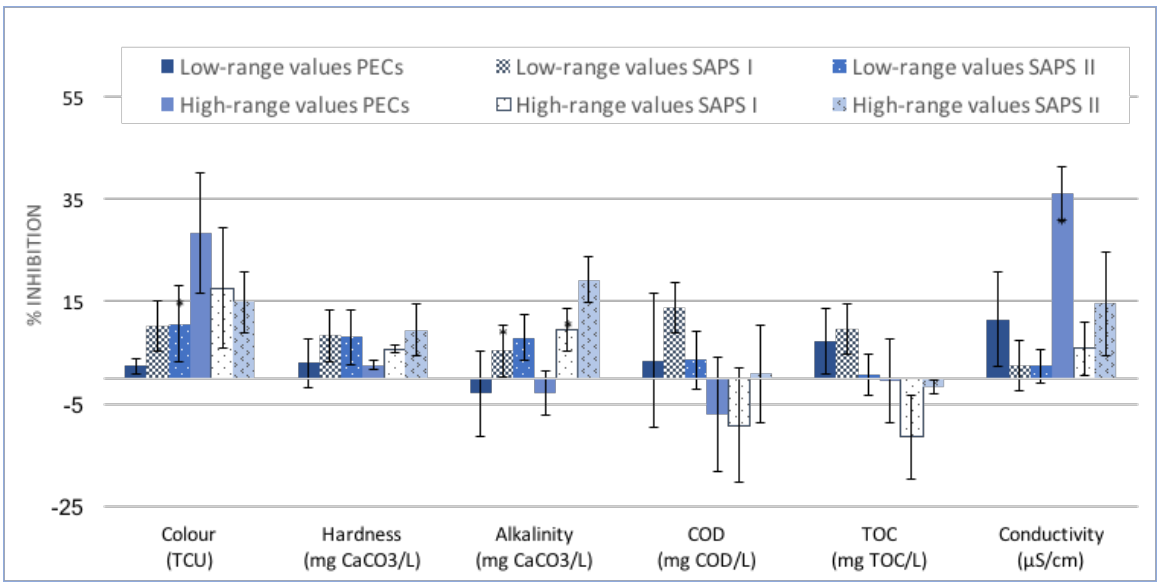


Figure 4 PECs, SAPS I and SAPS II inhibition of SWW samples having different secondary effluent characteristics. COD: Chemical Oxygen Demand; TOC: Total Organic Carbon. Sample exposure: 30 min light. Error bars represent one standard deviation, n=3. * p <0.05, compared to blanks not shown.

4.5.5 PCA on samples with different characteristics and biosensors

PCA was performed on the high-range inhibition values in Figure 5 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.

Figure 5 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 Figure 5, 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, Figure 5 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.

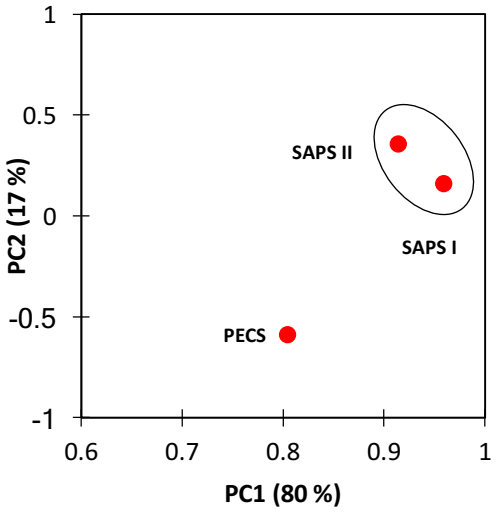


Figure 5 Scatter plot of the biosensor's principle component loading (PC1 and PC2). The variables that have been circled behave similarly. Variables are the biosensors, and observations are the high-range characteristics. PC1 and PC2 principle components comprise 97% of the variance.

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 and colleagues (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.

4.5.6 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 Figure 6. 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 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 vulgar*is in the Rebound 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 Rebound 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 15 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 ß-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 & Escher, 2011). Furthermore, Aquacion Inc. 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.

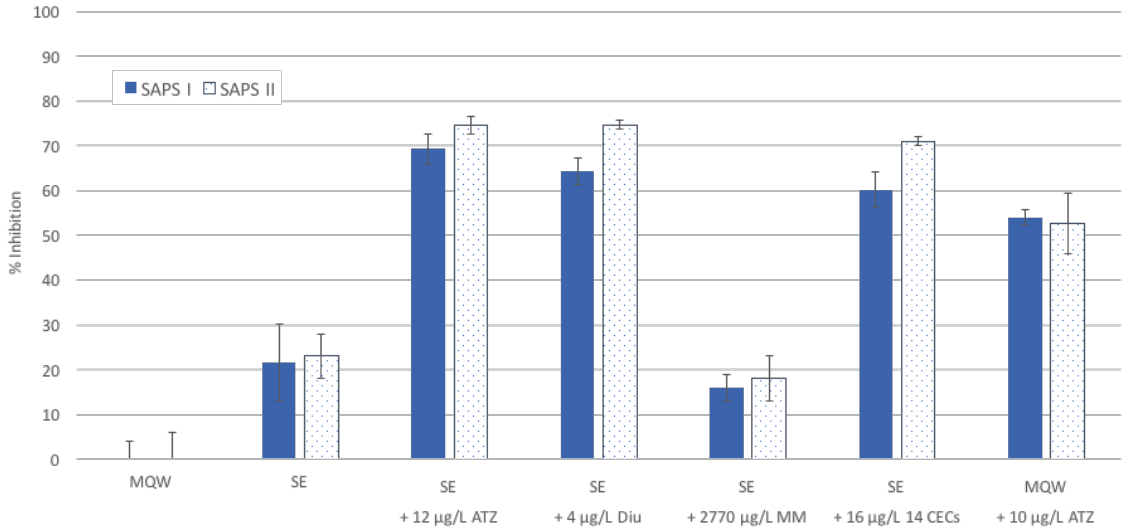


Figure 6 Sensitivity of the LuminoTox to different CECs with diverse modes of action (MOAs) present in SE. Exposure method: 30 min light. MM: metsulfuron methyl; ATZ: Atrazine. Error bars represent one standard deviation, n=3.

4.5.7 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 SE sample was performed to determine the concentration of the 14 target CECs and results are reported in Table 7. 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 6. The values obtained were comparable to data in the literature (Table 7).

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 7 indicated that 17α-ethinylestradiol and DEET were present at concentration ~2X 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 (~5X and ~4X 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 (Figure 4). 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.

4.6 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 mins 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.

4.7 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.

4.8 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.

5. MANUSCRIPT 2: IMPACT OF SUSPENDED SOLIDS ON THE USE OF LUMINOTOX TO DETECT TOXICITY OF MICROPOLLUTANTS

5.1 Preface

In the previous manuscript, the range of characteristics typical of those found in SE, including TOC, COD, alkalinity, EC, hardness, and colour were studied. This manuscript expands on this range of characteristics by exploring TSS which was of particular interest as other researchers have found that fines in freshwater natural sediments induced toxicity. This manuscript also addresses the recommendation of the previous manuscript to develop a sample pre-concentration method to increase the sensitivity of residual CECs in SE to the LuminoTox. This manuscript addresses objectives 1 and 2 of this PhD project.

Samples were made containing different concentrations of TS (recovered from the secondary clarifier of WWTP A) and spiked into SWW. Half of each sample was filtered and both the filtrate (with no TSS) and the TS samples were tested using SAPS I and SAPS II in the LuminoTox. For this experiment, chemical analysis was performed on the target CECs in both the TS samples and their filtrates in order to confirm the concentration of CECs in the samples. Using a particle size distribution analysis, the TS samples were also compared to the SE from WWTP A in order to determine if the particle sizes in the TS samples would be a good model for the distribution found in SE. In another experiment to assess the sensitivity of the LuminoTox to detect changes in atrazine concentrations in the presence of TS, atrazine was added at different concentrations to two different TS levels. Finally, a pre-concentration method using lyophilization was tested on samples with low levels of CECs spiked into SE.

It was determined that the TDS and not the TSS within the TS samples was responsible for inducing toxicity and that the LuminoTox could not detect changes in atrazine below 4 μg/L. Since native CECs in SE are typically below this concentration, this highlighted that sample pre-concentration was needed. Sample pre-concentration enabled toxicity that was not previously detected in the LuminoTox to illicit an inhibition in the mid-to high range. There were some limitations, however, with the method which involved the masking of toxicity by the concentrated

SE. Overall, the LuminoTox was shown to be a good tool for use in wastewater, however if the intent is specifically to determine the toxicity of residual CECs, significant method optimization is needed.

This manuscript was accepted in **Archives of Environmental Chemistry and Toxicology in October 2017**.

Impact of suspended solids on the use of LuminoTox to detect toxicity of micropollutants

Meghan Marshall, Marco Pineda, Viviane Yargeau[2]
Department of Chemical Engineering, McGill University, Montreal, Quebec, Canada H3A 0C5

5.2 Abstract

There is an increasing need for tools to monitor toxicity of contaminants of emerging concern (CECs) in wastewater. The purpose of this work was to assess interferences in the presence of total solids (TS) and total suspended solids (TSS) in the LuminoTox at concentrations typical of those found in municipal secondary effluent (SE), and to evaluate a simple sample enrichment method for increased CEC sensitivity. 4 µg/L or 10 µg/L atrazine in different TS concentrations and in corresponding filtrates (TSS removed) exhibited equivalent toxicities; since the only difference between these two fractions is TSS, this result demonstrates that, generally, this fraction does not induce toxicity nor interfere with the bioassay. At constant medium-low TS, the LuminoTox was able to detect the presence of 4 µg/L of atrazine but could not distinguish the change in atrazine concentration between 4 µg/L and 6 µg/L. No inhibition was observed in presence of a mix of 15 CECs each at 0.23 µg/L. However, upon sample enrichment by lyophilization (50X), an inhibition

[2] Viviane Yargeau: corresponding author. Email address: vivane.yargeau@mcgill.ca. Tel.:+1 514 398 2273; fax: +1 514 398 6678, ORCID: 0000-0002-7332-276X
Mailing address: 3610 University St. Department of Chemical Engineering, McGill University, Montreal, Quebec, Canada H3A 0C5

Abbreviations: SAPS: Stabilized Aqueous Photosynthetic Systems; CECs: Contaminants of Emerging Concern; SE: Secondary Effluent; SWW: Synthetic Wastewater; MQW: Milli Q water; ATZ: Atrazine; Φ: Photosystem II; OM: organic matter; HAs: Humic acids; PSD: Particle size distribution; Lum-SPA: LuminoTox Solid Phase Assay; Lum-LPA: LuminoTox Leachate Phase Assay; Lum-DCA: LuminoTox Direct Contact Assay.

of 81 ± 3%, was observed while the enriched SE alone (not spiked with CECs) led to an inhibition of 49 ± 1%, indicating the detection of the CEC contribution to toxicity after sample pre-concentration. The LuminoTox is a promising tool for monitoring SE, however, if the intent is to detect CECs, enrichment method optimization is required.

5.3 Acknowledgements

The authors acknowledge and thank the staff of each WWTP for their cooperation and help during sampling campaigns. We thank Marco Pineda for his assistance with chemical analysis of the target CECs and Andrew Golsztajn and Ranjan Roy for their help with the Particle Size Distribution Analysis. The authors thank the McGill Engineering Doctoral Award for supporting Meghan Marshall. A research grant was provided to Viviane Yargeau (PI) from the Natural Sciences and Engineering Research Council (NSERC) of Canada through the Discovery Grant Program (RGPIN/04635-2015).

Key words: total suspended solids, sample enrichment, contaminants of emerging concern, LuminoTox, *chlamydomonas reinhardtii*, *chlorella vulgaris*

5.4 Introduction

Wastewater treatment plants (WWTPs) were not traditionally designed to remove contaminants of emerging concern (CECs) from wastewater, resulting in many CECs being only partially removed (Rojas et al., 2013; Uslu et al., 2013). Consequently, CECs including herbicides, insecticides, endocrine disrupters, antibiotics, and prescription pharmaceuticals end up being released into the environment and are present at concentrations from parts per trillion to parts per billion (Carlson et al., 2013; Jasinska et al., 2015a; Lishman et al., 2006; Metcalfe et al., 2010; Miao et al., 2004; Uslu et al., 2013). CECs are of concern as they have been reported to induce toxicity in aquatic life, at environmentally relevant concentrations (Lonappan et al., 2016; Osorio et al., 2016; Uslu et al., 2013). There is a need for rapid, sensitive and inexpensive tools to monitor the toxicity of CECs present in wastewater (Connon et al., 2012; Krewski et al., 2010b; Maruya et al., 2016). The LuminoTox was assessed for its sensitivity to monitor CECs in secondary effluent (SE) and was shown to have potential for this purpose (Marshall & Yargeau, 2017).

The LuminoTox reports non-specific toxicity as photosynthetic inhibition. The LuminoTox is a programmed luminometer which determines the relative photosynthetic efficiency of Photosystem II (Φ) of different biosensors, including *chlorella vulgaris* (SAPS I); and *chlamidomonas reinhardtii* (SAPS II), when exposed to a sample. Different pollutants, including CECs, can bind specific sites within the biosensor's thylakoids which cause interferences within the photosynthetic electron transport chain (Conrad et al., 1993; Macedo et al., 2008; Maksymiec & Baszyński, 1988). This results in an altered chlorophyll a fluorescence emitted by Photosystems I and II which is measured by the LuminoTox. The Φ of a blank and sample of interest are used to determine the percentage inhibitory response of a sample.

In our previous work, we investigated the applicability of the LuminoTox tool in a range of five characteristics typical of municipal SE including TOC, COD, alkalinity, conductivity and hardness (Marshall & Yargeau, 2017). It was confirmed that, in most cases, the LuminoTox provides reliable results in the range of conditions representative of SE, but the impact of the presence of total solids (TS) considering both total suspended solids (TSS) and total dissolved solids (TDS) was not explored. Investigating the impact of TSS is of particular interest considering that in other studies investigating natural freshwater sediment toxicity and fines, defined as particles < 74 μm

(Burga Pérez), < 45 μm (max filter pore size, Dellematrice) and < 63 μm (Férard) were shown to contribute to the toxicity as measured by the LuminoTox (Burga Pérez et al., 2013; Dellamatrice et al., 2006; Férard et al., 2015). Toxicity caused by fines in natural freshwater sediments has also been reported in different bioassays including the Microtox Solid Phase Assay (M-SPA) (Péry et al., 2006; Ringwood et al., 1997; Tay et al., 1998) which prompted Environment Canada to establish of guidelines for the M-SPA (Environment Canada, 2002).

Our previous work also indicated the need for sample enrichment in order to improve the sensitivity of the LuminoTox to CECs for their detection at concentrations relevant to wastewater effluents (Marshall & Yargeau, 2017). Bioassay sample enrichment is most commonly achieved using solid phase extraction (SPE) (Prasse et al., 2015; Stalter et al., 2016; Yeh et al., 2014) and liquid-liquid extraction (LLE) (Neale et al., 2012; Pan et al., 2014; Plewa et al., 2012), while other methods include reverse osmosis concentration (Speth et al., 2008), purge and trap methods (Stalter et al., 2016), and passive sampling (Shaw et al., 2009). All of the aforementioned methods, however, may not be practical for the purposes of monitoring in municipal WWTPs as they require significant operator skill, require long sample preparation and analysis time and, in many cases, are costly. Lyophilization, another technique used for bioassay sample enrichment (Prasse et al., 2015), is of interest for the pre-concentration of CECs in wastewater due its method simplicity and ability to preserve delicate compounds and enrich samples non-selectively.

The objectives of this study were to determine if (1) the LuminoTox can be used to monitor atrazine in a range of TSS concentrations typical of those in municipal SEs; (2) TSS or TDS contribute more to toxicity, if any contribution is observed; (3) the LuminoTox is sensitive to changes in atrazine concentration at constant TS, and (4) lyophilization can be used as a simple SE sample enrichment method to increase the sensitivity of the LuminoTox for the detection of CECs at concentrations relevant to municipal SE.

5.5 Materials and methods

5.5.1 Overview

In all experiments exploring TS, samples were made using a synthetic wastewater (SWW) matrix, as it provided a controlled environment which allowed for the adjustment of wastewater composition achieved by spiking TS recovered from wastewater collected from the inlet to the secondary clarifier of WWTP A (see section 5.5.3). In the sample enrichment study, real SE was used in order to understand if sample enrichment of this wastewater was possible; samples were made by spiking a 14 CECs mix into SE from WWTP A (SE A).

5.5.2 LuminoTox: technology justification and protocol of use

The intent of the manufacturer of the LuminoTox, Aquacion Inc. was to eventually use the assay as an online monitoring tool for wastewater treatment plants with the intent of monitoring CECs. In this work, we further expand on our previous work (Marshall & Yargeau, 2017) in order to develop the tool for this use. SAPS I (product # LBLP15AA-L), SAPS II (product # LBLP16AA-L), and atrazine standards were SAPS I (product # LBLP15AA-L), SAPS II (product # LBLP16AA-L), and atrazine standards were obtained from Aquacion Inc (Montreal, Canada). SAPS were activated for 90 minutes prior to sample testing using a BAZZ lighting system (DC 12 V, 1.2 W, model # MK-B01-3528-0.25M) as per the manufacturer's instructions. 2 mL of each sample were added to a disposable borosilicate glass tube (Fisher Scientific, Fair Lawn, New Jersey). 100 µL of SAPS was subsequently added to each sample, in 30 second intervals. Samples were exposed to 30 minutes in the light. One at a time and in 30 second intervals, each sample was poured into a Fisherbrand disposable cuvette (Fisher Scientific, Fair Lawn, New Jersey), inserted in the LuminoTox Analyzer (Model LBLX01AA) and analyzed using the pesticide toxicity setting (for SAPS). F1 and F2 readings were recorded. For each experiment, a Milli Q water blank and a 10 µg/L atrazine in Milli Q water control were run to ensure that they pass the manufacturers specifications, which are a 35% to 45% average inhibition for the atrazine control and all F2 readings for the blank > 500,000. The photosynthetic efficiency and inhibition were computed.

5.5.3 Sampling sites for SE, sample collection and storage

WWTP A, with an average daily flow of 38,000 m^3 and serving a population of 95,000, was selected as a source of wastewater for the present study. The influent of the WWTP is composed of approximately 60% domestic wastewater and 40% of commercial sources. Wastewater samples were collected from the inlet and outlet of the secondary clarifier, immediately put on ice and frozen at -20^{o}C within 24h. Samples were thawed at room temperature prior to testing with the LuminoTox.

5.5.4 Preparation and characterization of samples of SWW spiked with recovered TS from wastewater

5.5.4.1 Synthetic wastewater recipe

Synthetic wastewater (SWW) was made as previously outlined (Marshall & Yargeau, 2017) as a matrix for the TS samples. Chemicals in the SWW include: 95+% sodium chloride granular (Fisher Scientific, Fair Lawn, New Jersey); magnesium sulphate anhydrous (AquaPhoenix Scientific Inc., Nazarath, Pennsylvania); 98+% urea (Fisher Science Education, Fair Lawn, New Jersey); 98+% calcium sulphate dehydrate; 99+% sodium bicarbonate, (Acros Organics, Fair Lawn, New Jersey); 99+%, potassium phosphate dibasic trihydrate, 99% calcium chloride dehydrate, 99+% potassium chloride and 99+% magnesium sulphate heptahydrate (Sigma Aldrich Canada, Oakville Ontario); BBL Biosate peptone (pancreatic digest of casein 65%, yeast extract, 35%) and BBL beef extract powder (BD Mississauga, Ontario).

5.5.4.2 TS recovery and spiking of SWW

TS was recovered by lyophilization using a FreeZone 4.5 Litre Benchtop Freeze Dry System (Labconco, Kansas City, MO). Wastewater collected from the inlet of the secondary clarifier was used as a source of TS considering the higher concentration of suspended solids in this stream facilitating the recovery of the large amounts required for spiking of SWW for multiple experiments. The wastewater was first frozen at -20 ^{o}C in 200 mL Fast-Freeze Flasks (Labconco, Kansas City, MO), then lyophilized for 24 h, desiccated for 24 h and stored at -20^{o}C for later use. A TSS range typical of that found in SE, which is 2 mg/L to 220 mg/L, was developed using literature values (Harris et al., 1987; Lishman et al., 2006; Ragush et al., 2015; Rusten et al., 1998)

and by characterizing samples from the WWTP A (see section 5.5.3) as well as from two other nearby WWTPs (WWTP B & C) (see Supplementary Information section 5.8.1). The SE TSS range was used to target 4 different levels of TSS which was achieved by first determining the TS:TSS ratio in the recovered TS and then using this ratio for sample preparation. The ratio of TS:TSS was determined by weighing a known amount of TS into SWW and then measuring the TSS of this sample. High TSS was made by adding 300 mg/L TS particles to SWW and each subsequent sample was made by applying a 50% dilution of SWW of the previous higher level. Dilutions were completed under constant stirring (with a magnetic stir bar) using a pipette with a wide head as specified for aliquot collection in method 2540 B for wastewater samples containing TSS (Rice et al., 2012). Samples made to meet objectives 1 and 2 have the following characteristics:

- **High TSS**: 259 mg/L TSS (300 mg/L TS; 374 µS/cm)
- **Medium-High (Med-Hi) TSS**: 129 mg/L TSS (150 mg/L TS; 187 µS/cm)
- **Medium-Low (Med-Lo) TSS:** 65 mg/L TSS (75 mg/L TS; 94 µS/cm)
- **Very Low TSS:** 32 mg/L TSS (38 mg/L TS; 47 µS/cm)

Samples made to meet objective 3 have the following characteristics:

- **High TSS**: 259 mg/L TSS (300 mg/L TS; 374 µS/cm)
- **Medium-Low (Med-Lo) TSS:** 68 mg/L TSS (75 mg/L TS; 98 µS/cm)

Med-lo TSS was selected for this study as it had a similar TSS to that found in SE from SE A while Hi TSS was selected as it had the highest TSS level typically exhibited in SE.

5.5.4.3 TS, TSS and EC analysis

TS was determined by weighing a known amount of recovered TS into SWW (see section 5.5.4.2). TSS analysis was performed using method 2540 B (Rice et al., 2012). Specific supplies and equipment for TSS included 0.45 µm glass fibre filters (Merck Milipore Ltd.; Etobicoke, Ontario) and a vacuum pump (Biorad, Hercules, CA). EC, used as an indication of TDS, was measured using a Hanna Instruments handheld DiST® 5 EC/TDS/Temperature Tester (HI 98311) which was calibrated with a 1413 µS/cm conductivity standard (HI 7031) (HACH, London, Ontario).

5.5.5 Particle size distribution analysis

Particle size distribution analysis (PSDA) of the SWW samples spiked with recovered TS, SWW and SE A was performed using a Mastersizer 2000 with a Hydro 2000S(A) pump accessory (stir/pump speed: 1640 rpm, ultrasonics: 51%) (Malvern Instruments Ltd., Malvern, United Kingdom) in which water was used as a dispersant. In the software (version 5.61), the optical model selected was General Purpose using the Particle Shape: Irregular setting. For each sample, 3 measurements were averaged to produce a particle size distribution that was reported as the particle volume percentage of 100 discrete size ranges from 0.2 μm to 1200 μm.

5.5.6 Target CECs and their internal standards

Table 8 presents target compounds and their internal standards, solvents used to prepare their stock solutions, purities, suppliers, and CEC concentrations reported in literature for wastewater effluents. 2500 mg/L single compound stock solutions (including atrazine) were prepared in methanol, with the exception of estrogens and their internal standards which were prepared in ratios of dimethylsulfoxide (DMSO) to methanol ranging from 30:70 to 100% DMSO. Stocks solutions of the 14 CECs mix (each at 1000 mg/L) and the 15 internal standards mix (each at 100 mg/L) were made in methanol from the individual stock solutions. All stock solutions were stored at -20^{o}C until use and working solutions were made from these stock solutions and immediately stored at -20^{o}C until required. The working solutions contained <1% of solvent and was shown not to cause a toxic response (data not shown). Atrazine was spiked into samples containing recovered TS in SWW and in their filtrates (containing no TSS) while the 14 CECs mix was spiked into SE A for sample enrichment. The 15 internal standards mix was used for the chemical analysis of the samples.

