Evaluating contaminants of emerging concern in municipal wastewater effluents

by

Emily McCann

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Author's Declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Abstract

Municipal wastewater treatment plant effluent (MWWE) is a complex matrix that acts as a significant source of contaminants to aquatic receiving environments. Contaminants of emerging concern (CECs) are known to affect aquatic organisms downstream of MWWE discharges. Past studies in the Grand River watershed of southern Ontario on the small-bodied, benthic rainbow darter (*Etheostoma caeruleum*) have shown altered gene expression, sex steroid levels, gonad size and expression of intersex (testis-ova) associated with wastewater outfalls. The Region of Waterloo is upgrading the two major wastewater treatment plants (WWTP) within the Grand River watershed (Waterloo, Kitchener) where biological impacts in the receiving waters have been observed. Although extensive research is currently being performed in the Grand River to determine the biological impacts of WWTP upgrades on exposed fish and benthos, there was no comprehensive work being done on the chemistry of the effluent itself. The objectives of the current study were to determine how process changes and temporal variability altered the concentration of select CECs present in the effluent as well as the total estrogenicity of the discharged effluent. Archived and current effluent samples from 2009 through to 2015 were analyzed for select CECs with LC-MS/MS as well as total estrogenicity with the Yeast Estrogen Screen assay. Concentrations of selected pharmaceuticals, such as ibuprofen and naproxen, are greatly reduced with nitrifying treatment while other contaminants such as carbamazepine and diclofenac remain recalcitrant. The removal of key CECs varies dependent on their physiochemical properties, with readily biotransformed CECs the most effectively removed by the WWTP after a transition to nitrifying treatment.

Increased understanding of how major upgrades to treatment plant infrastructure alter the contaminant concentrations in wastewater effluent will greatly improve our ability to inform future watershed regulatory decision-making. These improvements have potential significance for the environment downstream of the WWTPs where endocrine disruption has been documented, including high expression of intersex in fish. This data is a crucial piece of information supporting numerous studies examining the biological consequences of CECs in the aquatic receiving environment.

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Chapter 1 - Introduction

Over the last two decades it has become evident that a wide variety of chemicals are entering the environment that were not previously considered or assessed as environmental contaminants. These contaminants of emerging concern (CECs) include pharmaceuticals, natural and synthetic estrogens, and industrial chemicals, encompassing an array of individual contaminants with varying modes of action (Daughton and Ternes 1999). Effluent discharges from municipal wastewater treatment plants (WWTPs) are a major source of CECs to the environment (i.e. Ternes 1998; Kümmerer 2001; Kolpin et al. 2002). Chronic exposure to low levels of CECs has been linked to adverse impacts in aquatic organisms worldwide (Jobling et al. 1998; Mills & Chichester 2005). Although these impacts have been well documented, direct links between specific chemicals and effects observed in the environment are still not well established.

Numerous studies have now documented the distribution of these CECs in wastewaters around the world, including Canada (Metcalfe et al. 2003; Servos et al. 2005; Lishman et al. 2006). The influence of various treatment processes has been a very active area of research (Salveson et al., 2012). The composition of the final effluent is dependent on the inputs as well as the degree and type of effluent treatment (Joss et al. 2005; Clara et al. 2005; Salveson et al. 2012). Although each treatment plant is unique, patterns are emerging that are helping to explain the distribution of CEC in final effluents released into the environment (Salveson et al. 2012). Many of these studies have been short term in nature and there has been limited characterization of wastewater effluents over longer periods (month-years) of time to capture variation in inputs to aquatic receiving environments. The presence of CECs in effluents and associated receiving

environments, and their potential effects reported under laboratory settings means that they represent a potential risk to aquatic ecosystem health. Using two WWTPs that are undergoing major upgrades to the treatment process (Waterloo and Kitchener, ON), the current thesis examines how process changes and temporal variability impact the presence of CECs in municipal effluents. To characterize these effluents, changes in ammonia, nitrate, nitrite, selected pharmaceuticals as well as the total estrogenicity of the final effluent were examined over several years. This work supports several other ongoing studies on the impacts of wastewater effluents (and process upgrades) on fish responses in the Grand River, Ontario.

1.1 CECs in wastewater effluents

CECs have been reported in municipal wastewater effluents and associated surface waters worldwide (Pal et al. 2010; Petrie et al. 2014). Many of these compounds are human pharmaceuticals or natural estrogens that are not fully metabolized in the body and thus are excreted as waste (Escher & Fenner 2011; Vasquez et al. 2014). Other contaminants enter wastewater streams as byproducts of industrial or agricultural processes (Luo et al. 2014). A number of these contaminants are designed to be biologically active, and thus have the potential to impact non-target species in the receiving environment at low (ng/L to μ/L) concentrations (Fent et al. 2006; Arnold et al. 2014). Concentrations of CECs in effluent and the efficacy of their removal during conventional wastewater treatment vary widely depending on the properties of the individual contaminant as well as the treatment processes employed. As it is difficult to control the input of CECs to wastewater effluents, removal within the WWTP becomes a crucial step to limiting their presence in the environment. Improved understanding of CEC removal during treatment should therefore be of paramount concern, as both the input and removal efficacy of CECs is highly variable (Luo et al. 2014). An additional challenge to understanding

how these CECs behave is the accurate and precise quantification of contaminants at very low concentrations in complex matrices such as wastewater effluent. Significant work has been done since the early 1990s (and continues) to develop reliable and robust methodology for measuring these compounds in environmental samples.

1.2 LC-MS/MS Analysis of CECs

The detection and quantification of CECs in wastewater effluents is complicated by their low environmental concentrations, the wide variety of physiochemical properties, and the complexity of wastewater as a sample matrix. Although there is no standardized method for quantifying CECs in wastewater, extraction and analysis of these contaminants is frequently done with solid phase extraction (SPE) followed by liquid chromatography tandem mass spectrometry (LC-MS/MS). A comparison of analytical techniques found LC-MS/MS with isotope dilution was the most consistent method of analysis for the majority of CECs investigated (Vanderford et al. 2014). The use of LC-MS/MS typically provides sensitivity and accuracy when performing trace analysis on complex sample matrices. The addition of isotopically labelled standards compensates for matrix affects associated with co-extractives present in effluent samples. These methods typically use a deuterated version of the target analyte to account for extraction efficiencies and matrix effects that may occur during analysis.

Development of a method for LC-MS/MS is a difficult task that requires the consideration of numerous processes that must be optimized for each target analyte. Although there are published methods for the evaluation of many CECs in wastewater effluents, some analytes, including estrogens, are more difficult to measure accurately and consistently at environmentally relevant concentrations. Method development for a new compound on LC-MS/MS requires the consideration of a number of factors to ensure optimal sensitivity and

selectivity. LC parameters, such as mobile phase, mobile phase gradient, and column, must be selected to sufficiently separate target analytes while minimizing run-time. At the source of the mass spectrometer, a number of parameters, including source gas temperature and flow must be considered to optimally ionize target analytes. Finally, the fragmenter voltage, collision energy, and cell accelerator voltage within the mass spectrometer must be optimized for each target analyte. Once these optimization steps are complete, the method must be validated for accuracy and precision, and the detection and quantitation limits must be determined. A continuing quality assurance and quality control (QA/QC) plan must then be implemented, at which point analysis of samples can begin. Notably, when working with such a complex matrix at such low concentrations of analyte, samples which pass robust QA/QC processes can still present with unusable or nonsensical data, often due to the incomplete separation of compounds at suitable resolution. Furthermore, chemical analyses typically employed, such as LC-MS/MS, allow researchers to quantify the concentration of known compounds within an effluent sample, but are limited in that they can only provide information on known chemicals for which analytical standards are available (Nadzialek et al. 2010). Additional techniques for evaluating effluents, including biological assays, can provide a compliment to analytical data and enhance understanding of effluent contamination.

1.3 Biological assays for evaluation of effluent estrogenicity

Biological assays are often used in tandem with chemical analysis to allow for a more thorough characterization of effluents. While chemical analysis with LC-MS/MS is excellent at quantifying the presence of known contaminants, it can only report on the subset of CECs that the method is designed to measure, and thus may exclude some contaminants that have the ability to impact aquatic biota in the receiving environment. Evaluating effluents with bioassays

is an approach to characterization of samples that considers the integrated response induced by the whole effluent rather than quantifying specific individual compounds. It is also desirable to use bioassays that are mechanistically linked to the effects in aquatic biota that are of concern. A number of cellular bioassays have been designed to measure the estrogenicity of environmental matrices such as wastewater effluents (Nadzialek et al. 2010). Leusch et al. (2010) compared five common estrogen assays (ER-CALUX, E-SCREEN, MELN, T47D0kBluc, YES), and found the responses were consistent across the assays, particularly between 0.2- 20 ng/L. When the Yeast Estrogen Screen (YES) and E-SCREEN assays were applied to the same samples in two different laboratories there was no significant difference in the data, indicating quality control/quality assurance may be able to minimize inter-laboratory variation (Leusch et al. 2010). The YES assay has a higher quantification limit than the other assays used but performed particularly well in highly polluted and complex samples (Leusch et al. 2010).

The YES assay is a relatively straightforward and rapid way to determine the total estrogenicity of municipal wastewater treatment effluent (Nadzialek et al. 2010), and is often used in tandem with chemical analysis to characterize environmental samples (Beck et al. 2006; Viganò et al. 2008). This assay uses *Saccharomyces cerevisiae* yeast cells transfected with human estrogen receptor genes upstream of the β-galactosidase reporter gene (Gaido et al. 1997). These receptors are well conserved between species, allowing their activation in the YES assay (which is based on a human estrogen receptor) to indicate a similar response in exposed aquatic biota (Escher and Leusch 2012). After exposure to estrogenic compounds, the cells are suspended in a buffer that includes 2-nitrophenyl-β-D-galactosidase (ONPG), as well as compounds to induce cell lysis (Gaido et al. 1997). β-galactosidase will cleave ONPG to generate orthonitrophenol, and the resulting colour change can be measured with a microtiter

plate reader (Gaido et al. 1997). The relative speed and low cost of this assay paired with adequate sensitivity for determining estrogenic activity have made it a common tool for measuring estrogenicity in both wastewater and surface waters.

1.4 Adverse impacts associated with CECs in wastewater effluents

The ubiquitous presence of CECs in wastewater effluents has led to a large body of work investigating their potential impacts on aquatic life. Recent laboratory findings have demonstrated that chronic exposure to environmentally relevant levels of common pharmaceuticals can impact the metabolism, reproductive success, and development of fish (Ainter et al. 2009; David and Pancharatna 2009a; David and Pancharatna 2009b; Lister et al. 2009; Galus et al. 2013a; Galus et al. 2013b; Luo et al. 2014; Schoenfuss et al. 2015). Zebrafish exposed to ibuprofen and acetaminophen have shown increased developmental abnormalities and increased mortality (David and Pancharatna 2009a; David and Pancharatna 2009b), decreased spawning rates, and decreased clutch sizes (Lister et al. 2009). Galus et al. (2013a,b) found that adult female zebrafish exposed to four individual pharmaceuticals (carbamazepine, acetaminophen, venlafaxine, and gemfibrozil) as well as to a mixture of these pharmaceuticals produced fewer viable embryos and experienced increased oocyte atresia as well as altered kidney morphology. Likewise, the exposure of embryos to the same pharmaceuticals resulted in reduced survival and increased developmental abnormalities (Galus et al. 2013a,b). Goldfish exposed to the antidepressant fluoxetine exhibit altered behaviour, reproduction (Mennigen et al. 2008; Mennigen et al. 2010), and altered sex steroid levels (Mennigen et al. 2010). These findings indicate the potential for adverse impacts on fish exposed to wastewater effluents containing common pharmaceuticals.

More thoroughly documented are the adverse impacts associated with the natural and synthetic estrogens often found in wastewater effluents (Lange et al. 2001; Nash et al. 2004; Schäfers et al. 2007; Parrott & Blunt 2005; Kidd et al. 2007). These CECs act as estrogen receptor agonists and have the potential to impact aquatic life at very low (>1 ng/L) concentrations. Many studies have reported changes in the reproductive system or fitness of wild fish downstream of WWTP outfalls (Jobling et al. 1998; Woodling et al. 2006; Hinck et al. 2009; Tanna et al. 2013), and recent work has demonstrated that secondary-treated municipal effluents have the potential to cause changes in fish and fish populations in receiving environments (e.g. Tyler et al. 2008; Tetreault et al. 2011; 2013). Of particular note is the occurrence of intersex and changes in reproductive performance in fish associated with these wastewater effluent outfalls (Bahamonde et al. 2013; Sumpter & Jobling 2013); these changes may have associated impacts at the population levels (Tetreault et al. 2011; Fuzzen et al. 2015). In a whole lake experiment, Kidd et al. (2007) found exposure of fathead minnows (*Pimephales promelas*) to 5 – 6 ng/L of 17α-ethinylestradiol (EE2) resulted in feminized male fish with arrested gonadal development, which within two years led to recruitment failure in the population. These observations suggest that very low levels of EDCs in the environment can have adverse effects on exposed fish, and removal of these compounds from effluents should be an area of concern for environmental decision-makers.

1.5 Removal of CECs from wastewater

Although removal through treatment is crucial to limiting the entry of CECs into the environment, effluent quality monitoring does not typically include the analysis of these contaminants. Traditional measures of effluent quality focus instead on indicators such as total suspended solids, nutrient loading, and biological oxygen demand. Although these

indicators are important parameters to assist in the mitigation of environmental impact, they fail to account for the presence of CECs at trace (ng/L or μg/L) levels. No current Canadian legislation exists to specifically address these contaminants, and thus removal is dependent on processes implemented to meet conventional targets.

As wastewater treatment plants are not intentionally designed to remove CECs, removal efficacy varies between individual contaminants and treatment processes (Nakada et al. 2006; Salveson et al. 2012; Luo et al. 2014). Chemical processes, such as hydrolysis, volatilization, and photolysis, can also be important for the removal of some chemicals. However, these processes are generally responsible for very little CEC removal during conventional wastewater treatment. Sorption and biotransformation are the primary mechanisms for the removal of CECs from municipal wastewater (Salveson et al. 2012; Luo et al. 2014). Sorption is the process by which CECs are removed from wastewater through complexing with organic solid waste due to hydrophobic or electrostatic interactions (Salveson et al. 2012). Sorbable CECs are often hydrophobic, though the properties of the sludge in the system (e.g. organic content, charge) can also modify removal (Hyland et al. 2012). This is demonstrated by removal due to electrostatic interactions between negatively charged activated sludge and CECs that are positively charged at sludge pH (Jelic et al. 2012), indicating that estimation of a compound's sorption potential must consider both its hydrophobicity and its ionization state (Salveson et al. 2012). Historically, a compound's log K_{ow} value (octonal-water partitioning coefficient) was used to estimate sorption to sludge. However, this metric does not account for the altered sorption potential of weakly acidic or basic compounds in different ionization states. A pH-dependent partitioning coefficient (log D_{ow}) is a more accurate metric to estimate sorption potential for ionizable compounds (Salveson et al. 2012). However, the use of these metrics provides only a broad estimation of

sorption potential. To accurately assess the sorption of a CEC in a WWTP, the compound must be investigated within that particular system (determination of specific partition coefficients, K_p). This is in part due to the differences in the structure and properties of the organic matter in each matrix.

Biotransformation is the removal through biological processes and can be estimated based on a CEC's physiochemical properties and verified through laboratory assays (Salveson et al. 2012). Biodegradation potential can be represented by a first-order kinetic rate that is calculated for each specific compound in a particular sludge, and can vary widely between different processes (Salveson et al. 2012).The biodegradability of a compound is dependent on factors such as its physical structure (chain length or branching), size, and solubility, as well as process characteristics (Salveson et al. 2012). CECs that readily degrade in wastewater systems tend to be soluble in water, smaller, and have sites amenable to biological attack, though these metrics provide only a broad generalization with which removal efficacy may be estimated. The degree of removal achieved by sorption and biotransformation for any CEC is thus driven by a combination of the CEC's physiochemical properties and by the treatment processes it undergoes. Considering the number of chemicals and the diversity of treatment processes it is difficult to predict the composition of final wastewater effluents. Despite this complexity some general patterns have emerged from the literature. Although each treatment plant can vary considerably in design, they generally follow similar processes, and must meet similar legislated targets for effluent quality.

Table 1.5.1. Some physical and chemical properties of selected CECs.

Class	Compound	Most Common Use	Chemical Formula	Molecular Weight (g/mol)	pKa	Log K_{ow}	Log D_{ow} at pH 7[g]
Antiandrogens	Triclosan	Personal care products	$C_{12}H_7Cl_3O_2$	289.54	7.9	4.76	4.9
	Triclocarban	Personal care products	$C_{13}H_9Cl_3N_2O$	315.58	12.7[d]	4.2[d]	4.93
Antibiotics	Sulfamethoxazole	Veterinary medicine	$C_{10}H_{11}N_3O_3S$	253.28	pKa 1 = 1.6 pKa 2 = 5.7	0.89	0.14
	Trimethoprim	Veterinary medicine	$C_{14}H_{18}N_4O_3$	290.32	7.12	0.91	0.92
Anti-epileptics	Carbamazepine	Anti-epileptic	$C_{15}H_{12}N_2O$	236.27	13.9	2.45	2.5
Estrogens	17α-ethynylestradiol	Birth Control Pill	$C_{20}H_{24}O_2$	296.40	10.7[f]	4.2	3.81
	17β-estradiol	Natural Hormone	$C_{18}H_{24}O_2$	272.38	10.4[c]	4.1	3.75
	Estrone	Natural Hormone	$C_{18}H_{22}O_2$	270.36	10.4[c]	4.0	4.31
	Estriol	Natural Hormone	$C_{18}H_{24}O_3$	288.38	10.4[c]	3.13	2.67
Fibrates	Gemfibrozil	Hypolipidemic	$C_{15}H_{22}O_3$	250.33	4.5	4.77	1.85
NSAIDS	Diclofenac	Anti-inflammatory	$C_{14}H_{22}O$	296.16	4.15[e]	4.51[g]	1.37
	Ibuprofen	Anti-inflammatory	$C_{13}H_{18}O_2$	206.29	5.2	3.97	1.71
	Naproxen	Anti-inflammatory	$C_{14}H_{14}O_3$	230.25	4.15	3.18	0.25
SSRIs	Fluoxetine	Antidepressant	$C_{17}H_{18}F_3NO$	309.33	10.1[a]	4.05[b]	1.5
SNRIs	Venlafaxine	Antidepressant	$C_{17}H_{27}NO_2$	277.40	10.09	3.2	2.77
Statins	Atorvastatin p-hydroxy Atorvastatin	Lowering blood cholesterol Atorvastatin metabolite	$C_{33}H_{35}FN_2O_5$ $C_{33}H_{34}FN_2O_6$	558.64 573.65	4.46		

Adapted from "Hazardous Substances Data Bank" by United States National Library of Medicine. [a]Nakamura et al. 2008 [b]Adlard et al. 1995 [c] Trenholm et al. 2006 [d](Petrie et al. 2014) [e](Zhang et al. 2008) f(Clara et al. 2004) g(Salveson et al. 2012)

Primary treatment typically consists of the removal of suspended solids and some organic materials through the use of settling tanks and skimmers (Metcalfe and Eddy 2003). In some cases, a chemical coagulant may be used to improve sedimentation (Metcalfe and Eddy 2003). As this stage of treatment is primarily concerned with the settling out and removal of solids, CECs that are highly sorbable can show removal during this stage of treatment, while those that have low sorption potential show little to no removal (Salveson et al. 2012).

Secondary treatment uses biological and chemical agents to remove organic material and nutrients from wastewater. This is the stage of treatment that most influences CEC removal from wastewater, and is also the stage that shows the highest process variability among WWTPs. In Canada, conventional activated sludge (CAS) systems are the most common type of secondary treatment in municipal WWTPs (Canadian Water and Wastewater Association 2001). These systems are designed to remove organic contaminants through metabolic degradation by biological organisms followed by sedimentation in secondary clarifiers. Within a CAS system, operational parameters, such as temperature, hydraulic retention time (HRT), solids retention time (SRT) and redox conditions, result in varied bacteria communities and degrees of nitrification, which have a significant impact on CEC removal (Salveson et al. 2012; Luo et al. 2014).

Nitrifying conditions (ammonia conversion to nitrate) in WWTPs has been shown to improve removal efficacy for a number of CECs, including natural and synthetic estrogens (Servos et al. 2005; Suarez et al. 2010; Fernandez-Fontaina et al. 2012; Luo et al. 2014). Improved removal in nitrifying systems is particularly pronounced for compounds that are readily biotransformed, such as ibuprofen and naproxen (Suarez et al. 2010; Fernandez-Fontaina et al. 2012; Arlos et al. 2014). This could be due to the chemical conditions and/or the specific

bacterial communities present. Nitrification typically occurs alongside an increased SRT, resulting in a more diverse bacterial community that can more readily remove easily degradable compounds. CECs that are not readily biotransformed, including carbamazepine, tend to show little removal in conventional treatment plants even with nitrifying treatment (Salveson et al. 2012). The properties that make these compounds difficult to degrade still complicate degradation even when exposed to a more diverse bacterial community. As a result, improved removal efficacy under nitrifying conditions does not typically extend to recalcitrant compounds (Suarez et al. 2010; Fernandez-Fontaina et al. 2012; Luo et al. 2014).

