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Research Article

Evaluating Changes in Contaminants of Emerging Concern in Municipal Wastewater Effluents Following Treatment Plant Upgrades

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Abstract

Contaminants of emerging concern (CEC) are known to affect aquatic organisms downstream of wastewater treatment plant effluent discharges. Studies in the Grand River watershed on the small-bodied, benthic rainbow darter (Etheostoma caeruleum) have shown altered gene expression, sex steroid levels, gonad size and expression of intersex (testis-ova) associated with wastewater outfalls. Due to these observed biological impacts, over $450M has been spent by the municipal government to upgrade the two major wastewater treatment plants (WWTP) within the Grand River watershed (Waterloo, Kitchener). In this study we monitored process upgrades at each of the WWTPs between 2010 to 2019 for a suite of chemicals including nutrients, CECs, hormones and total estrogenicity. Effluent samples for select CECs and total estrogenicity were analyzed by LC-MS/MS and yeast estrogen screen (YES) assay, respectively. Estrogenicity of the effluent declined rapidly after upgrades were completed. The removal of key CECs varied depending on their physiochemical properties. Although treatment process upgrades lead to greatly reduced environmental exposure to many CECs such as naproxen, some remain at relatively high concentrations (i. e. carbamazepine) that may continue to represent a risk to the environment.

Keywords: Municipal Wastewater Treatment Plant Effluent (WWTP); Chemicals of Emerging Concern (CECs); Hormones; Yeast Estrogen Screen (YES); Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS).

HIGHLIGHTS

- The upgrades to the treatment processes at both the Kitchener and Waterloo WWTPs significantly improved the overall quality of the effluent discharged into the Grand River.
- There have been notable decreases in nutrient and estrogen concentrations at both Kitchener and Waterloo WWTPs.
- The impact of WWTPs upgrades on CEC removal is compound-dependent and is influenced by the physiochemical properties of each CEC and the specific treatment process within each plant.
- CECs classified as readily biotransformed with low sorption potential (ibuprofen, naproxen) showed a significant decrease in the effluents of the Kitchener and Waterloo WWTPs after major upgrades to the treatment process.
- CECs that were classified as having low biotransformation potential and low sorption rates such as carbamazepine were highly recalcitrant in effluents even after upgrades.

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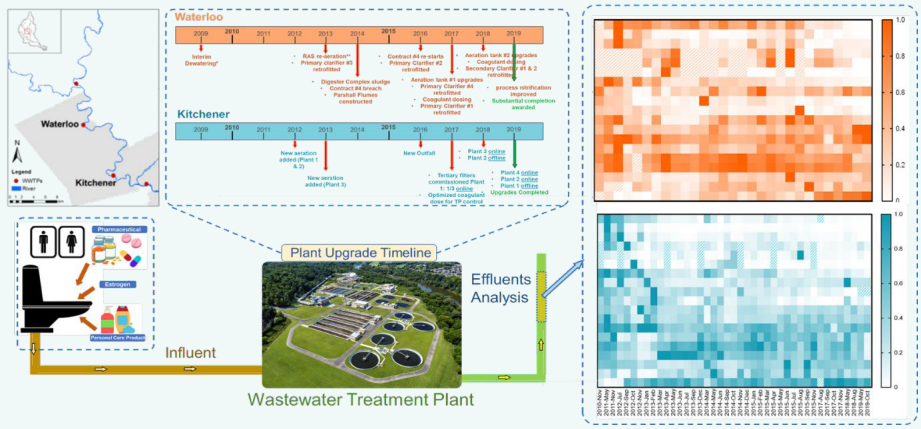
INTRODUCTION

Effluent discharges from municipal wastewater treatment plants (WWTPs) are a major source of contaminants of emerging concern (CECs) around the globe [1-3]. Although a variety of treatment processes can reduce their concentrations [4-6], many of these chemicals remain a concern for their possible impacts on aquatic ecosystems in the receiving waters [7,8]. Chronic exposure to low levels of CECs has been linked to adverse impacts in aquatic organisms worldwide [9,10], however direct links between specific chemicals and effects observed in the environment are difficult to establish.

Concentrations of CECs in effluents and the efficacy of CEC removal during wastewater treatment are dependent on the properties of the individual contaminant as well as the treatment processes employed [11-13]. In conventional WWTP, CEC removal is primarily dependent on sorption, biotransformation, and to a limited extent, chemical processes such as hydrolysis, volatilization and photolysis [13,14]. Sorption is the process by which many CECs are removed from wastewater through complexing with organic solids due to hydrophobic or electrostatic interactions [13]. The properties of the sludge (e.g. organic content, charge) can therefore modify removal [15]. CECs that readily biotransform in wastewater systems tend to be soluble in water, are good electron donors, and have sites amenable to biological attack, though many other factors can be important [16]. The degree of removal achieved by sorption and biotransformation for any CEC is thus driven by a combination of the CEC's physiochemical properties and by the treatment processes it undergoes.

In Canada, conventional activated sludge (CAS) systems are the most common type of secondary treatment in municipal WWTPs [17]. These systems are designed to remove organic contaminants through metabolic degradation by biological organisms followed by sedimentation in secondary clarifiers [18]. Within a CAS system, operational parameters, such as temperature, hydraulic retention time (HRT), solids retention time (SRT), and redox conditions, result in varied bacteria communities



Graphical Abstract

and degrees of nitrification [13,14].

