Analysis of Temporal Changes in Estrogenic Compounds Released from Municipal Wastewater Treatment Plants

by

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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

Wastewater treatment plants (WWTPs) are traditionally designed to target the removal of contaminants such as total suspended solids, phosphorous, biological oxygen demand, and ammonia. Recent changes to the Federal Wastewater Systems Effluent Regulations (WSER) in Canada require all WWTPs to be operating with secondary treatment or equivalent by 2021. Upgrades being implemented at WWTPs across the country will improve the quality of final effluent discharged into the receiving waters. However, over the past several years, contaminants of emerging concern such as pharmaceuticals, personal care products, and endocrine disrupting compounds have become widely prevalent in wastewater. These compounds are not monitored or targets for removal in Canada causing them to be routinely discharged into surface waters.

The Grand River watershed is the largest watershed in southern Ontario and receives effluent discharge from 30 WWTPs. Several studies have been conducted in the Grand River to assess the impacts of effluent discharge on fish found in the river. The two largest WWTPs are the Kitchener and Waterloo WWTPs, both of which having recently undergone upgrades to improve nitrification processes and improve the overall effluent quality. Studies linked effluent from the plant's pre-upgrade, to several adverse impacts on fish, such as severe cases of intersex and altered hormone production. Upgrades at the Kitchener WWTP were shown to reduce these impacts on fish. Effluent from both Waterloo and Kitchener have been collected and analyzed for pharmaceuticals and estrogens since before the upgrades providing the unique opportunity to evaluate the change in effluent quality and composition over time. In addition to the Kitchener and Waterloo WWTPs, nine secondary WWTPs across southern Ontario were studied to compare the composition of influent and effluent as well as evaluate the apparent removal of various pharmaceuticals and estrogens.

Despite all the plants being classified as having secondary level treatment there was a considerable amount of variability in their ability to treat the incoming influent. Pharmaceuticals of interest were ibuprofen, naproxen, carbamazepine, and venlafaxine because of their different behaviour during treatment. Ibuprofen and naproxen were significantly reduced at all plants, with an increased reduction at plants achieving better nitrification. Carbamazepine and venlafaxine are recalcitrant and remained untreated. Of the estrogens measured, estriol was significantly

reduced across all plants while 17α-ethinylestradiol had no difference post treatment. Estrone and 17β-estradiol were both reduced to varying degrees and were more influenced by external factors such as treatment type and biotransformation. Although there was compound specific variability, the total estrogenicity was significantly reduced post treatment at all plants except those with poor nitrification. Through the analysis of the pharmaceuticals and estrogens as well as nutrient data, nitrification was related to the apparent removal of these non-target compounds (although a direct relationship cannot be established). This correlates with the findings at the Kitchener and Waterloo WWTPs. With the introduction of nitrification at both plants there was a decrease in ammonia concentrations, improved treatment of ibuprofen, naproxen, estrone, and estradiol. There was also a decrease in the total estrogenicity of the effluent discharged from the plants. While venlafaxine, carbamazepine and ethinylestradiol concentrations remained unchanged post upgrades.

Understanding the composition and concentration of contaminants in influent and effluent can provide insight into treatment processes influencing the removal and biotransformation of these compounds. This information is important when deciding on the regulation of these contaminants in effluent discharge. Chemical analysis of these compounds is also critical in developing relationships between contaminant exposure to impacts found in the Grand River. This data can aid in validating predictive models linking contaminants to specific biological endpoints.

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Table of Contents

List of Figures

List of Tables

List of Equations

Chapter 1 – Introduction

Municipal wastewater is a major source of surface water contamination in several countries (Adeel et al. 2017; EC 2011; Holeton et al. 2011). In Canada, the specific treatment regime used in wastewater systems is very site specific and depends on factors such as influent characteristics, available infrastructure, required effluent quality, the sensitivity of the receiving environment and regulatory requirements (Ramalho 1983). The quality of the treatment can therefore be highly variable and effluent released from some plants may be a threat to the ecosystems in the receiving environments (Holeton et al. 2011). A growing concern in regards to wastewater effluent is the presence of endocrine disrupting compounds (EDCs) which can impact fish and other organisms in the environment across multiple levels of biological organization (Mills & Chichester 2005; Tyler et al. 1998). Trace levels of EDCs, particularly natural and synthetic hormones, have been associated with the alteration of hormone production, high incidences of intersex and reproductive success of fish (Kidd et al. 2007; Parrott et al. 2005; Andersen et al. 2003; Lange et al. 2001;). Although it has been documented that trace levels of these contaminants are found in final effluent (Servos et al. 2005; Ternes et al. 1999), they are difficult to quantitate due to the complexity of the sample matrix and the lack of reliable and validated methods to analyze these compounds. This thesis aimed to apply a robust analytical method to measure and characterize the concentration of natural and synthetic estrogens in the effluents from nine municipal WWTPs from southern Ontario. In addition, the concentrations of estrogens in two wastewater treatment plants in the Grand River watershed, that have recently undergone major infrastructure upgrades, have been followed over the past 10 years and analytical methods were used to explore and understand the fluctuations in concentrations.

1.1 Municipal Wastewater Treatment

Municipal wastewater is a complex matrix that originates from many sources including residential, commercial, industrial, urban and agricultural inputs. The various sources add a diversity of substances such as suspended solids, organic and inorganic contaminants, making the composition of raw influent very complex. Although treatment can reduce the presence of many of these contaminants, and their transformation products, many are still released into the environment in the final effluent (Tran et al. 2018).

Wastewaters can undergo a variety of different treatment processes that are generally classified as pre, primary, secondary, and tertiary treatment (Ramalho 1983). The pretreatment

and primary treatment of wastewater involves the removal of suspended solids typically using methods such as screening, sedimentation, and flotation. Secondary treatment utilizes biological processes to decrease the biological oxygen demand (BOD) and nitrogen levels, often performed mechanically using the activated sludge process or passively using other techniques such aerated lagoons, or trickling filters. Following secondary treatment, the effluent can enter a tertiary treatment system, which is considered advanced treatment and is not as common among municipal WWTPs (Ramalho 1983). This stage of treatment is used to further improve the quality of effluent before discharge into the receiving water and employs a diversity of processes such as filtration and various forms of advanced oxidation. Finally, depending on the receiving environment and season, the final effluent may undergo some form of disinfection (chlorine, UV light, etc.) before being released into the environment. Tertiary treatment is often a critical step in removing potentially harmful chemicals (e.g. EDCs) and nutrients (such as phosphorous) from final effluent to avoid adverse effects in vulnerable aquatic ecosystem. In recent years, additional treatment steps such as powdered carbon and ozone, have been introduced to remove specific contaminants of concern, such as pharmaceuticals (Eggen et al. 2014).

1.1.1 Regulations

In 2009, 80% of wastewater in Canada received at least secondary treatment and investment in wastewater has continued nationally (Mavinic et al. 2018; EC 2011). The introduction of the federal Wastewater Systems Effluent Regulations in 2012 under the Fisheries Act Canada has redirected the focus of municipalities to water quality (Ministry of Justice 2017). The new Fisheries Act Regulations on wastewater requires that all municipal wastewater treatment plants across Canada operate at a standard of at least secondary treatment or equivalent by 2021 to control and improve the quality of discharged effluent and most sites are working towards this goal (CCME 2014; Ministry of Justice 2017).

The regulation specifically targets carbonaceous biochemical oxygen demanding matter (CBOD), total suspended solids (TSS), total residual chlorine, and un-ionized ammonia and aims to attain the following effluent conditions (Ministry of Justice 2017):

1. average CBOD in the effluent does not exceed 25 mg/L;
2. average concentration of TSS does not exceed 25 mg/L;
3. average concentration of total residual chlorine does not exceed 0.02 mg/L;

4. maximum concentration of un-ionized ammonia does not exceed 1.25 mg/L, express as nitrogen (N), at 15°C ± 1°C;

The regulation also requires that the effluent discharged must not be acutely lethal to fish. Studies have found that even low concentrations of un-ionized ammonia can be toxic to aquatic species (Camargo & Alonso 2006).

In addition to federal regulations, Ontario treatment plants also need to comply with Provincial legislation, including Certificates of Approval (COA) (MOE 2016). The COA sets site specific criteria to be met for the release of wastewater for each treatment plant. The Ontario Ministry of the Environment Conservation and Parks (OMECP) has established Provincial Water Quality Objectives to provide guidelines on how to manage the quality and quantity of surface and ground waters (MOEE 1999). The goal is to ensure that the quality of surface water is sufficient to maintain aquatic life and recreation. These guidelines entail a much longer list of compounds with more specific water quality limits compared to the federal Wastewater Systems Effluent Regulations. However, the list does not include contaminants of emerging concern such as pharmaceuticals and personal care products (PPCPs) or EDCs.

Unlike Canada, the European community has introduced environmental quality standards (EQS) which is a list of chemicals selected for monitoring and control (Carvalho et al. 2015). Estrogenic hormones were recently added to the list in order to collect monitoring data to make more informed regulatory decisions. The European Commission has proposed the acceptable annual average of 17α-ethinylestradiol and 17β-estradiol in inland surface waters to be 0.035 ng/L and 0.4 ng/L respectively (European Commission 2011), despite very few methods being able to detect concentrations this low. In Canada, these compounds are being monitored under various research programs, however there are no regulations for their removal from effluent discharges. Despite this, upgrades to WWTPs to meet the new regulations have been found to also reduce many microcontaminants (e.g. PPCPs, EDCs) in final effluent (Hicks et al. 2017). Considerable research has examined how various treatment processes alter the removal of specific microcontaminants, PPCPs and EDCs in effluents (Ben et al. 2017; Joss et al. 2004; Johnson & Sumpter 2001). However, the distribution and removal of these contaminants, especially environmental estrogens, are poorly understood making them a growing concern (Cirja et al. 2008).

1.2.1 PPCPs in Wastewater

The fate of various pharmaceuticals and personal care products during wastewater treatment has been widely studied (Tran & Gin 2017; Yang et al. 2011; Kasprzyk-Hordern et al. 2009; Cirja et al. 2008; Kolpin et al. 2002;). The ability of these compounds to be removed or degraded is highly variable and depends heavily on their specific physiochemical properties such as structure and solubility (Tran et al. 2018). Research shows that compounds such as carbamazepine, diclofenac, and venlafaxine are not susceptible to biological treatment and remain very persistent (Tran & Gin 2017; Rua-Comez & Puttmann 2012). Compounds such as naproxen and salicylic acid are considered to have moderate removal, whereas compounds like ibuprofen, acetaminophen, and caffeine can easily be degraded (Tran & Gin 2017). It is suggested that compounds with high hydrophobicity and strong electron donating groups/weak electron withdrawing groups can readily undergo biotransformation and adsorb onto biomass. Compounds with moderate removal typically have the presence of both electron donating and withdrawing groups while persistent compounds have strong electron withdrawing groups/weak donating groups, making it much harder to degrade biologically (Tran & Gin 2017).

1.2.2 EDCs in Wastewater

EDCs are exogenous compounds that can interfere with natural processes, such as hormone biosynthesis, metabolism, and homeostasis (Lister & Van Der Kraak 2001). Estrogenic compounds are a potent subset of EDCs due to their high affinity to the estrogen receptor in aquatic organisms and therefore their ability to mimic and interfere with normal endocrine functions, especially reproduction and development (Gunatilake et al. 2016; Aris et al. 2014). Estrogenic compounds have been detected in industrial and municipal effluent, and urban and agricultural runoff, where concentrations as low as 5 ng/L have been documented to cause feminization of male fish (e.g. intersex) (Gunatilake et al. 2016; Kidd et al. 2007). Feminization entails the development of oocytes in testicular tissue (ova-testes), therefore the occurrence of intersex fish is likely to have adverse effects on the growth and reproduction of fish (Fuzzen et al. 2015; Ankley et al. 2010). Exposure to EDCs found in effluent discharge has been found to not only impact fish on an individual level, but also on a larger scale. The exposure of fish to effluent discharge has been shown to impact fish communities in terms of their abundance, diversity, as well as species and family richness (Tetreault et al. 2012). It has also been found that although fish collected near large urban areas and effluent discharge sites had little change in fertilization success, there was a reduced survival of the progeny to hatch (Fuzzen et al. 2015). A

study has also found that long term exposure to estrogens affects gonad development, resulting in the near collapse of a fish population (Kidd et al. 2007).

Although many chemicals can alter endocrine function in fish, the major estrogens responsible for the total estrogenicity of municipal effluent have been identified as natural estrogens (17β-estradiol (E2), estriol (E3), estrone (E1)), synthetic estrogens (17α-ethynylestradiol (EE2)), and alkylphenolic chemicals (Briciu et al. 2009; Desbrow et al. 1998). EE2 is a synthetic analog of E2 commonly used in birth control pills, however it has a high binding affinity to estrogen receptors and therefore trace concentrations of EE2 in effluent are environmentally relevant (Aris et al. 2014). However, natural estrogens are often the most dominant estrogens in both the influent and effluent (Ben et al. 2017).

Estrogens are excreted as glucuronide or sulfate conjugates but are quickly transformed to their unconjugated and more bioactive forms once they enter the WWTP collection systems (Adeel et al. 2017; Fernandez et al. 2007; Ternes et al. 1999). It was found that the transformation of conjugated estrogens begins in the collection system, with rapid cleavage occurring in the activated sludge system (Liu et al. 2015; Ternes et al. 1999). The presence of conjugated estrogens complicates interpretation of the analysis as they are not typically included in most analytical methods and therefore the total estrogen concentrations maybe underestimated.

Treatment plants implementing nitrification processes, higher temperatures, and longer solid (SRT) or hydraulic (HRT) retention times have higher levels of hormone removal and decreased estrogenicity (Ben et al. 2017; Servos et al. 2005; Johnson & Sumpter 2001). Generally, E3 has the highest removal followed by E2, E1 and EE2 (Ben et al. 2017; Johnson & Sumpter 2001). The estrogens can undergo degradation and transformation to other estrogens or forms during various treatment processes, as seen in Figure 1.1 (Adeel et al. 2017). Due to the hydrophobic properties of EE2, which has a K_{ow} of 3.9-4.1, it is predicted that approximately 80-90% of EE2 will bind to sludge and be removed from the final effluent (Johnson & Sumpter 2001; Joss et al. 2004). However, EE2 still makes a major contribution to the total estrogenicity of final effluent, along with the more water soluble natural estrogen (E2, E1, E3) (Desbrow et al. 1998). Elevated levels of total estrogenicity has been detected in the effluent discharged into the Grand River and has been associated with several cases of intersex in the native fish species found in this watershed (Arlos et al. 2018; Hicks et al. 2017; Fuzzen et al. 2015). Effects Directed Analysis has suggested that the total estrogenicity (based on the Yeast Estrogen Screen) is primarily associate with natural (E2, E1) and synthetic (EE2) estrogens (Arlos et al. 2018).

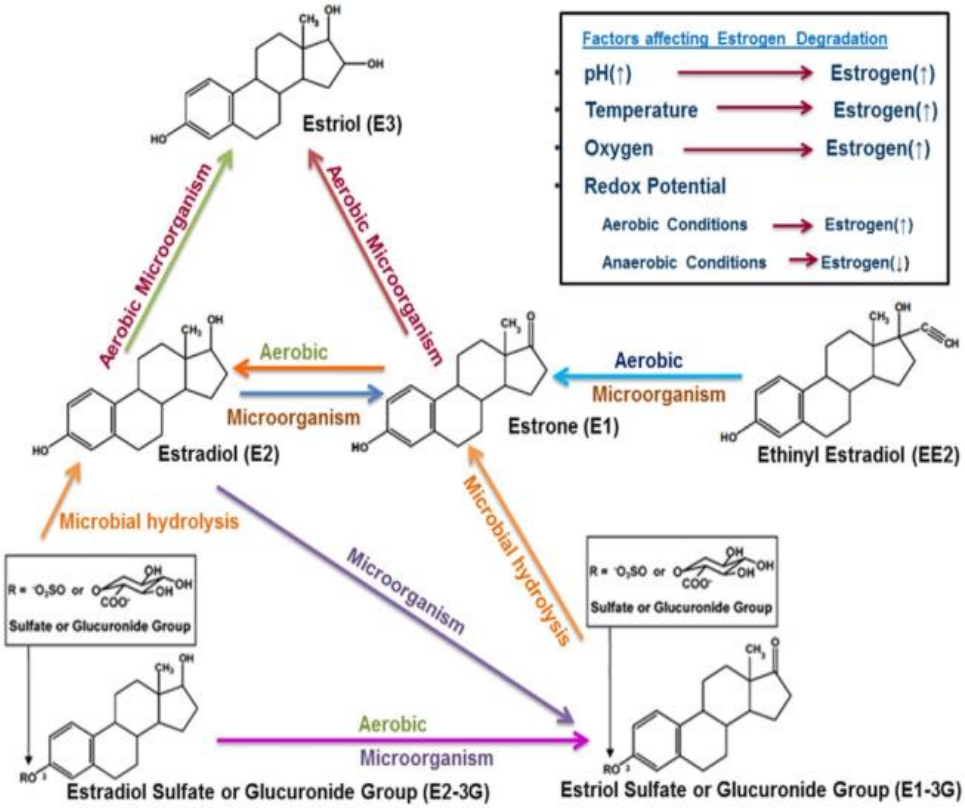


Figure 1. 1 Biotransformation of estrogenic compounds (Adeel et al. 2017)

Unfortunately, the lack of a validated analytical method at the time prevented confirmation of these chemicals in these effluents.

1.3 Grand River Watershed

The Grand River watershed covers 6965 km^2 of southern Ontario and is the recipient of wastewater effluent from 30 different WWTP (Figure 1.2) which all have varying levels of wastewater treatment and is also strongly influenced by rich agricultural lands, and urban developments (Cooke 2006). The upper Grand River watershed generally has good water quality due to minimal urban influences and the presence of low-intensity agriculture, wetlands, and forested lands (Cooke 2006). However, moving into the middle Grand River there is a discernible decrease in water quality attributed to the heavy agriculture and urban development. Three major agricultural tributaries drain into the middle Grand River adding to the increased levels of phosphorus, total ammonia, chloride, and nitrate. The lower Grand River receives the cumulative impacts from upstream but also receives inputs of groundwater that improve water quality, although there are increased levels of suspended solid as the river flows through the southern clay plain.

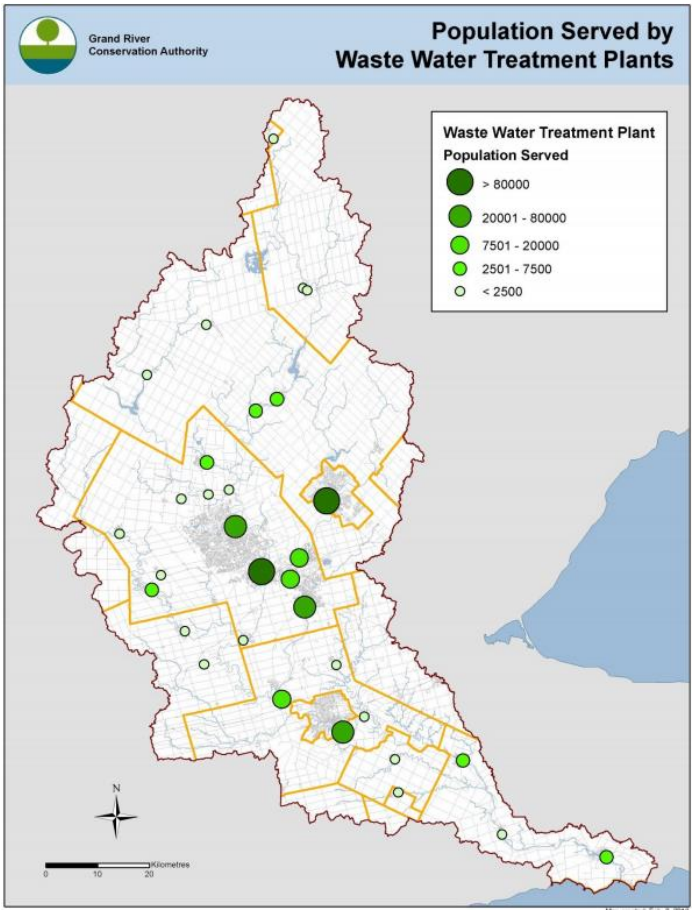


Figure 1. 4 Grand River Watershed WWTP and population served. Created Feb 8, 2016 by GRCA https://www.grandriver.ca/en/our-watershed/resources/Documents/Wastewater/2016-02-22_WatershedWWTPSummary2014.pdf

The two major WWTPs along the Grand River are the Kitchener WWTP and the Waterloo WWTP, both of which recently completed major upgrades to their infrastructure and processes (Bicudo et al. 2016). These plants were initially designed to treat wastewater using a conventional activated sludge system and continuous phosphorus removal followed by chlorine

disinfection to service a population of 138,271 and 54,157 people respectively in 1984 (AECOM 2012; OMOE 1984). As of 2017, these plants service 219,000 and 98,000 people, respectively. Both have undergone major upgrades to implement extended solids retention time, additional aeration (nitrification) and UV disinfection to improve the final effluent quality (Bicudo et al. 2016; AECOM 2012). The upgrades focus on three major issues that include improving effluent quality and odour control, decommissioning existing biosolids storage lagoons and improving process efficiency, and aim to meet the effluent requirements presented in Table 1.1.

Table 1.1 WWTP upgrade effluent objectives (Adapted from AECOM 2012; provided by Pam Law, Region of Waterloo)

		Kitchener				Waterloo			
		Effluent Limits		Operating Objectives		Effluent Limits		Operating Objectives	
Parameter		**Current**	**Future[1]**	**Current**	**Future**	**Current**	**Future[2]**	**Current**	**Future**
$CBOD_5$ (mg/L)		25	15	15	10	15	15	NA	10
TSS (mg/L)		25	15	15	10	15	15	NA	10
TP (mg/L)		1	0.4	0.6	0.2	0.8	0.6	NA	0.4
TAN (mg/L)	**May - Nov**	-	4	-	2	-	1.8	NA	1.5
	Dec - Apr	-	7	-	5				
pH		6-9.5	6-9.5	6.5-8.5	6.5-8.5	-	6-9.5	NA	6-9.5
E. coli (org/100 mL)		200	200	100	100	200	200	NA	100
Total chlorine residual (mg/L)		-	-	-	-	0.5	-	NA	-

1. Future limits apply upon substantial completion of Plants 3 and 4 (estimated for early 2019)
2. Future limits apply upon substantial completion of the current contract (estimated for June 2018)
3. NA, Not available

In addition to improvements in traditional water quality such as BOD, TSS, chloride and ammonia (Hicks et al. 2017; Bicudo et al. 2016;), a decline in the concentrations of pharmaceuticals and total estrogenicity of the final effluents released into the river have been observed as the upgrades were implemented (Hicks et al. 2017). Impacts on fish populations were very prominent downstream of the outfalls of these plants prior to the upgrades and included changes across several levels of biological organization (Fuzzen et al. 2016; Tetreault et

al. 2012, 2011). The recent upgrades have led to a major improvement in a number of endpoints including occurrence and severity of intersex (Hicks et al. 2017), and *in vitro* production of steroid hormones in fish (Marjan et al. 2018). These endpoints are suspected to be associated with exposure to estrogenic chemicals in the effluent (Bahamonde et al. 2013; Fuzzen et al. 2015). Arlos et al. (2018) has modelled the fate of estrogenic chemicals in the effluents and river and established a relationship between the predicted estrogenicity and endocrine responses (i.e. intersex) observed in fish downstream. The accurate quantification of estrogenic compounds in the effluent would aid in linking the changes in fish endpoints to the changes in effluent quality as well allow for the improvement and validation of model predictions.

1.4 Analysis of Estrogens in Wastewater

To characterize the quality of the effluent being discharged by WWTPs two different approaches can be taken. Bioassays can be used to measure the total estrogenic potency of the effluent or a chemical analysis can be performed to measure the concentrations of the individual target analytes (Könemann et al. 2018).

1.4.1 Biological Analysis

The total estrogenicity of a sample is a combination of the effects of various EDCs working through the same mechanism (i.e. binding to the estrogen receptor). Although there are many approaches to measuring estrogenicity (Leusch et al. 2010; Streck 2009; Drewes et al. 2005) the Yeast Estrogen Screen (YES) is a commonly used tool that is well suited to municipal wastewater (Bistan et al 2012; Leusch et al. 2010; Desbrow et al. 1998). The YES assay was originally developed by Gaido et al. and has been widely applied to measure total estrogenicity of wastewater extracts (Marjan et al. 2018; Sun et al. 2017; Volker et al. 2016), including the Grand River wastewater effluents (Hicks et al. 2017).

The YES assay uses a strain of *Saccharomyces cerevisiae* transfected with two plasmids. The first is an expression plasmid containing the CUP1 metallothionein promoter and the human estrogen receptor cDNA. The second is a reporter plasmid containing estrogen response elements upstream of the LacZ gene. The growth media of the yeast contains copper to induce the production of the estrogen receptors in the yeast cells. When the yeast cells are exposed to estrogen, the estrogens bind to the receptors and this complex will bind the estrogen response element on the second plasmid, inducing the transcription of the LacZ gene to produce the enzyme β-galactosidase. The amount of enzyme produced can be measured by introducing the colourless substrate ortho-nitrophenolgalactopyranoside (ONPG) and measuring the colour

change as the enzyme cleaves ONPG, forming ortho-nitrophenol (ONP), a yellow compound over time. The total estrogenicity of the sample can then be indirectly calculated from the change in absorbance measured from the colour change in the assay.

1.4.2 Chemical Analysis

In addition to measuring total estrogenicity, conducting a chemical analysis on the effluent to determine the concentration of the individual analytes provides a better understanding of the contribution of the individual compounds to the total estrogenicity.

Historically estrogen analyses were performed using gas chromatography – mass spectrometry (GC-MS), which has good sensitivity and separation of compounds (Petrovic et al. 2002). However, these compounds are not volatile by nature and require derivatization for analysis by GC-MS, leading to sample loss, and interferences that can out-weigh the advantages. Recently the analysis of estrogens using liquid chromatography with tandem mass spectrometry (LC-MS/MS) with the electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) interfaces, has increased in popularity due to the sensitivity, ruggedness, ease of use and avoiding the need to derivatize the samples.

LC uses a packed column to retain target analytes that are then eluted from the column in a specific order based on the mobile phase solvent composition and chemical properties of the analytes (Mikkelsen & Corton 2004). The eluted compounds are then introduced into the ion source, either ESI or APCI, of the mass spectrometer. ESI is a technique used to ionize the sample by creating very fine charged droplets (Cappiello 2007). APCI relies on gas-phase ions and solvent composition to undergo chemical reactions, typically acid-base chemistry, with the neutral analytes to form analyte ions (parent ion). ESI is the most commonly used ion source because of its low solvent consumption and its ability to ionize a wide variety of analytes. However, ESI is much more susceptible to matrix effects compared to APCI.

Once ionized, the analyte enters the MS where they are accelerated by an electrical potential towards a detector (Mikkelsen & Corton 2004). Due to the differences in charge to mass ratio, the ions are separated and focused for detection and quantification of analytes. The LC-MS/MS allows for fragmentation of the analyte using the tandem MS, providing increased selectivity. The parent ion collides with neutral gas molecules to induce fragmentation. Fragmentation is analyte specific and creates a unique mass-to-charge (m/z) ratio which is used to sort and detect the analytes, providing quantitative and qualitative data. The LC-MS/MS can

be operated under two modes, selected reaction monitoring (SRM) or multiple reaction monitoring (MRM) (Murray et al. 2013). SRM is a non-scanning method that uses two specific masses as filters to increase selectivity, whereas MRM will cycle through multiple mass transitions and measures the signal of all transitions at the different elution times. MRM therefore allows the monitoring of different mass transformations for a single analyte, increasing the selectivity.

The capabilities of the instrumentation are measured and reported as limit of detection (LOD), limit of quantification (LOQ), and method detection limit (MDL) (Armbruster & Pry 2008; Harris 2010). The LOD is the lowest concentration of an analyte that can be identified by an analytical instrument, producing a signal 3 times greater than the background (3:1 signal to noise ratio) (Armbruster & Pry 2008). The LOQ is the lowest concentration of an analyte that can be used to accurately quantify an analyte. This concentration should produce a signal to noise ratio of 10:1. The MDL is the minimum concentration of an analyte that can be quantified from a sample with a 99% confidence (Harris 2010).

LC-MS/MS using MRM is much more selective and sensitive than single quadrupole instruments and can achieve LODs of less than 1 ng/L, which is comparable to the sensitivity achieved using a GC-MS/MS (Petrovic et al. 2002). However, this is dependent on the instrument and sample matrix. Further resolution of compounds can be achieved using instruments such as an LC-MS/MS quadrupole time-of-flight (QTOF) that can achieve an exact mass of 0.001 or greater (Harris 2010). QTOF applies a high voltage to the ions to accelerate them through the ion source into a region with no electric or magnetic field. It is here that ions of different masses will separate since ideally, they will all have the same kinetic energy but due to mass differences the ions will travel at different speeds. The lighter ions will travel faster and reach the detector before the heavier ions, therefore this technique is more mass specific. This higher selectivity can be helpful when trying to identify compounds in complex samples.

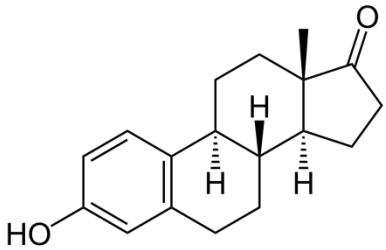
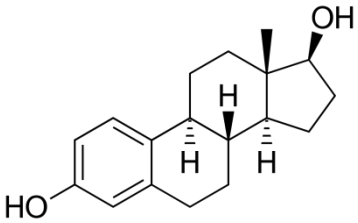
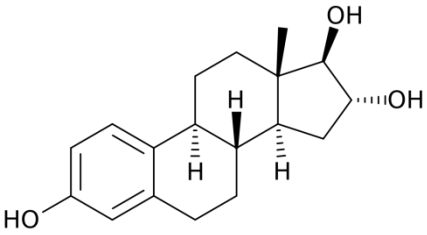
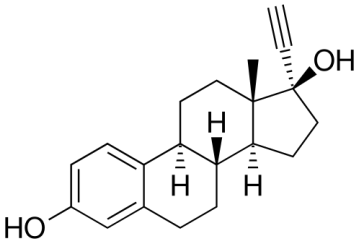
Using the calculated concentrations of the individual estrogens, an estimate of the total estrogenicity can be made by summing the contribution of each of the estrogens present (concentration x relative potency to E2) and expressed as an E2 equivalence (EEQ) (Arlos et al. 2018). The E2 equivalency factor for E1, E2, E3 and EE2 were reported as 0.3, 1.0, 0.03 and 1.23 respectively based on the YES assay. Ideally, the chemically determined estrogenicity

would align with the values determined using the biological assay, however there are many limitations to these analyses, as well as discrepancies between the methods.

1.4.2.1 Mass Fragment Patterns of Estrogens

Currently, LC-MS/MS is the most commonly used method to quantify specific estrogens in influent, effluent, and river water (Briciu et al. 2009). For the analysis of estrogens using mass spectrometry, the mass transition producing the most abundant product ion is used to quantify the compound, while less abundant but unique product ions are used as qualifiers to confirm the identity of the compound (Harris 2010). Natural and synthetic estrogens have very similar structures, as a result produce similar product ions (Table 1.2).

Table 1. 2 Estrogen structures and common fragment patterns

Estrogen	Structure	Parent Ion (m/z)	Product Ions (m/z)
Estrone (E1) (Magi et al. 2010)		296	253 183 159 145 143
Estradiol (E2) (Magi et al. 2010)		271	239 197 183 158 145 143
Estriol (E3) (Croley et al. 2000)		287	183 171 159 145 143
Ethinylestradiol (EE2) (Magi et al. 2010)		295	269 209 195 183 159 145 143

In the case of EE2, the product ion with the mass to charge ratio of 145 is most commonly used as the quantifier while 143 or 159 are used as the qualifiers (Magi et al. 2010; Vulliet et al. 2008; Croley et al. 2000). The 145 m/z is characteristic of the stable double phenol ring found in EE2 and is common among all estrogens and many steriods (Andrási et al. 2011; Magi et al. 2010).