As previously noted, the implementation of nitrification typically requires an extended SRT, allowing for the cultivation of a more diverse bacterial community. Literature surrounding the impact of SRT on removal of CECs indicates that it is compound and process dependent. Numerous studies have reported that increased SRTs resulted in improved removal efficacy for a broad range of micropollutants (Metcalfe et al. 2003; Servos et al. 2005; Clara, Strenn, et al. 2005; Suarez et al. 2010; Salveson et al. 2012), while others have found no correlation between SRT and CEC removal (Joss et al. 2005; Samaras et al. 2013). Salvenson et al. (2012) reported threshold SRT values for a number of compounds. When these SRTs were met or exceeded, 80% removal of the target CEC was achieved in their system (Salveson et al. 2012). These values ranged from 5 d for readily biotransformed CECs like ibuprofen and naproxen up to 30 d for trimethoprim. No threshold SRT could be determined for the most highly recalcitrant compounds such as carbamazepine. Servos et al. (2005) in a survey of Canadian WWTPs found that SRT > 5 d resulted in the effective removal of estrogenic compounds as well as effluent estrogenicity. As part of the same study, Metcalfe et al. (2003) found an SRT of > 5 d resulted in the removal of many, but not all, pharmaceuticals studied. Although the mechanism is unclear, increased SRT,

which is typically associated with better treatment, is frequently correlated with reduced release of a variety of CECs, though some contaminants remain very recalcitrant in final effluents. Although all plants in Canada are now required to implement secondary treatment processes (or equivalent), the conditions and operation may differ greatly leading to very different removal rates of CECs.

Tertiary treatment of various forms is often employed to further improve effluent quality to achieve desired receiving water quality. This type of advanced treatment is frequently employed when specific contaminants of concern are identified in influents or effluents. Tertiary treatment targeting CECs through the use of advance oxidation processes like UV:hydrogen peroxide and ozonation, are a current area of active research. Although UV light alone has minimal effect on most CEC, in combination with hydrogen peroxide it can be very effective at removing many CECs (Ternes et al. 2003; Rosario-Ortiz et al. 2010; Cesaro & Belgiorno 2015) Ozonation has been shown to successfully remove a number of CECs through oxidation, including those that are typically difficult to remove with conventional treatment processes (Prasse et al. 2015). The success of ozonation is dependent on dose as well as characteristics of the wastewater such as pH and organic matter content (Prasse et al. 2015). The primary concerns surrounding these methods of treatment are their high cost as well as the potential for the formation of reactive byproducts (Prasse et al. 2015; Semblante et al. 2015).

Overall, rapidly biotransformed CECs with low sorption potential typically have high removal efficacies, particularly in treatment plants with nitrifying treatment and SRTs above 5-7 days (Clara et al. 2005; Servos et al. 2005; Nakada et al. 2006; Salveson et al. 2012). In contrast, CECs that are resistant to biological transformation are more recalcitrant unless advanced processes are used, e.g. advanced oxidation (Salveson et al. 2012). Compounds with moderate

biotransformation vary widely in their removal depending on the operating parameters of the plant, with nitrification and increased SRTs typically associated with improved removal (Fernandez-Fontaina et al. 2012; Salveson et al. 2012). CEC removal also has been found to vary seasonally, with increased removal efficacy in summer and reduced efficacy in winter (Salveson et al. 2012). In Canada, where temperature can vary considerably throughout the year, treatment efficiencies, (e.g. nitrification) may be very important (Parker et al. 2014). As removal of CECs in wastewater treatment is a byproduct of processes intended to meet legislated targets, it is likely that CECs, particularly those resistant to biological transformation, will persist in effluents. It is therefore important to understand potential impacts to aquatic life in the receiving environment associated with chronic, low level exposure to CECs.

1.6 Wastewater effluents in the Grand River watershed

CECs have been detected in municipal wastewater effluents across Canada (Metcalfe et al. 2003; Servos et al. 2005; Lishman et al. 2006), including in the Grand River watershed (Tanna et al. 2013; Arlos et al. 2014). The Grand River is a highly impacted watershed, and receives inputs from more than 30 WWTPs (Chapman & Anderson 2011). Antimicrobials, antiandrogens, estrogens, artificial sweeteners, polycyclic musks and numerous pharmaceuticals have all been associated with major WWTP outfalls in the central Grand River (Servos et al. 2005; Smyth et al. 2008; Metcalfe et al. 2010; Spoelstra et al. 2013; Arlos et al. 2014; Couperus et al. 2016). Numerous studies evaluating impacts associated with these effluent outfalls in the Grand River have focused on the rainbow darter (*Etheostoma caeruleum*), a small-bodied benthic fish found throughout the watershed. When exposed to wastewater effluent in the Grand River, rainbow darter have exhibited effects at multiple levels of biological organization, including impacts often associated with EDCs. Rainbow darter present at sites downstream of

wastewater treatment plants in the Grand River have been found to have altered gene expression, sex steroid levels, gonad size and expression of elevated rates of intersex (testis-ova) associated with wastewater outfalls (Tetreault et al. 2011; Tanna et al. 2013; Bahamonde et al. 2015). Downstream of the Kitchener WWTP outfall, 80 – 100% of fish captured between 2009 and 2011 exhibited intersex (Tetreault et al. 2011; Tanna et al. 2013; Bahamonde et al. 2015). In addition, there is evidence that suggests changes in reproductive performance (Fuzzen et al. 2015) and altered fish assemblages (Tetreault et al. 2013). These changes may be related to a variety of contaminants found in the effluents including estrogenic and/or antiandrogenic compounds present in the wastewater effluent.

The Region of Waterloo is currently upgrading both the Waterloo (Table 1.6.1) and Kitchener (Table 1.6.2) WWTPs in order to improve effluent quality (Bicudo et al. 2016). Previous studies following municipal treatment plant upgrades in Boulder, Colorado showed that treatment upgrades (moving from trickling filters and solid contact to activated sludge) reduced effluent estrogenicity, and corresponding impacts, such as intersex, in fish downstream of the outfall (Barber et al. 2012). Prior to upgrades, the Kitchener and Waterloo WWTPs operated as secondary conventional activated sludge plants with minimal or no nitrification.

Table 1.6.1 Characteristics of the Waterloo WWTP

Treatment Plant	**Waterloo – Pre**	**Waterloo – Post (anticipated)**
Treatment Capacity Rate (m^3/d)	57,500	57,500
Treatment System	Conventional activated sludge (partial/non-nitrifying)	Conventional activated sludge (partial/non-nitrifying)
Primary Treatment	Bar screen, grit removal, primary clarifier	Bar screen, grit removal, primary clarifier
Secondary Treatment	Conventional activated sludge	Conventional activated sludge with return activated sludge
Advanced Treatment	Phosphorous removal	Phosphorous removal
Disinfection	Sodium hypochlorite	UV light

Table 1.6.2. Characteristics of the Kitchener WWTP before and after upgrades.

Treatment Plant	**Kitchener – Pre**	**Kitchener – Post (Current status)**
Treatment capacity rate (m^3/d)	122,000	122,000
Treatment System	Convectional activated sludge (non-nitrifying)	Convectional activated sludge (fully nitrifying)
Primary Treatment	Bar screen, grit removal, primary clarifier	Bar screen, grit removal, primary clarifier
Secondary Treatment	Conventional activated sludge[1]	Conventional activated sludge[3] with return activated sludge
Advanced Treatment	Phosphorous removal	Phosphorous removal
Disinfection	Sodium hypochlorite	UV light
Notes	[2]Two secondary treatment trains; shared headworks, primary clarifier; trains re-join before chlorination/dechlorination	[4]Two secondary treatment trains; shared headworks, primary clarifier; trains re-join before UV disinfection

[1] Mechanical surface aeration
[2] Both secondary treatment trains were operating as fully mixed bioreactors
[3] Fine bubble aeration installed in August 2012 (75% of the aeration tanks) and January 2013 (100% of aeration tanks); returning centrate passes through re-aeration zone
[4] Train 1 operating as a fully mixed bioreactor; Train 2 operating as a three-pass plug flow train as of Fall 2012

Upgrades at the Kitchener and Waterloo WWTPs began in 2007 and are scheduled to be completed in 2018 (Bicudo et al. 2016). At both plants, upgrades will move treatment towards extended solids retention times, nitrification, and UV disinfection of the effluents which is expected to greatly improve their quality. The Region of Waterloo's target for ammonia exiting the Kitchener WWTP is 7 mg/L year-round after the upgrades that occurred in 2012 and 2013, and 2 – 5 mg/L when all upgrades are completed in 2018 (Figure 1.6.1), far below the approximately 28 mg/L documented in effluents prior to upgrades (Bicudo et al. 2016).

The most significant upgrade impacting CEC removal at the Kitchener WWTP between 2007 and 2015 was the improvement to secondary treatment in late 2012 and early 2013 (Figure 1.6.1). The configuration of train 2 was altered from a completely mixed bioreactor to a three-step plug-flow system with a re-aeration zone for the return activated sludge entering the system (Bicudo et al. 2016). This change, paired with an upgrade from mechanical surface aeration to fine bubble aerators in both trains, allowed for a longer, stable SRT (an increase from <2 days to 5.4 days) and the growth of more diverse bacterial communities, resulting in nitrifying treatment in both treatment trains (Bicudo et al. 2016). In addition, these improvements increased the capacity of the secondary clarifiers (Bicudo et al. 2016).

Figure 1.6.1 Broad timeline of upgrades at the Kitchener WWTP adapted from Region of Waterloo (2010). UVDF = UV disinfection, EPS = Effluent pumping station.

The upgrades at the Waterloo WWTP, including major upgrades to include additional aeration to achieve nitrification, were originally expected to be completed in the fall of 2014 but have experienced delays in construction. The targeted upgrades were similar to those at the Kitchener WWTP, including implementation of a three-pass plug-flow system with fine bubble aeration for secondary treatment of effluents. Construction was halted after UV disinfection and return activated sludge processes were implemented but before improvements to aeration were made, resulting in high ammonia loading in the effluents (Figure A4.2). In mid-2014 a re-aeration zone was added for the return activated sludge (RAS) process, but the aeration in the rest of the secondary treatment train was not upgraded.

Previous work in the Grand River watershed indicated that treatment plants with greater nitrification led to greater removal of CECs (Tanna et al. 2013; Arlos et al. 2014). A number of ongoing studies in the Grand River watershed are working to characterize fish response to these changes in treatment on numerous levels of biological organization. An evaluation of impacts requires knowledge of what the organisms in the affected sites are exposed to. Wastewater effluents are extremely complex matrices, and their makeup varies significantly depending on the inputs to and processes employed within the plant. Effluents exiting a WWTP can also vary seasonally or due to process upsets. As a result of this variation, proper characterization of exposure requires a thorough understanding of the effluents exiting the WWTP upstream of impacted sites, and cannot be generalized from other WWTPs. As a result, it is imperative to characterize the specific effluents affecting an impacted site, and to understand their temporal variability.

1.7 Study Objectives

This study was intended to support the interpretation of biological work occurring downriver of the Kitchener and Waterloo WWTPs by providing data on potential exposure. Using two WWTPs undergoing major upgrades to the treatment process (Kitchener and Waterloo WWTPs), the current study examines how process upgrades and temporal variability impact the concentration of CECs in municipal effluents through:

1. Analysis of select CECs with LC-MS/MS in historical and current effluent samples.
2. Analysis of total estrogenicity in historical and current effluent samples via the YES assay.

1.8 Study Scope

To characterize the effluents of the Kitchener and Waterloo WWTPs over time, monthly grab samples of effluent were analyzed alongside limited archived samples for fifteen pharmaceuticals, three estrogens, and total estrogenicity.

Wastewater effluents may contain hundreds of CECs with widely varying physiochemical characteristics. The number of CECs selected for analysis must be therefore limited for practical reasons while still accurately characterizing the wastewater effluent. One way CECs can be categorized is by separation into broad categories based on the mechanisms by which they are removed in wastewater treatment (Salveson et al. 2012). A suite of indicator compounds was designed to allow for the evaluation of a broad spectrum of removal efficacy by sorption and biotransformation (Salveson et al. 2012). This allows for more general characterization of a WWTP's removal efficacy for CECs with similar physical and chemical properties (Salveson et al. 2012). The final consideration in the selection of CECs was the presence of a robust methodology and instrumentation for analysis and quantitation (Table 1.8.1). This was to ensure the data provided were accurate, precise, and sufficiently sensitive for environmental relevance.

Table 1.8.1 Selected CECs and their categorization based on expected mechanisms of removal in wastewater treatment.

		Biotransformation Potential		
	Level	Low	Medium	High
Sorption Potential	Low	Carbamazepine Diclofenac	Sulfamethoxazole Trimethoprim Gemfibrozil Venlafaxine	Ibuprofen Naproxen
	Medium		17-α ethinylestradiol Atorvastatin	Estradiol Estrone
	High	Triclocarban		Triclosan Fluoxetine

Chapter 2 - Materials and Methods

2.1 General Approach

Archived sample extracts of effluent from the Kitchener and Waterloo WWTPs in 2010, 2011, 2012, and 2013 were analyzed for CECs and estrogenic potency alongside effluent samples taken monthly from September 2014 to September 2015. Corresponding water quality data was collected at each effluent sampling (ammonia, nitrate, nitrite, chloride, conductivity). Samples were analyzed for select CECs with two LC-MS/MS methods and total estrogenic potency with the YES assay.

2.2 Materials

All solvents were of high performance liquid chromatography (HPLC) grade or higher. Methanol (MeOH), acetonitrile (ACN), ethyl acetate, and 10 M hydrochloric acid were purchased from Fisher Scientific (Toronto, ON, Canada). Methyl tert-butyl ether (MTBE), ammonium fluoride, and ammonium acetate were obtained from Sigma-Aldrich (Oakville, ON, Canada). Ultrapure water for mobile phase preparation was obtained from an EMD Milli-Q® Advantage A10 water purification system (Etobicoke, ON, Canada).

Atorvastatin and its metabolites, carbamazepine, diclofenac, fluoxetine, gemfibrozil, ibuprofen, naproxen, sulfamethoxazole, triclocarban, trimethoprim, venlafaxine, 4-nonylphenol, 4-octylphenol, estrone, 17α-ethynylestradiol, 17β-estradiol, estriol, lorazepam, and chloramphenicol were purchased from Sigma-Aldrich. Triclosan was purchased from Alfa Aesar (Wardhill, MA, USA). The isotopically labelled standards atorvastatin-d_5, p-hydroxy atorvastatin-d_5, carbamazepine-d_{10}, diclofenac-d_4, fluoxetine-d_5, gemfibrozil-d_6, ibuprofen-d_3, naproxen- d_3, sulfamethoxazole-d_4, triclosan-d_3, trimethoprim-d_3, triclocarban-d_4, venlafaxine-d_6, estrone-d_4, estriol-d_2, 17α-ethynylestradiol-d_4, 17β-estradiol-d_4, bisphenol A-d_{16}, 4-nonylphenol-

d_4, 4-octylphenol d_{-17}, and metformin-d_6 were purchased from CDN Isotopes Inc. (Pointe-Claire, QC, Canada). Stock solutions of all compounds were prepared in methanol.

Yeast β-galactosidase assay kits were purchased from ThermoFisher Scientific (Markham, ON, Canada). All other reagents for use in the YES assay were purchased from Sigma-Aldrich (Oakville, ON, Canada). A full list of these reagents can be found in Appendix A2: Reagents required for the YES Assay.

2.3 Wastewater Effluent Sampling

Grab samples were collected in triplicate directly from the effluent outflow just prior to release into the river at the Kitchener and Waterloo WWTPs. Samples were collected in pre-cleaned amber glass bottles with Teflon-lined screw caps and preserved with 1 g/L sodium azide and 50 mg/L ascorbic acid to prevent bacterial growth and analyte degradation. Samples were stored at 4 °C until extraction, usually within 24 h but always within 48 h of collection. Samples collected for LC-MS/MS analysis were taken in 125 mL bottles, while samples collected for analysis via the YES bioassay were taken in 500 mL bottles.

For analysis of nutrient data (ammonia, nitrite, nitrate), 250 mL grab samples were collected in triplicate in high density polyethylene (HDPE) bottles with 1 mL of 49% sulfuric acid as a preservative. For analysis of chloride and conductivity, 250 mL grab samples were collected in triplicate without preservation in 250 mL HDPE bottles.

2.4 Sample Preparation and Solid Phase Extraction

2.4.1 Solid Phase Extraction

Wastewater effluent samples were analyzed for select CECs with two LC-MS/MS methods as well as total estrogenicity with the YES assay. Three solid phase extraction (SPE)

methods were used, each optimized for the target analytes. The three solid phase extraction methods used are outlined in Table 2.4.1.

All samples were filtered through a glass fiber filter with a pore size of 1 µm (Pall Corporation, Mississauga, ON) prior to extraction. Isotopically labelled standards for each target CEC were added to samples prior to extraction for analysis with LC-MS/MS. Samples for bioassays were not spiked. A ThermoFisher AutoTrace™ (Dionex, Sunnyvale, CA) was used to extract the samples. All cartridges were preconditioned with solvents followed by water, and samples were passed through at a rate of approximately 5 mL/min. After elution, samples were evaporated to dryness under a gentle stream of nitrogen using a ThermoFisher SE 500 solvent evaporator at 30°C (Dionex, Sunnyvale, CA). After extraction, samples were stored at -20°C until analysis.

Table 2.4.1. Three SPE methods for the optimized extraction of different target analytes from wastewater effluents.

Target	**Cartridge**	**Volume extracted (mL)**	**Isotopically labelled surrogates (µg/L)**	**pH Adjustment**	**Wash**	**Elution**	**Reconstitution**
Pharmaceuticals, personal care products (PPCP)	Bond Elut Plexa[1]	100	20	To pH 2.0 ± 0.5 with 10 M HCl	5 mL water 5 mL 5% MeOH in water	3 mL MeOH 3 mL MeOH	500 µL MeOH with 75 µg/L lorazepam and chloramphenicol
Estrogenic Potency	Oasis HLB[2]	500	None used	None	5 mL water	5 mL 10:90 MeOH:MTBE 5 mL MeOH	80 µL MeOH
Estrogens	Superclean LC-18[3]	100	20	None	5 mL water 5 mL hexanes	5 mL ethyl acetate 5 mL ethyl acetate	500 µL MeOH with 75 µg/L lorazepam and chloramphenicol

[1]Bond Elut Plexa cartridges (6 cc, 500 mg, Agilent Technologies, Mississauga, ON) [2] Oasis HLB cartridges (6cc, 500 mg, Waters, Milford, MA) [3] Superclean LC-18 cartridges (6cc, 500 mg, Sigma-Aldrich)

With each batch of samples three quality assurance/quality control (QA/QC) samples were processed; one negative control (blank) and two positive controls (MS1, MS2)**.** All three QA/QC samples were prepared in MilliQ water. The blank was spiked with only isotopically labelled standards. The positive controls MS1 and MS2 are identical replicates spiked with both isotopically labelled standards and unlabeled chemicals at a concentration of 20 µg/L. As of February 2015, additional wastewater matrix QA/QC samples were added to the monthly extractions, spiked with both isotopically labelled standards and unlabeled chemicals at a concentration of 20 µg/L. These additional QA/QC samples help to account for matrix effects as well as to determine the efficiency of the extraction procedure.

2.5 Sample Analysis

2.5.1 LC-MS/MS analysis of select pharmaceuticals and personal care products

Analysis of pharmaceutical samples extracted with Bond Elut Plexa was performed with liquid chromatography and tandem mass spectrometry (LC-MS/MS). Separation of analytes was completed on an Agilent 1200 HPLC (Agilent, San Pedro, CA) using a 4.6 mm x 150 mm x 5 µm Agilent Eclipse XDB-C18 column. Detection of analytes was completed using multiple reaction monitoring (MRM) on a Sciex 3200 QTRAP mass spectrometer (ABSciex, Concord, ON, Canada) with electrospray ionization (ESI). Samples were run in both positive and negative ion mode to identify all target analytes. Analytes were identified based on the transitions listed in Table 2.5.1. Source-dependent and compound-specific parameters are listed in Appendix A, Table A1.1. The mobile phases used for this analysis were 5 mM ammonium acetate in MilliQ water (A) and 100% methanol (B). The mobile phase gradient was dependent on which ion mode was selected. For positive ion mode, the mobile phase gradient began at 50% B, increased to

100% B over 7.5 min, and was then held at 100% B for 2 min. In negative ion mode, the mobile phase gradient began at 40% B, increased to 100% B over 7.5 minutes, and was then held at 100% B for 3 min. In both cases, a re-equilibration period of 5 minutes occurred after each sample to return the mobile phase gradient to starting conditions.