Nitrifying conditions is a well established mechanism, in WWTPs and it have been shown to improve removal efficacy for a number of CECs [19-22]. Improved nitrification typically occurs alongside an increased SRT, resulting in a more diverse bacterial community that can more effectively remove readily biotransformed compounds, such as ibuprofen and naproxen [20-23]. Previous studies have shown that rapidly biotransformed CECs with low sorption potential typically have high removal efficacies, particularly in treatment plants with nitrifying treatment and SRTs above 5-7 days [13-25]. Improved removal efficacy under nitrifying conditions does not typically extend to recalcitrant compounds such as carbamazepine (bipolar and epilepsy treatment), diclofenac (non-steroidal anti-inflammatory drug NSAID, and sulpiride (schizophrenia treatment) [14-26]. These recalcitrant CECs are resistant to biological transformation unless advanced processes are used, e.g. advanced oxidation [13]. Compounds with moderate biotransformation capabilities vary widely in their removal depending on the operating parameters of the plant, with nitrification and increased SRTs typically associated with improved removal [13-21].

The Region of Waterloo invested approximately $450 million to upgrade the Waterloo and Kitchener WWTPs in order to improve effluent quality [27]. Prior to upgrades, the Kitchener and Waterloo WWTPs operated as conventional activated sludge plants with minimal or no nitrification. At both plants, upgrades have moved treatment towards extended, stable solids retention times (SRT from < 2 d to 5.4 d), nitrification, and UV disinfection, which is expected to greatly improve their effluent quality. Both Upgrades at the Kitchener and Waterloo WWTPs upgrades began in 2007 and were completed in 2019 (Figure 1). Although there was a disruption in treatment upgrades at the Waterloo WWTP, at all time the treatment plant met the regulatory effluent limit [27].

A number of studies in the Grand River watershed have measured fish responses to the changes in effluent quality as treatment upgrades progressed [29-34]. However, changes that occurred in the distribution of key CECs in the final effluents have not been systematically reported. This study documents the changes in nutrients, CECs and total estrogenicity over more than a decade at these two treatment plants as the process upgrades were implemented to include full nitrification. This work helps to better define how treatment upgrades shifted the composition of contaminants and led to improved fish health in the environment.

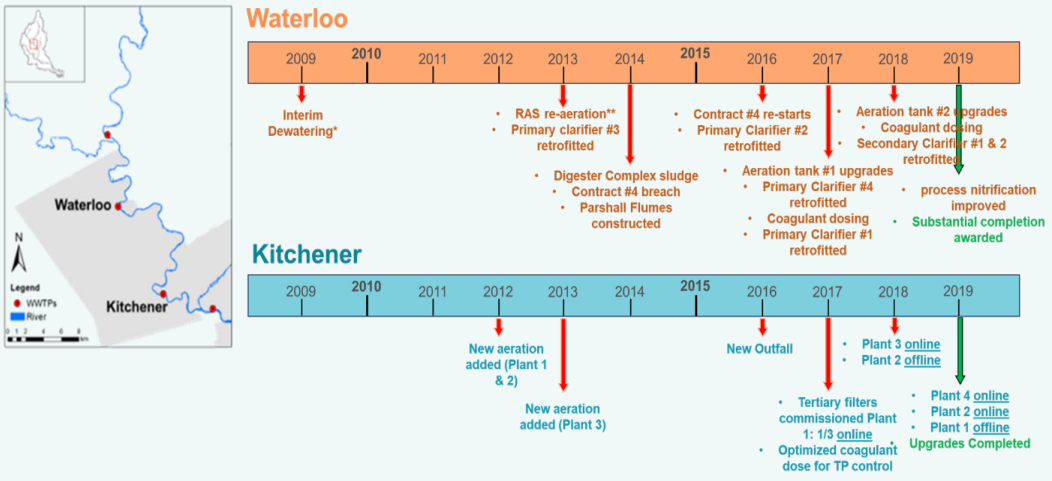


Figure 1: Timeline of major upgrades at the Kitchener and Waterloo WWTPs between 2009 and 2019, detailed information in SI [28].
*centrate sent to the Raw Sewage PS, aeration upgrades not completed, so temporary increase in effluent ammonia
** partially commissioned – required SCADA upgrades to be fully operational

MATERIALS AND METHODS

Materials

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

Select CECs were chosen based on anticipated removal mechanisms (Table 1) [35]. Atorvastatin and its metabolites, carbamazepine, diclofenac, fluoxetine, gemfibrozil, ibuprofen, naproxen, sulfamethoxazole, triclocarban, trimethoprim, venlafaxine, 4-nonylphenol, 4-octylphenol, estrone, 17α-ethynylestradiol, 17β-estradiol, estriol, lorazepam, and chloramphenicol were purchased from Sigma-Aldrich. Triclosan was purchased from Alfa Aesar (Wardhill, MA, USA). The isotopically labelled standards carbamazepine-d10, diclofenac-d4, fluoxetine-d5, gemfibrozil-d6, ibuprofen-d3, naproxen- d3, sulfamethoxazole-d4, triclosan-d3, trimethoprim-d3, triclocarban-d4, venlafaxine-d6, estrone-d4, estriol-d2, 17α-ethynylestradiol-d4, 17β-estradiol-d4, bisphenol A-d16, 4-nonylphenol-d4, 4-octylphenol d-17, and metformin-d6 were purchased from CDN Isotopes Inc. (Pointe-Claire, QC, Canada). Atovastatin-d5, o-hydroxy atorvastatin-d5, and p-hydroxy atorvastatin-d5 were from Toronto Research Chemicals (Toronto, ON, Canada). Stock solutions of all compounds were prepared in methanol. Descriptions of the physical and chemical properties of each CEC are described in the supplementary information (Table S1).

Yeast β-galactosidase assay kits were purchased from Fisher Scientific. All other reagents for use in the YES assay (yeast nutrient broth without amino acids, bactoagar, dextrose, copper II sulfate pentahydrate, adenine hydrochloride hydrate, L-histidine-HCl, L-arginine-HCl, L-methionine, L-tyrosine, L-isoleucine, L-lysine-HCl, L-phenylalanine, L-glutamic acid, L-aspartic acid, L-valine, L-threonine, L-serine, L-leucine, L-tryptophan, uracil) were purchased from Sigma-Aldrich.