1.4.3 Limitations

Despite the advancements made in the analysis of estrogens in effluent samples, there are still many analytical challenges to measuring these compounds, specifically E2 and EE2 (Konemann et al. 2018). A study comparing the analysis of estrogens in wastewater samples by various labs found that EE2 could only be quantified above the LOQ in less than 50% of the samples (Konemann et al. 2018). Currently the most attainable LOQ is 0.1 ng/L, however at this concentration the uncertainty of these results range between 50-100% (Heiss 2013). In addition, the highly complex nature of wastewater effluent results in matrix effects which raises the LOQ of these compounds, making analysis more difficult. Environmental samples are variable and consist of various compounds, such as humic acids, that can cause interferences during analysis (Vega-Morales et al. 2013). These compounds can co-elute with the compounds of interest causing ion suppression/enhancement and reduced sensitivity of the detection method (Gomes et al. 2005; Petrovic et al. 2002). Estrogens are found at low levels in the environment and the susceptibility of the LC-MS/MS to matrix effects makes accurate quantification difficult (Briciu et al. 2009).

To improve the resolution and sensitivity during the separation of the estrogen compounds studies have used Ultra-High Performance Liquid Chromatography (UHPLC) coupled with a tandem mass spectrometer (Ripolles et al. 2014; Kumar et al. 209). However, even with higher resolution, matrix effects can still be a problem. A study on the analysis of estrogens in surface water, influent, and effluent using UHPLC found high levels of background noise for the most commonly used EE2 transition of 295 m/z to 145 m/z (Ripolles et al. 2014). Therefore sample extraction and preparation are critical in the analysis of estrogens.

A method used to address the presence of matrix effect is standard addition (Harris 2010). This technique involves the addition of varying concentrations of standard solution to replicates of an unknown sample to determine how the matrix influences the signal response. The total concentration of analyte from the unknown and standard should increase linearly due to

the added standard. In the absence of matrix effect, the calculated analyte concentration in each of the samples will be the same, however, with matrix effect the calculated concentrations for the sample will be different. For each concentration added to the sample the amount of matrix effect is expected to be consistent, therefore by comparing the original sample to the spiked sample the relative amount of matrix effect can be determined and taken into account for future samples of a similar nature. Another commonly used method to account for matrix effects is the use of internal standards (Benijts et al. 2004). These can be isotopically labeled versions of the compound of interest or compounds that behave very similarly to the compound of interest and are not found in the sample itself. Known concentrations of internal standards are added to the collected samples prior to extraction, allowing these compounds to interact with the matrix and undergo the same processes as the sample. Therefore, quantification of the internal standard can account for any loss during sample analysis or suppression/enhancement due to the matrix, allowing for an appropriate adjustment for compounds originally found in the sample.

In addition to the limitations of the chemical analysis, there are also constraints to using the YES. This assay uses receptors to measures the total estrogenicity of the sample, however this can be influenced by the complexity of the sample matrix since a response will be induced by any chemical that binds and activates the receptor. Therefore, the specific chemicals causing a total estrogenic response in the YES assay is uncertain. In addition, other compounds (anti-estrogens) in effluents/extracts may block the receptor and prevent the estrogen response from occurring, resulting in the underestimation of the total estrogenicity of the sample (Jung et al. 2004). Consequently, each of the methods have specific biases that must be considered, and a weight of evidence approach is advisable.

1.5 Study Objectives

This study aimed to develop a reliable analytical method for the detection of estrogens in wastewater effluents and apply this method to quantify concentrations in selected plants to support other related studies modelling concentrations and effects in receiving waters. Samples for the study were from two sources. The first group of samples were composite influent and effluent samples collected from nine wastewater treatment plants across southern Ontario with varying degrees of treatment (including Kitchener and Waterloo) and were provided through a collaboration with Environment and Climate Change Canada. The second group of samples were grab samples from Waterloo and Kitchener and were collected during a period of major

treatment plant upgrades between 2010 and 2018. Archived extracts were available from previous studies and new samples were collected for the current study between 2017 and 2018.

The objectives were to: 1) Apply a robust methodology for estrogen analysis to influent and effluent samples from Canadian WWTPs; 2) contrast the concentrations of estrogens in influent and final effluent (i.e. removal); 3) determine the temporal pattern of estrogens in Waterloo and Kitchener over a period of treatment plant upgrades and 4) use various analytical techniques to explore and understand the fluctuations in estrogens. For each sample set analysis was completed for estrogens using LC-MS/MS (QQQ) and total estrogenicity using the YES assay. This was supported with analysis of nutrients and pharmaceuticals to characterize the effluent/treatment.

Chapter 2 – Analysis of PPCPs and EDCs in influent and effluent across nine Canadian WWTPs

WWTPs and their discharge has been a major area of study for several years as they have direct impacts on the surrounding environment. As new contaminants emerge the focus of study changes with time and currently some of the emerging contaminants of concern are pharmaceuticals and hormones. At very low concentrations, hormones such as 17α-ethinylestradiol (EE2) have been reported to have effects on aquatic species. Kidd et al. (2007) showed that concentrations as low as 5 ng/L can cause a collapse in fish community, while Parrott et al. (2005) showed that 1 ng/L impacted the reproductive success of fathead minnows.

Several studies have also been done on the impacts of hormones in the Grand River. EDCs and PPCPs found in effluent have been linked to impacts on fish health, including intersex (Hicks et al. 2017), and the *in vitro* production of steroid hormones in fish (Marjan et al. 2018). Although these compounds can have impacts on the environment and aquatic life downstream of discharge sites, they are not monitored or regulated in Canadian wastewater systems. Studies have been performed on the treatment and removal of PPCPs and EDCs, linking increased removal to nitrification and solid retention times, however they are only partially removed (Achermann et al. 2018; Servos et al. 2005; Ternes et al. 1999).

In Europe, an environmental quality standards (EQS) list, which includes several chemicals such as estrogenic hormones, was recently introduced for monitoring and regulatory decision making purposes (Carvalho et al. 2015). The European commission has set the acceptable annual average of EE2 as 0.035 ng/L (European Commission 2011). However very few methods can detect concentrations that low, especially considering the low initial concentrations and the influence of matrix interferences. Therefore, there is very little compiled data available for Canadian WWTPs. The objective of this study was to determine the presence of estrogens in effluents in several treatment plants using different processes and having different effluent quality. Influent and effluent samples from nine WWTPs across southern Ontario were characterized and the distribution of estrogen and total estrogenicity compared.

2.1 Methods

2.1.1 Materials

Reference compounds were purchased from Sigma Aldrich and CND Isotopes. Standard solutions were stored at -20℃. All solvents used were HPLC grade. Stock solutions of all the analytes were prepared at a concentration of 1 g/L in methanol which was used to prepare a calibration curve with the concentrations of 0, 0.5, 1, 10, 50, 100, 200, 500, 1000, 3000, 5000 µg/L. The YES assay was performed using the Yeast β-Galactosidase Assay Kit from Thermo Scientific.

2.1.2 Sample Collection

Composite and grab samples were collected just before the outfall from 9 wastewater treatment plants (WWTPs) in southern Ontario between May 2017 to August 2018 (Table 2.1). Influent and effluent were collected by Environment Canada and Climate Change in cooled (4℃) 24-h composite samplers on three sequential days. Three 1 L glass amber bottles were collected from the composite samples per day and preserved with 50 mg/L ascorbic acid and 1 g/L sodium azide on site. The samples were then transported to the University of Waterloo and filtered through a 47 mm glass fiber filter (Pall Corporation, Mississauga, ON) and stored at 4°C. Samples were extracted within 72 hours of collection.

Table 2. 1 Wastewater treatment plant characteristics

Plant	Treatment	Sampling Point	Watershed	Avg Flow (m^3/d)	% Residential Input	% Industrial-Commercial-Institutional Input	Major industry	Sampling Dates
A	Conventional activated sludge with nitrification[2]	Before UV	Grand River	37,900	85	15	Universities	Aug 2017
B	Conventional activated sludge plant with nitrification[2]	Before UV	Grand River	58,700	60	40	Food Manufacturing	Aug 2017
C	Aerated lagoon, continuous phosphorous removal with sand filters and nitrification[2]	Before filters	Grand River	510	90	10	Sandpaper Manufacturing	Aug 2017
D	Oxidation ditch extended aeration with nitrification[2] and sand filters	After filters, before UV	Grand River	1,040	90	10	Tourism	Aug 2017
E	Conventional activated sludge, continuous phosphorous removal	Before chlorination/ dechlorination	Bay of Quinte	22,900	60	40	Food Manufacturing, Automotive	Oct 2017
F	Conventional activated sludge	Before chlorination/ dechlorination	Bay of Quinte	5,000	90	10	Leachate	Oct 2017

Plant	Treatment	Sampling Point	Watershed	Avg Flow (m^3/d)	% Residential Input	% Industrial-Commercial-Institutional Input	Major industry	Sampling Dates
G	Conventional activated sludge	After chlorination, before dechlorination	Grand River	34,062	50	55	Chemicals, Paper Recycling	May 2018
H	Conventional activated sludge	After UV	Grand River	27,125	-	-	-	Jul 2018
I	Conventional activated sludge Extended aeration	Before chlorination/ dechlorination	Grand River	3,990 3,368	80	20	PVC Manufacturing, Chicken Processing	May 2017 (a) Jul 2018 (b)

1. Day 3 influent results were strange, sent for re-analysis, therefore not included in the average.
2. In these cases, nitrification means the Certificate of Approval for the WWTP includes requirement to nitrify

All samples were collected during dry weather and all plants were operating normally

Data provided by Shirley Anne Smyth, Environment and Climate Change Canada (ECCC))

2.1.3 Sample Extraction

Prior to extractions, the filtered 1 L effluent samples were split into the appropriate volumes for the extractions of hormones, total estrogenicity, and pharmaceuticals using solid phase extractions (SPE). The analytes of interest in this study are listed in Table A2.1. Extractions were done manually or using the ThermoFisher AutoTrace™ (Dionex, Sunnyvale, CA). With each batch of samples extracted, quality assurance/quality control (QA/QC) samples were also processed. Each batch was extracted with two matrix spikes (MS1, MS2) as positive controls and a blank as a negative control. These samples were prepared using 100 mL of MilliQ water and spiked with 100 µL of 100 µg/L regular and deuterated standards for matrix spikes, or 100 µg/L deuterated standards for the blanks.

2.1.3.1 Hormones

The hormone extraction was performed using Superclean LC-18 cartridges (6 cc, 500 mg, Sigma Aldrich) preconditioned with methanol and MilliQ water. A 100 mL sample, spiked with 100 µL deuterated internal standard, was loaded onto the cartridge. The cartridge was then allowed to dry before eluting the analytes with ethyl acetate. The samples were evaporated to dryness under nitrogen before being reconstituted in 500 µL of methanol with 75 µg/L lorazepam and chloramphenicol, two compounds which are not found in the samples and therefore used as QA/QC. Samples were stored at -20°C.

2.1.3.2 Total Estrogenicity

Extractions for total estrogenicity were performed using Oasis HLB cartridges (6cc, 500mg, Waters) preconditioned with methyl tert-butyl ether (MTBE), methanol, and MilliQ water. A 500 mL or 1 L of unspiked sample was loaded onto the cartridge. The cartridges were then rinsed with MilliQ water before eluting the analytes with methanol and 10% methanol in MTBE. The samples were evaporated to dryness under nitrogen before being reconstituted in 500 µL of methanol then stored at -20°C.

2.1.3.3 Pharmaceuticals and Personal Care Products

The pharmaceutical extraction was performed using Bond Elut Plexa cartridges (6 cc, 500 mg, Agilent) preconditioned with methanol and MilliQ water. A 100 mL sample was brought to pH 2 ± 0.05 and then spiked with 100 µL deuterated internal standard before loading onto the cartridge. The cartridges were then rinsed with MilliQ water and 5% methanol in water before eluting the analytes with methanol. The samples were evaporated to dryness under nitrogen before being reconstituted in 500 µL of methanol with 75 µg/L lorazepam and

chloramphenicol. Lorazepam and chloramphenicol were used as instrument controls to ensure proper injection of the samples. Samples were stored at -20°C.

Table 2.2 Optimized SPE methods for the extraction of analytes from wastewater

Target Analytes	Sample Volume	Cartridge	Conditioning	Elution	Final Volume
Hormones	100 mL	Superclean LC-18 cartridges (6 cc, 500 mg, Sigma Aldrich)	5 mL Methanol 5 mL MilliQ Water	5 mL Ethylacetate 5 mL Ethylacetate	0.5 mL
Total Estrogenicity	500 mL	Oasis HLB cartridges (6cc, 500mg, Waters)	5 mL methyl tert-butyl ether (MTBE) 5 ml Methanol 5 mL MilliQ Water	5 mL Methanol 5 mL 90% MTBE in methanol	0.5 mL
PPCPs	100 mL	Bond Elut Plexa cartridges (6 cc, 500 mg, Agilent)	5 mL Methanol 5 mL MilliQ Water	3 mL Methanol 3 mL Methanol	0.5 mL

2.1.4 Sample Analysis

Following the extraction of final effluent samples, the reconstituted extracts were analyzed for hormones, pharmaceuticals and total estrogenicity using different analytical techniques optimized for the analytes of interest. Detection limits for the chemical analyses were determined by McCann (2016) and are reported in Appendix A5. Instrument detection limits (IDL) and quantification limits (IQL) were determined by repeated injections of blanks (n=7) and the calibration curve. The IDLs and IQLs were calculated as three times and ten times the standard deviation of the blanks respectively. Method detection limits (MDLs) were determined by measuring multiple wastewater samples spiked at various concentrations (n=7). Values were calculated using a student's t-test value at 99% confidence multiplied by the standard deviation. The YES assay kit used had a linear absorbance range of 0.02-1.00 and samples are analyzed relative to the standard curve.

2.1.4.1 Nutrients

Nutrient analysis was provided by Environment and Climate Change Canada using the methods listed in Table 2.3.

Table 2. 3 Methods used to measure various nutrients in influent and effluent samples

Analyte	Method	Detection Limit (mg/L)
Alkalinity, Total (CaCO3)	Titrimetry	0.3
Ammonia as N	Colorimetry – Phenate	0.250
Nitrate as N	Chem Suppress IC	0.02
Nitrate/Nitrite as N	Chem Suppress IC	0.03
Nitrite as N	Chem Suppress IC	0.006
BOD	B_BOD_Water	2.0
COD	Spectrophotometry	4.8
pH	Potentiometry	-
Nitrogen, Total Kjeldahl	Colorimetry -Black Digest Phenate	0.700
Solids, Total Suspended (TSS)	Gravimetry	5.0
Solids, Volatile Suspended (VSS)	Gravimetry	6.0

The un-ionized ammonia concentration in the final effluent was calculated from the measured ammonia concentration, pH and temperature of the sample. Equation 1 was used to calculate the dissociation constant (pKa), where T is temperature in Kelvin. The pKa was used to determine the fraction of total un-ionized ammonia (Equation 2). These equations were based on the Canadian Water Quality Guidelines for the Protection of Aquatic Life (CCME 2010).

Equation 1. pKa calculation

$$pKa = 0.0901821 + \frac{2729.92}{T}$$

Equation 2. Fraction of total un-ionized ammonia

$$f = {}^{1}/_{[10^{(pKa-pH)}+1]}$$

2.1.4.2 Hormones - LC-MS/MS QQQ

Analysis of hormones was performed using an Agilent 1260 HPLC, equipped with a HiP Sampler, binary pump, and an Agilent 6460 triple quadrupole (QQQ) mass spectrometer with an Agilent Jet Stream (AJS) electrospray ionization. 10 µL of reconstituted hormone extract was injected onto an Agilent ZORBAX Eclipse Plus C18 (2.1 mm x 50 mm x 1.8 µm) HPLC column, held at 35℃, to chromatographically separate the analytes of interest, achieved with a flow rate of 300 µl/min and the mobile phase gradient described in Table 2.3.

Table 2. 4 Hormone LC-MS/MS QQQ mobile phase parameters

Mobile Phase A:	5 mM Ammonium fluoride in MilliQ
Mobile Phase B:	100% Acetonitrile

Time (min)	% Mobile Phase A	% Mobile Phase B
0	90	10
10	0	100
13	0	100
13.10	90	10

Following separation, analytes were identified and quantified using a dynamic multiple reaction method optimized on the triple quadruple. The method was run in a negative ionization mode with a source temperature of 700℃, gas temperature of 250℃, gas flow 10 L/min, nebulizer at 30 psi, and a capillary voltage of 4000V. The compound parameters were optimized to the values indicated in Table 2.4. With each batch of samples analyzed on the triple quadrupole a calibration curve ranging from 0.5 ng/L to 5000 ng/L was also injected.

Table 2. 5 Optimized compound parameters

Compound	Precursor Ion	Product Ion	Frag (V)	CE (V)	Cell Acc (V)	Ret Time (min)
4 NP – d4	223.27	110.1	79	18	4	12.09
4 NP Linear	219.34	106.1	98	18	4	12.13
4 NP Mixture	219.34	147.1	130	26	4	11.46
4 NP Mixture	219.34	133.1	130	30	4	11.46
4 OP	205.3	106.1	90	14	4	11.53
4 OP – d17	222.4	108.1	90	18	4	11.46
BPA	227.3	212.3	128	14	4	7.86
BPA	227.3	133.2	128	26	4	7.86
BPA – d16	241.28	223.3	140	16	4	7.82
BPA – d16	241.28	142.2	140	24	4	7.82
E1	269.4	145.1	155	38	4	8.3
E1	269.4	143.1	155	45	4	8.3
E1 – d2	271.2	147.1	170	38	4	8.3
E1 – d2	271.2	145.1	170	58	4	8.3
E1 – d4	273.4	147.1	187	36	4	8.3
E1 – d4	273.4	145.1	187	50	4	8.3
E2	271.4	145.1	200	40	4	7.99

Compound	Precursor Ion	Product Ion	Frag (V)	CE (V)	Cell Acc (V)	Ret Time (min)
E2	271.4	143.1	200	56	4	7.99
E2 – d4	275.2	187.2	147	42	4	7.95
E2 – d4	275.2	145.3	147	34	4	7.95
E3	287.4	171.2	170	30	4	6.23
E3	287.4	145.1	170	38	4	6.23
E3 – d2	289.39	173.2	200	33	4	6.23
E3 – d2	289.39	147.1	200	37	4	6.23
E3 – d2	289.39	145.1	200	53	4	6.23
EE2	295.39	144.9	170	38	4	8.37
EE2 – d4	299.4	161.2	170	34	4	8.37
EE2 – d4	299.4	147.2	170	38	4	8.37
TCS	286.99	35	90	10	4	10.5
TCS – d3	289.99	35	90	9	4	10.46

2.1.4.3 Pharmaceuticals and Personal Care Products - LC-MS/MS QTRAP

PPCPs were analyzed using an Agilent 1200 HPLC (Agilent, San Pedro, CA) connected to a Sciex 3200 Triple Quadrupole Linear Ion Trap (QTRAP) mass spectrometer (ABSciex, Concord, ON, Canada) with electrospray ionization (ESI). Compound separation was achieved by injecting 20 µL of reconstituted PPCPs extract onto an Agilent Eclipse XDB-C18 (4.6 mm x 150 mm x 5 µm) column using the mobile phase gradient described in Table 2.4 at a flow rate of 800 µL/min.

Table 2. 4 PPCPs LC-MS/MS QTRAP Mobile Phase Parameters

Mobile Phase A: 5 mM Ammonium acetate in MilliQ
Mobile Phase B: 100% Methanol

Negative Ionization Mode			Positive Ionization Mode		
Time (min)	% Mobile Phase A	% Mobile Phase B	Time (min)	% Mobile Phase A	% Mobile Phase B
0.00	90.0	10.0	0.00	90.0	10.0
0.50	90.0	10.0	0.50	90.0	10.0
0.51	60.0	40.0	0.51	50.0	50.0
8.00	0.0	100.0	8.00	0.0	100.0
11.00	0.0	100.0	10.00	0.0	100.0
11.50	90.0	10.0	10.01	90.0	10.0
16.00	90.0	10.0	15.00	90.0	10.0

Following separation, the compounds were identified and quantitated on the QTRAP using a multiple reaction method (MRM) with optimized instrument parameters for the analytes of interest identified by McCann (2016) (Table 2.5, Table 2.6). With each batch of samples analyzed on the QTRAP a calibration curve ranging from 0.5 ng/L to 5000 ng/L was also injected.

Table 2. 5 LC-MS/MS QTRAP Optimized Source Parameters (McCann 2016)

	Positive Ionization	Negative Ionization
CUR	30	10
CAD	Med	Med
IS Voltage	5500	-4500
Temp	750	750
GS1	50	60
GS2	30	40

Table 2. 6 Optimized PPCP Parameters (McCann 2016)

ID	Q1	Q3	Time (ms)	DP	EP	CEP	CE	CXP	RT (mins)
Positive Ionization Mode									
ACE	152.096	109.9	100	51	7.5	10	21	3	4.009
ACE-d4	156.106	113.9	100	51	10	10	23	4	4.009
ATEN	267.2	145.1	100	51	3	30	36	5	3.779
ATEN-d7	274.3	145.2	100	49.81	3.7	41.4	35.6	3.7	3.779
ATOR	559.3	440.2	100	83	5.9	18.91	32	22	6.988
ATOR-d5	564.3	445.3	100	45.6	4	25.943	30	16	6.988
ATRZ	216.2	174.3	100	66.9	3.8	13.5	27	2.4	7.233
ATRZ-d5	221.1	179.3	100	67.9	4.1	16.334	22.1	3	7.233
CAFF	195.2	123	100	47	3.4	8.8	41	5	4.415
CAFF-d3	198.2	123.1	100	42	3	15	42	4	4.415
CBZ	237.1	193.3	100	55	4.9	14.3	51	2.7	6.565
CBZ-d10	247.2	204.4	100	60.9	4.3	17.065	28	3.31	6.565
Desven	264.2	58.2	100	21	2	16	39	8	4.704
Desven-d6	270.2	64	100	21	7	40	39	8	4.704
e-CBZ	253.2	180.3	100	26	5.5	17.233	33	4	5.454
e-CBZ-d10	263.2	190.3	100	53	3.5	20	34	5	5.454
FLX	310.3	44.3	100	48	2.9	12.08	44	7	7.38

ID	Q1	Q3	Time (ms)	DP	EP	CEP	CE	CXP	RT (mins)
FLX-d5	315.2	44.2	100	50	4	18.969	38.2	3.1	7.38
LIN	407.3	126	100	60	9	21.547	49.7	4	6.398
LIN-d3	410.28	129.2	100	36	8.5	18	43	4	6.398
Lorazapam[1]	321.1	275.1	100	60.1	5.1	19.134	32.8	3	7.02
MET	130.132	71.1	100	36	3.5	10	29	4	2.249
MET-d6	136.145	77	100	36	4	10	29	4	2.249
MON	693.4	675.4	100	124.7	9.6	29.558	55.9	10.9	10.234
NFLX	296.1	134.1	100	23	3	9.5	9	5	7.213
NFLX-d5	301.2	139.2	100	23	3	10	9	5	7.213
o-ATOR	575.229	440.3	100	46	7.5	20	25	14	6.679
o-ATOR-d5	580.2	445.2	100	64	4	19	32	5	6.679
Oxybenzone	229.053	151	100	56	5.5	14	27	4	8.807
Oxybenzone-d5	234.1	151.1	100	51	8	14	27	4	8.807
p-ATOR	575.23	440.301	100	46	7.5	20	25	14	5.839
p-ATOR-d5	580.201	445.201	100	64	4	19	32	5	5.839
SMZ	279.084	92	100	36	4.5	14	41	5	4.27
SMZ-d4	283.14	96	100	41	4	14	43	8	4.27
SULFA	254.1	156.2	100	41	3	9	22.1	3	4.222
SULFA-d4	258.122	160.1	100	36	12	14	21	4	4.222
TRIM	291.1	261.2	100	59	4	12	32	3	4.537
TRIM-d3	294.2	230.3	100	46	8.5	22	31	6	4.537
VEN	278.3	58.1	100	38.2	2.9	21	42	8	6.161
VEN-d6	284.271	64.1	100	26	11	8	35	8	6.161
Negative Ionization Mode									
BPA	227	211.9	200	-53	-10	-20.055	-28	-5	8.092
BPA-d16	241	142	200	-50	-10	-20.573	-50	-3	8.092
BPA2	227.036	212.1	200	-60	-2	-10	-26	-2	8.092
Chloramphenicol	321	151.9	200	-53	-1	-23.533	-23.1	-1	
DCF	293.9	250	200	-46	-2.5	-22.53	-15	-1.7	7.814
DCF-d5	298.2	253.8	200	-25.8	-6.9	-22.689	-16.9	-6.1	7.814
EE2	295.1	144.9	200	-71.8	-7	-10	-54	-3	8.669
EE2-d4	299.1	146.9	200	-72.9	-5	-28.23	-51.8	-15.1	8.669
GFZ	249.1	121.1	200	-55	-2	-20.873	-17	-3	9.488
GFZ-d6	255	120.7	200	-46.5	-11	-21.091	-19.24	-2	9.488
IBU	204.9	160.9	200	-41	-2.6	-19.237	-11	-0.5	8.209
IBU-d3	207.9	164.1	200	-25.1	-7.6	-19.348	-10	-3	8.209

ID	Q1	Q3	Time (ms)	DP	EP	CEP	CE	CXP	RT (mins)
NPX	229	170	200	-29	-1.9	-20.129	-25	-3.8	6.69
NPX-d3	232.1	172.8	200	-15	-5	-10	-20	-3	6.69
NPX-d4	233	169.9	200	-36.8	-2	-20.277	-25.7	-1	6.69
TCCB	314.8	161.6	200	-50	-3	-12	-20	-13	10.319
TCCB-d2	316.9	159.9	200	-50	-2.5	-23.381	-18	-2	10.319
TCS	286.9	35	200	-33	-2	-7	-30	-3	10.284
TCS-d3	289.9	35	200	-28.5	-2	-11.31	-25.3	-2.3	10.284

1. Lorazepam was used as an internal standard for monensin

2.1.4.4 Total Estrogenicity - YES Assay

Total estrogenicity of the samples was measured using the Yeast Estrogen Screen (YES) assay as outlined by Arlos et al. (2018.) Buffers and media required for this analysis were prepared as outlined in Appendix A2 (Table A2.1, Table A2.2). *Saccharomyces cerevisiae* cells (Receptor: ER_{trp} (YePtrpER), Reporter E2.ura ($YRpE2_{ura}$)), stored in 30% glycerol stock at -80^{o}C, were provided by Heidi Engelhardt, University of Waterloo (originally from K. Gaido, Research Triangle Park).

A stock of *S. cerevisiae* was thawed at 4^{o}C and streaked onto an agar plate. Plates were incubated at 30^{o}C, shaking at 300 rpm for 3 d or until individual colonies were visible on the plate. The plates were then stored at 4^{o}C for up to 14 d. A single colony from the streak plate was used to inoculate 1mL of selective GOLD media and incubated at 30^{o}C, 300 rpm for 24 h to allow cell growth. The cells were then diluted 1:10 with minimal media and incubated at the same conditions for another 24 h. A further 1:1 dilution of the cells was made using minimal media and incubated for 4-6 h at the same conditions. After 4-6 h, cells were diluted with a solution of copper II sulfate and minimal media until an optical density (OD) of 0.03 was obtained at 660 nm.

To expose the cells, 10 µL of standard (68.10 µg/L – 0.03 µg/L E2) or sample (serially diluted 1:1 up to 1024x dilution) was transferred to a 2 mL amber glass vial in duplicate and left in the laminar flow hood to allow the methanol to evaporate. Along with the calibration curve and samples, QA/QC samples were also run in duplicate. The blank consisted of an empty vial, positive control had 10 µL of standard level 8 (2.13 µg/L E2), and the negative control had 10 µL of methanol. Once the methanol had evaporated, 200 µL of the diluted cells (OD 0.03) was added to each vial. Cells were exposed for 18-24 h at 30^{o}C, shaking at 300 rpm.

To measure the estrogenicity, a yeast β-Galactosidase assay kit (Thermo Scientific) was used. The 2x yeastβ-Galactosidase assay buffer, stored at -20°C was thawed at 4°C. After the cells had been exposed for 18-24 h, 25 µL aliquots from each vial was transferred into a well on a 96-well plate and diluted with 75 µL of minimal media. The cell density of each well was measured at 660 nm before adding 100 µL of a 1:1 solution of 2x yeastβ-Galactosidase assay buffer and yeast protein extraction reagent (Y-PER). The reaction was measured at 420 nm every 25 secs, for 30 mins on a Molecular Devices Max 3 spectrophotometer plate reader (Sunnydale, CA, USA). Samples below the detection limit of the assay were further concentrated to 80 µL and analyzed again using the YES assay.

2.1.4.5 Data Analyses

Analyte peaks produced by the QQQ and QTOF were integrated using Agilent MassHunter Quantitative Analysis software version B.06.00, peak data from the QTRAP were integrated using the Analyst software version 1.6.2. Analyte concentrations were determined by comparing the ratio between the peak area of the analyte and the peak area of the deuterated internal standard peak area to the ratios of the calibration curve injected with the batch of samples. Dilution factors were accounted for to determine the concentration in the initial sample volume. The apparent percent reduction (difference) between corresponding influent and effluent samples was calculated using Equation 3. The calculated total estrogenicity of the sample was determined using Equation 4 by taking the EEQ of each estrogen and their concentration into account.

Equation 3. Percent Reduction

$$Percent\ Reduction = \frac{influent\ concentration - effluent\ concentration}{influent\ concentration} \times 100\%$$

Equation 4. Calculated Total Estrogenicity (ng/L E2 equivalents)

$$Total\ Estrogenicity = ([E2] \times EEQ_{E2}) + ([E1] \times EEQ_{E1}) + ([E3] \times EEQ_{E3}) + ([EE2] \times EEQ_{EE2})$$

From the YES Assay, absorbance values measured per well over the course of 30 mins were used to determine the rate of B-galactosidase activity. The absorbance measured per well was corrected for the number of cells in the well using the OD_{660} measurement and the β-galactosidase activity was calculated using Equation 5, where t represents time (min) of incubation and v is the volume (mL) of cells used in the assay. The assay kit used for this

analysis is linear between an OD_{420} of 0.02 and 1.0, therefore time points with absorbances outside of this range were not included in the analysis. Wells with cell death were also not included in the analysis.

Equation 5. B-galactosidase Activity

$$\boldsymbol{Activity = \frac{1000 \times A_{420}}{t \times v \times OD_{660}}}$$

The β-galactosidase activity of the calibration curve and the samples were normalized to the maximum and minimum response of the calibration curve and fit to a regression model (Sigmoidal, Hill, 4 Parameter) using Sigma Plot (Equation 6). Using this model, the normalized β-galactosidase activity of the samples was calculated and subsequently the total estrogenicity of each well was expressed as an E2 equivalence in ng/L using Equation 7.

Equation 6. Four Parameter Logistic

$$x = y_0 + \frac{ax^b}{c^b + x^b}$$

Equation 7. YES Assay Final Concentration (ng/L E2 equivalents)

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

Statistical analysis was performed on all data using SigmaPlot version 13.0. A two-way ANOVA along with a pairwise multiple comparison (Tukey test) was used to identify statistical differences due to site or sample type in total estrogenicity, estrogen concentrations, and pharmaceutical concentrations ($P<0.05$). A two-way ANOVA was also used to identify statistical differences in the percent difference of the estrogens and pharmaceuticals between influent and effluent samples from the various sites ($P<0.05$). Additionally, a paired t-test was used to find statistical differences in the calculated and measured total estrogenicity.

2.2 Results

2.2.1 Effluent quality

The treatment plants sampled represented a wide variety of plants with differing treatment processes (Table 2.1) and therefore had different effluent quality (Table 2.5). The percent reduction of select parameters are described in Table 2.6. The total Suspended Solids (TSS) had over 97% reduction with over 200 mg/L in influent and effluent samples ranging from <5.0 to 12.2 mg/L. pH and temperature of the samples collected remained consistent. pH ranged from 7-9 for all influent and effluent samples, with the temperature of effluent samples being between 16-21°C. Biological oxygen demand (BOD) and chemical oxygen demand (COD) are both traditionally targeted nutrient parameters and both were reduced by over 90% and 80% respectively. Final effluent concentrations of BOD were below 9 mg/L while COD concentrations were between 30-110 mg/L. Ammonia had over 99% removal, except at Plants A (89%), B (95%), E (93%), F (53%), and I-a (90%). Similar patterns were seen in the removal of total Kjeldahl nitrogen (TKN), with over 95% removal except at Plants A, B, E, F, and I-a. With lower removal of ammonia, it was expected that these plants would have lower concentrations of nitrate produced, however it was not always the case. Despite the high concentration of ammonia at Plants A and B, these plants had high levels of nitrate in the effluent, 25 and 29 mg/L respectively. In addition, Plant C which had 99% removal of ammonia had only 0.1 mg/L of nitrate detected in the final effluent, whereas Plant F with 53% removal of ammonia had 9.7 mg/L of nitrate.