Table 2.5.1 Parameters for analysis of select PPCPs

Analyte	**Q1**	**Q3**	**Polarity**
Atorvastatin	559.3	440.2	Positive
p-hydroxy atorvastatin	575.2	440.3	Positive
Carbamazepine	237.1	193.3	Positive
Diclofenac	293.9	250	Negative
Fluoxetine	310.3	44.3	Positive
Gemfibrozil	249.1	121.1	Negative
Ibuprofen	204.9	160.9	Negative
Naproxen	229.0	170.0	Negative
Sulfamethoxazole	254.1	156.2	Positive
Triclocarban	314.8	161.6	Negative
Triclosan	286.9	35.0	Negative
Trimethoprim	291.1	261.2	Positive
Venlafaxine	278.3	58.1	Positive
Surrogate	**Q1**	**Q3**	**Polarity**
Atorvastatin-d_5	564.3	445.3	Positive
p-hydroxy atorvastatin-d_5	580.2	445.2	Positive
Carbamazepine-d_{10}	247.2	204.4	Positive
Diclofenac-d_4	298.2	253.8	Negative
Fluoxetine-d_5	315.2	44.2	Positive
Gemfibrozil-d_6	255	120.7	Negative
Ibuprofen-d_3	207.9	164.1	Negative
Naproxen-d_3	232.1	172.8	Negative
Sulfamethoxazole-d_4	258.1	160.1	Positive
Triclocarban-d_4	316.9	159.9	Negative
Triclosan-d_3	286.9	35.0	Negative
Trimethoprim-d_3	294.2	230.3	Positive
Venlafaxine-d_6	288.4	58.1	Positive

Q1=quadrupole 1; Q3=quadropole 3

2.5.2 LC-MS/MS analysis of selected estrogens

Analysis of select estrogens extracted with Superclean LC-18 was performed with LC-MS/MS. Separation of analytes via liquid chromatography was completed on an Agilent 1260 HPLC with a 2.1 mm x 50 mm x 1.8 μm Agilent ZORBAX Eclipse Plus C18 column. Detection of analytes was completed using dynamic multiple reaction monitoring (dMRM) on an Agilent 6460 triple quadrupole (QQQ) mass spectrometer with Agilent Jet Stream (AJS) electrospray ionization. Samples were run in both negative ion mode to identify all target analytes. Analytes were identified based on the transitions listed in Table 1.5.2. Source-dependent and compound-specific parameters are listed in Appendix A, Table A1.2.

The mobile phases used for this analysis were 0.5 mM ammonium fluoride in MilliQ water (A) and 100% acetonitrile (B). The mobile phase gradient started at 10% B, increased to 100% B over 10 minutes, and was then held at 100% B for 3 minutes. An 8 minute re-equilibration period occurred after each sample to establish the mobile phase gradient at its starting conditions.

Table 2.5.2. Parameters for the analysis of select estrogens via LC-MS/MS

Analyte	Q1	Q3	Polarity
Bisphenol A	227.3	133.2	Negative
		212.3	Negative
Estrone	269.4	145.1	Negative
		143.1	Negative
Estradiol	271.4	145.1	Negative
		143.1	Negative
Estriol	287.4	171.2	Negative
		145.1	Negative
Ethinylestradiol	295.39	158.9	Negative
		144.9	Negative
Triclosan	286.99	35	Negative
Surrogate	**Q1**	**Q3**	**Polarity**
Bisphenol A - d16	241.28	223.3	Negative
		142.2	Negative
Estrone - d2	271.2	147.1	Negative
		145.1	Negative
Estrone-d4	273.4	147.1	Negative
		145.1	Negative
Estradiol-d4	275.2	187.2	Negative
		145.3	Negative
Estriol-d2	289.39	173.2	Negative
		147.1	Negative
Estriol-d3	290.2	173.2	Negative
		145.3	Negative
Ethinylestradiol - d4	299.4	161.2	Negative
		147.2	Negative
Triclosan-d3	289.99	35	Negative

2.5.3 Measuring estrogenic potency with the YES assay

Analysis of the total estrogenicity of samples extracted with the Oasis HLB method was performed with the YES assay. Buffers and other materials for the YES assay were prepared as outlined in Appendix A2: Reagents required for the YES Assay, Table A2.1.

Saccharomyces cerevisiae cells (Receptor: ER_{trp} (YePtrpER), Reporter E2.ura ($YRpE2_{ura}$)) provided by Heidi Engelhardt, University of Waterloo (originally from K. Gaido, Research Triangle Park) were stored in 30% glycerol stock at -80°C until use. After thawing at 4°C, cells were streaked on agar plates and placed in a 30°C incubator at 300 rpm for 3-4 days, or until identifiable colonies were present. Plates were then stored at 4°C until use for no more than 14 d.

A single yeast colony was selected from the agar plate, placed into 1 mL GOLD media in a 25 mL centrifuge tube, and incubated at 30°C and 300 rpm for 24 h. Cells were then diluted 1:10 in minimal media and incubated at 30°C and 300 rpm for 24 h. After 24 h, cells were diluted 1:1 in minimal media and incubated at 30°C and 300 rpm for 6 h. When the incubation period was complete, cells were diluted in minimal media and 50 µM copper sulfate to an optical density (OD) of 0.03 at 660 nm.

Standards or samples were transferred in 10 µL aliquots in duplicate into 2 mL amber glass vials and left open in the flow hood to dry. Three pseudoreplicates from each annual sampling event were run in duplicate in a 2x serial dilution curve in MeOH from undiluted effluent to 1024x dilution. Once the sample or standard was dry, 200 µL of the cells in minimal media and copper sulfate was added to each vial. Vials were capped and incubated for 18 - 24 h at 30°C and 300 rpm.

After the 18 - 24 h incubation period, 25 µL of each exposed cell solution was transferred to a 96-well plate and 75 µL of minimal media was added to each well. The OD_{660} of each well was taken with a Molecular Devices Max 3 spectrophotometer plate reader (Sunnydale, CA, USA) to determine cell density. β-galactose and Yeast Protein Extraction Reagent (YPER) from a ThermoFisher Yeast β-galactosidase assay kit were diluted 1:1 and 100 µL was added to each well. The plate was then immediately read every 15 s for 30 min at 420 nm. The β-galactosidase activity of each well was reported in E2 equivalents (E2eq).

2.5.4 Nutrient Analysis

Analysis for nitrite, nitrate, total nitrogen, chloride, and conductivity was performed by Maxxam Analytics (Mississauga, ON) as outlined in Table 2.5.3.

Table 2.5.3 Nutrient analysis methods and MDLs from Maxxam Analytics (Mississauga, ON)

Parameter	Analysis	MDL
Ammonia	Colourimetry	0.05 mg/L
Nitrate	Determined by subtraction of nitrite value from total oxidized nitrogen value	0.5 mg/L
Nitrite	Colourimetry	0.01 mg/L
Total oxidized nitrogen	Cadmium column reduction	0.5 mg/L
Chloride	Colourimetry	4 mg/L
Conductivity	Conductivity meter	1.0 umho/cm

MDL = Method Detection Limit

2.6 Detection Limits and Quantitation

2.6.1 Quantitation of LC-MS/MS samples

Each LC-MS/MS method was optimized to achieve the best possible detection for the selected analytes. The chromatographic conditions were optimized for the best separation and ionization into the mass spectrometer.

Data analysis for the PPCP method was completed with Analyst software version 1.6.1 (Applied Biosystems). For the estrogen method Agilent MassHunter Quantitative Analysis version B.05.02 was used for quantitation. A series of calibration standards at concentrations of 0, 0.5, 1, 10, 50, 100, 200, and 500 µg/L were run prior to each batch of samples. An additional two calibration points at 0.1 and 5 µg/L were added to this for the estrogen analysis. Samples were quantified based on the ratio of analyte peak area to isotopically labelled standard peak area.

The instrument detection (IDL) and quantification limits (IQL) were determined by running a series of blanks (n=7) as well as a calibration curve with concentrations of 0, 0.1, 0.5, 1.0, 10, 50, 100, 200, and 500 µg/L. The IDLs were reported as three times the standard deviation of the blanks. The IQLs were calculated based on ten times the standard deviation of the blanks.

The method detection limit (MDL) was determined by running a series of wastewater samples that had been spiked with various concentrations of standards (0, 5, 10, and 50 ng/L). MDLs were calculated at a 99% confidence using a student's t-test value (n-1) multiplied by the standard deviation of 7 samples. The instrument detection and quantification limits as well as the method detection limit for each analyte are listed in Table 2.6.1.

Table 2.6.1. LC-MS/MS method detection limits (MDL) for CECs in wastewater effluents.

Analyte	Surface water MDL (ng/L)	Wastewater MDL (ng/L)
Triclosan	11.2	56[a]
Triclocarban	9.6	48[a]
Sulfamethoxazole	1.2	6[a]
Trimethoprim	1.2	6[a]
Carbamazepine	1.54	10.8
17α-ethynylestradiol	1	3.2
17β-estradiol	0.5	3
Estrone	1	4
Estriol	1	3.6
Gemfibrozil	3.3	16.5[a]
Diclofenac	7.3	36.5[a]
Ibuprofen	2.2	21.4
Naproxen	2.6	16.4
Fluoxetine	10.33	51.7[a]
Venlafaxine	1.36	8.2
Atorvastatin	8.2	41[a]
p-hydroxy Atorvastatin	8.2	41[a]
o-hydroxy Atorvastatin	8.2	41[a]

[a]Wastewater MDL was calculated as 5x surface water MDL

2.6.2 YES Assay

The YES assay was previously validated in the Servos lab for use on wastewater effluent samples from the Kitchener and Waterloo WWTPs (Tanna et al. 2013). On each plate, a calibration curve of 17β-estradiol standards at concentrations of 1.25E-08, 6.25E-09, 3.13E-09, 1.56E-09, 7.81E-10, 3.91E-10, 1.95E-10, 9.77E-11, 4.88E-11, 2.44E-11, 1.22E-11 M was run in duplicate. QA/QC samples were also run in duplicate on each plate in the form of one positive

control (cells and a calibration curve standard), one negative control (MeOH and cells), and a blank (cells only).

The β-galactosidase activity of each well was determined by comparing the rate of chromogen production at OD_{420} by the samples relative to the 17β-estradiol calibration curve standards. Readings were taken every 30 s over a 30 m period. Standards or samples that were cytotoxic were not considered in calculations. The OD_{660} of each well was used to correct for the volume of cells present. As indicated by the ThermoFisher yeast β-galactosidase assay kit, only values between 0.2 and 1 were included in these calculations. The equation used to determine β-galactosidase activity is as follows, where t = time in minutes of incubation and V = volume of cells in mL used in the assay (Equation 1).

$$\beta - \text{galactosidase activity} = \frac{1000 \times \Delta \mathrm{OD}_{420}}{t \times V \times \mathrm{OD}_{660}} \qquad \text{Equation 1}$$

Once $\beta - \text{galactosidase activity}$ was determined, the final E2eq of each well in ng/L was calculated with the following equation (Equation 2):

$$Final\ Conc\ \left(\frac{ng}{L} E2\ equivalents\right) = \frac{Conc.\ of\ E2\ \left(\frac{mol}{L}\right) \times Vol\ of\ cells\ per\ well\ (\mu L)}{Vol\ of\ extract\ the\ cells\ are\ exposed\ to\ (\mu l)} \times MW\ of\ E2\left(\frac{g}{mol}\right) \times \frac{final\ reconstituted\ volume\ after\ drying (mL)}{vol\ of\ samples\ extracted\ (mL)} \times serial\ dilution$$

Equation 2

2.7 Statistics

Most statistical analysis was done in Sigma-Plot v. 13 (Systat Software, San Jose, CA). Dose-response curves for the YES assay were calculated with a four-parameter Hill equation. All

error bars represent standard deviation as sample sizes were not equal. One-way ANOVAs were performed on pharmaceutical and estrogenicity data after it had been log-transformed to ensure normality (Shapiro-Wilk test) and equal variances (Brown-Forsythe test) and followed by Tukey post-hoc tests. Primer-E v. 7 (Auckland, NZ) was used to perform PCAs on each WWTP.

Chapter 3 – Results

3.1 Nitrate and Ammonia in the Kitchener WWTP effluents

Ammonia and nitrate concentrations determined from grab samples at the Kitchener WWTP aligned with the monitoring data released by the Region of Waterloo (Appendix A4). Ammonia concentrations (Figure 3.1.1) were significantly higher ($p < 0.001$) in the Kitchener WWTP in 2010 and 2011 (24.5 ± 6.6 mg/L) than in 2012 (10.7 ± 1.3 mg/L), 2013 (2.1 ± 0.9 mg/L), 2014 (4.7 ± 5.9 mg/L), and 2015 (2.5 ± 1.9 mg/L). Ammonia levels were consistently low from March – December 2013, becoming less consistent in 2014 and 2015, with sudden increases occurring in the early spring months (March – May). Nitrate concentrations (Figure 3.1.1) were significantly lower ($p < 0.001$) before the upgrades in 2010 and 2011 (1.6 ± 1.6 mg/L) than during upgrade implementation in 2012 (18.0 ± 3.8 mg/L) or after in 2013 (19.4 ±

2.8 mg/L), 2014 (16.2 ± 5.3 mg/L), and 2015 (18.8 ± 3.7 mg/L). From 2013 – 2015, nitrate levels were lowest in spring 2014 and spring 2015.

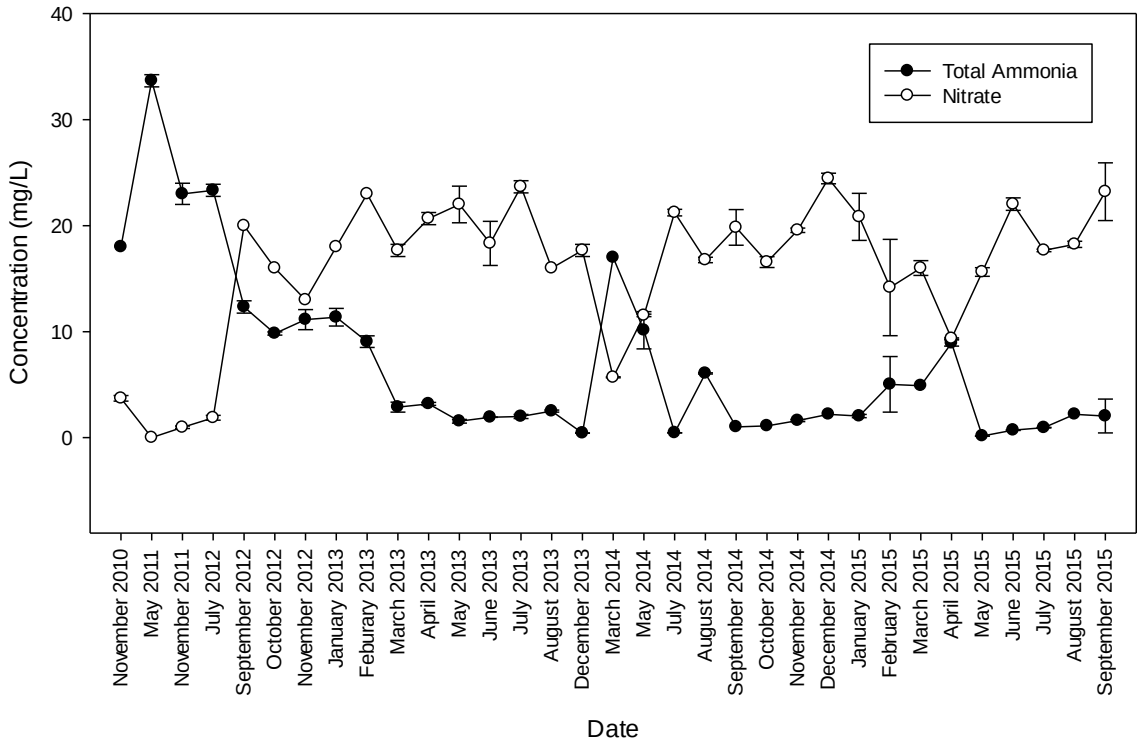

Figure 3.1.1. Ammonia and nitrate concentrations in the Kitchener WWTP November 2010 - November 2015. Sampling events corresponded with when samples were taken for CEC analysis.

3.2 Select Pharmaceuticals in the Kitchener WWTP

The two pharmaceuticals (ibuprofen, naproxen) that were previously identified as having high biotransformation potential and low sorption potential were readily removed by the WWTP after upgrades were implemented. The concentration of ibuprofen in the Kitchener WWTP (Figure 3.2.1) was significantly lower ($p < 0.001$) in 2013 (26.3 ± 29.6 ng/L), 2014 (75.3 ± 42.4 ng/L), and 2015 (111 ± 77.7 ng/L) compared to pre-upgrade conditions (3540 ± 2790 ng/L), though the increase in concentration after 2013 was also significant ($p < 0.001$). Similarly, the concentration of naproxen (Figure 3.2.1) in the Kitchener WWTP effluent was

significantly higher (p = 0.005) before upgrades (507 ± 353 ng/L) than during (163 ± 144 ng/L) or after in 2013 (25.5 ± 18.9 ng/L), 2014 (56.0 ± 33.9 ng/L), and 2015 (71.8 ± 53.1 ng/L).

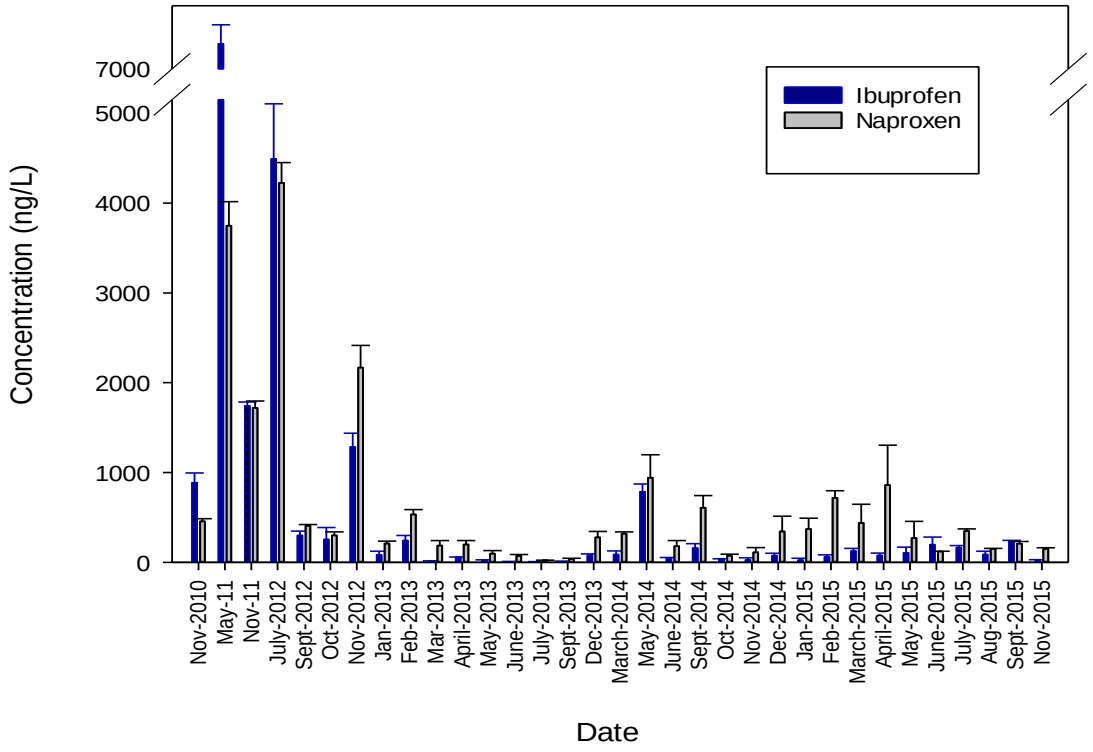

Figure 3.2.1. Select pharmaceuticals with high biotransformation and low sorption rates in the Kitchener WWTP effluents November 2010 - November 2015. Concentrations of ibuprofen and Naproxen were reduced significantly (p < 0.001) after the implementation of upgrades in 2012.

In contrast, the two pharmaceuticals previously identified as having low sorption potential and low biotransformation potential (carbamazepine, diclofenac) were largely recalcitrant in the effluents even after upgrades. The concentration of carbamazepine (Figure 3.2.2) was significantly reduced (p < 0.001) only in 2013 (236 ± 46.3 ng/L) when compared to before upgrades occurred (619 ± 121 ng/L), and was not significantly different in 2014 (434 ± 221 ng/L) or 2015 (603 ± 313 ng/L). The concentration of diclofenac (Figure 3.2.2) in the WWTP effluent was not significantly different from 2010 (413 ± 285 ng/L) to 2015 (830 ± 401

ng/L) except for the period in 2012 while upgrades were being implemented (193 ± 278 ng/L), when concentrations were significantly lower ($p < 0.001$).