Wastewater Effluent Sampling

Grab samples were collected in triplicate directly from the effluent outflow prior to release into the river at the Kitchener and Waterloo WWTPs at various time points from 2010 - 2019. These collections were related to different projects so were opportunistic across time. Details of each treatment plant before and after upgrades are listed in supplementary info (Tables S2 and S3). Samples were collected in 125 mL or 500 mL pre-cleaned amber glass bottles with Teflon-lined screw caps and preserved with 1 g/L sodium azide and 50 mg/L ascorbic acid to prevent bacterial growth and analyte degradation. Samples were stored at 4°C and extracted within 48h of collection. Additional samples were collected for nitrite, nitrate, and total nitrogen analysis, which were performed by Bureau Veritas Labs (Mississauga, ON, Canada) as outlined in Table S6.

Solid Phase Extraction

All samples were filtered through a glass fiber filter with a pore size of 1 µm (Pall Corporation, Mississauga, ON, Canada) prior to extraction. Isotopically labelled standards for each target CEC were added to samples prior to extraction. Samples for bioassays were not spiked. A ThermoFisher/Dionex AutoTrace™ (Sunnyvale, CA, USA) or a manual vacuum manifold were used to extract the samples. The solid phase extraction methods are outlined in Table S5. Briefly, all cartridges were preconditioned with solvents followed by water, and samples were passed through at a rate of approximately 5 mL/min. After elution, samples were evaporated to dryness under a gentle stream of nitrogen using a Dionex SE 500 solvent evaporator at 30°C (Sunnyvale, CA, USA). Samples were reconstituted in methanol (with internal standards, lorazepam and chloramphenicol). After extraction, samples were stored at -20°C until analysis. With each batch of samples three quality assurance/quality control (QA/QC) samples were processed; one negative control (blank) and two positive controls. All three QA/QC samples were prepared in MilliQ water. The blank was spiked with only isotopically labelled standards. The positive controls are identical replicates spiked with both isotopically labelled standards and unlabeled chemicals at a concentration of 20 µg/L.

Table 1: Selected CECs and their categorization based on expected mechanisms of removal in wastewater treatment [35].

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

LC-MS/MS Analysis

Analysis of pharmaceutical samples extracted for CEC analysis was performed with LC-MS/MS. Separation of analytes was completed at 0.8 mL/min on an Agilent 1200 HPLC using a 4.6 x 150 mm x 5 µm Agilent Zorbax Eclipse XDB-C18 column. Detection of analytes was completed using multiple reaction monitoring (MRM) on a Sciex 3200 QTRAP mass spectrometer (ABSciex, Concord, ON, Canada) with electrospray ionization (ESI). Samples were run in both positive and negative ion mode to identify all target analytes. Analytes were identified based on the transitions listed in Table S12. Analyst 1.6.2 software was used for data analysis. Source-dependent and compound-specific parameters are listed in Table S13. The mobile phases used for this analysis were 5 mM ammonium acetate in MilliQ water (A) and 100% methanol (B). The mobile phase gradient was dependent on which ion mode was selected and is described in Table S14.

Analysis of pharmaceutical samples from 2019 was completed on an Agilent 1260 HPLC and 6460 triple quadrupole (QQQ) mass spectrometer with an Agilent Jet Stream (AJS) electrospray ionization. The separation was done on a 2.1 x 50 mm x 1.8 µm Agilent Zorbax Eclipse XDB-C18 column at a flow rate of 0.3 mL/min with an injection volume of 10 µL (NEG) or 2 µL (POS) and the same mobile phases used as above. Method details are listed in Table S7.

Analysis of hormones was performed using an Agilent 1260 HPLC and 6460 triple quadrupole (QQQ) mass spectrometer with an Agilent Jet Stream (AJS) electrospray ionization. Chromatographic separation of hormones was completed using an Agilent Zorbax Eclipse Plus C18 (2.1 x 50 mm x 1.8 µm) HPLC column, held at 35°C, at a flow rate of 0.30 mL/min. Mobile phase A was 0.5 mM ammonium fluoride in water and mobile phase B was 100% acetonitrile. Mobile phase gradients are listed in Table S8. Analytes were identified based on the transitions listed in Table S9 and analyzed using Mass Hunter B.06.00 Source parameters for the individual compounds can be found in Table S10.

A series of calibration standards at concentrations of 0, 0.5, 1, 10, 50, 100, 200, and 500 µg/L were run prior to each batch of samples. Samples were quantified based on the ratio of analyte peak area to isotopically labelled standard peak area. The instrument detection (IDL) and quantification limits (IQL) were determined by running a series of blanks (n=7). The IDLs were reported as three times the standard deviation of the blanks. The IQLs were calculated based on ten times the standard deviation of the blanks. The method detection limit (MDL) was determined by running a series of wastewater samples that had been spiked with various concentrations of standards (0, 5, 10, and 50 ng/L). MDLs were calculated at a 99% confidence using a student's t-test value (n-1) multiplied by the standard deviation of 7 samples. The instrument detection and quantification limits as well as the method detection limit for each analyte are listed in Table S11.

YES Assay

Unspiked 500 mL samples were extracted for analysis of the total estrogenicity of the wastewater treatment plant samples. Buffers, materials and details for the Yeast Estrogen Screen (YES) assay are outlined in supplementary information and Table S15 and S16. Saccharomyces cerevisiae cells (Receptor: ERtrp (YePtrpER), Reporter E2.ura (YRpE2ura)) were provided by H. Engelhardt, University of Waterloo (originally from K. Gaido, Research Triangle Park, North Carolina, USA). The YES assay was previously validated for use on wastewater effluent samples from the Kitchener and Waterloo WWTPs [30].

