Investigation of the presence and endocrine activities of pesticides found in wastewater effluent using yeast-based bioassays

Paul Westlund, Viviane Yargeau

Department of Chemical Engineering, McGill University, Montreal, Canada, H3A0C5

Abstract

This study investigated the presence of a variety of pesticides (herbicides, fungicides, and insecticides) in effluent of three wastewater treatment plants as well as their endocrine activities using yeast-based *in vitro* assays. Although the presence of these contaminants of emerging concern are frequently reported to be present throughout the environment, their presence in wastewater treatment plants have been seldom studied. Of the 18 compounds investigated in this study, imidacloprid was the only compound not detected in all three WWTPs. Concentrations measured ranged from 3 ng/L to 27 ug/L for fluconazole. The yeast estrogenic and yeast androgenic screen assays were performed on target compounds in order to investigate their endocrine disruption and potential environmental risks to receiving waters. It was found that of the 14 compounds investigated 12 showed either antiestrogenic or antiandrogenic activity and seven compounds showed pleiotropic effects. In addition to confirming endocrine activities of pesticides using the yeast-based assays this study is one of the first to report activities for novel compounds including three neonicotinoids.

Keywords: pesticides, wastewater, endocrine disruption, *in-vitro* bioassays

Abbreviations
CEC's, contaminants of emerging concern; CPRG, chlorophenol red-β-D-galactopyranoside; DHT, dihydrotestosterone; E2, estradiol; EC_{50}, effective median concentration; IC_{50}, inhibitory median concentration; LC_{50}, lethal median concentration; ONPG, ortho-Nitrophenyl-β-galactoside; WWTP, wastewater treatment plant; YAS, yeast androgen screen; YES, yeast estrogen screen

1.0 Introduction

While urban and industrial wastewater treatment continues to meet current legislation on water quality standards there has been many recent studies with overwhelming evidence demonstrating the incomplete removal of contaminants of emerging concern (CECs) (Bollmann et al., 2014; Lindberg, Östman, Olofsson, Grabic, & Fick, 2014; Loos et al., 2013). These emerging contaminants are not yet regulated and include a variety of compounds such as pharmaceuticals, personal care products, hormones, and industrial chemicals. These compounds often end up at wastewater treatment plants (WWTPs) via different pathways including combined sewer systems. The incomplete removal of CECs at WWTPs have been shown to be a significant contributor of micropollutants entering aquatic environments resulting

in an increase of water pollution (Kock-Schulmeyer et al., 2013; Loos et al., 2013; Morasch et al., 2010; Muller, Bach, Hartmann, Spiteller, & Frede, 2002).

A group of CECs that has received less attention in comparison to pharmaceuticals and personal care products regarding the occurrence in WWTPs are pesticides. This may be due to the popular belief that the diffuse of agricultural field-run off containing pesticides is more relevant for the contamination of aquatic environments (Loos et al., 2013). The limited studies that do focus on the fate and occurrence of pesticides in conventional WWTPs have reported concentrations up to μg/L range as well as negative or low removals for a variety of compounds including atrazine, mecoprop, propiconazole, and prochloraz among others (Kock-Schulmeyer et al., 2013; Masiá et al., 2013; Morasch et al., 2010). These studies have consistently highlighted the importance of monitoring these compounds and as noted by Kock-Schulmeyer et at., (2013) the contamination of aquatic environments by pesticides in urban areas cannot be neglected.

A major concern for pesticides present in effluent wastewater is their potential indirect bioactivity (as a parent structure or their related transformation products) towards aquatic species. In comparison to investigating the toxicity, studies focusing on endocrine disruption of pesticides are less established and for some compounds completely absent with most of the published literature focused on legacy compounds such as DDT, which are no longer registered for use (F. Orton, Rosivatz, Scholze, & Kortenkamp, 2011). Although there have been several bioassays developed for monitoring endocrine activity of environmental samples and other CECs, most studies have an emphasis on measuring estrogenic activity with limited focus on potential androgenic effects (Kojima, Katsura, Takeuchi, Niiyama, & Kobayashi, 2003; F. Orton et al., 2011; Sohoni & Sumpter, 1998; Urbatzka et al., 2007). In this context an analysis conducted by Kojima et al. (2004) using the *in vitro* CHO-K1 assay showed that many organochlorines and organophosphorous had both estrogenic and antiandrogenic activities, highlighting the importance of measuring both androgenic and estrogenic activities of CECs.

The main objective of this study was to assess the occurrence of a variety of fungicides, herbicides, and insecticides in WWTP's as well as their potential estrogenic and androgenic activities using yeast-based *in vitro* assays. Three WWTPs located in the vicinity of Montreal, Canada were sampled, eighteen chemicals were monitored and four different endocrine activity endpoints were conducted (estrogenic, anti-estrogenic, androgenic and anti-androgenic) for each individual target pesticides.

2.0 Material and Methods

2.1 Target compounds:

Analytical standards of all target chemicals and internal standards are listed in Table 1 (purity >99%). Positive controls required for yeast-based *in vitro* assays were flutamide (>99% pure) and β-Estradiol (E2) (98% pure) purchased from Sigma-Aldrich, 4-Hydroxytamoxifen (>97% pure) purchased from Abcam, and Dihydrotestosterone (DHT) (98% pure) purchased from Steraloids Inc. DHT and

flutamide were used as positive controls in the YAS assay and E2 and 4-Hydroxytamoxifen were used as positive controls in the YES assay.

Stock solutions for each pesticide were made to 0.2M in ethanol, methanol, or DMSO based on their solubility in organic solvents. Stock solutions for propiconazole, myclobutanil, dicamba, 2,4-D, and mecoprop were made in EtOH. Stock solutions for climbazole, tebuconazole, atrazine, and diuron were made in DMSO. Stock solutions for irgarol and terbutryn were made in MeOH. Neonicotinoid stock solutions were made to 10,000 mg/L in MeOH. In cases where no labeled surrogates were available at the time of chemical analysis, a qualitative analysis was performed.