The unionized form of ammonia is very toxic and therefore important. However, the percentage of total ammonia that exists as unionized ammonia is a function of both temperature and pH. The CCME environmental quality guideline for unionized ammonia is 0.019 mg/L (CCME 2010) while the Wastewater Effluent Systems Regulation targets 1.25 mg/L, expressed as nitrogen (N), at 15°C ± 1°C. But it also states that the effluent must not be acutely lethal to fish. Plants A, B, I-a slightly exceed the proposed limits and guidelines for ammonia while plant F is well over (Table 2.6).

Table 2. 6 Nutrient parameters of influent and effluent collected from various Ontario WWTPs

		NH_3		NO_3^-		TKN		TSS		BOD		COD		pH		Temp. (°C)	Un-ionized NH_3 $(mg/L)^3$
		Avg. (mg/L)	SE	Avg. (mg/L)	SE	Avg. (mg/L)	SE	Avg. (mg/L)	SE	Avg. (mg/L)	SE	Avg. (mg/L)	SE	Avg. (mg/L)	SE		
A	Inf	30.8	2.3	<0.02	0.0	46.6	5.1	366.7	72.7	176.0	19.4	492.3	134.2	7.6	0.0	-	
	Eff	3.2	1.7	29.3	1.5	6.0	2.3	6.6	0.2	8.3	1.3	63.7	7.8	7.6	0.1	19.0	0.05
B	Inf	29.1	1.5	<0.02	0.0	50.5	0.1	294.0	39.5	262.0	21.2	614.3	94.0	7.6	0.0	-	
	Eff	1.5	0.7	24.9	3.4	4.0	0.9	<5.0		5.9	0.5	48.3	3.8	7.7	0.1	21.0	0.03
C	Inf	53.0	4.7	<0.02	0.0	86.4	2.5	358.7	44.5	221.0	5.6	614.7	50.5	8.3	0.0	-	
	Eff	0.3	0.1	0.1	0.0	2.8	0.1	5.7		8.6	3.1	109.7	7.5	7.9	0.1	20.0	0.01
D	Inf	25.1	2.4	0.5	0.2	38.9	3.8	716.0	278.7	192.7	13.3	504.3	83.8	7.6	0.1	-	
	Eff	0.1	0.0	19.5	1.2	1.1	0.1	<5.0		4.7	0.1	37.0	3.0	8.1	0.0	18.0	0.01
E	Inf	16.8	1.6	<0.02	0.0	29.1	3.8	298.3	93.6	278.0	71.4	542.0	144.0	7.0	0.1	-	
	Eff	1.2	0.6	14.5	2.9	2.9	1.0	<5.0		5.9	0.1	42.3	1.5	7.7	0.1	17.0	0.02
F	Inf	21.7	1.4	<0.02	0.0	37.3	1.2	312.0	45.7	206.7	12.2	545.3	84.3	7.1	0.1	-	
	Eff	10.1	1.2	9.7	1.1	16.9	0.5	<5.0		5.6	0.1	30.0	0.6	7.5	0.0	17.0	0.11
G	Inf	22.3	1.0	0.1	-	33.5	0.8	210.0	9.9	182.7	1.8	467.0	9.7	7.5	0.0	-	
	Eff	0.0	0.0	24.7	0.7	2.2	0.3	<5.0		3.9	0.2	46.3	0.3	7.9	0.1	21.0	0.00
H	Inf	35.6	2.9	<0.02	0.0	50.5	4.6	314.0	15.7	266.0	7.2	580.7	18.9	7.5	0.0	-	
	Eff	0.2	0.1	31.4	2.4	2.9	0.3	<5.0		4.7	0.8	47.3	5.0	7.9	0.0	20.0	0.00
I-a	Inf	24.4	2.1	<0.02	0.0	42.4	3.2	290.0	73.5	250.0	4.0	533.7	74.0	7.4	0.0	-	
	Eff	2.4	0.6	3.2	0.3	4.1	0.6	10.0		6.0	0.4	52.3	2.7	7.8	0.0	16.0	0.05
I-b	Inf	23.7	3.2	<0.02	0.0	43.2	1.1	379.0	39.3	304.0	17.1	638.7	50.3	7.4	0.0	-	
	Eff	0.1	0.0	25.0	0.7	1.2	0.2	12.2	5.6	5.4	0.5	36.0	2.5	7.9	0.0	21.0	0.00

1. Nutrient Parameters: Ammonia as N (NH_3), Nitrate as N (NO_3^-), Nitrogen, Total Kjeldahl (TKN), Total suspended solids (TSS), Biological Oxygen Demand (BOD), Chemical Oxygen Demand (COD), pH, Temperature
2. Day 3 influent sample had very high results. Re-checking with lab, not included in average
3. Un-ionized ammonia calculated from effluent ammonia concentrations, taking pH and temperature into consideration
4. Nutrient data was collected and provided by Shirley Anne Smyth at Environment and Climate Change Canada (ECCC)

Table 2. 7 Percent removal of select nutrients at various Ontario WWTPs

Plant	% BOD Removal	% COD Removal	% TSS Removal	% TKN Removal	% Ammonia Removal
A	95	89	98	88	89
B	97	93	99	91	95
C	97	82	99	97	99
D	97	92	99	97	99
E	98	92	99	93	93
F	97	94	99	54	53
G	98	90	99	94	100
H	98	92	99	95[1]	100
I-a	98	89	99	89	90
I-b	98	94	97	97	100

1. Day 3 influent sample had very high results. Re-checking with lab, not included in average

2.2.2 Pharmaceuticals

Both influent and effluent samples were analyzed for the 24 pharmaceuticals listed in Appendix A1 (Table A1.1) using the LC-MS/MS QTRAP. The percent difference in concentration of each pharmaceutical after treatment was calculated and can be found in Table 2.8. Lincomycin (LIN) and monensin (MON) could not be detected in either influent or effluent. Atrazine (ATRZ) was only detected in influent from plants G, however concentrations ranging from 4-30 ng/L was found in effluent from all plants sampled. As a result, a difference could not be accurately determined. Among the different pharmaceuticals measured, caffeine (CAFF), ibuprofen (IBU), and naproxen (NPX) had consistently higher percent differences between influent and effluent at all plants whereas compounds such as carbamazepine (CBZ), venlafaxine (VEN), sulfamethoxazole (SULFA), and fluoxetine (FLX) and consistently low differences (<80%). However, unlike the other plants sampled, Plant C had increased differences of all compounds, including compounds that were not easily removed by other plants. This plant had over 80% difference in concentration of all compounds analyzed except for carbamazepine (CBZ), however it still had the highest percent difference compared to all the other plants at 67%.

Table 2. 8 Percent difference of pharmaceuticals

	Percent Difference (%)									
	A	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I-a**	**I-b**
ATRZ	ND	ND	ND	ND	ND	ND	27	ND	ND	ND
CBZ	-43	-40	67	-1	-8	-72	-9	0	-77	-80
FLX	8	55	100	54	40	13	2	31	52	80
ATOR	-16	38	100	96	89	20	100	73	92	100
VEN	4	5	98	16	19	16	38	3	-5	-1
LIN[1]	-	-	-	-	-	-	-	-	-	-
MON[1]	-	-	-	-	-	-	-	-	-	-
SULFA	18	13	99	39	49	-35	91	16	42	48
TRIM	-16	24	100	100	17	10	94	7	49	97
NFLX	22	88	100	100	87	51	100	83	100	100
CAFF	100	100	100	100	100	64	100	100	100	100
e-CBZ	-29	-5	86	-2	27	18	17	-24	29	-3
p-ATOR	-10	33	100	99	88	14	100	78	95	100
o-ATOR	4	37	100	99	87	10	100	76	89	100
SMZ	79	74	90	96	51	-168	100	78	24	100
ACE	100	100	100	100	100	100	NQ	NQ	100	NQ
DES	-44	32	99	-31	60	55	95	-45	-22	-47
IBU	94	100	100	96	100	90	99	99	98	100
BPA	96	100	89	67	100	43	90	88	60	90
NPX	99	99	100	99	98	98	100	99	99	100
GFZ	72	97	ND	98	97	39	95	96	59	ND
DCF	-33	28	97	38	3	-3	82	35	-10	61
TCS	60	91	99	97	76	58	74	90	236	95
TCCB	40	86	84	100	52	63	NQ	NQ	78	NQ

1.No LIN or MON detected in either influent or effluent
ND – Not detected in influent samples but quantifiable in effluent
NQ – Not quantified

The concentrations of CBZ in influent and effluent were only significantly different at plants C, F, I-a, and I-b ($p = <0.001, 0.013, 0.008, 0.007$) (Fig. 2.1). Plant C had a significant decrease in concentrations whereas plants F and I both had significantly higher concentrations in the final effluent compared to influent samples. It can also be seen that concentrations of VEN in influent and effluent were only significantly different at plants C and G, with decreased

concentrations in the effluent samples (p= <0.001, 0.002). The concentration of IBU in influent and effluent were significantly different at all plants sampled (p<0.001). Concentrations of NPX in influent and effluent were statistically different at all plants (p<0.001).

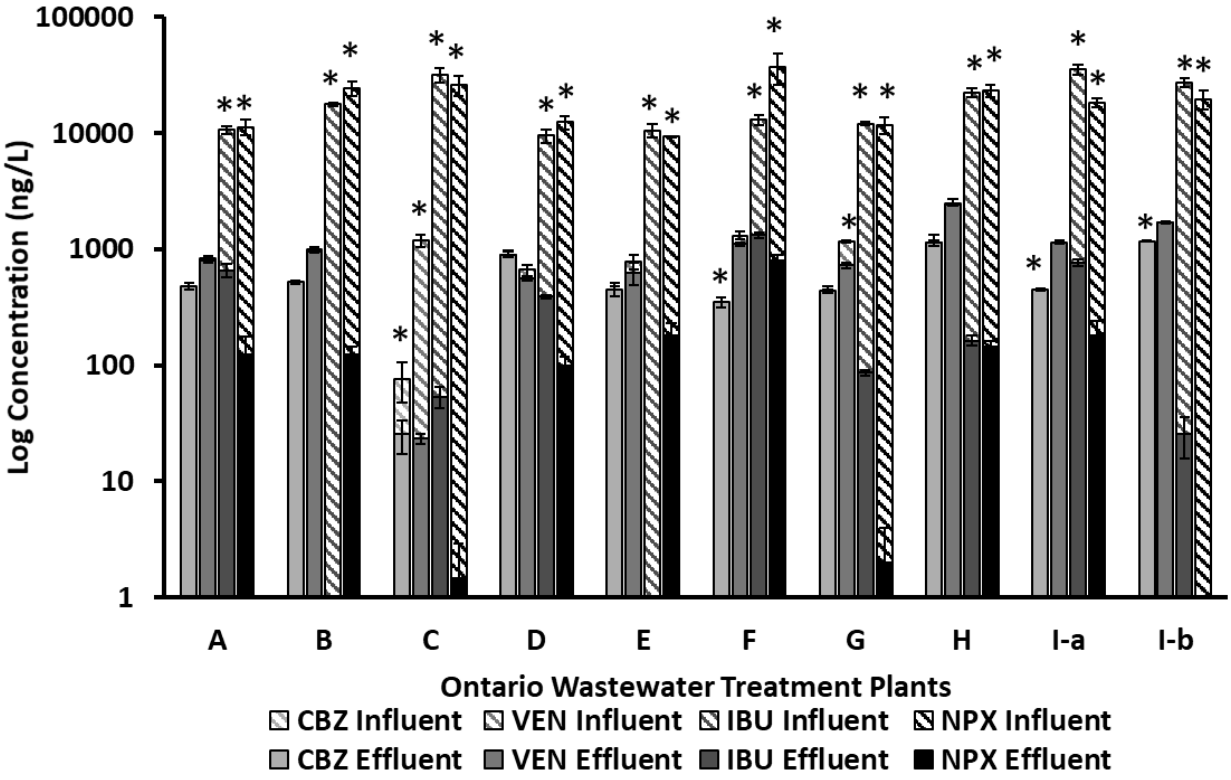


Figure 2.1 Mean concentration of select pharmaceuticals in influent and effluent. Carbamazepine, venlafaxine, ibuprofen, and naproxen concentrations were measured in influent (striped) and effluent (solid) samples from various WWTPs. The log concentration along with standard error was plotted to show the difference between the samples from each site. Significant differences (p<0.05) between influent and effluent concentrations of the pharmaceuticals measured from the different WWTPs are indicated by *.

2.2.3 Hormones

Influent and effluent samples from the various WWTPs were analyzed on the LC-MS/MS QQQ to determine the concentrations of the estrogens E1, E2, EE2, and E3 (Appendix A.4). It was found that the concentration of E2 detected in influent (9-28 ng/L) was significantly different from effluent concentrations (1-6 ng/L) at each plant (p < 0.001). The percent difference of the samples ranged from 62 to 92% across the different plants (Table 2.9).

Significant differences were also found in influent and effluent concentrations between the plants sampled (Figure 2.2). Based on the mean influent concentrations Plants B, C, I-b and H were significantly different from Plant E (p=<0.001, 0.010, <0.001, <0.001). While Plants D,

F, and G were significantly different from Plants B (p=0.001, 0.002, 0.020) and H (p=0.002, 0.003, 0.030). Plants A and I-a were not significantly different from any of the plants sampled. Plant B had the highest influent concentration of E2 at 28.02 ng/L while Plant E had the lowest concentration (8.71 ng/L).

Effluent concentrations of E2 at Plants A vs I-a, H vs I-b, B vs G, and C vs D were not statistically different from each other (p>0.05). E2 concentrations in the effluent from Plants A and I-a were statistically higher than the concentrations measured in effluent from Plants E (p<0.001). Concentrations measured at Plants B, G, H, and I-b were statistically different from Plant E (p=0.006, 0.012, <0.001, <0.001) but not different from Plants A (p=0.522, 0.365, 1.000, 1.000) and I-a (p=0.332, 0.211, 0.999, 0.995). Concentrations from Plants C and D were statistically different from concentration measured at Plants A (p=<0.001, 0.004) and I-a (p=<0.001, 0.002) but not from Plant E (p=0.995, 0.600). Plant F however was only statistically different from Plant A and I-a. Plant A had the highest concentration of E2 in the effluent (5.41 ng/L) while Plant E had the lowest concentration (1.22 ng/L). However, Plant C had the greatest percent difference between the influent and effluent samples (92%).

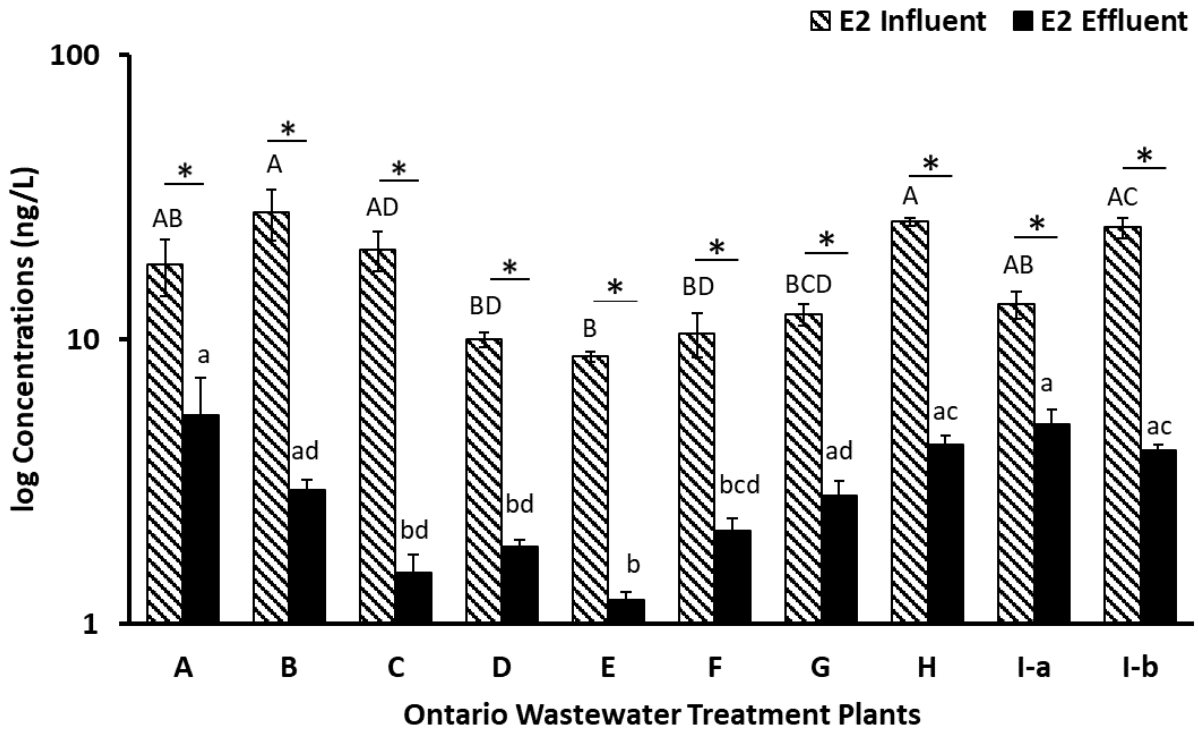


Figure 2. 2 Mean estradiol (E2) concentrations in influent and effluent from various wastewater treatment plants. Estradiol concentrations were measured in influent (striped) and effluent (solid) samples from various WWTPs. The log concentration along with standard error was plotted to show the difference between the samples from each site. Significant differences between influent and effluent concentrations were found at all plants sampled (*). Significant differences in influent concentrations between plants are indicated using upper case letters, while differences in effluent concentrations are shown using lower case letters.

E1 concentrations ranged from 42 to 104 ng/L in influent and 0.7 to 52 ng/L in effluent. This compound had the most varied percent difference across the different plants sampled, with -3% difference at Plant I-a to 98% difference at Plants C and D (Table 2.9). E1 concentrations were significantly different between influent and effluent samples at all plants ($p<0.001$) except plants F ($p=0.090$) and I-a ($p=0.961$) (Figure 2.3). Influent E1 concentrations at Plants A, C, H, and I-b were not statistically different from each other ($p>0.05$), however they were statistically higher than concentrations measured at Plant G ($p=0.002$, 0.014, <0.001, 0.001). Plants B, D, E, F, and I-b were not statistically different from each other or from any of the other plants ($p>0.05$). E1 concentrations in the effluent sampled varied significantly between the plants. Plants C, D, E, and G were not statistically different ($p=1.000$) with concentrations of E1 around 1 ng/L. However, the concentrations at these plants were statistically different from all other

plants sampled at which had concentrations above 5 ng/L ($p<0.05$). Plant I-a had a statistically different concentration of E1 in the effluent compared to all other plants ($p<0.05$) except for Plant A ($p=0.743$) and Plant F ($p=0.690$), which were also not statistically different from Plants B, H, and I-b ($p>0.200$).

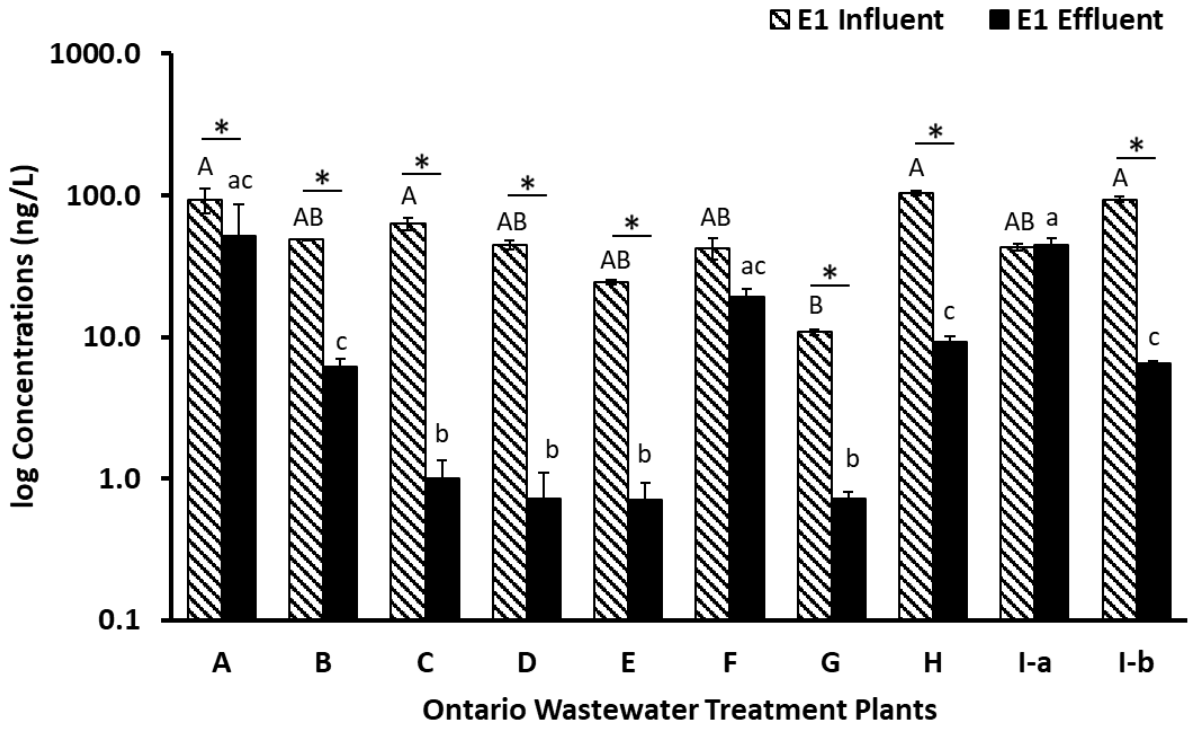


Figure 2. 3 Mean estrone (E1) concentrations in influent and effluent from various wastewater treatment plants. Estrone concentrations were measured in influent (striped) and effluent (solid) samples from various WWTPs. The log concentration along with standard error was plotted to show the difference between the sample from each site. Significant differences ($p<0.05$) between influent and effluent concentrations from the different WWTPs are indicated by *. Significant differences in influent concentrations between plants are indicated using upper case letters, while differences in effluent concentrations are shown using lower case letters.

As predicted E3 concentrations in influent (91 – 395 ng/L) and effluent (0 – 7 ng/L) (Figure 2.4) were significantly different at all plants sampled ($p<0.001$). This compound had the greatest and most significant percent difference between influent and effluent samples, with over 95% across all treatment plants sampled. Influent concentrations were found to be significantly different between plants. Plant H was significantly different from Plants D ($p<0.001$), E ($p=0.003$), and F ($p=0.018$). Plants B, C, and I-b were significantly different from Plant D ($p=0.003$, 0.031, 0.011), but not Plants E ($p= 0.068$, 0.353, 0.180) and F ($p=0.288$, 0.353,0.552).

Influent concentrations at Plants A, G, and I-a were not significantly different from any of the plants sampled ($p>0.05$).

Patterns of E3 effluent concentrations are much more variable and unpredictable. Plant I-a was statistically different from all other plants sampled, with no E3 measured in the effluent samples collected ($p<0.020$). Plants A and D had statistically different concentrations of E3 in the effluent compared to Plants F ($p<0.001$) and H ($p<0.001$). Concentrations found at Plants B and C are not significantly different ($p=1.000$).

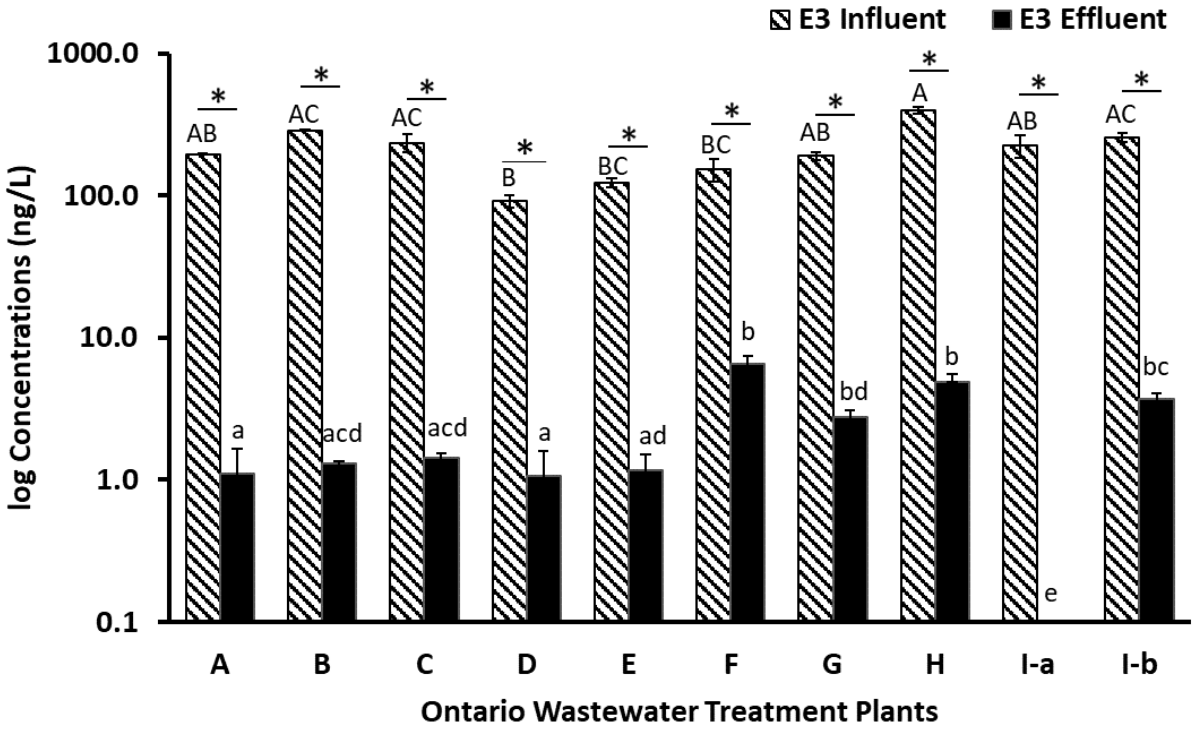


Figure 2. 4 Mean estriol (E3) concentrations in influent and effluent from various wastewater treatment plants. Estriol concentrations were measured in influent (striped) and effluent (solid) samples from various WWTPs. The log concentration along with standard error was plotted to show the difference between the sample from each site. Significant differences ($p<0.05$) between influent and effluent concentrations from the different WWTPs are indicated by *. Significant differences in influent concentrations between plants are indicated using upper case letters, while differences in effluent concentrations are shown using lower case letters.

For EE2 there was no significant difference between influent (0.3-7 ng/L) and effluent (0.7-5 ng/L) concentrations at plants A-E and I-a ($p > 0.05$), however significant differences were detected at plants F, G, H, and I-b ($p < 0.001$) (Figure 2.5). At plants G and H, the influent concentrations were below the detection limit of 0.5 ng/L, whereas at Plant I-b the influent

concentrations were significantly lower than effluent concentrations but still within the detection limit. At Plant F, the effluent concentration was significantly lower than influent concentrations.

Influent EE2 concentrations at Plants G, H and I-b were not significantly different from each other ($p>0.05$), however they are statistically different from all other plants sampled ($p<0.001$). EE2 concentrations from Plants C and F were not significantly different from each other but were significantly different from Plants A ($p=0.011, 0.015$), G ($p<0.001$), H ($p<0.001$), and I-b ($p<0.001$).

Effluent concentrations at Plants B and D were not significantly different from any of the other plants sampled($p>0.05$). Plants A, H, I-b were only significantly different from Plants C ($p=0.025, 0.023, 0.034$), E ($p=0.020, 0.018, 0.027$), F ($p<0.001$), which had lower concentrations of EE2. Plants C and E were not different from Plants G ($p=0.396, 0.346$) and I-a ($p=0.364, 0.317$), however Plant F ($p=0.005, 0.004$) was significantly different from those plants.

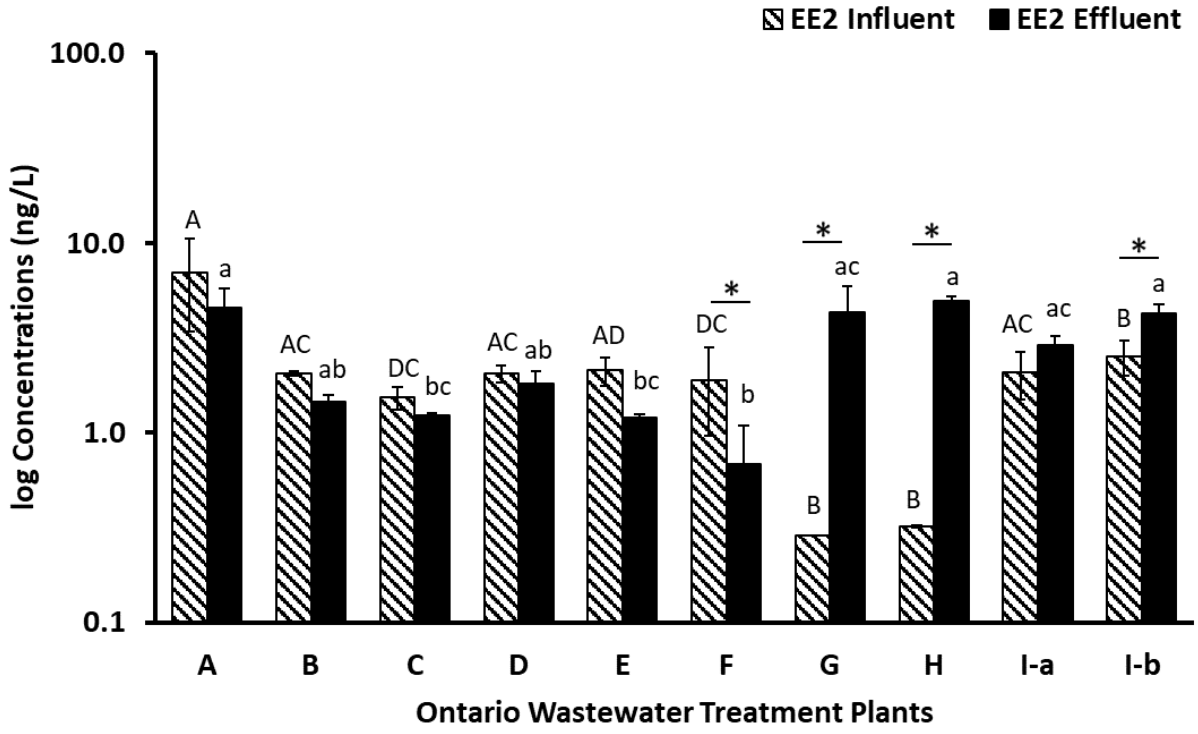


Figure 2. 5 Mean ethinyl estradiol (EE2) concentrations in influent and effluent from various wastewater treatment plants. Ethinyl estradiol concentrations were measured in influent (striped) and effluent (solid) samples from various WWTPs. The log concentration along with standard error was plotted to show the difference between the sample from each site. Significant differences ($p<0.05$) between influent and effluent concentrations from the different WWTPs are indicated by *. Significant differences in influent concentrations between plants are indicated using upper case letters, while differences in effluent concentrations are shown using lower case letters.

Percent reductions ranged from 19-100% post treatment at all WWTPs, expect Plant I-a which had a 3% increase in E1 concentrations and an 81% increase in EE2, and a 88% increase in 2018 (Table 2.5). E3 had the highest and most consistent percent reduction across all treatment plants, however the reduction at plant F was significantly different (One-way ANOVA, $p<0.05$) to the other plants analyzed. EE2 had the lowest reduction with no significant differences between plants. EE2 was below the detection limit in the influent from Plants G and H, therefore an accurate percent reduction could not be determined. E2 and E1 had the most variable reductions in concentrations between plants. Although reductions were variable at most plants, Plant C had consistently high reductions of all the natural estrogens, while one of the lowest reductions of EE2.

Table 2.9 Percent reduction of estrogen concentration at various wastewater treatment plants. Percent reduction was calculated using the measured estrogen concentrations in influent and effluent samples from the various WWTPs. Statistical differences in reduction between the plants for each estrogen measured is shown using superscripts ($p<0.05$).