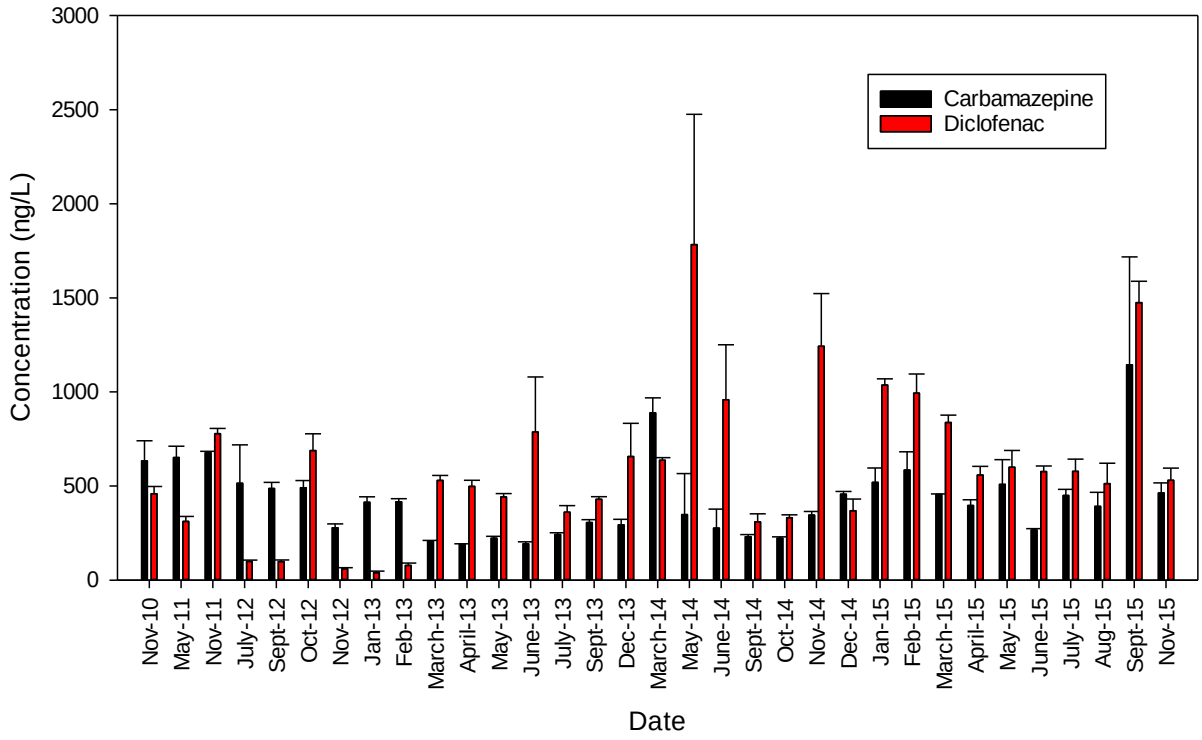

Figure 3.2.2. Select pharmaceuticals with low biotransformation and sorption rates in the Kitchener WWTP effluents November 2010 - November 2015. Concentrations of carbamazepine and diclofenac did not change significantly after the implementation of upgrades in 2012.

The four pharmaceuticals previously identified as having moderate biotransformation potential and low sorption potential (sulfamethoxazole, trimethoprim, gemfibrozil, venlafaxine) varied in their response to the WWTP upgrades. The concentration of venlafaxine (Figure 3.2.3) decreased significantly in the effluent ($p < 0.001$) in 2013 (911 ± 150 ng/L) and 2014 (953 ± 227 ng/L) but not in 2015 (1149 ± 309.1 ng/L) compared to before upgrades were implemented (1631 ± 231.5 ng/L). Sulfamethoxazole concentrations (Figure 3.2.3) were reduced in Kitchener WWTP effluents ($p = 0.03$) only in 2014. Concentrations of trimethoprim and gemfibrozil

(Figure 3.2.3) in the effluent were not significantly different ($p > 0.05$) across the years from 2010 – 2015.

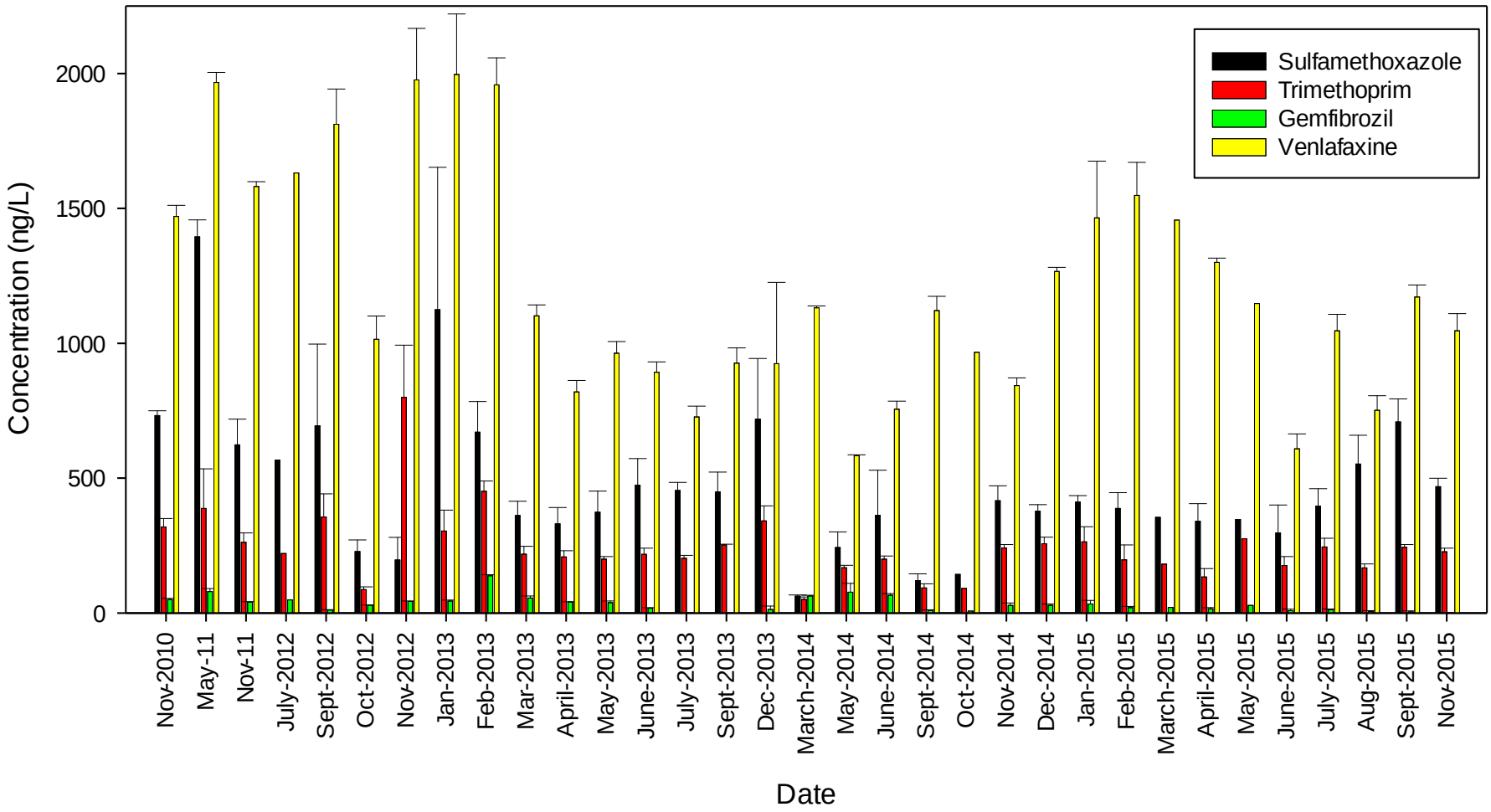

Figure 3.2.3. Select pharmaceuticals with moderate removal through biotransformation and low sorption at the Kitchener WWTP 2010 - 2015. The concentrations of sulfamethoxazole, trimethoprim, gemfibrozil, and venlafaxine were variable over time.

The concentration of triclosan in the Kitchener WWTP effluents (Figure 3.2.4) was significantly lower ($p < 0.001$) in 2013 (206 ± 86.9 ng/L), 2014 (319 ± 227 ng/L), and 2015 (226 ± 137 ng/L) compared to before-upgrade conditions (818 ± 164 ng/L). The concentration of fluoxetine was significantly lower ($p = 0.035$) than before upgrades (60.6 ± 46.6 ng/L) in 2013 (14.8 ± 5.24 ng/L) and 2014 (19.6 ± 10.8 ng/L), but not in 2015 (22.9 ± 7.71 ng/L).

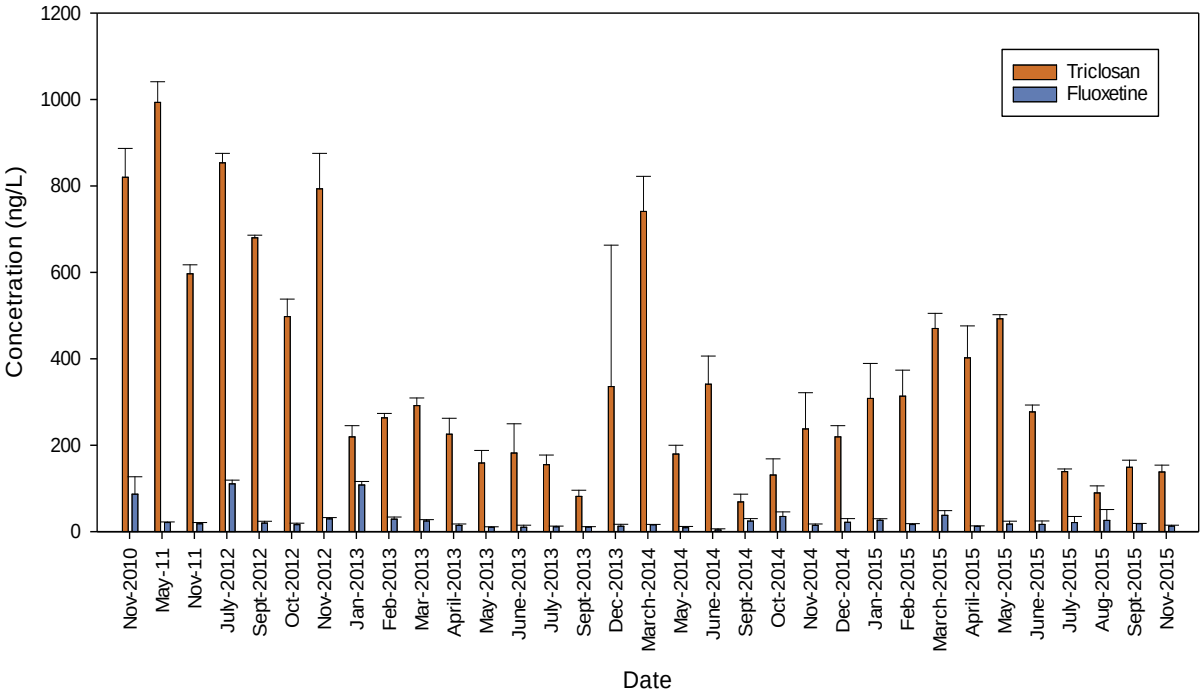

Figure 3.2.4. Select pharmaceuticals with high biotransformation and sorption rates in the Kitchener WWTP effluents 2010 - 2015. The concentrations of triclosan and fluoxetine were significantly reduced ($p < 0.035$) in 2013 and 2014; triclosan was also significantly reduced in 2015 ($p < 0.001$).

The concentration of atorvastatin and its metabolite p-hydroxy atorvastatin in the Kitchener WWTP effluents (Figure 3.2.5) were significantly reduced ($p < 0.001$) only while upgrades were being implemented (Sept 2012 – Feb 2013) compared to before-upgrade conditions. Levels of atorvastatin and p-hydroxy atorvastatin were also significantly higher ($p < 0.001$) in 2013, 2014, and 2015 than in 2012.

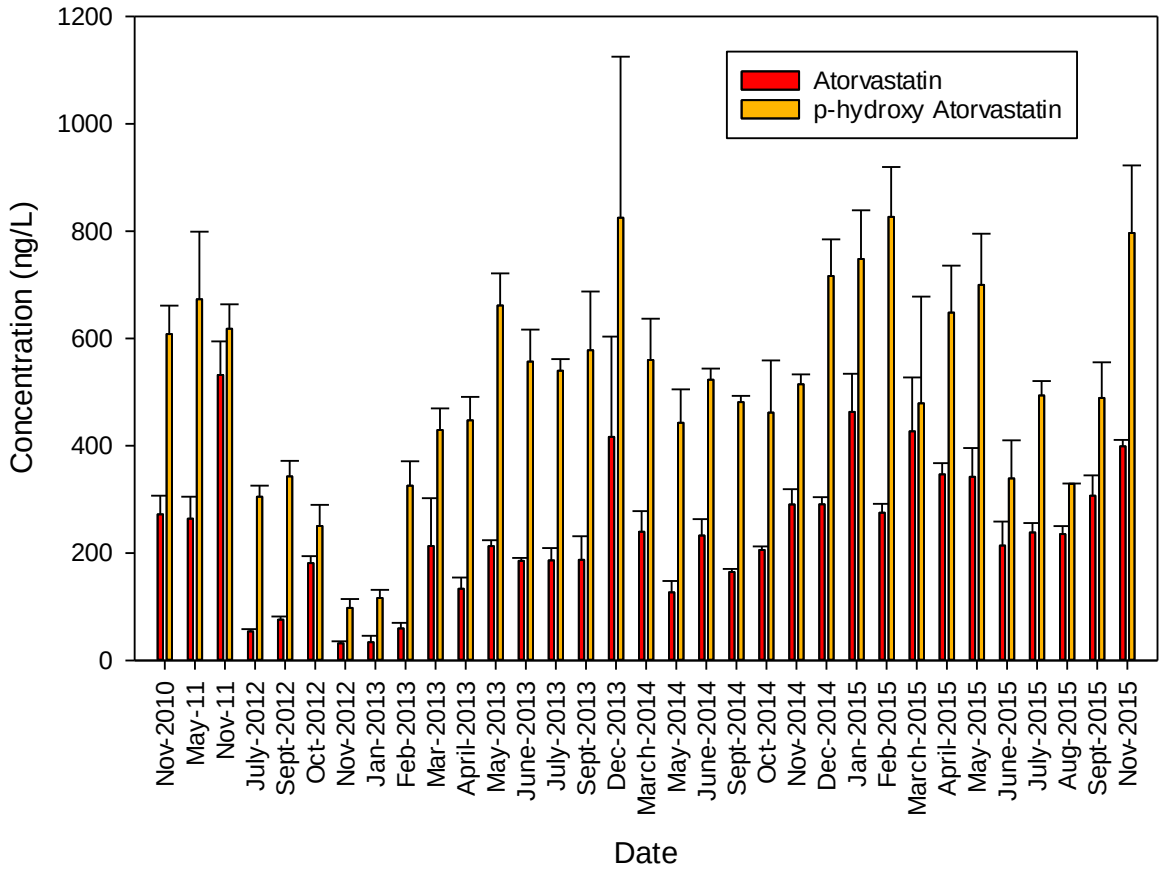

Figure 3.2.5. Select pharmaceuticals with moderate biotransformation and sorption potential in the Kitchener WWTP, 2010 – 2015. Atorvastatin and its metabolite p-hydroxy atorvastatin were significantly reduced ($p < 0.001$) only during the period of upgrade implementation.

The concentration of triclocarban in the Kitchener WWTP effluents (Figure 3.2.6) was significantly reduced ($p < 0.035$) in 2014 (31.5 ± 20.8 ng/L) and ($p < 0.001$) 2015 (17.3 ± 6.44 ng/L) compared to before-upgrade conditions (64.7 ± 17.7 ng/L).

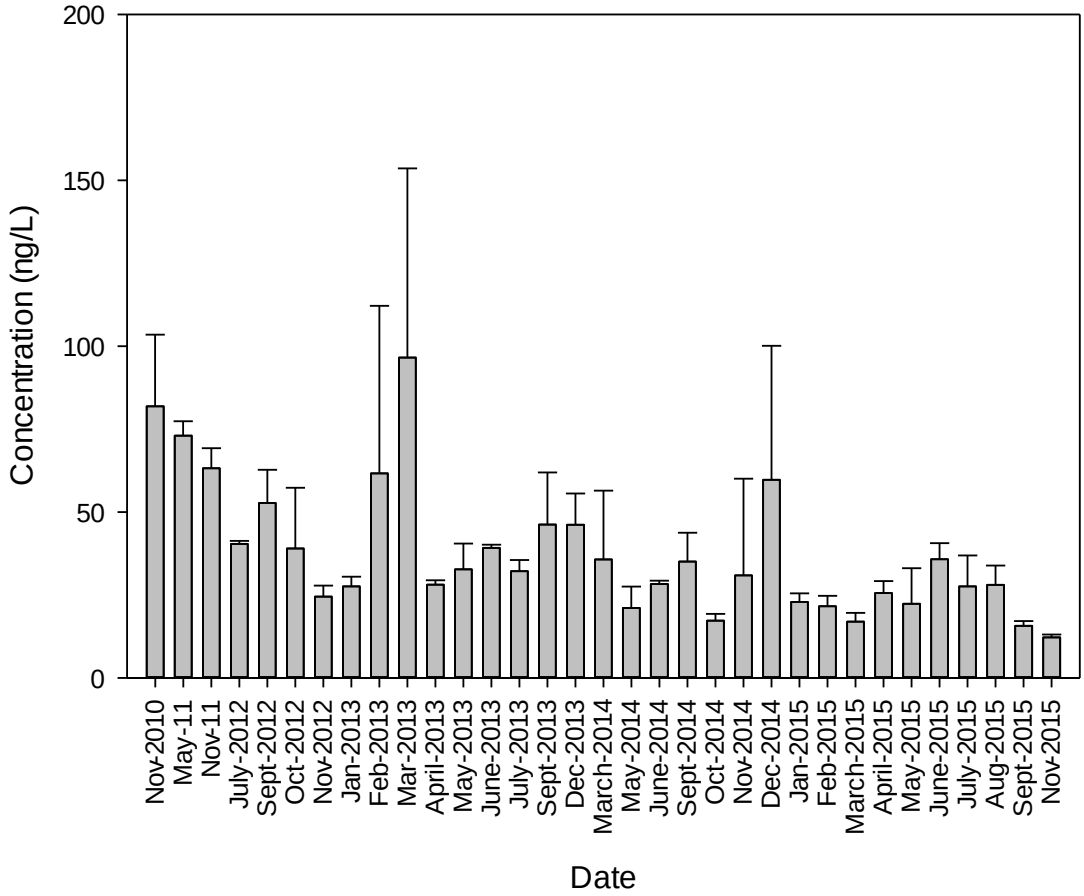

Figure 3.2.6. Select pharmaceuticals with low biotransformation and high sorption rates, 2010 – 2015. Triclocarban was significantly reduced ($p < 0.035$) in 2014 and 2015 compared to pre-2012 conditions.

Linear regressions were used to model the relationship between each pharmaceutical and ammonia or nitrate concentrations in the WWTP effluent (n=33 for all tests). Moderate relationships existed between nitrate and triclosan ($r^2 = 0.61$, $p < 0.001$), ibuprofen ($r^2 = 0.50$, $p < 0.001$), and naproxen ($r^2 = 0.49$, $p < 0.001$). Moderate inverse relationships exist between ammonia concentrations and concentrations of ibuprofen ($r^2 = 0.68$, $p < 0.001$), naproxen ($r^2 = 0.62$, $p < 0.001$), and triclosan ($r^2 = 0.73$, $p < 0.001$) in effluents. Full linear regression results can be found in Appendix A3.

3.3 Total estrogenicity in the Kitchener WWTP effluents

The estrogenicity of the Kitchener WWTP effluent (Figure 3.3.1) was significantly higher ($p < 0.001$) in 2010 (22.5 ± 6.52 ng/L E2eq) and 2011 (10.4 ± 1.9 ng/L E2eq) than during and after upgrades in Fall 2012 (2.4 ± 1.6 ng/L E2eq), 2013 (0.88 ± 0.53 ng/L E2eq), 2014 (2.0 ± 0.22 ng/L E2eq), and 2015 (1.5 ± 0.77 ng/L E2eq). Effluent estrogenicity was not significantly different from 2012 – 2015. One effluent sample (February 2010, 22.9 ng/L E2eq) was excluded from this analysis as it was the only sample that was not collected during autumn (September – December). Linear regression analysis showed that total estrogenicity had a relationship with both ammonia (r^2=0.79, $p < 0.01$) and nitrate (r^2=0.88, $p < 0.005$).