Table 1: Target compounds analyzed by LC-HRMS, molecular weights (MW; g/mol), and internal standards used for quantification.

2.2 Sampling sites:

Wastewater samples were taken from the effluent of three WWTPs from Southern Quebec, Canada between the months of April to September 2015. 24hr composite samples were collected in amber bottles at treatment plants and stored at -4^{o}C until they were filtered and extracted for chemical analysis.

The WWTP's studied receive mainly domestic wastewater and urban runoff. WWTP 1 has advanced primary treatment and receives an average flow rate of 2.0E06 m^3/day from approximately 40% separate and 60% combine sewers providing treatment services for a metropolitan area consisting of an approximate population of 1.6E06 people. WWTP 2 has secondary activated sludge treatment and receives an average flow rate of 5.6E04 m^3/day providing treatment services for a rural area consisting of an approximate population of 5.0E04 people. The influent load comprises of approximately 75% industrial and 25% residential sources from 10% separate and 90% combined sewers. WWTP 3 has secondary activated sludge treatment and receives an average flow rate of 6.5E04 m^3/day providing treatment services for an urban area consisting of an approximate population of 9.3E04 people. The influent load comprises of approximately 55% industrial and 45% residential sources.

2.3 Sample preparation:

The analysis of pesticides in wastewater effluent samples was performed after pre-concentration by solid phase extraction. Briefly, the wastewater effluent samples were vacuum filtered and the pH was adjusted for the corresponding extraction process, pH=8 and pH=2.5 for MAX and MCX, respectively. Each 110 mL sample was spiked with 100 µL of 400 µg/L equimolar mixture of surrogates (Table 1) to reach a post-extraction concentration of 100 µg/L. The samples were extracted in triplicates using two types of Oasis cartridges, MAX and MCX. The use of both cartridges allows for the extraction of both basic, neutrals and acidic compounds of interest and has been proven to effectively extract pesticides in various

environmental matrices. The MAX cartridges were pre-conditioned using a mixture of methanol, sodium hydroxide, and distilled water. The MCX cartridges were pre-conditioned using a mixture of acetone, methanol, and distilled water. To obtain high recoveries the extraction flow rate was kept below 5 mL/min. After the samples had been extracted the elution step consisted of eluting 3 mL of a mixture consisting of 2% formic acid in methanol for MAX cartridges and 3 mL of a mixture consisting of 2% ammonium hydroxide in methanol for MCX cartridges. The elution step for both MAX and MCX cartridges were performed in triplicates with a flow rate of 1 mL/min. The organic solvents were evaporated under vacuum and reconstituted in 0.4 mL of HPLC grade methanol.

2.4 Chemical analysis:

All extracts were analyzed by liquid chromatography using an Accela 600 system coupled to a mass spectrometry Thermo Scientific LTQ XL equipped with a high resolution Orbitrap detector. Column configuration consisted of a Thermo in-line filter hardware unit with a 2.1mm ID and 0.2µm filter cartridge PN: 22180 (Bellefonte, PA. USA) followed by an Agilent UHPLC guard column Zorbax Eclipse plus C18 2.1 x 5mm and 1.8µm PN: 821725-901 while resolving of the pesticides was carried on an Agilent analytical column Zorbax Eclipse plus C18 RRHD 2.1 x 5mm and 1.8µm PN: 959757-902 (Santa Clara, Cal. USA). The initial mobile phase composition was aqueous 2mM ammonium formate 0.1% formic acid buffer and methanol 0.1% formic acid at a 85:15 reaching 17.5%B at 2.5min followed by a ramp to 100%B in 4.5min and held for 3 additional minutes. Column buffer composition was then brought immediately to initial conditions for equilibration. 25 µL of sample or its dilution were injected for a total run time of 15min at a constant flow rate of 0.3 mL/min. MS detection was performed by FT-IT positive and negative modes. Optimization of the instrument parameters was performed by direct infusion of standard solutions at 10 µL/min, while source optimization conditions was done by infusion flow analysis. Nitrogen gas was used for all sheath, auxiliary and sweep gasses, while helium gas was used as the collision gas. The LODs and LOQs for the target analytes in wastewater are listed in supplemental material.

2.5 Yeast estrogen screen assay:

A recombinant yeast strain (*Saccharomyces cerevisiae*) that had been stably transfected with the human estrogen receptor (hERα) and an expression plasmid carrying the reporter gene *lac-Z* was used to perform the assay (Routledge & Sumpter, 1996). Techniques for yeast culturing and YES assay procedures were performed as described in Beresford, Routledge, Harris, and Sumpter (2000); Routledge and Sumpter (1996) with minor adjustments.

Each 96-well plate contained a triplicate 12 serial dilution of a sample of interest and separate rows consisting of a solvent blank, yeast culture control, and a 12 serial dilution of a positive control (E2). The validity of the YES assay was confirmed by ensuring that a median effective concentration (EC_{50}) of 3.55nM (n=24) was obtained for the E2 control, as reported in previous studies (Larcher,

Delbes, Robaire, & Yargeau, 2012). Rows containing samples were prepared by reconstituting in their appropriate solvents, transferring 10, 25, or 50 μL to assay plate, evaporating, and reconstituting in assay medium enriched with yeast and CPRG. Rows containing E2 standard were prepared similarly. The plates were sealed with film and shaken for two minutes and placed in an incubator for 72 hrs. After the incubation period, the plates were transferred to a plate reader where the absorbance was measured.