Statistics

Statistical analysis was done in Sigma-Plot v. 13 (Systat Software, San Jose, CA). Dose-response curves for the YES assay were calculated with a four-parameter Hill equation. All error bars represent standard deviation as sample sizes were not equal. A Kruskal-Wallis One Way Analysis of Variance on Ranks was performed, followed by Dunn's test for pairwise comparison. Heat maps were graphed using GraphPad V 9.5.1. The concentrations were normalized by dividing the monthly concentration to the maximum.

RESULTS AND DISCUSSION

Select Nutrients before and after upgrades

Ammonia concentrations were significantly higher before upgrades were implemented in 2013 ($p < 0.001$) at the Kitchener WWTP, compared to post upgrade levels between 2013 and 2019 (Figure 2). Ammonia levels were consistently low from March – December 2013 but showed sudden increases occurring in the early spring months (March – May) in 2014 and 2015. Nitrate concentrations were significantly different between pre, during, and post upgrades ($p<0.05$) (Figure 2). Nitrite concentrations were significantly higher ($p<0.001$) prior to upgrades in 2013 ($p < 0.001$) (Figure S1).

In the Waterloo WWTP a high level of ammonia (9 – 37 mg/L) with substantial variability was observed from 2011 – 2015 (Figure 3). However, the total ammonia concentrations in the effluent decrease significantly ($p<0.001$) following upgrades to the infrastructure in 2015. Corresponding to the change in total ammonia, there was a significant increase in nitrate during all three phases of construction ($p<0.001$) (Figure 3) and no significant difference in nitrite ($p=0.301$) (Figure S1-Figure S5).

The upgrades to the Kitchener WWTP in late 2012 and early 2013, including improved aeration and secondary treatment train reconfiguration, were implemented to increase the SRT and introduce more diverse bacterial communities to the secondary treatment process [27]. Train 2 was upgraded to fully nitrify in 2012-13 but the smaller original Train 1 was not nitrifying. In 2018, the new Train 3 came online and Train 2 closed (to allow other construction). In 2019, a new Train 4 came online, Train 2 came back online and Train 1 was permanently taken offline. The resulting nitrification is reflected in a significant decrease in ammonia levels and increase in nitrate levels in the final effluents from the plant after upgrades were implemented with a major change occurring in 2012-13 (Train 2 nitrifying), although the final upgrades were completed in 2019. This is consistent with expectations as well as typical outcomes associated with improved aeration in CAS systems [27-36]. Increased nitrification is typically associated with higher quality treatment, and nitrifying plants have been shown to improve removal of a number of CECs and reduce effluent estrogenicity, although the mechanisms are not clear [19-21].

The Waterloo WWTP performed inconsistently from 2010-2015, with ammonia remaining high rather than dropping as nitrate increased. This was likely due to the disruptions related to construction at the Waterloo WWTP. Though a return activated sludge (RAS) system was implemented, upgrades to aeration were not complete, resulting in high ammonia loads even when partial nitrification was occurring. Between 2009 and 2014, the Waterloo WWTP received centrate from the biosolids dewatering system, with high concentrations of ammonia. Overloading the system with ammonia explains the increase in ammonia despite the implementation of improved treatment systems. Ammonia dropped in 2014 when a re-aeration zone was introduced to the RAS system. In 2017, the treatment plant was operating with two aeration tanks, providing year-round nitrification, which is reflected in the significant decrease in ammonia concentrations.

Select CEC before and after upgrades

The two NSAIDs (ibuprofen, naproxen) identified as having high biotransformation potential and low sorption potential were readily removed by the Kitchener WWTP after upgrades were implemented to

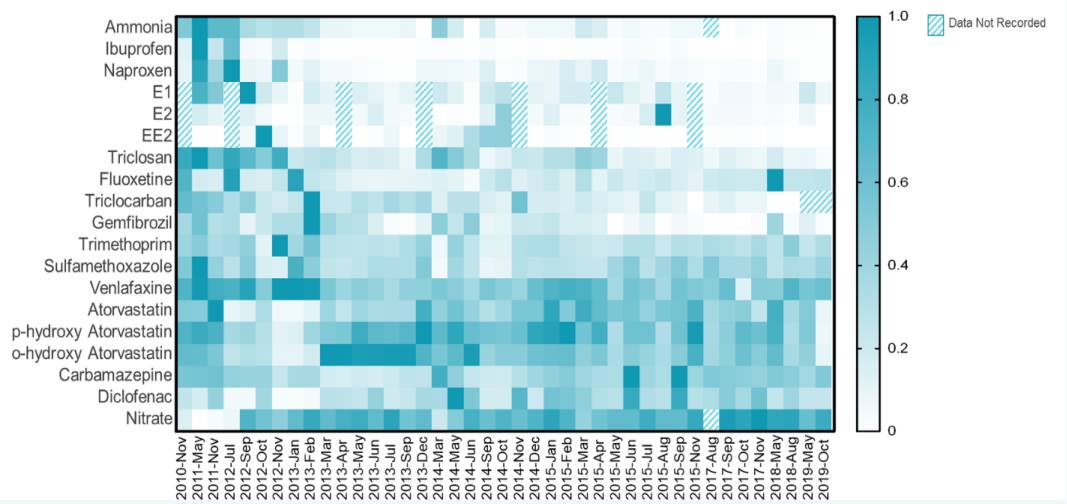


Figure 2: Heat map illustration of concentration of Select CECs, nutrients and estrone (E1), estradiol (E2), and ethinylestradiol (EE2) in the final effluent samples from the Kitchener WWTP since 2010. Scale shows the normalized concentration of each CEC. Data are available in Figure S1-S2.

Train 2 in 2012 (Figure 2). The concentration of ibuprofen in the Kitchener WWTP was significantly lower ($p < 0.001$) in 2013 - 2019 compared to pre-upgrade conditions. Similarly, the concentration of naproxen in the Kitchener WWTP effluent was significantly higher ($p = 0.005$) before upgrade (2010 – 2011) than during or after (2012 – 2019).