WWTP	Percent difference (%)			
	E2	E1	E3	EE2
A	68 [ab]	51 [ab]	99	18
B	89 [a]	87 [ab]	100	29
C	92 [a]	98 [a]	99	15
D	81 [ab]	98 [a]	99	7
E	86 [ab]	97 [ab]	99	39
F	78 [ab]	52 [ab]	95 [a]	37
G	76 [ab]	93 [ab]	99	-
H	84 [ab]	91 [ab]	99	-
I-a	62 [b]	-3 [b]	100	-81
I-b	83 [ab]	93 [ab]	99	-88

The total estrogenicity (E2 equivalency) was calculated for each sample using the individual estrogen concentrations and the E2 equivalency factor proposed by Arlos et al. (2018) for each estrogen. The percent composition of the total calculated estrogen concentration and total calculated estrogenicity for influent and effluent are shown in Figure 2.6 and Figure 2.7 respectively.

The total estrogen concentration in influent samples ranged from 200 to 500 ng/L (Table 2.10). A consistent trend in the composition of the influent was found across all treatment plants, as shown in Figure 2.6(A). The total estrogen concentration in influent samples was composed of over 50% E3, followed by E1, E2, and less than 5% EE2. The calculated influent estrogenicity ranged from 20-70 ng/L E2eq (Table 2.10). Although E3 was a major contributor to influent estrogen concentration, it has less than a 30% contribution to the total estrogenicity of the influent across all treatment plants. The major drivers of the influent estrogenicity are E1 and E2.

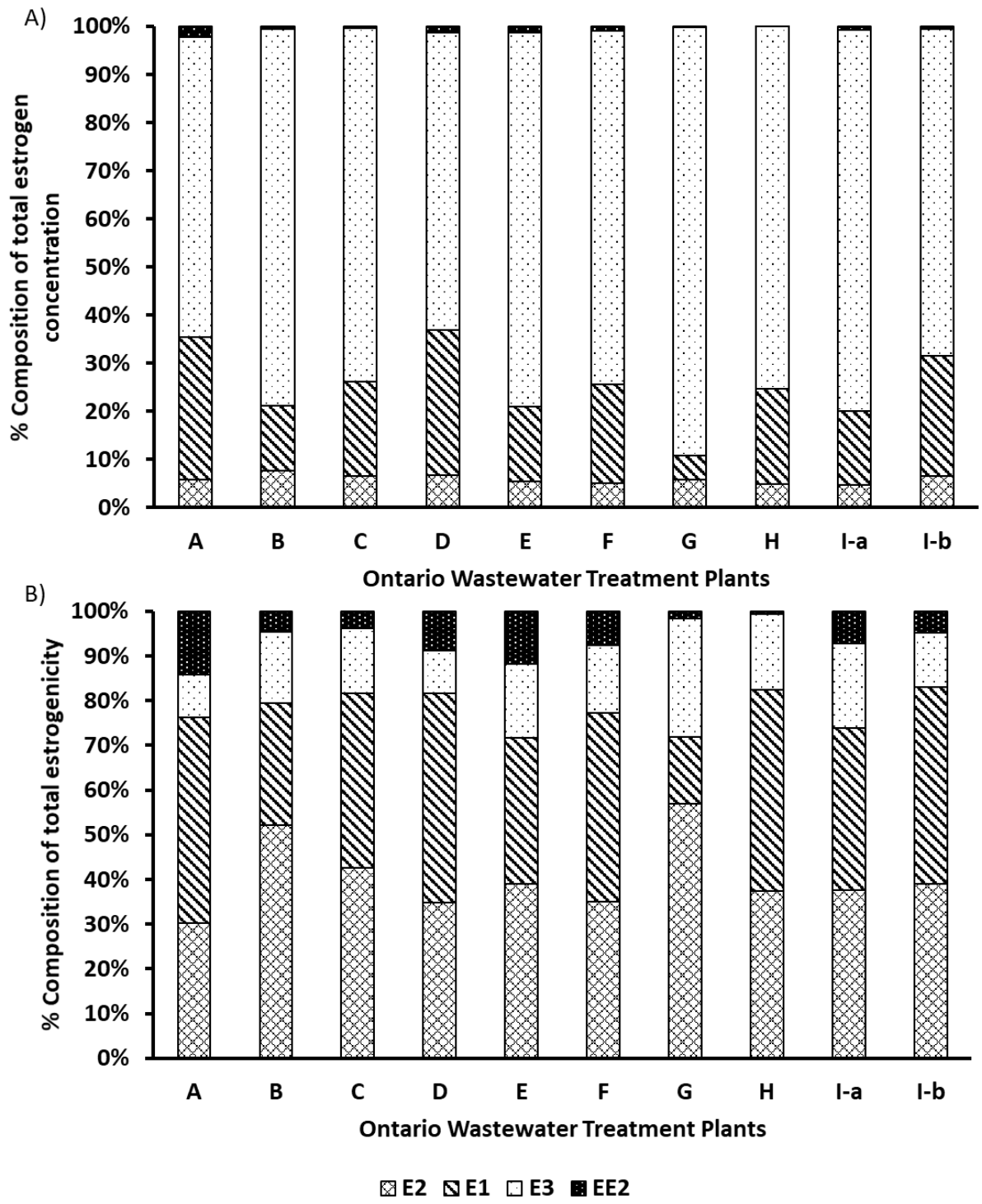


Figure 2. 6 Percent composition of total estrogen concentration (A) and total estrogenicity(B) in influent samples from various Ontario wastewater treatment. The concentration of E1, E2, E3, and EE2 were measured in influent samples and summed to determine the total estrogen concentration. The percent of individual estrogen in the total concentration was plotted to demonstrate the change in estrogen concentration ratio before treatment.

Table 2. 10 Total estrogen concentration and total estrogenicity of influent samples

WWTP	Total estrogen concentration (ng/L)	Calculated Total Estrogenicity (ng/L E2eq)
A	313.58	60.7
B	364.64	53.8
C	320.20	48.5
D	148.49	28.7
E	158.19	22.3
F	206.39	30.1
G	214.11	21.6
H	524.74	69.3
I-a	281.87	35.5
I-b	376.55	63.7

The total estrogen concentration of effluent samples ranged from 4 to 63 ng/L (Table 2.11). Unlike the influent samples, the composition of the effluent was not uniform across the different treatment plants (Figure 2.7 (A)). E3 accounted for less than 30% of the total concentration despite being the largest component of influent, therefore the drop in total concentration can be attributed to the efficient reduction of E3. At plants A, B, F, H and I E1 was the biggest contributor, while E2 was the largest contributor of concentration at plants C, D, and E. At plant G, EE2 was the largest contributor of concentration. The total calculated estrogenicity of these effluent samples ranged from 2 to 27 ng/L E2eq (Table 2.11). Similar to the influent samples, E3 was the smallest contributor to estrogenicity, accounting for less than 5% of the total estrogenicity of the effluent samples across all the plants sampled. E1 accounted for a small portion of the total estrogenicity at plants C, D, E, G, H, and I-b. Plants A, B, F, and I-a had greater contributions from E1, which correlates with the higher proportion of E1 in the effluent samples at these sites and the higher total estrogen concentrations. E2 and EE2 are the largest contributors to the total estrogenicity of the effluent from plants B, C, D, E, G, H, and I-b, whereas the estrogenicity of plants A, F, and I-a are dominated by E1 (Figure 2.7 (B)).

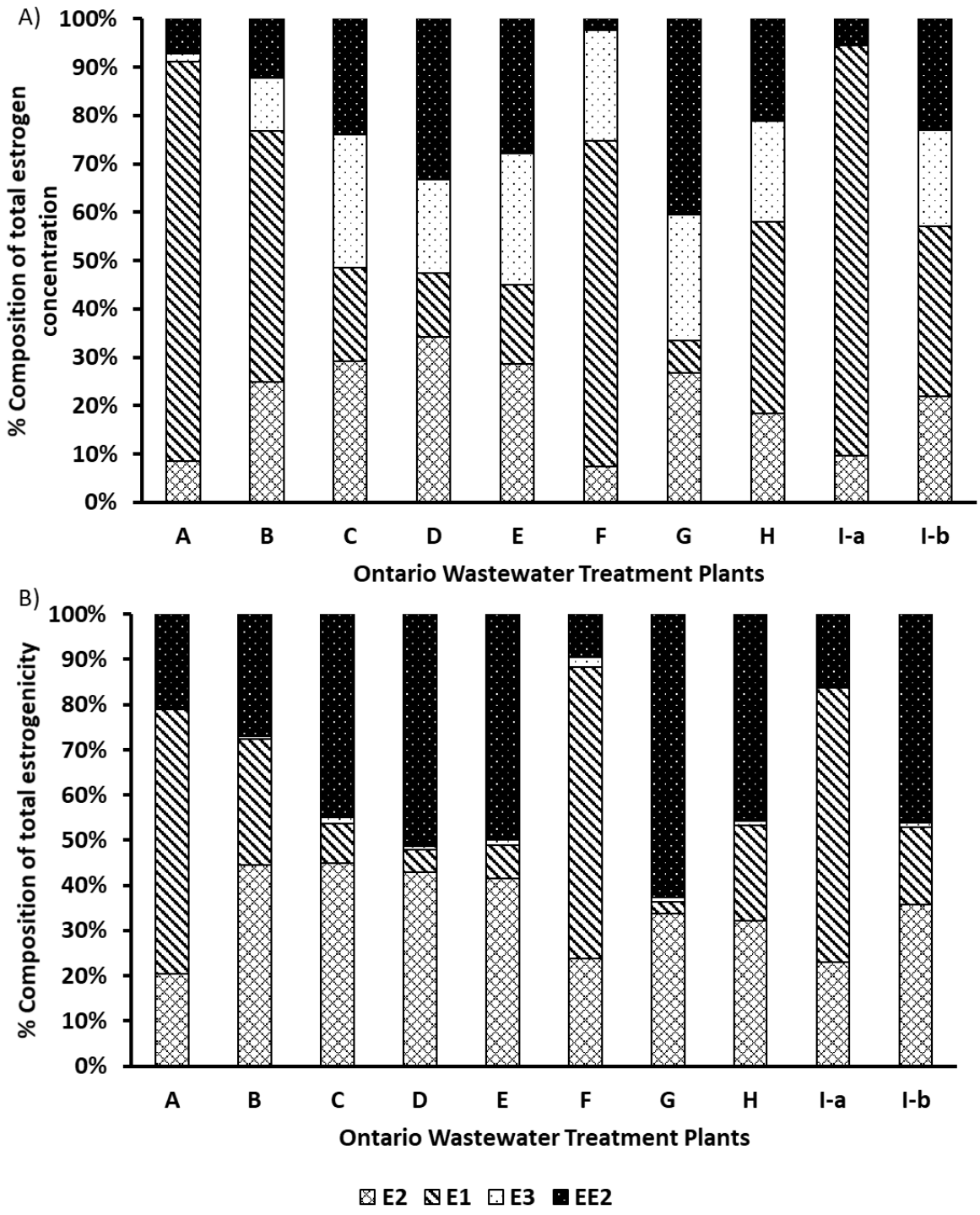


Figure 2. 7 Percent composition of total estrogen concentration (A) and total estrogenicity(B) in effluent samples from various Ontario wastewater treatment plants. The concentration of E1, E2, E3, and EE2 were measured in effluent samples and summed to determine the total estrogen concentration. The percent of individual estrogen in the total concentration was plotted to demonstrate the change in estrogen concentration ratio after treatment.

Table 2. 11 Total estrogen concentration and total estrogenicity of effluent samples

WWTP	Total estrogen concentration (ng/L)	Calculated Total Estrogenicity (ng/L E2eq)
A	62.95	26.60
B	11.89	6.65
C	5.18	3.38
D	5.47	4.36
E	4.29	2.94
F	28.37	8.88
G	10.60	8.41
H	23.30	13.26
I-a	52.28	21.89
I-b	18.59	11.41

v2.2.4 Estrogenicity

The total estrogenicity of the various WWTP samples was measured using a Yeast Estrogen Screen assay (YES). Initial estrogenicity in the influent ranged between 5-14 ng/L E2eq with no significant difference between plants ($p>0.05$). After treatment, estrogenicity was significantly ($p<0.05$) reduced to levels between 0.25-12 ng/L E2eq (Figure 2.5). However, the total estrogenicity of the influent was not significantly different from that of the effluent at plants A, F, and I-a (Two-way ANOVA, $p<0.05$). The reduction of estrogenicity at these plants was less than 20% while the other plants were achieving reductions greater than 80%, except Plant B which only had a 55% decrease (Appendix 4). Similar to the results in pharmaceuticals and estrogen concentration reduction, Plant C had the highest reduction of total estrogenicity (93%). Sampling of Plant I over two years shows a drastic difference. In 2017 there was a 23% increase in estrogenicity while 2018 had a 77% decrease.

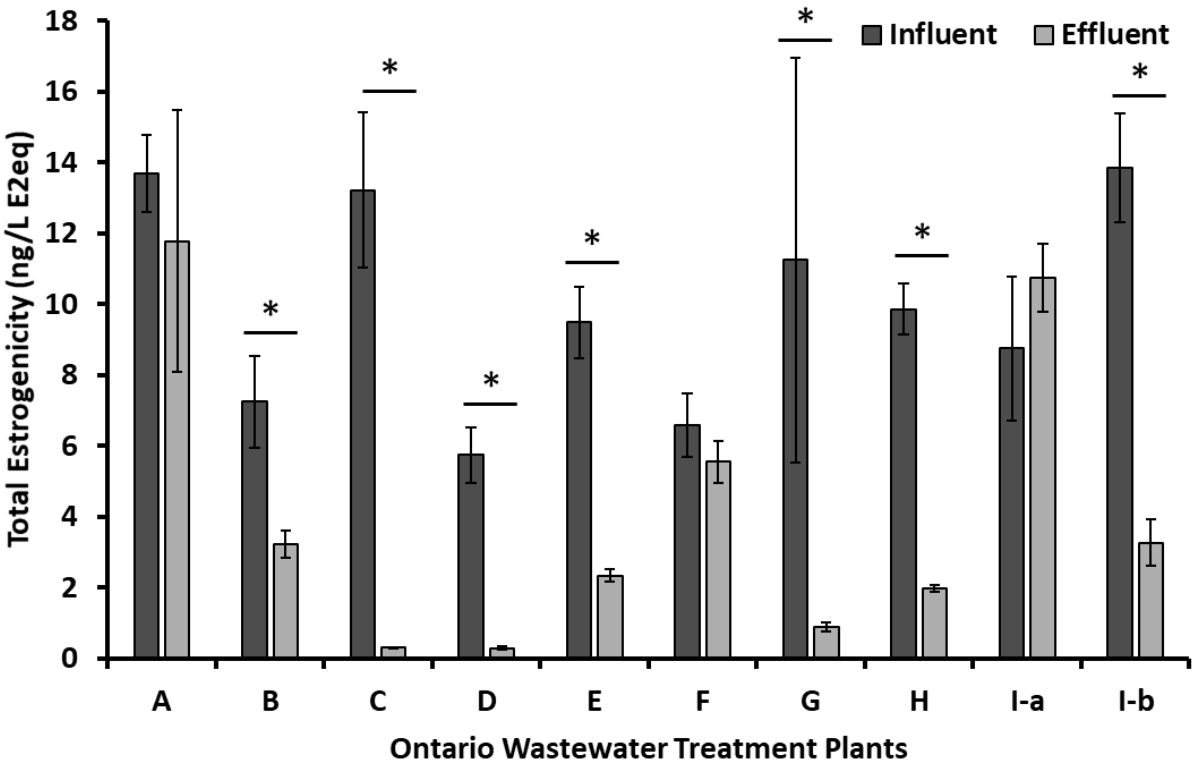


Figure 2. 8 Mean total estrogenicity (based on YES) of influent and effluent from various wastewater treatment plants. Total estrogenicity of the influent and effluent samples was measured using the YES assay and expressed as estrogenicity relative to E2. This data is plotted with standard error to show the difference in estrogenicity before and after treatment. Significant differences in estrogenicity of influent and effluent from the different WWTPs are indicated with *.

Compared to the calculated total estrogenicity of the influent and effluent samples (Table 2.10, Table 2.11), the measured total estrogenicity was significantly lower (p= <0.001, 0.001). The total estrogenicity determined using both techniques were plotted to determine if there was a positive correlation between the methods (Figure 2.9). Total estrogenicity of influent samples had poor correlation (R=0.49) with chemical measurements while effluent samples had a higher positive correlation (R=0.90).

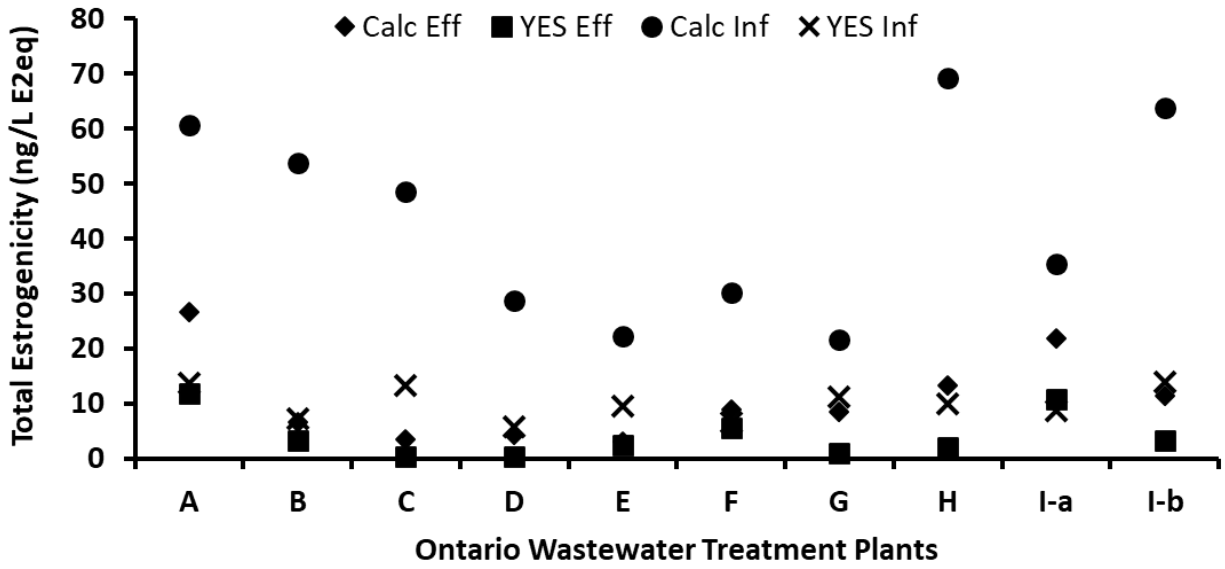


Figure 2. 9 Calculated and measured total estrogenicity from various Ontario wastewater treatment plants. Calculated total estrogenicity was determined using the concentrations and E2 EEQ factor of E1, E2, E3, and EE2. Measured total estrogenicity was determined by running the YES assay on whole sample extracts.

2.3 Discussion

Although all treatment plants sampled (A, B, E, F, G, H, I) operated using conventional activated sludge they varied greatly in size and operation (Table 2.1). Plants C and D were very small plants operated as an aerated lagoon (510 m^3/s) and oxidation ditch (1,040 m^3/s), respectively. All the plants would be classified as secondary treatment, which meets Canadian regulatory requirements. Conventional activated sludge (CAS) systems are biological processes that aerate the wastewater to reduce organic matter and produce flocculate sludge. This flocculate is a heterogenous mix of microorganisms which changes continuously in response to the composition of the wastewater and environmental changes (Ramalho 1983). This sludge is recycled and added to new wastewater influent to promote increased biological degradation of contaminants. Extended aeration (Plant I), and oxidation ditch (Plant D) are modifications of CAS systems, and typically have longer residence times and reduced the need for sludge handling, which is better suited for smaller scale plants (Ramalho 1983). Aerated lagoons (Plant C) also have longer residence times, however are not CAS systems as they are flow through systems with no recycling of sludge.

All the plants sampled were below the regulations for effluent total suspended solids (TSS) of 25 mg/L. Regulations also require effluent to have less than 25 mg/L of carbonaceous biochemical oxygen demand (CBOD), which is a subset of biological oxygen demand (BOD). Effluent from all plants were measuring below 9 mg/L BOD and therefore met the requirements outlined. Of the nine treatment plants sampled, only four plants (Plants A, B, C, D) were required to nitrify according to their COA. Of these plants, Plants A and B were above the CCME guidelines for un-ionized ammonia (0.019 mg/L). Despite being required to have nitrification by its C of A, Plant A only had 89% removal of ammonia. Plant F only had 53% removal of ammonia and consequently had the highest concentration of un-ionized ammonia. Therefore, this plant is expected to have the poorest effluent quality. The new Wastewater Systems Effluent Regulations state that effluent discharged from WWTPs must not exceed 1.25 mg/L of un-ionized ammonia at 15°C ± 1°C when they come into force in 2021. The removal of ammonia is a critical parameter for wastewater treatment as it has been associated with acute toxicity. In addition, it has been shown although there is not direct causal link established there appears to a relationship between nitrification and the removal of key pharmaceuticals and estrogen (Achermann et al. 2018).

Removal of pharmaceuticals by various WWTPs has been widely studied in recent years. Due to the wide range of structures and chemical properties of pharmaceuticals, removal and degradation of these compounds varies greatly among WWTPs (Clara et al. 2004). In a similar way, the concentration and apparent removal of different pharmaceuticals varied considerably among the plants included in the present study in Ontario.

Some select pharmaceuticals (e.g. atrazine (ATRZ), carbamazepine (CBZ)) were not detected in influent samples but were quantifiable in effluent or had increased concentrations in effluent compared to the corresponding influent samples. This has been reported in other studies and can be a result of several factors (Clara et al. 2004; Behera et al. 2011). Some compounds are excreted in their inactive conjugate forms which can become deconjugated by microorganisms during the treatment processes (Tran et al. 2018). The method used for this study only extracted unconjugated compounds, therefore influent concentrations could be underestimated since conjugated compounds are missed. The deconjugation of compounds that are not easily removed can increase effluent concentrations above influent levels. Another possibility is that compounds can be absorbed or entrapped in particulate matter as the samples

are filter through a 1 µm filter prior to extraction. Although these compounds will not be detected in the influent, throughout the treatment process the compounds can be released back into the aqueous phase and therefore detected in the final effluent. The detection of compounds in effluent and not influent could also be a result of the matrix and detection limits. Influent is a dirtier sample, making detection of compounds more difficult compared to in effluent samples which should be cleaner.

Several representative compounds were selected for further discussion (e.g. CBZ, VEN, IBU, NPX) due to their specific characteristics. CBZ and VEN have been characterized as persistent pharmaceutical that are difficult to remove using traditional wastewater treatment processes, suggesting that influent and effluent concentrations of these compounds should remain fairly consistent (Tran & Gin 2017). Therefore, these compounds can be used to account for environmental changes such as increased flow due to rainfall (e.g. dilution). IBU and NPX are compounds that are easily removed or degraded, therefore the presence of these compounds in final effluent can be used as an indicator of poor treatment at the WWTPs.

Concentrations of CBZ in influent and final effluent were not significantly different at six of the ten WWTPs sampled, with an increase in concentration in the final effluent at all plants except C which had a 67% reduction. This minimal change in concentration was expected due to its well-known persistence of CBZ during biological degradation and low octanol/water partition coefficient (K_{ow} <4.00) (Tran & Gin 2017). There was a was a statistically significant increase of CBZ at Plants F and I-(a/b). An increase in pharmaceutical concentration between influent and effluent has previously been documented at wastewater treatment plants and can be a result of the factors mentioned above. Plant C was the only WWTP to see a significant reduction in CBZ concentration post treatment. VEN is another compound characterized as being persistent throughout biological treatment processes, with reductions ranging from -5 to 98%. Similar to CBZ, it was also found to have significantly different concentrations in influent and effluent at Plant C (98% reduction) and in Plant G (38% reduction). Plant C is the only treatment plant that used an aerated lagoon. Aerated lagoons, with extended solids retention times (SRT) have been previously demonstrated to be effective removal of pharmaceuticals (Metcalfe et al. 2003; Li et al. 2013).

As expected, a significant difference in IBU was measured between influent and effluent samples at all WWTPs with no difference in effluent concentrations among any of the plants.

IBU has a low sorption coefficient and is easily degraded when nitrification is applied (Joss et al. 2004). This matches the nutrient data collected from these samples, which shows that Plants A-E and G-I were achieving over 80% total nitrogen removal. Plant F had the lowest IBU difference between influent and effluent which corresponds to its low nitrogen removal. NPX concentrations in influent and effluent were significantly different at all WWTPs sampled. NPX is a compound that is easily removed due to its molecular structure, therefore it is not surprising that plants with secondary treatment are able to significantly reduce the concentration. There was a slight difference in IBU (98% to 100%) and NPX (99% to 100%) concentrations in Plant I sampled at different times (2017, 2018). This correlates with an improvement seen at the plant in terms of improved TKN removal, increasing from 89% to 97%, and supports the relationship between increased nitrification and increased pharmaceutical degradation (Joss et al. 2004).

All WWTPs were sampled during dry weather and what was reported as normal operation. The stable nature of CBZ and the similar concentration in influent and effluent suggests that there were no major external factors influencing the wastewater (dilution, etc.). Other than Plant F which had low ammonia removal and correspondingly lower removal of the pharmaceuticals measured, indicating poor treatment, the high degradation of IBU and NPX at all other plants sampled indicate that the treatment plants are operating efficiently. This was further supported by the effective removal of other endpoints such as TSS and COD/BOD.

In general, the influent concentrations of estrogens greatly depend on the treatment plant and the type of influent the treatment plants were receiving. Influent concentrations of E1, E2, and E3 at Plants D, E, F, and G were similar (not significantly different) while Plants A, B, C, H, and I were also similar to each other. Concentrations of EE2 entering these plants however was very unpredictable. Despite all the treatment plants sampled operating with secondary treatment, there was significant variability in the estrogen concentrations in final effluent and percent reduction (difference) among the nine treatment plants.

Several recent studies have looked at the concentrations of estrogens in influent and effluent samples from various types of WWTPs as summarized in Table 2.12. The values of E1, E2, and EE2 found in influent and effluent samples fall in the current study in Ontario fall within range of other studies that have been reported. However, very high concentrations of E3 were found in influent samples compared to other studies. Discrepancies between the range of influent and effluent concentrations measured can be attributed to sampling location or sampling

methods. Baronti et al. (2000) and Johnson et al. (2000) measured concentrations in European WWTPs which have different collection and treatment plant configurations (although similar processes) as well as different regulations on effluent discharge compared to Canadian WWTPs. Sampling method can also impact the concentrations of estrogens measured. Grab samples give an immediate snap shot of what is in the sample at that specific time point with the samples being preserved immediately. However, when collecting composite samples, collection is done over a 24 h period and samples are not preserved until after the sample was collected. This could lead to degradation and biotransformation during the sample collection period.

Table 2. 12 Range of estrogen concentrations in influent and effluent samples from previous studies

		Estrogen Concentration Range (ng/L)			
Source	**Sample Type**	**E1**	**E2**	**E3**	**EE2**
Servos et al. (2005)	Influent	19-78	2.4-26	-	-
	Effluent	1-96	0.2-14.7	-	-
Baronti et al. (2000)	Influent	25-132	4-25	24-188	0.4-13
	Effluent	3.3-82.1	0.4-3.3	0.43-18	0 – 1.1
Johnson et al. (2000)	Influent	<0.5 – 140	<0.5 – 48	2-120	<0.2 – 10
	Effluent	<0.5 - 54	<0.5 - 12	<0.5-28	<0.2 – 4.5
Fernandez et al. (2007)	Influent	0-33	0-11	0-22	0-2
	Effluent	8-56	0-158	0-29	0-178
This Study	Influent	10-100	8-28	90-400	0.3-7
	Effluent	0.7-52	1-6	0-7	0.7-5

As expected, there was a significant difference in E2 and E3 concentrations between influent and effluent at all WWTPs sampled. These plants all utilize aerobic processes, which promotes the oxidation of E2 to E1 (Ternes et al. 1999), resulting in the reduction of E2 but an increase in E1 concentrations. This could explain why no significant difference in E1 concentration was measured at Plants F and I-a. In addition to the increased concentration as a result of biotransformation, conjugated E1 compounds not detected in the influent sample could have become deconjugated during the treatment process, offsetting the reduction of the initial concentration measured in the influent sample. E1 concentrations in influent and effluent from

Plant A were significantly different but were not different from concentrations found at Plants F and I-a. Although the influent and effluent concentrations of E2 were significantly different at each plant, Plants A, F, and I-a had the lowest percent difference in concentration. These are also the plants with the highest residual ammonia and TKN in the final effluent (suggesting poor treatment).

E1 and E2 have increased removal when there is good nitrification being applied to the influent. Estrogen removal does seem to be related to activity of ammonia oxidizing bacterium (AOB) (Ren et al. 2007). However, other recent studies have found that there is no direct link to nitrifying activity (Achermann et al. 2018). Out of all the plants sampled Plants A, F, and I-a had less than 90% removal of TKN, indicating poorer nitrification. These plants also had slightly lower percent differences in IBU and NPX between influent and effluent. The lower level of nitrogen removal can be attributed to a lower activity of AOB in the activated sludge. Nitrifying conditions apparently also promote the activity of bacteria/conditions that may be responsible for the removal of estrogen. This may explain why the E1 concentrations in the final effluent of the poorly operating plants were not different from the final effluent and there was also a lower percent apparent removal of E2 concentration in these plants. E1 is further converted to E3 which is the least hydrophobic of the four estrogens measured, making it the more accessible for degradation. There was over a 95% difference between E3 concentrations in influent and effluent at all plants sampled, which is the highest and most consistent difference among the different estrogens. Unlike the other estrogens, the main degradation pathway for E3 is via direct use as an electron donor for heterotrophs which are readily found in CAS systems (Ren et al. 2007).

EE2 had the lowest apparent removal (i.e. percent difference; <40%) between influent and effluent samples across all the plants. Significant differences were found at plants G, H, and I-b, however these differences were a result of increased effluent concentrations. Plant F had the only significant decrease in EE2 concentrations, however this plant had the poorest treatment of pharmaceuticals and nutrients. EE2 is more metabolically stable than the naturally produced estrogens (Shi et al. 2004; Ren et al. 2007). Ternes et al. (1999) had found that there was no significant reduction in EE2 concentration in activated sludge batch reactions and that despite the higher K_{ow}, sorption did not play a big role in their experiments. Batch experiments with nitrifying activated sludge have shown that the enzyme ammonium monooxygenase produced by AOB (or related bacteria) may co-metabolize EE2 resulting in the hydroxylation of the

compound (Shi et al. 2004; Vader et al. 2000). This produces a hydrophilic degradation product that has been shown to be non-estrogenic (Vader et al. 2000). These experiments however were conducted with initial concentrations of EE2 that are above concentrations measured in influent samples and are performed for individual compounds. Vader et al. (2000) showed a 50% decrease in EE2 (initial concentration of 50 µg/L) after 25 hours, while Shi et al. (2004) showed a 50% decrease from a concentration of 1 mg/L after approximately 20 hours. EE2 was found at concentrations around 10 ng/L in influent and is the smallest contributor to the total estrogens. Conditions that promote AOB (and related bacteria) will lead to the removal of ammonia, and possibly other estrogens, or the various other pharmaceuticals and hormones found in wastewater. Therefore, the degradation of EE2 may be enhanced by conditions that promote AOB (even if they are not directly responsible) and longer SRTs.

Although EE2 is poorly treated in the CAS systems studied in this study in Ontario, EE2 is a synthetic hormone and is not produced as a by product during the treatment process so the increased effluent concentrations at Plants G, H, I-a, and I-b may be a result of deconjugation and/or incomplete extraction techniques. This study was designed to measure the concentration of free estrogens, however it is possible that most of the EE2 in the influent at these plants was in the conjugated form and not accounted for during the extraction, which under estimates the concentrations. The conjugated EE2 could have become deconjugated throughout the treatment process and then detected in the final effluent. Another possibility is that as the extraction of estrogens used the same method for both influent and effluent samples, much dirtier influent samples could cause overloading of the cartridges. This can lead to the breakthrough of estrogens in the sample (differently from the surrogate spikes) through the cartridges resulting in the underestimation of the influent concentration.