2.6 Yeast anti-estrogen screen assay:

The yeast anti-estrogenic screen assay was performed using the same techniques as previously described for the YES assay with the addition of minor modifications. The anti-estrogenic activity of samples was measured by quantifying the suppression of β-galactosidase activity induced by an E2 standard. An E2 standard concentration of 5nM, which results in a sub maximal response of estrogenic activity at the receptor, was added to each well of the 12 serial dilution of sample. A dose-dependent decrease in E2 activity indicates anti-estrogenic activity. Each 96-well plate contained triplicates of 12 serial dilutions of samples, and separate rows consisting of a solvent blank, 5nM E2 control, and a 12 serial dilution of a positive control. The validity of the anti-estrogenic screen assay was confirmed by selecting 4-Hydroxytamoxifen as a reference compound and positive control, which maintained a median inhibitory concentration (IC_{50}) of 1.10 μM (n=23). These results are within a range of concentrations previously reported (0.1-2.1 μM) (Buckley, 2010). The serial dilution of samples was prepared as previously described, however, the evaporated wells were reconstituted with assay medium enriched with yeast, CPRG and spiked with E2. Rows containing 4-Hydroxytamoxifen were prepared similar to that of E2 standard, but were reconstituted with yeast spiked with E2. The plates were sealed with film and shaken for two minutes and placed in an incubator for 72 hrs. After the incubation period, the plates were transferred to a plate reader where the absorbance was measured.

2.7 Yeast androgen screen assay:

A recombinant yeast strain (*Saccharomyces cerevisiae*) that had been stably transfected with the human androgen receptor (hAR) and an expression plasmid carrying the reporter gene *lac-Z* was used to perform the assays (Sohoni & Sumpter, 1998). Techniques for yeast culturing and YAS assay procedures were performed as described in Beresford et al. (2000); Routledge and Sumpter (1996) with minor adjustments.

The YAS assay plates were constructed in a similar fashion as described for the YES assay, such that each 96-well plate contained a triplicate 12 serial dilution of a sample of interest and separate rows consisting of a solvent blank, yeast culture control, and a 12 serial dilution of a positive control. The validity of the YAS assay was confirmed by selecting DHT as a reference compound and positive control,

which maintained a median effective concentration (EC_{50}) of 2.35 nM (n=28), in the range of values previous reported (Akram et al., 2011; Larcher et al., 2012; Svobodova & Cajthaml, 2010). Rows containing samples were prepared by reconstituting in their appropriate solvents, transferring 10, 25 or 50 µL to assay plate, evaporating, and reconstituting with 200 µL of yeast culture. Rows containing controls were prepared similar to that of samples. The plates were sealed with film and shaken for two minutes and placed in an incubator for 24 hrs. After 24 hrs of exposure an assay buffer was prepared containing ONPG. The incubated plates containing the exposed yeast were then reconstituted and 100 µL is transferred to a final plate where 100 µL of the assay buffer is added. The plates were shaken for two minutes and incubated for 30 minutes until color change is observed and transferred to a plate reader where the absorbance was measured.

2.8 Yeast anti-androgen screen assay:

The yeast anti-androgenic screen assay was performed using the same techniques as previously described for the YAS assay with the addition of minor modifications. The anti-androgenic activity of samples was measured by quantifying the suppression of β-galactosidase activity induced by a DHT standard. A DHT standard concentration of 10nM, which results in a sub maximal response of androgenic activity at the receptor, was added to each well of the 12 serial dilution of sample. A dose-dependent decrease in DHT activity indicates anti-androgenic activity. Each 96-well plate contained triplicates of 12 serial dilutions of samples, and separate rows consisting of a solvent blank, 10nM DHT control, and a 12 serial dilution of a positive control. The validity of the anti-androgenic screen assay was confirmed by selecting flutamide as a reference compound and positive control, which maintained a median inhibitory concentration (IC_{50}) of 164.84 µM (n=27). Although the experimentally obtained IC_{50} for flutamide is higher than values reported in literature (6.1 µM Chatterjee, Majumder, and Roy (2007)), results for flutamide were consistently reproducible. The serial dilution of samples was prepared as previously described, however, the evaporated wells were reconstituted with yeast culture spiked with the DHT standard. Rows containing flutamide standard were prepared similar to that of DHT standard, but were reconstituted with yeast spiked with DHT standard. The plates were sealed with film and shaken for two minutes and placed in an incubator for 24 hrs. The plates were shaken for two minutes and incubated for 30 minutes until color change is observed and transferred to a plate reader where the absorbance was measured.

2.9 Cytoxicity assay:

Considering the large range of concentrations of target pesticides, a Microbial Viability Assay Kit-WST purchased from PromoKine was used to identify concentrations that were cytotoxic to the yeast. The wells that show cytoxicity may lead to inhibition responses that are not caused by the compounds interaction at the receptor site, but rather the result of no living yeast cells. The viability assay was

performed in two separate experiments as directed in the technical manual provided with the kit. The dilutions that showed cytotoxicity were not considered in the determination of endocrine activity for pesticides. The results for the viability assay are provided in the supplemental material (Figure S.2).

2.10 Data analysis:

Data analysis for YES and YAS assays were completed using the same calculation method. Briefly, absorbance values obtained by a microplate reader were corrected for yeast turbidity within the plates (in the yeast control) and normalized to positive controls for the determination of endocrine activity.

The corrected absorbance for the YES and YAS assays were determined by the following equations:

$$\text{YES Corrected Value} = \text{Sample}_{540nm} - (\text{Sample}_{630nm} - \text{Blank}_{630nm})$$

$$\text{YAS Corrected Value} = \text{Sample}_{415nm} - (\text{Sample}_{595nm} - \text{Blank}_{595nm})$$

The agonist endocrine activity of the samples for the YES and YAS assays were determined by dividing the corrected absorbance values of the sample by the corrected absorbance of the positive control that represents 100% activity (highest stable absorbance).

$$\text{Estrogenic Activity (\%)} = (\text{Sample}_{\text{corrected absorbance}} / \text{E2}_{\text{corrected absorbance}}) * 100$$

$$\text{Androgenic Activity (\%)} = (\text{Sample}_{\text{corrected absorbance}} / \text{DHT}_{\text{corrected absorbance}}) * 100$$

The antagonist activity for the YES and YAS assays for each sample were obtained in a similar fashion as the agonist activity. Corrected absorbance of the sample was divided by the positive control, however, the positive control is the corrected absorbance values for 5nM E2 and 10nM DHT for the YES and YAS assays, respectively.