Triclosan (antibacterial agent) and fluoxetine (antidepressant) were identified as having both high biotransformation and sorption potential. The concentration of triclosan in the Kitchener WWTP effluents was significantly lower ($p < 0.001$) in 2013 - 2019 compared to pre-upgrade conditions. The concentration of fluoxetine was significantly lower ($p = 0.035$) than pre-upgrades in 2013 and 2014, but not in 2015. Triclocarban was identified as having a high sorption potential but low biotransformation potential. Its concentration in the Kitchener WWTP effluents was significantly reduced ($p < 0.035$) in 2014 and ($p < 0.001$) 2015-2019 compared to pre-upgrade conditions.

The four pharmaceuticals identified as having moderate biotransformation potential and low sorption potential (sulfamethoxazole (antibiotic), trimethoprim (antibiotic), gemfibrozil (cholesterol treatment), venlafaxine (antidepressant)) varied in their response to the WWTP upgrades. The concentrations of gemfibrozil were higher prior to 2013 and decreased significantly after the upgrades completed in 2019. The concentrations of trimethoprim were not significantly different ($p > 0.05$) across the years from 2010 – 2019. Sulfamethoxazole concentrations were reduced in Kitchener WWTP effluents ($p = 0.03$) in 2014 - 2015. The concentration of venlafaxine decreased significantly ($p < 0.001$) in 2013 and 2014 but not in 2015 compared to before when the upgrades were implemented.

Atorvastatin (cholesterol treatment) and its metabolites are moderately removed via biotransformation and sorption. Their concentrations in the Kitchener WWTP effluents were significantly reduced ($p < 0.001$) only while upgrades were being implemented (Sept 2012 – Feb 2013) compared to before-upgrade conditions. Levels of atorvastatin and its metabolites were also significantly higher ($p < 0.001$) in 2013 - 2015 than in 2012.

Carbamazepine and diclofenac which identified as having low sorption potential and low biotransformation potential were largely recalcitrant in the effluents even after upgrades. The concentration of carbamazepine in the Kitchener WWTP was significantly reduced ($p < 0.001$) only in 2013 compared to before upgrades occurred. The concentration of diclofenac in the effluent was not significantly different from 2010 to 2015 except for the period in 2012 to early 2013 while upgrades were being implemented, when concentrations were significantly lower ($p < 0.001$).

In Waterloo, effluent concentrations of ibuprofen were significantly lower ($p < 0.03$) in 2014 - 2019 than prior to 2014 (Figure 3). Effluent concentrations of naproxen were also significantly lower ($p = 0.025$) in 2019 compared to pre-2014 conditions. Triclosan concentrations in Waterloo were significantly lower ($p = 0.02$) in 2014 - 2019 ($p < 0.001$) compared to pre-2014 conditions. Fluoxetine was significantly lower ($p < 0.009$) in 2014 and 2015 ($p = 0.03$) compared to pre-2014. Triclocarban concentration in the Waterloo WWTP effluents were significantly reduced after 2012.

Sulfamethoxazole, trimethoprim, gemfibrozil, and venlafaxine also showed the highest variability of any group for the Waterloo effluent. Gemfibrozil concentrations decreased significantly ($p = 0.003$) in the effluent compared to both pre-2014 as well as 2014 conditions. Trimethoprim concentrations were reduced in Waterloo ($p < 0.04$) only in 2014. The concentration of sulfamethoxazole decreased significantly in the effluent ($p < 0.001$) in 2014 and 2015 compared to pre-2014 conditions. Venlafaxine concentrations did not significantly change in the effluent during the duration of the study period ($p > 0.05$).

Atorvastatin and its metabolites were significantly reduced ($p < 0.001$) in Waterloo WWTP effluents in 2014 and 2015 compared to pre-2014 conditions. Carbamazepine and diclofenac levels at the Waterloo WWTP in 2014 – 2019 were not significantly different than the pre-2014 before-upgrade conditions.

CECs that were classified as readily biotransformed with low sorption potential (ibuprofen, naproxen) showed a significant decrease in the effluents of the Kitchener WWTP after major upgrades to the treatment process. Fluoxetine and triclosan, which demonstrate both high biotransformation and sorption potentials, were likewise reduced after upgrades, though the concentration of fluoxetine was not significantly different in 2019 compared to before upgrade conditions. This is consistent with literature showing ibuprofen, naproxen, and other compounds with similar physiochemical properties that promote rapid biotransformation are readily removed from CAS systems with nitrifying treatment and an SRT of over 5 days [11-13]. Though there is no clear consensus on whether longer SRTs improve removal of highly sorbable CECs such as triclosan and fluoxetine, there is indication that sludge age could influence removal of CECs by sorption due to changes in the biomass (percent active fraction, specific microbial population) [11-37].

