

# Biological Activity of Wastewater Assessed Using *in Vitro* Cell-Based Assays

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## Research Article

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## Abstract

Bioanalytical tools, namely *in vitro* bioassays, can be employed in tandem with chemical analyses to assess the efficacy of wastewater treatment and the potential for adverse effects from the discharges of wastewater into receiving waters. In the present study, samples of untreated wastewater (i.e. influent) and treated wastewater (i.e. effluent) were collected from two wastewater treatment plants and a wastewater treatment lagoon serving municipalities in southern Ontario, Canada. In addition, grab samples of surface water were collected downstream of the lagoon discharge. After solid phase extraction (SPE) using ion-exchange columns for basic/neutral and acidic compounds, respectively, the extracts were analyzed for a suite of 16 indicator compounds. The two SPE extracts were combined for analysis of biological responses in four *in vitro* cell-based bioassays. The concentrations of several indicator compounds, including the estrogens, 17 $\beta$ -estradiol and 17 $\alpha$ -ethinylestradiol, were below the limits of detection. However, androstenedione and estrone were detected in several influent samples. The concentrations of these steroid hormones and some of the other indicator compounds declined during treatment but acesulfame K, carbamazepine, trimethoprim and DEET persisted in the effluent. The MTS- CellTiter 96<sup>®</sup> AQueous One Solution Cell Proliferation Assay (MTS) indicated that cell viability was not affected by exposure to the extracts. The Qiagen Nuclear Receptors 10-Pathway Reporter Array indicated that several cellular pathways were upregulated, with the greatest upregulation observed with the estrogen receptor (i.e. induction ratios 12 to 47) and the liver X receptor (i.e. induction ratios 10 to 45). The ER $\alpha$  CALUX assay indicated that estrogenic activity was lower in effluents compared to influents, with the greatest estrogenic activity observed for grab samples of influent from the lagoon (i.e. 56-215 ng L<sup>-1</sup> 17 $\beta$ -estradiol equivalents). Finally, the Nrf2 Luciferase Luminescence Assay indicated a lower oxidative stress in the effluent samples. Overall, the present study demonstrates that chemical analyses are limited in their ability to predict or explain reductions in the toxicity of treated wastewater. There are thus advantages to using a combination of chemical analyses and *in vitro* bioassays to monitor the treatment efficiency of wastewater treatment plants and to predict the potential impacts of wastewater discharges into receiving waters.

## Introduction

A range of potentially toxic substances are discharged from municipal wastewater treatment plants (WWTPs), including pharmaceuticals and personal care products (Metcalf et al. 2003, Carballa et al. 2004, Ratola et al. 2012), illicit drugs (Rodayan et al. 2015), estrogens (Servos et al. 2005, Ternes 2006, Liu et al. 2015), pesticides (Kahle et al, 2008) and toxic metals (Marcogliese et al. 2015). For many organic contaminants, there are also transformation products of the parent compounds that are present in wastewater. Effective wastewater treatment can reduce the discharges of various substances from municipal effluents, but removals may be influenced by the type of treatment and the operating conditions, seasonal changes in temperature, and other factors (Eggen et al. 2014, Baalbaki et al. 2017).

Due to advances in analytical methods, it is now possible to monitor thousands of chemical contaminants in water and wastewater (Richardson and Ternes 2018). Regulating wastewater discharges based on the analysis of a wide range of target compounds is an expensive and time-consuming approach (Jia et al. 2015). In addition, limiting contaminant monitoring to a number of known pollutants may underestimate the risks to the environment and possible hazards to human health (Smital et al. 2013) and do not take into account mixture effects (Snyder and Leusch 2018). A complementary approach is to use bioanalytical tools to screen for different modes of toxicity in samples of water and wastewater. These screening assays can be combined with subsequent analytical approaches to assess the effectiveness of wastewater treatment (Escher and Leusch 2012, Jia et al. 2015).