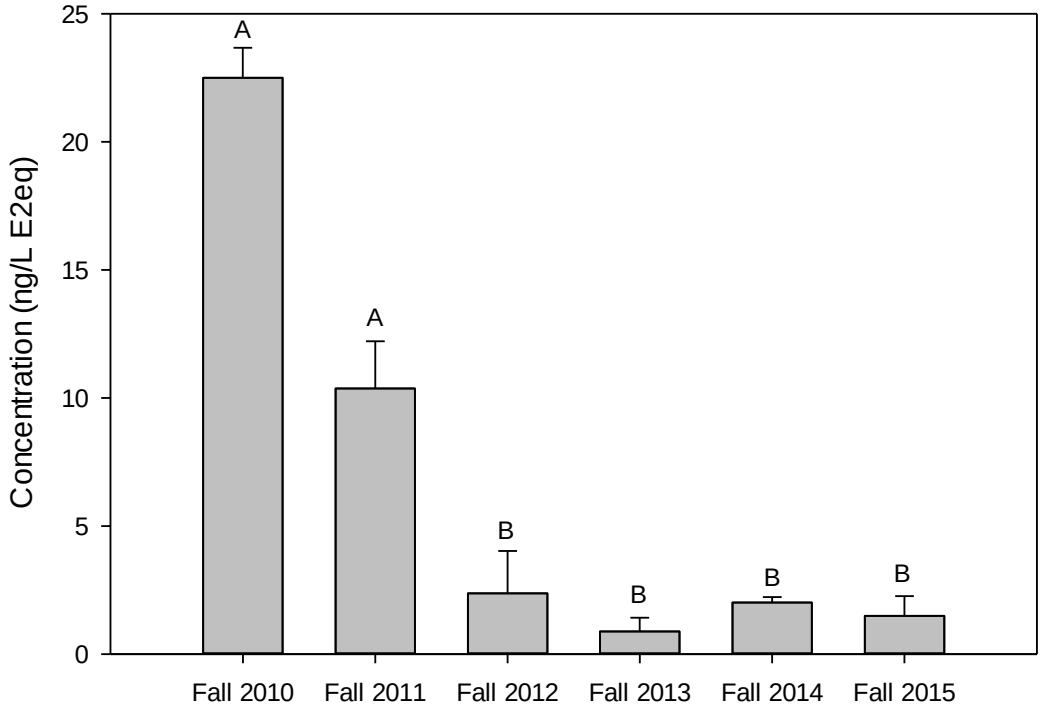

Figure 3.3.1. Estrogenicity in E2 equivalents in the Kitchener WWTP, Fall 2010 - 2015. Total estrogenicity was reduced ($p < 0.001$) in 2012 – 2014 compared to pre-2012 conditions.

3.4 Principle components analysis of the Kitchener WWTP effluents, 2010 – 2015

A principle components analysis (PCA) (Figure 3.4.1) was performed with the pharmaceutical and nutrient data in the Kitchener WWTP. Principle component 1 (PC1) explained 41.2% of the variability and was primarily driven by total ammonia, followed by triclosan, ibuprofen, naproxen, and nitrate in approximately equal quantities. PC2 explained an additional 15.3% of the variability and was primarily driven by diclofenac, carbamazepine, and nitrate. Additional information can be found in Appendix A5: Principle components analysis details.

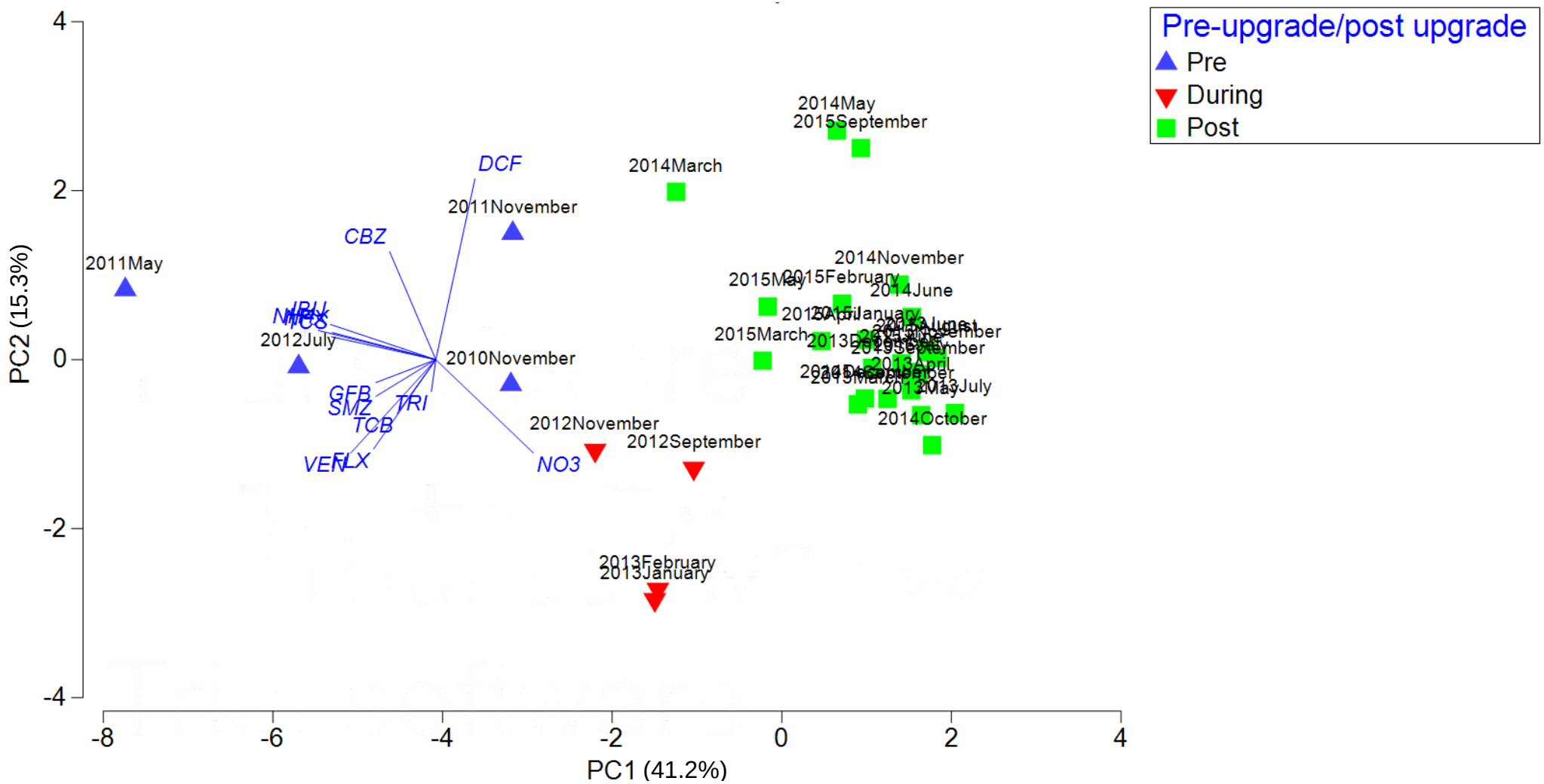

Figure 3.4.1. Principle components analysis for the Kitchener WWTP, 2010 - 2015.

3.5 Nitrate and Ammonia in the Waterloo WWTP

A high level of ammonia (9 – 37 mg/L) with substantial variability was observed in the Waterloo WWTP (Figure 3.5.1) from 2011 – 2015. Ammonia levels did not change significantly over this time period, though nitrate levels (Figure 3.5.1) were significantly higher ($p = 0.015$) in 2015 (9.69 ± 3.52 mg/L) than in pre-2014 (2.36 ± 3.16 mg/L).

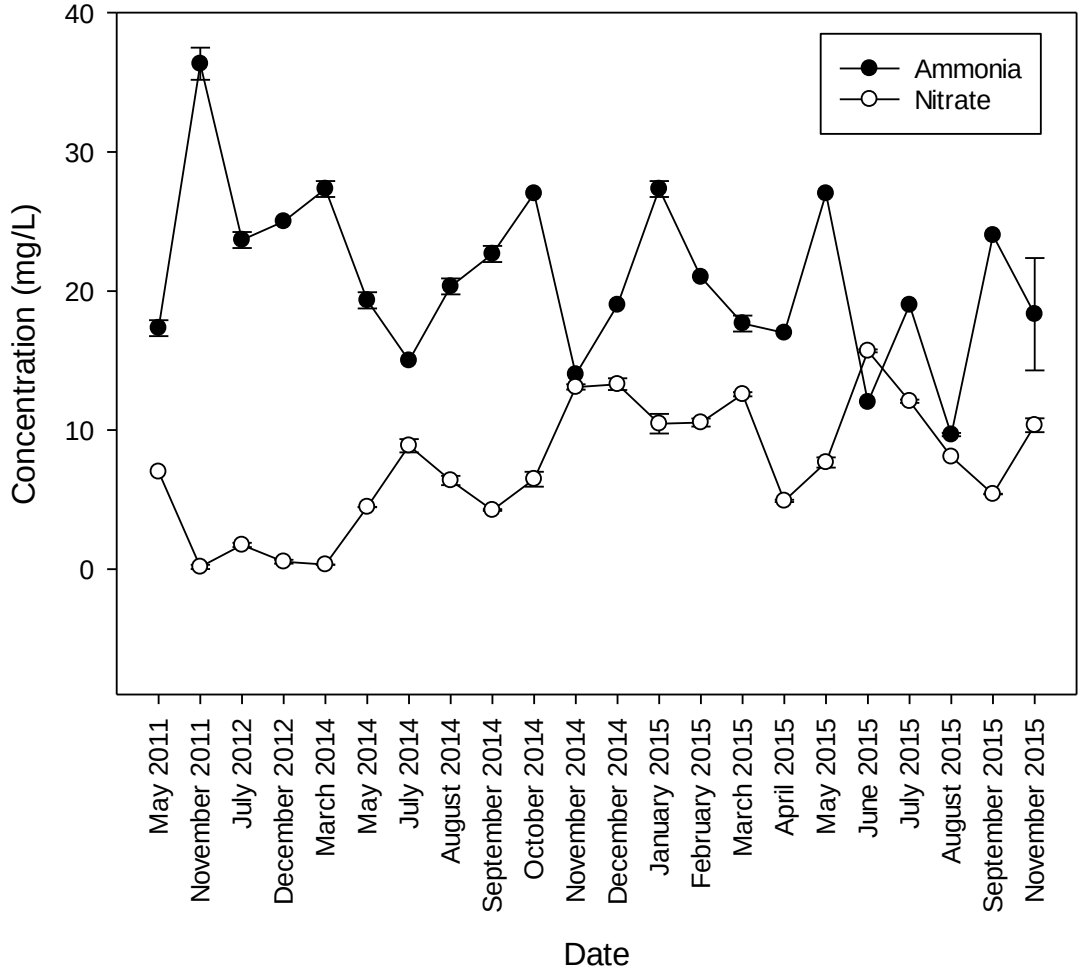

Figure 3.5.1. Ammonia and nitrate concentrations in the Waterloo WWTP 2011 – 2015. Sampling events correspond with sample collections for CEC analysis.

3.6 Select Pharmaceuticals in the Waterloo WWTP

Effluent concentrations of ibuprofen (Figure 3.6.1) were significantly lower ($p < 0.03$) in 2014 (332 ± 398 ng/L) and 2015 (531 ± 485 ng/L) compared to pre-2014 conditions (1729 ±

1744 ng/L). Effluent concentrations of naproxen (Figure 3.6.1) were also significantly lower (p = 0.025) in 2014 (467 ± 253 ng/L) and 2015 (816 ± 595 ng/L) compared to pre-2014 conditions (1850 ± 940 ng/L).

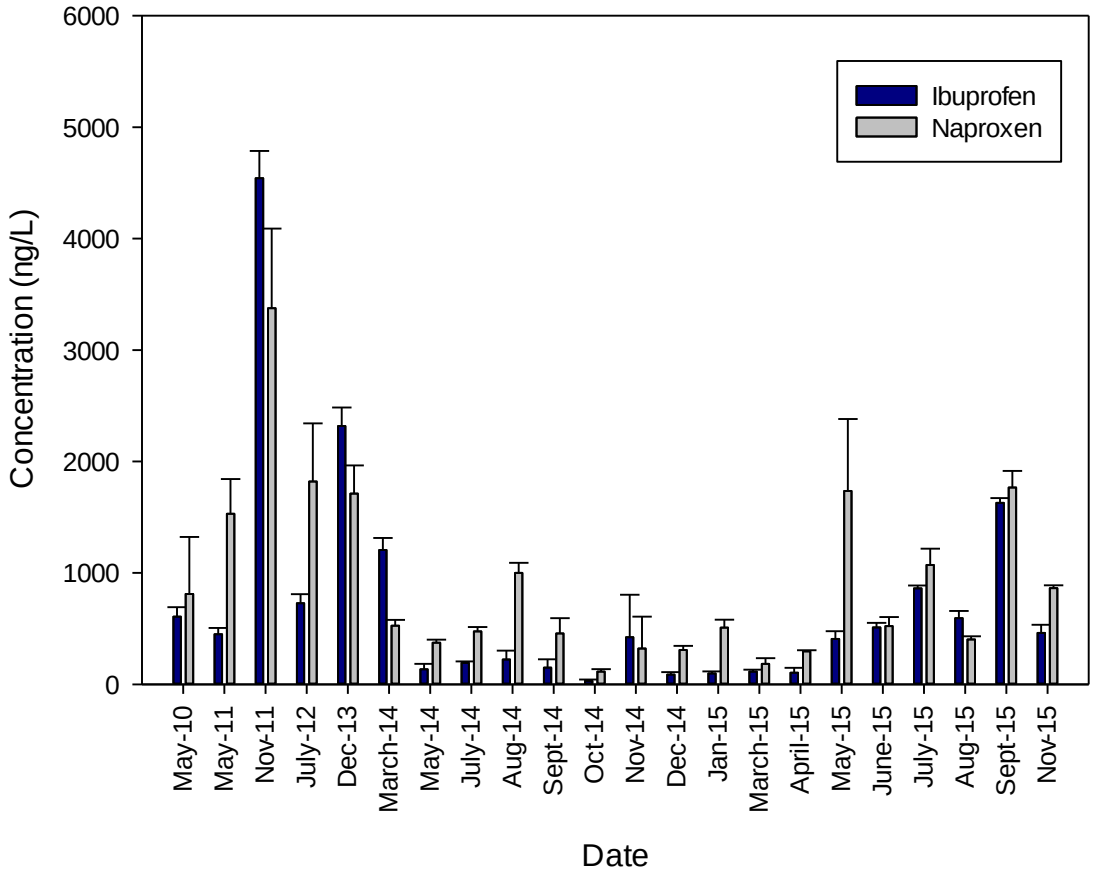

Figure 3.6.1. Select pharmaceuticals with high biotransformation and low sorption rates in the Waterloo WWTP effluents, 2010 – 2015. Ibuprofen and naproxen were significantly reduced (p < 0.03) after mid-2014.

Carbamazepine levels (Figure 3.6.2) were significantly higher (p < 0.028) in the Waterloo WWTP effluents in 2015 (448 ± 278 ng/L) than in 2014 (181 ± 91.3 ng/L), though neither 2014 nor 2015 were significantly different than pre-2014 (341 ± 106 ng/L). Similarly, diclofenac levels (Figure 3.6.2) were significantly higher (p < 0.04) in 2015 (552 ± 153 ng/L)

than in 2014 (315 ± 191 ng/L), though neither was significantly different from pre-2014 conditions (307 ± 235 ng/L).

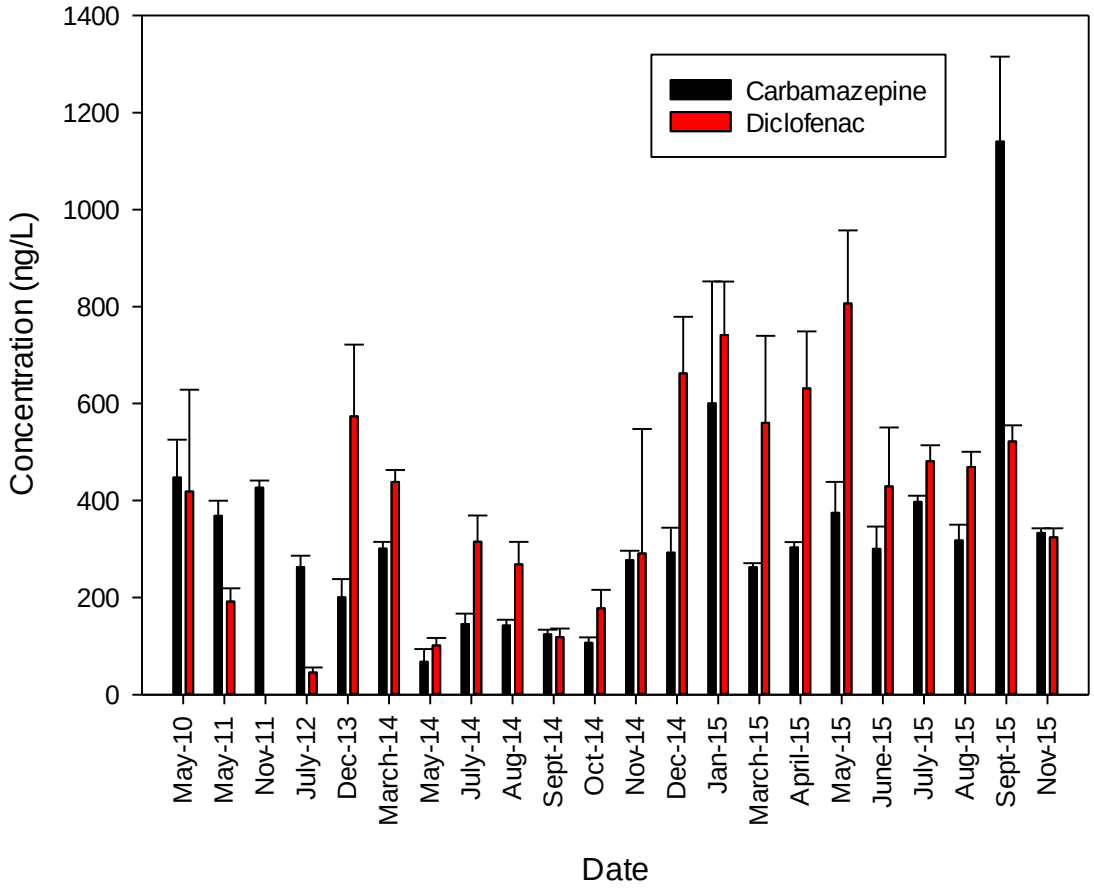

Figure 3.6.2. Select pharmaceuticals with low biotransformation and sorption rates in the Waterloo WWTP, 2010 - 2015. Carbamazepine and diclofenac did not change significantly in response to treatment plant upgrades.

Sulfamethoxazole, trimethoprim, gemfibrozil, and venlafaxine showed the highest variability of any group. Gemfibrozil concentrations decreased significantly ($p = 0.003$) in the effluent in 2015 (55.1 ± 17.3 ng/L) compared to both pre-2014 (99.5 ± 7.14 ng/L) as well as 2014 conditions (81.8 ± 14.0 ng/L). The concentration of sulfamethoxazole decreased significantly in the effluent ($p < 0.001$) in 2014 (186 ± 124 ng/L) and 2015 (173 ± 135 ng/L)

compared to pre-2014 conditions (524 ± 106 ng/L). Trimethoprim concentrations were reduced in effluents ($p < 0.04$) only in 2014. Venlafaxine was not significantly changed in the effluent ($p > 0.05$) over the years from 2010 – 2015.