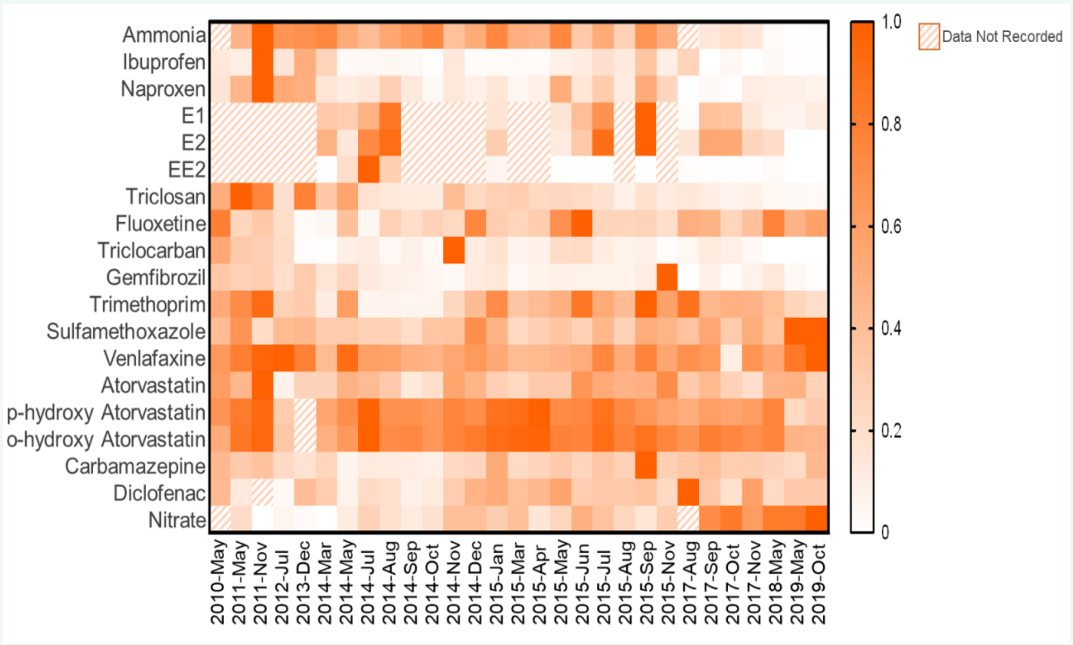


Figure 3: Heat map illustration of concentration of Select CECs, nutrients and estrone (E1), estradiol (E2), and ethinylestradiol (EE2) in the final effluent samples from the Waterloo WWTP since 2011. Scale shows the normalized concentration available in Figure S1 &S3.

Similar to the Kitchener WWTP, there was a reduction in compounds with high biotransformation potential (ibuprofen, naproxen, triclosan) in 2019 after the implementation of a RAS re-aeration zone at the Waterloo WWTP. Their presence in effluents at higher levels than in the Kitchener WWTP may be due to challenges associated with the operation of the RAS re-aeration process. Infrastructure upgrades completed in 2018 addressed the deficiencies of this process. The one CEC that typically shows low sorption and high biotransformation potential (triclocarban) was significantly reduced in effluents when the upgrades completed in 2019.

Unlike CECs with high biotransformation potential, those with moderate biotransformation potential showed high variability in removal between compounds within the same group. Those with moderate biotransformation and low sorption potential were responsible for this variability. While venlafaxine was significantly decreased in effluents in 2013 and 2014, sulfamethoxazole was only reduced in 2014, and trimethoprim and gemfibrozil showed no significant reduction over the years 2010 – 2015. Previous work shows CEC removal varying substantially based on the specific compound being investigated and the operating parameters of the WWTP [13].

Atorvastatin and its metabolites, which represented the moderate sorption and moderate biotransformation group, were only significantly reduced during the period where upgrades were coming online (Sept 2012 – Feb 2013), but not afterwards (March 2013 – November 2015). At the Waterloo WWTP the moderately biotransformed pharmaceuticals were also highly variable, with sulfamethoxazole significantly reduced in 2014 and 2015, trimethoprim reduced only in 2014, gemfibrozil reduced only in 2015, venlafaxine unchanged, and atorvastatin and its metabolite reduced in 2014 and 2015. The variability of the CECs during this time period is due to some challenges with the processes that were brought online at the time (Table 1 & 2 in SI).

CECs that were classified as having low biotransformation potential and low sorption rates were highly recalcitrant in effluents even after upgrades, with diclofenac significantly reduced only while upgrades were being implemented and carbamazepine significantly reduced only in 2013. Numerous studies have shown that both of these compounds are difficult to remove from wastewater even with advanced treatment in nitrifying systems with long SRTs [21-39].

Select hormones before and after upgrades

Concentrations of select estrogens were measured in the Kitchener WWTP effluent from 2011 to 2019, spanning the pre, during, and post upgrade periods (Figure 2). When comparing concentrations during these three time periods, at the Kitchener WWTP, there was a significant decrease in estrone (E1) ($p<0.001$) and estradiol (E2) ($p=0.021$) concentrations once upgrades were completed in 2019. The final E1 concentrations pre-upgrades were 65-75 ng/L and were decreased to 10-15 ng/L post-upgrades in 2019. Though there was no significant difference in ethinylestradiol (EE2) concentrations between 2014 and 2015, when the plant was undergoing upgrades there was a significant increase in EE2 ($p<0.001$). EE2 concentrations increased to over 100 ng/L during 2014 and decreased again after when the upgrades were completed in 2019. Similar trends in EE2 removal have been seen in other studies and was related to the upgrade methods [40-42]. Estrogen concentrations in the effluent from the Waterloo WWTP was measured from 2014 to 2019, spanning the start and completion of the upgrades to infrastructure and treatment (Figure 3). Once upgrades were completed in 2019, there was a significant decrease in E1 ($p=0.002$), E2 ($p<0.001$), and EE2 ($p<0.001$) concentration in discharged effluent.

Total estrogenicity of the effluent via YES assay

The estrogenicity of the Kitchener WWTP effluent (Figure 4) was significantly higher ($p < 0.001$) in 2010 and 2011 than 2012 – 2015. The estrogenicity of the Waterloo WWTP effluent was highly variable over the years 2009 – 2015 (Figure 4) and decreased after 2018, when the process upgrades were completed. When grouped similarly to the pharmaceutical data, pre-2014 was significantly higher ($p < 0.001$) than 2014, but not 2015. Fall 2018-2019 had the lowest estrogenicity and was significantly lower ($p < 0.001$) than any other year.