Using OriginPro software a sigmoidal dose response curve for each plate was constructed by plotting the endocrine activity percentage over a serial logarithmic concentration. EC_{50} or IC_{50} values were only determined when a full dose response curve was observed. Quantification of a full or partial antagonist activity should not be made for a partial dose curve (no established lower or upper asymptote) as a result of inaccuracies for EC_{50} or IC_{50} values (Campbell et al., 2012). To compare all compounds, including those without a complete dose-response curve (ie. without and EC_{50} or IC_{50} value), the endocrine activities were compared qualitatively after being classified as having a slight activity (+) for compounds having partial dose responses and a moderate activity (++) when compounds had a full dose response curve.

3.0 Results and Discussion

3.1 Presence of pesticides in wastewater treatment plant effluents:

Of the 18 compounds investigated in this study, imidacloprid was the only compound not detected in all three WWTPs (Table 2). WWTP 1 effluent contained 17 compounds of interest while effluent samples from WWTP 2 and WWTP 3 contained 13 and 14 compounds, respectively. All fungicides except climbazole and myclobutanil were quantifiable and/or detectable in the three WWTP's, where climbazole was not detected in WWTP 2 and myclobutanil was not detected in WWTP 3 samples. 2,4-D and mecoprop were the only two herbicides not detected in WWTP 2 & 3 samples. In regards to the presence of neonicotinoids thiamethoxam was only present in WWTP 1 effluent sample, thiacloprid was present in the effluent samples of the other two plants, and acetamiprid was detected in all three effluent samples. In addition to containing the majority of compounds investigated, WWTP 1 effluent sample had the highest concentrations measured for propiconazole (1815 ng/L), 2,4-D (751 (ng/L) and atrazine (44.1 ng/L), while the concentration for dicamba (184 ng/L) was comparable to what was found in WWTP 2 (184 ng/L).

Table 2: Measured concentrations of pesticides in three WWTPs sampled and comparison to values reported in literature.

Of the azole compounds investigated in this study (fluconazole, clotrimazole, climbazole, propiconazole, tebuconazole, prochloraz, and myclobutanil) fluconazole was most prominent and found in all three WWTPs at concentrations of 27606 ng/L in WWTP 2, 23324 ng/L in WWTP 3, and 1348 ng/L in WWTP 1 effluent samples. These results are approximately 35 times higher than reported in literature (Kahle et al., 2008; Lindberg et al., 2014; Loos et al., 2013) suggesting there are additional inputs (e.g. industrial and agricultural) of this antifungal in waterways.

As pointed out by previous studies, limited information is available on the occurrence of pesticides in WWTPs in comparison to other CECs such as pharmaceuticals and personal care products (De la Cruz et al., 2012; Loos et al., 2013; Singer et al., 2010). Dicamba was present at all three WWTPs sampled with concentrations ranging from 116 ng/L to 184 ng/L and myclobutanil was detected in WWTP 2 and WWTP 1. Although myclobutanil has not been reported in literature for wastewater effluents it has been identified in storm water runoff along with propiconazole, tebuconazole and atrazine (Morace, 2012). Since all three WWTPs studied are combined systems, the presence of pesticides in storm water runoff suggests this is an important pathway of contaminants entering in wastewater systems and may also provide an explanation for the high levels of fluconazole observed in the present study. Recently it has been reported that biocides are found to leach out of fungicide treated wood both in dry and wet conditions and detected in combined sewage systems (Bollmann et al., 2014).

A group of insecticides that has received increased attention due to their potential toxic effects on indirect organisms and rise in global usage are neonicotinoids. A recent study determined that 74% of global surface waters exhibited concentrations of individual neonicotinoids (Morrissey et al., 2015;

Sadaria, 2016) yet few studies have focused on the their presence in wastewater. No literature was found detecting thiamethoxam in wastewater effluent with limited published data on acetamiprid, imidacloprid, and thiacloprid. A recent study identified acetamiprid, thiacloprid, and imidacloprid in wastewater effluents and concluded that neonicotinoids showed the highest ecological relevance of the composition of invertebrate communities (Münze et al., 2017). Of the four neonicotinoids selected for the present study, three compounds (thiamethoxam, thiacloprid, and acetamiprid) were detected in at least one WWTP indicating the need for further investigation regarding their prevalence in wastewater treatment. To the best of our knowledge this is the first study to detect thiamethoxam in wastewater effluent.

3.2 YES/YAS results:

Each pesticide was tested in two or more separate assay runs that contained triplicate serial dilutions of target compounds resulting in a $n > 6$ for each compound. The assays were validated using positive controls and a cytoxicity test was performed to investigate the cause of inhibitory responses of compounds in the Anti-YES and Anti-YAS assays. It was determined that the first two dilutions of each fungicide and herbicide with an initial concentration of 0.2M showed cytoxicity, while the three neonicotinoids showed no signs of cytoxicity. Taking into consideration results obtained from the microbial viability assay, the concentrations within the dilution range of each compound that showed cytoxicity were removed from the analysis and the analysis considered only the range of concentration for which any inhibited activity is the result of the suppression of β-galactosidase.

Endocrine activities were investigated for all compounds by plotting percent activity over the log concentration of pesticides. Most of the pesticides showed antagonist activities at either the estrogen or androgen receptor (Figure 1) and no pesticide showed agonist activities (provided in supplemental material, Figure S.1). The lack of agonist activities is in agreement with results obtained in recent studies that performed the YES and YAS bioassays for of subset of the target compounds: 2,4-D, diuron, mecoprop, atrazine, and irgarol (Table 3) (Kojima et al., 2003; Noguerol et al., 2006; Frances Orton, Lutz, Kloas, & Routledge, 2009b). Upon review of the antagonist activities of fungicides (Figure 1a,b), herbicides (Figure 1c,d), and neonicotinoids (Figure 1e,f) most compounds have a partial inhibitory response making it challenging to accurately determine IC_{50} values. IC_{50} values were determined for 2,4-D, diuron, dicamba, and mecoprop using the YES assay and 2,4-D, mecoprop, terbutryn, and acetamiprid using the YAS assay. The remaining compounds investigated either had partial or no inhibitory response. To compare the endocrine activities of all pesticides, the inhibitory responses were summarized qualitatively, which slight activity (partial inhibition) and moderate activity (full inhibition) being represented by + and ++, respectively (Table 3). No comparable data are available in literature.