Characterizing the change in composition of estrogens after treatment provided some indication of which estrogens were more persistent than others. The ratio of estrogens in the raw influent didn't vary between treatment plants which was surprising considering the major sources of organic load varied between the WWTPs. Natural estrogens are excreted in their conjugated forms by men and women, with excretion rates ranging from 1.8-550 µg/d E1, 1-395 µg/d of E2, and a maximum of 64 µg/d of E3 (Johnson & Williams 2004; Ternes et al. 1999). Birth control pills are the major contributors to EE2 in influent and are excreted at an average rate of 11.3 µg/d (Johnson & Williams 2004). Therefore, the expected ratio of estrogens is E1>E2>E3>EE2 in raw

influent. Although the major estrogens excreted are E1 and E2, E3 has the greatest presence in raw influent as a result of the biotransformation of E1 and E2 into E3 (Ternes et al. 1999). Taking biotransformation into consideration, the excretion rates are consistent with the ratios of estrogens found in the raw influent.

Comparing the ratio of estrogens in the influent to the effluent, there is a shift in the estrogen composition that varies among plants. The removal efficiencies for E3, E2, E1, and EE2 decrease in that order (Ben et al. 2017; Johnson & Sumpter 2001). Based on the removal efficiencies it would be expected that EE2 and E1 would be the biggest contributors to the total estrogen concentration (E1, E2, E3, EE2), however this is dependent on the level of treatment applied at the various plants. Fernandez et al. (2007) found E1>E2>E3 to have the greatest concentration of estrogen in final effluent with EE2 concentration ranging widely between plants. This is similar to the results found in the present study.

Aligning with the removal efficiencies and the degradation pathway of E3, it contributes the least to the total estrogen concentration in the final effluent and has an even smaller contribution to the total estrogenicity of the sample. Effluents from Plants A, F, and I-a were mainly composed of E1, with E1 being the major contributor to total estrogenicity. As discussed earlier, these plants had lower levels of nitrification, which could result in inefficient removal of E1. Plants B, H, and I-b had similar effluent compositions. These plants had higher levels of calculated total estrogenicity, which is proportional to the higher concentrations of total estrogens in the effluent. Plants C, D, E, and G had similar effluent compositions. A larger proportion of the effluent was composed of E2 and EE2 which were also the largest contributors to total estrogenicity as expected, as E2 and EE2 are the more potent estrogens. These four plants range in treatment processes from lagoons, oxidation ditches to conventional activated sludge.

Based on the concentrations and EEQ of E1, E2, E3, and EE2, the calculated total estrogenicity of influent samples ranged from 20 to 70 ng/L E2eq. However, the total estrogenicity measured using the YES assay had significantly lower results, ranging from 5 to 14 ng/L ($p<0.001$). This was not expected since the YES assay is performed using whole sample extracts and contains additional weakly estrogenic compounds that are not accounted for in the calculated total estrogenicity (Yu & Chu 2009). Unlike Fernandez et al. (2007) who showed a positive trend between calculated and measured total estrogenicity of the samples, the calculated

total estrogenicity did not follow a similar trend to the measured estrogenicity and had poor correlation (R=0.48).

The calculated total estrogenicity of the final effluent ranged from 2 to 27 ng/L E2eq, whereas the measured estrogenicity using the YES assay ranged from 0.2 to 12 ng/L E2eq. These two sets of values had a positive correlation (R=0.90) and were more similar than influent samples. These effluent samples also had significantly lower measured total estrogenicity than what was predicted from the estrogen concentrations ($p = 0.001$). The discrepancies between the calculated and measured total estrogenicity of the samples can be attributed to several factors. Firstly, the YES assay is conducted with whole sample extracts. Wastewater samples, especially influent, are very dirty and contain a variety of compounds such as anti-estrogens (Beresford et al. 2000) such as BPA (Fernandez et al. 2007) which could inhibit the estrogen receptors in the assay. There are also compounds found in both influent and effluent which can be toxic to yeast from various pathways, for example triclosan (Yu & Chu 2009), which can prevent the growth and result in a lower response on the YES assay. On the other hand, the calculated total estrogenicity is only based on the concentrations of the four extracted compounds, so it doesn't account for the interaction of other components of wastewater. Since influent samples are dirtier and more loaded with contaminants than effluent samples the inhibitory affects could be amplified in the influent YES assay. Although a serial dilution is used to dilute the influent before exposure to the cells, the toxicity of the sample could lead to inhibitions in cell growth. Although cell density is accounted for when calculating the response, a low cell density would not give accurate results and therefore underestimate the total toxicity since viable cells are needed for this assay. Calculated and measured effluent total estrogenicity values were much closer because the effluent is treated, and it is expected to be cleaner than influent (possibly removing some of the interfering compounds). The YES assay also indicated a significant decrease in total estrogenicity at all WWTPs except Plants A, F, and I-a, which was expected as these were the plants that had low percent apparent removal (differences between influent and effluent concentrations) as well as poorer ammonia and %TKN removal.

In conclusion, the results from the analysis of influent and effluent from nine secondary WWTPs in southern Ontario show that certain pharmaceuticals can be used as indicators of treatment and changes to the treatment process. Recalcitrant compounds such as CBZ and VEN can be used to assess seasonal flow changes while highly treatable compounds such as IBU and

NPX can be indicators of efficient treatment processes. Analysis of estrogens in influent and effluent supported the importance of efficient nitrification and longer SRTs in the reduction of these compounds. As expected E2 and E3 had significant differences (i.e. reductions) between influent and effluent. E1 varied between plants and was more dependent on treatment processes, while EE2 had very little difference across the treatment plants. Overall the total estrogenicity of the influent was decreased significantly when there was over 90% removal of TKN. Although the total estrogenicity is reduced, the final effluent discharged into surface waters has a larger proportion of EE2, the more persistent and potent estrogen.

Despite these secondary treatment plants meeting the current regulations (with the exception of Plants A, B, F, and I-a for unionized ammonia), there was not complete removal of environmental contaminants of concern. Meeting these standards does have a positive impact on the treatment of most of these compounds and reduces the total estrogenicity of the final effluent. However, low but detectable concentrations of estrogens and estrogenicity remain especially for the more recalcitrant EE2. Plants with poorer treatment processes, as indicated by the lower removal of nitrogen and more treatable pharmaceuticals, also do not readily remove all the estrogens or total estrogenicity. It demonstrates the importance of continuously aiming to achieve improved treatment standards to ensure the reduction of a contaminants to minimize impacts on the surrounding environment.

Chapter 3 – Tracking effluent quality changes associated with upgrades at two Canadian WWTPs (Kitchener and Waterloo)

Emerging contaminants are a big concern as these compounds are always changing with time and are typically not targeted by traditional wastewater treatment plants (WWTPs). Compounds such as pharmaceuticals and personal care products as well as endocrine disrupting compounds have been detected in effluent discharged from WWTPs and have been linked to adverse impacts on fish downstream of these discharge sites (Gunatilake et al. 2016; Fuzzen et al. 2015; Vega-Morales et al. 2013; Kidd et al. 2007). Improving treatment plants for the removal of these compounds across Canada could cost billions of dollars. A better understanding of the behaviour of these compounds in wastewater treatment plants is therefore needed.

High concentrations of pharmaceuticals and estrogenic hormones have been detected in effluent discharged from the two largest WWTPs in the Grand River watershed (Kitchener and Waterloo). Both plants have undergone major upgrades to improve the quality of effluent discharged, specifically targeting improving nitrification to decrease ammonia concentrations. Upgrades have continued at the Kitchener plant since 2012, and the Waterloo WWTP began major upgrades in 2017. Studies on fish in the Grand River have shown that the upgrades at the Kitchener WWTP in 2012 have led to a reduction in intersex (Hicks et al. 2017) and steroid responses (Marjan et al. 2018). The upgrades have resulted in a decline in total estrogenicity of the effluent discharged since 2012 (based on YES). Unfortunately, there was no data for individual estrogen concentrations as a reliable method was not available, however a limited number of samples were archived with surrogate spikes. This presented the unique opportunity to document the changes at the two WWTPs over time. Using achieved samples (since 2010) and new collections (2017 and 2018) specific estrogens (EE2, E2, E1) are determined in the final effluents of Kitchener and Waterloo. This is compared to measurement of effluent quality, pharmaceuticals and total estrogenicity overtime.

3.1 Method

Grab samples were collected in 1 L amber glass bottles just before the outfall at the Waterloo WWTP and before UV disinfection at the Kitchener WWTP. These samples were preserved, extracted, and analyzed for hormones, total estrogenicity, and pharmaceuticals as outlined in Chapter 2.1. In addition to these analyses, the final effluent was collected to

determine nutrient levels. Although these samples were not collected as part of a planned monitoring program they were collected and analysed under the same or very similar protocols over time. The timing of the samples is therefore not regular as they were collected for other purposes but were all spiked with surrogate standards before being stored at -20°C.

3.1.1 Nutrients

Nutrient analysis of the final effluent at the Kitchener and Waterloo WWTPs were done by Maxxam Labs (Waterloo, Ontario). Grab effluent samples were collected in 250 mL in high density polyethylene (HDPE) bottles with 1 mL of 49% sulfuric acid as a preservative for the analysis of ammonia, nitrate, and nitrite. Grab effluent samples were collected in 250 mL HDPE bottles with no preservatives for the analysis of dissolved chloride and conductivity. Effluent nutrient quality parameters were measured by a third party, Maxxam Analytics using the approved methods listed in Table 3.1.

Table 3. 1 Effluent nutrient analysis methods

Analyte	Method	Method Description	Reportable Detection Limit (RDL
Nitrate (NO3)	SM 4500 NO3-I	Colourimetry	0.50 mg/L
Nitrite (NO2)	SM4500 NO2-B	Colourimetry	0.010 mg/L
Total Ammonia - N	EPA GS I-2522-90 m	Colourimetry	0.05 mg/L
Conductivity	SM 23 2510 m	Conductivity Meter	1.0 umho/cm
Dissolved Chloride (Cl-)	EPA 325.2 m	Automated Colourimetry	6.0 mg/L

In addition, the Region of Waterloo provided effluent quality data for the duration of the study period at Kitchener and Waterloo.

3.1.2 Kitchener and Waterloo WWTP Upgrades

The Kitchener WWTP was initially designed with two plants in the early 1960s to mid-1970s. As of 2007 the treatment process involved flow through four primary clarifiers which is then split between two plants for ferrous chloride injection and aeration. Secondary clarifiers were used for settling. Effluent was disinfected with sodium hypochlorite before being discharged into the Grand River. In 2010 dechlorination was implemented. The largest process changes started in 2012 with upgrades to a fine bubble aeration system to introduce partial nitrification in Plant 1 and full nitrification in Plant 2 (Table 3.2). UV disinfection replaced chlorination as a disinfection technique. In 2015 the construction of Plant 3 and 4 began. These plants are designed to provide full nitrification and tertiary treatment for advanced phosphorous removal and will eventually replace Plant 1. In addition, Plant 2 received return activated sludge.

In 2016 a new outfall to the Grand River was commissioned followed by tertiary filters in 2017. However, wastewater flowing through Plant 1 is not currently being filtered and is only partially nitrifying. As a result, the effluent discharged from the outfall is a mixture of effluent receiving varying levels of treatment.

Table 3. 2 Summary of Kitchener WWTP upgrades since 2012 (Pam Law, Region of Waterloo)

Upgrade Description	Commissioning Date
New aeration – Plant 1(new diffusers/blowers, but not fully nitrifying)	October 2012
New tanks/aeration – Plant 2 – Pass 1	August 2012
New tanks/aeration – Plant 2 – Passes 2 and 3	January 2013
New Outfall	November 2016
Tertiary Filters Commissioned (Plant 1 not being filtered)	August 2017
Optimized coagulant dose for TP control	October 2017
Plant 3 online	
Plant 4 online	
Plant 1 offline	

The Waterloo WWTP is a conventional activated sludge system. Wastewater goes through primary clarifiers before entering aeration cells with mechanical aerators. Ferrous chloride was the added for phosphorous removal before going through final clarifies and being disinfected using sodium hypochlorite. Effluent from this plant is discharged into the Grand River. From 2009 to 2014 the WWTP began receiving centrate which was high in ammonia from the biosolids dewatering system. In 2012 UV disinfection was commissioned to replace chlorine disinfection. In 2014 upgrades to primary and secondary treatment were initiated.
In March 2017 aeration tank 1 was upgraded and in March 2018 a second aeration tank was brought online, providing year-round nitrification (Table 3.3).

Table 3. 3 Summary of Waterloo WWTP upgrades since 2009 (Pam Law, Region of Waterloo)

Upgrade Description	Commissioning Date
Interim Dewatering (note: centrate sent to the Raw Sewage PS, aeration upgrades not completed, so temporary increase in effluent ammonia)	2009 – 2014
RAS re-aeration online	
Aeration tank 1 upgrades	March 2017
Aeration tank 2 online	March 2018

3.2 Results

3.2.1 Nutrients

Between 2010 and 2018 both the Kitchener and Waterloo WWTP effluents have observed decreases in ammonia concentrations in their final effluent discharged and subsequently an increase in nitrate concentrations. Kitchener effluent was measuring above 20 mg/L of ammonia prior to 2012 at which time there was a major drop in concentration (10 mg/L) (Figure. 3.1) that corresponds with the implementation of major plant upgrades. The ammonia concentrations continued to decrease after the beginning of 2013 and generally remained below 5 mg/L. During the continuation of plant construction there were several treatment upsets. In mid-2014 there was a large spike in ammonia concentrations suggesting a major change in treatment operation and effectiveness (Figure 3.2). Daily flow at the Kitchener WWTP have been relatively consistent since 2007, with yearly peaks corresponding to increases in flow due to snow and rain events (Figure 3.3). This shows that when the first treatment upgrades occurred in 2012 (increased aeration) there was an improvement (based on ammonia removal) although several upsets occurred, and the plant has been running effectively since 2016.

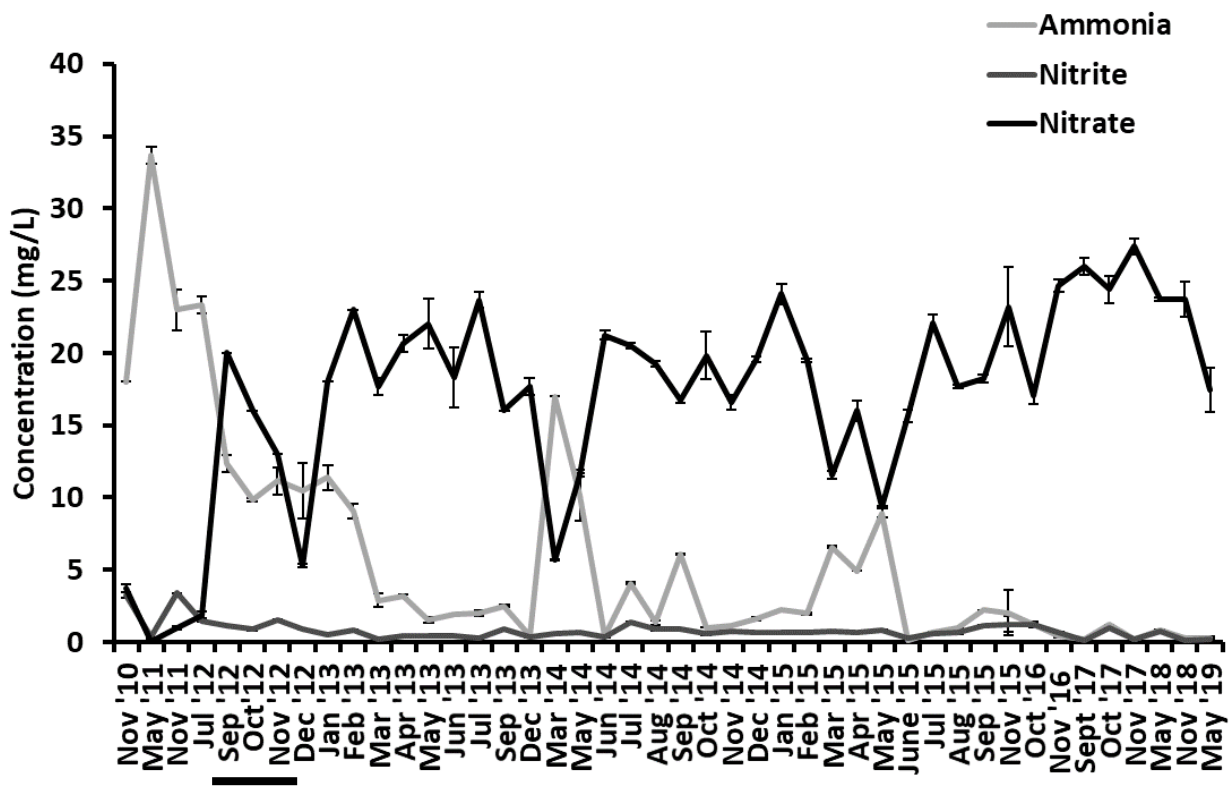


Figure 3.1 Select nutrients in final effluent grab samples from the Kitchener WWTP. Ammonia, nitrite, and nitrate concentrations were measured in effluent grab samples from Kitchener since 2010. Upgrades occurred at this plant in the fall of 2012 and are indicated on the x-axis.

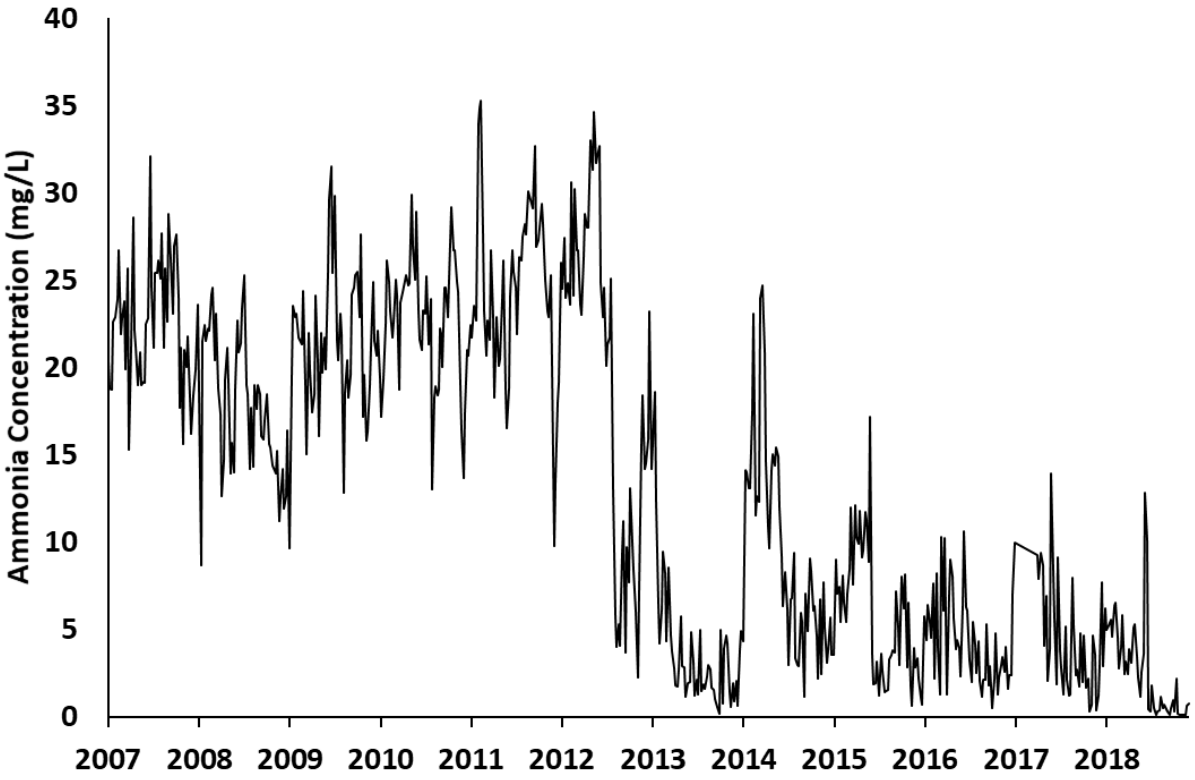


Figure 3. 2 Weekly ammonia concentrations in final effluent from the Kitchener WWTP (data provided by Pam Law, Region of Waterloo)

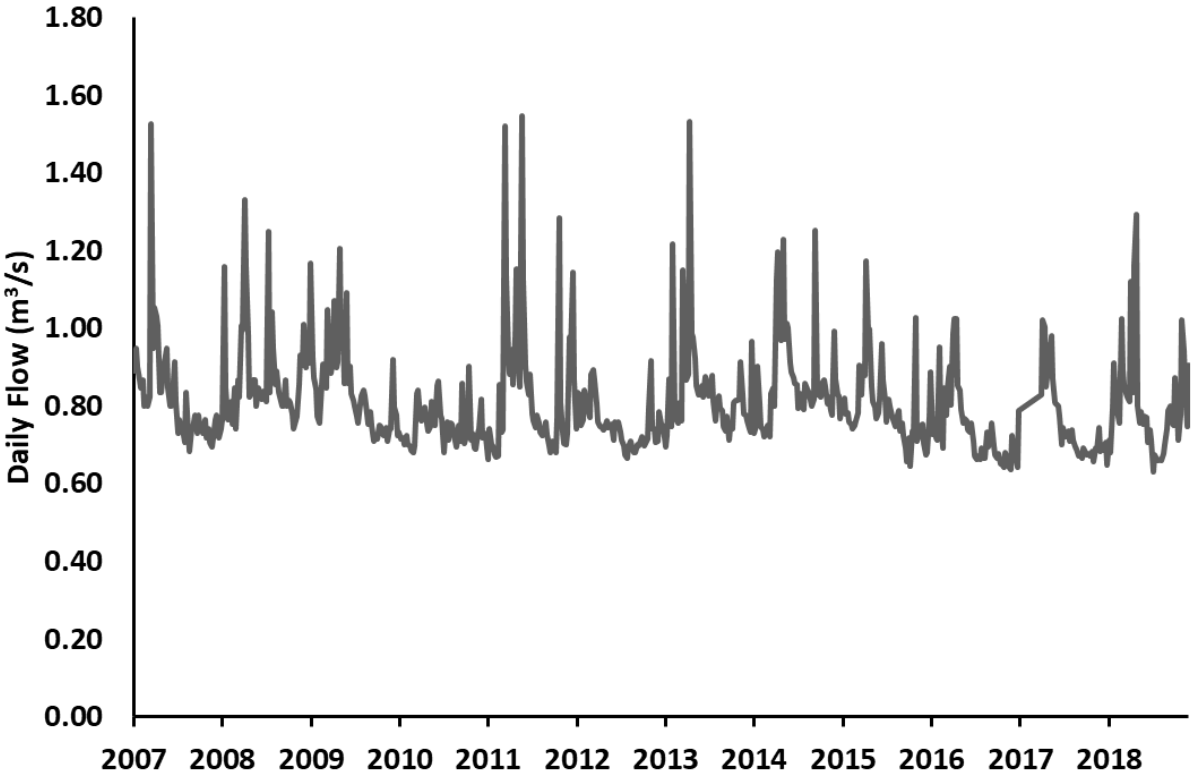


Figure 3. 3 Daily Flow at the Kitchener WWTP (provided by Pam Law, Region of Waterloo)

The Waterloo WWTP experienced a similar trend to Kitchener after the upgrades with a reduction in ammonia concentrations and increase in nitrate concentrations being observed, beginning in 2016 (Figure 3.4). Grab samples were analyzed since mid-2011 and analysis of these samples indicate a gradual decrease in initially high levels of ammonia. However, analysis of weekly effluent data from 2007 (Figure 3.5) reveals that prior to 2011 ammonia levels were generally well below 20 mg/L and progressively increased, reaching the highest concentrations of ammonia in 2012-2013. Daily flow at the Waterloo WWTP shows relatively consistent flows since 2007 with yearly peaks corresponding to increases in flow due to snow and rain events (Figure 3.6). This suggest that there was a reduced treatment effectiveness during construction but a rapid change with the completion of the plant in late 2017.

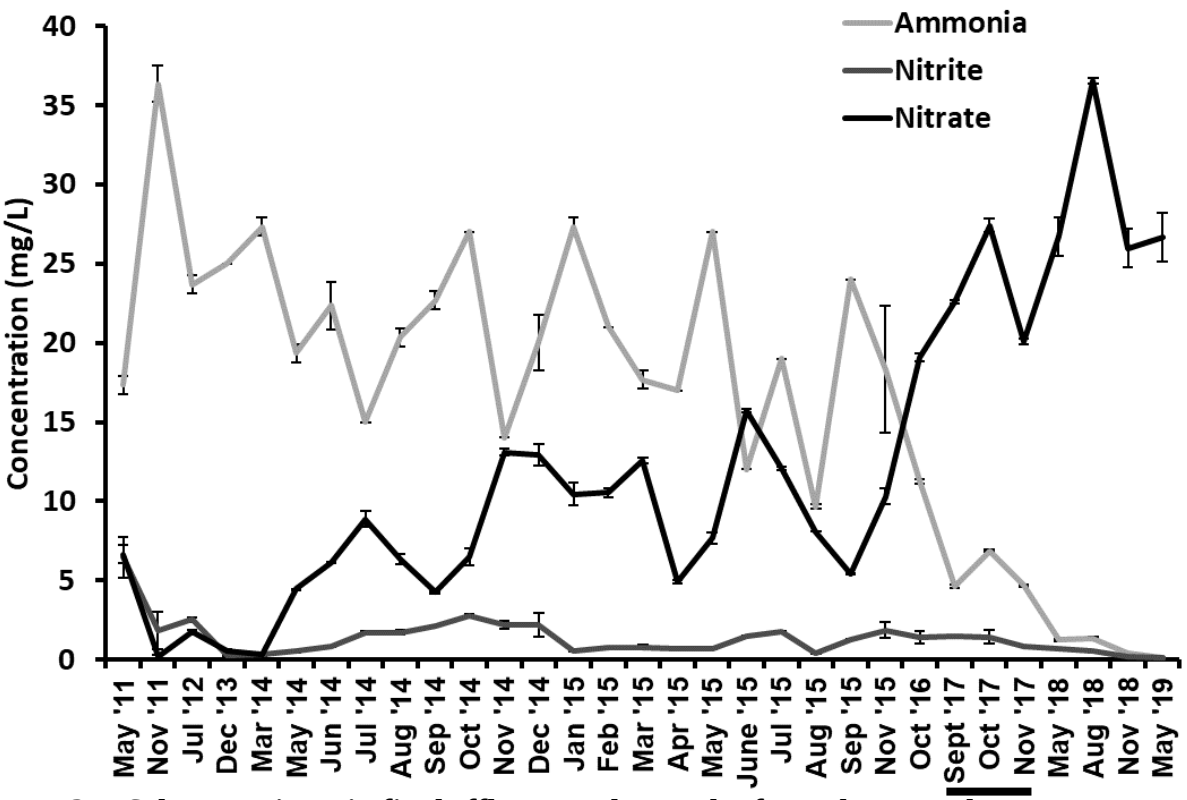


Figure 3. 4 Select nutrients in final effluent grab samples from the Waterloo WWTP. Ammonia, nitrite, and nitrate concentrations were measured in effluent grab samples from Waterloo since 2011. Major upgrades occurred at this plant in the fall of 2017 and are indicated on the x-axis.

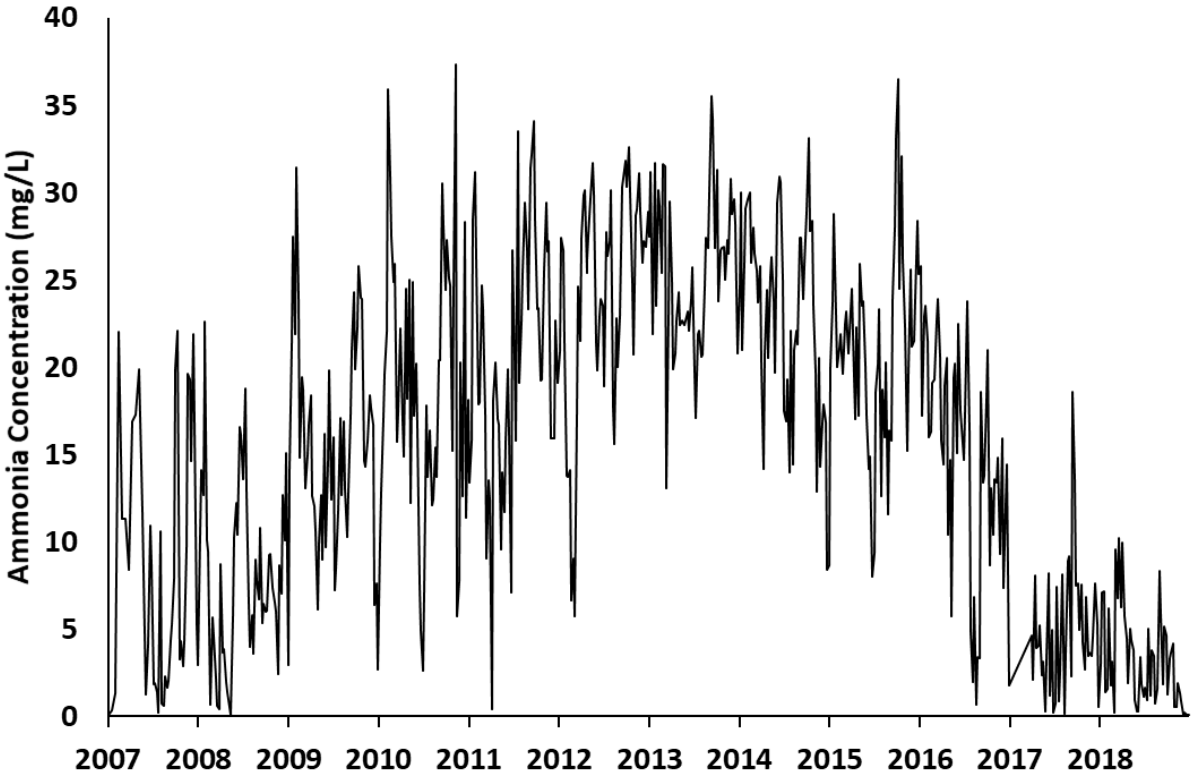


Figure 3. 5 Daily ammonia concentrations in final effluent from the Waterloo WWTP (data provided by Pam Law, Region of Waterloo)

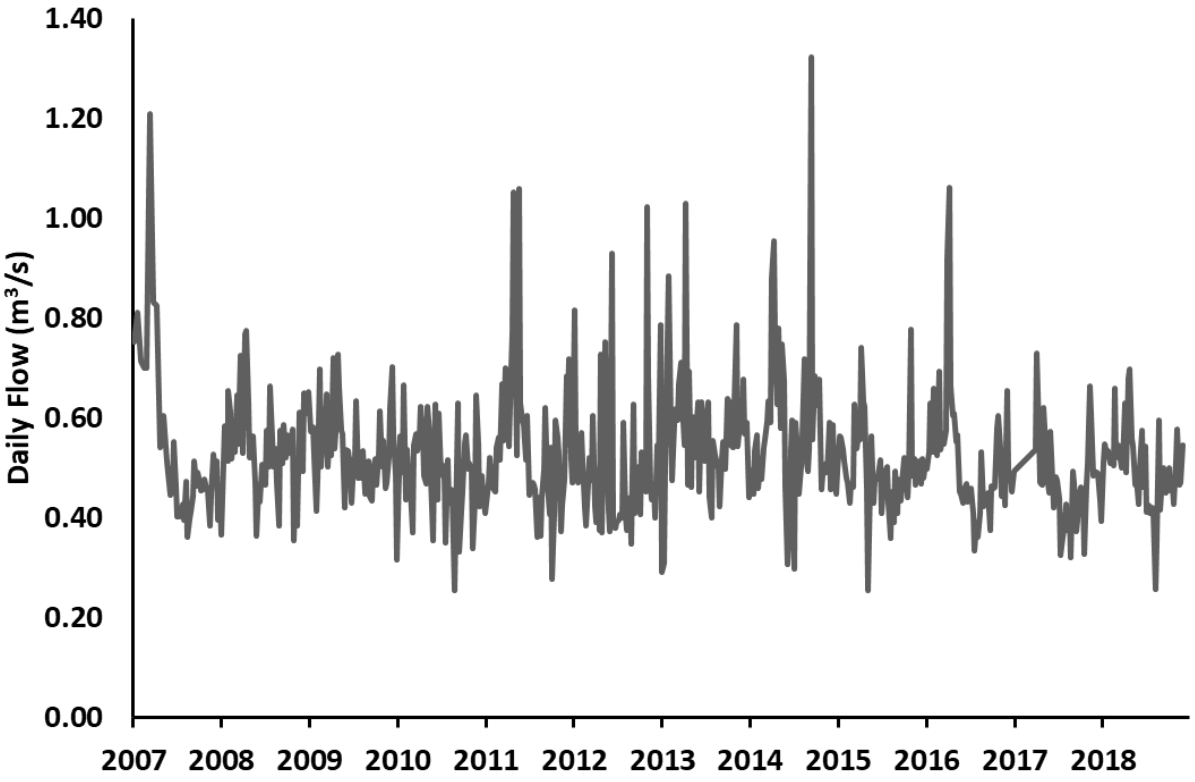


Figure 3. 6 Daily Flow at the Waterloo WWTP (provided by Pam Law)

3.2.2 Pharmaceuticals

Select pharmaceuticals (Table A2.1) have been monitored in the final effluent from the Kitchener and Waterloo WWTPs since 2010, however on the basis of the results from Chapter 2, CBZ, VEN, IBU, and NPX were used to characterize the effluent quality over time. The Kitchener WWTP began upgrades in the fall of 2012, therefore samples collected between November 2010 to July 2012 were used to determine a pre-upgrade mean and 95% confidence interval for each compound (Table 3.4). CBZ and VEN concentrations remain close to pre-upgrade levels following the upgrades (Figure 3.7 (A, B)). Some variation is observed in the CBZ and VEN concentrations over the years, bringing it outside of the 95% confidence interval. IBU and NPX concentrations show significant decreases in concentrations post upgrades (Figure 3.7 (C, D)).