The total estrogenicity of the effluent as measured by the YES assay

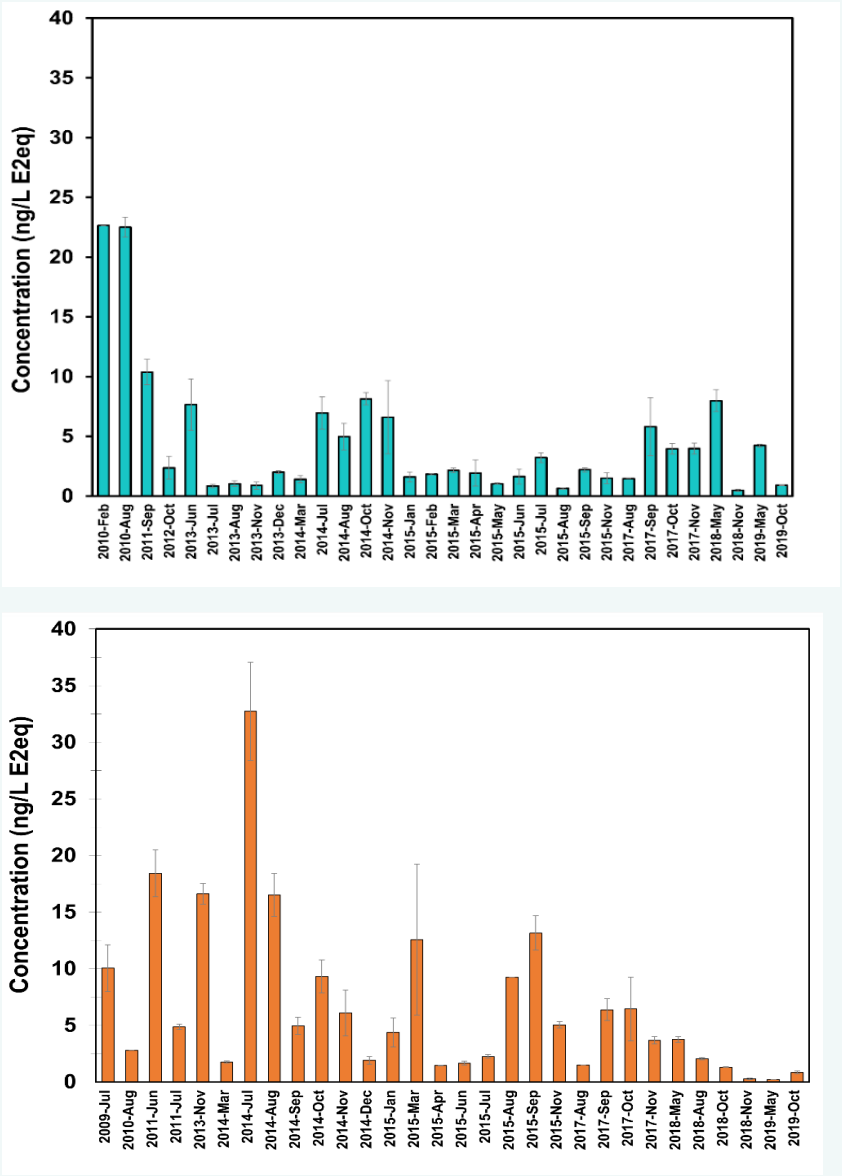


Figure 4: Total estrogenicity from the Kitchener and Waterloo WWTPs.

was significantly reduced after the implementation of upgrades at the Kitchener WWTP. Williams et al. [43], modeled the risk of endocrine disruption in over 10,000 reaches impacted by estrogenic WWTP effluents using the Predicted No-Effect Concentrations (PNECs) of E1, E2, and EE2 as well as their relative potency. Their parameters for low, moderate, and high risk were < 1.0 ng/L E2eq, > 1.0 ng/L E2eq, and > 10.0 ng/L E2eq respectively in impacted surface waters. Following this model, direct exposure to the levels of estrogenicity in the Kitchener WWTP effluents would put populations at high risk before upgrades were implemented, no risk in 2013, and moderate risk in 2014 and 2015. It is important to note that the dilution that occurs when effluent enters the receiving environment will reduce these concentrations in surface water. The increase in 2014 and 2015 may have been due to issues with the operation of the new aerators as well as disruption of operations due to ongoing construction at the WWTP [27]. The reduction in the estrogenicity of the WWTP effluents in association with upgrades to nitrifying treatment is consistent with literature [14-19]. As estrone and 17β-estradiol were two of the main species responsible for the estrogenicity of the Kitchener WWTP effluents [44] it is also consistent with other readily biotransformable CECs in the Kitchener WWTP, all of which showed significant reduction after upgrades with the lowest levels occurring in 2013. Of all the CECs that showed significant reduction when upgrades were implemented, total estrogenicity had the closest relationship with both total ammonia and nitrate concentrations in the effluents.

Total estrogenicity in the Waterloo WWTP effluents was highly variable prior to 2018 due to interruptions in the treatment upgrades (Figure 4). Total estrogenicity in 2014 and 2015 was lower when compared to pooled pre-2014 data but looking at year-to-year comparisons illustrates high variability between years before 2014. Fall 2009, 2010, and 2013 had high total estrogenicity (above 10 ng/L E2eq), while 2011, 2014, and 2015 were lower (above 1 ng/L E2eq but below 10 ng/L E2eq). The Waterloo WWTP has significantly lower daily flow

than the Kitchener WWTP, serving roughly half the number of people. This needs to be considered when predicting exposure and effects, such as intersex in fish, in the receiving environment.

However, it is clear that treatment changes reduced the exposure of some key CECs (such as estrogens) to fish downstream of the outfalls of and were associated with recovery of several endpoints, including intersex [28-34].There has been a decline in intersex in rainbow darter downstream of the Kitchener WWTP [32] and more recently the Waterloo treatment plant [34-49], consistent with the YES assay performed on these effluents which shows a significant decline in estrogenicity after the implementation of upgrades.