Figure 1 *Antiandrogenic activity (Figure 1a) and antiestrogenic activity (Figure 1b) of fungicides, antiandrogenic activity (Figure 1c) and antiestrogenic activity (Figure 1d) of herbicides, antiandrogenic activity (Figure 1e) and antiestrogenic activity (Figure 1f) of neonicotinoids. Figure 1a and 1b share the same legend for target compounds, Figure 1c and 1d share the same legend for target compounds, and Figure 1e and 1f share the same legend for target compounds. Error bars are standard of error of triplicate samples.*

Table 3: YES and YAS results obtained for individual compounds and comparison to bioactivities reported in literature. Bioactivities measured are agonist and antagonist activities at the estrogenic and androgenic receptor. The activities are represented as qualitative results as a result of partial dose responses.

To the best of our knowledge this is the first study to investigate the estrogenic and androgenic activity using *in-vitro* based bioassays for climbazole, dicamba, terbutryn, acetamiprid, thiacloprid, and thiamethoxam. The results from this study showed that twelve out of fourteen compounds elicited antagonist responses in either the YES and/or YAS assay. Two fungicides (myclobutanil and propiconazole) and five herbicides (2,4-D, dicamba, diuron, mecoprop, and terbutryn) showed pleiotropic effects, acting as both antiestrogenic and antiandrogenic. This pleiotropic behavior has been documented for other pesticides not selected for this study including pyrethroids and other fungicides and herbicides (Kojima et al., 2003; Li & Gramatica, 2010; Frances Orton et al., 2009a).

Two compounds (climbazole and atrazine) showed no observable activity either as an agonist and antagonist for both YES and YAS assays within the range of concentrations studied. The results for atrazine are in agreement with non-effective activities found using both yeast (Hurst & Sheahan, 2003; Frances Orton et al., 2009b) and other *in vitro* cell-based assays (Aït-Aïssa et al., 2010; Kojima et al., 2003). No literature was found measuring the estrogenic or androgenic activity of climbazole using *in vitro* assays.

When full dose response curves were observed the IC_{50} values of compounds were determined. Using the YES assay, IC_{50} values were obtained for 2,4-D (257.0 μM), diuron (31.4 μM), dicamba (1048.0 μM), and mecoprop (88.4 μM). Frances Orton et al. (2009) reported an IC_{50} value for diuron (0.93-31.3 μM) and mecoprop (1000 μM) using the YES assay. Diuron IC_{50} value reported by Frances Orton et al. (2009) is almost identical yet the IC_{50} values for mecoprop are quite different. To the best of our knowledge this is the first study to quantify the IC_{50} value for 2,4-D and dicamba using the YES assay. From these results the ranking of antiestrogenic potency is diuron>mecoprop>2,4-D>dicamba, where diuron and dicamba are the most and least potent, respectively. Using the YAS assay, IC_{50} values were obtained for 2,4-D (71.4 μM), mecoprop (140.0 μM), terbutryn (23.8 μM), and acetamiprid (266.9 μM). Frances Orton et al. (2009) did not observe antiandrogenic activity for mecoprop but determined an IC_{50} value for diuron (15.6-31.3 μM) using the YAS assay. To the best of our knowledge this is the first study to quantify the IC_{50} value for terbutryn and acetamiprid using the YAS assay. From these results the ranking of

antiandrogenic potency is terbutryn>2,4-D>mecoprop>acetamiprid, where terbutryn and acetamiprid are the most and least potent, respectively.

Three compounds were shown to have conflicting results with activities reported in studies using the yeast-based bioassays. Myclobutanil and propiconazole were shown to have a weak positive estrogenic activity by Hurst & Sheahan (2003), while our results indicate anti-estrogenic activity. The anti-estrogenic activity of myclobutanil has also been shown using the MCF-7 assay conducted by Okubo et al. (2004). Mecoprop was shown to have no effect as an anti-androgen using the YAS conducted by Frances Orton et al. (2009) while our results showed anti-androgenic activity.

There are few studies that investigate potential endocrine disrupting effects of neonicotinoids and no studies have been found for acetamiprid, thiacloprid, and thiamethoxam that focuses on their interaction at the estrogen or androgen receptor sites. The endocrine activity of imidacloprid was investigated by Kojima et al. (2003) using Chinese Hamster Ovary Cells, however, no estrogenic or androgenic activity was found at the ERα, ERβ, and AR sites. The results gathered from the YES and YAS performed in this study suggests an antiestrogenic activity for thiacloprid and weak antiandrogenic activity for acetamiprid and thiamethoxam. No agonist activity at either estrogen or androgen receptor sites were detected for neonicotinoids investigated in the present study.

Due to the limitations regarding the measurable concentrations of pesticides in wastewater effluent as well as the ability to quantify the IC_{50} of antagonist behavior at the estrogenic and androgenic receptors, the concentrations of three herbicides could be evaluated. There are notable differences between effluent concentrations and IC_{50} concentrations for antagonist activities of dicamba, diuron and 2,4-D. For instance, the IC_{50} value for antiestrogenic activity for diuron is 31.4 μM and the average effluent concentration is 0.012 μM. The comparison of effluent concentrations and IC_{50} values of endocrine activities does not provide enough information required for a accurate risk assessment, however there is still concern regarding the presence of these compounds in effluent waters as these compounds often behave in a synergistic manner with other pesticides (Kretschmann, Gottardi, Dalhoff, & Cedergreen, 2015) thus causing indirect effects at low concentrations.

