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

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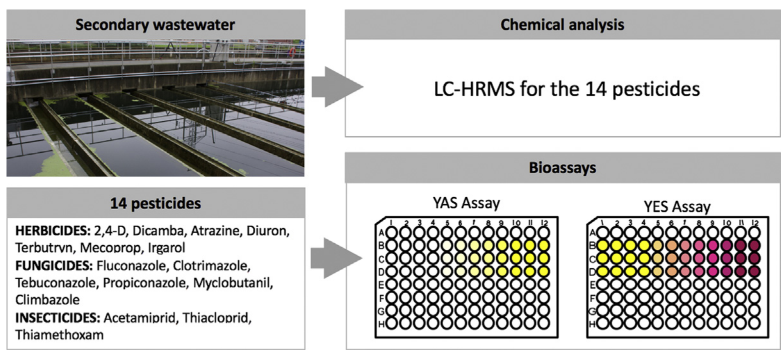
HIGHLIGHTS

- One of the first studies to identify select fungicides and neonicotinoids in wastewater effluent
- First study to report on endocrine activity of select neonicotinoids using yeast-based assays
- 17 out of 18 pesticides investigated detected in WWTP effluents with concentrations from 3 ng/L to 27 μg/L
- Most pesticides showed anti-estrogenic or anti-androgenic activity and seven had pleiotropic effects.

GRAPHICAL ABSTRACT



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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 is frequently reported to be present throughout the environment, their presence in wastewater treatment plants has 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 μg/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.

1. 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 et al., 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

Abbreviations: CECs, 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.

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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 et al., 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 al. (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 et al., 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 et al., 2003; F. Orton et al., 2011; Sohoni and Sumpter, 1998; Urbatzka et al., 2007). In this context an analysis conducted by Kojima et al. (2003) 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. 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 0.2 M 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.

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

NA: not applicable – qualitative analysis only.
[a] Sigma-Aldrich.
[b] Abcam.
[c] Santa Cruz Biotechnology.
[d] C/D/N isotopes.
[e] Dr. Ehrenstorfer GmbH (Augsburg, Germany).

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. 24 h composite samples were collected in amber bottles at treatment plants and stored at −4 °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