The Waterloo WWTP implemented significant upgrades in the fall of 2017, so samples from August 2015 to August 2017 were used to determine a pre-upgrade mean and 95% confidence interval (Table 3.2). As shown in Figure 3.8 (A, B) CBZ and VEN concentrations in final effluent from Waterloo remain unchanged post upgrades. In contrast, IBU and NPX

concentrations have significantly decreased since the upgrades were implemented (Figure 3.8 (C, D)). Analysis of these compounds also shows fluctuations in concentrations which follow the fluctuations in ammonia concentrations.

Table 3. 4 Pre-upgrade mean and 95% confidence intervals of select pharmaceuticals and estrogens in the final effluent from the Kitchener and Waterloo WWTPs

	Concentration (ng/L) ± 95% Confidence			
Pharmaceuticals	**CBZ**	**VEN**	**IBU**	**NPX**
Kitchener WWTP	619 ± 1580	1631 ± 997	3538 ± 41	2537 ± 131
Waterloo WWTP	560 ± 226	678 ± 73	950 ± 308	828 ± 400
Hormones	**E1**	**E2**	**EE2**	
Kitchener WWTP	47.1 ± 12.5	6.4 ± 2.0	5.8 ± 6.6	
Waterloo WWTP	25.6 ± 14.4	6.2 ± 2.5	1.0 ± 0.6	

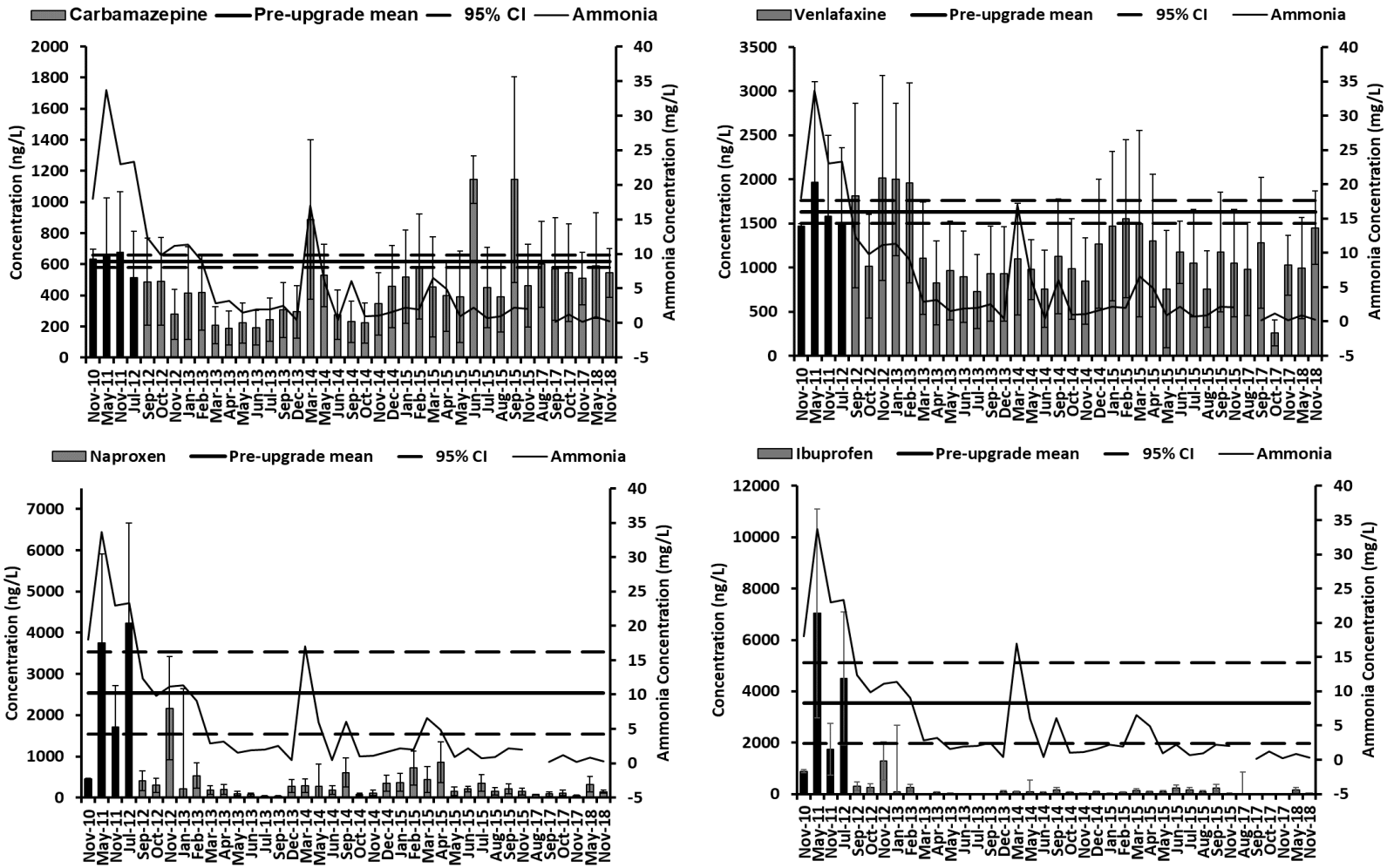


Figure 3. 7 Concentrations of carbamazepine (A), venlafaxine (B), naproxen (C), and ibuprofen (D) measured in final effluent grab samples from Kitchener WWTP over time. Concentrations of select pharmaceuticals (ng/L) in final effluent grab samples from Kitchener and standard error are plotted along with the corresponding ammonia concentrations (mg/L). The solid line indicates the pre-upgrade mean calculated using the samples indicated with black bars. The dotted lines are the 95% confidence intervals for the pre-upgrade mean.

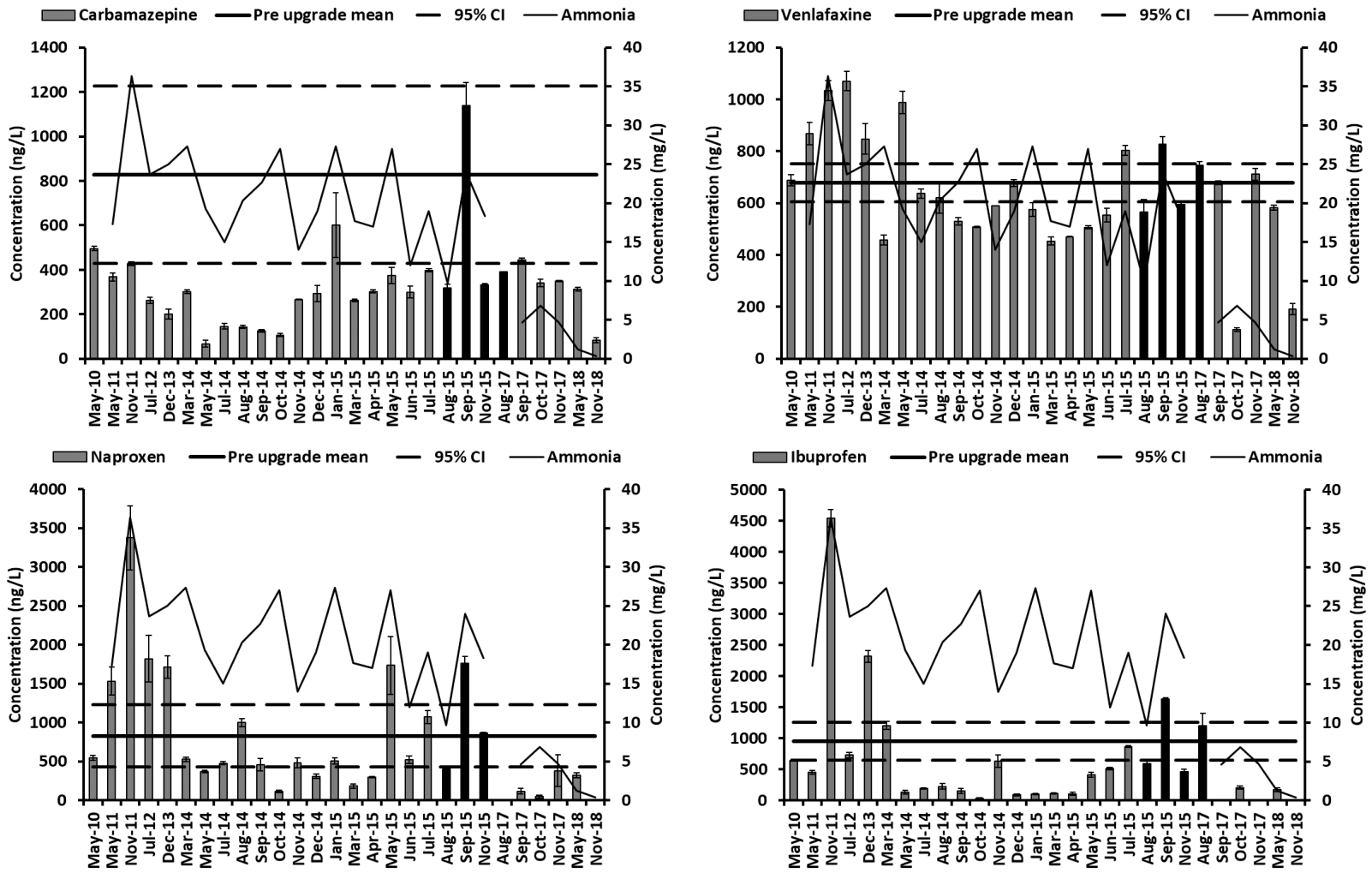


Figure 3. 8 Concentrations of carbamazepine (A), venlafaxine (B), naproxen (C), and ibuprofen (D) measured in final effluent grab samples from Waterloo WWTP over time. Concentrations of select pharmaceuticals (ng/L) in final effluent grab samples from Kitchener and standard error are plotted along with the corresponding ammonia concentrations (mg/L). The solid red line indicates the pre-upgrade mean calculated using the samples indicated with black bars (during a period of poor operation prior to the major upgrade. The dotted lines are the 95% confidence intervals for the pre-upgrade mean.

3.2.3 Hormones

Concentrations of estrogens (E1, E2, EE2) have also been measured in final effluent grab samples from the Kitchener and Waterloo WWTPs since 2010. As done for the pharmaceuticals the 95% confidence intervals were calculated for each plant immediately prior to the major upgrades, e.g. 2012 Kitchener and 2017 Waterloo (Table 3.4). Samples collected for this study were only included in the analysis if specific criteria were met. These criteria include good QA/QC recoveries, appropriate qualifier ion ratios and retention times, and consistent peak area for the internal standard spiked into each sample analyzed. Recoveries for the QA/QC samples were required to be ± 20% of the expected 20 µg/L. Qualifier ion ratios and retention times are compound specific and were determined using estrogen standards during method development. Finally, the peak area of the internal standard was observed and required to maintain ± 20% of the peak area between samples and QA/QC samples throughout the analysis. All samples used for this study met all of these requirements to ensure proper extraction, analysis, and compound quantification and qualification.

At the Kitchener WWTP E2 concentrations showed a significant decrease post upgrades, however there were periods of elevated results (Figure 3.9 (A)). In early 2013, E2 concentrations were back to pre-upgrade levels, while in the summer of 2014 and 2015 concentrations were much higher than pre-upgrade concentrations. A drop in E1 concentrations was also observed over the years post-upgrade, however there was a spike in Aug 2014, brining concentrations back to pre-upgrade levels (Figure 3.9 (B)). Like E2, increases in E1 concentrations were also seen in 2013 and 2015, however these concentrations were still lower than the pre-upgrade concentration.

E2 concentrations at the Waterloo WWTP began to decrease just after the upgrades in fall 2017, however they increased back to pre-upgrade concentrations in August 2018 (Figure 3.10 (A)). A similar response was observed in E1 concentrations (Figure 3.10 (B)), however further sample collection and analysis is needed post-upgrade to determine if this was an upset/event or a trend.

As shown in Figure 3.9 (C) and Figure 3.10 (C), concentrations of EE2 at both the Kitchener and Waterloo WWTPs remain unchanged post upgrades. Final effluent concentrations have been below 5 ng/L, except for major spikes observed in 2012 and 2014 when the concentration of EE2 ranged from 50-120 ng/L.

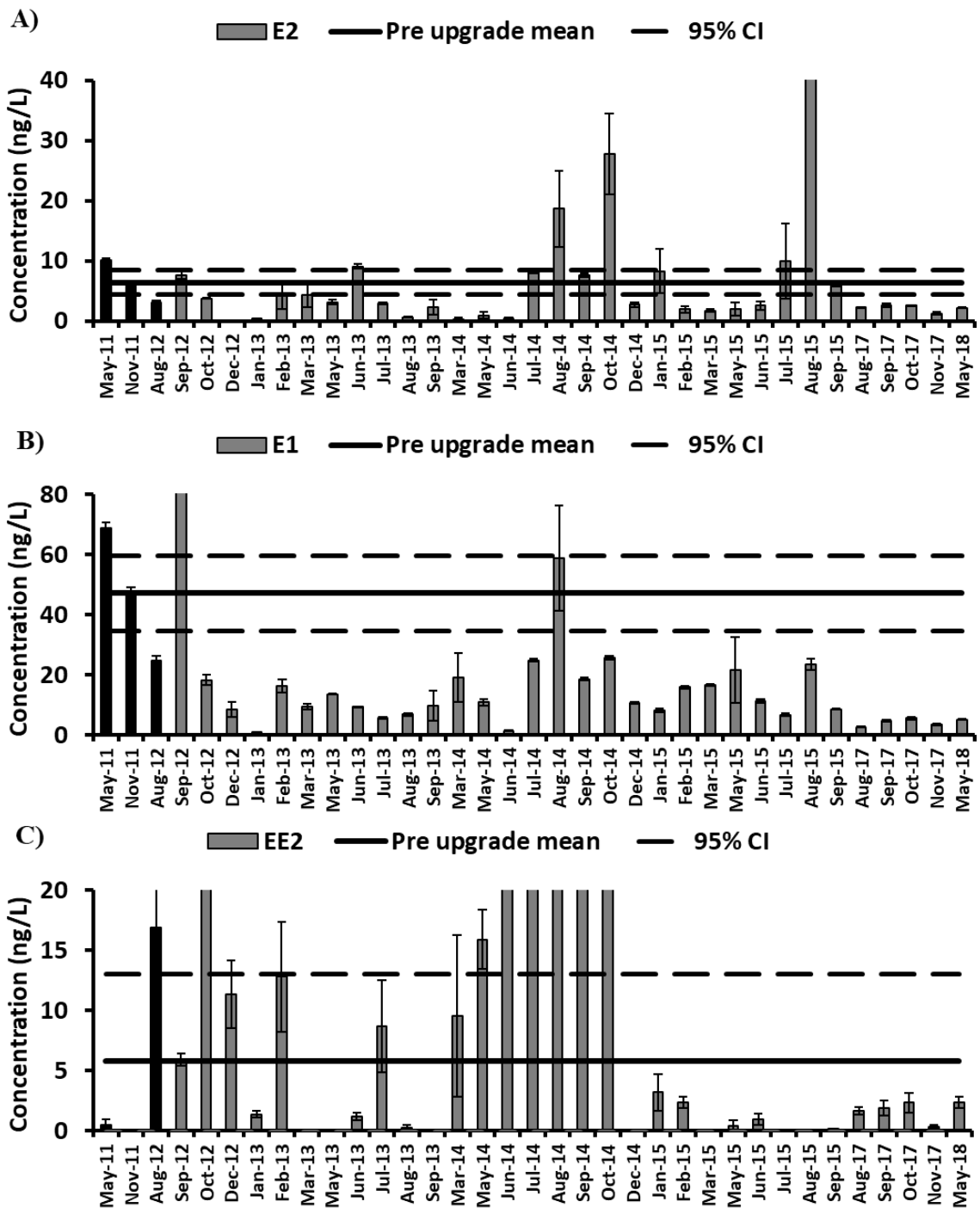


Figure 3. 9 Concentrations of E2 (A), E1 (B), and EE2 (C) in final effluent grab samples from the Kitchener WWTP over time. Estrogen concentrations in effluent grab samples have been analyzed since 2011in Kitchener and plotted with standard error. To determine if concentrations have changed since upgrades were implemented, a pre-upgrade mean (solid line) was calculated from samples collected pre-upgrade (black samples). The 95% confidence interval for this mean is indicated by the dotted line. Samples indicated with * are above the average concentration. The concentrations can be found in Appendix 7.

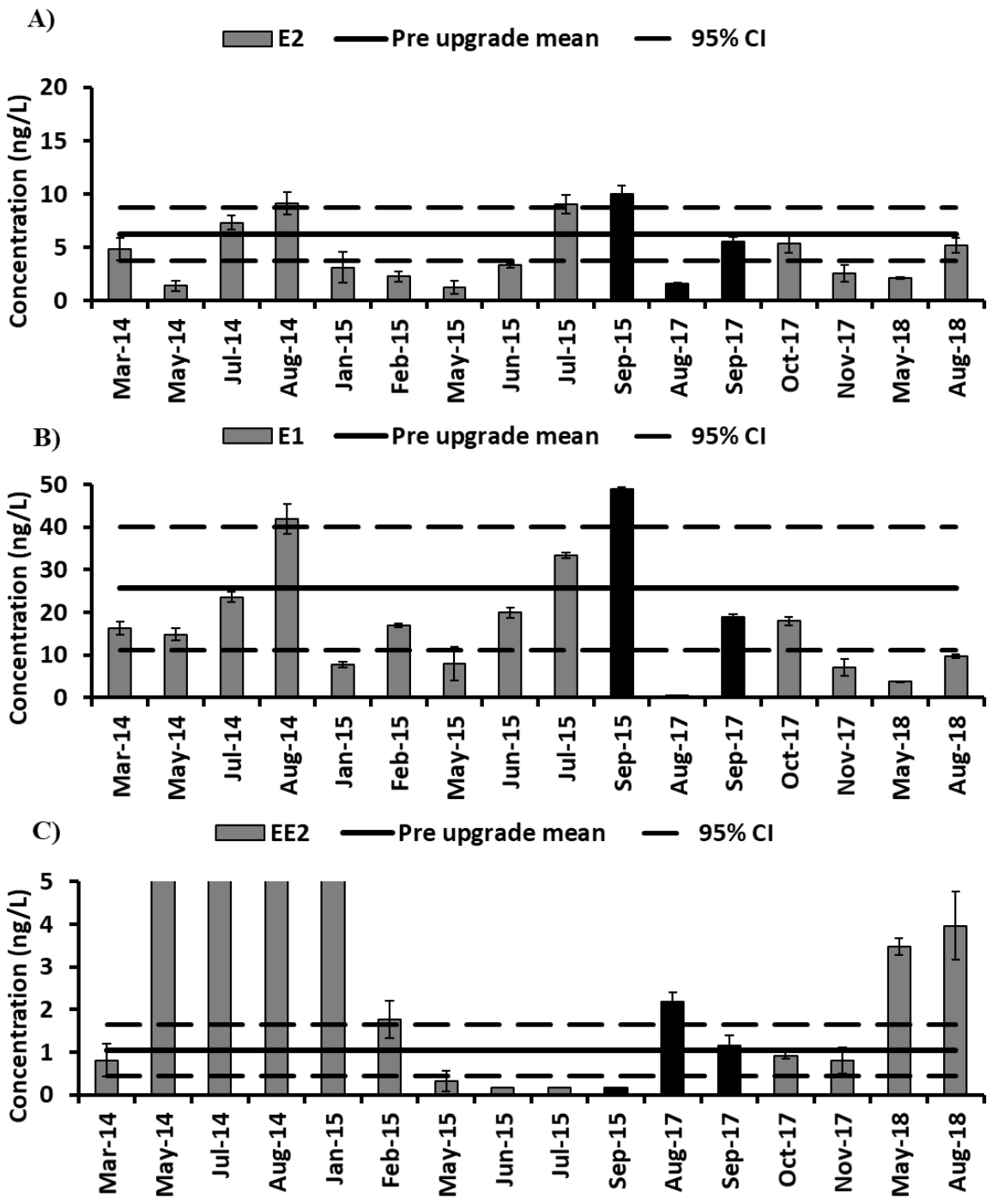


Figure 3. 10 Concentrations of E2 (A), E1 (B), and EE2 (C) in final effluent grab samples from the Waterloo WWTP over time. Estrogen concentrations in effluent grab samples have been analyzed since 2014 in Waterloo and plotted with standard error. To determine if concentrations have changed since upgrades were implemented, a pre-upgrade mean (solid line) was calculated from samples collected pre-upgrade (black samples). The 95% confidence interval for this mean is indicated by the dotted line. Samples indicated with * are above the average concentration. The concentrations can be found in Appendix 7.

3.2.4 Estrogenicity

The total estrogenicity of final effluent grab samples from Kitchener and Waterloo were measured using the YES assay since 2009/2010. This assay determines the estrogenicity of the whole matrix, considering all of the various components that can bind to the estrogen receptor. Post-upgrades at Kitchener a significant reduction of total estrogenicity has been found in the final effluent (Figure 3.11). Concentrations are outside the 95% confidence interval of the pre-upgrade average. Although the estrogenicity has been reduced, spikes were detected in the summer and fall of 2014 with concentrations equivalent to pre-upgrade conditions.

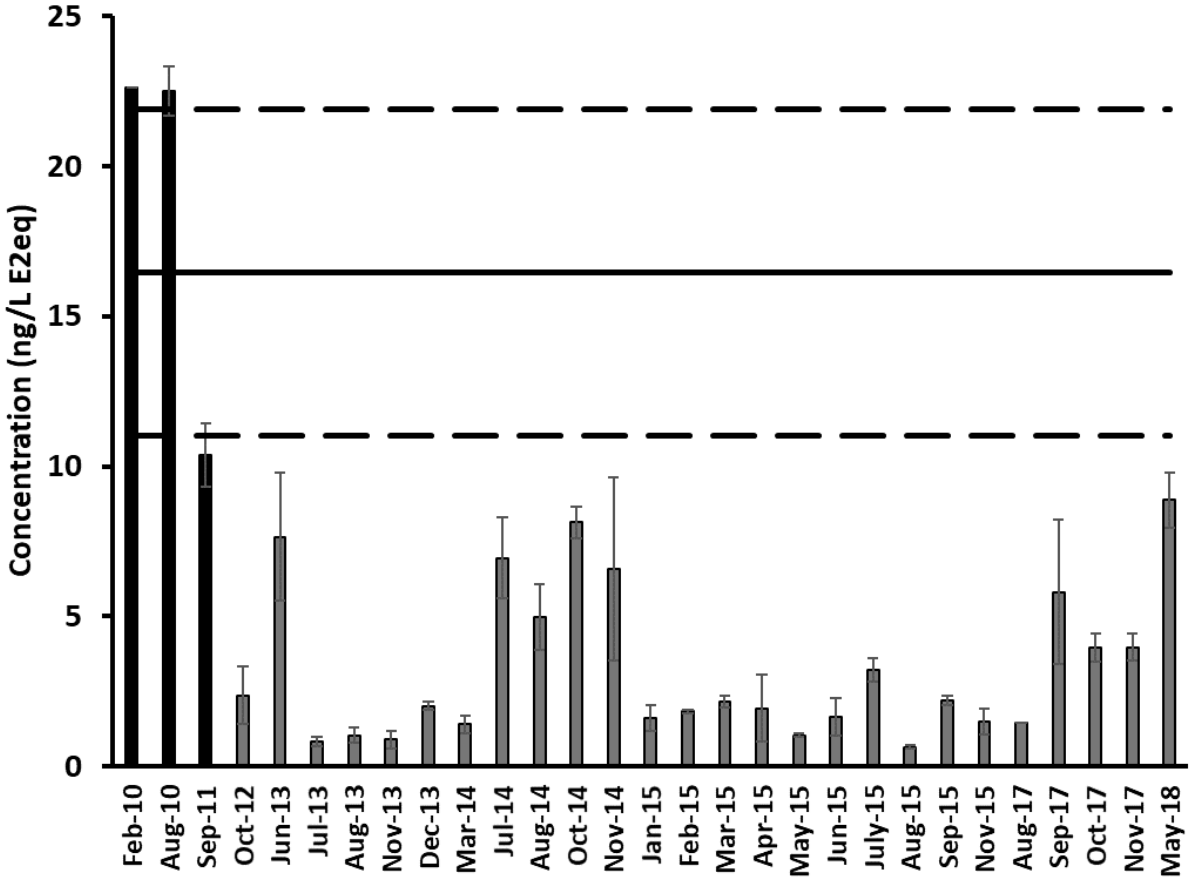


Figure 3. 11 Total estrogenicity measured in final effluent grab samples from the Kitchener WWTP over time. Total estrogenicity of final effluent grab samples from Kitchener were measured using the YES assay since 2010. The average estrogenicity was plotted with the standard error. The average pre-upgrade estrogenicity was calculated using samples from 2 years prior to the upgrades, indicated in black. The solid line indicates the pre-upgrade average total estrogenicity with the 95% confidence interval indicated by the dotted lines.

Over time at the Waterloo WWTP the total estrogenicity of the final effluent has been highly variable. Major upgrades were implemented in the fall of 2017 and therefore samples

from two years prior to the upgrade were used to determine a pre-upgrade mean. Several samples from 2014 measured very highly estrogenicity, with concentrations greater than 10 ng/L E2eq (Figure 3.12). However, effluent grab samples analyzed post upgrades do not fall outside the 95% confidence interval of the pre-upgrade average.

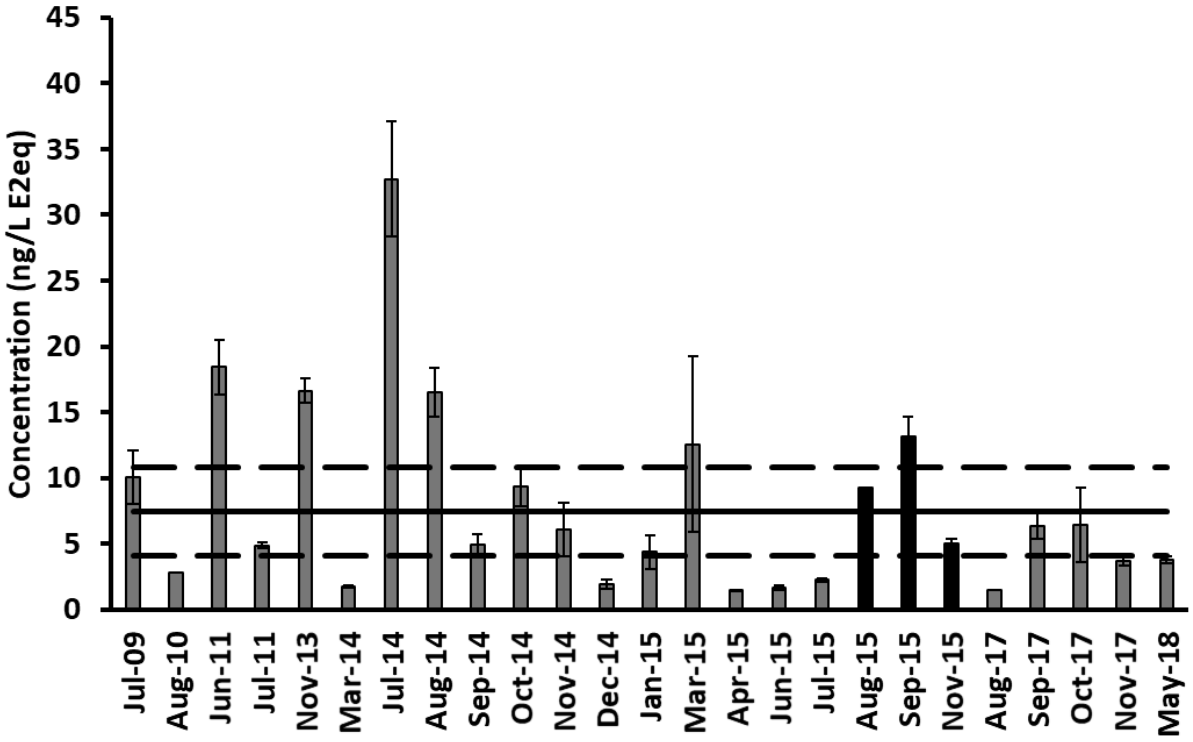


Figure 3. 12 Total estrogenicity measured in final effluent from the Waterloo WWTP over time. Total estrogenicity of final effluent grab samples from Waterloo were measured using the YES assay since 2009. The average estrogenicity was plotted with the standard error. The average pre-upgrade estrogenicity was calculated using samples from 2 years prior to the upgrades, indicated in black. The solid line indicates the pre-upgrade average total estrogenicity with the 95% confidence interval indicated by the dotted lines.

3.3 Discussion

The main focus of the upgrades at both the Kitchener and Waterloo WWTPs was to implement full nitrification to address concerns of water quality in the receiving environment and to address emerging regulatory requirements. The upgrades to date have shown to be successful, with increased removal of ammonia and other parameters. A major decrease in ammonia concentration and corresponding increase in nitrate was observed in 2012 at the Kitchener WWTP corresponding with the upgrades to the aeration system that introduced improved nitrification. The spike in ammonia concentrations in 2014 correlates with various

upsets documented at the plant. At Waterloo, the centrate from the biosolids dewatering introduced to the wastewater treatment plant was high in ammonia. This resulted in the temporary increase in final effluent ammonia concentrations seen in Figure 3.4. The decrease in ammonia concentrations in the final effluent beginning in 2017 indicates improvements to treatment process and increased nitrification. This corresponds with the upgrades and installments of the aeration tanks. Through the analysis of ammonia/nitrate at these two plants during the upgrade process, it is evident that ammonia (and nitrate) levels are a good indicator of treatment (e.g. nitrification). Improved treatment at the plants can be seen by the decreased concentrations of ammonia, and the times of treatment upsets are reflected by increased ammonia concentrations.

Although the upgrades at these two plants being designed to improve traditional effluent quality targets such as those in the Wastewater Effluent Systems Regulation (outlined in Chapter 1.1.1), studies have found that improving nitrification and extending solid retention time (SRT) can indirectly improve the removal of various micropollutants (Achermann et al. 2018, Metcalfe et al. 2003; Servos et al. 2005). CBZ and VEN are recalcitrant compounds that can be used as indicators of external factors that can influence treatment and concentration while IBU and NPX can be used to indicate the effectiveness of the treatment applied during the treatment process (Chapter 2). At both the Kitchener and Waterloo WWTPs the final effluent CBZ concentration remained relatively consistent throughout the years and a similar pattern was seen with VEN concentrations. The slight fluctuations in the concentrations seen between November and April of each year correspond with fluctuations in flow at the plants (Figure 3.3, 3.6) and are most likely due to snow melt and rain events.

IBU concentrations in the effluent from the Kitchener WWTP have significantly decreased since the upgrades in the fall of 2012 when additional aeration (i.e. nitrification) was introduced. At the Waterloo WWTP a significant decrease in effluent IBU concentration also occurred once nitrification was implemented in 2017. NPX concentrations have also significantly decreased since the implementation of nitrification at both plants, however unlike IBU it is influenced more by the treatment level. IBU is very responsive to treatment however, NPX concentrations had a stronger correlation to increases in ammonia concentrations compared to IBU. A similar observation was made by Joss et al. (2005). Both of these compounds increased in concentration at the Kitchener WWTP in 2014 when there was a spike in ammonia

concentrations. Overall, the significant decrease in both IBU and NPX final effluent concentrations over time at the Kitchener WWTP highlights the effectiveness of the new treatment processes being utilized.