Table 8 Target compounds and internal standards along with solvents used for stock solutions, purities, suppliers and their concentrations reported in different wastewater effluents

Type	Subtype	Compound	Internal standard	Solvent: (Compound, internal standard[1])	Standard or Purity (%): (Compound, internal standard)	Supplier: (Compound, internal standard)	CEC concentrations in different types of effluent reported in the literature	
							Maximum concentration found in wastewater effluents (μg/L)	Type of municipal effluent and references
Pharma-ceutical	Antibiotic	Sulfamethoxazole	Sulfamethoxazole-d4	MeOH	VETRANA L, 98	S, I	0.871	8 WWTPs including PE and SE[a]
	Antibiotic	Trimethoprim	Trimethoprim-d9	MeOH	VETRANA L, 99.9	S, S	0.011, 0.344 ± 0.081	L[b] SE[c]
	Lipopenic	Gemfibrozil	Gemfibrozil-d4 (2,2-dimethyl-d6)	MeOH	99.98, 99	S, I	0.192 ± 0.020 0.436 1.3	12 WWTPs including: SE and L[e] 18 WWTP effluents including: SE, TE, PE and L[f]
	Neurphathic/ epileptic	Carbamazepine	Carbamazepine-d10 (rings-d10)	MeOH	98+, 98	S, I	0.135 0.344 ± 0.005 1.036 ± 0.279 2.3	L[b] SE[c] SE[d] 18 WWTP effluents including: SE, TE, PE and L[f]
	Anti-depressant	D, L Venlafaxine	(±)-Venlafaxine-d6 HCl (N,N-dimethyl-d6)	MeOH	95, 99	T, I	1.8 0.8	SE[g] TE[h]
	Anti-inflammatory	Naproxen	(±)-Naproxen-d3 (α-methyl-d3)	MeOH	98, 99	T, I	0.599 ± 0.258 0.180 ± 0.036 1.189 33.9	SE[c] SE[d] 12 WWTPs including: SE and L[e] 18 WWTP effluents including: SE, TE, PE and L[f]
	Anti-inflammatory	Ibuprofen	(±)-Ibuprofen-d3 (α-methyl-d3)	MeOH	98, 99	T, I	0.105 ± 0.041 0.444 ± 0.214 0.773 24.6	SE[c] SE[d] 12 WWTPs including: SE and L[e] 18 WWTP effluents including: SE, TE, PE and L[f]

Table 8 Continued

Type	Subtype	Compound	Internal standard	Solvent: (Compound, internal standard [1])	Standard or Purity (%): (Compound, internal standard)	Supplier: (Compound, internal standard)	CEC concentrations in different types of effluent reported in the literature	
							Maximum concentration found in wastewater effluents (µg/L)	Type of municipal effluent and references
	Estrogen hormone	Estrone	Estrone 16, 16-d2	MeOH :DMSO, 1:1	99+, 98	S, I	0.038 0.1	12 WWTPs including: SE and L[e] 18 WWTPs including: PE, SE, TE and L[i]
	Estrogen hormone	17β-estradiol	17β-estradiol-2, 4- d2	dimethyl sulfoxide	98+, 99	S, I	0.1 0.016	4 WWTPs including SE and TE[j] 18 WWTPs including: PE, SE, TE and L[i]
	Estrogen hormone	17α-ethinylestradiol	17α-ethinylestradiol-2,4,16,16-d4	MeOH :DMSO, 7:3	98, 98	T, I	0.00763 ± 0.00301 0.017	TE[k] 5 SE WWTPs[l]
Pesticide	Herbicide	Atrazine	Atrazine-d5	MeOH, DMSO: MeOH, 1:9	98, 98	T, T	0.055 0.175	L[b] SE [c]
	Herbicide	MCPA (4-Chloro-2-methylphenoxyacetic acid)	4-Chloro-2-methylphenoxy-d3 acetic Acid	MeOH	99.8, 98	S, I	0.004 ± 0.003	SE[d]
	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	0.860	TE[m]
Anti-microbial agent	Antibacterial/ antifungal agent	Triclosan	Triclosan-d3	MeOH	98, 98.1	T, T	0.183 ± 0.005, 0.324	SE[n] 12 WWTPs including: SE and L[e]

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. a: (Miao et al., 2004); b: (Carlson et al., 2013); c: (Hua et al., 2006b); d: (Kerr et al., 2008); e: (Lishman et al., 2006); f: (Metcalfe et al., 2003); g: (Lajeunesse et al., 2012); h: (Metcalfe et al., 2010); i: (Servos et al., 2005); j: (Metcalfe et al., 2013); k: (Cicek et al., 2007); l: (Fernandez et al., 2007); m: (Sengupta et al., 2014); n: (Buth et al., 2011)

5.5.7 Sample enrichment for the LuminoTox and chemical analysis

Samples for chemical analysis were concentrated using lyophilization as previously described in Marshall and Yargeau, using a FreeZone 4.5 Litre Benchtop Freeze Dry System (Labconco, Kansas City, MO) (Marshall & Yargeau, 2017). For the LuminoTox sample enrichment, the three samples, each consisting of 500 mL SE A were spiked with different concentrations of the 14 CEC mix. 400 mL of each sample was then put into an 800 mL Fast-Freeze Flask (Labconco, Kansas City, MO) and frozen at -20^{o}C, while 10 mL of leftover sample was stored at -20^{o}C until further required. The 400 mL samples were then lyophilized, reconstituted with 8 mL of Milli Q water (resulting in a 50X concentration), and frozen at -20^{o}C until further required. Each sample was thawed at room temperature and filtered using a Fisherbrand 25 mm, 0.2 μm diameter PTFE syringe filter (Fisher Science Education, Fair Lawn, New Jersey). A 0.14 μg/L atrazine in SE A method control was made using the same procedure. Concentrated and un-concentrated samples were then tested in the LuminoTox. Samples were also made containing the 14 CEC mix at concentrations equivalent to those of the 50X samples spiked into un-enriched SE A and filtered (using the same supplies as those for the enriched samples).

5.5.8 Quantification of target analytes

Chemical analysis was completed using an Accela 600 LC System (Thermo Scientific, Waltham MA, USA) coupled to an LTQ XL Orbitrap mass spectrometer, both controlled using Thermo Xcalibur 2.0 software (Thermo Scientific, San Jose CA, USA). Chromatographic separation was performed as previously described in Marshall and Yargeau 2017. Detection of target compounds and their surrogates was performed using an electrospray ionization source (ESI) as also previously described in Marshall and Yargeau 2017.

5.5.9 Statistical analysis

LuminoTox, chemical, and TSS analyses were performed in triplicate and results are reported as averages along with one standard deviation. T tests (Paired Two Sample for Means) using a two-tailed distribution and $p < 0.05$ were performed in Excel to compare the toxicity of samples containing increasing concentrations of TS with their filtrates at equivalent atrazine concentrations

and to compare changes in atrazine toxicity at constant TSS. T tests were performed to compare the toxicity of samples containing different concentrations of the 14 CECs mix in SE A for enriched and un-enriched samples.

5.6 Results and discussion

5.6.1 Particle size distribution analysis

PSDA was completed (see Figure 7) in order to understand if the samples containing TS spiked in SWW would contain particle sizes representative of those found in real SE. Samples containing TS spiked in SWW had larger particle size distributions (PSDs) (Med-Lo TSS: 4 μm to 924 μm and High TSS: 4 μm to 1008 μm) compared to that of SE A (3 μm to 549 μm) but within their distributions, contained a fraction of particles that fell within the same size range as those of SE A. Larger particles are expected in the samples containing TS spiked in SWW because it was recovered from the inlet of the secondary clarifier where the larger particles have not yet has time to settle and be eliminated. Particles in the upper half of the SE A distribution are large compared to SE PSDs reported by others which ranged from 2 μm to 200 μm (Baek & Chang, 2009) and from 2 μm to 80 μm (Wang et al., 2014). Differences may be attributed to the highly variable composition of wastewater. The size of particles coming into the wastewater treatment plant can dictate the size exiting; particles that are < 1 μm biochemically degrade faster compared to those larger than this size (Levine et al., 1985). SE A and the work completed by Baek & Chang as well as that by Wang and colleagues all used activated sludge for secondary treatment (Baek & Chang, 2009; Wang et al., 2014), however, the particle size can also vary with the specific design of each facility which is another possible factor for the differences observed. Further, samples prepared by Baek and Chang, were left to settle for 90 minutes prior to the supernatant collection which was subsequently used for TSS analysis; this could further explain the significantly smaller particle sizes observed in their distribution. Despite the differences observed between SE A and those reported in the literature, a fraction of the PSD for TS samples spiked in SWW includes the PSDs of SE A and those in the literature. Furthermore, the TS samples spiked in SWW comprise particles defined as fines by others which were shown to induce LuminoTox toxicity (Burga Pérez et al., 2013; Dellamatrice et al., 2006; Férard et al., 2015). This fines range is also exhibited by SE A and the PSDs of others (Baek & Chang, 2009; Wang et al., 2014). Thus, the TS samples spiked in

SWW contain the size range of interest for further study and may serve as good models for toxicity assessment in the presence of TS.

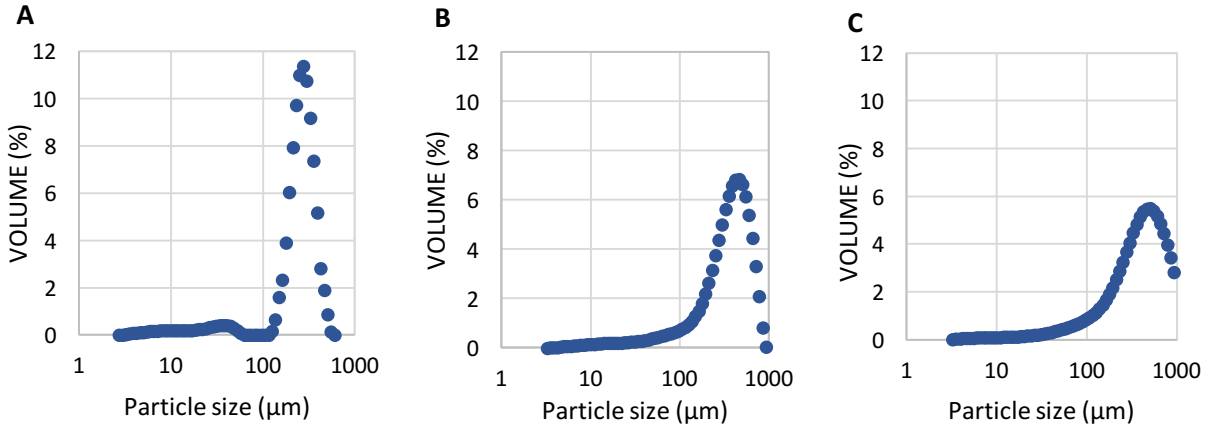


Figure 7 Particle size distribution analysis. PSD: particle size distribution; A: Secondary effluent A (SE A), PSD: 3 µm to 549 µm; B: Med-Lo TSS (65mg/L TSS), PSD: 4 µm to 924 µm; C: High TSS (259mg/L TSS) PSD: 4 µm to 1008 µm.

5.6.2 Assessment of toxicity or interferences associated with the TS fractions

Figure 8 reports the inhibition of SAPS I and SAPS II when exposed to 4 µg/L and 10 µg/L atrazine in increasing concentrations of TS spiked in SWW and their respective filtrates (TSS > 0.45 µm removed). The following sections discuss these results by first focusing on the impact of TSS on toxicity measurements for atrazine in samples containing TS spiked in SWW (section 5.6.2.1), and then evaluating the impact of the dissolved fraction (section 5.6.2.2).

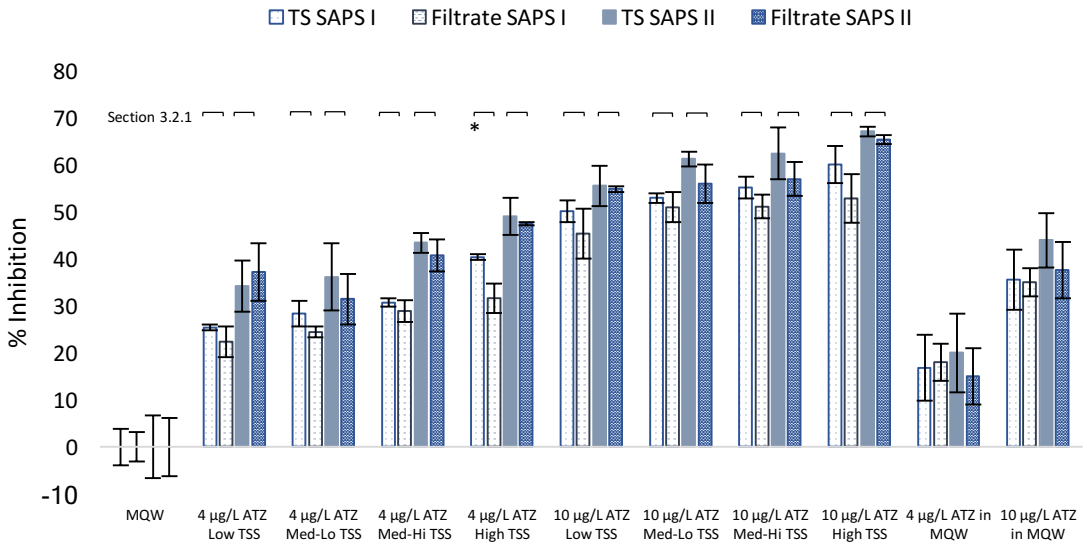


Figure 8 Inhibition of SAPS I and SAPS II by exposure to samples containing atrazine (ATZ) in different concentrations of TS and their filtrates (TSS removed). MQW: Milli Q water. Error bars = standard deviation of triplicates. * $p < 0.05$.

5.6.2.1 Toxicity or interferences associated to TSS

The potential for TSS to induce toxicity was explored by comparing the toxicity of samples containing TS in SWW and their respective filtrates. For both biosensors, in all cases but one (indicated by a * on Figure 8), the inhibitions of the atrazine in the TS in SWW samples were statistically equivalent to their filtrates as confirmed by Paired T-tests. Since the only difference between the TS in SWW samples and their filtrates is the TSS fraction, this suggests that the TSS does not contribute to toxicity. The exception to this result was the SAPS I inhibition of the samples containing 4 µg/L atrazine and High TSS which exhibited a higher inhibition compared to that of its filtrate ($p = 0.046$). We could not find an explanation for this higher inhibition but overall the LuminoTox toxicity measurements would not be affected by the presence of TSS in the range of concentrations typically present in secondary effluent.

The lack of contribution of SE fine particulates to LuminoTox toxicity contradicts other findings regarding sediment fines (Burga Pérez et al., 2013; Dellamatrice et al., 2006; Férard et al., 2015). Dellamatrice and colleagues reported that toxicity increased with increasing amounts of kaolin clay (grain size < 4 µm) relative to silica sand (grain size: 125 µm to 250 µm). Furthermore, a

significant inverse correlation was demonstrated between IC50s and fines content (Burga Pérez et al., 2013; Férard et al., 2015) in natural freshwater sediment samples using the LuminoTox Solid Phase Assay (Lum-SPA). Burga Pérez and colleagues also concluded that the LuminoTox toxicity of natural freshwater sediments containing fines will be dependent on the characteristics of the clay particles themselves (adsorption to PECs, composition, sedimentation rate, and photosynthetic inhibition) and also on their ability to adsorb to contaminants thereby decrease their bioavailability and toxicity (Burga Pérez et al., 2013). The contrast between our finding and that of others is likely due to a different sample composition considering that generally, municipal wastewater TSS has more natural organic matter (NOM) and less mineral content compared to suspended natural freshwater sediments (Shon et al., 2006; Yao et al., 2015).

5.6.2.2 Toxicity or interferences associated with the TDS (other than atrazine)

The comparison of the inhibition reported for the TS in SWW samples and their filtrates with the inhibition for their respective atrazine controls in Milli Q water indicated that the matrix contributed to a higher toxicity. Considering than the TSS was shown to have no effect, the increased toxicity is likely caused by unknown contaminants of various natures already present in the TDS fraction (estimated as EC), including CECs and inorganic species, as previous observed in other work (Bellemare et al., 2006; Boucher & Carpentier, 1999b; Dewez et al., 2007; Mamindy-Pajany et al., 2011; Muller et al., 2008; Souza et al., 2013). Humic acids (HAs) within the TS particles may be another potential source of toxicity; evidence exists that low molecular weight, soluble HAs have the potential to behave as xenobiotics and have been reported to inhibit the production of photosynthetic oxygen in *Ceratophyllum demersum* thylakoids (Pflugmacher et al., 2006; Steinberg et al., 2003). To evaluate the possible contribution of the target CECs to this increased toxicity, samples were analyzed by LC-HRMS. The target CECs are presented in Table 9 along with the range of concentrations measured in the various samples. The highest concentrations of CECs observed were for carbamazepine, 17-alpha-ethinylestradiol and atrazine which were present at concentrations up to 35 µg/L, 27 µg/L and 27 µg/L, respectively. These concentrations are respectively ~6X, ~4X and ~5X the concentration at which a 20% inhibition was observed for a mixture of 15 target CECs spiked in SE (Marshall & Yargeau, 2017). Although it is not possible to determine if the measured toxicity

was caused by these CECs or other unknown constituents present, the measured concentrations of the target analytes were confirmed to be sufficient to trigger a response in the LuminoTox.

Table 9 Concentration of the target CECs in the TS samples and their filtrates tested in Figure 8

Target CEC	Concentration range (μg/L)
Trimethoprim	0.01-16.4
Sulfamethoxazole	0.27-10.0
Ibuprofen	0.08-0.29
Venlafaxine	0.18-20.0
Carbamazepine	0.41-35.0
17α ethinylestradiol	1.16-26.8
Atrazine	5.50-27.2
DEET	1.19-12.0
Naproxen	0.80-18.0
Estrone	1.02-15.7
17β Estradiol	0.89-11.1
Gemfibrozil	1.36-13.0
Triclosan	1.14-2.56
MCPA	0.44-1.42

5.6.3 Sensitivity of the LuminoTox to increasing concentrations of atrazine at constant TS

Results reported in Figure 9 indicate that both biosensors were sensitive to changes in spiked atrazine concentrations at constant Med-Lo TSS and High TSS. SAPS I and SAPS II were both sensitive to changes in atrazine concentration, inducing a mid- to high-level of inhibition. Paired T-tests confirmed significant differences between at the atrazine concentrations tested at High TSS: 0 μg/L < 4 μg/L < 10 μg/L, and most of the extended range of concentrations of atrazine tested at Med-Lo TSS: 0 μg/L < 4 μg/L = 6 μg/L < 10 μg/L < 200 μg/L as confirmed by Paired T-tests. This compares well to another LuminoTox study which used atrazine in water; increasing inhibition was observed for similar increasing concentrations: 1 μg/L < 10 μg/L < 200 μg/L (Dewez et al., 2007). Thus, the LuminoTox was sensitive to the detection of changes in atrazine concentration in the mid- to high-range of inhibition in the presence of TSS but with limited resolution for similar concentrations (4 μg/L and 6 μg/L). Limited information is available in literature about the limit of detection of LuminoTox for atrazine. Dewez and colleagues (2007), examined the response of atrazine at 1 μg/L (which elicited no response) and 10 μg/L (which

elicited a ~15% inhibition), without any other concentrations examined in between these values; Souza and colleagues (2013) used an initial concentration of 100 µg/L atrazine spiked in SE and reported inhibition of about 10% at concentrations of ~ 5 µg/L and ~3 µg/L but without reporting the exact values and commenting on differences of inhibition at these low concentrations; lastly, Bellemare and colleagues (2006) determined that the threshold value (~10% inhibition, exposure 10 mins dark) of SAPS I and SAPS II exposed to atrazine in water was 0.7 µg/L, however, there were no other concentrations examined to determine the resolution of the measurements. The lack of sensitivity of both sensors to a change in atrazine concentrations of 2 µg/L further suggests that the LuminoTox might not be able to distinguish the toxicity of the native CECs in wastewater, which are typically present at concentrations below 3 µg/L (see Table 8), highlighting the importance of sample enrichment to increase the LuminoTox sensitivity towards CECs for monitoring changes in CEC at concentrations relevant to those native to wastewater.

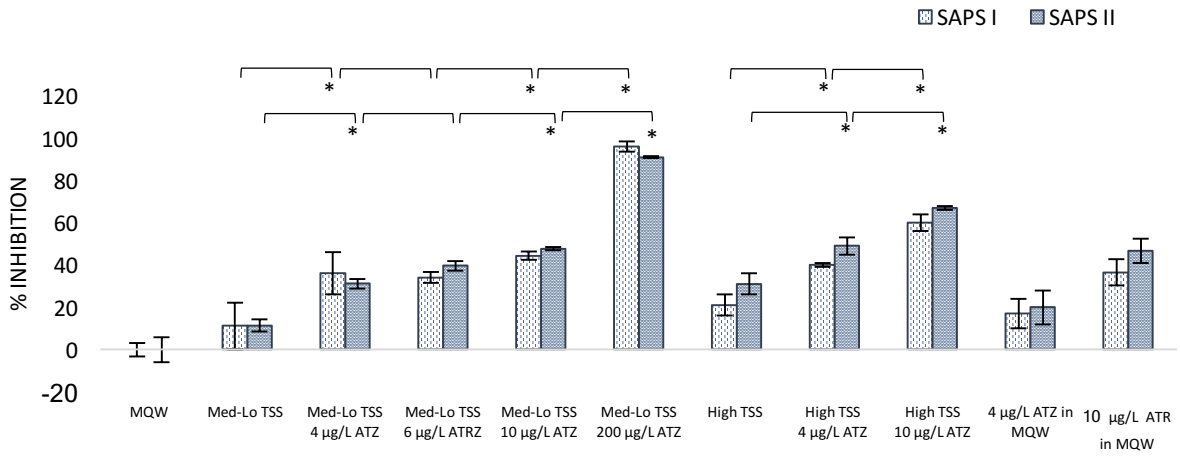


Figure 9 Inhibition of SAPS I and SAPS II to atrazine (ATZ) concentrations at constant Med-Lo TSS and High TSS. MQW: Milli Q water. Error bars = standard deviation of triplicates. * $p < 0.05$.

5.6.4 Sample enrichment for the detection of CECs

Figure 10 presents the inhibition measured for three different types of samples: SE A spiked with CECs; SE A spiked with CECs and pre-concentrated 50X; and SE A spiked with a higher concentration of CECs equivalent to those of the 50X concentration. The spiking of the 14 CECs in SE A was performed at three different levels of individual compound concentrations: 0.12 µg/L, 0.17 µg/L and 0.23 µg/L. Results for atrazine are also shown as quality controls. SE A spiked with

the higher concentration of CECs were analyzed in order to compare the response to CECs in enriched versus un-unenriched samples and also to evaluate the effect of pre-concentrating the matrix by lyophilization.

Results indicate that SE A spiked with CECs was not more toxic that SE A alone, indicating that the LuminoTox was unable to detect the presence of CECs at these environmentally relevant concentrations. However, after enrichment (50X CECs in SE A) inhibitions of 51 ± 2%, 70 ± 3% and 81 ± 3% were obtained for the three concentration levels, respectively. When comparing the results for the enriched samples (50X CECs in SE A) to the SE A spiked with CECs at concentrations equivalent to the ones present in the pre-concentrated samples, it is observed that the inhibition in the pre-concentrated samples are significantly higher. This can be explained by the pre-concentration of other constituents present in the matrix such as metals, nitrogen ammonia, phenols, and other organic compounds which have been previously reported to be detectable in the LuminoTox (Bellemare et al., 2006; Boucher & Carpentier, 1999a; Hiriart-Baer et al., 2006; Rai et al., 1981; Rai et al., 1991; Waldemar & Tadeusz, 1988; Winner & Owen, 1991). The enrichment approach thus increased the sensitivity of the LuminoTox towards spiked CECs as indicated by the increasing toxicity with CEC concentration.

The inhibitions measured for 50X 0.17 µg/L 14 CECs mix in SE A, and 50 X 0.23 µg/L14 CECs mix in SE A were statistically higher compared to that of pre-concentrated un-spiked SE A (50X SE A, inhibition of 49 ± 1%), demonstrating that upon enrichment certain levels of spiked CECs (at 0.17 µg/L and above in this case) can be differentiated from the matrix. These two highest concentrations levels, however, were statistically equivalent to each other. If they are compared to the samples containing equivalent 14 CEC mix concentrations (8.5 µg/L and 11.5 µg/L) in un-enriched SE A, there is a significant difference in inhibition of the latter pair compared to those of the enriched samples, highlighting that sample concentration is masking the effects of toxicity. Furthermore, the enrichment method was not able to distinguish between concentrations exhibiting high toxicities (≥ 70% inhibition). The pre-concentration of the matrix seems to lead to a masking of the toxicity response. Another example of the masking of toxicity response is seen between the 50X 0.12 µg/L 14 CECs mix in SE A sample which was statistically equivalent to that of 50X SE A alone, however, the toxicity response of an equivalent 14 CEC mix concentration (6 µg/L) in

the un-enriched SE A was shown to be statistically different than that of un-enriched SE A alone. There are several possible explanations for the masking of toxicity response. There could be a decrease in sensitivity due to toxic interferences caused by the increased concentration of toxic compounds in the enriched samples. Toxic interferences could involve mixture effects such as synergism, antagonism, or toxicity addition, which can be concentration-dependent and have been reported by others to occur for certain CEC mixtures (Altenburger et al., 2013; Boltes et al., 2012; Jonker et al., 2005). Furthermore, there could be a decrease in the bioavailability of toxic compounds due to the increased presence of NOM compared to that of the un-concentrated samples. Finally, as the toxicity approaches its endpoint, a decrease in dose response occurs, as was noted by LBI Innovations Inc. for the LuminoTox toxicity of atrazine in water.