4.0 Conclusion

This study further supports the work of Kock-Schulmeyer et al. (2013) and other studies demonstrating that the contamination of aquatic environments by pesticides in urban areas cannot be neglected. Seventeen of the eighteen compounds investigated in the present study were present in one or more WWTP and four compounds (myclobutanil, dicamba, thiamethoxam, and thiacloprid) were detected for the first time in wastewater effluent. In addition, these results demonstrate the presence of a variety of pesticides at considerably high concentrations (up to 27606 ng/L for fluconazole) in wastewater effluents collected from three WWTPs. Detection of compounds such as neonicotinoids also raise environmental concerns and require additional investigation regarding their prevalence in WWTPs. The variety of pesticides found as mixtures in the effluent samples also raises

environmental concerns regarding bioactivity. Even though there has been numerous studies emphasizing the advantages and applicability of yeast-based bioassays for the screening of contaminants of emerging concern (Akram et al., 2011; Seeger, Klawonn, Bekale, & Steinberg, 2016a; Soto, Maffini, Schaeberle, & Sonnenschein, 2006), there still remains a lack of information regarding the endocrine activities of pesticides using such assays (F. Orton et al., 2011; Seeger et al., 2016a). As demonstrated by the results obtained using the YES and YAS assay, most of the individual compounds were shown to have antiestrogenic and/or antiandrogenic activities. This study is the first to report endocrine activities for six compounds (climbazole, dicamba, terbutryn, acetamiprid, thiamethoxam, and thiacloprid) using *in-vitro* bioassays. Although endocrine activities are relatively weak in comparison to natural hormones, pesticides at environmentally relevant concentrations are known to have synergistic and additive effects as mixtures in terms of endocrine activity and toxicity (Chen, Wang, Qian, Zhao, & Wang, 2015; Seeger et al., 2016a; Seeger, Klawonn, Bekale, & Steinberg, 2016b). The combination of the presence of pesticides found in the effluents of three WWTPs studied and the results gathered from investigating potential endocrine activities of individual compounds, strongly suggest there are environmental risks towards receiving waterways.

Acknowledgments

We gratefully acknowledge the assistance of the staff of the three wastewater treatment plants for collecting the samples. We also like to thank Marco Pineda for his help developing the required analytical method and performing chemical analysis, and Linda Taylor for performing sample extractions. Funding for this study was provided by a research grant to Viviane Yargeau (PI) from the Natural Sciences and Engineering Research Council (NSERC) of Canada through the Discovery Grant Program (RGPIN/04635-2015) and by the McGill Engineering Doctoral Award (MEDA).

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Table 1: Target compounds analyzed by LC-HRMS, molecular weights (MW; g/mol), and internal standards used for quantification.

Target compound	MW	Internal Standards
Herbicides		
2,4-D[1]	221.0	2,4-D-d_3 [4]
Dicamba[3]	221.0	Dicamba- d_3 [4]
Atrazine[1]	215.7	Atrazine-d_5 [4]
Diuron[3]	233.1	Diuron-d_6 [4]
Terbutryn[3]	241.3	NA
Mecoprop[3]	214.6	NA
Irgarol 1051[3]	253.3	NA
Fungicides		
Fluconazole[3]	306.3	Fluconazole-d_4 [4]
Clotrimazole[3]	344.8	Clotrimazole- d_5 [4]
Tebuconazole[2]	307.8	Tebuconazole- d_6 [4]
Propiconazole[1]	342.2	Propiconazole- d_5 [5]
Myclobutanil[2]	288.8	NA
Climbazole[2]	292.7	NA
Insecticides		
Acetamiprid[3]	226.6	NA
Thiacloprid[3]	252.7	NA
Thiamethoxam[3]	291.7	NA

1: Sigma-Aldrich; 2: Abcam; 3: Santa Cruz Biotechnology; 4: C/D/N Isotopes; 5: Dr. Ehrenstorfer GmbH (Augsburg, Germany)
NA: Not applicable – Qualitative analysis only

Table 2: Measured concentrations of pesticides in three WWTPs sampled and comparison to values reported in literature.

Compounds	Concentration measured in effluent WW (ng/L)			Literature data (ng/L)	
	WWTP 1	WWTP 2	WWTP 3	Influent	Effluent
Propiconazole	1815	7.29	14.7	86[a] 32.1[b] 4-27[c]	220[a] 36.6[b] 5-40[c]
Tebuconazole	7.74	8.57	7.08	1-8[c] 564.1[d]	1-10[c] 691.1[d] 22.89[e]
Fluconazole	1348	27606	23324	34-109[c] 583[f]	28-83[c] 793[f] 108[g]
Clotrimazole	1.75	2.99	3.91	20[h]	9[h] 5.3[g]
2,4-D	751	<LOD	<LOD	88.5[i]	42.9[i] 27.1[g]
Atrazine	44.1	28.2	27.4	52[a] 1.24[i] 6.0-22.9[j]	250[a] 124[i] 5.1-23.4[j] 4.2[g]
Dicamba	184	184	116	Not reported	
Diuron	9.70	2.64	24.1	 93.0[i] 28.4-2526.1[j]	190[a] 127[i] 29.1-2393.1[j] 61.7[g]
Compounds	**Compounds without surrogates – Qualitative analysis only**			**Literature data (ng/L)**	
	WWTP 1	**WWTP 2**	**WWTP 3**	**Influent**	**Effluent**
Climbazole	Detected	<LOD	Detected	475[k]	312[k]
Myclobutanil	Detected	Detected	<LOD	Not reported	
Irgarol	Detected	Detected	Detected	6[a]	200[a] 10[l]
Mecoprop	Detected	<LOD	<LOD	170[a] 106[i]	500[a] 17.3[i] 127[g] 870[l]
Terbutryn	Detected	Detected	Detected	11[a] 5.0-182.9[j]	390[a] 8.8-45.8[j] 70[l]
Prochloraz	Detected	Detected	Detected	63.2[j] 26.5[e]	2.9-59.1[j] 22.2[e]
Thiamethoxam	Detected	<LOD	<LOD	Not reported	
Acetamiprid	Detected	Detected	Detected	3.7[m]	3.4[m] 3[o]
Thiacloprid	Detected	<LOD	Detected		6[o]
Imidacloprid	<LOD	<LOD	<LOD	2.0-5.2[j] 54.7[m] 2.7[e]	2.0-6.7[j] 58.4[m] 5.3[e] 36[o]