In the present study, extracts from samples of treated and untreated wastewater, and surface water were analyzed for the concentrations of 16 target analytes (i.e. indicator compounds), which included specific compounds selected from the classes of estrogens, androgens, antibiotics and pharmaceuticals, pesticides, artificial sweeteners and personal care products and results were analyzed using two approaches described in Neale et al. (2020): comparison between influent and effluent for the evaluation of treatment process efficiency and comparison to effect-based trigger values for the evaluation of treated water quality. The extracts were also tested for toxicity using four mammalian cell-based *in vitro* assays. In the case of *in vitro* toxicity testing, extracts from water and wastewater were initially screened using the Qiagen Nuclear Receptors 10-Pathway Reporter Array to provide information regarding the regulation of multiple nuclear receptors. The estrogenic activity of the extracts was subsequently tested with the ER $\alpha$  CALUX assay. The capacity of extracts to induce oxidative stress was tested with a Nrf2 Luciferase Luminescence Assay. Finally, the MTS assay was employed to evaluate non-specific cytotoxicity in order to ensure that responses observed with the reporter gene-based assays were not influenced by decreased cell viability. In tandem with chemical analyses, the *in vitro* toxicity data were used to evaluate whether toxic substances are removed effectively from wastewater and to evaluate the sensitivity of the assays to detect changes in the levels of specific classes of chemical

contaminants in water and wastewater. Samples of wastewater were collected from two WWTPs and a wastewater lagoon (WL) serving municipalities in the southern region in the province of Ontario, Canada, and from river water 2.0 km downstream of the lagoon discharge.

## Materials And Methods

### Indicator Compounds

The 16 indicator compounds analyzed in extracts of water and wastewater are listed in Table 1, along with information on the class of the contaminant, the supplier, and the stable-isotope labelled surrogate used as an internal standard for quantification. An external standard stock solution (1000 ppm) for these indicator compounds was prepared in methanol (Fisher Optima, LC/MS grade) and stored in the dark at 4°C for preparation of fresh analytical standards.

### Sample Collection

In 2014, samples of wastewater were collected at two WWTPs in the West Central Region of southern Ontario (WWTP 1 and WWTP 2). Both treatment plants employ conventional activated sludge wastewater treatment, but WWTP 2 also has a tertiary treatment train. At these WWTPs, 24-h composite samples of untreated wastewater (i.e. influent) and treated wastewater (i.e. effluent) were collected once a month for 5 months from April to August. A wastewater lagoon located in the Central Region of southern Ontario (WL) was also sampled in the spring and fall of 2014 during periods of intermittent discharge. In this case, grab samples of influent and effluent were collected in May, June and twice in September. Grab samples of surface water were also collected from a river approximately 2.0 km downstream of the lagoon during discharge. Information regarding the WWTPs and WL is summarized in Table S1 in Supplementary Information.

The Laboratory Services Branch of the Ontario Ministry of the Environment, Conservation and Parks (Etobicoke, ON, Canada) analyzed all samples of water and wastewater for a wide range of water quality parameters. Samples were analyzed for a range of cations and metals, chemical oxygen demand (COD), carbonaceous biochemical oxygen demand (cBOD), pH, nitrogen species, phosphorous species, total and dissolved organic carbon, suspended and dissolved solids, etc. Samples were analyzed according to standard protocols developed by the Ministry.

### Sample Extraction

Subsamples of water and wastewater were extracted using two solid phase extraction (SPE) methods, including extraction with Oasis® MCX cartridges (6 mL, 150 mg) to concentrate base/neutral compounds and extraction with MAX cartridges (6 mL, 400 mg) to concentrate acidic compounds. The SPE cartridges were purchased from Waters (Milford, MA, USA). Prior to extraction, samples (110 mL) were filtered through 1 µm glass-fiber filters (Fisher Scientific, Ottawa, ON, Canada) and the pH was adjusted to pH = 2.5 for extraction using MCX cartridges and to pH = 8.0 for extraction using MAX cartridges. For subsamples prepared for analysis of contaminants (n = 3), the samples were spiked with the internal standard solution of labeled surrogates. Subsamples prepared for bioassays (n = 3) were not spiked. The methods for SPE extraction were previously described by Baalbaki et al. (2017). Following extraction with both types of cartridges, elution solvents were evaporated to near dryness and reconstituted in either 0.4 mL of a 1:1 methanol-water solution (Fisher Optima HPLC grade) for chemical analyses, or in 0.4 mL of DMSO (Sigma-Aldrich Bioreagent, molecular biology grade) for bioassays. The MCX and MAX extracts were analyzed separately for contaminants, as described below. Prior to conducting the *in vitro* bioassays, the MCX and MAX extracts were pooled for each sample. Extracts were placed into amber high performance liquid chromatography (HPLC) vials with polytetrafluoroethylene (PTFE) tops and stored at -20°C until analysis. The overall pre-concentration factor was 275× (i.e. 0.11 L of sample concentrated to 400 µL extract). Chemical analysis and bioassays were run on triplicate samples.