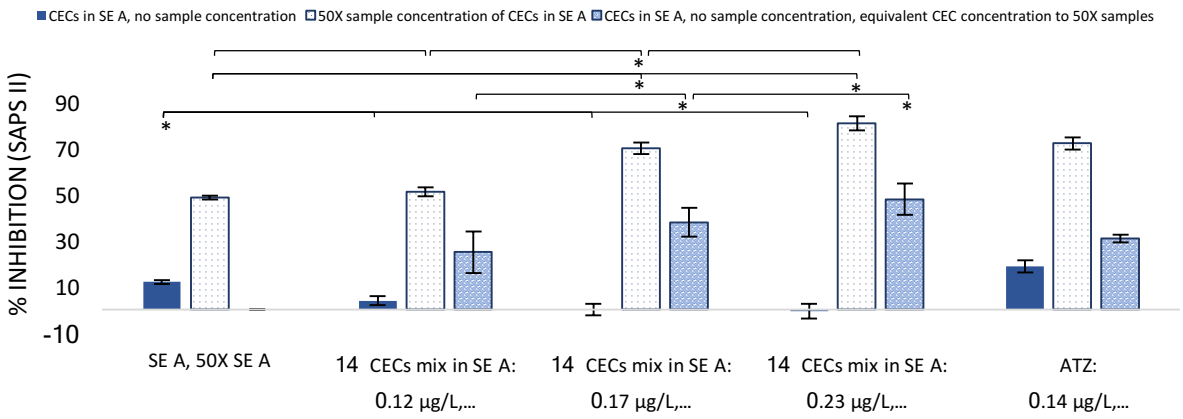


Figure 10 Inhibition of a mix of 14 CECs in secondary effluent A (SE A) tested using an enrichment method and comparison to inhibition of samples not subjected to pre-concentration. Controls and blanks were not presented to improve the readability of the graph. Error bars= standard deviation of triplicates. * $p < 0.05$.

5.7 Conclusions

The toxicity of 4 µg/L or 10 µg/L atrazine in different TS concentrations and in their filtrates revealed that, in all samples but one, samples containing TS in SWW were statistically equivalent to their filtrates which demonstrated that, in most cases, TSS did not induce toxicity in the LuminoTox. The LuminoTox sample enrichment method was able to increase the sensitivity of a mixture of 14 CECs and, in most cases, to distinguish between the spiked 14 CECs mix and the enriched SE A. It was not, however, able to differentiate between the two highest concentrations

of the 14 CECs mix, and a decrease in toxic response of enriched samples compared to those containing equivalent CEC concentrations in unenriched SE A was observed; both of these results are limitations for monitoring changing concentrations of CECs in SE using the LuminoTox. Therefore, overall, the LuminoTox is a promising tool for toxicity monitoring of municipal SE, however, if the intent is to focus on the detection of residual CECs, significant development and optimization of the sample enrichment method is required.

5.8 Supplementary information

5.8.1 Characteristics of WWTPs B and C

WWTPs B and C have average daily flows of 50,2600 m^3, and 50,755 m^3, respectively and serve populations of 54,000 and 134,894, respectively. Influents of WWTPs B and C consisted of the following types of wastewater: approximately 50% industrial and 50% domestic, and approximately 25% industrial and 75% domestic respectively.

5.8.2 Limits of detection and limits of quantification of the target analytes

Table 10 Limits of Detection (LODs) and Limits of Quantification (LOQs) of the target analytes

Compound	LOD, LOQ (µg/L)
Sulfamethoxazole	1, 4
Trimethoprim	1, 4
Gemfibrozil	1, 4
Carbamazepine	1, 4
D, L Venlafaxine	1, 4
Naproxen	1, 3
Ibuprofen	1, 4
Estrone	1, 4
17β estradiol	1, 4
17α ethinylestradiol	1, 4
Atrazine	1, 4
MCPA (4-Chloro-2-methylphenoxyacetic acid)	1, 3
DEET (N,N-Diethyl-3-methylbenzamide)	1, 4
Triclosan	1, 3

1

6. MANUSCRIPT 3: LUMINOTOX AS A TOOL TO OPTIMIZE OZONE DOSES FOR THE REMOVAL OF CONTAMINANTS AND THEIR ASSOCIATED TOXICITY

6.1 Preface

Over the past two decades, ozone treatment for municipal wastewater has been of increasing interest for the removal of CECs. In many studies, CEC removal has been shown to be associated with a decrease in toxicity for diverse endpoints; on the other hand, some studies have shown an increase in toxicity after ozone treatment. Therefore, it is critical to monitor the success of ozone treatment using bioassays. In the previous two manuscripts, the LuminoTox has been shown to be a promising tool for this application. This manuscript addresses objectives 3 and 4 of this PhD project through exploring the use of the LuminoTox as a monitoring tool, and ozone as a technology for toxicity removal associated with CECs and their TPs.

Two experiments were conducted for this manuscript using different wastewater mixtures. The first mixture was made by spiking atrazine into SWW, and the second by spiking a mixture of 14 CECs into SE collected from municipal WWTP A. These samples were exposed to ozone using a semi-batch ozone reactor and collected at different exposure times (and hence ozone doses) for further analysis. LuminoTox analysis was performed on all samples, using SAPS I and SAPS II. Chemical analysis was performed on samples exposed to select doses of ozone. In the case of atrazine spiked into SWW, samples were analyzed for atrazine and four of its primary TPs, and in the case of the 14 CECs mixture spiked in SE, samples were analyzed for these 14 CECs.

The LuminoTox was shown to be an excellent tool to monitor changes in toxicity in ozone treatment, and ozone proved to be an excellent technology for toxicity reduction. A decrease in CECs, due to the application of ozone, was found to correspond with a decrease in toxicity in all samples analyzed, which expands the literature for current toxicity assessment using ozone. It was determined that how the ozone is applied has an impact on the toxicity removal; for an equivalent ozone dose, a lower ozone feed concentration applied for a longer time showed to be more efficient at toxicity reduction in the atrazine in SWW experiment. This toxicity reduction also corresponded to better CEC removal rates, and after a certain ozone dose, better TP removal rates. For the 14

CECs in SE, however, the opposite was found to be true; a higher ozone feed concentration applied for a shorter period of time proved to be more efficient at toxicity and CEC removal. The efficiency of toxicity removal, therefore, appears to be dependent on the way in which the ozone dose is applied, and specific to the wastewater mixture being treated.

This manuscript is in review after submission of minor revisions in **Chemosphere, October 2017.**

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

Meghan Marshall, Viviane Yargeau

Department of Chemical Engineering, Room 4180, McGill University, 3610 University, Montreal, Quebec, Canada, H3A 0C5. Email: meghan.marshall2@mail.mcgill.ca; viviane.yargeau@mcgill.ca *(corresponding author: tel: 515-398-2273)*

6.2 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% for LuminoTox algae biosensors SAPS I and SAPS II inhibition, respectively, for statistically equivalent ozone dose pairs of 43 ± 1.15 mg (5 ppm ozone feed) and 36 ± 3.51 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 ± 3.79 mg (5 ppm ozone feed) and 42 ± 1.77 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*

6.3 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., 2005b; 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). 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, 1999a; 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).

6.4 Materials and methods

6.4.1 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^oC 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.

6.4.2 Target CECs and internal standard stock solutions

The CECs were selected for this work because we have previously detected them in several SEs (data not shown), and ATZ is the positive control used in 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 11 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; DMSO:MeOH, 1:9[1]	98, 98	T, T	1, 4
	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	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.

6.4.3 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 so 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 treatments in the inlet gas of the O_3 reactor: 5 ppm O_3 and 15 ppm O_3 respectively. 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^{o}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., 2005b; 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.

6.4.4 LuminoTox

6.4.4.1 Justification of use and theory

The intention of Aquacion Inc., the company that produces LuminoTox, was to eventually use the LuminoTox as an online monitoring tool at WWTPs with goal of eventually using the technology to monitor CECs. As such, in this work, the LuminoTox was selected to explore its ability to monitor wastewaters containing CECs. This test could be used alone or along with a battery of bioassays. 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).

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

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

6.4.4.2 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.

6.4.5 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). 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. Due to the lack of analytical standards,

ATZ transformation products were reported as counts, which were used only to determine relative removal. CECs from the 14 CEC mix were reported as relative removal.

6.4.6 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.

6.4.7 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 $p<0.05$.

6.5 Results and discussion

6.5.1 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.

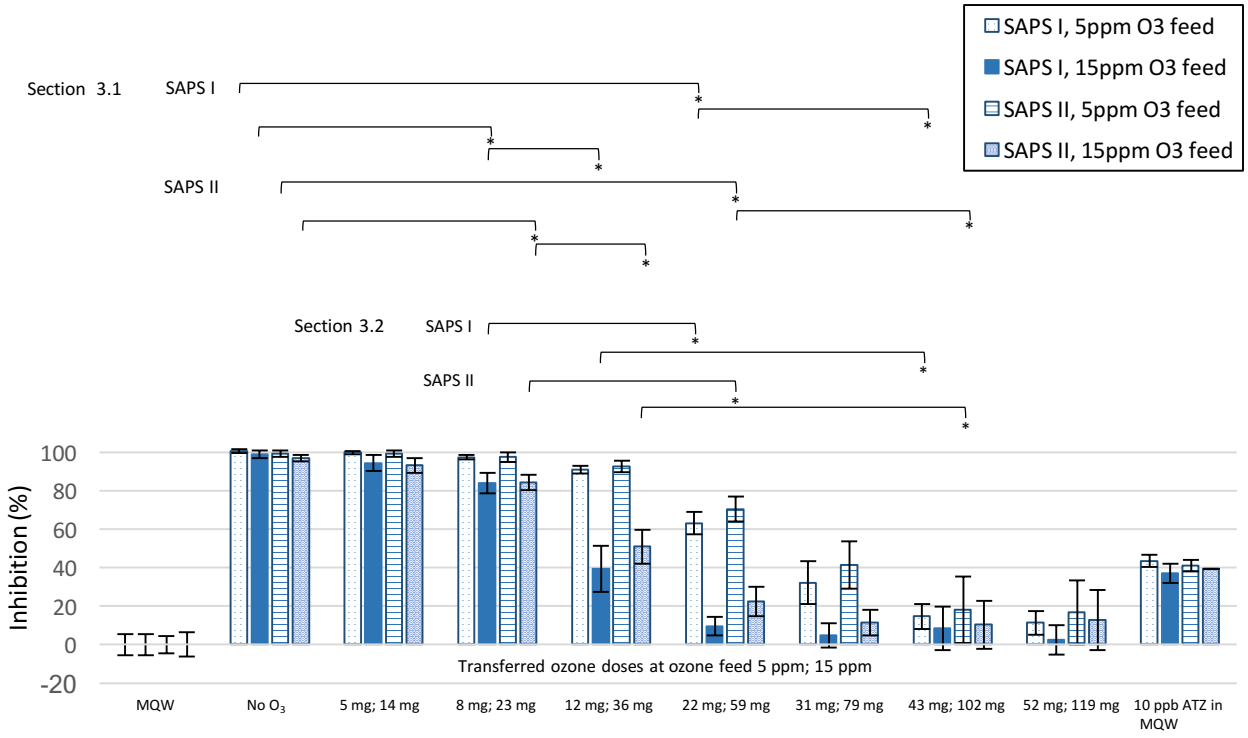


Figure 11 SAPS I and SAPS II toxicity of atrazine in synthetic wastewater exposed to different transferred ozone doses using 5 ppm and 15 ppm ozone feed concentrations

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 ($p < 0.05$).

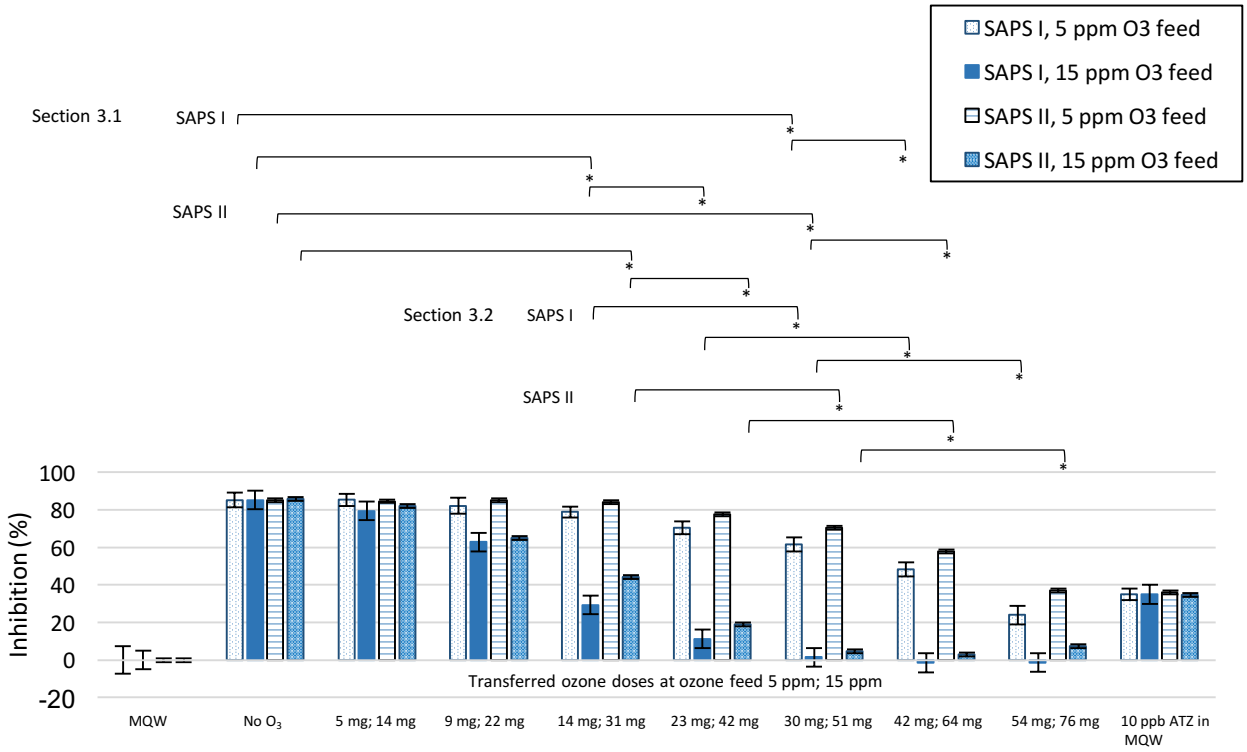


Figure 12 SAPS I and SAPS II toxicity of a mixture of 14 CECs in in secondary effluent exposed to different transferred ozone doses using 5 ppm and 15 ppm ozone feed concentrations

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 12 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., 2005b); 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., 2003a) 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., 2004a) 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)

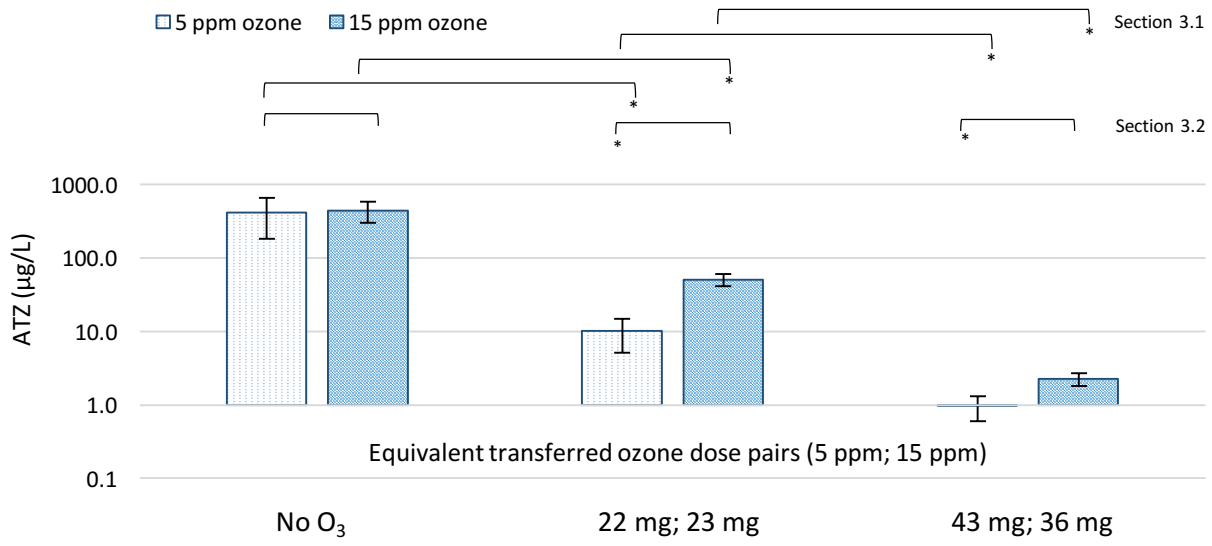


Figure 13 Chemical analysis of atrazine in samples containing atrazine in synthetic wastewater exposed to different equivalent transferred ozone dose pairs for 5 ppm and 15 ppm ozone feed

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 13: Range of 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).

6.5.2 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 14 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)**	
	Difference in inhibition of SAPS I %	Difference in inhibition of SAPS II %
Atrazine in SWW (Figure 1)		
22; 23 43; 36	22% 25%	16% 35%
14 CECs mix in SE (Figure 2)		
30; 31 42; 42 54; 51	-33% -37% -23%	-28% -40% -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: deethylatrazine (DEA), deisopropylatrazine (DIA), 4-acetamido-2-chloro-6-isopropylamino- s-triazine (CDIT) and 2-chloro-4-ethylimino-6-isopropylamino-s-triazine (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 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.

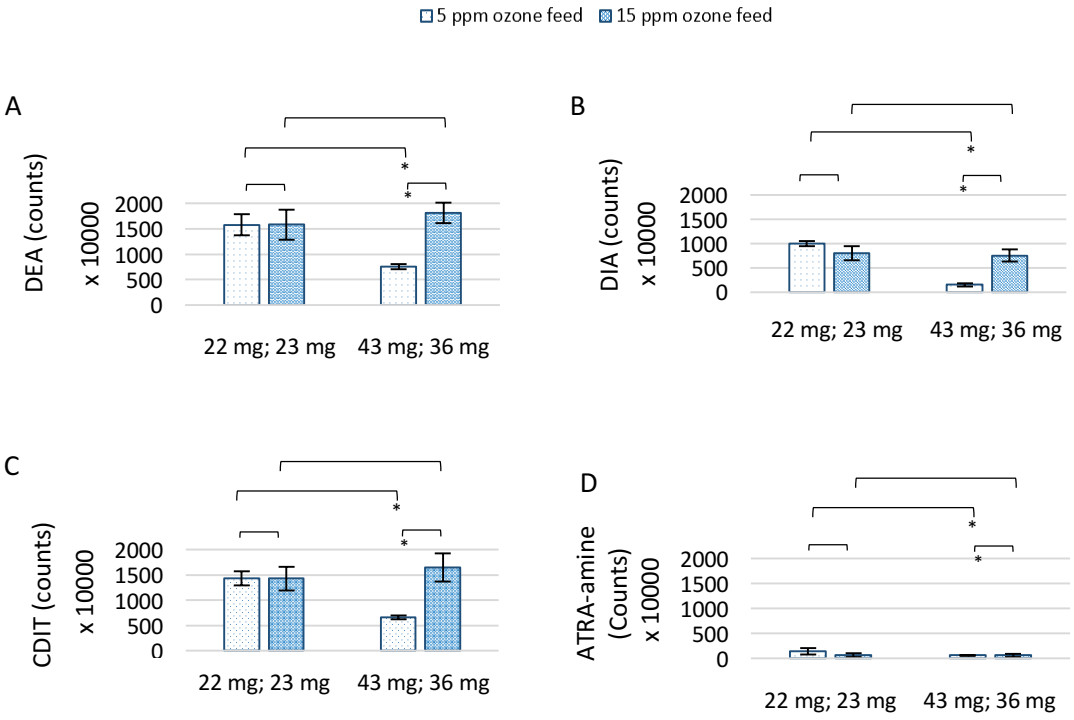


Figure 14 Chemical analysis of four atrazine transformation products in samples containing atrazine in synthetic wastewater exposed to different transferred ozone doses

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., 2013b). 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 15 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, ie. 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 16 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 using *Thamnoephalus platyurus* (% immobility)	12	Figure 3	30; 15	3	Petala 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

6.6 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.

6.7 Acknowledgements

The authors thank the WWTP staff for their assistance with the sampling campaign. We thank Marco Pineda for his help with chemical analysis and Deniz Nasuhoglu for his guidance with the ozone experiments. We are thankful for the McGill Engineering Doctoral Award which has supported Meghan Marshall and a research grant awarded to Viviane Yargeau (PI) from the Natural Sciences and Engineering Research Council (NSERC) of Canada through the Discovery Grant Program (RGPIN/04635-2015), which supported this work.

7. ORIGINAL CONTRIBUTIONS

The original contributions of this PhD project are:

1. **The exploration of toxic interferences by the LuminoTox for ranges of characteristics typical of those found in municipal SE**. For the first time, wastewater ranges characteristic of those found in municipal SE, including TOC, COD, alkalinity, EC, hardness, colour, and TSS were explored for toxic interferences, in order to determine the applicability of the use of the LuminoTox as a tool for toxicity evaluation of municipal SE. While LBI Innovations Inc. established ranges of characteristics applicable to all water samples, none were specifically developed for municipal SE, nor were their methods for validation of these ranges described. Furthermore, there is no literature published on the assessment of these ranges.
2. **Evaluation of a new algae biosensor called SAPS II (*chlamydomonas reinhardtii*) for monitoring toxicity of municipal SE.** There is no literature published on SAPS II toxicity; for the first time the applicability of SAPS II for use of detecting CECs in different wastewater matrices and monitoring ozone was evaluated. Overall, SAPS II behaved similarly to SAPS I in terms of sensitivity towards CECs in different wastewater matrices, and in terms of monitoring changes in toxicity during wastewater treatment by ozone.
3. **Mapping the limits of sensitivity of the LuminoTox towards CECs.** For the first time,
    a. the sensitivity of SAPS I and SAPS II to a mixture of CECs at different exposures to light and dark was explored. There has been no literature published on the sensitivity of SAPS I and SAPS II exposure to different light and dark conditions, nor has there been any literature published on exploring the lower limits of sensitivity of the LuminoTox towards CECs in wastewater.
    b. the range of concentrations detectable in the LuminoTox, and the lower limits of sensitivity towards atrazine spiked in a SWW sample containing TS particles recovered from secondary wastewater, and in a mixture of 14 CECs in SE was explored.
    c. it was determined that the LuminoTox demonstrated limited sensitivity towards select CECs at environmentally relevant concentrations.

d. it was determined that by using a pre-concentration method it was possible to increase the sensitivity of the LuminoTox toward CECs at environmentally relevant concentrations.

4. **Expanding on the current literature by determining that the LuminoTox is more sensitive to CECs that act directly on photosynthesis at the Q_B binding site within PS II, compared to CECs that act at different locations within PS II or those that act on indirectly photosynthesis.** In the bigger picture, the LuminoTox will be more sensitive to herbicides that act at the Q_B binding site than to other classes of CEC. In the existing literature it was reported, using the LuminoTox, that herbicides were more sensitive than insecticides, and the authors reported that this had to do with the specific site of action of the CEC.
5. **Demonstration that the LuminoTox is an excellent tool to monitor changes in toxicity of wastewaters during ozone treatment.** Although one paper in the literature reported monitoring changes in LuminoTox toxicity by treatment with ozone, there was no statistical analysis included, hence it did not conclusively demonstrate changes in toxicity. The research in the thesis expands upon the existing literature by exploring and confirming the applicability of LuminoTox as a wastewater monitoring tool during ozone treatment.
6. **Demonstration that ozone is an excellent wastewater treatment technology for toxicity reduction associated with CEC removal as measured by the LuminoTox**. As in Contribution 5 above, the paper in the literature that reported monitoring changes in LuminoTox toxicity by treatment with ozone does not conclusively demonstrate a toxicity reduction.
7. **Demonstration that the efficiency of toxicity removal by ozone is dependent on the way in which the ozone dose is applied, and appears to be specific to the wastewater mixture being treated**. For the first time, this was demonstrated in two different wastewater mixtures: SWW spiked with atrazine, and SE spiked with a mixture of 14 CECs. In the literature, differences in the efficiency of toxicity removal from wastewaters using ozone at different feed concentrations have been demonstrated, however, no work has been published on the efficiency of toxicity removal at equivalent ozone doses using different feed treatments and exposure times.

8. **Demonstration that in the range of TSS typical of that found in municipal SE, TSS does not elicit toxicity in the LuminoTox.** While there has been literature published on fines natural in fresh water sediments inducing toxicity as measured by the LuminoTox, there has been no literature previously published on LuminoTox toxicity related to particulate matter in wastewater samples.

8. CONCLUSIONS

This thesis examined the sensitivity of the LuminoTox as a tool to monitor the toxicity of SEs, as well as that of CECs within this wastewater and during ozone treatment. The LuminoTox demonstrated excellent potential in this role, however, if it is to be used for detecting toxicity related to CECs, work remains to be done on a sample pre-concentration method to make this a practical tool for use in municipal WWTPs. Using the LuminoTox it was demonstrated that the efficiency of toxicity removal during ozone treatment of wastewater containing CECs depends on the way in which the ozone feed is applied, implying that ozone system optimization may be required to minimize toxicity.