Although the upgrades at both the Kitchener and Waterloo WWTPs have had a significant impact on the reduction of ammonia concentrations and select pharmaceuticals from the final effluent being discharged into the Grand River, the removal of estrogens remains more complex and ambiguous. When interpreting estrogen concentrations, it is important to consider two factors that can strongly influence the measurements: conjugation and biotransformation (including into other estrogens).

With the upgrades at Kitchener (2012) and Waterloo (2017), significant reductions in E2 and E1 concentrations in the final effluent were observed. The concentrations of E2 were consistently lower than those of E1 which was expected because of their high removal efficiencies and the biotransformation of E2 to E1 under aerobic conditions (Ternes et al. 1999) (further explained in Chapter 2). Overall, the patterns in E2 and E1 concentrations follow the fluctuations in ammonia concentrations similar to the indicator compounds ibuprofen and naproxen. However, spikes in concentration of E2 and E1 were seen at similar time points for both these compounds at the Kitchener WWTP, which followed a large increase in ammonia (Dec 2012, March 2014, May 2015). These fluctuations indicate issues with the nitrification and possibly a disturbance to the nitrifying conditions (and related bacteria) found in the sludge. A relationship has been previously reported between degradation of micropollutants and ammonia oxidizing bacteria (AOB) (Chapter 2) but the degradation does not appear to be directly related to the AOB but rather to the conditions that enhance nitrification as well as solids retention time (SRT) (Achermann et al. 2018). Servos et al. (2005) also reported that wastewater plants in Canada with longer SRT had higher apparent removal of estrogens (E2, E1). After an upset the bacteria may need time to recover back to their full abundance/activity to be able to degrade estrogens. Arlos et al. (2018) estimated influent concentrations of E1 and E2 at the Kitchener WWTP to be 37 ± 5 ng/L and 17± 2 ng/L, while at Waterloo they were 32 ± 7 ng/L and 13 ± 3 ng/L. The spikes in E1 and E2 at both plants fall below the predicted influent range and are not unreasonable concentrations if there was poor treatment. E3 was not measured over time at the Kitchener and Waterloo WWTPs. In eight Ontario treatment plants, 95% removal of E3 was

observed (Chapter 2). E3 concentrations in final effluent have been reported to be very low in a variety of other studies (Fernandez et al. 2007; Johnson et al. 2002; Baronti et al.)

Unlike the natural estrogens, EE2 concentrations are very low in influent (as shown in Chapter 2), therefore concentrations in final effluent are also low and remain below 5 ng/L post upgrades, with no obvious trends. This aligns with the findings of other studies (Table 2.12) and is expected because of its higher stability and low removal efficiency (Ternes et al. 1999).

With the introduction of nitrification, there was a decrease in total estrogenicity at the Kitchener and Waterloo WWTPs. Total estrogenicity is a measure of the cumulative estrogenic impacts of the components in the final effluent, so a decrease was expected as there was a decrease in several estrogenic compounds measured (e.g. E1, E2). There can also be other estrogenic compounds in the effluent, such as alkyphenols, but the effects directed assessment (EDA) reported by Arlos et al. (2018) indicated that the natural estrogens along with EE2 explain most of the estrogenicity in the Kitchener effluent. The YES assay data (total estrogenicity) also generally follow the patterns of estrogen concentration measured by mass spectrometry. However, they tend to be lower than that predicted during major upsets by the analytical chemistry and application of equivalency factors (see Chapter 2). This could be a result of the presence of anti-estrogens or other compounds which could be inhibiting the YES, especially in poorly treated effluent (Buckley et al. 2010; Ihara et al. 2014).

Although there was no apparent decrease in EE2 concentrations post upgrades at either plant, some anomalies/inconsistencies in the results (2012, 2014) have led to questions about the estimated concentrations that need further study. The concentrations of EE2 in final effluent samples (post-upgrades) were typically below 2 ng/L which is similar to other report in the literature (Table chapter 2). However, there was an elevation in the apparent effluent EE2 concentrations ranging from 25 ng/L to 130 ng/L during the months of June to October of 2014 at both WWTPs. The same samples had a corresponding increase in total estrogenicity (YES), however to a much lower extent than what would be expected based on the chemically detected estrogen concentrations. It is possible that the matrix inferred with the YES assay and underestimated the total estrogenicity. Based on the concentrations of E1, E2, and EE2 found in those samples, a YES assay response at approximately 150 ng/L EEQ was expected, whereas the biologically measured estrogenicity peaked at 32 ng/L EEQ. The value of 130 ng/L of EE2 (150 ng/L total EEQ) is much higher than what would be expected (but not impossible) based on the

literature and predictive models. Arlos et al. (2018) predicted that the concertation in influent would typically be less than 6 ng/L in these treatment plants but there are many factors that could alter this estimate. These differences could be related to artifacts of the methodologies and this methodological problem needs to be resolved to better understand the environmental exposure and biological implications of these chemicals. However, it is possible that some event occurred during the upgrades that led to elevated EE2 and estrogen values during these periods.

This very high EE2 concentrations are not consistent with the biological responses in reported in downstream fish published by Hicks et al. (2017). Downstream of the Waterloo WWTP there were minimal changes in percent (%) incidence or severity of intersex between the years of 2007 and 2015 whereas a significant decrease in the % incidence and severity of intersex (testes-ova) was observed downstream the Kitchener WWTP post upgrades in 2012 (Hickset al., 2017). However, with a spike in EE2 concentrations in the effluent over several months, an increase in incidence and severity might have been expected; although timing of exposure is also important, and the elevated exposure might have occurred outside of the sensitive development window. To further complicate the results, during the period of interest, there were no major upsets or spills reported at either and there are no significant changes in the weekly effluent quality measurements of total Kjeldahl nitrogen, phosphorous, suspended solids, or carbonaceous biochemical oxygen demand (5 day). However, just prior to these events of high EE2 concentrations, there were spikes in ammonia levels at both plants. It may have taken time for the biological community to re-establish. Despite the estrogen samples meeting all normal QA/QC these discrepancies raise the concern that the results obtained are biased in some way by analytical interferences. Although these elevated concentrations of EE2 are not typical effluent concentrations, they are not unheard of. Fernandez et al. (2007) also studied influent and effluent samples from Canadian WWTPs and reported measuring concentrations of up to 178 ng/L. Considering the importance of environmental estrogens in the responses observed in fish associated with wastewater outfalls, resolving these analytical issues and obtaining accurate measurements of their concentrations in wastewater is an important priority. Only 5 ng/L in an experimental lake led t o fish population collapse (Kidd et al. 2007) and Arlos et al (2018) suggested that estrogens at <20 ng/L could lead to severe intersex in fish in the Grand River. As most of the effluent extracts in the previous studies at the Kitchener and Waterloo WWTP are available the opportunity existed to re-examine these samples using alternate methods.

Historical samples from Kitchener and Waterloo were re-analyzed using a more selective analytical approach, the LC-MS/MS QTOF, to try to determine whether the spikes measured on the LC-MS/MS QQQ were actually a result of EE2 or compounds analogous to EE2 (See Appendix B). Samples were also re-analyzed on the LC-MS/MS QQQ using a different quantification ion to determine whether a more compound specific fragment would be able to distinguish between EE2 and other components of the matrix (See Appendix B). Attempts to verify the original results were hampered by the low remaining volumes of the historical samples. These additional analyses suggest that these historical samples most likely did have high concentrations of EE2, however several factors (e.g. very low concentrations, limited number of samples) still make it diffiuclt to completely rule out an analaytical issue.

The reason for the high concentrations of EE2 at the Waterlooo WWTP can be a result of the on-going construction at the plant. EE2 sorption onto sludge in the wastewater system is both physical and chemical, however it is dominated by physical sorption (Xu et al. 2008). Therefore this reaction can easily be reversed and can have implication during the handling of sludge, specifically during dewatering (Matsui et al. 2000). Matsui et al. (2000) measured the total estrogenicity of samples from various points throughout a wastewater treatment plant and found that the filtrate post dewatering of the sludge had the highest total estrogenicity (474 ng/L E2 equivalence), much greater than either effluent or influent levels.

In 2009, the Waterloo WWTP began to receive centrate which was high in ammonia and caused a major spike in effluent ammonia concentrations. Since the dewatering process can release EE2 and other contaminants, it can be assumed that these compounds were also entering the WWTP at very high concentrations. Spikes in EE2 concentrations follow spikes in ammonia concentations in the effluent and appear to occur at points of seasonal changes. This could indicate issues with the bacteria culture in the activated sludge, due to weather changes, plant proceses (e.g. changes were made to the air supply in 2014), or possibly contaminant overload. There could be a delayed response in the estrogens, where the bacteria are not able to properly treat the increased concentrations of estrogens as they recover. In Chapter 2, it was shown that the total estrogenicity of the influent measured using the YES assay were muted compared to the chemically calculated values. Considering that dewatering centrate can be more estrogenic than influent and increase the effluent concentrations by a factor of nine (Yi & Harper 2007; Matsui et al. 2000), the YES assay may have understimated the effluent concentrations, as was reported in

Chapter 2. If this was the case, it would be expected that there would be an increase of other hydrophilic compounds with sorption coefficients (log K_D) between 2 and 4 that would have increased in concentration during these time periods as well since compounds within this range can be removed by both degradation and sorption (Racz & Goel 2010). Compounds such as triclocarban, triclosan, fluoxetine, ibuprofen, and venlafaxine (Figure 3.8, Figure A6.2) had increases in concentration but not as evident as the estrogens. Although this could partially explain the occurrence at the Waterloo WWTP, the Kitchener WWTP is independent of the Waterloo plant but experienced spikes of EE2 in the same time period.

Between 2012 and 2014 the Kitchener WWTP was undergoing major construction proceses, including the decommissioning of the lagoon, which involved transfer pumping the digested sludge. This involves a processes similar to dewatering and returning the centrate to the WWTP. However, contaminants bound to the sludge could have been released into the cenrate when the lagoon was being decommissioned. Both Kitchener and Waterloo experienced spikes in ammonia concentrations in March 2014 followed by increases in EE2 concentrations. Therefore it is possible there was a weather event impacting the bacteria culture at both plants, and resulted in poor treatment of the estrogens entering the system during the recovery period. As seen in Waterloo, there were similar changes in triclocarban, triclosan, fluoxetine, ibuprofen, venlafaxine (Figure 3.7, Figure A5.2), and in the total estrogenicity of the samples. Unfortunately, additional samples are not available to allow further analysis.

In conclusion, the upgrades to the treatment processes at both the Kitchener and Waterloo WWTP have been significant in improving the overall quality of the effluent discharged into the Grand River. There have been notable decreases in ammonia, pharmaceutical concentrations and estrogen concentrations at both plants. The quantification of EE2 is difficult due to its low environmental concentrations and influence by matrix effects. However, although analytical artifacts can not be completely ruled out, detection of large concentrations of this compound is possible when there are upsets or major changes at the treatment plants high concentrations can occur; this is something to consider when studying treatment plants and may have implication of aquatic organisms in the receiving waters.

Chapter 4 – Conclusion and Recommendations

The analysis of influent and effluent from various WWTP has shown that despite all the plants in the study being classified as secondary treatment and meeting new regulations (with the exception of ammonia at some plants) they all had varying levels of effectiveness in regard to treating the influent for pharmaceuticals and estrogens. However, the improved treatment of select PPCPs, EDCs, and total estrogenicity with conditions that promoted nitrification (e.g. increased aeration) was apparent. The concentration and removal of select pharmaceuticals such as ibuprofen and naproxen can be used as indicators of effective treatment as they are easily degraded in those conditions. However, compounds such as carbamazepine and venlafaxine are very recalcitrant and remain untreated even with increased nitrification. Therefore, fluctuations in the concentrations of these compounds can be used as indicators of external factors influencing the treatment of PPCPs and EDCs.

The treatment patterns of these pharmaceuticals and estrogenic hormones were also seen in the temporal study of the Kitchener and Waterloo WWTPs, both of which underwent major upgrades to their treatment processes. The upgrades were specifically intended to improve nitrification at both plants, which as shown in the first part of this study also fosters conditions to improve the removal of other contaminants of concern. These upgrades have resulted in decreased concentrations of ammonia, and many pharmaceuticals (e.g. ibuprofen, naproxen). As expected, compounds such as carbamazepine and venlafaxine remained unchanged post upgrades.

A major method of degradation of estrogens is through biotransformation which makes the quantification difficult as they get converted into different estrogens. The biotransformation can be influenced by the treatment processes applied and solid retention times causing variability among the different plants. Of the estrogens studied estriol had the highest removal whereas 17α-ethinylestradiol had the poorest removal. Due to the consistently low concentrations of estriol in effluent samples, it was not measured at the Kitchener and Waterloo WWTPs. Estrone and 17β-estradiol show decreases with improved nitrification at both plants. However, the upgrades show now impact on the concentration of 17α-ethinylestradiol measured at these plants. Despite this, there was a decrease in the total estrogenicity of the effluent discharged from the treatment plants

post upgrades. The estrogenicity of the effluent discharged is a combination of concentration and the potency of the compounds present in the effluent.

The most estrogenic compound is 17α-ethinylestradiol and has impacts at low concentrations. However very few studies are reporting significant concentrations of 17α-ethinylestradiol which brings to question whether regulations are needed. Although these contaminants are not currently regulated, working towards achieving improved nitrification shows that it would continue to reduce the risk of PPCPs and EDCs on the environment. Some recommendations to consider are:

1. Influent and effluent composite samples were handled (e.g. extraction) using the same methods, however influent samples are much dirtier and more concentrated compared to final effluent. Using the same extraction method may have led to loss of compounds or compromised the detection. It would be recommended to use either a smaller sample volume, sample clean up, or use tandem SPE columns to avoid these issues.
2. Continued method development for estrogens, especially EE2 is recommended. A clean-up method for estrogen samples should be developed for future analysis as it would result in clearer extracts with less potential matrix interferences. The 269 m/z fragement should be included in future EE2 analyis as it is more specific fragment and may potentially help to eliminate background in some samples.
3. Influent samples may have a higher proportion of conjugated compounds which were not extracted or accounted for. Using an enzymatic deconjugation step prior to sampling can help account for all forms of compounds and give a more accurate representation of total concentrations. A study should be conducted to determine if conjugated estrogens are present in the influent and final effluent of treatment plants.
4. The YES assay response to the influent composite samples resulted in lower than expected values. Influent samples are very dirty, and a sample clean-up step could help prevent over loading the yeast cells in the assay.
5. An effects directed assessment of the effluent along with chemical measurement would be very informative. This would provide information on which compounds are causing the most impact and provide a better understanding between impact and concentration.

6. Lastly, when conducting studies on WWTPs it is important to not only consider the current conditions of the plant but the history of the plant as well. Like Kitchener and Waterloo disruption at the plants can lead to delayed impacts on the treatment.

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Appendices

Appendix A1: Analytes of Interest

Table A1. 1 Chemical properties of the pharmaceuticals of interest

Abbr.	Compound Name	Class	Molecular Formula	Molecular Weight (g/mol)	Log K_{ow}	Ionization Mode
IBU	Ibuprofen	NSAID	$C_{13}H_{18}O_2$	127.071	3.97	Negative
BPA	Bisphenol A	Industrial	$C_{15}H_{16}O_2$	228.286	3.32	Negative
NPX	Naproxen	NSAID	$C_{14}H_{14}O_3$	230.258	3.18	Negative
GFZ	Gemfibrozil	Lipid regulator	$C_{15}H_{22}O_3$	250.332	4.77	Negative
DCF	Diclofenac	NSAID	$C_{14}H_{11}Cl_2NO_2$	296.148	4.51	Negative
TCS	Triclosan	Antibacterial	$C_{12}H_7Cl_3O_2$	289.541	4.76	Negative
TCCB	Triclocarban	Antibacterial	$C_{13}H_9Cl_3N_2O$	315.582	4.34	Negative
ATRZ	Atrazine	Herbicide	$C_8H_{14}ClN_5$	215.683	2.61	Positive
CBZ	Carbamazepine	Anti-epileptic	$C_{15}H_{12}N_2O$	236.258	2.45	Positive
FLX	Fluoxetine	Antidepressant	$C_{17}H_{18}F_3NO$	309.326	4.05	Positive
ATOR	Atorvastatin	Lipid regulator	$C_{33}H_{35}FN_2O_5$	556.622	5.7	Positive
VEN	Venlafaxine	Antidepressant	$C_{17}H_{27}NO_2$	277.401	3.2	Positive
LIN	Lincomycin	Antibiotic	$C_{18}H_{34}N_2O_6S$	406.535	0.2	Positive
MON	Monensin	Antibiotic	$C_{36}H_{62}O_{11}$	670.866	5.43	Positive
SULF	Sulfamethoxazole	Antibiotic	$C_{10}H_{11}N_3O_3S$	253.276	0.89	Positive
TRIM	Trimethoprim	Antibiotic	$C_{14}H_{18}N_4O_3$	290.317	0.91	Positive
NFLX	Norfluoxetine	Metabolite of FLX	$C_{16}H_{16}F_3NO$	295.299	-	Positive
ATEN	Atenolol	Betablocker	$C_{14}H_{22}N_2O_3$	266.335	0.16	Positive
e-CBZ	10, 11-epoxide Carbamazepine	Metabolite of CBZ	$C_{15}H1_2N_2O_2$	252.273	-	Positive
p-ATOR	p-hydroxy Atorvastatin	Metabolite of ATOR	$C_{33}H_{34}FN_2O_6$	573.24	-	Positive

o-ATOR	o-hydryxy Atorvastatin	Metabolite of ATOR	$C_{33}H_{34}FN_2O_6$	573.24	-	Positive
ACE	Acetominophen	Analgesic	$C_8H_9NO_2$	151.162	0.46	Positive
SMZ	Sulfamethazine	Antibacterial	$C_{12}H_{14}N_4O_2S$	278.329	0.14	Positive
DESVEN	Desvenlafaxine	Metabolite of VEN	$C_{16}H_{25}NO_2$	263.374	2.72	Positive

Table A1. 2 Chemical properties of the hormones of interest

Abbr.	**Compound Name**	**Class**	**Molecular Formula**	**Molecular Weight (g/mol)**	**Log K_{ow}**	**Ionization Mode**
4-NP	4-nonylphenol	Industrial	$C_{15}H_{24}O$	220.35	5.76	Negative
4-OP	4-octylphenol	Industrial	$C_{14}H_{22}O$	206.323	-	Negative
BPA	Bisphenol A	Industrial	$C_{15}H_{16}O_2$	228.286	3.32	Negative
E1	Estrone	Estrogen	$C_{18}H_{22}O_2$	270.365	3.13	Negative
E2	Estradiol	Estrogen	$C_{18}H_{24}O_2$	272.381	4.01	Negative
E3	Estriol	Estrogen	$C_{18}H_{24}O_3$	288.38	2.45	Negative
EE2	Ethynyl estradiol	Estrogen	$C_{20}H_{24}O_2$	296.403	3.67	Negative
TCS	Triclosan	Antibacterial	$C_{12}H_7Cl_3O_2$	289.541	4.76	Negative

Table A1. 3 Chemical properties of the internal standards

Abbr.	Compound	Formula	Molecular Weight (g/mol)	Ionization Mode
d3-IBU	d3-Ibuprofen	$C_{13}H_{15}O_2$-d_3	209.28	Negative
4 NP – d4	d4-4-nonylphenol	$C_{15}H_{20}O$-d_4	216.35	Negative
4 OP – d17	d17-4-n-octylphenol	$C_{14}H_5O$-d_{17}	223.323	Negative
d3-NPX	d3-Naproxen	$C_{14}H_{11}O_3$-d_3	230.258	Negative
d4--NPX	d4-Naproxen	$C_{14}H_{10}O_3$-d_4	234.258	Negative
d14-BPA	d14-Bisphenol A	$C_{15}H_2O_2$-d_{14}	242.286	Negative
BPA – d16	d16-Bisphenol A	$C_{15}O_2$-d_{16}	244.286	Negative
d6-GFZ	d6-Gemfibrozil	$C_{15}H_{16}O_3$-d_6	256.332	Negative
E1 – d4 (new)	d4-Estrone	$C_{18}H_{18}O_2$-d_4	266.365	Negative
E1 – d2 (old)	d2-Estrone	$C_{18}H_{20}O_2$-d_2	268.365	Negative
E2 – d4	d4-Estradiol	$C_{18}H_{20}O_2$-d_4	268.381	Negative
E3 – d2 (new)	d2-Estriol	$C_{18}H_{22}O_3$-d_2	290.38	Negative
E3 – d3 (old)	d3-Estriol	$C_{18}H_{21}O_3$-d_3	291.38	Negative
d3-TCS	d3-Triclosan	$C_{12}H_4Cl_3O_2$-d_3	292.541	Negative
d4-EE2	d4-Ethinylestradiol	$C_{20}H_{20}O_2$-d_4	300.403	Negative
d5-DCF	d5-Diclofenac	$C_{14}H_6Cl_2NO_2$-d_5	301.148	Negative
d2-TCCB	d2-Triclocarban	$C_{13}H_7Cl_3N_2O$-d_2	317.582	Negative
d-4-CP	d4- Chlorophene			Negative
d6-MET	d6-metformin	$C_4H_5N_5$	135.164	Positive
ACE	Acetominophen	$C_8H_9NO_2$	151.162	Positive

d4-ACE	d4- Acetominophen	$C_8H_5NO_2$-d_4	155.162	Positive
d3-CAFF	d3-Caffeine	$C_8H_7N_4O_2$-d_3	197.19	Positive
d5-ATRZ	d5-Atrazine	$C_8H_9ClN_5$-d_5	220.683	Positive
d5-Oxybenzone	d5-Oxybenzone	$C_{14}H_7O_3$-d_5	233.242	Positive
d10-CBZ	d10-Carbamazepine	$C_{15}H_2N_2O$-d_{10}	246.258	Positive
d4-SULFA	d4-sulfamethoxazole	$C_{10}H_7N_3O_3S$-d_4	257.276	Positive
d10-e-CBZ	d10-10,11 epoxide carbamazepine	$C_{15}H_2N_2O_2$-d_{10}	262.273	Positive
d6-Desven	d6-desvenlafaxine	$C_{16}H_{19}NO_2$-d_6	269.374	Positive
d7-ATEN	d7-Atenolol	$C_{14}H_{15}N_2O_3$-d_7	273.335	Positive
d4-SMZ	d4-sulfamethazine	$C_{12}H_{10}N_4O_2S$-d_4	282.329	Positive
d6-VEN	d6-venlafaxine	$C_{17}H_{21}NO_2$-d_6	283.401	Positive
d3-TRIM	d3-trimethoprim	$C_{14}H_{15}N_4O_3$	293.317	Positive
d5-NFLX	d5-Norfluoxetine	$C_{16}H_{11}F_3NO$-d_5	300.299	Positive
d5-FLX	d5-Fluoxetine	$C_{17}H_{13}F_3NO$-d_5	314.326	Positive
d3-LIN	d3-lincomycin	$C_{18}H_{31}N_2O_6S$	409.535	Positive
d5-ATOR	d5-Atorvastatin	$C_{33}H_{30}FN_2O_5$-d_5	561.622	Positive
d5-p-ATOR	d5-p-hydroxyl-atorvastatin	$C_{33}H_{29}FN_2O_6$-d_5	583.3106	Positive
d5-o-ATOR	d5-o-hydroxyl-atorvastatin	$C_{33}H_{29}FN_2O_6$-d_5	583.3106	Positive

Appendix A2: YES Assay Reagents

Table A2. 1 Yes assay reagent preparation

Reagent	Preparation	Sterilization and Storage
10x Yeast Nitrogen Base without Amino Acids	67g YNB wo amino acids in 1L of MilliQ water	0.2 µm filter sterilized 4 °C
20% Dextrose Stock	700 mL MilliQ water 200 g of dextrose added slowly to the milliQ while stirring brought up to 1L	0.2 µmfilter sterilized 4 °C
10mM copper sulfate solution	250 mg copper II sulfate pentahydrate in 100 mL MilliQ water	0.2 µm filter sterilized Room temperature
GOLD Concentration Stock	25 mL L-arginine-HCl, L-methionine, L-isoleucine, L-valine, L-threonine, L-tyrosine, L-phenylalanine, L-glutamic acid, L-aspartic acid, L-leucine and uracil 50 mL of L-histidine-HCl, L-serine and L-tryptophan 75 mL of adenine sulfate 100 mL of L-lysine-HCL	0.2 µm filter sterilized 4 °C
GOLD Media	60 mL 20% dextrose, 60 mL 10X YNB wo AA, 110 mL GOLD concentrate, 370 mL MilliQ water	0.2 µm filter sterilized 4 °C
Minimal Media	100 mL 10X YNB wo AA, 100 mL 20% dextrose, 10mL L-lysine, 10 mL L-histidine, 78	0.2 µm filter sterilized 4 °C
Minimal Media Agar Plates	Autoclave 10 mL 10X YNB wo AA, 2 g bactoagar, 78mL MilliQ Cool to touch Add 1mL L-histidine, 1mL L-lysine, 10 mL 20% dextrose Swirl to mix and pour into plates and cool to solidify at room temperature	Inverted at 4 °C

Table A2. 2 Amino acid stock solutions

Amino Acid	Sterilization and Storage	Amino Acid	Sterilization and Storage
1.2g/L adenine sulfate	Autoclave, RT	6.0 g/L L-glutamic acid	Autoclave, RT
2.4 g/L L-histidine-HCl	Autoclave, 4℃	4.0 g/L L-aspartic acid	Autoclave, RT
2.4 g/L L-arginine	Autoclave, 4℃	18.0 g/L L-valine	Autoclave, 4℃
2.4 g/L L-methionine	Autoclave, 4℃	24.0 g/L L-threonine	Autoclave, 4℃
0.9 g/L L-tyrosine	Autoclave, RT	45.0 g/L L-serine	Autoclave, 4℃
3.6 g/L L-isoleucine	Autoclave, 4℃	3.6 g/L L-leucine	Autoclave, RT
3.6 g/L L-lysine-HCl	Autoclave, 4℃	4.8 g/L L-tryptophan	Filter, 4℃
3.0 g/L L-phenyalanine	Autoclave, RT	2.4 g/L uracil	Autoclave, RT

Appendix A3: Analytical Detection Limits

Table A3. 1 Method detection limits of compounds of interest on the LC-MS/MS QQQ (McCann 2016)

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-ethinylestradiol	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

Appendix A4: Influent and Effluent Data

Table A4. 1 Mean estrogen concentrations in influent and effluent from various Ontario WWTPs

	Average (ug/L)							
	E3		E2		EE2		E1	
Site	Influent	Effluent	Influent	Effluent	Influent	Effluent	Influent	Effluent
A	195.35	1.10	18.34	5.41	6.99	4.54	92.90	51.90
B	285.74	1.29	28.02	2.96	2.05	1.46	48.83	6.18
C	235.07	1.43	20.69	1.51	1.53	1.24	62.91	1.00
D	91.58	1.05	10.02	1.87	2.04	1.82	44.86	0.72
E	123.01	1.16	8.71	1.22	2.13	1.20	24.34	0.71
F	151.57	6.49	10.52	2.12	1.88	0.69	42.41	19.07
G	190.72	2.75	12.30	2.83	0.29	4.29	10.80	0.72
H	394.82	4.84	25.97	4.28	0.32	4.93	103.63	9.25
I-a	223.56	0.00	13.30	5.03	2.08	2.89	42.93	44.36
I-b	255.49	3.69	24.78	4.07	2.53	4.28	93.76	6.54
Average	**214.69**	**2.38**	**17.27**	**3.13**	**2.18**	**2.73**	**56.74**	**14.05**

Table A4. 2 Standard error of estrogen concentrations in influent and effluent from various Ontario WWTP

	Standard Error							
	E3		E2		EE2		E1	
Site	Influent	Effluent	Influent	Effluent	Influent	Effluent	Influent	Effluent
A	4.86	0.69	0.82	0.10	1.25	0.60	5.20	0.58
B	3.45	0.31	1.07	0.32	0.34	0.07	0.90	0.13
C	2.34	0.81	0.51	0.27	0.31	0.14	2.52	0.35
D	2.14	0.74	0.56	0.09	0.17	0.27	1.49	0.16
E	2.66	0.29	0.39	0.25	0.48	0.17	1.25	0.24
F	2.25	0.35	1.31	0.25	0.31	0.73	1.16	0.75
G	9.69	0.47	0.42	0.19	0.00	0.26	0.20	0.10
H	14.65	1.12	1.43	0.30	0.00	0.96	4.24	0.19
I-a	5.09	0.00	0.96	0.38	0.49	0.31	2.48	0.32
I-b	7.48	0.65	1.26	0.22	0.41	1.11	0.90	0.51

Table A4. 3 Total estrogenicity in influent and effluent composite samples from Ontario WWTPs

WWTP	Measured Total Estrogenicity (ng/L E2eq)		
	Influent	Effluent	% Difference
A	13.68 ± 1.08	11.78 ± 3.71	14
B	7.25 ± 1.29	3.22 ± 0.39	55
C	13.21 ± 2.19	0.30 ± 0.02	98
D	5.74 ± 0.79	0.29 ± 0.04	95
E	9.48 ± 1.02	2.32 ± 0.17	75
F	6.59 ± 0.89	5.54 ± 0.60	16
G	11.24 ± 5.71	0.88 ± 0.14	92
H	9.86 ± 0.72	1.98 ± 0.09	80
I-a	8.75 ± 2.04	10.74 ± 0.95	-23
I-b	13.85 ± 1.53	3.25 ± 0.65	77