Table 1

List of indicator compounds, their compound class and respective labeled surrogates, with chemical suppliers identified.

COMPOUND (Manufacturer)	COMPOUND CLASS	LABELED SURROGATE (Manufacturer)
Acesulfame Potassium (Sigma-Aldrich, Fluka)	artificial sweetener	Acesulfame K-d <sub>4</sub> (Santa Cruz)
Androstenedione (Sigma-Aldrich, Cerilliant)	androgen	Androstenedione- <sup>13</sup> C <sub>3</sub> (Sigma-Aldrich, Cerilliant)
Atrazine (AccuStandard, Inc.)	herbicide	Atrazine-d <sub>5</sub> (Sigma-Aldrich, Fluka)
Bentazon (AccuStandard, Inc.)	herbicide	Bentazon-d <sub>7</sub> (Sigma-Aldrich)
Carbamazepine (Sigma-Aldrich)	pharmaceutical	Carbamazepine-D <sub>10</sub> (Cambridge Isotope Laboratories, Inc.)
2,4-D <sup>1</sup> (AccuStandard, Inc.)	herbicide	2,4-D-d <sub>3</sub> (CDN Isotopes)
DEET <sup>2</sup> (AccuStandard, Inc.)	pest repellent	DEET-D <sub>7</sub> (CDN Isotopes)
17β-Estradiol (Sigma-Aldrich)	estrogen	17β-Estradiol- <sup>13</sup> C <sub>2</sub> (Cambridge Isotope Laboratories, Inc.)
Estrone (Sigma-Aldrich)	estrogen	Estrone- <sup>13</sup> C <sub>2</sub> (Cambridge Isotope Laboratories, Inc.)
17α-Ethinylestradiol (Sigma-Aldrich)	estrogen	17α-Ethinylestradiol- <sup>13</sup> C <sub>2</sub> (Cambridge Isotope Laboratories, Inc.)
Gemfibrozil (Sigma-Aldrich, Fluka)	pharmaceutical	Gemfibrozil-d <sub>6</sub> (Cambridge Isotope Laboratories, Inc.)
Ibuprofen (Sigma-Aldrich)	pharmaceutical	Ibuprofen- <sup>13</sup> C <sub>3</sub> (Cambridge Isotope Laboratories, Inc.)
MCPA <sup>3</sup> (AccuStandard, Inc.)	herbicide	MPCA-d <sub>3</sub> (CDN Isotopes)
Sulfamethoxazole (Sigma-Aldrich, Fluka)	antibiotic	Sulfamethoxazole- <sup>13</sup> C <sub>6</sub> (Cambridge Isotope Laboratories, Inc.)
Triclosan (Sigma-Aldrich)	personal care product	Triclosan- <sup>13</sup> C <sub>12</sub> (Cambridge Isotope Laboratories, Inc.)
Trimethoprim (Sigma-Aldrich)	antibiotic	Trimethoprim- <sup>13</sup> C <sub>3</sub> (Santa Cruz)

<sup>1</sup>2,4-D: 2,4-dichlorophenoxyacetic acid

<sup>2</sup>DEET: *N,N*-diethyl-*meta*-toluamide

<sup>3</sup>MCPA: 2-methyl-4-chlorophenoxyacetic acid

To evaluate whether there was contamination from the extraction process and to allow for the calculation of SPE recoveries, procedural blanks were prepared by extracting 110 mL of Milli-Q water. Prior to extraction, the procedural blank was spiked with the internal standard solution of labeled surrogates and the pH was adjusted for extraction using MCX and MAX cartridges, as described for the water and wastewater samples.

## Chemical Analysis

The concentrations of the 16 indicator compounds in procedural blanks and in extracts from water and wastewater were quantified by liquid chromatography with high resolution mass spectrometry (LC–HRMS) or by liquid chromatography with tandem mass spectrometry (LC-MS/MS). An Accela HPLC coupled to an LTQ Orbitrap XL hybrid linear ion trap-orbital trap instrument purchased from Thermo Fisher Scientific (Waltham, MA, USA) was used for LC-HRMS analysis. Analysis by LC–MS/MS was conducted with an AB Sciex QTrap 5500 instrument equipped with an Agilent 1100 series HPLC purchased from Applied Biosystems-Sciex (Mississauga, ON, Canada) and operated in multiple reaction monitoring mode (MRM).