The results show that:

1. In most cases, the LuminoTox would be unbiased (elicit no or minimal toxic interferences) for wastewater characteristic ranges typical of those found in SE, including TOC, COD, EC, alkalinity, hardness, colour, and TSS. Exceptions included SAPS I toxicity towards low and high alkalinity, high TSS, as well as SAPS II toxicity towards high colour and EC. For samples that exhibit characteristics similar to those that elicited toxic interferences, care should be taken when interpreting their toxicity data.
2. Overall, SAPS II behaved similarly to SAPS I in terms of sensitivity towards CECs in different wastewater matrices and in terms of monitoring changes in toxicity during wastewater treatment by ozone. The benefit of using SAPS II over SAPS I remains a topic for further investigation.

3. To obtain the highest LuminoTox method sensitivity (without sample pre-concentration) towards the 14 CECs in different waste water matrices, an exposure time of 20 minutes in the dark or 30 minutes in the light is recommended with biosensors including SAPS I and SAPS II. Using these conditions, however, LuminoTox demonstrated limited sensitivity towards atrazine in samples containing TS and no sensitivity towards a mixture of 14 CECs in SE at environmentally relevant concentrations.

4. The LuminoTox is more sensitive to CECs that act directly on photosynthesis at the Q_B binding site within PS II, compared to CECs that act at different locations within PS II or

those that act on indirectly photosynthesis. Since many herbicides are designed to act at the Q_B binding site, the LuminoTox will be most sensitive to this class of CEC. The LuminoTox will be less sensitive to classes of CECs, such as pharmaceuticals, that are not specifically designed to target that mode of action.

5. With the sample pre-concentration method it is possible to increase the sensitivity of the LuminoTox to a mixture of 14 CECs in the range of environmentally relevant concentrations. However, significant optimization of the method would be required for monitoring CECs in SE.

6. The LuminoTox is an excellent tool to monitor changes in toxicity during ozone treatment of wastewater spiked with CECs. A decrease in CEC concentration, due to the application of ozone, corresponded with a decrease in toxicity.

7. Ozone is an excellent technology for toxicity reduction associated with CEC removal as measured by the LuminoTox. How the ozone is applied has an impact on the toxicity removal; for an equivalent ozone dose, a lower ozone feed concentration applied for a longer time is more efficient at toxicity reduction of atrazine in SWW. Conversely, for the 14 CECs in SE, the opposite is true; a higher ozone feed concentration applied for a shorter period of time is more efficient at toxicity and CEC removal. The efficiency of toxicity removal, therefore, is dependent on the way in which the ozone dose is applied, and appears to be specific to the wastewater mixture being treated. If WWTPs use this technology in the future, the optimization of toxicity removal will have to be completed for each specific treatment facility.

9. RECOMMENDATIONS

The following recommendations are made as a result of insights gained from work performed and conclusions drawn in this PhD thesis, as well as research reported in the literature:

1. Expand the range of characteristics typical of those found in SE to include analysis on influent wastewater and primary effluent wastewater, so that
    a. toxicity removal can be monitored over course of treatment, and
    b. potential toxicity issues can be detected before the end of the treatment train.
2. Given that it was demonstrated that for all biosensors, as inhibition decreases the standard deviation of the sample tends to increase, samples exhibiting < 15% inhibition should be interpreted with care, particularly since municipal SE alone tends to exhibit minimal to no inhibition.
3. Since the efficiency of toxicity removal was found to be potentially dependent on the specific wastewater mixtures, more study on the efficiency of toxicity removal for equivalent ozone doses on wastewaters containing different mixtures of CECs is warranted, both to confirm this theory, and to better understand the differences in the efficiencies of toxicity removal seen in this PhD. This might include testing:
    a. different ozone feed concentrations to build upon the existing data about the efficiency of toxicity reduction
    b. different mixtures of CECs in different SEs to further investigate if the efficiency of toxicity removal is specific to the wastewater composition.

    If this theory is confirmed, WWTPs using ozonation could, by means of the LuminoTox and a battery of bioassays, optimize this treatment for toxicity removal instead of CEC removal,
4. Further exploration of the sensitivity of current and new biosensors towards different CECs in wastewater is warranted. Results from this PhD thesis indicated that SAPS I and SAPS II had similar sensitivities for certain CECs in wastewater, however a larger range of CECs could be tested. Aquacion Inc. conducted preliminary minimum threshold level studies using new biosensors SAPS III (*Dunaliella tertiolecta*), a marine algae species, and SAPS IV (*Anabaena flos-aquae*), a cyanobacteria. These algae have demonstrated a higher sensitivity to certain CECs over currently available biosensors. Furthermore, SAPS III

could expand the use of the LuminoTox for applications involving seawater, for example, the reclamation of wastewater from sea.

5. Further development and method optimization of sample pre-concentration for the LuminoTox is warranted. This might include investigating
    a. different concentration factors
    b. the masking of toxicity of spiked CECs in the concentrated SE
    c. the ability to concentrate and to distinguish native CECs from their SE matrix; this would bring the sample pre-concentration process closer to being practical for use with the LuminoTox in WWTPs.

10. BIBLIOGRAPHY

Abdelmelek, S. B., Greaves, J., Ishida, K. P., Cooper, W. J., & Song, W. (2011). Removal of Pharmaceutical and Personal Care Products from Reverse Osmosis Retentate Using Advanced Oxidation Processes. *Environmental Science & Technology, 45*(8), 3665-3671. doi:http://dx.doi.org/10.1021/es104287n

Acero, J. L., Benitez, F. J., Leal, A. I., Real, F. J., & Teva, F. (2010a). Membrane filtration technologies applied to municipal secondary effluents for potential reuse. *Journal of Hazardous materials, 177*(1–3), 390-398. doi:http://dx.doi.org/10.1016/j.jhazmat.2009.12.045

Acero, J. L., Benitez, F. J., Teva, F., & Leal, A. I. (2010b). Retention of emerging micropollutants from UP water and a municipal secondary effluent by ultrafiltration and nanofiltration. *Chemical Engineering Journal, 163*(3), 264-272. doi:http://dx.doi.org/10.1016/j.cej.2010.07.060

Acero, J. L., Stemmler, K., & von Gunten, U. (2000). Degradation Kinetics of Atrazine and Its Degradation Products with Ozone and OH Radicals: A Predictive Tool for Drinking Water Treatment. *Environmental Science & Technology, 34*(4), 591-597. doi:http://dx.doi.org/10.1021/es990724e

Adeel, M., Song, X., Wang, Y., Francis, D., & Yang, Y. (2017). Environmental impact of estrogens on human, animal and plant life: A critical review. *Environment International, 99*, 107-119. doi:https://doi.org/10.1016/j.envint.2016.12.010

Adolfsson-Erici, M., Pettersson, M., Parkkonen, J., & Sturve, J. (2002). Triclosan, a commonly used bactericide found in human milk and in the aquatic environment in Sweden. *Chemosphere, 46*(9–10), 1485-1489. doi:http://dx.doi.org/10.1016/S0045-6535(01)00255-7

Ahel, M., & Jeličić, I. (2001). Phenazone Analgesics in Soil and Groundwater below a Municipal Solid Waste Landfill *Pharmaceuticals and Care Products in the Environment* (Vol. 791, pp. 100-115): American Chemical Society.

Al Qasmi, A. M. K. (2013). *The relative effects of the herbicide atrazine on selected microalgae.* (Doctorate of Philosophy), Tasmania.

Ali, N. S., Mo, K., & Kim, M. (2012). A case study on the relationship between conductivity and dissolved solids to evaluate the potential for reuse of reclaimed industrial wastewater. *KSCE Journal of Civil Engineering, 16*(5), 708-713. doi:http://dx.doi.org/10.1007/s12205-012-1581-x

Altenburger, R., Backhaus, T., Boedeker, W., Faust, M., & Scholze, M. (2013). Simplifying complexity: Mixture toxicity assessment in the last 20 years. *Environmental Toxicology and Chemistry, 32*(8), 1685-1687. doi:http://dx.doi.org/10.1002/etc.2294

Andreozzi, R., Caprio, V., Ciniglia, C., de Champdoré, M., Lo Giudice, R., Marotta, R., & Zuccato, E. (2004). Antibiotics in the environment: occurrence in Italian STPs, fate, and preliminary assessment on algal toxicity of amoxicillin. *Environmental Science & Technology, 38*(24), 6832-6838. doi:http://dx.doi.org/10.1021/es049509a

Angthararuk, D., Sutthivaiyakit, P., Blaise, C., Gagné, F., & Sutthivaiyakit, S. (2015). Photo-catalysis of bromacil under simulated solar light using Au/TiO2: evaluation of main

degradation products and toxicity implications. *Environmental Science and Pollution Research, 22*(2), 1468-1479. doi:http://dx.doi.org/10.1007/s11356-014-3425-3

Aquacion Inc. (2014). Micropollutant Threshold Values. In M. T. values (Ed.), (pp. PDF provided by company.).

Aronson, D., Weeks, J., Meylan, B., Guiney, P. D., & Howard, P. H. (2012). Environmental release, environmental concentrations, and ecological risk of N,N-Diethyl-m-toluamide (DEET). *Integrated Environmental Assessment and Management, 8*(1), 135-166. doi:http://dx.doi.org/10.1002/ieam.271

Arvai, A., Klecka, G., Jasim, S., Melcer, H., & Laitta, M. T. (2014). Protecting our Great Lakes: assessing the effectiveness of wastewater treatments for the removal of chemicals of emerging concern. *Water Quality Research Journal of Canada, 49*(1), 23-31. doi:http://dx.doi.org/10.2166/wqrjc.2013.104

Ascar, L., Ahumada, I., López, A., Quintanilla, F., & Leiva, K. (2013). Nonsteroidal anti-inflammatory drug determination in water samples by HPLC-DAD under isocratic conditions. *Journal of the Brazilian Chemical Society, 24*, 1160-1166. doi:http://dx.doi.org/10.5935/0103-5053.20130150

Ashikawa, I., Miyata, A., Koike, H., Inoue, Y., & Koyama, Y. (1986). Light-Induced Structural Change of beta Carotene in Thylakoid Membranes. *Journal of Biochemistry, 25*, 6154-6160. doi:http://dx.doi.org/10.1021/bi00368a049

Atekwana, E. A., Atekwana, E. A., Rowe, R. S., Werkema, D. D., & Legall, F. D. (2004). The relationship of total dissolved solids measurements to bulk electrical conductivity in an aquifer contaminated with hydrocarbon. *Journal of Applied Geophysics, 56*(4), 281-294. doi:http://dx.doi.org/10.1016/j.jappgeo.2004.08.003

Audenaert, W. (2014). *(Future) Regulation of TOrCs in WWTP Effluents as a Driver of Advanced Wastewater Treatment*

. Paper presented at the IOA-PAG Conference

, Montreal

Baalbaki, Z., Sultana, T., Maere, T., Vanrolleghem, P. A., Metcalfe, C. D., & Yargeau, V. (2016). Fate and mass balance of contaminants of emerging concern during wastewater treatment determined using the fractionated approach. *Science of the Total Environment, 573*, 1147-1158. doi:http://dx.doi.org/10.1016/j.scitotenv.2016.08.073

Baalbaki, Z., Sultana, T., Metcalfe, C., & Yargeau, V. (2017a). Estimating removals of contaminants of emerging concern from wastewater treatment plants: The critical role of wastewater hydrodynamics. *Chemosphere, 178*, 439-448. doi:https://doi.org/10.1016/j.chemosphere.2017.03.070

Baalbaki, Z., Torfs, E., Maere, T., Yargeau, V., & Vanrolleghem, P. A. (2017b). Dynamic modelling of solids in a full-scale activated sludge plant preceded by CEPT as a preliminary step for micropollutant removal modelling. *Bioprocess and Biosystems Engineering, 40*(4), 499-510. doi:http://dx.doi.org/10.1007/s00449-016-1715-5

Baalbaki, Z., Torfs, E., Yargeau, V., & Vanrolleghem, P. A. (2017c). Predicting the fate of micropollutants during wastewater treatment: Calibration and sensitivity analysis. *Science of the Total Environment, 601–602*, 874-885. doi:https://doi.org/10.1016/j.scitotenv.2017.05.072

Babić, S., Barišić, J., Višić, H., Klobučar, R. S., Popović, N. T., Strunjak-Perović, I., . . . Klobučar, G. (2017). Embryotoxic and genotoxic effects of sewage effluents in zebrafish embryo using multiple endpoint testing. *Water Research, 115*, 9-21. doi:http://doi.org/10.1016/j.watres.2017.02.049

Baek, S.-O., & Chang, I.-S. (2009). Pretreatments to control membrane fouling in membrane filtration of secondary effluents. *Desalination, 244*(1-3), 153-163. doi:http://dx.dio.org/10.1016/j.desal.2008.04.043

Baldwin, A. K., Corsi, S. R., De Cicco, L. A., Lenaker, P. L., Lutz, M. A., Sullivan, D. J., & Richards, K. D. (2016). Organic contaminants in Great Lakes tributaries: Prevalence and potential aquatic toxicity. *Science of the Total Environment, 554–555*, 42-52. doi:https://doi.org/10.1016/j.scitotenv.2016.02.137

Barber, J. (2012). *Photosystem II: The water-splitting enzyme of photosynthesis.* Paper presented at the Cold Spring Harbor Symposia on Quantitative Biology.

Behera, S. K., Kim, H. W., Oh, J.-E., & Park, H.-S. (2011). Occurrence and removal of antibiotics, hormones and several other pharmaceuticals in wastewater treatment plants of the largest industrial city of Korea. *Science of the Total Environment, 409*(20), 4351-4360. doi:http://dx.doi.org/10.1016/j.scitotenv.2011.07.015

Belfroid, A. C., van Drunen, M., Beek, M. A., Schrap, S. M., van Gestel, C. A. M., & van Hattum, B. (1998). Relative risks of transformation products of pesticides for aquatic ecosystems. *Science of the Total Environment, 222*(3), 167-183. doi:https://doi.org/10.1016/S0048-9697(98)00298-8

Bellemare, F., Rouette, M.-E., Lorrain, L., Perron, E., & Boucher, N. (2006). Combined use of photosynthetic enzyme complexes and microalgal photosynthetic systems for rapid screening of wastewater toxicity. *Environmental Toxicology, 21*(5), 445-449. doi:http://dx.doi.org/10.1002/tox.20205

Bendz, D., Paxéus, N. A., Ginn, T. R., & Loge, F. J. (2005). Occurrence and fate of pharmaceutically active compounds in the environment, a case study: Höje River in Sweden. *Journal of Hazardous materials, 122*(3), 195-204. doi:http://dx.doi.org/10.1016/j.jhazmat.2005.03.012

Benitez, F. J., Acero, J. L., Garcia-Reyes, J. F., Real, F. J., Roldan, G., Rodriguez, E., & Molina-Díaz, A. (2013). Determination of the Reaction Rate Constants and Decomposition Mechanisms of Ozone with Two Model Emerging Contaminants: DEET and Nortriptyline. *Industrial & Engineering Chemistry Research, 52*(48), 17064-17073. doi:http://dx.doi.org/10.1021/ie402916u

Benitez, F. J., Acero, J. L., Real, F. J., & Roman, S. (2004a). Oxidation of MCPA and 2,4-d by UV Radiation, Ozone, and the Combinations UV/H2O2 and O3/H2O2. *Journal of Environmental Science and Health, Part B, 39*(3), 393-409. doi:http://dx.doi.org/10.1081/PFC-120035925

Benitez, F. J., Acero, J. L., Real, F. J., & Roman, S. (2004b). Oxidation of MCPA and 2, 4-D by UV radiation, ozone, and the combinations UV/H2O2 and O3/H2O2. *Journal of Environmental Science and Health, Part B, 39*(3), 393-409. doi:http://dx.doi.org/10.1081/PFC-120035925

Bernier, M., & Carpentier, R. (1995). The action of mercury on the binding of the extrinsic polypeptides associated with the water oxidizing complex of photosystem II. *FEBS Letters, 360*(3), 251-254. doi:http://dx.doi.org/10.1016/0014-5793(95)00101-E

Bernier, M., Popovic, R., & Carpentier, R. (1993). Mercury inhibition at the donor side of photosystem II is reversed by chloride. *FEBS Letters, 321*(1), 19-23. doi:http://dx.doi.org/10.1016/0014-5793(93)80612-X

Berthod, L., Roberts, G., Sharpe, A., Whitley, D. C., Greenwood, R., & Mills, G. A. (2016). Effect of sewage sludge type on the partitioning behaviour of pharmaceuticals: a meta-analysis. *Environmental Science: Water Research & Technology, 2*(1), 154-163. doi:http://dx.doi.org/10.1039/C5EW00171D

Bettinetti, R., Giarei, C., & Provini, A. (2003). Chemical analysis and sediment toxicity bioassays to assess the contamination of the River Lambro (Northern Italy). *Archives of Environmental Contamination and Toxicology, 45*(1), 72-78. doi:http://dx.doi.org/10.1007/s00244-002-0126-6

Bharti, S., & Banerjee, T. K. (2013). Bioassay analysis of efficacy of phytoremediation in decontamination of coal mine effluent. *Ecotoxicology and Environmental Safety, 92*, 312-319. doi:http://dx.doi.org/10.1016/j.ecoenv.2013.03.004

Blair, B. D., Crago, J. P., Hedman, C. J., & Klaper, R. D. (2013). Pharmaceuticals and personal care products found in the Great Lakes above concentrations of environmental concern. *Chemosphere, 93*(9), 2116-2123. doi:http://dx.doi.org/10.1016/j.chemosphere.2013.07.057

Blaise, C., Gagne, F., Ferard, J., & Eullaffroy, P. (2008). Ecotoxicity of selected nano-materials to aquatic organisms. *Environmental Toxicology, 23*(5), 591-598. doi:http://dx.doi.org/10.1002/tox.20402

Bolong, N., Ismail, A. F., Salim, M. R., & Matsuura, T. (2009). A review of the effects of emerging contaminants in wastewater and options for their removal. *Desalination, 239*(1), 229-246. doi:http://dx.doi.org/10.1016/j.desal.2008.03.020

Boltes, K., Rosal, R., & García-Calvo, E. (2012). Toxicity of mixtures of perfluorooctane sulphonic acid with chlorinated chemicals and lipid regulators. *Chemosphere, 86*(1), 24-29. doi:http://dx.doi.org/10.1016/j.chemosphere.2011.08.041

Boreen, A. L., Arnold, W. A., & McNeill, K. (2003). Photodegradation of pharmaceuticals in the aquatic environment: a review. *Aquatic Sciences, 65*(4), 320-341. doi:http://dx.doi.org/10.1007/s00027-003-0672-7

Boucher, N., & Carpentier, R. (1999a). Hg2+, Cu2+, and Pb2+ -induced changes in Photosystem II photochemical yield and energy storage in isolated thylakoid membranes: A study using simultaneous fluorescence and photoacoustic measurements. *Photosynthesis Research, 59*(2), 167-174. doi:http://dx.doi.org/10.1023/a:1006194621553

Boucher, N., & Carpentier, R. (1999b). Hg2+, Cu2+, and Pb2+-induced changes in photosystem II photochemical yield and energy storage in isolated thylakoid membranes: A study using simultaneous fluorescence and photoacoustic measurements. *Photosynthesis Research, 59*(2-3), 167-174. doi:http://dx.doi.org/10.1023/a:1006194621553

Bound, J. P., & Voulvoulis, N. (2005). Household disposal of pharmaceuticals as a pathway for aquatic contamination in the United Kingdom. *Environmental Health Perspectives*, 1705-1711. doi:http://dx.doi.org/10.1289/ehp.8315

Brain, R. A., Ramirez, A. J., Fulton, B. A., Chambliss, C. K., & Brooks, B. W. (2008). Herbicidal Effects of Sulfamethoxazole in Lemna gibba: Using p-Aminobenzoic Acid As a Biomarker of Effect. *Environmental Science & Technology, 42*(23), 8965-8970. doi:http://dx.doi.org/10.1021/es801611a

Brown, P. L., & Markich, S. J. (2000). Evaluation of the free ion activity model of metal-organism interaction: extension of the conceptual model. *Aquatic Toxicology, 51*(2), 177-194. doi:http://dx.doi.org/10.1016/S0166-445X(00)00115-6

Brozinski, J.-M., Lahti, M., Meierjohann, A., Oikari, A., & Kronberg, L. (2013). The Anti-Inflammatory Drugs Diclofenac, Naproxen and Ibuprofen are found in the Bile of Wild Fish Caught Downstream of a Wastewater Treatment Plant. *Environmental Science & Technology, 47*(1), 342-348. doi:http://dx.doi.org/10.1021/es303013j

Brun, G. L., Bernier, M., Losier, R., Doe, K., Jackman, P., & Lee, H.-B. (2006). Pharmaceutically active compounds in Atlantic Canadian sewage treatment plant effluents and receiving waters, and potential for environmental effects as measured by acute and chronic Aquatic Toxicity. *Environmental Toxicology and Chemistry, 25*(8), 2163-2176. doi:http://dx.doi.org/10.1897/05-426R.1

Brust, K., Licht, O., Hultsch, V., Jungmann, D., & Nagel, R. (2001). Effects of terbutryn on aufwuchs and Lumbriculus variegatus in artificial indoor streams. *Environmental Toxicology and Chemistry, 20*(9), 2000-2007. doi:http://dx.doi.org/10.1002/etc.5620200919

Buffle, M.-O., Schumacher, J., Meylan, S., Jekel, M., & von Gunten, U. (2006). Ozonation and Advanced Oxidation of Wastewater: Effect of O3 Dose, pH, DOM and HO•-Scavengers on Ozone Decomposition and HO• Generation. *Ozone: Science & Engineering, 28*(4), 247-259. doi:http://dx.doi.org/10.1080/01919510600718825

Burga Pérez, K. F., Charlatchka, R., & Férard, J.-F. (2013). Assessment of the LuminoTox leachate phase assay as a complement to the LuminoTox solid phase assay: Effect of fine particles in natural sediments. *Chemosphere, 90*(3), 1310-1315. doi:http://dx.doi.org/10.1016/j.chemosphere.2012.09.078

Burga-Perez, K. F., Toumi, H., Cotelle, S., Ferard, J.-F., & Radetski, C. M. (2012). Sensitivity of different aquatic bioassays in the assessment of a new natural formicide. *Journal of Environmental Science and Health, Part B, 48*(1), 57-62. doi:http://dx.doi.org/10.1080/03601234.2012.716731

Burkhardt-Holm, P., Segner, H., Burki, R., Peter, A., Schubert, S., Suter, M. J. F., & Borsuk, M. E. (2008). Estrogenic Endocrine Disruption in Switzerland: Assessment of Fish Exposure and Effects. *CHIMIA International Journal for Chemistry, 62*(5), 376-382. doi:http://dx.doi.org/10.2533/chimia.2008.376

Buth, J. M., Ross, M. R., McNeill, K., & Arnold, W. A. (2011). Reprint of: Removal and formation of chlorinated triclosan derivatives in wastewater treatment plants using chlorine and UV disinfection. *Chemosphere, 85*(2), 284-289. doi:http://dx.doi.org/10.1016/j.chemosphere.2011.09.003

Cabeza, Y., Candela, L., Ronen, D., & Teijon, G. (2012). Monitoring the occurrence of emerging contaminants in treated wastewater and groundwater between 2008 and 2010. The Baix Llobregat (Barcelona, Spain). *Journal of Hazardous materials, 239–240*, 32-39. doi:https://doi.org/10.1016/j.jhazmat.2012.07.032

Cahill, M. G., Caprioli, G., Stack, M., Vittori, S., & James, K. J. (2011). Semi-automated liquid chromatography–mass spectrometry (LC–MS/MS) method for basic pesticides in wastewater effluents. *Analytical and Bioanalytical Chemistry, 400*(2), 587-594. doi:http://dx.doi.org/10.1007/s00216-011-4781-1

Calabrese, E. J., & Baldwin, L. A. (2002). Applications of hormesis in toxicology, risk assessment and chemotherapeutics. *Trends in Pharmacological Sciences, 23*(7), 331-337. doi:http://dx.doi.org/10.1016/S0165-6147(02)02034-5

Campos, D., Gravato, C., Quintaneiro, C., Koba, O., Randak, T., Soares, A. M. V. M., & Pestana, J. L. T. (2016). Are insect repellents toxic to freshwater insects? A case study using caddisflies exposed to DEET. *Chemosphere, 149*, 177-182. doi:https://doi.org/10.1016/j.chemosphere.2016.01.098

Cao, J. J., & Govindjee. (1990). Chlorophyll a fluorescence transient as an indicator of active and inactive Photosystem II in thylakoid membranes. *Biochimica et Biophysica Acta, 1015*(2), 180-188. doi:https://doi.org/10.1016/0005-2728(90)90018-Y

Carballa, M., Omil, F., Lema, J. M., Llompart, M. a., García-Jares, C., Rodríguez, I., . . . Ternes, T. (2004). Behavior of pharmaceuticals, cosmetics and hormones in a sewage treatment plant. *Water Research, 38*(12), 2918-2926. doi:http://dx.doi.org/10.1016/j.watres.2004.03.029

Carlson, J. C., Anderson, J. C., Low, J. E., Cardinal, P., MacKenzie, S. D., Beattie, S. A., . . . Hanson, M. L. (2013). Presence and hazards of nutrients and emerging organic micropollutants from sewage lagoon discharges into Dead Horse Creek, Manitoba, Canada. *Science of the Total Environment, 445–446*, 64-78. doi:http://dx.doi.org/10.1016/j.scitotenv.2012.11.100

Cassidy, D. P., Hudak, A. J., Werkema, D. D., Atekwana, E. A., Rossbach, S., Duris, J. W., . . . William, A. (2002). In Situ Rhamnolipid Production at an Abandoned Petroleum Refinery. *Soil and Sediment Contamination: An International Journal, 11*(5), 769-787. doi:http://dx.doi.org/10.1080/20025891107087

Celiz, M. D., Tso, J., & Aga, D. S. (2009). Pharmaceutical metabolites in the environment: analytical challenges and ecological risks. *Environmental Toxicology and Chemistry, 28*(12), 2473-2484. doi:http://dx.doi.org/10.1897/09-173.1

Chen, C., Wang, Y., Zhao, X., Wang, Q., & Qian, Y. (2013). The combined toxicity assessment of carp (Cyprinus carpio) acetylcholinesterase activity by binary mixtures of chlorpyrifos and four other insecticides. *Ecotoxicology*, 1-8. doi:https://doi.org/10.1371/journal.pone.0096580

Chen, X., Richard, J., Liu, Y. J., Dopp, E., Tuerk, J., & Bester, K. (2012). Ozonation products of triclosan in advanced wastewater treatment. *Water Research, 46*(7), 2247-2256. doi:http://dx.doi.org/10.1016/j.watres.2012.01.039

Christou, A., Karaolia, P., Hapeshi, E., Michael, C., & Fatta-Kassinos, D. (2017). Long-term wastewater irrigation of vegetables in real agricultural systems: Concentration of pharmaceuticals in soil, uptake and bioaccumulation in tomato fruits and human health risk assessment. *Water Research, 109*, 24-34. doi:https://doi.org/10.1016/j.watres.2016.11.033

Chusaksri, S., Sutthivaiyakit, P., Blaise, C., & Gagne, F. (2010). Pesticide toxicity screening of aqueous samples: insites into an appropriate test battery *Fresenius Environmental Bulletin, 19*(10), 2253-2258. doi:http://www.psp-parlar.de/

Cicek, N., Londry, K., Oleszkiewicz, J. A., Wong, D., & Lee, Y. (2007). Removal of Selected Natural and Synthetic Estrogenic Compounds in a Canadian Full-Scale Municipal Wastewater Treatment Plant. *Water Environment Research, 79*(7), 795-800. doi:http://dx.doi.org/10.2175/106143007X17

Cleuvers, M. (2003). Aquatic ecotoxicity of pharmaceuticals including the assessment of combination effects. *Toxicology Letters, 142*(3), 185-194. doi:http://dx.doi.org/10.1016/S0378-4274(03)00068-7

Clijsters, H., & Van Assche, F. (1985). Inhibition of photosynthesis by heavy metals. *Photosynthesis Research, 7*(1), 31-40. doi:http://dx.doi.org/10.1007/bf00032920

Connon, R. E., Geist, J., & Werner, I. (2012). Effect-Based Tools for Monitoring and Predicting the Ecotoxicological Effects of Chemicals in the Aquatic Environment. *Sensors (Basel, Switzerland), 12*(9), 12741-12771. doi:http://dx.doi.org/10.3390/s120912741

Conrad, R., Büchel, C., Wilhelm, C., Arsalane, W., Berkaloff, C., & Duval, J.-C. (1993). Changes in yield ofin-vivo fluorescence of chlorophyll a as a tool for selective herbicide monitoring. *Journal of Applied Phycology, 5*(5), 505-516.