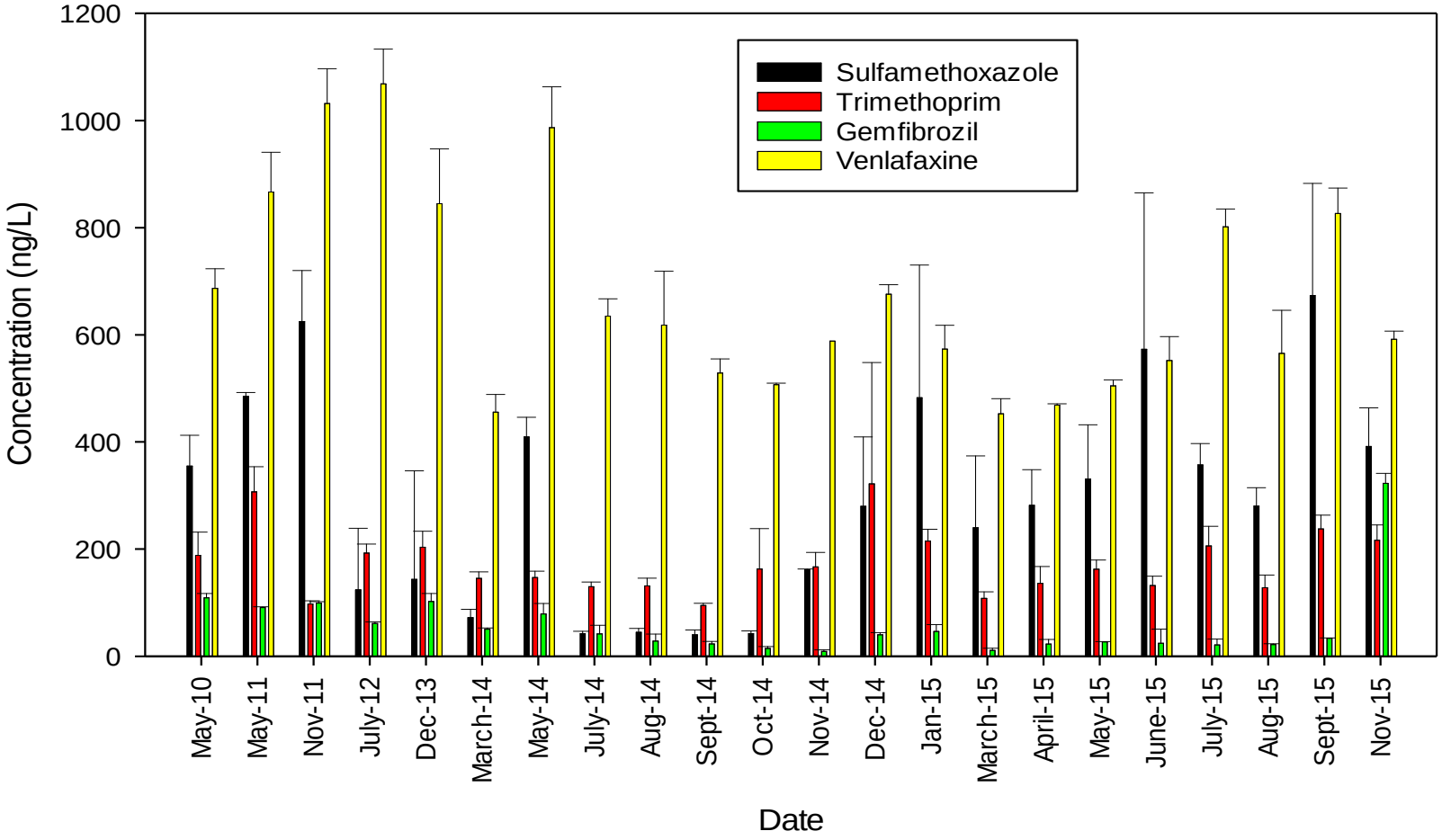

Figure 3.6.3. Select pharmaceuticals with moderate removal through biotransformation and sorption in the Waterloo WWTP, 2010 – 2015. Sulfamethoxazole, trimethoprim, gemfibrozil, and venlafaxine were variable in effluents between 2010 and 2015.

Triclosan (Figure 3.6.4) was significantly lower ($p = 0.02$) in 2014 (735 ± 260 ng/L) and 2015 ($p < 0.001$) (491 ± 219 ng/L) compared to pre-2014 conditions (1150 ± 267.3 ng/L). Similarly, fluoxetine was significantly lower ($p < 0.009$) in 2014 (7.14 ± 5.49 ng/L) and 2015 ($p = 0.03$) (10.2 ± 6.91 ng/L) compared to pre-2014 (22.3 ± 12.3 ng/L).

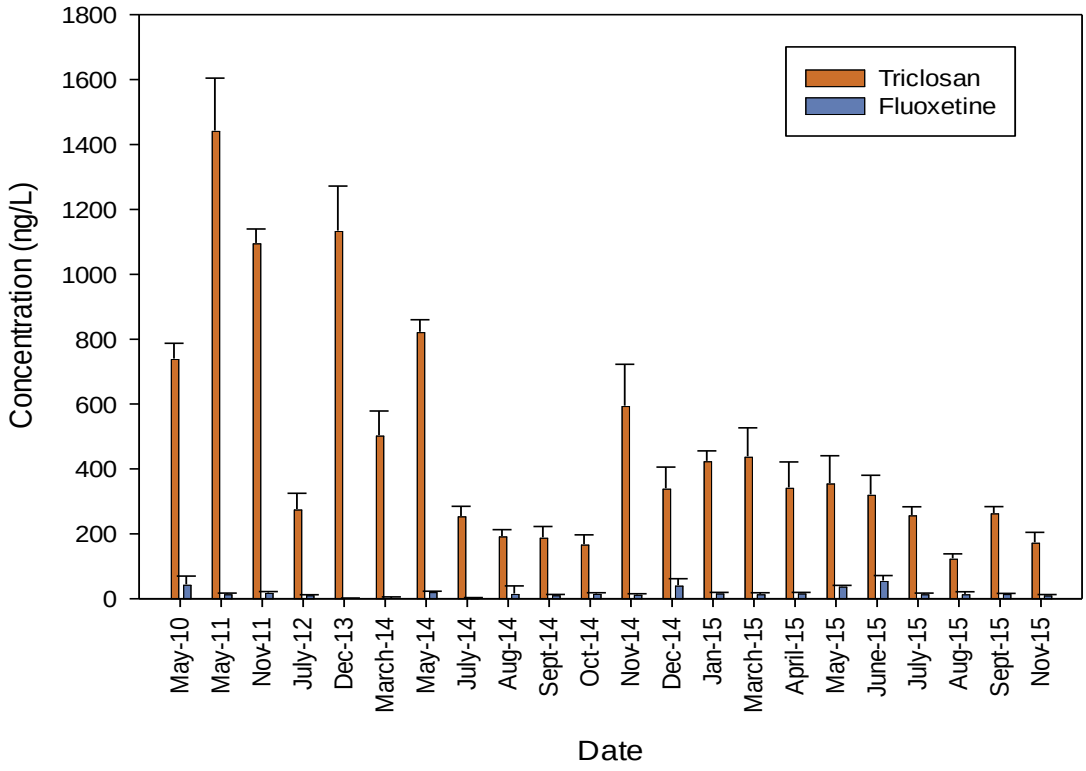

Figure 3.6.4. Select pharmaceuticals with high biotransformation and sorption rates in the Waterloo WWTP effluents 2010 - 2015. Triclosan and fluoxetine were reduced in effluents in 2014 and 2015 compared to pre-2014 conditions.

Atorvastatin was significantly reduced ($p < 0.001$) in Waterloo WWTP effluents in 2014 (113 ± 80.0 ng/L) and 2015 (156 ± 27.6 ng/L) compared to pre-2014 conditions (297 ± 84.9 ng/L). The metabolite p-hydroxy atorvastatin was likewise reduced ($p < 0.001$) in 2014 (113 ± 89.6 ng/L) and 2015 (271 ± 65.3 ng/L) compared to pre-2014 (336 ± 18.2 ng/L).

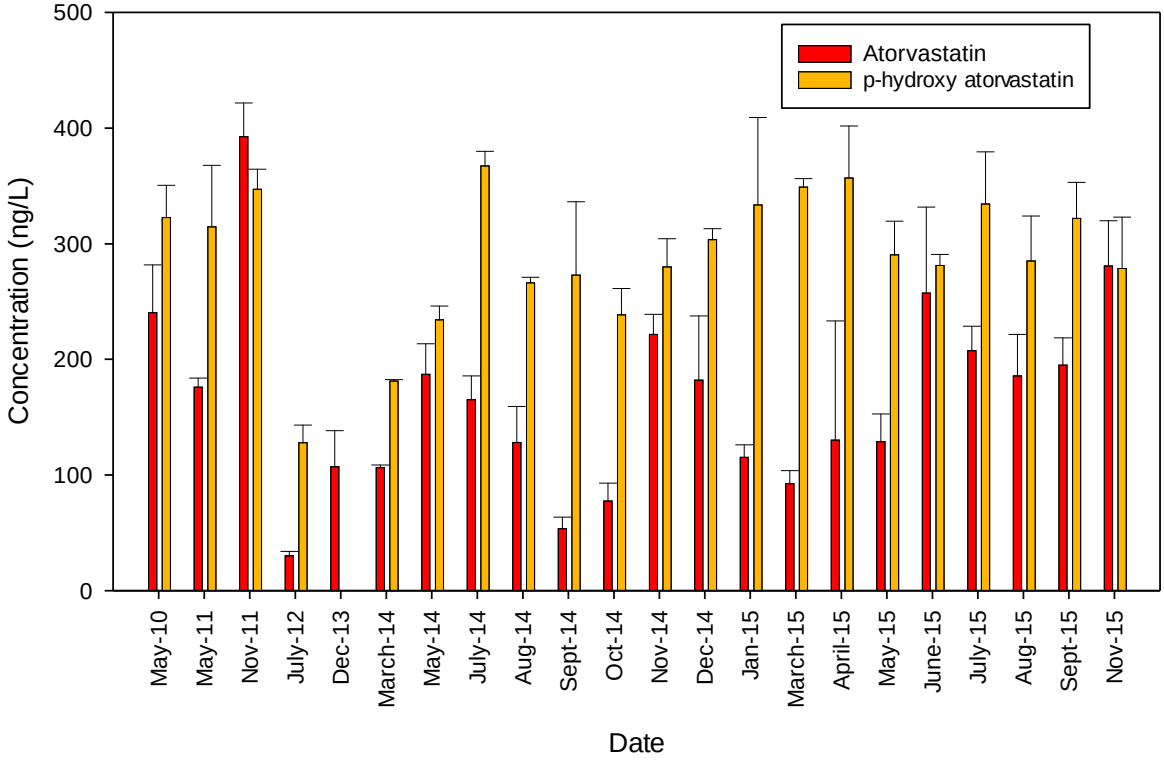

Figure 3.6.5. Select pharmaceuticals with moderate biotransformation and sorption rates in the Waterloo WWTP effluents, 2010 - 2015. Atorvastatin and p-hydroxy atorvastatin were reduced in effluents in 2014 and 2015 compared to pre-2014 conditions.

Linear regression analyses were performed to model the relationship between each pharmaceutical and ammonia or nitrate concentrations in the WWTP effluent (n=21 for all tests). When the relationships between nitrate and select CECs were modeled, the strongest present were with nitrate and sulfamethoxazole ($r^2 = 0.43$, $p = 0.001$) and venlafaxine ($r^2 = 0.40$, $p = 0.002$). When the relationships between ammonia and select CECs were considered, the strongest present were between ammonia and triclocarban ($r^2=0.50$, $p < 0.001$), ibuprofen ($r^2=0.34$, $p < 0.001$), and naproxen ($r^2=0.36$, $p < 0.001$). Full linear regression results can be found in Appendix A3.

3.7 Total estrogenicity in the Waterloo WWTP

The estrogenicity of the Waterloo WWTP effluent (Figure 3.7.1) was highly variable over the years 2009 – 2015. Fall 2014 had the lowest estrogenicity (2.3 ± 0.60 ng/L E2eq), and was significantly lower ($p < 0.001$) than any other year. Similarly, Fall 2011 (5.3 ± 0.90 ng/L E2eq) was significantly lower ($p < 0.001$) than every other year except for Fall 2015 (10.8 ± 2.89 ng/L E2eq). When grouped similarly to the pharmaceutical data, pre-2014 (13.8 ± 1.89 ng/L E2eq) is significantly higher ($p < 0.001$) than 2014, but not 2015.

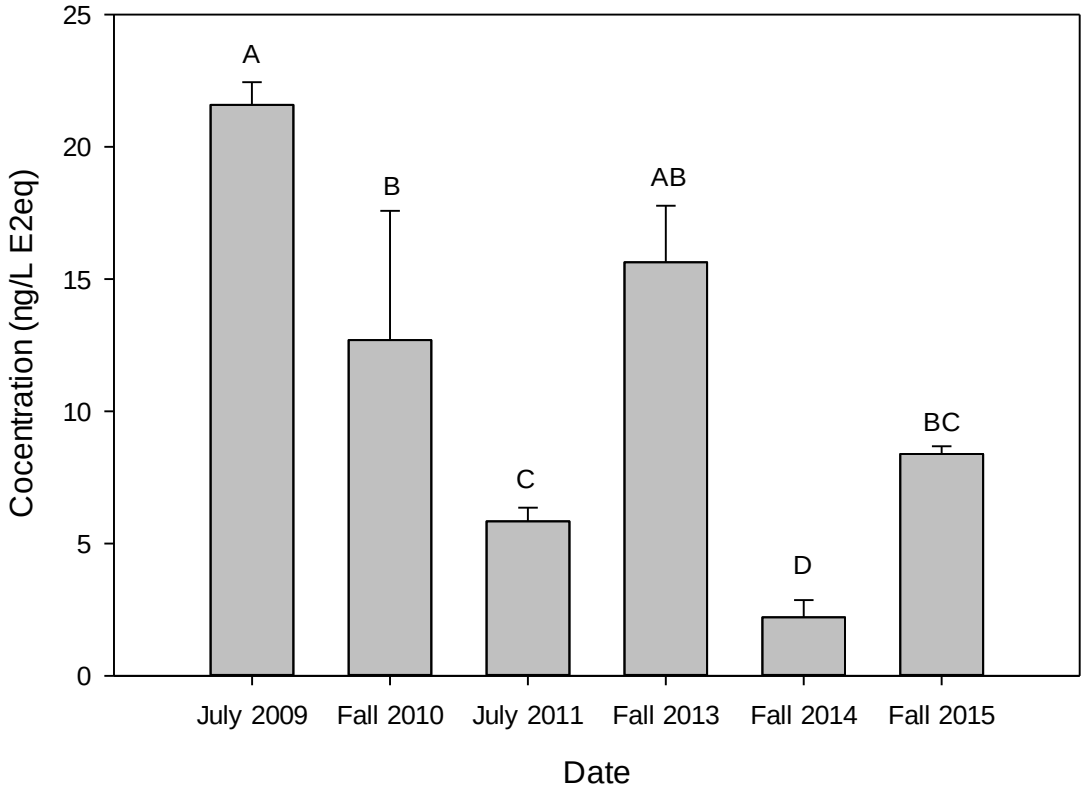

Figure 3.7.1. Total estrogenicity in E2 equivalents at the Waterloo WWTP, 2009 - 2015. The total estrogenicity of the Waterloo WWTP effluent was highly variable over time.

3.8 Principle Components Analysis of the Waterloo WWTP, 2010 – 2015

A principle components analysis was performed with the pharmaceutical and nutrient data in the Kitchener WWTP. PC1 explained 31.2% of the variability and was primarily driven by ibuprofen, naproxen, and venlafaxine. PC2 explained an additional 20.0% of the variability and was primarily driven by nitrate, sulfamethoxazole, and fluoxetine. Additional information can be found in Appendix A5: Principle components analysis details.

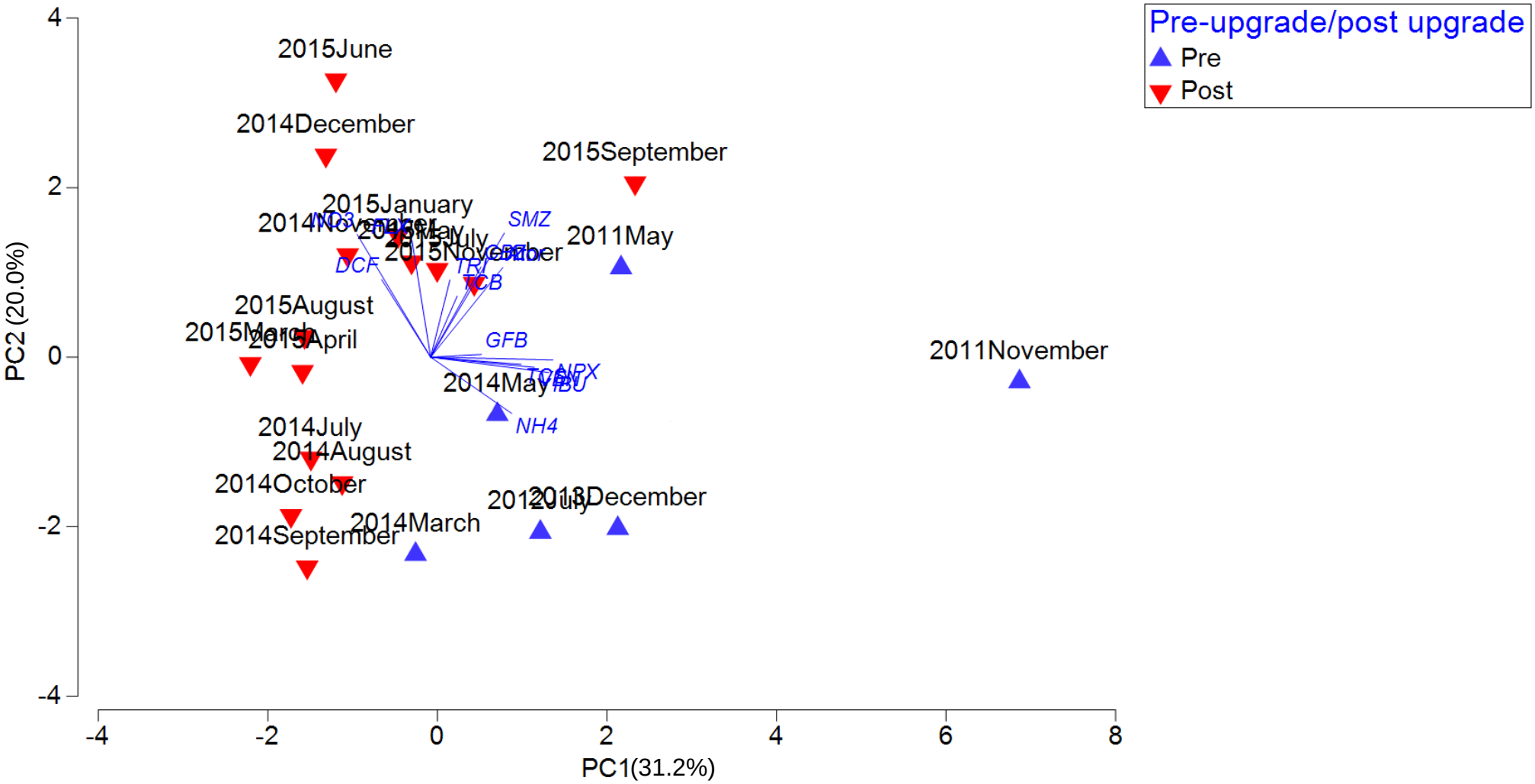

Figure 3.8.1. Principle Components Analysis of the Waterloo WWTP, 2011 - 2015.

3.9 Select estrogens in the Kitchener and Waterloo WWTP effluents

The method developed to measure select estrogens in WWTP effluents worked well with low detection limits (< 1 ng/L) in clean water, and passed all necessary validation requirements (specificity, selectivity, reproducibility, etc.). However, when this method was applied to effluents from the Kitchener and Waterloo WWTPs, matrix effects resulted in data that was suspect and requires additional analysis on a more mass selective instrument (accurate rather than nominal mass resolution) before it can be published.

Chapter 4 – Discussion

The presence of CECs in wastewater effluents during major upgrades to the treatment process was investigated at two treatment plants in the Grand River watershed. The results provide evidence that upgrades to wastewater treatment (resulting in longer SRTs and nitrification) will decrease the concentrations of key pharmaceuticals and estrogenicity in wastewater effluents. Specifically, this work demonstrates that the impact of upgrades to wastewater treatment plants on CEC removal is compound-dependent, and is influenced by the physiochemical properties of each CEC and the specific treatment process within each plant. It is important to note that CECs are reported here as concentrations rather than as loads due to the relevance of concentration when estimating potential exposure. Flow rates for both the Kitchener and Waterloo WTTPs are provided in Appendix A6: Flow Data for the Kitchener and Waterloo WWTPs.

4.1 Nutrients in the Kitchener WWTP before and after upgrades

The upgrades to the Kitchener WWTP in late 2012 and early 2013, including improved aeration and secondary treatment train reconfiguration, were implemented to increase the SRT (from <2 days to 5.4 days) and introduce more diverse and slow-growing bacterial communities to the secondary treatment process (Bicudo et al. 2016). The resulting nitrification is reflected in a significant decrease in ammonia levels and increase in nitrate levels in effluents from the plant after upgrades were implemented. The changes in ammonia and nitrate were reflected both in grab samples taken at each CEC sampling event and in the biweekly monitoring data from the Region of Waterloo (Figure 3.1.1, Figure A4.1). This is consistent with expectations as well as typical outcomes associated with improved aeration in CAS systems (Bicudo et al. 2016).

Increased nitrification is typically associated with higher quality treatment, and nitrifying plants have been shown to improve removal of a number of CECs and reduce effluent estrogenicity (Servos et al. 2005; Suarez et al. 2010; Fernandez-Fontaina et al. 2012).