For LC-MS/MS, the target compounds of estrone, 17 $\beta$ -estradiol and 17 $\alpha$ -ethinylestradiol were separated by liquid chromatography using a Genesis C18 column (150 mm x 2.1 mm ID; 4 mm particle size) purchased from Chromatographic Specialties (Brockville, ON, Canada), coupled with a guard column with the same packing material (4 mm x 2.0 mm) purchased from Phenomenex (Torrance, CA, USA). The solvents used for chromatographic separations were: [A] 10 mM ammonium acetate with 0.1% acetic acid and [B] 100% acetonitrile, using the gradient previously described by Metcalfe et al. (2014).

For LC-HRMS, chromatographic separation of the indicator compounds was accomplished with a Thermo Scientific Hypersil Gold C18 column (100 x 2.1 mm, 1.9  $\mu$ m particle size) heated to 30°C which was connected to an in-line UHPLC filter (0.2  $\mu$ m). The chromatography solvents were [A] 0.1% acetic acid in water, and [B] 100% acetonitrile. The flow rate in the column was 200  $\mu$ L/min.

Three different analytical methods were used for the various indicator compounds: Method 1: LC-HRMS with electrospray ionization (ESI) and positive ion monitoring; Method 2: LC-HRMS with ESI and negative ion monitoring; Method 3: LC-MS/MS with ESI and positive ion monitoring in MRM. Table S2 in Supplementary Information describes which of these three analytical methods was employed for each of the indicator compounds. For LC-HRMS, a 6-point calibration curve covering the range of anticipated analyte concentrations was used for external calibration. The concentrations were adjusted according to the recovery of the internal standards in order to compensate for the effects of the sample matrix on ionization and for variations in the recoveries during sample extraction.

The limits of detection (LODs) and limits of quantification (LOQs) were determined by analyzing serial dilutions of a 1:1 methanol-water standard solution containing all compounds. LOD and LOQ are defined as the indicator compound concentration producing a peak with a signal-to-noise ratio of 3 and 10, respectively. The LODs and LOQs for all analytes are listed in Table S3 in Supplementary Information. Two samples spiked with the indicator compounds at concentrations of 12 and 30  $\mu$ g L<sup>-1</sup> were extracted and analyzed for quality control purposes. For these spiked samples, the relative error between expected and measured quality control concentrations was  $\leq$  20% for all indicator compounds.

## Nuclear Receptors 10-Pathway Reporter Array

The Nuclear Receptors 10-Pathway Reporter Array purchased from Qiagen (Germantown, MD, USA), hereafter referred to as the 10-Pathway Reporter Array, was used as a screening assay to determine which cellular signalling pathways are affected or regulated by exposure to sample extracts. MCF7 breast cancer cells were selected to carry out this assay. This cell line expresses a majority of the transcription factors monitored by the Qiagen 10-Pathway Reporter Array. Recently, Kittler et al. (2013) systematically “mapped the genomic binding sites of all nuclear receptors expressed in MCF-7 breast cancer cells”. In total, they “mapped the genomic binding sites of a total of 33 proteins whose corresponding genes are expressed at moderate to high levels in MCF-7 cells”. Amongst these proteins, 9 of the 10 transcription factors were identified, whose activity can be monitored by the Qiagen reporter array. The only transcription factor that will likely not be encountered in the MCF7 cells is HNF4 (hepatocyte nuclear factor 4). However, in Escher et al. (2014), HNF4 (hepatocyte nuclear factor 4) that is not expressed by the MCF7 cell line is not a major player in the MOA in any of the toxicity pathways. All cell lines employed in this study were cultured at 37°C, 5% CO<sub>2</sub> and 95% humidity. All cell culture materials manufactured by Life