Costanzo, S. D., Watkinson, A. J., Murby, E. J., Kolpin, D. W., & Sandstrom, M. W. (2007). Is there a risk associated with the insect repellent DEET (N,N-diethyl-m-toluamide) commonly found in aquatic environments? *Science of the Total Environment, 384*(1–3), 214-220. doi:http://dx.doi.org/10.1016/j.scitotenv.2007.05.036

Couling, D. J., Bernot, R. J., Docherty, K. M., Dixon, J. K., & Maginn, E. J. (2006). Assessing the factors responsible for ionic liquid toxicity to aquatic organisms via quantitative structure–property relationship modeling. *Green Chemistry, 8*(1), 82-90. doi:http://dx.doi.org/10.1039/B511333D

Čučak, D. I., Spasojević, J. M., Babić, O. B., Maletić, S. P., Simeunović, J. B., Rončević, S. D., . . . Radnović, D. V. (2017). A chemical and microbiological characterization and toxicity assessment of the Pančevo industrial complex wastewater canal sediments, Serbia. *Environmental Science and Pollution Research, 24*(9), 8458-8468. doi:http://dx.doi.org/10.1007/s11356-017-8513-8

Czerpak, R., Piotrowska, A., Dobrzyń, P., Tatur, A., & Marczuk, M. (2003). The effect of biochanin A on the chlorophylls and carotenoids content in the alga Chlorella vulgaris Beijerinck. *Acta Physiologiae Plantarum, 25*(3), 271-278. doi:http://dx.doi.org/10.1007/s11738-003-0008-1

Daughton, C. G. (2004). Non-regulated water contaminants: emerging research. *Environmental Impact Assessment Review, 24*(7–8), 711-732. doi:http://dx.doi.org/10.1016/j.eiar.2004.06.003

De la Cruz, N., Dantas, R., Giménez, J., & Esplugas, S. (2013). Photolysis and TiO 2 photocatalysis of the pharmaceutical propranolol: solar and artificial light. *Applied Catalysis B: Environmental, 130*, 249-256. doi:http://dx.doi.org/10.1016/j.apcatb.2012.10.003

De Luca, A., Dantas, R. F., Simões, A. S. M., Toscano, I. A. S., Lofrano, G., Cruz, A., & Esplugas, S. (2013). Atrazine Removal in Municipal Secondary Effluents by Fenton and Photo-Fenton

Treatments. *Chemical Engineering & Technology, 36*(12), 2155-2162. doi:http://dx.doi.org/10.1002/ceat.201300135
De Nicola, E., Meriç, S., Gallo, M., Iaccarino, M., Della Rocca, C., Lofrano, G., . . . Pagano, G. (2007). Vegetable and synthetic tannins induce hormesis/toxicity in sea urchin early development and in algal growth. *Environmental Pollution, 146*(1), 46-54. doi:http://dx.doi.org/10.1016/j.envpol.2006.06.018
Debenest, T., Gagné, F., Petit, A.-N., André, C., Kohli, M., & Blaise, C. (2010). Ecotoxicity of a brominated flame retardant (tetrabromobisphenol A) and its derivatives to aquatic organisms. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology, 152*(4), 407-412. doi:http://dx.doi.org/10.1016/j.cbpc.2010.06.009
Dellamatrice, P. M., Monteiro, R. T. R., Blaise, C., Slabbert, J. L., Gagné, F., & Alleau, S. (2006). Toxicity assessment of reference and natural freshwater sediments with the LuminoTox assay. *Environmental Toxicology, 21*(4), 395-402. doi:http://dx.doi.org/10.1002/tox.20186
Dewez, D., Boucher, N., Bellemare, F., & Popovic, R. (2007). Use of different fluorometric systems in the determination of fluorescence parameters from spinach thylakoid membranes being exposed to atrazine and copper. *Toxicological and Environ Chemistry, 89*(4), 655-664. doi:http://dx.doi.org/10.1080/02772240701561577
Di Toro, D. M., McGrath, J. A., & Hansen, D. J. (2000). Technical basis for narcotic chemicals and polycyclic aromatic hydrocarbon criteria. I. Water and tissue. *Environmental Toxicology and Chemistry, 19*(8), 1951-1970. doi:http://dx.doi.org/10.1002/etc.5620190803
Diamond, J. M., Latimer, H. A., Munkittrick, K. R., Thornton, K. W., Bartell, S. M., & Kidd, K. A. (2011). Prioritizing contaminants of emerging concern for ecological screening assessments. *Environmental Toxicology and Chemistry, 30*(11), 2385-2394. doi:http://dx.doi.org/10.1002/etc.667
Dodd, M. C., Buffle, M.-O., & von Gunten, U. (2006). Oxidation of Antibacterial Molecules by Aqueous Ozone: Moiety-Specific Reaction Kinetics and Application to Ozone-Based Wastewater Treatment. *Environmental Science & Technology, 40*(6), 1969-1977. doi:http://dx.doi.org/10.1021/es051369x
Doussantousse, É. (2014, July 11, 2014) *Facts and History surronding the LuminoTox assay/Interviewer: M. Marshall*. McGill University, Montreal QC.
Dyer, S. D., White-Hull, C. E., & Shephard, B. K. (2000). Assessments of chemical mixtures via toxicity reference values overpredict hazard to Ohio fish communities. *Environmental Science & Technology, 34*(12), 2518-2524. doi:http://dx.doi.org/10.1021/es991160h
Environment Canada. (2002). *Biological test method: solid-phase reference method for determining the toxicity of sediment using luminescent bacteria (Vibrio fischeri), Environmental Protection Series, Report EPS 1/RM/42*. Retrieved from Ottawa ON:
Environment Canada. (2005). *Luminotox: A tool for rapid toxicity testing*. (ISBN 0-662-39710-X). Montreal, Quebec: Minister of the Environment.
EPA. (1999). *Guidance Mannual, Alternative Disinfectants and Oxidants; Ozone (EPA-R-99-014)*.
EPA. (2010). *Treating Contaminant of Emmerging Concern, A litterature review database*. Retrieved from Washington DC:
Escher, B. I., Allinson, M., Altenburger, R., Bain, P. A., Balaguer, P., Busch, W., . . . Leusch, F. D. L. (2013). Benchmarking Organic Micropollutants in Wastewater, Recycled Water and

Drinking Water with In Vitro Bioassays. *Environmental Science & Technology, 48*(3), 1940-1956. doi:http://dx.doi.org/10.1021/es403899t

Escher, B. I., Bramaz, N., Eggen, R. I. L., & Richter, M. (2005). In Vitro Assessment of Modes of Toxic Action of Pharmaceuticals in Aquatic Life. *Environmental Science & Technology, 39*(9), 3090-3100. doi:http://dx.doi.org/10.1021/es048590e

Escher, B. I., Bramaz, N., Mueller, J. F., Quayle, P., Rutishauser, S., & Vermeirssen, E. L. M. (2008a). Toxic equivalent concentrations (TEQs) for baseline toxicity and specific modes of action as a tool to improve interpretation of ecotoxicity testing of environmental samples. *Journal of Environmental Monitoring, 10*(5), 612-621. doi:http://dx.doi.org/10.1039/B800949J

Escher, B. I., Bramaz, N., Quayle, P., Rutishauser, S., & Vermeirssen, E. L. M. (2008b). Monitoring of the ecotoxicological hazard potential by polar organic micropollutants in sewage treatment plants and surface waters using a mode-of-action based test battery. *Journal of Environmental Monitoring, 10*(5), 622-631. doi:http://dx.doi.org/10.1039/b800951a

Escher, B. I., Lawrence, M., Macova, M., Mueller, J. F., Poussade, Y., Robillot, C., . . . Gernjak, W. (2011). Evaluation of Contaminant Removal of Reverse Osmosis and Advanced Oxidation in Full-Scale Operation by Combining Passive Sampling with Chemical Analysis and Bioanalytical Tools. *Environmental Science & Technology, 45*(12), 5387-5394. doi:http://dx.doi.org/10.1021/es201153k

Escher, B. I., Pronk, W., Suter, M. J. F., & Maurer, M. (2006). Monitoring the removal efficiency of pharmaceuticals and hormones in different treatment processes of source-separated urine with bioassays. *Environmental Science & Technology, 40*(16), 5095-5101. doi:http://dx.doi.org/10.1021/es060598w

Fai, P. B., Grant, A., & Reid, B. (2007). Chlorophyll a fluorescence as a biomarker for rapid toxicity assessment. *Environmental Toxicology and Chemistry, 26*(7), 1520-1531. doi:http://dx.doi.org/10.1897/06-394R1.1

Fang, Y., Karnjanapiboonwong, A., Chase, D. A., Wang, J., Morse, A. N., & Anderson, T. A. (2012). Occurrence, fate, and persistence of gemfibrozil in water and soil. *Environmental Toxicology and Chemistry, 31*(3), 550-555. doi:http://dx.doi.org/10.1002/etc.1725

Feito, R., Valcárcel, Y., & Catalá, M. (2013). Preliminary data suggest that venlafaxine environmental concentrations could be toxic to plants. *Chemosphere, 90*(7), 2065-2069. doi:https://doi.org/10.1016/j.chemosphere.2012.09.028

Fenske, M., Maack, G., Schäfers, C., & Segner, H. (2005). An environmentally relevant concentration of estrogen induces arrest of male gonad development in zebrafish, Danio rerio. *Environmental Toxicology and Chemistry, 24*(5), 1088-1098. doi:http://dx.doi.org/10.1897/04-096R1.1

Férard, J., Burga Pérez, K., Blaise, C., Péry, A., Sutthivaiyakit, P., & Gangné, F. (2015). Microscale ecotoxicity testing of Moselle river watershed (Lorraine Province, France) sediments. *Journal of Xenobiotics, 5*(1), 1-7. doi:https://dx.doi.org/10.4081/xeno.2015.5125

Fernandez, M. P., Ikonomou, M. G., & Buchanan, I. (2007). An assessment of estrogenic organic contaminants in Canadian wastewaters. *Science of the Total Environment, 373*(1), 250-269. doi:http://dx.doi.org/10.1016/j.scitotenv.2006.11.018

Francs, C. G. (2006). *Phytoremediation of Pharmaceuticals With Salix exigua.* (Master of Applied Science), University of Lethbridge, Lethbridge, Canada.

Franz, S., Altenburger, R., Heilmeier, H., & Schmitt-Jansen, M. (2008). What contributes to the sensitivity of microalgae to triclosan? *Aquatic Toxicology, 90*(2), 102-108. doi:http://dx.doi.org/10.1016/j.aquatox.2008.08.003

Fuerst, E. P., & Norman, M. A. (1991). Interactions of herbicides with photosynthetic electron transport. *Weed Science, 39*(3), 458-464. doi: http://www.jstor.org/stable/4044979

Gaultier, J., Farenhorst, A., & Cathcart, J. (2008). Regional Assessment of Herbicide Sorption and Degradation in Two Sampling Years. *Journal of Environmental Quality, 37*(5), 1825-1836. doi:http://dx.doi.org/10.2134/jeq2007.0578

Gerrity, D., Pisarenko, A. N., Marti, E., Trenholm, R. A., Gerringer, F., Reungoat, J., & Dickenson, E. (2015). Nitrosamines in pilot-scale and full-scale wastewater treatment plants with ozonation. *Water Research, 72*, 251-261. doi:http://dx.doi.org/10.1016/j.watres.2014.06.025

Gesuale, G., Liechti, P.-A., Fournier, M., Payment, P., Gagnon, C., & Hausler, R. (2010). Ozone: A wastewater disinfectant of the future. *Water Environment & Technology*. doi:https://doi.org/10.2175/193864710798182637

Ghayenia, S. B. S., Beatsona, P. J., R.P.Schneiderb, & Fanea, A. G. (1998). Water reclamation from municipal wastewater using combined microfiltration-reverse osmosis (ME-RO): Preliminary performance dataandmicrobiological aspects of

system operation. *Desalination, 116*, 65-80. doi:http://dx.doi.org/10.1016/S0011-9164(98)00058-7

Gimeno, O., Aguinaco, A., Rey, A., Beltrán Novillo, F. J., & Toledo, J. R. (2014). Ozonation of 4-chloro-2-methylphenoxyacetic acid (MCPA) in an activated sludge system. *Journal of Chemical Technology and Biotechnology, 89*(8), 1219-1227. doi:http://dx.doi.org/10.1002/jctb.4351

Glaze, W. H., & Kang, J.-W. (1988). Advanced oxidation processes for treating groundwater contaminated with TCE and PCE: laboratory studies. *Journal (American Water Works Association)*, 57-63.

Göbel, A., McArdell, C. S., Joss, A., Siegrist, H., & Giger, W. (2007). Fate of sulfonamides, macrolides, and trimethoprim in different wastewater treatment technologies. *Science of the Total Environment, 372*(2–3), 361-371. doi:http://dx.doi.org/10.1016/j.scitotenv.2006.07.039

Gomes, J., Costa, R., Quinta-Ferreira, R. M., & Martins, R. C. (2017). Application of ozonation for pharmaceuticals and personal care products removal from water. *Science of the Total Environment, 586*, 265-283. doi:https://doi.org/10.1016/j.scitotenv.2017.01.216

Guang, Y., Maohong, F., & Guangming, Z. (2014). Emerging contaminants in surface waters in China—a short review. *Environmental Research Letters, 9*(7), 074018. doi:http://dx.doi.org/10.1088/1748-9326/9/7/074018

Gunnarsson, L., Adolfsson-Erici, M., Björlenius, B., Rutgersson, C., Förlin, L., & Larsson, D. G. J. (2009). Comparison of six different sewage treatment processes—Reduction of estrogenic substances and effects on gene expression in exposed male fish. *Science of the Total Environment, 407*(19), 5235-5242. doi:http://dx.doi.org/10.1016/j.scitotenv.2009.06.018

Haase, S. M., Panas, P., Rath, T., & Huchzermeyer, B. (2015). Effects of Carbamazepine on Two Microalgae Species Differing in Stress Resistance. *Water, Air, & Soil Pollution, 226*(10), 328. doi:http://dx.doi.org/10.1007/s11270-015-2562-8

Halling-Sørensen, B., Nors Nielsen, S., Lanzky, P. F., Ingerslev, F., Holten Lützhøft, H. C., & Jørgensen, S. E. (1998). Occurrence, fate and effects of pharmaceutical substances in the environment- A review. *Chemosphere, 36*(2), 357-393. doi:http://dx.doi.org/10.1016/S0045-6535(97)00354-8

Harding, L. B., Schultz, I. R., da Silva, D. A. M., Ylitalo, G. M., Ragsdale, D., Harris, S. I., . . . Swanson, P. (2016). Wastewater treatment plant effluent alters pituitary gland gonadotropin mRNA levels in juvenile coho salmon (Oncorhynchus kisutch). *Aquatic Toxicology, 178*, 118-131. doi:http://dx.doi.org/10.1016/j.aquatox.2016.07.013

Harris, G. D., Adams, V. D., Sorensen, D. L., & Dupont, R. R. (1987). The Influence of Photoreactivation and Water Quality on Ultraviolet Disinfection of Secondary Municipal Wastewater. *Journal (Water Pollution Control Federation), 59*(8), 781-787.

Heberer, T. (2002). Occurrence, fate, and removal of pharmaceutical residues in the aquatic environment: a review of recent research data. *Toxicology Letters, 131*(1–2), 5-17. doi:http://dx.doi.org/10.1016/S0378-4274(02)00041-3

Hem, J. D. (1985). *Study and Interpretation of the Chemical Characteristics of Natural Water* (3rd ed. Vol. 2254). Washington DC: U.S. Geological Survey Water-Supply Paper.

Hemachandra, C. K., & Pathiratne, A. (2017). Cytogenotoxicity screening of source water, wastewater and treated water of drinking water treatment plants using two in vivo test systems: Allium cepa root based and Nile tilapia erythrocyte based tests. *Water Research, 108*, 320-329. doi:http://dx.doi.org/10.1016/j.watres.2016.11.009

Henze, M., Loosdrecht, M. C. M. v., & Brdjanovic, G. A. E. D. (2008). *Biological Wastewater Treatment: Principles in Modelling and Design*. London: IWA Publishing.

Hiriart-Baer, V. P., Fortin, C., Lee, D.-Y., & Campbell, P. G. C. (2006). Toxicity of silver to two freshwater algae, Chlamydomonas reinhardtii and Pseudokirchneriella subcapitata, grown under continuous culture conditions: Influence of thiosulphate. *Aquatic Toxicology, 78*(2), 136-148. doi:http://dx.doi.org/10.1016/j.aquatox.2006.02.027

Hirsch, R., Ternes, T., Haberer, K., & Kratz, K.-L. (1999). Occurrence of antibiotics in the aquatic environment. *Science of the Total Environment, 225*(1), 109-118.

Hoigné, J., & Bader, H. (1976). The role of hydroxyl radical reactions in ozonation processes in aqueous solutions. *Water Research, 10*(5), 377-386. doi:http://dx.doi.org/10.1016/0043-1354(76)90055-5

Hollender, J., Zimmermann, S. G., Koepke, S., Krauss, M., McArdell, C. S., Ort, C., . . . Siegrist, H. (2009). Elimination of organic micropollutants in a municipal wastewater treatment plant upgraded with a full-scale post-ozonation followed by sand filtration. *Environmental Science & Technology, 43*(20), 7862-7869. doi:http://dx.doi.org/10.1021/es9014629

Holm, J. V., Ruegge, K., Bjerg, P. L., & Christensen, T. H. (1995). Occurrence and Distribution of Pharmaceutical Organic Compounds in the Groundwater Downgradient of a Landfill (Grindsted, Denmark). *Environmental Science & Technology, 29*(5), 1415-1420. doi:http://dx.doi.org/10.1021/es00005a039

Hörsing, M., Ledin, A., Grabic, R., Fick, J., Tysklind, M., Jansen, J. l. C., & Andersen, H. R. (2011). Determination of sorption of seventy-five pharmaceuticals in sewage sludge. *Water Research, 45*(15), 4470-4482. doi:http://dx.doi.org/10.1016/j.watres.2011.05.033

Hua, W. Y., Bennett, E. R., & Letcher, R. J. (2006a). Ozone treatment and the depletion of detectable pharmaceuticals and atrazine herbicide in drinking water sourced from the upper Detroit River, Ontario, Canada. *Water Research, 40*(12), 2259-2266. doi:https://doi.org/10.1016/j.watres.2006.04.033

Hua, W. Y., Bennett, E. R., Maio, X.-S., Metcalfe, C. D., & Letcher, R. J. (2006b). Seasonality effects on pharmaceuticals and s-triazine herbicides in wastewater effluent and surface water from the Canadian side of the upper Detroit River. *Environmental Toxicology and Chemistry, 25*(9), 2356-2365. doi:http://dx.doi.org/10.1897/05-571R.1

Huang, X., Tu, Y., Song, C., Li, T., Lin, J., Wu, Y., . . . Wu, C. (2016). Interactions between the antimicrobial agent triclosan and the bloom-forming cyanobacteria Microcystis aeruginosa. *Aquatic Toxicology, 172*, 103-110. doi:http://dx.doi.org/10.1016/j.aquatox.2016.01.002

Huber, M. M., Canonica, S., Park, G., & Gunten, U. v. (2003a). Oxidation of Pharmaceuticals during Ozonation and Advanced Oxidation Processes. *Environmental Science & Technology, 37*(5), 1016-1024. doi:http://dx.doi.org/10.1021/es025896h

Huber, M. M., Canonica, S., Park, G. Y., & von Gunten, U. (2003b). Oxidation of pharmaceuticals during ozonation and advanced oxidation processes. *Environ. Sci. Technol, 37*, 1016-1024. doi:http://dx.doi.org/10.1021/es025896h

Huber, M. M., Göbel, A., Joss, A., Hermann, N., Löffler, D., McArdell, C. S., . . . von Gunten, U. (2005a). *Environ. Sci. Technol, 39*, 4290. doi:http://dx.doi.org/10.1021/es048396s

Huber, M. M., Göbel, A., Joss, A., Hermann, N., LÖffler, D., McArdell, C. S., . . . von Gunten, U. (2005b). Oxidation of Pharmaceuticals during Ozonation of Municipal Wastewater Effluents: A Pilot Study. *Environmental Science & Technology, 39*(11), 4290-4299. doi:http://dx.doi.org/10.1021/es048396s

Huber, W. (1993). Ecotoxicological relevance of atrazine in aquatic systems. *Environmental Toxicology and Chemistry, 12*(10), 1865-1881. doi:http://dx.doi.org/10.1002/etc.5620121014

Ikehata, K., Gamal El-Din, M., & Snyder, S. A. (2008). Ozonation and Advanced Oxidation Treatment of Emerging Organic Pollutants in Water and Wastewater. *Ozone: Science & Engineering, 30*(1), 21-26. doi:http://dx.doi.org/10.1080/01919510701728970

International Trade Administration. (2016). 2016 Top Markets Report Pharmaceuticals; Overview and key findings. Retrieved from http://trade.gov/topmarkets/pdf/Pharmaceuticals_Executive_Summary.pdf

James, C. P., Germain, E., & Judd, S. (2014). Micropollutant removal by advanced oxidation of microfiltered secondary effluent for water reuse. *Separation and Purification Technology, 127*, 77-83. doi:http://dx.doi.org/10.1016/j.seppur.2014.02.016

Jarošová, B., Erseková, A., Hilscherová, K., Loos, R., Gawlik, B. M., Giesy, J. P., & Bláha, L. (2014). Europe-wide survey of estrogenicity in wastewater treatment plant effluents: the need for the effect-based monitoring. *Environmental Science and Pollution Research, 21*(18), 10970-10982. doi:http://dx.doi.org/10.1007/s11356-014-3056-8