4.2 Pharmaceuticals in the Kitchener WWTP before and after upgrades

A suite of key CECs was investigated to determine the impact of WWTP upgrades on a number of pharmaceuticals with varying physiochemical properties (Table 1.8.1). CECs were categorized based on their expected removal through biotransformation and sorption, the two main mechanisms by which CECs are removed in WWTPs (Salveson et al. 2012).

CECs that were classified as readily biotransformed in this system with low sorption potential (ibuprofen, naproxen) showed a significant decrease in the effluents of the Kitchener WWTP after major upgrades to the treatment process, including nitrification. This is consistent with literature that shows ibuprofen, naproxen, and other compounds with similar physiochemical properties are readily removed from CAS systems with nitrifying treatment and an SRT of over 5 days (Clara et al. 2005; Joss et al. 2005; Salveson et al. 2012). These compounds are both small molecules with structural segments that are amenable to biological attack (i.e. an aliphatic region) and low sorption potential, and are therefore excellent candidates for rapid biotransformation (Salveson et al. 2012).

Biotransformation is also integral in the removal of triclosan and fluoxetine from this system. These compounds were used to determine the behavior of CECs classified as having high biotransformation and sorption potential. The concentrations of both triclosan and fluoxetine were significantly reduced in Kitchener WWTP effluents in 2013 and 2014, though the concentration of fluoxetine was not significantly different in 2015 compared to before upgrade conditions. Results from other studies demonstrate that readily biodegradable CECs,

including fluoxetine, show improved removal in nitrifying conditions (Fernandez-Fontaina et al. 2012), and that triclosan is readily biodegraded by ammonia oxidizing bacteria (Roh et al. 2009). Though there is no clear consensus on whether longer SRTs improve removal of highly sorbable CECs, there is indication that sludge age could influence removal of CECs by sorption due to changes in the biomass (percent active fraction, specific microbial population) (Clara et al. 2005; Joss et al. 2005; Joss et al. 2006; Hyland et al. 2012).

Unlike CECs with high biotransformation rates, those with moderate biotransformation rates showed high variability in removal success between compounds within the same group. Those with moderate biotransformation and low sorption potential were responsible for this variability. While venlafaxine was significantly decreased in effluents in 2013 and 2014, sulfamethoxazole was only reduced in 2014, and trimethoprim and gemfibrozil showed no significant reduction over the years 2010 – 2015. Previous work shows CECs within this group may be removed from 0 – 100% in CAS conditions, varying substantially based on the specific compound being investigated and the operating parameters of the WWTP (Salveson et al. 2012). A more accurate estimate of removal for a particular compound within this group may be achieved by considering the SRT of the WWTP in question (Clara et al. 2005; Salveson et al. 2012). Atorvastatin and p-hydroxy atorvastatin, which represented the moderate sorption and moderate biotransformation group, were only significantly reduced during the period where upgrades were coming online (Sept 2012 – Feb 2013), but not afterwards (March 2013 – November 2015).

CECs that were classified as having low biotransformation rates and low sorption rates were highly recalcitrant in effluents even after upgrades occurred, with diclofenac significantly reduced only while upgrades were being implemented and carbamazepine significantly reduced

only in 2013. Numerous studies have shown that both of these compounds are difficult to remove from wastewater even with advanced treatment in nitrifying systems with long SRTs (Zhang et al. 2008; Fernandez-Fontaina et al. 2012; Vieno & Sillanpaa 2014). Finally, the one CEC that typically shows low biotransformation and sorption rates (triclocarban) was significantly reduced in effluents after the implementation of upgrades.

Overall, CECs that had high biotransformation potential were most significantly impacted by the upgrades implemented at the Kitchener WWTP, with all CECs in this category showing significant decline after their implementation. Moderately biotransformed CECs were highly variable, with some showing significant reduction after upgrades and others remaining recalcitrant in effluents. CECs that are slowly biotransformed and not readily amenable to biological attack remained recalcitrant in effluents. The Kitchener WWTP experienced an upset in early 2014 that resulted in a spike in ammonia and a reduction in nitrate in the effluents. This upset was reflected in some of the readily biotransformed CECs that have an inverse relationship with ammonia concentration, particularly triclosan. Based on the PCA, the three time periods (before, during, and after upgrades) are clearly separated from one another when all CECs and nitrate/ammonia concentrations in effluent are taken into account, and the upset months (March 2014, May 2014) are also distinct from the majority of post-upgrade samples.

4.3 Total estrogenicity of the Kitchener WWTP effluent

The total estrogenicity of the effluent as measured by the YES assay was significantly reduced after the implementation of upgrades. Williams *et al*. (2009) modeled the risk of endocrine disruption in over 10,000 reaches impacted by estrogenic WWTP effluents using the PNECs of E1, E2, and EE2 as well as their relative potency. Their parameters for low, moderate, and high risk were < 1.0 ng/L E2eq, > 1.0 ng/L E2eq, and > 10.0 ng/L E2eq respectively in

impacted surface waters. Following this model, direct exposure to the levels of estrogenicity in the Kitchener WWTP effluents would put populations at high risk before upgrades were implemented (12.3 ± 4.14 ng/L E2eq), no risk in 2013 (0.57 ± 0.08 ng/L E2eq), and moderate risk in 2014 (1.7 ± 0.48 ng/L E2eq) and 2015 (2.1 ± 0.21 ng/L E2eq). It is important to note that the dilution that occurs when effluent enters the receiving environment will reduce these concentrations in surface water. The increase in 2014 and 2015 may be due to issues with the operation of the new aerators as well disruption of operations due to ongoing construction at the WWTP (Bicudo et al. 2016). The reduction in the estrogenicity of the WWTP effluents in association with upgrades to nitrifying treatment is consistent with literature, which demonstrates reduced estrogenicity in the effluents of nitrifying plants (Servos et al. 2005; Luo et al. 2014). As estrone and 17β-estradiol were two of the main species responsible for the estrogenicity of the Kitchener WWTP effluents (Smith 2013), it is also consistent with other readily biotransformable CECs in the Kitchener WWTP, all of which showed significant reduction after upgrades with the lowest levels occurring in 2013. Of all the CECs that showed significant reduction when upgrades were implemented, total estrogenicity had the closest relationship with both total ammonia and nitrate concentrations in the effluents.

4.4 Nutrients in the Waterloo WWTP from 2010 - 2015

The Waterloo WWTP performed inconsistently from 2010-2015. Ammonia levels remained high within the plant over this time period, though nitrate levels did increase significantly in 2015 compared to pre-2014 conditions. Typically, if an increase in nitrate is as a result of nitrifying treatment, it would be associated with a decrease in ammonia. In the Waterloo WWTP from 2011 - 2015, ammonia remains high even as nitrate increases, with the exception of 2014 concentrations. This is likely due to the delays in construction and upgrades at the WWTP.

Though a return activated sludge (RAS) was implemented, upgrades to aeration were not, resulting in high ammonia loads even when partial nitrification is occurring during secondary treatment. In 2014, a re-aeration zone was introduced to the RAS system, resulting in a drop in ammonia and an increase in nitrate. However, the plant is still operating with mechanical surface aeration in the remainder of the secondary treatment train. Improvements to aeration are crucial to the reduction of ammonia in WWTP effluents, so the ammonia at this plant will likely remain elevated until additional upgrades are implemented.

4.5 Pharmaceuticals and total estrogenicity in the Waterloo WWTP from 2010 – 2015

The concentrations of all pharmaceuticals in the Waterloo WWTP were highly variable over time, but followed the same general patterns of those at the Kitchener WWTP when process changes are considered. Similarly to the Kitchener WWTP, there was a reduction in readily biodegradable compounds (ibuprofen, naproxen, triclosan, fluoxetine) in 2014 and 2015 after the implementation of a RAS re-aeration zone at the Waterloo WWTP. Their continued presence in effluents at higher levels than in the Kitchener WWTP may be due to the incomplete nature of upgrades at the Waterloo WWTP, where secondary treatment still employs surface mechanical aeration with a relatively short SRT (<5 days). The moderately biotransformed pharmaceuticals were once again highly variable, with sulfamethoxazole significantly reduced in 2014 and 2015, trimethoprim reduced in 2014 only, gemfibrozil reduced in 2015 only, venlafaxine unchanged across the years, and atorvastatin and its metabolite reduced in 2014 and 2015. The recalcitrant compounds carbamazepine and diclofenac were unchanged in 2014 and increased in the effluents in 2015 compared to pre-2014 conditions.

Total estrogenicity in the Waterloo WWTP effluents was likewise highly variable. Total estrogenicity in 2014 and 2015 was lower when compared to pooled pre-2014 data, but looking

at year-to-year comparisons illustrates high variability between years before 2014. Fall 2009, 2010, and 2013 had high total estrogenicity (above 10 ng/L E2eq), while 2011, 2014, and 2015 were lower (above 1 ng/L E2eq but below 10 ng/L E2eq). The Waterloo WWTP has significantly lower daily flow than the Kitchener WWTP, serving roughly half the number of people. This needs to be considered when predicting exposure and effects, such as intersex in fish, in the receiving environment.

4.6 Treatment upgrades and their impacts on CECs

Since effluents are complex mixtures potentially containing hundreds of different CECs and other contaminants, it is not feasible to measure each individual contaminant. To be useful, indicator compounds should be present in wastewater effluents and surface waters in measurable quantities, relatively straightforward to analyse, and commonly used so they are present on a wide scale in WWTPs (Salveson et al. 2012). Ibuprofen, naproxen, and triclosan typically meet all of these conditions, and their removal was significantly increased as the plant moved from non-nitrifying to partially/fully nitrifying treatment. Total estrogenicity had the strongest relationship with ammonia and nitrate levels in the Kitchener WWTP, clearly indicating improvements to effluent quality. These compounds, as well as total estrogenicity, may therefore be useful as key indicators of high-quality CAS plants employing nitrification in the secondary treatment process. In the case of this research, influent concentrations were not measured in the WWTP. Though this information would have been beneficial to more accurately determine the change in removal over time, historical influent samples were not available. Additionally, the quantitation of CECs in influents is difficult due to the extremely complex matrix, and would likely require the development of additional extraction and analysis methods. As influent

concentrations were not quantitated, the reduction of these key CECs in effluents cannot be causatively linked to the changes in treatment process.

4.7 Conclusions

The upgrades implemented in the Kitchener WWTP significantly reduced the presence of highly biodegradable pharmaceuticals in and total estrogenicity of the effluents, while the inconsistent treatment at the Waterloo WWTP is reflected in many of the pharmaceuticals as well as total estrogenicity. Key pharmaceuticals with high biodegradation rates (ibuprofen, naproxen, triclosan) may be useful as indicators for the quality of and upgrades to the secondary treatment process within an associated WWTP. This work has implications for the interpretation of biological assessments currently being performed downstream of these major WWTPs in the Grand River watershed. These studies are examining the impacts of these two major WWTPs on biota living downstream of effluent discharges at multiple levels of biological organization. Manuscripts currently in preparation show a decline in intersex in rainbow darter downstream of the Kitchener WWTP (Hicks et al., University of Waterloo, personal communication), consistent with the YES assay performed on these effluents which shows a significant decline in estrogenicity after the implementation of upgrades. A thorough characterization of the effluent chemistry is a crucial element in determining the potential exposure of downstream biota for studies looking at exposure, impact, or recovery.

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Appendices

A1: Additional LC-MS/MS Parameters for analysis of CECs

A number of parameters must be optimized for each contaminant analysed with LC-MS/MS to ensure optimal sensitivity and selectivity.

Table A1.1. Source parameters for the detection of select analytes on a AB Sciex 3200 Qtrap mass spectrometer.

Analyte	DP	EP	CEP	CE	CXP
Atorvastatin	83	5.9	18.91	32	22
p-hydroxy atorvastatin	46	7.5	20	25	14
Carbamazepine	55	4.9	14	51	2.7
Diclofenac	-22.5	-22.5	-15	-15	-1.7
Fluoxetine	48	2.9	12.08	44	7
Gemfibrozil	-20.9	-20.9	-17	-17	-3
Ibuprofen	-41	-2.6	-19	-11	-0.5
Naproxen	-29	-1.9	-20	-25	-3.8
Sulfamethoxazole	41	3	9	22.1	3
Triclocarban	-12	-12	-20	-20	-13
Triclosan	-33	-2.0	-7	-30	-3.0
Trimethoprim	59	4	12	32	3
Venlafaxine	38	2.9	21	42	8.0
Surrogate					
Atorvastatin	45.6	4	25.9	30	16
p-hydroxy atorvastatin	64	4	19	32	5
Carbamazepine-d_{10}	61	4.3	17	28	3.1
Diclofenac	-22.7	-22.7	-16.9	-16.9	-6.1
Fluoxetine- d_5	50	4	12.9	38.2	3.1
Gemfibrozil	-21.1	-21.1	-19.2	-19.2	-2
Ibuprofen-d_3	-25	-7.6	-19	-10	-3.0
Naproxen-d_3	-15	-5.0	-10	-20	-3.0
Sulfamethoxazole-d_4	36	12	14	21	4
Triclocarban	-23.4	-23.34	-18	-18	-2
Triclosan-d_3	-33	-2.0	-7	-30	-3.0
Trimethoprim-d_3	46	8.5	22	31	6
Venlafaxine-d_6	45	3.3	18	45	2.4

DP=declustering potential; EP=entrance potential; CEP=collision cell entrance potential; CE=collision energy; CXP=collision exit potential.

Table A1.2 Analysis parameters for select Estrogens on the QQQ

Analyte	Q1	Q3	Polarity	Ret Time (min)	Fragmentor	Collision Energy	Cell Accelerator Voltage
Bisphenol A	227.3	133.2	Negative	7.86	128	26	4
		212.3	Negative	7.86	128	14	4
Estrone	269.4	145.1	Negative	8.3	155	38	4
		143.1	Negative	8.3	155	45	4
Estradiol	271.4	145.1	Negative	7.99	200	40	4
		143.1	Negative	7.99	200	56	4
Estriol	287.4	171.2	Negative	6.23	170	30	4
		145.1	Negative	6.23	170	38	4
Ethinylestradiol	295.39	158.9	Negative	8.37	170	32	4
		144.9	Negative	8.37	170	38	4
Triclosan	286.99	35	Negative	10.5	90	10	4
Surrogates							
Bisphenol A - d16	241.28	223.3	Negative	7.82	140	16	4
		142.2	Negative	7.82	140	24	4
Estrone - d2	271.2	147.1	Negative	8.3	170	38	4
		145.1	Negative	8.3	170	58	4
Estrone-d4	273.4	147.1	Negative	8.3	187	36	4
		145.1	Negative	8.3	187	50	4
Estradiol-d4	275.2	187.2	Negative	7.95	147	42	4
		145.3	Negative	7.95	147	34	4
Estriol-d2	289.39	173.2	Negative	6.23	200	33	4
		147.1	Negative	6.23	200	37	4
		145.1	Negative	6.23	200	53	4
Estriol-d3	290.2	173.2	Negative	6.23	147	38	4
		145.3	Negative	6.23	147	50	4
Ethinylestradiol - d4	299.4	161.2	Negative	8.37	170	34	4
		147.2	Negative	8.37	170	38	4
Triclosan-d3	289.99	35	Negative	10.46	90	9	4

A2: Reagents required for the YES Assay

This appendix provides additional details regarding the preparation and storage of reagents required to run the Yeast Estrogen Screen (YES) assay. Reagents for the YES assay were prepared no more than three weeks prior to use. All reagent storage bottles were autoclaved for 35 minutes on a liquid cycle at 120°C and allowed to cool before use to ensure sterility. A laminar flow hood was used when possible to reduce potential contamination. When suction filtration necessitated work on an open bench, a Bunsen burner was used to provide a sterile work area. All solutions were sterilized with a 0.2 µm suction filter into an autoclaved glass screw top bottle.

Table A2.1. Reagents and media required for the YES Assay.

Material	**Contents**	**Storage**
Agar Plate	10 mL 10X YNB, 2 g Bactoagar, 28 mL MilliQ H_2O	4°C, inverted
GOLD Concentrate	600 mL dd H_2O, amino acids (See table 2.8.2)	4°C
GOLD Media	60 mL 20% dextrose, 60 mL 10x YNB, 110 mL GOLD concentrate, 370 mL MilliQ H_2O	4°C
Minimal Media	100 mL 10X YNB, 100 mL 20% dextrose, 10 mL L-lysine, 10 mL L-histidine, 780 mL MilliQ H_2O	4°C
20% Dextrose	200 g dextrose, 700 mL MilliQ H_2O	4°C
Copper II Sulfate	250 mg copper II sulfate pentahydrate, 100 mL MilliQ H_2O	4°C

YNB = Yeast Nitrogen Base without amino acids

Table A2.2. Amino acid stock solutions, storage conditions, and volume used in GOLD concentrate.

Compound	**Stock solution (g/L)**	**Storage**	**Volume for GOLD concentrate (mL)**
Adenine Sulfate	1.2	RT	75
L-Histidine-HCl	2.4	4°C	50
L-Arginine-HCl	2.4	4°C	25
L-Methionine	2.4	4°C	25
L-Tyrosine	0.9	RT	25
L-Isoleucine	3.6	4°C	25
L-Lysine-HCl	3.6	4°C	100
L-Phenylalanine	3.0	RT	25
L-Glutamic Acid	6.0	RT	25
L-Aspartic Acid	4.0	RT	25
L-Valine	18.0	4°C	25
L-Threonine	24.0	4°C	25
L-Serine	45.0	4°C	50
L-Leucine	3.6	RT	25
L-Tryptophan	4.8	4°C	50
Uracil	2.4	RT	25

RT = Room Temperature

A3: Linear regression analysis of CECs, Nitrate, and Ammonia

Linear regressions were used to model the relationship between key CECs and ammonia or nitrate in the WWTP effluents. The result of all regressions are listed below for each WWTP (Kitchener, Waterloo).

Table A3.1. Linear regression r^2 values and p-values for select pharmaceuticals vs nitrate in the Kitchener WWTP, n = 33 for each test.

Pharmaceutical	R^2	p-value
Atorvastatin	n/a	> 0.05
p-hydroxy Atorvastatin	n/a	> 0.05
Carbamazepine	0.12	0.048
Diclofenac	n/a	> 0.05
Fluoxetine	0.16	0.023
Gemfibrozil	n/a	> 0.05
Ibuprofen	0.50	< 0.001
Naproxen	0.49	< 0.001
Sulfamethoxazole	n/a	> 0.05
Trimethoprim	n/a	> 0.05
Triclocarban	n/a	> 0.05
Triclosan	0.61	< 0.001
Venlafaxine	0.13	0.041

Table A3.2. Linear regression r^2 values and p-values for select pharmaceuticals vs total ammonia in the Kitchener WWTP, n = 33 for each test.

Pharmaceutical	R^2	p-value
Atorvastatin	n/a	> 0.05
p-hydroxy Atorvastatin	n/a	> 0.05
Carbamazepine	n/a	> 0.05
Diclofenac	n/a	> 0.05
Fluoxetine	0.19	< 0.01
Gemfibrozil	0.24	0.004
Ibuprofen	0.68	< 0.001
Naproxen	0.62	< 0.001
Sulfamethoxazole	0.18	0.013
Trimethoprim	n/a	> 0.05
Triclocarban	0.18	0.012
Triclosan	0.73	< 0.001
Venlafaxine	0.13	< 0.001

Table A3.3. Linear regression r^2 values and p-values for select pharmaceuticals vs nitrate in the Waterloo WWTP, n = 21 for each test.

Pharmaceutical	R^2	p-value
Atorvastatin	0.34	0.006
p-hydroxy Atorvastatin	n/a	> 0.05
Carbamazepine	n/a	> 0.05
Diclofenac	n/a	> 0.05
Fluoxetine	n/a	> 0.05
Gemfibrozil	0.31	< 0.001
Ibuprofen	0.27	0.02
Naproxen	0.25	0.02
Sulfamethoxazole	0.43	0.001
Trimethoprim	n/a	> 0.05
Triclocarban	n/a	> 0.05
Triclosan	0.21	0.035
Venlafaxine	0.40	0.002

Table A3.4. Linear regression r^2 values and p-values for select pharmaceuticals vs total ammonia in the Waterloo WWTP, n = 21 for each test.