<LOD, Below limit of detection

[a]Morasch et al., 2010,[b]Van De Steene, Stove, & Lambert, 2010,[c]Kahle, Buerge, Hauser, Muller, & Poiger, 2008,[d]Stamatis, Hela, & Konstantinou, 2010,[e]Campo, Masiá, Blasco, & Picó, 2013,[f]Lindberg et al., 2014,[g]Loos et al., 2013,[h]Peschka, Roberts, & Knepper, 2007,[i]Kock-Schulmeyer et al., 2013,[j]Masiá et al., 2013,[k]Wick, Fink, & Ternes, 2010,[l]Singer et al., 2010,[m]Sadaria, 2016,[o]Münze et al., 2017

Table 3: YES and YAS results obtained for individual compounds and comparison to bioactivities reported in literature. Bioactivities measured are agonist and antagonist activities at the estrogenic and androgenic receptor. The activities are represented as qualitative results as a result of partial dose responses.

Compound	Measured bioactivity				Endocrine Activity reported in literature (*in vitro* cell models)			
	YES Result		YAS Result		Yeast-based			
	E	AE	A	AA	E	AE	A	AA
Climbazole	n.o.	n.o.	n.o.	n.o.	NR			
Myclobutanil	n.o.	+	n.o.	+	+[a]	NR	NR	NR
Propiconazole	n.o.	+	n.o.	+	+[a]	NR	NR	NR
Tebuconazole	n.o.	n.o.	n.o.	+	NR			
2,4-D	n.o.	++	n.o.	++	n.o.[a,b]	NR	n.o.[b]	NR
Atrazine	n.o.	n.o.	n.o.	n.o.	n.o.[a]	NR	NR	NR
Dicamba	n.o.	++	n.o.	+	NR			
Diuron	n.o.	++	n.o.	+	n.o.[a,b]	+[b]	n.o.[b]	+[b]
Irgarol	n.o.	+	n.o.	n.o.	n.o.[c]	NR	NR	NR
Mecoprop	n.o.	++	n.o.	++	n.o.[a,b]	+[b]	n.o.[b]	n.o.[b]
Terbutryn	n.o.	+	n.o.	+	NR			
Acetamiprid	n.o.	n.o.	n.o.	+	NR			
Thiacloprid	n.o.	+	n.o.	n.o.	NR			
Thiamethoxam	n.o.	n.o.	n.o.	+	NR			

E, estrogenic; A, androgenic; AE, anti-estrogenic; AA, anti-androgenic; n.o., not observed; NR, not reported; ++, positive response; +, slightly positive response.
[a]Hurst & Sheahan, 2003, [b]Frances Orton, Lutz, Kloas, & Routledge, 2009a, [c]Noguerol et al., 2006

Supplemental Material

Table S.1 LODs and LOQs of the target analytes

Compound	LOD, ppb	LOQ, ppb
Atrazine	1.09	3.63
Diuron	1.12	3.73
Climbazole	1.13	3.77
Clotrimazole	1.12	3.74
Terbutryn	1.10	3.66
Myclobutanil	1.08	3.60
Irgarol	1.20	4.01
Tebuconazole	1.05	3.51
Fluconazole	1.09	3.62
Propiconazole	1.14	3.79
Mecoprop	1.59	5.29
Dicamba	1.01	3.36
2,4-D	0.94	3.12
IS-Diuron-d6	1.13	3.77
IS-Atrazine-d5	1.12	3.74
IS-Clotrimazole-d5	1.17	3.89
IS-Fluconazole-d4	1.09	3.63
IS-Tebuconazole-d6	1.09	3.64
IS-Propiconazone-d5	1.06	3.53
IS-2,4-D-d3	1.01	3.36
IS-Dicamba-d3	1.01	3.37