Jasinska, E. J., Goss, G. G., Gillis, P. L., Kraak, G. J. V. D., Matsumoto, J., Machado, A. A. d. S., . . . Metcalfe, C. D. (2015a). Assessment of biomarkers for contaminants of emerging concern on aquatic organisms downstream of a municipal wastewater discharge. *Science of the Total Environment, 530-531*, 140-153. doi:http://dx.doi.org/10.1016/j.scitotenv.2015.05.080

Jasinska, E. J., Goss, G. G., Gillis, P. L., Van Der Kraak, G. J., Matsumoto, J., de Souza Machado, A. A., . . . Metcalfe, C. D. (2015b). Assessment of biomarkers for contaminants of emerging concern on aquatic organisms downstream of a municipal wastewater discharge. *Science of the Total Environment, 530–531*, 140-153. doi:http://dx.doi.org/10.1016/j.scitotenv.2015.05.080

Jelic, A., Gros, M., Ginebreda, A., Cespedes-Sánchez, R., Ventura, F., Petrovic, M., & Barcelo, D. (2011). Occurrence, partition and removal of pharmaceuticals in sewage water and sludge during wastewater treatment. *Water Research, 45*(3), 1165-1176. doi:http://dx.doi.org/10.1016/j.watres.2010.11.010

Jia, A., Escher, B. I., Leusch, F. D. L., Tang, J. Y. M., Prochazka, E., Dong, B., . . . Snyder, S. A. (2015). In vitro bioassays to evaluate complex chemical mixtures in recycled water. *Water Research, 80*, 1-11. doi:http://dx.doi.org/10.1016/j.watres.2015.05.020

Jiang, J.-Q., Zhou, Z., & Sharma, V. K. (2013). Occurrence, transportation, monitoring and treatment of emerging micro-pollutants in waste water — A review from global views. *Microchemical Journal, 110*, 292-300. doi:http://dx.doi.org/10.1016/j.microc.2013.04.014

Jones, O. A. H., Voulvoulis, N., & Lester, J. N. (2002). Aquatic environmental assessment of the top 25 English prescription pharmaceuticals. *Water Research, 36*(20), 5013-5022. doi:http://dx.doi.org/10.1016/S0043-1354(02)00227-0

Jonker, M. J., Svendsen, C., Bedaux, J. J. M., Bongers, M., & Kammenga, J. E. (2005). Significance testing of synergistic/antagonistic, dose level-dependent, or dose ratio-dependent effects in mixture dose-response analysis. *Environmental Toxicology and Chemistry, 24*(10), 2701-2713. doi:http://dx.doi.org/10.1897/04-431R.1

Juneau, P., Qiu, B., & Deblois, C. P. (2007). Use of chlorophyll fluorescence as a tool for determination of herbicide toxic effect: Review. *Toxicological and Environmental Chemistry, 89*(4), 609-625. doi:http://dx.doi.org/10.1080/02772240701561569

Kang, S. K., Choo, K. H., & Lim, K. H. (2003). Use of Iron Oxide Particles as Adsorbents to Enhance Phosphorus Removal from Secondary Wastewater Effluent. *Separation Science and Technology, 38*(15), 3853-3874. doi:http://dx/doi.org/10.1081/SS-120024236

Karthikeyan, K. G., & Meyer, M. T. (2006). Occurrence of antibiotics in wastewater treatment facilities in Wisconsin, USA. *Science of the Total Environment, 361*(1–3), 196-207. doi:http://dx.doi.org/10.1016/j.scitotenv.2005.06.030

Kasprzyk-Hordern, B., Dinsdale, R. M., & Guwy, A. J. (2009). The removal of pharmaceuticals, personal care products, endocrine disruptors and illicit drugs during wastewater treatment and its impact on the quality of receiving waters. *Water Research, 43*(2), 363-380. doi:http://dx.doi.org/10.1016/j.watres.2008.10.047

Kerr, J. L., Guo, Z., Smith, D. W., Goss, G. G., & Belosevic, M. (2008). Use of goldfish to monitor wastewater and reuse water for xenobiotics. *Journal of Environmental Engineering and Science, 7*(4), 369-383. doi:http://dx.doi.org/10.1139/S08-011

Khairy, M., Muir, D., Teixeira, C., & Lohmann, R. (2014). Spatial Trends, Sources, and Air–Water Exchange of Organochlorine Pesticides in the Great Lakes Basin Using Low Density Polyethylene Passive Samplers. *Environmental Science & Technology, 48*(16), 9315-9324. doi:http://dx.doi.org/10.1021/es501686a

Khan, G. A., Berglund, B., Khan, K. M., Lindgren, P.-E., & Fick, J. (2013). Occurrence and Abundance of Antibiotics and Resistance Genes in Rivers, Canal and near Drug Formulation Facilities – A Study in Pakistan. *PloS One, 8*(6), e62712. doi:http://dx.doi.org/10.1371/journal.pone.0062712

Kharariian, H. A., Siddiqi, R. H., & Farooq, S. (1981). Chemical treatment for reuse of waste water effluents in saudi arabia. *Desalination, 38*, 437-438. doi:http://dx.doi.org/10.1016/S0011-9164(00)86088-9

Kidd, K. A., Blanchfield, P. J., Mills, K. H., Palace, V. P., Evans, R. E., Lazorchak, J. M., & Flick, R. W. (2007). Collapse of a fish population after exposure to a synthetic estrogen. *Proceedings of the National Academy of Sciences, 104*(21), 8897-8901. doi:http://dx.doi.org/10.1073/pnas.0609568104

Kiguchi, O., Sato, G., & Kobayashi, T. (2016). Source-specific sewage pollution detection in urban river waters using pharmaceuticals and personal care products as molecular indicators. *Environmental Science and Pollution Research, 23*(22), 22513-22529. doi:http://dx.doi.org/10.1007/s11356-016-7437-z

Kinman, R. N., & Rempel, G. (1975). Water and wastewater disinfection with ozone: A critical review. *C R C Critical Reviews in Environmental Control, 5*(1), 141-152. doi:http://dx.doi.org/10.1080/10643387509381625

Klamerth, N. (2011). *Application of a solar photo-fenton for the treatment of contaminants in municipal watewater effluents* (Doctorate), University of Almeria, Almeria, Spain.

Knauert, S., Singer, H., Hollender, J., & Knauer, K. (2010). Phytotoxicity of atrazine, isoproturon, and diuron to submersed macrophytes in outdoor mesocosms. *Environmental Pollution, 158*(1), 167-174. doi:http://dx.doi.org/10.1016/j.envpol.2009.07.023

Köck-Schulmeyer, M., Villagrasa, M., López de Alda, M., Céspedes-Sánchez, R., Ventura, F., & Barceló, D. (2013). Occurrence and behavior of pesticides in wastewater treatment plants and their environmental impact. *Science of the Total Environment, 458–460*, 466-476. doi:http://dx.doi.org/10.1016/j.scitotenv.2013.04.010

Kolpin, D. W., Furlong, E. T., Meyer, M. T., Thurman, E. M., Zaugg, S. D., Barber, L. B., & Buxton, H. T. (2002). Pharmaceuticals, Hormones, and Other Organic Wastewater Contaminants in U.S. Streams, 1999−2000: A National Reconnaissance. *Environmental Science & Technology, 36*(6), 1202-1211. doi:http://dx.doi.org/10.1021/es011055j

Kontana, A., Papadimitriou, C. A., Samaras, P., Zdragas, A., & Yiangou, M. (2009). Effectiveness of ozonation and chlorination on municipal wastewater treatment evaluated by a battery of bioassays and biomarkers. *Water Science and Technology, 60*(6), 1497-1505. doi:http://dx.doi.org/10.2166/wst.2009.478

Krewski, D., Acosta, D., Andersen, M., Anderson, H., Bailar, J. C., Boekelheide, K., . . . Assessment of Environmental, A. (2010a). Toxicity Testing in the 21st Century: A Vision and a Strategy. *Journal of Toxicology and Environmental Health, Part B, 13*(2-4), 51-138. doi:http://dx.doi.org/10.1080/10937404.2010.483176

Krewski, D., Acosta, D., Jr., Andersen, M., Anderson, H., Bailar, J. C., 3rd, Boekelheide, K., . . . Zeise, L. (2010b). Toxicity testing in the 21st century: a vision and a strategy. *Journal of Toxicology and Environmental Health. Part B: Critical Reviews, 13*(2-4), 51-138. doi:http://dx.doi.org/10.1080/10937404.2010.483176

Kristiana, I., Tan, J., Joll, C. A., Heitz, A., von Gunten, U., & Charrois, J. W. A. (2013). Formation of N-nitrosamines from chlorination and chloramination of molecular weight fractions of natural organic matter. *Water Research, 47*(2), 535-546. doi:http://dx.doi.org/10.1016/j.watres.2012.10.014

Krupa, Z. (1999). Cadmium against higher plant photosynthesis - a variety of effects and where do they possibly come from? *ZEITSCHRIFT FUR NATURFORSCHUNG C-A JOURNAL OF BIOSCIENCES, 54*(9-10), 723-729. doi:https://doi.org/10.1515/znc-1999-9-1017

Kümmerer, K. (2003). Significance of antibiotics in the environment. *Journal of Antimicrobial Chemotherapy, 52*(1), 5-7. doi:http://dx.doi.org/10.1093/jac/dkg293

Lab Bell. (2006). *Luminotox Rapid Toxicity Testing*.

Lajeunesse, A., Smyth, S. A., Barclay, K., Sauvé, S., & Gagnon, C. (2012). Distribution of antidepressant residues in wastewater and biosolids following different treatment processes by municipal wastewater treatment plants in Canada. *Water Research, 46*(17), 5600-5612. doi:http://dx.doi.org/10.1016/j.watres.2012.07.042

Landrum, P. F., Nihart, S. R., Eadie, B. J., & Herche, L. R. (1987). Reduction in bioavailability of organic contaminants to the amphipod pontoporeia hoyi by dissolved organic matter of sediment interstitial waters. *Environmental Toxicology and Chemistry, 6*(1), 11-20. doi:http://dx.doi.org/10.1002/etc.5620060102

Landrum, P. F., Reinhold, M. D., Nihart, S. R., & Eadie, B. J. (1985). Predicting the bioavailability of organic xenobiotics to Pontoporeia hoyi in the presence of humic and fulvic materials and natural dissolved organic matter. *Environmental Toxicology and Chemistry, 4*(4), 459-467. doi:http://dx.doi.org/10.1002/etc.5620040406

Langdon, K. A., Warne, M., Smernik, R. J., Shareef, A., & Kookana, R. S. (2014). persistence of estrogenic activity in soils following land application of biosolids. *Environmental Toxicology and Chemistry, 33*(1), 26-28. doi:http://dx.doi.org/10.1002/etc.2395

Larcher, S., Delbès, G., Robaire, B., & Yargeau, V. (2012). Degradation of 17α-ethinylestradiol by ozonation—Identification of the by-products and assessment of their estrogenicity and toxicity. *Environment International, 39*(1), 66-72. doi:http://dx.xoi.org/10.1016/j.envint.2011.09.008

Larcher, S., & Yargeau, V. (2013a). Biodegradation of 17α-ethinylestradiol by heterotrophic bacteria. *Environmental Pollution, 173*, 17-22. doi:https://doi.org/10.1016/j.envpol.2012.10.028

Larcher, S., & Yargeau, V. (2013b). The effect of ozone on the biodegradation of 17α-ethinylestradiol and sulfamethoxazole by mixed bacterial cultures. *Applied Microbiology and Biotechnology, 97*(5), 2201-2210.

Larsson, D. G. J., de Pedro, C., & Paxeus, N. (2007). Effluent from drug manufactures contains extremely high levels of pharmaceuticals. *Journal of Hazardous materials, 148*(3), 751-755. doi:http://dx.doi.org/10.1016/j.jhazmat.2007.07.008

Lassonde, G., Nasuhoglu, D., Pan, J. F., Gaye, B., Yargeau, V., & Delbes, G. (2015). Ozone treatment prevents the toxicity of an environmental mixture of estrogens on rat fetal

testicular development. *Reproductive Toxicology, 58*, 85-92. doi:http://dx.doi.org/10.1016/j.reprotox.2015.09.001

Latch, D. E., Packer, J. L., Stender, B. L., VanOverbeke, J., Arnold, W. A., & McNeill, K. (2005). Aqueous photochemistry of triclosan: Formation of 2,4-dichlorophenol, 2,8-dichlorodibenzo-p-dioxin, and oligomerization products. *Environmental Toxicology and Chemistry, 24*(3), 517-525. doi:http://dx.doi.org/10.1897/04-243R.1

LBI Innovations Inc. (2014). Luminotox : a biosensor of toxicity; Integrated solution for optimized management of our resources.

Lee, Y., Gerrity, D., Lee, M., Bogeat, A. E., Salhi, E., Gamage, S., . . . von Gunten, U. (2013). Prediction of Micropollutant Elimination during Ozonation of Municipal Wastewater Effluents: Use of Kinetic and Water Specific Information. *Environmental Science & Technology, 47*(11), 5872-5881. doi:http://dx.doi.org/10.1021/es400781r

Lee, Y., & von Gunten, U. (2012). Quantitative structure–activity relationships (QSARs) for the transformation of organic micropollutants during oxidative water treatment. *Water Research, 46*(19), 6177-6195. doi:http://dx.doi.org/10.1016/j.watres.2012.06.006

Leusch, F. D. L., de Jager, C., Levi, Y., Lim, R., Puijker, L., Sacher, F., . . . Chapman, H. F. (2010). Comparison of Five in Vitro Bioassays to Measure Estrogenic Activity in Environmental Waters. *Environmental Science & Technology, 44*(10), 3853-3860. doi:http://dx.doi.org/10.1021/es903899d

Leusch, F. D. L., Khan, S. J., Gagnon, M. M., Quayle, P., Trinh, T., Coleman, H., . . . Reitsema, T. (2014a). Assessment of wastewater and recycled water quality: A comparison of lines of evidence from in vitro, in vivo and chemical analyses. *Water Research, 50*, 420-431. doi:https://doi.org/10.1016/j.watres.2013.10.056

Leusch, F. D. L., Khan, S. J., Gagnon, M. M., Quayle, P., Trinh, T., Coleman, H., . . . Reitsema, T. (2014b). Assessment of wastewater and recycled water quality: A comparison of lines of evidence from invitro, invivo and chemical analyses. *Water Research, 50*, 420-431. doi:http://dx.doi.org/10.1016/j.watres.2013.10.056

Leusch, F. D. L., Khan, S. J., Laingam, S., Prochazka, E., Froscio, S., Trinh, T., . . . Humpage, A. (2014c). Assessment of the application of bioanalytical tools as surrogate measure of chemical contaminants in recycled water. *Water Research, 49*, 300-315. doi:http://dx.doi.org/10.1016/j.watres.2013.11.030

Leusch, F. D. L., & Snyder, S. A. (2015). Bioanalytical tools: half a century of application for potable reuse. *Environmental Science: Water Research & Technology, 1*(5), 606-621. doi:http://dx.doi.org/10.1039/C5EW00115C

Levine, A. D., Tchobanoglous, G., & Asano, T. (1985). Characterization of the Size Distribution of Contaminants in Wastewater: Treatment and Reuse Implications. *Journal (Water Pollution Control Federation), 57*(7), 805-816. doi:http://www.jstor.org/stable/25042701

Lewis, K. M., & Archer, R. D. (1979). pKa values of estrone, 17β-estradiol and 2-methoxyestrone. *Steroids, 34*(5), 485-499. doi:http://dx.doi.org/10.1016/S0039-128X(79)80011-2

Li, J. Y., Tang, J. Y. M., Jin, L., & Escher, B. I. (2013). understanding bioavailability and toxicity of sediment-associated contaminants by combining passive sampling with in vitro bioassays in an urban river catchment. *Environmental Toxicology and Chemistry, 32*(12), 2888-2896. doi:http://dx.doi.org/10.1002/etc.2387

Lippert, C., Seeger, H., Mueck, A. O., & Lippert, T. H. (2000). The effects of A-ring and D-ring metabolites of estradiol on the proliferation of vascular endothelial cells. *Life Sciences, 67*(13), 1653-1658. doi:http://dx.doi.org/10.1016/S0024-3205(00)00747-5

Lishman, L., Smyth, S. A., Sarafin, K., Kleywegt, S., Toito, J., Peart, T., . . . Seto, P. (2006). Occurrence and reductions of pharmaceuticals and personal care products and estrogens by municipal wastewater treatment plants in Ontario, Canada. *Science of the Total Environment, 367*(2–3), 544-558. doi:http://dx.doi.org/10.1016/j.scitotenv.2006.03.021

Liu, H.-t. (2016). Achilles heel of environmental risk from recycling of sludge to soil as amendment: A summary in recent ten years (2007–2016). *Waste Management, 56*, 575-583. doi:http://dx.doi.org/10.1016/j.wasman.2016.05.028

Liu, X., Shi, J., Bo, T., Meng, Y., Zhan, X., Zhang, M., & Zhang, Y. (2017). Distributions and ecological risk assessment of estrogens and bisphenol A in an arid and semiarid area in northwest China. *Environmental Science and Pollution Research, 24*(8), 7216-7225. doi:http://dx.doi.org/10.1007/s11356-017-8434-6

Lloyd, J. W. (1985). *Natural Inorganic Hydro- chemistry in Relation to Groundwater*. Oxford, England: Clarendon Press.

Lonappan, L., Brar, S. K., Das, R. K., Verma, M., & Surampalli, R. Y. (2016). Diclofenac and its transformation products: Environmental occurrence and toxicity - A review. *Environment International, 96*, 127-138. doi:https://doi.org/10.1016/j.envint.2016.09.014

Luo, Y., Guo, W., Ngo, H. H., Nghiem, L. D., Hai, F. I., Zhang, J., . . . Wang, X. C. (2014). A review on the occurrence of micropollutants in the aquatic environment and their fate and removal during wastewater treatment. *Science of the Total Environment, 473–474*, 619-641. doi:http://dx.doi.org/10.1016/j.scitotenv.2013.12.065

Luster-Teasley, S. L., Ganey, P. E., DiOrio, M., Ward, J. S., Maleczka, R. E., Trosko, J. E., & Masten, S. J. (2005). Effect of byproducts from the ozonation of pyrene: Biphenyl-2,2′,6,6′-tetracarbaldehyde and biphenyl-2,2′,6,6′-tetracarboxylic acid on gap junction intercellular communication and neutrophil function. *Environmental Toxicology and Chemistry, 24*(3), 733-740. doi:http://dx.doi.org/10.1897/04-679.1

Ma, J., Xu, L., Wang, S., Zheng, R., Jin, S., Huang, S., & Huang, Y. (2002). Toxicity of 40 Herbicides to the Green Alga Chlorella vulgaris. *Ecotoxicology and Environmental Safety, 51*(2), 128-132. doi:http://dx.doi.org/10.1006/eesa.2001.2113

Ma, R., Wang, B., Yin, L., Zhang, Y., Deng, S., Huang, J., . . . Yu, G. (2017). Characterization of pharmaceutically active compounds in Beijing, China: occurrence pattern, spatiotemporal distribution and its environmental implication. *Journal of Hazardous materials, 323*, 147-155. doi:https://doi.org/10.1016/j.scitotenv.2016.03.053

MacBean, C. (2008-2010). *e-Pesticide Manual, version 5.1* (15 ed.). Alton, UK: British Crop Protection Council.

Macedo, R. S., Lombardi, A. T., Omachi, C. Y., & Rörig, L. R. (2008). Effects of the herbicide bentazon on growth and photosystem II maximum quantum yield of the marine diatom Skeletonema costatum. *Toxicology in Vitro, 22*(3), 716-722. doi:http://dx.doi.org/10.1016/j.tiv.2007.11.012

Macfie, S., & Welbourn, P. (2000). The cell wall as a barrier to uptake of metal ions in the unicellular green alga Chlamydomonas reinhardtii (Chlorophyceae). *Archives of*

Environmental Contamination and Toxicology, 39(4), 413-419. doi:http://dx.doi.org/10.1007/s002440010122

Macova, M., Escher, B. I., Reungoat, J., Carswell, S., Chue, K. L., Keller, J., & Mueller, J. F. (2010). Monitoring the biological activity of micropollutants during advanced wastewater treatment with ozonation and activated carbon filtration. *Water Research, 44*(2), 477-492. doi:http://dx.doi.org/10.1016/j.watres.2009.09.025

Magdeburg, A., Stalter, D., Schlusener, M., Ternes, T., & Oehlmann, J. (2014). Evaluating the efficiency of advanced wastewater treatment: Target analysis of organic contaminants and (geno-)toxicity assessment tell a different story. *Water Research, 50*, 35-47. doi:http://dx.doi.org/10.1016/j.watres.2013.11.041

Maksymiec, W., & Baszyński, T. (1988). The effect of Cd2+ on the release of proteins from thylakoid membranes of tomato leaves. *Acta Societatis Botanicorum Poloniae, 57*(4), 465-474. doi:http://dx.doi.org/10.5586/asbp.1988.044

Mamindy-Pajany, Y., Hamer, B., Roméo, M., Géret, F., Galgani, F., Durmiši, E., . . . Marmier, N. (2011). The toxicity of composted sediments from Mediterranean ports evaluated by several bioassays. *Chemosphere, 82*(3), 362-369. doi:http://dx.doi.org/10.1016/j.chemosphere.2010.10.005

Manusadžianas, L., Caillet, C., Fachetti, L., Gylytė, B., Grigutytė, R., Jurkonienė, S., . . . Férard, J.-F. (2012). Toxicity of copper oxide nanoparticle suspensions to aquatic biota. *Environmental Toxicology and Chemistry, 31*(1), 108-114. doi:http://dx.doi.org/10.1002/etc.715

Margot, J., Kienle, C., Magnet, A., Weil, M., Rossi, L., de Alencastro, L. F., . . . Barry, D. A. (2013). Treatment of micropollutants in municipal wastewater: Ozone or powdered activated carbon? *Science of the Total Environment, 461*, 480-498. doi:http://dx.doi.org/10.1016/j.scitotenv.2013.05.034

Marshall, M., & Yargeau, V. (2017). Sensitivity of the LuminoTox tool to monitor contaminants of emerging concern in municipal secondary wastewater effluent. *Science of the Total Environment, 598*, 1065–1075. doi:https://doi.org/10.1016/j.scitotenv.2017.04.118

Martín, J., Camacho-Muñoz, M. D., Santos, J. L., Aparicio, I., & Alonso, E. (2012). Distribution and temporal evolution of pharmaceutically active compounds alongside sewage sludge treatment. Risk assessment of sludge application onto soils. *Journal of Environmental Management, 102*, 18-25. doi:http://dx.doi.org/10.1016/j.jenvman.2012.02.020

Martinez, E., Vélez, S. M., Mayo, M., & Sastre, M. P. (2016). Acute toxicity assessment of N,N-diethyl-m-toluamide (DEET) on the oxygen flux of the dinoflagellate Gymnodinium instriatum. *Ecotoxicology, 25*(1), 248-252. doi:http://dx.doi.org/10.1007/s10646-015-1564-z

Maruya, K. A., Dodder, N. G., Mehinto, A. C., Denslow, N. D., Schlenk, D., Snyder, S. A., & Weisberg, S. B. (2016). A tiered, integrated biological and chemical monitoring framework for contaminants of emerging concern in aquatic ecosystems. *Integrated Environmental Assessment and Management, 12*(3), 540-547. doi:http://dx.doi.org/10.1002/ieam.1702

Max, S., & Nathalie, H. (2016). Switzerland's first facility for the removal of micropollutants, WWTP Neugut - rst full-scale ozonation. In A. Neugut (Ed.). Dübendorf Switzerland.

Maxwell, K., & Johnson, G. N. (2000). Chlorophyll fluorescence—a practical guide. *Journal of Experimental Botany, 51*(345), 659-668. doi:http://dx.doi.org/10.1093/jexbot/51.345.659

Mehinto Alvine, C., Jayasinghe, B. S., Vandervort, D. R., Denslow, N. D., & Maruya, K. A. (2016). Screening for Endocrine Activity in Water Using Commercially-available In Vitro Transactivation Bioassays. *Journal of Visualized Experiments*(118). doi:http://dx.doi.org/10.3791/54725

Mendonça, E., Picado, A., Paixão, S. M., Silva, L., Cunha, M. A., Leitão, S., . . . Brito, F. (2009). Ecotoxicity tests in the environmental analysis of wastewater treatment plants: Case study in Portugal. *Journal of Hazardous materials, 163*(2–3), 665-670. doi:https://doi.org/10.1016/j.jhazmat.2008.07.012

Meng, J., Yang, B., Zhang, Y., Dong, X., & Shu, J. (2010). Heterogeneous ozonation of suspended malathion and chlorpyrifos particles. *Chemosphere, 79*(4), 394-400. doi:https://doi.org/10.1016/j.chemosphere.2010.02.008

Mestankova, H., Escher, B., Schirmer, K., von Gunten, U., & Canonica, S. (2011). Evolution of algal toxicity during (photo)oxidative degradation of diuron. *Aquatic Toxicology, 101*(2), 466-473. doi:https://doi.org/10.1016/j.aquatox.2010.10.012

Mesut, A. K., & (2013). *Determination of quality changes in treated wastewater during percolation through soil media.* Dokuz Eylül University, İzmir, Turkey. Retrieved from http://dspace.deu.edu.tr/xmlui/handle/12345/9105

Metcalfe & Eddy Inc., Tchobanoglous, G., Burton, F. L., & Stensel, H. D. (2002). *Wastewater Engineering: Treatment and Reuse* (4th Edition ed.): McGraw Hill Higher Education.