Appendix A5: Kitchener Additional Graphs Showing Full Range of Data

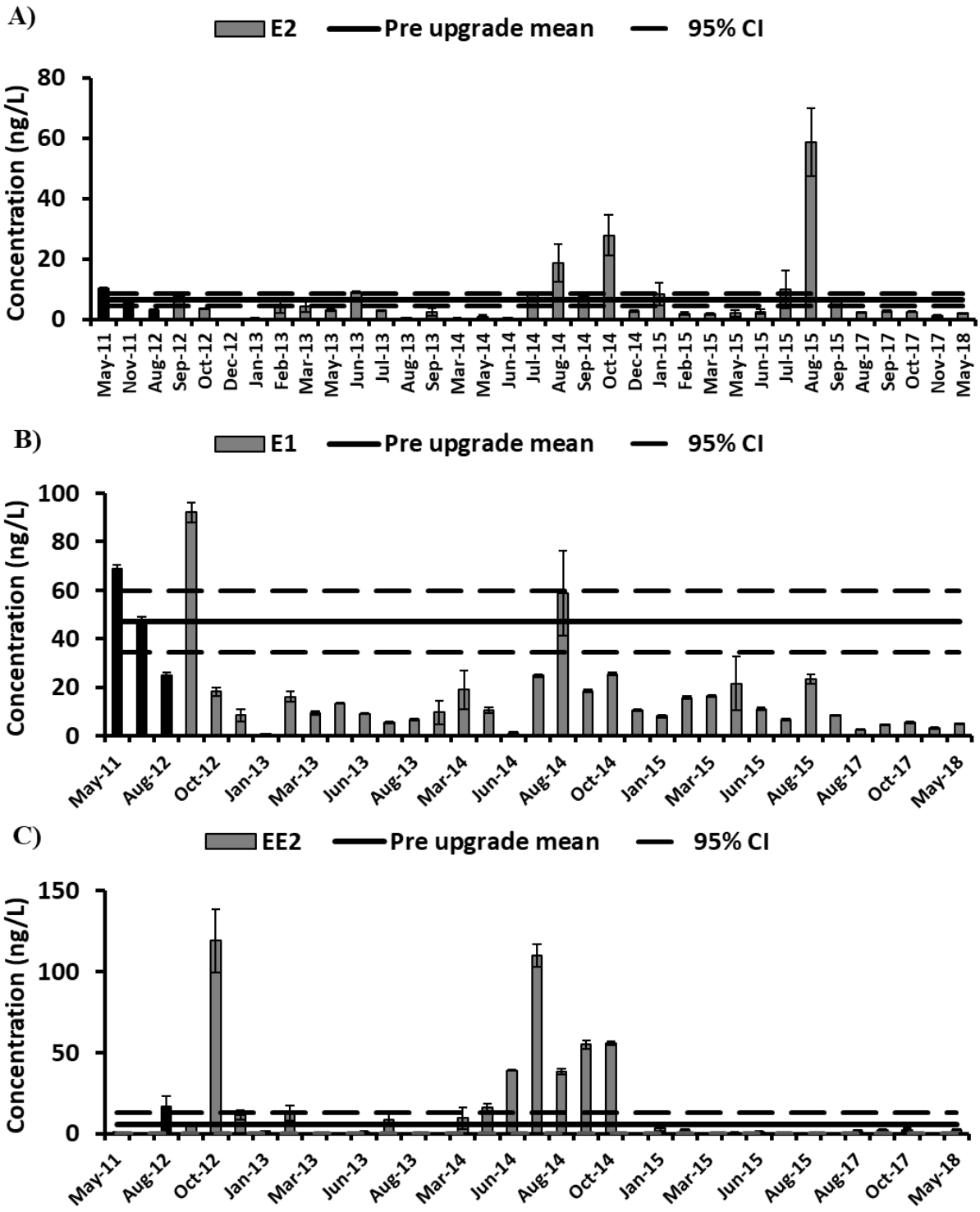

Figure A5. 1 Estrogen concentrations at the Kitchener WWTP over time

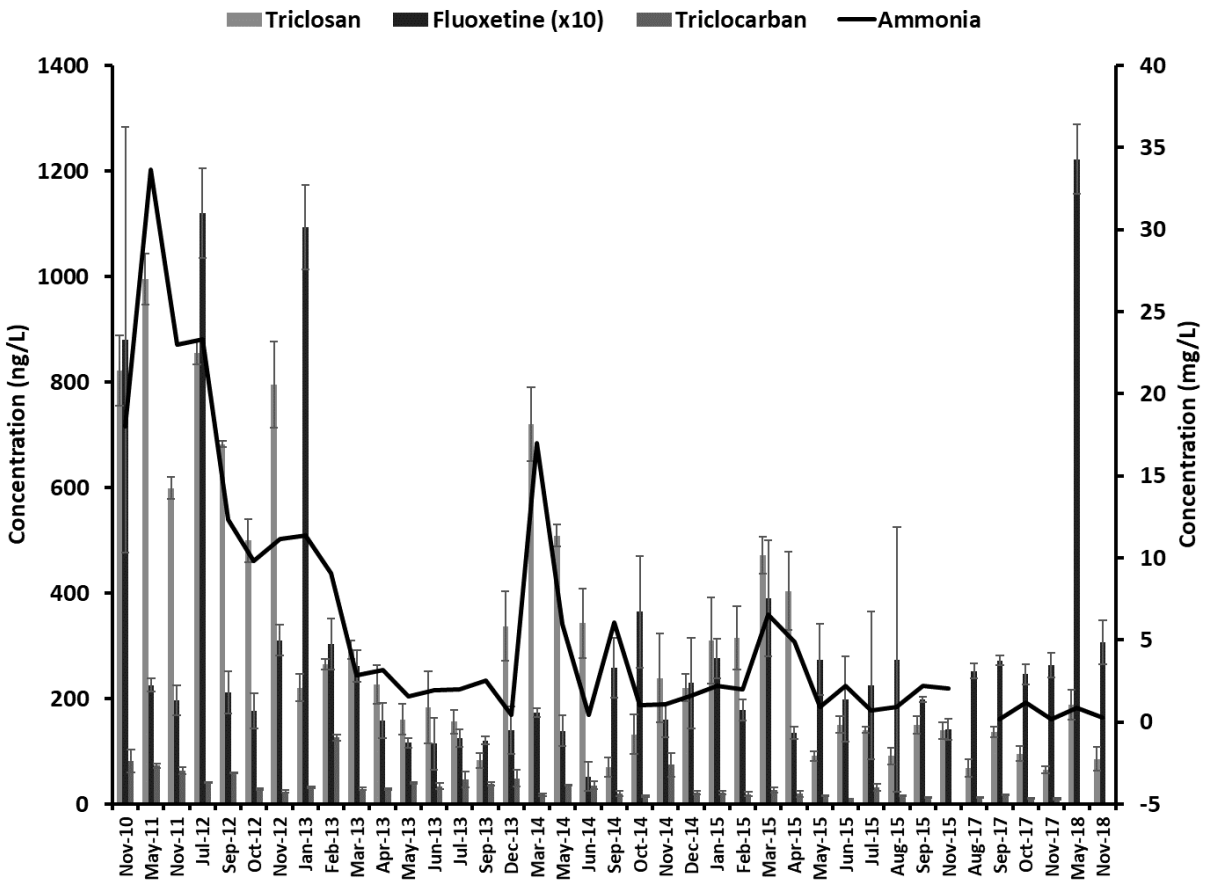

Figure A5. 2 Other pharmaceuticals measured in the effluent from the Kitchener WWTP over time

Appendix A6: Waterloo Additional Graphs Showing Full Range of Data

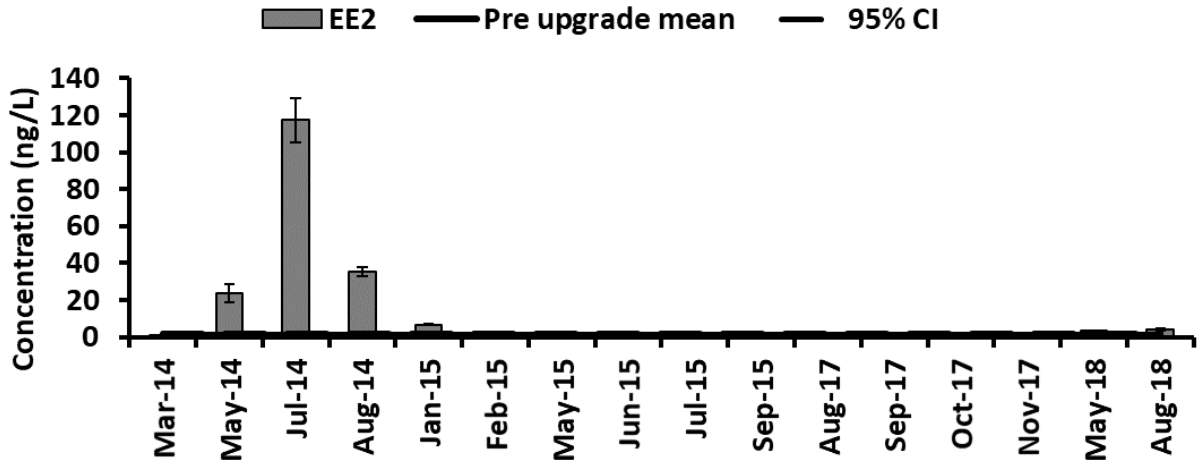

Figure A6. 1 EE2 concentrations measured in the effluent from the Waterloo WWTP over time

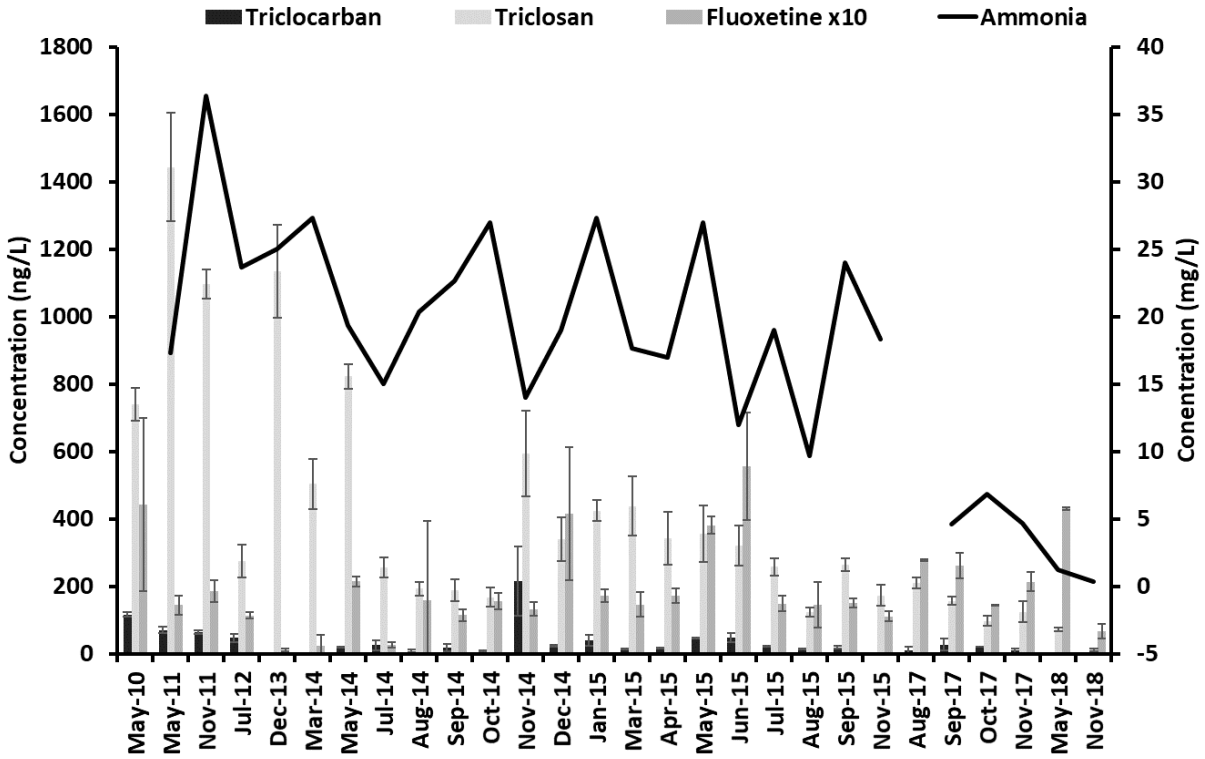

Figure A6. 2 Other pharmaceuticals measured in the effluent from the Waterloo WWTP over time

Appendix B: Potential analytical uncertainty in the analysis of EE2

B.1 Methods

Ethinyl Estradiol is typically found in low concentrations (<1 ng/L) in the environment, making accurate identification and quantification difficult. Select effluent samples analyzed in Chapter 3 had measured concentrations greater than 20 ng/L of EE2 using the LC-MS/MS QQQ. Based on previous studies these concentrations are well above the normally expected concentration for final effluent samples. Samples had been extracted for estrogens using methods described in Chapter 1and stored at -20°C. Therefore, historical samples with high concentrations of EE2 were available and used for re-analysis using an Agilent 1200 series HPLC with an Agilent 6520 quadrupole time-of-flight (QTOF) mass spectrometer in negative-mode ESI since the QTOF has increased selectivity to compounds compared to the QQQ.

B.1.1 Ethinyl Estradiol - LC-MS/MS QTOF

The QTOF method was optimized by injecting concentrated standards of regular and deuterated EE2 onto an Agilent ZORBAX Eclipse Plus C18 (2.1 mm x 50 mm x 1.8 µm) HPLC column in full scan mode. Once the instrument was optimized for the compound, a calibration curve (10 µg/L to 3000 µg/L) was injected multiple times to determine the linearity and detection limit of the instrument. Following optimization of the standard, effluent samples seen to have high levels of matrix when analyzed on the QQQ were used to optimize the method to minimize the matrix effect. The method and mobile phase parameters are described in Table B.1 and Table B.2.

Table B. 1 LC-MS/MS QTOF Method Parameters

Mass Spec Parameters			
MS Abs Threshold	500	**MS Scan Rate (spectra/sec)**	3.00
MS/MS Abs Threshold	100	**MS/MS Scan Rate (spectra/sec)**	1.00
Max Time between MS (sec)	0.0		
Source Parameters			
Gas Temp (℃)	300	**Nebulizer (psig)**	30
Gas Flow (l/min)	10		
EE2 Parameters			
m/z	295.17041	**Collision Energy (V)**	45
z	1	**Detection Window (ppm)**	20
Quantifier Ion (m/z)	145.0659	**Min Height (counts)**	600
Qualifier Ion (m/z)	159.0815	**Retention Time (min)**	6.03

Table B. 2 Estradiol LC-MS/MS QTOF Mobile Phase Parameters

Mobile Phase A:	5mM Ammonium fluoride in MilliQ
Mobile Phase B:	100% Acetonitrile

Time (min)	% Mobile Phase A	% Mobile Phase B
0	90	10
10	0	100
13	0	100
13.10	90	10

Using the optimized method described in Table B.1, 10 µL of the hormone extracts were injected onto the column with the mobile phase gradient indicated in Table B.2 at a flow rate of 300 µl/min. In addition, samples were injected as a sandwich injection, where 1µL of 500 µg/L EE2 was injected with 10 µl of the sample. A standard addition test was also performed to determine the impact of sample matrix on the accurate quantification of EE2. For this analysis a series of sandwich injections were done using a sample that had high background peaks and matrix, but no detected EE2 on the QQQ (Doon 2H – 15Sept17) and various concentrations of EE2 ranging from 0 – 100 000 µg/L.

B.1.2 Ethinyl Estradiol - LC-MS/MS QQQ

Historical samples analyzed on the QQQ were reanalyzed for EE2 using the method described in Chapter 2.1 with the additional transition of 296 m/z → 269 m/z. Samples as well as the calibration curve were quantified using two different methods. The first method used the fragment 145 m/z as the quantifier and the fragment 269 m/z was used as an additional qualifier ion. While the second method quantified EE2 using the 269 m/z fragment as the quantifier and 145 m/z as a qualifier.

B.2 Results & Discussion

B.2.1 QTOF Method Validation

Repeat injections of an EE2 standard and the calibration curve was used to determine the retention time and fragmentation patterns of the compound. The retention time of EE2 was found to be 6.03 minutes and is shown in Figure B.1. Injections of the calibration curve also allowed for the determination of the limit of detection (LOQ) on the QTOF. Figure B.2 demonstrates the linearity of EE2.

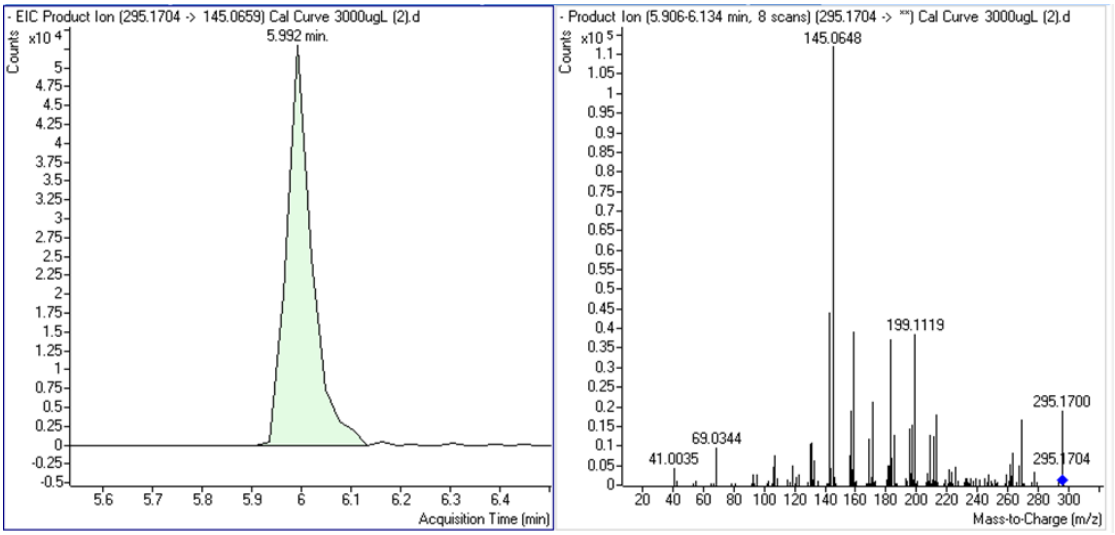

Figure B. 1 Retention time and product ions of the 3000 µg/L calibration point

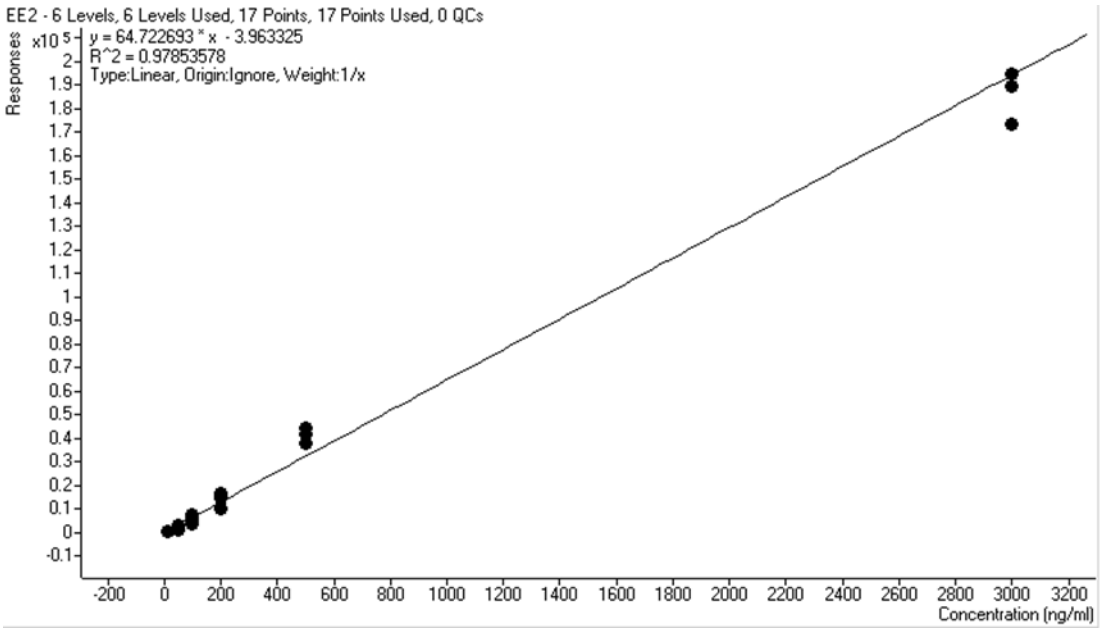

Figure B. 2 EE2 calibration curve. The calibration points (n=3) were used to determine the linear range of EE2 on the QTOF. A 1/x weighting was applied since the effluent samples are expected to have concentrations at the lower end of the calibration curve.

Key fragments produced by EE2, listed in Table B.3, have been identified by several studies (Croley et al. 2000; Magi et al. 2010). A full scan analysis of EE2 at various concentrations produced the fragment patterns seen in Figure B.3. Qualitative analysis of the mass spectrum produced shows that the parent ion of EE2 and the most common fragments can be identified when the concentration is greater than 10 µg/L. When the 10 µg/L calibration point was analyzed, no parent ion was detected and the only product ions of EE2 found were 143 m/z

and 145 m/z, the most abundant fragments of EE2. At higher concentrations, the mass accuracy of the parent ion was within 20ppm and a range of fragments were detected. The most abundant fragment was 145 m/z, while 143, 159, 199 and 183 m/z were similar in intensity. The 269 m/z fragment had the lowest intensity.

Table B. 3 Common EE2 Fragments

m/z	Loss	Product Ion
295	-	$C_{20}H_{24}O_2$
199	C_6H_8O	$C_{14}H_{16}O$
183	$C_7H_{12}O$	$C_{13}H_{12}O$
159	$C_9H_{12}O$	$C_{11}H_{12}O$
145	$C_{10}H_{14}O$	$C_{10}H_{10}O$
143	$C_{10}H_{16}O$	$C_{10}H_8O$
269	C_2H_2	$C_{18}H_{20}O_2$

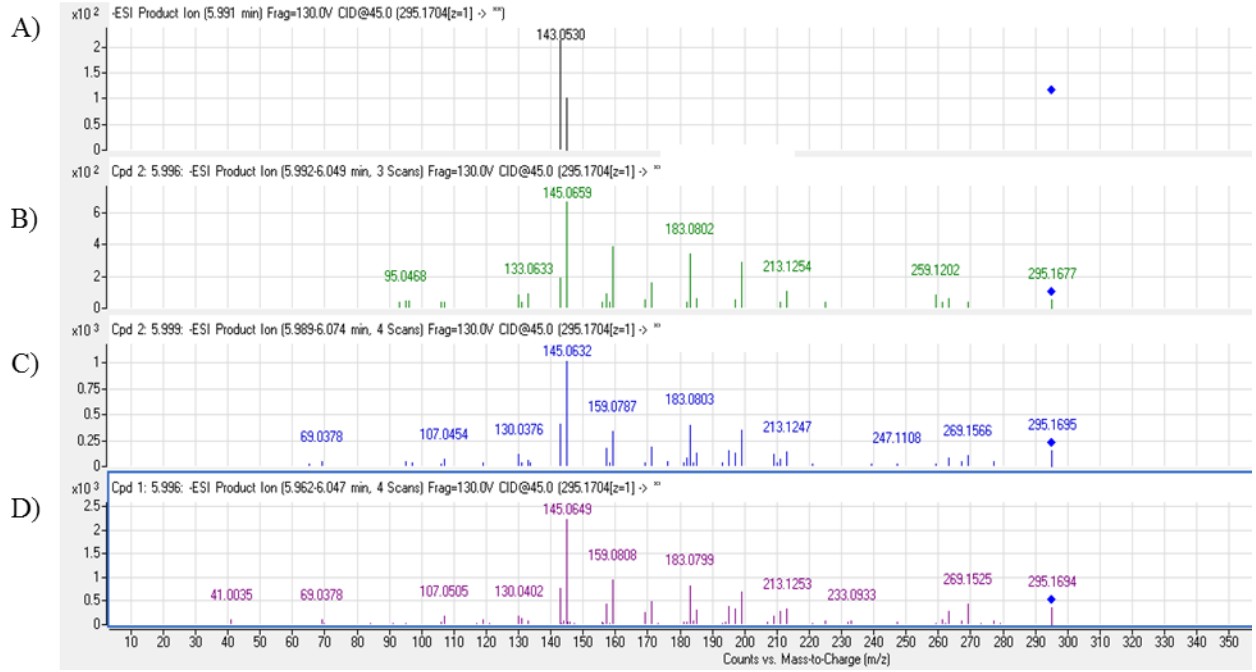

Figure B. 3 EE2 calibration standard fragmentation patterns. A calibration curve with concentrations from 10-3000 µg/L was analyzed using a full scan method on MS/MS to determine the product ion ratios. In this figure the product ion spectrum for the concentrations of 10 µg/L (A), 50 µg/L (B), 100 µg/L (C), and 200 µg/L (D) are shown. The parent ion is indicated by the blue diamond at 295 m/z.

Although the parent ion was not detected in the 10 µg/L calibration point, the calibration curve was quantified with an accuracy of 80% or above when the 145 m/z fragment was used as the quantifier (Table B.4). However, the qualifier fragment of 159 m/z was not found for the 10 µg/L point. The other fragments of EE2 were also difficult to detect in both the 10 and 50 µg/L

standards, which are environmentally relevant concentrations. The 143 m/z fragment had the highest abundance relative to the parent ion followed by 159, 183, and finally 269 m/z.

Table B. 4 Average Calibration Curve Response

Calibration Point (µg/L)	Response	Conc.	Accuracy	Qualifier Ratio 159	Qualifier Ratio 269	Qualifier Ratio 183	Qualifier Ratio 143
10[1]	669	10	104	-	-	-	94
50	2614	40	81	44	-	74	41
100	5522	85	85	39	16	53	61
200	13906	215	107	41	15	42	51
500	41435	640	128	29	15	32	38
3000	185669	2869	96	34	16	33	40

1. N of 2 used. No EE2 detected in the sample.

B.2.2 QTOF Sample Analysis

The standard addition was performed to determine the affect of high matrix and background on the detection of EE2. A historical sample with high levels of background noise but no detectable EE2 was spiked with various concentrations of EE2. Compared to the expected concentration after spiking, the concentration of EE2 detected by the QTOF was consistently higher. Measured concentrations when spiked with 500 µg/L EE2 was off by 78%, but when the concentrations were increased, there was improved accuracy (Table B.5). It can be concluded that at higher concentration the matrix has minimal impact on the detection of EE2, however samples with lower concentrations (<20 µg/L) could be influenced and difficult to detect accurately. This becomes a problem because the environmentally relevant concentrations of EE2 are below 20 µg/L.

Table B. 5 EE2 standard addition and measured concentrations.

EE2 Spike Solution Conc (µg/L)	Expected Conc EE2 in Sample (µg/L)	Conc EE2 Measured in Sample (µg/L)	Percent Difference (%)
0	0.0	0	0
200	18.2	0	-100
500	45.5	80.8	78
1000	90.9	111.1	22
2000	181.8	270.4	49
5000	454.5	584.3	28
10000	909.1	1075.1	18

In addition, when comparing samples spiked with a high (10,000 ug/L) and low concentration (500 ug/L) spike of EE2, it clear that the mass accuracy was lost in the sample

with the low level spike (Figure B.4). When spiked with 10,000 ug/L EE2, the mass accuracy of the EE2 detected was -13.06 ppm off of the actual mass, within 20 ppm, whereas the mass of the parent ion in the low spike was -85.9 ppm off from the actual mass.

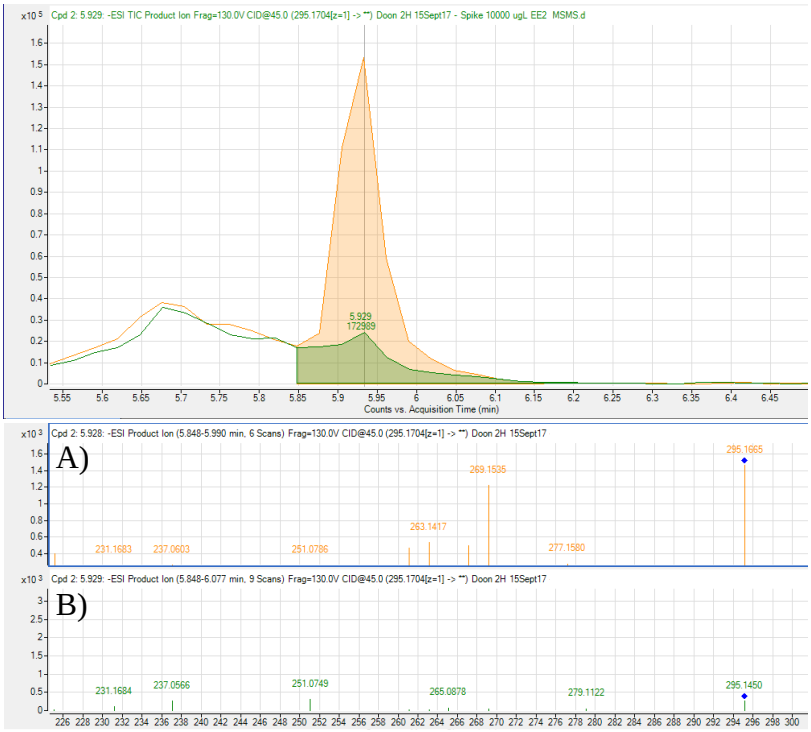

Figure B. 4 Mass accuracy on QTOF in samples with EE2 spikes. Environmental samples were spiked with a high concentration of 10 000 ug/L EE2 (A) and low concentration of 500 ug/L EE2 (B) to determine the mass accuracy of the parent ion detected by the QTOF when influenced by matrix.

The qualification of EE2 in environmental samples was based on three factors, the correct retention time (6.00 ± 1.00 min), a detected mass to charge ratio (m/z) within 20 ppm of the expected value (295.17041 m/z), and the correct product fragment ratios. As described in Chapter 3, select grab effluent samples analyzed from the Kitchener and Waterloo WWTPs measured EE2 concentrations over 100 ng/L. These concentrations are much greater than most previously reported effluent concentrations, therefore these samples were selected and further analyzed using the QTOF to verify the identity of the compound being quantified using the QQQ method described in Chapter 2. Samples with low levels of EE2 were also analyzed on the QTOF and are listed in Table B.6.

Table B. 6 Comparison of EE2 quantification using an LC-MS/MS QQQ and QTOF

Sample ID	EE2 Concentration (µg/L)		
	QQQ	QTOF	
		20 ppm	50 ppm
Doon 12Oct29	78.9	9.3119	20.4465
Doon 2 13Mar07	0	6.5812	6.4524
Doon 1 13Jun07	0.1	No Peak	No Peak
Doon 1 13Jul31	0.8	No Peak	3.7228
Doon 14Jul24	25.9	No Peak	35.0307
Doon 2 14Aug20	8.3	No Peak	9.9007
Doon 3H 14Sept25	11.3	10.0444	11.6133
Doon 2E 14Oct22	-	5.7656	5.75
Doon 2H 14Oct22	10.3	12.6303	25.5573
Doon 9H 15Jan21	-	No Peak	7.5455
Doon 1H 15Mar18	0	No Peak	No Peak
Doon 2H 15Sept17	0.03	No Peak	No Peak
Waterloo 2E 14Oct22	-	11.8693	19.1187
Waterloo 1H 15Jan21	54.4	48.1087	51.6716
Waterloo 2H 15Jan21	1.8	No Peak	5.8114
Waterloo 1H 15Feb11	0.4	4.2582	8.4035

When samples were analyzed on the QTOF peaks were quantified within a 20 ppm (295.1645 – 295.1763) and 50 ppm (295.1556 – 295.1852) range of EE2. Comparing the quantification results from the QQQ method to the QTOF showed very varied results. When analyzed using a 20 ppm restriction, many of the samples had no EE2 detected. These samples were ones that had low concentrations detected on the QQQ (<10 µg/L), except for the Doon 14Jul24 sample which was quantified at 25.9 µg/L. When the range was widened to 50 ppm, which brings the selectivity of the QTOF close to the selectivity of the QQQ, a peak was detected and quantified at 35 µg/L. The Doon 12Oct29 sample showed much lower concentrations using the QTOF method, whereas the Doon 2H 14Oct22 and Waterloo 1H 15Jan21 sample had similar concentrations using all methods.

This indicated that despite the QTOF is more selective and mass accurate, it is more susceptible to matrix effect and the mass accuracy is lost at the lower concentrations. Therefore, although this shows that historical samples with high concentrations of EE2, possible have high concentrations, it can not be said with certainty due to the matrix effects. This method also might not be the most ideal method for analyzing environmental samples since concentrations are generally low and mass accuracy is lost at those concentrations.

B.2.3 QQQ Sample Analysis

Historical samples with high concentrations of EE2 were re-run on the LC-MS/MS QQQ and quantified using a different transition to determine whether the instrument was detecting EE2 or a different compound. This was suggested by Ripolles et al. (2014) who found that the most intense fragment of EE2 (145 m/z) was found in the background and therefore resulted in increased results. The calibration curve and historical samples were quantifed using the fragments 145 m/z and 269 m/z to determine if there was a difference (Table B.7). Although the 269 m/z fragment is less intense, analysis of the calibration curve shows that it can give an accurate measure of EE2.

Comparisons of the samples between the two quantification methods shows that although there are some differences between the two methods, samples that had higher amounts of EE2 detected using the 145 m/z method generally had high levels of EE2 when quantified using the 269 m/z fragment as well.

Table B. 7 EE2 concentration measured on the LC-MS/MS QQQ using different quantification methods

		EE2 Concentration (µg/L)	
	Quantification Ion:	**145 m/z**	**269 m/z**
Calibration Curve	0 µg/L	0.00	0.79
	0.5 µg/L	0.39	1.01
	1 µg/L	0.76	1.36
	10 µg/L	8.29	7.89
	50 µg/L	27.63	27.44
	100 µg/L	95.98	94.37
	200 µg/L	194.23	195.37
	500 µg/L	533.51	534.65
Historical Samples	MS1-H July 7/15	21.91	22.75
	Spk Blank-H July 7/15	5.54	5.07
	Waterloo 2E_Oct24/14	1560.71	103.80
	Waterloo 1H Jan 21/15	30.51	47.86
	Waterloo 2H Jan 21/15	111.63	5.93
	Waterloo 1H Feb 11/15	0.49	1.37
	Waterloo Eff-1 Day 2 Aug 16/17	1.24	1.38
	Doon STP 1/3 Oct 29/12	21.76	21.17
	Doon 1 July 31/13	1.00	1.20
	Doon 2H Sep 25/14	27.40	10.90
	Doon 3H Sept 25/14	12.68	11.43
	Doon 2E Oct 22/14	28.00	9.55
	Doon 9H Jan 21/15	0.00	0.97
	Doon STP 1/3 Oct 29/12	22.14	20.33