Principal Components Analysis

A principal components analysis (PCA) (Figure 5) was performed with the pharmaceutical and nutrient data in the Kitchener and waterloo WWTPs. Principal component 1 (PC1) in Figure 5A explained 42.2% of the variability and was primarily driven by total ammonia, followed by triclosan, ibuprofen, naproxen, and nitrate in approximately equal quantities in Kitchener WWTP. PC2 explained an additional 13.2% of the variability and was primarily driven by diclofenac, carbamazepine, and nitrate. For the Waterloo WWTP, PC1 explained 27.6% of the variability and was primarily driven by ibuprofen, naproxen, and venlafaxine. PC2 explained an additional 18.8% of the variability and was primarily driven by nitrate, sulfamethoxazole, and fluoxetine. It can be seen that in Kitchener WWTP PCA, the three-time period (before upgrades, during and after upgrades are clearly separated from one another when all CECs and nitrate ammonia concentrations in effluents are taken into account. However, the Waterloo WWTP PCA does not distinguish the time periods due to disruptions in treatment upgrades. More details for the PCA are provided in supplementary information (SI Table S14-26).

CONCLUSIONS

The presence of CECs in wastewater effluents during major upgrades to the treatment process was investigated at two treatment plants in the Grand River watershed. The results provide evidence that upgrades to wastewater treatment, decrease the concentrations of key CECs and the total estrogenicity in wastewater effluents. This work demonstrates that the impact of upgrades to wastewater treatment plants on CEC removal is compound-dependent and is influenced by the physiochemical properties of each CEC and the specific treatment process within each plant. The upgrades implemented in the Kitchener WWTP significantly reduced the presence of CECs that have high biotransformation potential and total estrogenicity of the effluents, while the disruption in treatment upgrades at the Waterloo WWTP is reflected in many of the CECs as well as total estrogenicity. By the completion of the Waterloo upgrades, the reduction in CECs and estrogenicity were similar to those at Kitchener. CECs that had high biotransformation potential were most significantly impacted by the upgrades implemented at the Kitchener WWTP, with all CECs in this category showing significant decline after their implementation. Moderately biotransformed CECs were highly variable, with some showing significant reduction after upgrades and others remaining recalcitrant in effluents. CECs that are slowly biotransformed remained recalcitrant in effluents. Total estrogenicity had the strongest relationship with ammonia and nitrate levels in the Kitchener WWTP, indicating an association with improvements to effluent quality. Based on the Kitchener WWTP PCA, the three time periods (before, during, and after upgrades) are separated from one another when all CECs and nitrate/ammonia concentrations in effluent are taken into account, and the months during which the WWTP experienced process upsets (March 2014, May 2014) are also distinct from the majority of post-upgrade samples.

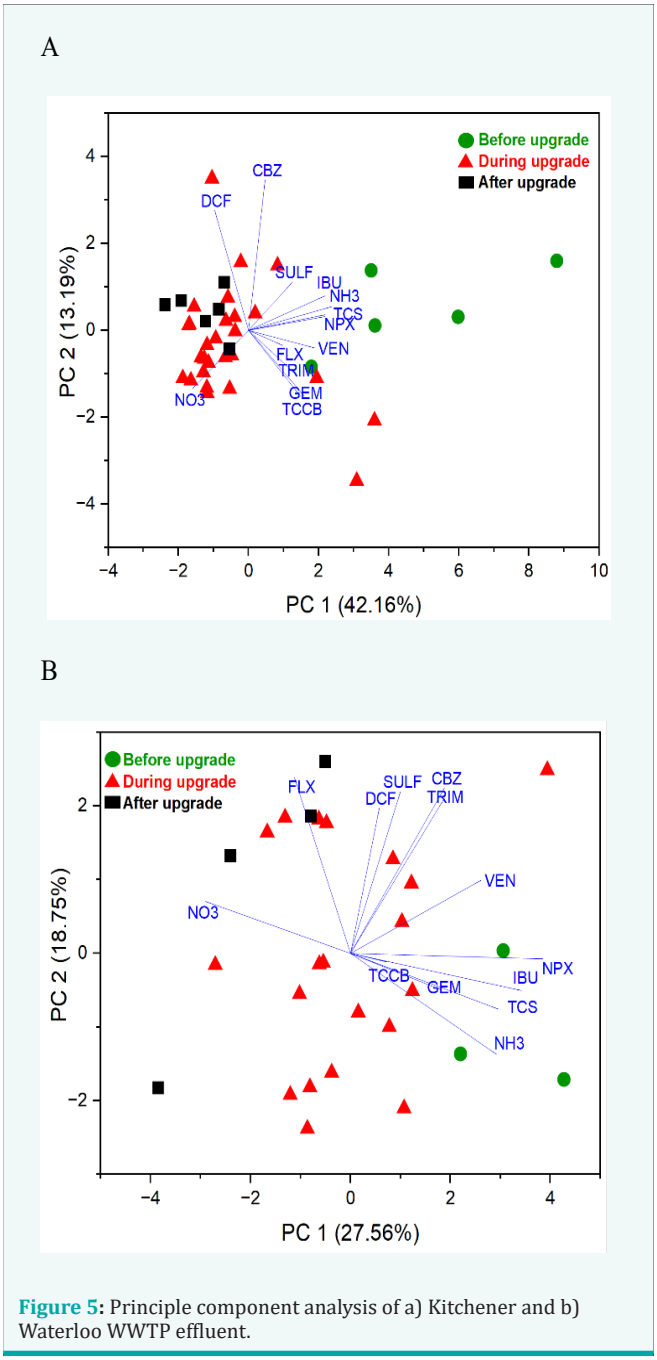


Figure 5: Principle component analysis of a) Kitchener and b) Waterloo WWTP effluent.

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