Metcalfe, C. D., Chu, S., Judt, C., Li, H., Oakes, K. D., Servos, M. R., & Andrews, D. M. (2010). Antidepressants and their metabolites in municipal wastewater, and downstream exposure in an urban watershed. *Environmental Toxicology and Chemistry, 29*(1), 79-89. doi:http://dx.doi.org/10.1002/etc.27

Metcalfe, C. D., Kleywegt, S., Letcher, R. J., Topp, E., Wagh, P., Trudeau, V. L., & Moon, T. W. (2013). A multi-assay screening approach for assessment of endocrine-active contaminants in wastewater effluent samples. *Science of the Total Environment, 454–455*(0), 132-140. doi:http://dx.doi.org/10.1016/j.scitotenv.2013.02.074

Metcalfe, C. D., Koenig, B. G., Bennie, D. T., Servos, M., Ternes, T. A., & Hirsch, R. (2003). Occurrence of neutral and acidic drugs in the effluents of Canadian sewage treatment plants. *Environmental Toxicology and Chemistry, 22*(12), 2872-2880. doi:http://dx.doi.org/10.1897/02-469

Miao, X. S., Bishay, F., Chen, M., & Metcalfe, C. D. (2004). Occurrence of antimicrobials in the final effluents of wastewater treatment plants in Canada. *Environ. Sci. Technol, 38*(13), 3533-3541. doi:http://dx.doi.org/10.1021/es030653q

Miik, M., Knasmueller, S., Ferk, F., Cichna-Markl, M., Grummt, T., Schaar, H., & Kreuzinger, N. (2011). Impact of ozonation on the genotoxic activity of tertiary treated municipal wastewater. *Water Research, 45*(12), 3681-3691. doi:http://dx.doi.org/10.1016/j.watres.2011.04.015

Mitchell, J., Pabon, N., Collier, Z. A., Egeghy, P. P., Cohen-Hubal, E., Linkov, I., & Vallero, D. A. (2013). A Decision Analytic Approach to Exposure-Based Chemical Prioritization. *PloS One, 8*(8), 1-13. doi:http://dx.doi.org/10.1371/journal.pone.0070911

Montaseri, H., & Forbes, P. B. C. (2016). A review of monitoring methods for triclosan and its occurrence in aquatic environments. *TRAC Trends in Analytical Chemistry, 85, Part C*, 221-231. doi:https://doi.org/10.1016/j.trac.2016.09.010

Morton, J., Hall, J., Smith, P., Akita, F., Koua, F. H. M., Shen, J.-R., & Krausz, E. (2014). Determination of the PS I content of PS II core preparations using selective emission: A new emission of PS II at 780 nm. *Biochimica et Biophysica Acta (BBA) - Bioenergetics, 1837*(1), 167-177. doi:https://doi.org/10.1016/j.bbabio.2013.09.008

Muller, R., Schreiber, U., Escher, B. I., Quayle, P., Bengtson Nash, S. M., & Mueller, J. F. (2008). Rapid exposure assessment of PSII herbicides in surface water using a novel chlorophyll a fluorescence imaging assay. *Science of the Total Environment, 401*(1–3), 51-59. doi:http://dx.doi.org/10.1016/j.scitotenv.2008.02.062

Murata, N., Takahashi, S., Nishiyama, Y., & Allakhverdiev, S. I. (2007). Photoinhibition of photosystem II under environmental stress. *Biochimica et Biophysica Acta (BBA) - Bioenergetics, 1767*(6), 414-421. doi:https://doi.org/10.1016/j.bbabio.2006.11.019

Nakonechny, M., Ikehata, K., & Gamal El-Din, M. (2008). Kinetics of Estrone Ozone/Hydrogen Peroxide Advanced Oxidation Treatment. *Ozone: Science & Engineering, 30*(4), 249-255. doi:http://dx.doi.org/10.1080/01919510802084570

Neale, P. A., Antony, A., Bartkow, M. E., Farré, M. J., Heitz, A., Kristiana, I., . . . Escher, B. I. (2012). Bioanalytical Assessment of the Formation of Disinfection Byproducts in a Drinking Water Treatment Plant. *Environmental Science & Technology, 46*(18), 10317-10325. doi:http://dx.doi.org/10.1021/es302126t

Neale, P. J., & Melis, A. (1989). Salinity-stress Enhances Photoinhibition of Photosynthesis in Chlamydomonas reinhardtii. *Journal of Plant Physiology, 134*(5), 619-622. doi:http://dx.doi.org/10.1016/S0176-1617(89)80158-0

Neuwoehner, J., & Escher, B. I. (2011). The pH-dependent toxicity of basic pharmaceuticals in the green algae Scenedesmus vacuolatus can be explained with a toxicokinetic ion-trapping model. *Aquatic Toxicology, 101*(1), 266-275. doi:http://dx.doi.org/10.1016/j.aquatox.2010.10.008

Nöthe, T., Fahlenkamp, H., & Sonntag, C. v. (2009). Ozonation of wastewater: rate of ozone consumption and hydroxyl radical yield. *Environmental Science & Technology, 43*(15), 5990-5995. doi:http://dx.doi.org/10.1021/es900825f

Nyström, B., Becker-Van Slooten, K., Bérard, A., Grandjean, D., Druart, J.-C., & Leboulanger, C. (2002). Toxic effects of Irgarol 1051 on phytoplankton and macrophytes in Lake Geneva. *Water Research, 36*(8), 2020-2028. doi:http://dx.doi.org/10.1016/S0043-1354(01)00404-3

Office Fédérale de l'Environnement Suisse. (2012). Micropolluants: fonds pour l'équipement des stations d'épuration en consultation. Retrieved from https://www.admin.ch/gov/fr/accueil/documentation/communiques.msg-id-44263.html

Okuda, T., Yamashita, N., Tanaka, H., Matsukawa, H., & Tanabe, K. (2009). Development of extraction method of pharmaceuticals and their occurrences found in Japanese wastewater treatment plants. *Environment International, 35*(5), 815-820. doi:http://dx.doi.org/10.1016/j.envint.2009.01.006

Osorio, V., Larrañaga, A., Aceña, J., Pérez, S., & Barceló, D. (2016). Concentration and risk of pharmaceuticals in freshwater systems are related to the population density and the

livestock units in Iberian Rivers. *Science of the Total Environment, 540*, 267-277. doi:https://doi.org/10.1016/j.scitotenv.2015.06.143

Packer, J. L., Werner, J. J., Latch, D. E., McNeill, K., & Arnold, W. A. (2003). Photochemical fate of pharmaceuticals in the environment: Naproxen, diclofenac, clofibric acid, and ibuprofen. *Aquatic Sciences, 65*(4), 342-351. doi:http://dx.doi.org/10.1007/s00027-003-0671-8

Pan, Y., Wang, Y., Li, A., Xu, B., Xian, Q., Shuang, C., . . . Zhou, Q. (2017). Detection, formation and occurrence of 13 new polar phenolic chlorinated and brominated disinfection byproducts in drinking water. *Water Research, 112*, 129-136. doi:https://doi.org/10.1016/j.watres.2017.01.037

Pan, Y., Zhang, X., Wagner, E. D., Osiol, J., & Plewa, M. J. (2014). Boiling of Simulated Tap Water: Effect on Polar Brominated Disinfection Byproducts, Halogen Speciation, and Cytotoxicity. *Environmental Science & Technology, 48*(1), 149-156. doi:http://dx.doi.org/10.1021/es403775v

Panagiota Paraskeva, N. J. D. G. (2002). Ozonation of Municipal Wastewater Effluents. *Water Environment Research,, 74*(6), 13. doi:https://doi.org/10.2175/106143002X140387

Papa, M., Pedrazzani, R., & Bertanza, G. (2013). How green are environmental technologies? A new approach for a global evaluation: The case of WWTP effluents ozonation. *Water Research, 47*(11), 3679-3687. doi:https://doi.org/10.1016/j.watres.2013.04.015

Pape-Lindstrom, P. A., & Lydy, M. J. (1997). Synergistic toxicity of atrazine and organophosphate insecticides contravenes the response addition mixture model. *Environmental Toxicology and Chemistry, 16*(11), 2415-2420. doi:http://dx.doi.org/10.1002/etc.5620161130

Paraskeva, P., & Graham, N. J. D. (2002). *Water Environment Research, 74*, 569. doi:https://doi.org/10.2175/106143002X140387

Paraskeva, P., Lambert, S., & Graham, N. (1998). Influence of ozonation conditions on the treatability of secondary effluents. *Journal of the International Ozone Association, 20*(2), 133-150. doi:http://dx.doi.org/10.1080/01919519808547282

Perkins, E. J., Ankley, G. T., Crofton, K. M., Garcia-Reyero, N., LaLone, C. A., Johnson, M. S., . . . Villeneuve, D. L. (2013). Current perspectives on the use of alternative species in human health and ecological hazard assessments. *Environmental Health Perspectives, 121*(9), 1002-1010. doi:http://dx.doi.org/10.1289/ehp.1306638

Perron, M.-C., Qiu, B., Boucher, N., Bellemare, F., & Juneau, P. (2012). Use of chlorophyll a fluorescence to detect the effect of microcystins on photosynthesis and photosystem II energy fluxes of green algae. *Toxicon, 59*(5), 567-577. doi:http://dx.doi.org/10.1016/j.toxicon.2011.12.005

Perron, M. C. (2011). *Élaboration des bioessais basés sur la fluorescence clorophyllienne du phytoplancton dans l'étude de problématiques environnementales émergentes.* (Master's of Biology), Université de Québec à Montréal

Perron, M. C., & Juneau, P. (2011). Effect of endocrine disrupters on photosystem II energy fluxes of green algae and cyanobacteria. *Environmental Research, 111*(4), 520-529. doi:http://dx.doi.org/10.1016/j.envres.2011.02.013

Personal Care Products Council. (2010). *Economic and Social Contributions of the U.S. Personal Care Products Industry*. Retrieved from

http://www.personalcarecouncil.org/sites/default/files/PCPC FINAL Economic & Social Contributions Report - Web.pdf

Péry, A. R. R., Babut, M. P., Mons, R., & Garric, J. (2006). Deriving effects on Chironomus population carrying capacity from standard toxicity tests. *Environmental Toxicology and Chemistry, 25*(1), 144-148. doi:http://dx.doi.org/10.1897/05-199R.1

Petala, Samaras, P., Zouboulis, A., Kungolos, A., & Sakellaropoulos, G. (2006). Ecotoxicological properties of wastewater treated using tertiary methods. *Environmental Toxicology, 21*(4), 417-424. doi:http://dx.doi.org/10.1002/tox.20188

Petala, Samaras, P., Zouboulis, A., Kungolos, A., & Sakellaropoulos, G. P. (2008). Influence of ozonation on the in vitro mutagenic and toxic potential of secondary effluents. *Water Research, 42*(20), 4929-4940. doi:http://dx.doi.org/10.1016/j.watres.2008.09.018

Petrie, B., Barden, R., & Kasprzyk-Hordern, B. (2015). A review on emerging contaminants in wastewaters and the environment: Current knowledge, understudied areas and recommendations for future monitoring. *Water Research, 72*, 3-27. doi:http://dx.doi.org/10.1016/j.watres.2014.08.053

Petrovic, M., Eljarrat, E., Lopez de Alda, M. J., & Barceló, D. (2004). Endocrine disrupting compounds and other emerging contaminants in the environment: A survey on new monitoring strategies and occurrence data. *Analytical and Bioanalytical Chemistry, 378*(3), 549-562. doi:http://dx.doi.org/10.1007/s00216-003-2184-7

Pfannschmidt, T., Bräutigam, K., Wagner, R., Dietzel, L., Schröter, Y., Steiner, S., & Nykytenko, A. (2009). Potential regulation of gene expression in photosynthetic cells by redox and energy state: approaches towards better understanding. *Annals of Botany, 103*(4), 599-607. doi:http://dx.doi.org/10.1093/aob/mcn081

Pflugmacher, S., Pietsch, C., Rieger, W., & Steinberg, C. E. W. (2006). Dissolved natural organic matter (NOM) impacts photosynthetic oxygen production and electron transport in coontail Ceratophyllum demersum. *Science of the Total Environment, 357*(1–3), 169-175. doi:http://dx.doi.org/10.1016/j.scitotenv.2005.03.021

Phillips, P. J., Smith, S. G., Kolpin, D. W., Zaugg, S. D., Buxton, H. T., Furlong, E. T., . . . Stinson, B. (2010). Pharmaceutical Formulation Facilities as Sources of Opioids and Other Pharmaceuticals to Wastewater Treatment Plant Effluents. *Environmental Science & Technology, 44*(13), 4910-4916. doi:10.1021/es100356f

Plewa, M. J., Wagner, E. D., Metz, D. H., Kashinkunti, R., Jamriska, K. J., & Meyer, M. (2012). Differential Toxicity of Drinking Water Disinfected with Combinations of Ultraviolet Radiation and Chlorine. *Environmental Science & Technology, 46*(14), 7811-7817. doi:http://dx.doi.org/10.1021/es300859t

Polock, T. (2014). Negative Impact on Growth and Photosynthesis in the Green Alga Chlamydomonas reinhardtii in the Presence of the Estrogen 17α-Ethynylestradiol. . *PLoS ONE 9(10), 9*(10: e109289). doi: https://dx.doi.org/10.1371/journal.pone.0109289

Prasse, C., Stalter, D., Schulte-Oehlmann, U., Oehlmann, J., & Ternes, T. A. (2015). Spoilt for choice: A critical review on the chemical and biological assessment of current wastewater treatment technologies. *Water Research, 87*, 237-270. doi:http://dx.doi.org/10.1016/j.watres.2015.09.023

Prosser, R. S., & Sibley, P. K. (2015). Human health risk assessment of pharmaceuticals and personal care products in plant tissue due to biosolids and manure amendments, and

wastewater irrigation. *Environment International, 75*, 223-233. doi:https://doi.org/10.1016/j.envint.2014.11.020

Quero-Pastor, M., Valenzuela, A., Quiroga, J. M., & Acevedo, A. (2014). Degradation of drugs in water with advanced oxidation processes and ozone. *Journal of Environmental Management, 137*, 197-203. doi:https://doi.org/10.1016/j.jenvman.2014.02.011

Radjenović, J., Petrović, M., & Barceló, D. (2009). Fate and distribution of pharmaceuticals in wastewater and sewage sludge of the conventional activated sludge (CAS) and advanced membrane bioreactor (MBR) treatment. *Water Research, 43*(3), 831-841. doi:http://dx.doi.org/10.1016/j.watres.2008.11.043

Ragush, C. M., Schmidt, J. J., Krkosek, W. H., Gagnon, G. A., Truelstrup-Hansen, L., & Jamieson, R. C. (2015). Performance of municipal waste stabilization ponds in the Canadian Arctic. *Ecological Engineering, 83*, 413-421. doi:http://dx.doi.org/10.1016/j.ecoleng.2015.07.008

Rai, L. C., Gaur, J. P., & Kumar, H. D. (1981). Protective effects of certain environmental factors on the toxicity of zinc, mercury, and methylmercury to Chlorella vulgaris. *Environmental Research, 25*(2), 250-259. doi:http://dx.doi.org/10.1016/0013-9351(81)90026-8

Rai, L. C., Singh, A. K., & Mallick, N. (1991). Studies on Photosynthesis, the Associated Electron Transport System and Some Physiological Variables of Chlorella vulgaris Under Heavy Metal Stress. *Journal of Plant Physiology, 137*(4), 419-424. doi:http://dx.doi.org/10.1016/S0176-1617(11)80310-X

Rashid, A., Camm, E. L., & Ekramoddoullah, A. K. M. (1994). Molecular mechanism of action of Pb2+ and Zn2+ on water oxidizing complex of photosystem II. *FEBS Letters, 350*(2-3), 296-298. doi:http://dx.doi.org/10.1016/0014-5793(94)00789-6

Rashid, A., & Popovic, R. (1990). Protective role of CaCl2 against Pb2+ inhibition in photosystem II. *FEBS Letters, 271*(1-2), 181-184. doi:http://dx.doi.org/10.1016/0014-5793(90)80401-4

Ray, P., Zhao, Z., & Knowlton, K. F. (2013). Sustainable animal agriculture. In E. Kebreab (Ed.), (pp. 268-283). Boston MA, USA: Cabi International.

Razavi, B., Song, W., Cooper, W. J., Greaves, J., & Jeong, J. (2009). Free-Radical-Induced Oxidative and Reductive Degradation of Fibrate Pharmaceuticals: Kinetic Studies and Degradation Mechanisms. *The Journal of Physical Chemistry A, 113*(7), 1287-1294. doi:http://dx.doi.org/10.1021/jp808057c

Razmi, K., Naji, T., Alizadeh, M., & Sahafi, H. H. (2011). Hormonal sex reversal of rainbow trout (Oncorhynchus mykiss) by ethynylestradiol-17 alpha (EE2). *Iranian journal of fisheries sciences, 10*(2), 304-315. doi:http://jifro.ir/article-1-178-en.html

Reboud, X. (2002). Response of Chlamydomonas reinhardtii to Herbicides: Negative Relationship Between Toxicity and Water Solubility Across Several Herbicide Families. *Bulletin of Environmental Contamination and Toxicology, 69*(4), 554-561. doi:http://dx.doi.org/10.1007/s00128-002-0097-3

Renger, G. (2011). Light induced oxidative water splitting in photosynthesis: Energetics, kinetics and mechanism. *Journal of Photochemistry and Photobiology B: Biology, 104*(1–2), 35-43. doi:https://doi.org/10.1016/j.jphotobiol.2011.01.023

Renger, G., & Renger, T. (2008). Photosystem II: The machinery of photosynthetic water splitting. *Photosynthesis Research, 98*(1), 53-80. doi:http://dx.doi.org/10.1007/s11120-008-9345-7

Reungoat, J., Escher, B. I., Macova, M., Argaud, F. X., Gernjak, W., & Keller, J. (2012). Ozonation and biological activated carbon filtration of wastewater treatment plant effluents. *Water Research, 46*(3), 863-872. doi:https://doi.org/10.1016/j.watres.2011.11.064

Reungoat, J., Macova, M., Escher, B. I., Carswell, S., Mueller, J. F., & Keller, J. (2010). Removal of micropollutants and reduction of biological activity in a full scale reclamation plant using ozonation and activated carbon filtration. *Water Research, 44*(2), 625-637. doi:http://dx.doi.org/10.1016/j.watres.2009.09.048

Rice, E. W., Baird, R. B., Eaton, A. D., & Clesceri, L. S. (2012). Standard Methods for the Examination of Water and Wastewater (22 ed., pp. 5:21-25:25): American Public Health Association, American Water Works Association, Water Environment Federation.

Ringwood, A. H., DeLorenzo, M. E., Ross, P. E., & Holland, A. F. (1997). Interpretation of Microtox® solid-phase toxicity tests: The effects of sediment composition. *Environmental Toxicology and Chemistry, 16*, 1135–1140. doi:http://dx.doi.org/10.1002/etc.5620160607

Rizzo, L. (2011). Bioassays as a tool for evaluating advanced oxidation processes in water and wastewater treatment. *Water Research, 45*(15), 4311-4340. doi:http://dx.doi.org/10.1016/j.watres.2011.05.035

Roberts, J., Bain, P. A., Kumar, A., Hepplewhite, C., Ellis, D. J., Christy, A. G., & Beavis, S. G. (2015). Tracking multiple modes of endocrine activity in Australia's largest inland sewage treatment plant and effluent- receiving environment using a panel of in vitro bioassays. *Environmental Toxicology and Chemistry, 34*(10), 2271-2281. doi:http://dx.doi.org/10.1002/etc.3051

Rodayan, A., Segura, P. A., & Yargeau, V. (2013). Ozonation of wastewater: Removal and transformation products of drugs of abuse. *Science of the Total Environment*. doi:http://dx.doi.org/10.1016/j.scitotenv.2013.11.023

Rodayan, A., Segura, P. A., & Yargeau, V. (2014). Ozonation of wastewater: Removal and transformation products of drugs of abuse. *Science of the Total Environment, 487*, 763-770. doi:http://dx.doi.org/10.1016/j.scitotenv.2013.11.023

Rojas, M. R., Leung, C., Bonk, F., Zhu, Y., Edwards, L., Arnold, R. G., . . . Klečka, G. (2013). Assessment of the Effectiveness of Secondary Wastewater Treatment Technologies to Remove Trace Chemicals of Emerging Concern. *Critical Reviews in Environmental Science and Technology, 43*(12), 1281-1314. doi:http://dx.doi.org/10.1080/10643389.2011.644221

Rosal, R., Gonzalo, M. S., Boltes, K., Letón, P., Vaquero, J. J., & García-Calvo, E. (2009). Identification of intermediates and assessment of ecotoxicity in the oxidation products generated during the ozonation of clofibric acid. *Journal of Hazardous materials, 172*(2), 1061-1068. doi:http://dx.doi.org/10.1016/j.jhazmat.2009.07.110

Rosenfeldt, E. J., & Linden, K. G. (2004). Degradation of Endocrine Disrupting Chemicals Bisphenol A, Ethinyl Estradiol, and Estradiol during UV Photolysis and Advanced Oxidation Processes. *Environmental Science & Technology, 38*(20), 5476-5483. doi:http://dx.doi.org/10.1021/es035413p

Russom, C. L., Bradbury, S. P., Broderius, S. J., Hammermeister, D. E., & Drummond, R. A. (1997). Predicting modes of toxic action from chemical structure: Acute toxicity in the

fathead minnow (Pimephales promelas). *Environmental Toxicology and Chemistry, 16*(5), 948-967. doi:http://dx.doi.org/10.1002/etc.5620160514

Rusten, B., xf, rn, McCoy, M., Proctor, R., & Siljudalen, J. G. (1998). The Innovative Moving Bed Biofilm Reactor/Solids Contact Reaeration Process for Secondary Treatment of Municipal Wastewater. *Water Environment Research, 70*(5), 1083-1089. doi:http://www.jstor.org/stable/25045121

Ryu, J., Oh, J., Snyder, S. A., & Yoon, Y. (2014). Determination of micropollutants in combined sewer overflows and their removal in a wastewater treatment plant (Seoul, South Korea). *Environmental Monitoring and Assessment, 186*(5), 3239-3251. doi:http://dx.doi.org/10.1007/s10661-013-3613-5

Sabat, S. C. (1996). Copper ion inhibition of electron transport activity in sodium chloride washed photosystem II particle is partially prevented by calcium ion. *ZEITSCHRIFT FUR NATURFORSCHUNG C-A JOURNAL OF BIOSCIENCES, 51*(3-4), 179-184. doi: https://doi.org/10.1515/znc-1996-3-408

Santoke, H., Song, W., Cooper, W. J., & Peake, B. M. (2012). Advanced oxidation treatment and photochemical fate of selected antidepressant pharmaceuticals in solutions of Suwannee River humic acid. *Journal of Hazardous materials, 217–218*, 382-390. doi:http://dx.doi.org/10.1016/j.jhazmat.2012.03.049

Sarafi, E., Chatzissavvidis, C., & Therios, I. (2017). Response of two pomegranate (Punica granatum L.) cultivars to six boron concentrations: Growth performance, nutrient status, gas exchange parameters, chlorophyll fluorescence, and proline and carbohydrate content. *Journal of Plant Nutrition, 40*(7), 983-994. doi:http://dx.doi.org/10.1080/01904167.2016.1262403

Schrank, S., Bieling, U., José, H., Moreira, R., & Schröder, H. F. (2009). Generation of endocrine disruptor compounds during ozone treatment of tannery wastewater confirmed by biological effect analysis and substance specific analysis. *Water Science & Technology, 59*(1). doi:http://dx.doi.org/10.2166/wst.2009.762

Schreiner, V. C., Szöcs, E., Bhowmik, A. K., Vijver, M. G., & Schäfer, R. B. (2016). Pesticide mixtures in streams of several European countries and the USA. *Science of the Total Environment, 573*, 680-689. doi:http://dx.doi.org/10.1016/j.scitotenv.2016.08.163

Schultz, M. M., Furlong, E. T., Kolpin, D. W., Werner, S. L., Schoenfuss, H. L., Barber, L. B., . . . Vajda, A. M. (2010). Antidepressant Pharmaceuticals in Two U.S. Effluent-Impacted Streams: Occurrence and Fate in Water and Sediment, and Selective Uptake in Fish Neural Tissue. *Environmental Science & Technology, 44*(6), 1918-1925. doi:http://dx.doi.org/10.1021/es9022706

Sellin, M. K., Snow, D. D., Akerly, D. L., & Kolok, A. S. (2009). Estrogenic Compounds Downstream From Three Small Cities in Eastern Nebraska: Occurrence and Biological Effect1. *JAWRA Journal of the American Water Resources Association, 45*(1), 14-21. doi:http://dx.doi.org/10.1111/j.1752-1688.2008.00285.x

Sengupta, A., Lyons, J. M., Smith, D. J., Drewes, J. E., Snyder, S. A., Heil, A., & Maruya, K. A. (2014). The occurrence and fate of chemicals of emerging concern in coastal urban rivers receiving discharge of treated municipal wastewater effluent. *Environmental Toxicology and Chemistry, 33*(2), 350-358. doi:http://dx.doi.org/10.1002/etc.2457

Servos, M. R., Bennie, D. T., Burnison, B. K., Jurkovic, A., McInnis, R., Neheli, T., . . . Ternes, T. A. (2005). Distribution of estrogens, 17β-estradiol and estrone, in Canadian municipal wastewater treatment plants. *Science of the Total Environment, 336*(1–3), 155-170. doi:http://dx.doi.org/10.1016/j.scitotenv.2004.05.025

Sharma, S., Rupare, J. P., & Patel, M. L. (2011). *A general review on Advanced Oxidation Processes for waste water treatment*. Paper presented at the International conference in current trends in technology Nuicone, Ahmedabad, India.