Technologies (Rockville, MD, USA) were supplied by Thermo Fisher. The MCF7 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) media supplemented with 10% fetal bovine serum (FBS), 1x GlutaMAX supplement, 100 units mL<sup>-1</sup> penicillin and 100 µg mL<sup>-1</sup> streptomycin. To carry out this assay, cells were transfected with Qiagen Reporter Array DNA. This was achieved employing the Attractene Transfection Reagent (Qiagen) as described by the manufacturer. Prior to transfection, 75–95% confluent MCF7 cells growing in T75 plates were trypsinized with 0.25% trypsin-EDTA and plated onto white, opaque 96-well plates at a density of 4×10<sup>4</sup> cells mL<sup>-1</sup> (i.e. 100 µL per well) in Opti-MEM® I Reduced Serum Media (Invitrogen) supplemented with 5% FBS and 1% NEAAs. Cells were plated onto transparent polypropylene 96-well plates. Following 24-h growth, media was removed from the cells and the cells were rinsed with 100 µL of phosphate buffered saline (PBS) prior to the addition of 88 µL per well fresh antibiotic and Opti-MEM® I Reduced Serum Media (FBS-free). At this point, cells were exposed to the transfection materials.

Transfection materials were prepared in 15 mL polypropylene centrifuge tubes. The total volume of the transfection materials prepared was determined by the number of wells on the 96-well plate to be transfected. Briefly, into each centrifuge tube, 0.08 µg per well (i.e., 0.8 µL per well) of Reporter Array DNA was added to 12 µL per well of Opti-MEM® I Reduced Serum Media. Next, 0.4 µL per well of Attractene Transfection Reagent was added to the DNA and the mixture was vortexed. The DNA complexes were then incubated at room temperature for 15 minutes, before being added to the MCF7 cells in the 96-well plates. Cells were then incubated under normal growth conditions for 48-h. Following incubation with the transfection reagents, the Dual-Luciferase Reporter Assay System (Promega) was utilized to monitor luminescence. As described by the supplier for the Dual-Luciferase Reporter Assay System, firefly luminescence and *Renilla* luminescence were quantified on the luminometer, which was a Berthold Technologies Mitras LB 940 Multimode Microplate Reader. Positive and negative control transfections were also prepared and included on each plate.

Responses for the various reporters in the array were quantified in terms of Induction Ratios (IR), which in luminescence-based assays is defined as the ratio of the relative light units (RLU) measured for an experimental sample relative to a suitable control sample, as described in Eq. 1 (Jia et al. 2015). For the assay used, an IR value > 5 is recommended as evidence of a significant response. This value was later compared to Escher et al. (2014) who reported an IR of 1.5 as evidence of a positive biological response in other *in vitro* assays and further suggest that the ratio was sufficient to ensure that a significant effect was observed.

$$IR = \frac{RLU_{sample}}{RLU_{control}} \quad (1)$$

## ERα CALUX Assay

The ERα Chemically Activated LUciferase eXpression® assay, hereafter referred to as the ERα CALUX assay was employed to determine the estrogenic activity of sample extracts. This reporter gene assay is based on the ERα U2OS.Luc cell line, which can be stably transfected with ERα or ERβ and a luciferase reporter gene and was obtained under license from Biodetection Systems B.V. (Amsterdam, the Netherlands). The recombinant construct for this reporter-gene assay and the basis for the response in the assay has been previously described (Quaedackers et al. 2001, Sonneveld et al. 2005, Wang et al. 2014).

The ERα U2OS.Luc cells were cultured using the methods recommended by the supplier, with minor modifications. As stated previously, all cell lines employed in this study were cultured at 37°C, 5% CO<sub>2</sub> and 95% humidity. All cell culture materials were from Life Technologies. Briefly, the cells were cultured in T75 flasks containing 22 mL of DMEM/F12 media supplemented with 10% Fetal Bovine Serum (FBS), 100 units mL<sup>-1</sup> of penicillin and 100 µg mL<sup>-1</sup> of streptomycin, 0.8 mg mL<sup>-1</sup> geneticin and 1x non-essential amino acids (NEAAs). All assays were carried out in 96-well plates containing assay medium without phenol red and supplemented with 10% USDA-approved charcoal stripped FBS, 100 units mL<sup>-1</sup> penicillin and 100 µg mL<sup>-1</sup> streptomycin and 1x NEAAs (note: no geneticin was added to the assay media). Phenol red-free media was employed because of the ER-agonist activity of phenol red (Berthois et al. 1986). Furthermore, charcoal stripped FBS was employed to minimize exposure to any estrogens present in the culture media.