Pharmaceutical	R^2	p-value
Atorvastatin	n/a	> 0.05
p-hydroxy Atorvastatin	n/a	> 0.05
Carbamazepine	n/a	> 0.05
Diclofenac	n/a	> 0.05
Fluoxetine	n/a	> 0.05
Gemfibrozil	0.20	0.04
Ibuprofen	0.34	< 0.001
Naproxen	0.36	< 0.001
Sulfamethoxazole	0.22	0.03
Trimethoprim	n/a	> 0.05
Triclocarban	0.50	< 0.001
Triclosan	0.22	0.03
Venlafaxine	n/a	> 0.05

A4: Ammonia and Nitrate data from the Region of Waterloo

The data from monthly grab samples taken at the Kitchener and Waterloo WWTP and analysed for total ammonia and nitrate concentrations was compared to the average monthly data released by the Region of Waterloo (ROW) monitoring programs at both WWTPs. Data (nitrate, total ammonia) has been supplied under agreement with the Regional Municipality of Waterloo. Sampling data matched well with the ROW for the Kitchener WWTP, both showing a sharp decline in ammonia and a corresponding increase in nitrate after 2012. The data for the Waterloo WWTP was significantly more variable on a month-to-month basis, so the yearly averages do not as closely mirror the data collected on a monthly basis, though the drop in ammonia in 2014 from pre-2014 conditions and the following increase in ammonia in 2015 is captured in both

datasets.

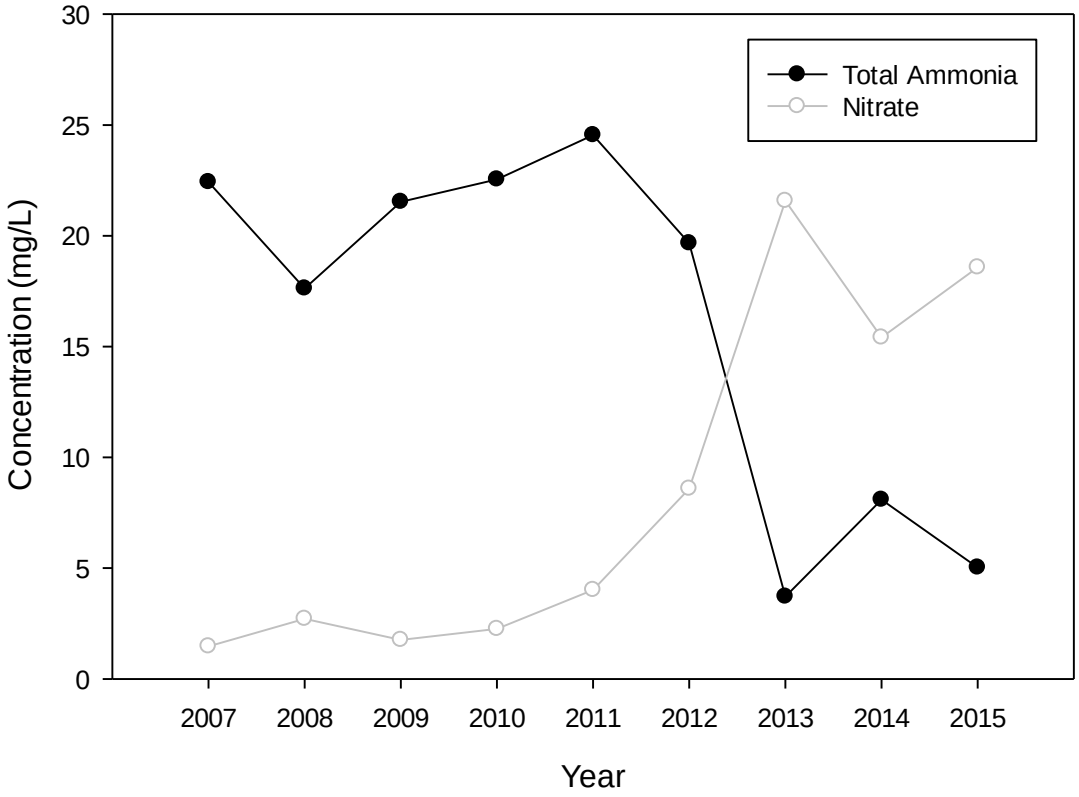

Figure A4.1. Annual mean ammonia and nitrate concentrations at the Kitchener WWTP 2007 - 2015, data provided by the Region of Waterloo.

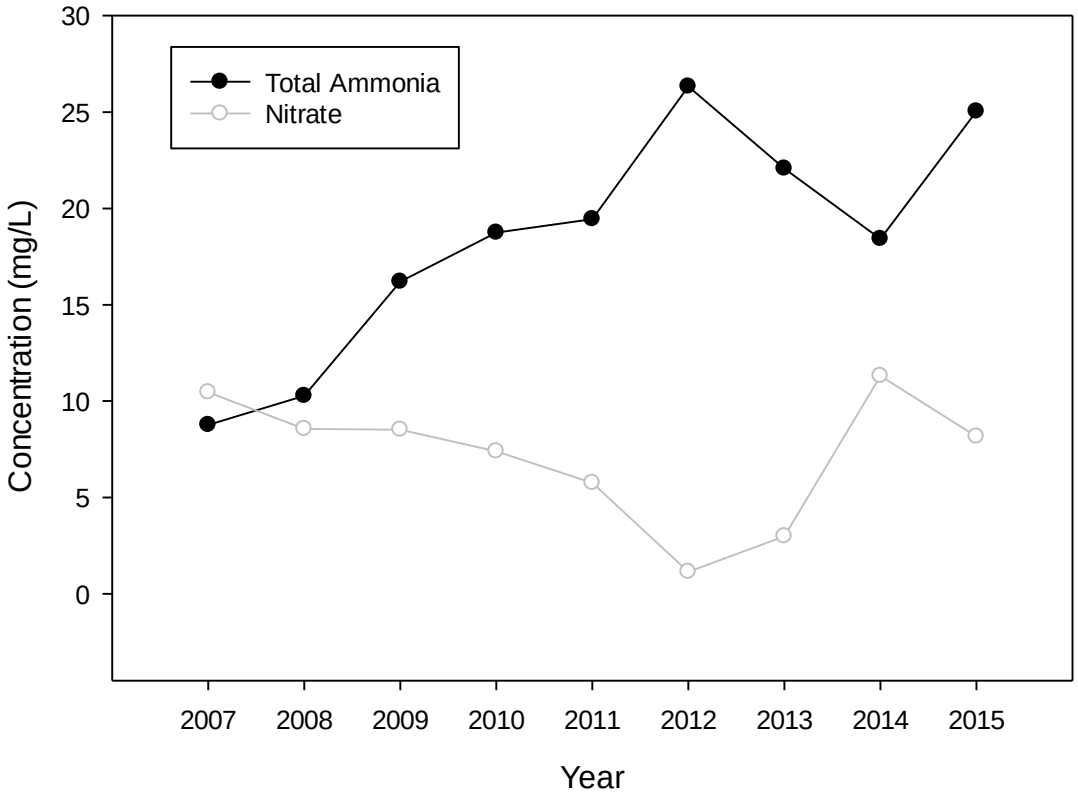

Figure A4.2. Annual mean ammonia and nitrate data for the Waterloo WWTP 2007 – 2015; data provided by the Region of Waterloo.

A5: Principle components analysis details

A principle components analysis (PCA) was performed for each of the WWTPs (Kitchener and Waterloo). This analysis covered all sampling dates and considered all CECs measured as well as total ammonia and nitrate.

Table A5.1. Principle components analysis details for the Kitchener WWTP, 2010 - 2015.

Principle component	Eigenvalues	%variation	Cum. %variation
1	5.77	41.2	41.2
2	2.15	15.3	56.6
3	1.28	9.1	65.7
4	1.16	8.3	74.0
5	0.833	5.9	79.9
6	0.807	5.8	85.7
7	0.622	4.4	90.1
8	0.434	3.1	93.2
9	0.393	2.8	96.0
10	0.221	1.6	97.6

Table A5.2. Eigenvectors (coefficients in the linear combinations of variables making up principle components (PCs)) for the Kitchener WWTP, 2010 – 2015.

Variable	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8	PC9	PC10
NH4	-0.385	0.147	-0.064	0.113	-0.066	0.063	-0.059	0.222	-0.038	0.226
NO3	0.309	-0.317	0.161	-0.226	0.029	-0.132	0.247	0.209	0.060	-0.309
IBU	-0.353	0.120	-0.077	0.144	0.344	0.155	0.340	0.121	0.016	-0.210
NPX	-0.350	0.075	-0.225	0.158	0.289	-0.087	0.280	-0.084	0.120	-0.452
CBZ	-0.156	0.435	0.128	-0.332	-0.415	-0.186	0.063	0.143	-0.451	-0.350
VEN	-0.309	-0.152	0.065	-0.408	-0.125	-0.321	-0.093	0.170	0.244	-0.112
DCF	0.160	0.449	0.286	0.194	-0.143	-0.165	0.413	-0.474	0.016	0.129
TCS	-0.354	0.125	-0.093	0.083	0.032	-0.205	-0.369	-0.026	-0.162	0.282
TCB	-0.175	-0.210	0.556	0.238	-0.002	0.358	-0.363	-0.216	-0.242	-0.401
SMZ	-0.228	-0.008	0.393	-0.416	0.145	0.450	0.348	0.127	-0.022	0.401
TRI	-0.224	-0.317	0.194	-0.196	0.302	-0.490	0.043	-0.480	-0.131	0.145
FLX	-0.226	-0.157	-0.335	-0.244	-0.478	0.349	0.050	-0.511	0.275	-0.088
GFB	-0.218	-0.139	0.378	0.414	-0.396	-0.215	0.126	0.224	0.470	0.068
Ator	0.084	0.490	0.216	-0.263	0.294	0.035	-0.388	-0.083	0.564	-0.141

Table A5.3. Principal component scores by date for the Kitchener WWTP, 2010 – 2015.

Sample	Score 1	Score 2	Score 3	Score 4	Score 5	Score 6	Score 7	Score 8	Score 9	Score 10
2010November	-3.42	0.331	0.452	-0.504	-1.45	1.13	-1.42	-1.27	-0.187	0.59
2011May	-7.99	1.44	1.19	0.306	2.06	0.85	1.25	1.33	0.0112	0.265
2011November	-2.96	2.8	0.951	-0.1	0.645	0.127	-1.25	-0.112	0.587	-0.129
2012Ju1y	-5.84	-0.138	-3.19	0.983	-0.318	0.766	0.771	-0.923	0.119	-1.09
2012September	-1.48	-1.6	0.243	-1.21	0.172	-0.355	-0.639	0.723	-1.48	0.368
2012October	0.623	0.682	-0.705	0.798	-0.672	-0.109	-0.283	0.687	-0.538	0.133
2012November	-3.16	-2.7	-1.25	-0.0636	1.25	-3.46	-0.28	-1.09	-0.444	0.359
2013January	-1.96	-2.6	-0.548	-2.47	-1.99	1.38	1.01	-0.114	0.578	0.822
2013February	-2.32	-3.7	3.43	0.71	-1.43	-0.464	-0.256	0.674	0.425	-0.963
2013March	0.909	-0.844	-0.0758	0.65	-0.188	-0.119	0.0023	0.167	0.795	0.455
2013April	1.51	-1.13	-0.338	0.944	0.124	0.0479	0.38	0.364	0.0481	0.384
2013May	1.65	-1.03	0.213	0.499	0.308	0.192	0.0488	0.492	0.282	-0.0591
2013June	1.72	-0.497	0.0754	0.443	0.553	0.35	0.448	-0.181	-0.165	0.585
2013July	2	-1.06	-0.101	0.0327	0.837	0.87	-0.00382	0.252	-0.695	-0.129
2013September	1.54	-0.641	-0.245	-0.137	0.714	0.54	-0.0237	-0.0387	-0.724	0.181
2013December	0.95	0.15	1.1	-0.736	1.48	0.435	-0.41	-0.736	0.281	0.417
2014March	-0.779	2.38	-0.813	1.14	-2.09	-0.97	-1.14	1.27	-0.656	0.258
2014May	0.874	1.33	0.529	2.91	-0.839	-0.496	1.92	-0.761	0.163	0.635
2014June	1.6	-0.121	0.858	1.34	-0.0705	-0.41	0.477	0.0979	0.454	0.346
2014September	1.5	-0.607	-1.68	0.521	0.0214	0.4	-0.0799	0.627	0.194	-0.413
2014October	2.07	-0.675	-1.59	0.186	-0.159	0.493	-0.148	0.347	0.448	-0.272
2014November	1.38	0.418	1.49	0.853	0.16	0.419	-0.0416	-1.25	-0.241	-0.197
2014December	0.963	-0.273	-0.221	-0.738	0.108	-0.442	-0.112	0.351	0.341	-0.307
2015January	1.09	0.993	0.77	-1.22	-0.00834	-0.981	0.117	-0.365	1.23	-0.495
2015Februahy	0.831	0.857	0.0387	-0.796	-0.309	-0.949	0.508	0.0688	0.0612	-0.446
2015March	0.141	1.44	-0.345	-0.612	-0.235	-0.154	-0.886	-0.439	0.978	0.081
2015April	0.771	0.819	-0.639	-0.249	0.449	-0.244	-0.517	0.528	0.504	-0.151
2015May	0.0191	1.13	-0.144	0.0877	-0.0483	-0.22	-1.05	0.0251	-0.0161	0.399
2015June	1.4	-0.574	0.615	1.26	0.509	1.41	-1.04	-0.77	-1.01	-0.703
2015July	1.48	-0.313	-0.0878	-0.546	0.217	-0.029	0.37	-0.031	-0.262	-0.522
2015August	1.88	0.32	-0.644	-0.742	0.489	0.953	0.358	0.0118	-0.0966	0.163
2015September	0.997	2.79	1.03	-2.1	-1.09	-0.71	1.71	-0.381	-1.43	-0.485
2015November	2	0.631	-0.378	-1.44	0.798	-0.25	0.215	0.44	0.445	-0.0816

Table A5.4. Principle components analysis details for the Waterloo WWTP, 2010 - 2015.

Principle component	**Eigenvalues**	**%variation explained**	**Cum. %variation explained**
1	4.37	31.2	31.2
2	2.79	20.0	51.1
3	1.58	11.3	62.4
4	1.29	9.2	71.7
5	1.16	8.3	80.0
6	0.742	5.3	85.3
7	0.644	4.6	89.9
8	0.518	3.7	93.6
9	0.328	2.3	95.9
10	0.235	1.7	97.6

Table A5.5. Eigenvectors (coefficients in the linear combinations of variables making up principle components (PCs)) for the Waterloo WWTP, 2011 – 2015.

Variable	**PC1**	**PC2**	**PC3**	**PC4**	**PC5**	**PC6**	**PC7**	**PC8**	**PC9**	**PC10**
NH4	0.288	-0.200	0.341	-0.210	0.037	-0.238	0.041	-0.568	0.295	-0.345
NO3	-0.261	0.435	-0.185	0.039	-0.051	0.048	-0.041	-0.133	-0.147	-0.237
IBU	0.419	-0.054	0.053	-0.241	-0.156	0.023	-0.201	0.057	-0.375	-0.245
NPX	0.434	-0.010	0.078	-0.132	0.031	-0.016	0.085	-0.151	-0.365	0.443
CBZ	0.179	0.320	0.457	-0.067	0.064	0.519	0.103	0.078	0.216	0.021
VEN	0.368	-0.036	-0.178	0.178	0.240	0.027	0.479	0.239	-0.259	0.028
DCF	-0.174	0.275	0.518	-0.037	0.055	-0.199	-0.419	0.072	-0.361	0.250
TCS	0.321	-0.026	-0.146	0.158	0.309	-0.403	-0.494	0.344	0.277	-0.028
TCB	0.094	0.216	-0.442	-0.230	0.422	0.302	-0.295	-0.428	0.089	0.259
SMZ	0.262	0.440	0.073	-0.042	-0.120	-0.015	0.103	0.281	0.467	0.104
TRI	0.069	0.274	0.160	0.576	0.429	-0.053	0.080	-0.219	-0.181	-0.337
FLX	-0.066	0.413	-0.099	-0.264	-0.024	-0.607	0.378	-0.135	0.002	0.148
GFB	0.180	0.009	-0.042	0.598	-0.493	-0.034	-0.105	-0.343	0.114	0.372
ATOR	0.256	0.318	-0.270	-0.055	-0.436	0.060	-0.176	0.021	-0.159	-0.390

Table A5.6. Principal component scores by date for the Waterloo WWTP, 2011 – 2015.

Sample	**Score 1**	**Score 2**	**Score 3**	**Score 4**	**Score 5**	**Score 6**	**Score 7**	**Score 8**	**Score 9**	**Score 10**
2011May	2.17	1.06	-1.22	1.99	2.09	-0.313	-0.664	0.487	0.726	0.142
2011November	6.87	-0.29	-0.979	-2.02	-1.22	-0.283	-0.31	-0.346	-0.0735	-0.42
2012July	1.21	-2.06	-0.458	0.431	1.45	0.564	1.86	-0.571	-0.414	0.849
2013December	2.13	-2.02	1.1	1.07	0.588	-0.966	-1.24	0.638	-0.952	0.205
2014March	-0.258	-2.33	1.42	-0.213	-0.278	-0.0435	-0.925	-0.137	0.358	-0.436
2014May	0.707	-0.676	-1.66	0.736	-0.0469	-0.936	0.882	1.1	0.871	-0.0594
2014July	-1.49	-1.19	-0.867	0.217	-0.398	0.82	-0.317	0.345	-0.588	-0.155
2014August	-1.12	-1.48	-0.229	-0.342	-0.348	-0.122	0.561	-0.12	-0.454	0.0225
2014September	-1.54	-2.48	-0.3	-0.701	-0.171	0.126	0.505	-0.281	0.586	0.0877
2014October	-1.73	-1.87	0.121	-0.258	0.0881	-0.393	0.61	-0.841	0.544	-0.914
2014November	-1.06	1.2	-2.56	-0.758	1.4	1.66	-1.31	-1.02	-0.122	-0.0378
2014December	-1.32	2.38	0.413	1.08	0.846	-1.35	0.588	-0.545	-0.857	-0.87
2015January	-0.465	1.43	1.85	0.02	0.68	0.145	-0.433	-0.513	0.87	-0.0978
2015March	-2.21	-0.0788	0.305	-0.571	-0.224	-0.107	-0.71	0.624	0.276	0.0395
2015Apr11	-1.59	-0.171	0.747	-0.428	-0.249	-0.123	-0.565	0.69	0.251	0.444
2015May	-0.305	1.12	1.36	-1.42	0.382	-1.23	-0.247	-1.07	-0.235	1.05
2015June	-1.2	3.27	-1.47	-1.36	-0.98	-1.05	0.562	0.476	0.141	0.245
2015Ju1y	-0.00269	1.03	0.162	0.105	0.0485	0.524	0.412	0.343	-0.855	-0.584
2015August	-1.57	0.241	-0.238	-0.324	-0.86	0.791	-0.0812	1.23	-0.543	0.218
20155eptember	2.33	2.05	2.68	-0.238	0.269	1.83	1.01	0.623	0.308	-0.111
2015November	0.434	0.867	-0.176	2.98	-3.07	0.451	-0.179	-1.12	0.163	0.385

A6: Flow Data for the Kitchener and Waterloo WWTPs

This study reported the concentrations of select CECs in wastewater effluents. To allow for conversion to load, flow rates for the Kitchener and Waterloo WWTPs over time are provided below. Data has been supplied under agreement with the Regional Municipality of Waterloo.

Table A6.1. Average monthly flow at the Kitchener WWTP, 2010 – 2014 reported as 1000 m^3/d. Data provided by the Region of Waterloo.

Month	2010	2011	2012	2013	2014
Jan	62.33571	61.36439	70.461	71.417	70.58483
Feb	59.77989	63.15564	69.194	67.095	64.65795
Mar	69.97555	83.21655	72.681	79.369	71.57371
Apr	69.43803	83.37637	64.744	90.149	94.71034
May	65.7219	87.33552	64.303	72.424	84.92745
Jun	67.50947	71.6509	65.54	74.571	73.04732
Jul	64.72787	62.7379	60.658	70.025	70.13452
Aug	61.03403	61.73148	59.194	66.158	70.40039
Sep	62.2614	60.36391	62.148	64.935	78.18814
Oct	62.924	66.78358	67.659	71.623	72.28735
Nov	62.563	66.43421	64.654	73.558	75.41153
Dec	63.379	76.4606	66.942	67.79	71.55768

Table A6.2. Average monthly flow at the Waterloo WWTP, 2010 – 2014 reported as 1000 m^3/d. Data provided by the Region of Waterloo.

Month	**2010**	**2011**	**2012**	**2013**	**2014**
Jan	42.26545	40.77855	44.595	50.00926	47.356
Feb	40.01164	41.5695	40.293	45.1985	43.408
Mar	47.97874	56.94481	42.21	53.31981	51.81
Apr	44.69987	57.196	39.059	69.45137	61.95
May	41.11919	56.50074	38.443	57.86919	45.469
Jun	43.563	45.96687	46.632	41.70617	46.441
Jul	40.51148	38.05665	35.551	42.74698	48.528
Aug	36.99581	36.97284	37.377	46.92991	40.838
Sep	41.16117	40.03507	39.775	49.94497	45
Oct	43.02774	44.31113	52.006	47.95755	52.676
Nov	42.691	41.3807	39.774	45.7445	52.791
Dec	39.992	46.46223	47.96	41.58629	46.457