Shaw, M., Negri, A., Fabricius, K., & Mueller, J. F. (2009). Predicting water toxicity: Pairing passive sampling with bioassays on the Great Barrier Reef. *Aquatic Toxicology, 95*(2), 108-116. doi:http://dx.doi.org/10.1016/j.aquatox.2009.08.007

Shon, H. K., Vigneswaran, S., & Snyder, S. A. (2006). Effluent Organic Matter (EfOM) in Wastewater: Constituents, Effects, and Treatment. *Critical Reviews in Environmental Science and Technology, 36*(4), 327-374. doi:http://dx.doi.org/10.1080/10643380600580011

Siefermann-Harms, D. (1978). Light-induced Changes of the Carotenoid Levels in Chloroplast Envelopes. *Journal of LPlant Physiology, 61*(4), 530-533. doi:http://dx.doi.org/10.1104/pp.61.4.530

Singh, S. (2012). *Ozone treatment of municipal wastewater effluent for oxidation of emerging contaminants and disinfection.* (Master's), University of Windsor.

Singh, S., Seth, R., Tabe, S., & Yang, P. (2015). Oxidation of Emerging Contaminants during Pilot-Scale Ozonation of Secondary Treated Municipal Effluent. *Ozone: Science & Engineering, 37*(4), 323-329. doi:http://dx.doi.org/10.1080/01919512.2014.998755

Sipes, N. S., Martin, M. T., Kothiya, P., Reif, D. M., Judson, R. S., Richard, A. M., . . . Knudsen, T. B. (2013). Profiling 976 ToxCast Chemicals across 331 Enzymatic and Receptor Signaling Assays. *Chemical Research in Toxicology, 26*(6), 878-895. doi:http://dx.doi.org/10.1021/tx400021f

Smith, P. D., Brockway, D. L., & Stancil, F. E. (1987). Effects of hardness, alkalinity and pH on the toxicity of pentachlorophenol to selenastrum capricornutum (printz). *Environmental Toxicology and Chemistry, 6*(11), 891-900. doi:http://dx.doi.org/10.1002/etc.5620061110

Snyder, S. A., Wert, E. C., Rexing, D. J., Zegers, R. E., & Drury, D. D. (2006). Ozone Oxidation of Endocrine Disruptors and Pharmaceuticals in Surface Water and Wastewater. *Ozone: Science & Engineering, 28*(6), 445-460. doi:https://doi.org/10.1080/01919510601039726

Solís, R. R., Rivas, F. J., Pérez-Bote, J. L., & Gimeno, O. (2015). Photocatalytic ozonation of 4-chloro-2-methylphenoxyacetic acid and its reaction intermediate 4-chloro-2-methyl phenol. *Journal of the Taiwan Institute of Chemical Engineers, 46*, 125-131. doi:http://dx.doi.org/10.1016/j.jtice.2014.09.010

Song, W., Cooper, W. J., Peake, B. M., Mezyk, S. P., Nickelsen, M. G., & O'Shea, K. E. (2009). Free-radical-induced oxidative and reductive degradation of N,N'-diethyl-m-toluamide (DEET): Kinetic studies and degradation pathway. *Water Research, 43*(3), 635-642. doi:http://dx.doi.org/10.1016/j.watres.2008.11.018

Souza, B. S., Dantas, R. F., Agulló-Barceló, M., Lucena, F., Sans, C., Esplugas, S., & Dezotti, M. (2013). Evaluation of UV/H2O2 for the disinfection and treatment of municipal secondary effluents for water reuse. *Journal of Chemical Technology and Biotechnology, 88*(9), 1697-1706. doi:http://dx.doi.org/10.1002/jctb.4021

Speth, T. F., Miltner, R. J., Richardson, S. D., & Simmons, J. E. (2008). Integrated Disinfection By-Products Mixtures Research: Concentration by Reverse Osmosis Membrane Techniques of Disinfection By-Products from Water Disinfected by Chlorination and Ozonation/Postchlorination. *Journal of Toxicology and Environmental Health, Part A, 71*(17), 1149-1164. doi:http://dx.doi.org/10.1080/15287390802182219

Staehelin, J., & Hoigne, J. (1985). Decomposition of ozone in water in the presence of organic solutes acting as promoters and inhibitors of radical chain reactions. *Environmental Science & Technology, 19*(12), 1206-1213. doi:http://dx.doi.org/10.1021/es00142a012

Stalter, D., Magdeburg, A., Wagner, M., & Oehlmann, J. (2011). Ozonation and activated carbon treatment of sewage effluents: Removal of endocrine activity and cytotoxicity. *Water Research, 45*(3), 1015-1024. doi:http://dx.doi.org/10.1016/j.watres.2010.10.008

Stalter, D., Magdeburg, A., Weil, M., Knacker, T., & Oehlmann, J. (2010). Toxication or detoxication? In vivo toxicity assessment of ozonation as advanced wastewater treatment with the rainbow trout. *Water Research, 44*(2), 439-448. doi:http://dx.doi.org/10.1016/j.watres.2009.07.025

Stalter, D., Peters, L. I., O'Malley, E., Tang, J. Y.-M., Revalor, M., Farré, M. J., . . . Escher, B. I. (2016). Sample Enrichment for Bioanalytical Assessment of Disinfected Drinking Water: Concentrating the Polar, the Volatiles, and the Unknowns. *Environmental Science & Technology, 50*(12), 6495-6505. doi:http://dx.doi.org/10.1021/acs.est.6b00712

Stasinakis, A. S., Thomaidis, N. S., Arvaniti, O. S., Asimakopoulos, A. G., Samaras, V. G., Ajibola, A., . . . Lekkas, T. D. (2013). Contribution of primary and secondary treatment on the removal of benzothiazoles, benzotriazoles, endocrine disruptors, pharmaceuticals and perfluorinated compounds in a sewage treatment plant. *Science of the Total Environment, 463–464*, 1067-1075. doi:http://dx.doi.org/10.1016/j.scitotenv.2013.06.087

Steinberg, C. E. W., Paul, A., Pflugmacher, S., Meinelt, T., Klöcking, R., & Wiegand, C. (2003). Pure humic acid substances have the potential to act as xenobiotic chemicals- a review *Fresenius Environmental Bulletin, 12*(5), 391-401. doi:http://www.jstor.org/stable/25043350

Stevens-Garmon, J., Drewes, J. E., Khan, S. J., McDonald, J. A., & Dickenson, E. R. V. (2011). Sorption of emerging trace organic compounds onto wastewater sludge solids. *Water Research, 45*(11), 3417-3426. doi:http://dx.doi.org/10.1016/j.watres.2011.03.056

Strasserf, R. J., & Srivastava, A. (1995). polyphasic chlorophyll a fluorescence transient in plants and cyanobacteria*. *Photochemistry and Photobiology, 61*(1), 32-42. doi:http://dx.doi.org/10.1111/j.1751-1097.1995.tb09240.x

Stratton, G. W. (1984). Effects of the herbicide atrazine and its degradation products, alone and in combination, on phototrophic microorganisms. *Archives of Environmental Contamination and Toxicology, 13*(1), 35-42. doi:https://doi.org/10.1007/bf01055644

Suarez, S., Dodd, M. C., Omil, F., & von Gunten, U. (2007). Kinetics of triclosan oxidation by aqueous ozone and consequent loss of antibacterial activity: Relevance to municipal wastewater ozonation. *Water Research, 41*(12), 2481-2490. doi:http://dx.doi.org/10.1016/j.watres.2007.02.049

Sui, Q., Cao, X., Lu, S., Zhao, W., Qiu, Z., & Yu, G. (2015). Occurrence, sources and fate of pharmaceuticals and personal care products in the groundwater: A review. *Emerging Contaminants, 1*(1), 14-24. doi:https://doi.org/10.1016/j.emcon.2015.07.001

Sullivan, K. B., & Spence, K. M. (2003). Effects of sublethal concentrations of atrazine and nitrate on metamorphosis of the African clawed frog. *Environmental Toxicology and Chemistry, 22*(3), 627-635. doi:http://dx.doi.org/10.1002/etc.5620220323

Sun, J., Wang, J., Zhang, R., Wei, D., Long, Q., Huang, Y., . . . Li, A. (2017). Comparison of different advanced treatment processes in removing endocrine disruption effects from municipal wastewater secondary effluent. *Chemosphere, 168*, 1-9. doi:http://dx.doi.org/10.1016/j.chemosphere.2016.10.031

Sundaram, V., Emerick, R. W., & Shumaker, S. E. (2014). Advanced Treatment Process for Pharmaceuticals, Endocrine Disruptors, and Flame Retardants Removal. *Water Environment Research, 86*(2), 111-122. doi:http://dx.doi.org/10.2175/106143013X13807328849053

Tabata, A., Kashiwada, S., Ohnishi, Y., Ishikawa, H., Miyamoto, N., Itoh, M., & Magara, Y. (2001). Estrogenic influences of estradiol-17b, p-nonylphenol and bis-phenol-A on Japanese Medaka (Oryzias latipes) at detected environmental concentrations. *Water Science and Technology, 43*(2), 109-116.

Tang, J. Y. M., Aryal, R., Deletic, A., Gernjak, W., Glenn, E., McCarthy, D., & Escher, B. I. (2013a). Toxicity characterization of urban stormwater with bioanalytical tools. *Water Research, 47*(15), 5594-5606. doi:http://dx.doi.org/10.1016/j.watres.2013.06.037

Tang, J. Y. M., McCarty, S., Glenn, E., Neale, P. A., Warne, M. S. J., & Escher, B. I. (2013b). Mixture effects of organic micropollutants present in water: Towards the development of effect-based water quality trigger values for baseline toxicity. *Water Research, 47*(10), 3300-3314. doi:http://dx.doi.org/10.1016/j.watres.2013.03.011

Tay, K. L., Doe, K. G., MacDonald, A. J., & Lee, K. (1998). *Microscale Testing in Aquatic Toxicology—Advances, Techni- ques and Practice.* (P. G. Wells, K. Lee, & C. Blaise Eds.). New York, NY: CRC Press.

Ternes, T. A., Joss, A., & H, S. (2004). Scrutinizing Pharmaceuticals and Personal Care Products in Wastewater Treatment. *Environmental Science and Technology*, 393-399. doi:http://dx.doi.org/10.1021/es040639t

Ternes, T. A., Meisenheimer, M., McDowell, D., Sacher, F., Brauch, H.-J., Haist-Gulde, B., . . . Zulei-Seibert, N. (2002). Removal of Pharmaceuticals during Drinking Water Treatment. *Environmental Science & Technology, 36*(17), 3855-3863. doi:http://dx.doi.org/10.1021/es015757k

Ternes, T. A., Stüber, J., Herrmann, N., McDowell, D., Ried, A., Kampmann, M., & Teiser, B. (2003). Ozonation: a tool for removal of pharmaceuticals, contrast media and musk fragrances from wastewater? *Water Research, 37*(8), 1976-1982. doi:http://dx.doi.org/10.1016/S0043-1354(02)00570-5

Thomas, R. S., Philbert, M. A., Auerbach, S. S., Wetmore, B. A., Devito, M. J., Cote, I., . . . Nong, A. (2013). Incorporating New Technologies Into Toxicity Testing and Risk Assessment: Moving From 21st Century Vision to a Data-Driven Framework. *Toxicological Sciences, 136*(1), 4-18. doi:http://dx.doi.org/10.1093/toxsci/kft178

Tischer, W., & Strotmann, H. (1977). Relationship between inhibitor binding by chloroplasts and inhibition of photosynthetic electron transport. *Biochimica et Biophysica Acta (BBA) - Bioenergetics, 460*(1), 113-125. doi:http://dx.doi.org/0.1016/0005-2728(77)90157-8

Tong, A. Y. C., Peake, B. M., & Braund, R. (2011). Disposal practices for unused medications around the world. *Environment International, 37*(1), 292-298. doi:http://dx.doi.org/10.1016/j.envint.2010.10.002

Toumi, H., Cotelle, S., Ferard, J., & Radetski, C. (2013). Sensitivity of different aquatic bioassays in the assessment of a new natural formicide. *Journal of Environmental Science and Health, Part B, 48*(1), 57-62. doi:http://dx.doi.org/10.1080/03601234.2012.716731

Toxnet. (2016, April 11, 2016). Venlafaxine CASRN: 93413-69-5. Retrieved from https://toxnet.nlm.nih.gov/cgi-bin/sis/search2/f?./temp/~V1yFnK:3

US EPA. (2011). Pesticides Industry Sales and Usage; 2006 and 2007 Market Estimates. Retrieved from https://www.epa.gov/sites/production/files/2015-10/documents/market_estimates2007.pdf

Uslu, M., Seth, R., Jasim, S., Tabe, S., & Biswas, N. (2015). Reaction Kinetics of Ozone with Selected Pharmaceuticals and Their Removal Potential from a Secondary Treated Municipal Wastewater Effluent in the Great Lakes Basin. *Ozone: Science & Engineering, 37*(1), 36-44. doi:http://dx.doi.org/10.1080/01919512.2014.929520

Uslu, M. O., & Balcioglu, I. A. (2008). Ozonation of animal wastes containing oxytetracycline. *Ozone: Science and Engineering, 30*(4), 290-299. doi:http://dx.doi.org/10.1080/01919510802143947

Uslu, M. O., Jasim, S., Arvai, A., Bewtra, J., & Biswas, N. (2013). A survey of occurrence and risk assessment of pharmaceutical substances in the Great Lakes Basin. *Ozone: Science & Engineering, 35*(4), 249-262. doi:http://dx.doi.org/10.1080/01919512.2013.793595

van Rensen, J. J. S. (1982). Molecular mechanisms of herbicide action near photosystem II. *Physiologia Plantarum, 54*(4), 515-521. doi:http://dx.doi.org/10.1111/j.1399-3054.1982.tb00719.x

Veeranjaneyulu, K., Charlebois, D., N'soukpoé-Kossi, C. N., & Leblanc, R. M. (1992). Sulfite inhibition of photochemical activity of intact pea leaves. *Photosynthesis Research, 34*(2), 271-278. doi:http://dx.doi.org/10.1007/bf00033444

Veli, S., & Alyüz, B. (2007). Adsorption of copper and zinc from aqueous solutions by using natural clay. *Journal of Hazardous materials, 149*(1), 226-233. doi:http://dx.doi.org/10.1016/j.jhazmat.2007.04.109

Vidal-Dorsch, D. E., Bay, S. M., Maruya, K., Snyder, S. A., Trenholm, R. A., & Vanderford, B. J. (2012). Contaminants of emerging concern in municipal wastewater effluents and marine receiving water. *Environmental Toxicology and Chemistry, 31*(12), 2674-2682. doi:http://dx.doi.org/10.1002/etc.2004

Vilfan, N., van der Tol, C., Muller, O., Rascher, U., & Verhoef, W. (2016). Fluspect-B: A model for leaf fluorescence, reflectance and transmittance spectra. *Remote Sensing of Environment, 186*, 596-615. doi:https://doi.org/10.1016/j.rse.2016.09.017

Villeneuve, D. L., Jensen, K. M., Cavallin, J. E., Durhan, E. J., Garcia-Reyero, N., Kahl, M. D., . . . Ankley, G. T. (2017). Effects of the antimicrobial contaminant triclocarban, and co-exposure with the androgen 17β-trenbolone, on reproductive function and ovarian transcriptome of the fathead minnow (Pimephales promelas). *Environmental Toxicology and Chemistry, 36*(1), 231-242. doi:http://dx.doi.org/10.1002/etc.3531

von Gunten, U. (2003). Ozonation of drinking water: Part I. Oxidation kinetics and product formation. *Water Research, 37*, 1443-1467. doi:https://doi.org/10.1016/S0043-1354(02)00457-8

Waldemar, M., & Tadeusz, B. (1988). The effect of Cd2+ on the release of proteins from thylakoid membranes of tomato leaves. *Acta Societalis Botanicorum Poloniae, 57*(4), 465-474. doi:http://dx.doi.org/10.5586/asbp.1988.044

Wang, P., Wong, M. H., & Tam, N. F. Y. (2013). Antioxidant responses of two microalgae, Selenastrum capricornutum and Chlorella sp., to estradiol and ethinylestradiol. *Journal of Applied Phycology, 25*(3), 891-903. doi:http://dx.doi.org/10.1007/s10811-012-9959-1

Wang, S., Zhang, X., Wang, Z., Li, X., & Ma, J. (2014). In-depth characterization of secondary effluent from a municipal wastewater treatment plant located in northern China for advanced treatment. *Water Science and Technology, 69*(7), 1482-1488. doi:http://dx.doi.org/10.2166/wst.2014.040

Wang, Z., Hu, F., Song, W., Guo, J., He, W., & Ding, F. (2011, 19-22 Aug. 2011). *Chronic toxic effect of three estrogens to algae (Scenedesmus obliquus).* Paper presented at the Proceedings 2011 International Conference on Human Health and Biomedical Engineering.

Waters, M., & Jackson, M. (2008). Databases applicable to quantitative hazard/risk assessment-Towards a predictive systems toxicology. *Toxicology and Applied Pharmacology, 233*(1), 34-44. doi:http://dx.doi.org/10.1016/j.taap.2007.12.036

Wert, E. C., Gonzales, S., Dong, M. M., & Rosario-Ortiz, F. L. (2011). Evaluation of enhanced coagulation pretreatment to improve ozone oxidation efficiency in wastewater. *Water Research, 45*(16), 5191-5199. doi:http://dx.doi.org/10.1016/j.watres.2011.07.021

Wert, E. C., Rosario-Ortiz, F. L., & Snyder, S. A. (2009). Effect of ozone exposure on the oxidation of trace organic contaminants in wastewater. *Water Research, 43*(4), 1005-1014. doi:http://dx.doi.org/10.1016/j.watres.2008.11.050

Wezel, A. P. v., & Opperhuizen, A. (1995). Narcosis due to environmental pollutants in aquatic organisms: residue-based toxicity, mechanisms, and membrane burdens. *Critical Reviews in Toxicology, 25*(3), 255-279. doi:http://dx.doi.org/10.3109/10408449509089890

Wilkinson, A. D., Collier, C. J., Flores, F., & Negri, A. P. (2015). Acute and additive toxicity of ten photosystem-II herbicides to seagrass. *Scientific Reports, 5*, 17443. doi:http://dx.doi.org/10.1038/srep17443

Winner, R. W., & Owen, H. A. (1991). Toxicity of copper to Chlamydomonas reinhardtii (Chlorophyceae) and Ceriodaphnia dubia (Crustacea) in relation to changes in water chemistry of a freshwater pond. *Aquatic Toxicology, 21*(3), 157-169. doi:http://dx.doi.org/10.1016/0166-445X(91)90070-P

Wood, R. J., Mitrovic, S. M., Lim, R. P., & Kefford, B. J. (2016). How benthic diatoms within natural communities respond to eight common herbicides with different modes of action. *Science of the Total Environment, 557–558*, 636-643. doi:https://doi.org/10.1016/j.scitotenv.2016.03.142

Wood, W. W. (1976). *Guidelines for collection and field analyses of groundwater samples for selected unstable constituents. Techni- ques of Water-Resources Investigations of the*

United States Geological Survey, Book 1, Chapter D2. Washington, DC: U.S. Geological Survey.

Xu, P., Janex, M.-L., Savoye, P., Cockx, A., & Lazarova, V. (2002). Wastewater disinfection by ozone: main parameters for process design. *Water Research, 36*(4), 1043-1055. doi:http://dx.doi.org/10.1016/S0043-1354(01)00298-6

Yan, Q., Gao, X., Chen, Y.-P., Peng, X.-Y., Zhang, Y.-X., Gan, X.-M., . . . Guo, J.-S. (2014a). Occurrence, fate and ecotoxicological assessment of pharmaceutically active compounds in wastewater and sludge from wastewater treatment plants in Chongqing, the Three Gorges Reservoir Area. *Science of the Total Environment, 470–471*, 618-630. doi:http://dx.doi.org/10.1016/j.scitotenv.2013.09.032

Yan, Z., Zhang, Y., Yuan, H., Tian, Z., & Yang, M. (2014b). Fish larval deformity caused by aldehydes and unknown byproducts in ozonated effluents from municipal wastewater treatment systems. *Water Research, 66*, 423-429. doi:http://dx.doi.org/10.1016/j.watres.2014.08.019

Yang, L., Ying, G., Su, H., Stauber, J., Adams, M., & Binet, M. (2008). Growth-inhibiting effects of 12 antibacterial agents and their mixtures on the freshwater microalga Pseudokirchneriella subcapitata. *Environmental Toxicology and Chemistry, 27*(5), 1201-1208. doi:http://dx.doi.org/10.1897/07-471.1

Yang, M., & Zhang, X. (2013). Comparative Developmental Toxicity of New Aromatic Halogenated DBPs in a Chlorinated Saline Sewage Effluent to the Marine Polychaete Platynereis dumerilii. *Environmental Science & Technology, 47*(19), 10868-10876. doi:http://dx.doi.org/10.1021/es401841t

Yao, Q., Wang, X., Jian, H., Chen, H., & Yu, Z. (2015). Characterization of the Particle Size Fraction associated with Heavy Metals in Suspended Sediments of the Yellow River. *International Journal of Environmental Research and Public Health, 12*(6), 6725-6744. doi:http://dx.doi.org/10.3390/ijerph120606725

Yargeau, V., & Danylo, F. (2015). Removal and transformation products of ibuprofen obtained during ozone- and ultrasound-based oxidative treatment. *Water Science and Technology, 72*(3), 491-500. doi:http://dx.doi.org/10.2166/wst.2015.234

Yargeau, V., & Leclair, C. (2007). Potential of ozonation for the degradation of antibiotics in wastewater. *Water Science & Technology, 55*(12). doi:http://dx.doi.org/10.2166/wst.2007.427

Yargeau, V., & Leclair, C. (2008). Impact of operating conditions on decomposition of antibiotics during ozonation: a review. *Ozone: Science and Engineering, 30*(3), 175-188. doi:http://dx.doi.org/10.1080/01919510701878387

Yavuz, Ö., Altunkaynak, Y., & Güzel, F. (2003). Removal of copper, nickel, cobalt and manganese from aqueous solution by kaolinite. *Water Research, 37*(4), 948-952. doi:http://dx.doi.org/10.1016/S0043-1354(02)00409-8

Yeh, R. Y. L., Farré, M. J., Stalter, D., Tang, J. Y. M., Molendijk, J., & Escher, B. I. (2014). Bioanalytical and chemical evaluation of disinfection by-products in swimming pool water. *Water Research, 59*, 172-184. doi:http://dx.doi.org/10.1016/j.watres.2014.04.002

Zhang, H., Yamada, H., & Tsuno, H. (2008). Removal of Endocrine-Disrupting Chemicals during Ozonation of Municipal Sewage with Brominated Byproducts Control. *Environmental Science & Technology, 42*(9), 3375-3380. doi:http://dx.doi.org/10.1021/es702714e

Zhu, Y. (2014). *Sorption of Microconstituents onto Primary and Activated Sludge to which Alum Has Been Added.* (Master of Applied Science), University of Ottawa.

Zimmermann, S. G., Wittenwiler, M., Hollender, J., Krauss, M., Ort, C., Siegrist, H., & von Gunten, U. (2011). Kinetic assessment and modeling of an ozonation step for full-scale municipal wastewater treatment: Micropollutant oxidation, by-product formation and disinfection. *Water Research, 45*(2), 605-617. doi:http://dx.doi.org/10.1016/j.watres.2010.07.080

Zouboulis, A. I., Xiao-Li, C., & Katsoyiannis, I. A. (2004). The application of bioflocculant for the removal of humic acids from stabilized landfill leachates. *Journal of Environmental Management, 70*(1), 35-41. doi:http://dx.doi.org/10.1016/j.jenvman.2003.10.003

Zurita, J. L., Repetto, G., Jos, Á., Salguero, M., López-Artíguez, M., & Cameán, A. M. (2007). Toxicological effects of the lipid regulator gemfibrozil in four aquatic systems. *Aquatic Toxicology, 81*(1), 106-115. doi:https://doi.org/10.1016/j.aquatox.2006.11.007

Zushi, K., & Matsuzoe, N. (2017). Using of chlorophyll a fluorescence OJIP transients for sensing salt stress in the leaves and fruits of tomato. *Scientia Horticulturae, 219*, 216-221. doi:https://doi.org/10.1016/j.scienta.2017.03.016

Zwiener, C., & Frimmel, F. H. (2000). Oxidative treatment of pharmaceuticals in water. *Water Research, 34*(6), 1881-1885. doi:http://dx.doi.org/10.1016/S0043-1354(99)00338-3