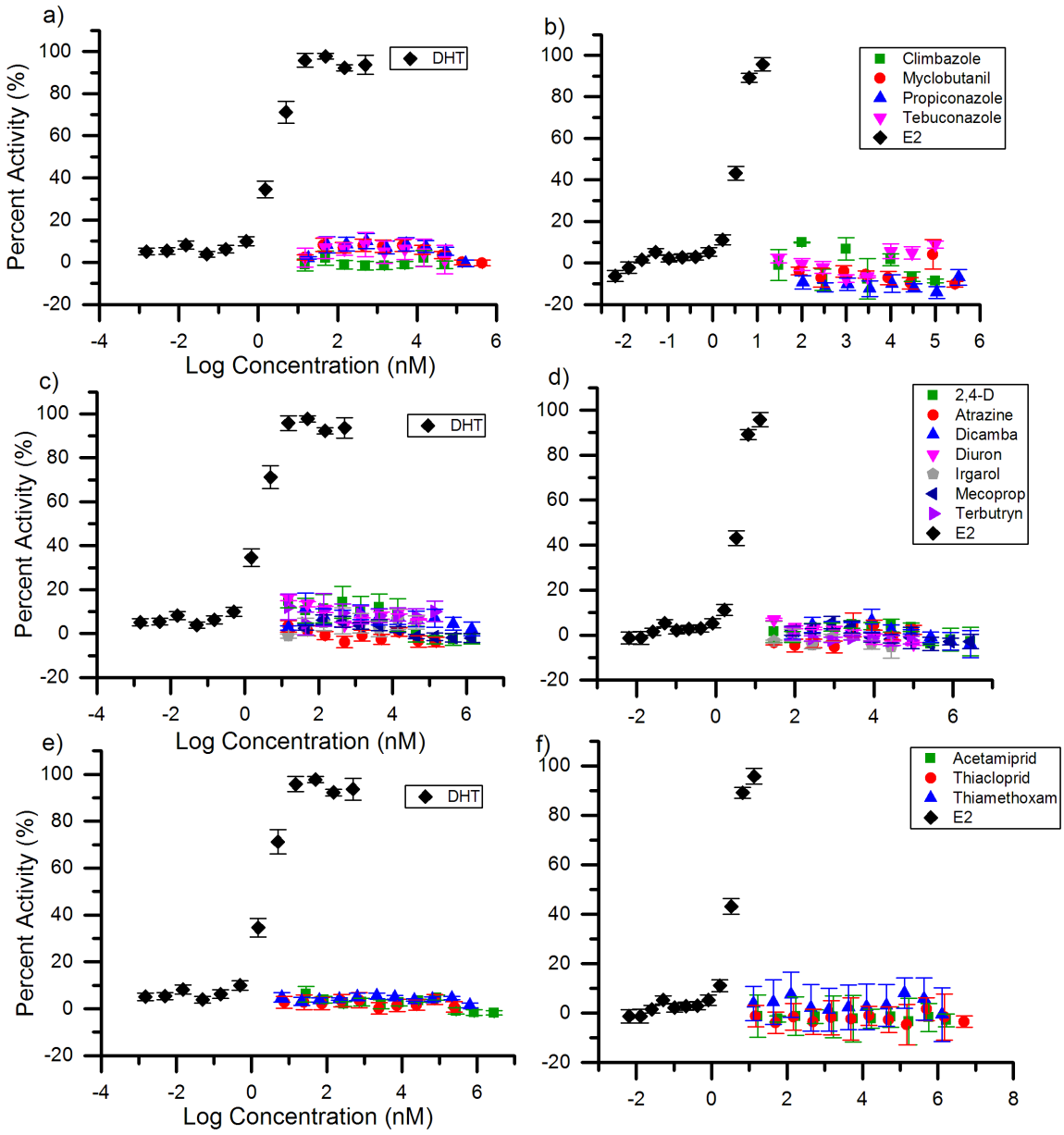

Figure S.1 Agonist activities for the YES and YAS assay: androgenic (a) and estrogenic (b) activity for fungicides, androgenic (c) and estrogenic (d) activity for herbicides, androgenic (e) and estrogenic (f) activity for neonicotinoids. Error bars are standard of error of triplicate samples.

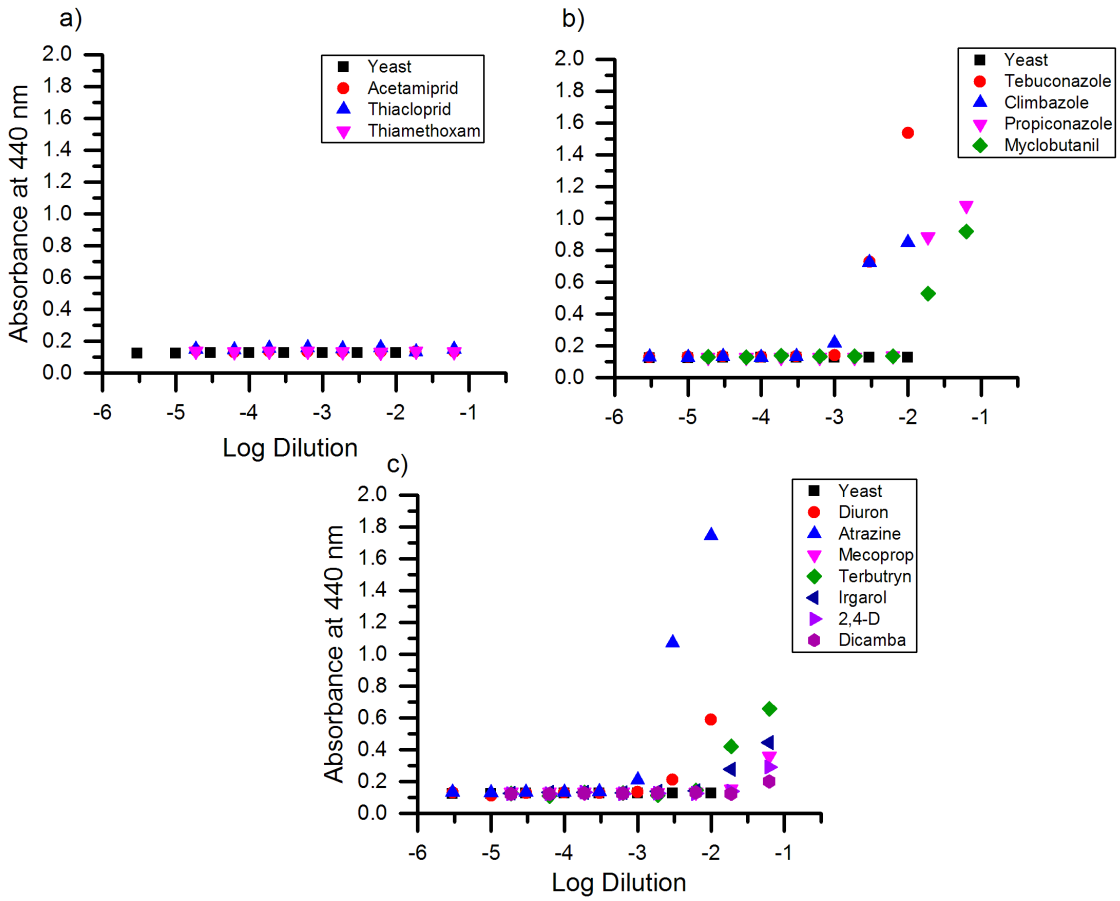

Figure S.2 Colorimetric Cell Viability Test (WST-1, Kit II) results for neonicotinoids (a), fungicides (b), and herbicides (c). The increase in absorbance measure at 440nm indicates cytoxicity. The dilutions that resulted in an increase of absorbance values were not considered in the determination of endocrine activity using YES and YAS assays.