Once plated, 75–95% confluent cells were trypsinized with phenol red-free, 0.05% trypsin-EDTA. Next, 100 µL of cells at a 100,000 cells mL<sup>-1</sup> concentration were plated onto 96-well white opaque plates (Nunc, Thermo Scientific). The Bright-Glo™ Luciferase Assay System (Promega) was employed to quantitate firefly (*Photinus pyralis*) luciferase expression following 24-h exposure to sample extracts. A 17β-estradiol standard curve at concentrations ranging from 1.0×10<sup>-13</sup> to 1.0×10<sup>-10</sup> M was included on each opaque plate, allowing for quantitation of estrogenic activity in terms of ng L<sup>-1</sup> of 17β-estradiol (E2) equivalents. All luminescence measurements were performed on a Berthold Technologies Mitras LB 940 Multimode Microplate Reader.

## Nrf2 Luciferase Luminescence Assay

The assay with the Nrf2 luciferase reporter MCF7 stable cell line, hereafter referred to as the Nrf2 assay was employed to determine whether exposure to sample extracts resulted in an oxidative stress response. Nrf2 cells were obtained from Signosis Inc. (Santa Clara, CA, USA) and were cultured in Dulbecco's Modified Eagle medium (DMEM) supplemented with 10% FBS, 1× GlutaMAX, 0.8 mg mL<sup>-1</sup> geneticin (G418 sulfate), 100 units mL<sup>-1</sup> penicillin and 100 µg mL<sup>-1</sup> streptomycin. All media components were from Life Technologies. Once 75–95% confluent in T75 flasks, cells were trypsinized with 0.25% trypsin-EDTA (Life Technologies) and plated onto white, opaque 96-well plates at a density of 5×10<sup>4</sup> cells mL<sup>-1</sup> (i.e. 100 µL of cells added per well) for luminescence analyses. Cells were allowed to grow for 24-h prior to the addition of sample extracts. Following 24-h exposure to the extracts, the Bright-Glo™ Luciferase Assay System (Promega) was employed to quantitate firefly luciferase expression. Once more, luminescence measurements were performed on a Berthold Technologies Mitras LB 940 Multimode Microplate Reader.

For quantitation of the oxidative stress response, a dilution series of *tert*-butylhydroquinone (*t*-BHQ, Sigma-Aldrich) at concentrations ranging from 1×10<sup>-5</sup> – 1.6×10<sup>-7</sup> M was included on each opaque plate. Oxidative stress was expressed in terms *t*-BHQ equivalents.

## MTS Assay

The cytotoxicity of the sample extracts to all mammalian cell lines employed in this study was assessed with the CellTiter 96® AQueous One Solution Cell Proliferation Assay manufactured by Promega (Madison, WI, USA). In this colorimetric assay, the MTS reagent (3-[4,5-dimethylthiazol-2-yl]-5-[3-carboxy methoxyphenyl]-2-[4-sulfophenyl]-2H-tetrazolium, inner salt) is bio-reduced to a soluble formazan product when it is exposed to actively respiring cells. For every 96-well plate of cells tested, 2.5 mL of MTS reagent was diluted in 12.5 mL of Opti-MEM® I Reduced Serum Media. Following 24-h exposure of the cells plated on transparent polypropylene 96-well plates (Greiner) to the sample extracts, the media was removed, and the cells rinsed twice with sterile PBS. Next, 120 µL of the MTS reagent diluted in Opti-MEM® media was added to the cells, and then allowed to incubate for 2-h. Formazan product formation was detected with a Biorad (Hercules, CA, USA) UV-visible microplate reader (Benchmark Plus with Microplate Manager 5.2.1 software) by measuring the optical density at λ = 490 nm.

## Statistical Analysis

For all data on the concentrations of indicator compounds and the responses in *in vitro* assays, the statistical analysis was done using the statistical package available on Excel.

## Results And Discussion

### Wastewater Quality Results

A wide range of wastewater quality parameters were monitored in the influent and effluent of WWTP 1, WWTP 2 and WL. As illustrated with a selected number of these parameters, there was a marked improvement in water quality as a result of wastewater treatment (Fig. 1). WWTP2 was particularly effective at treating the wastewater, possibly because of the tertiary treatment system at this plant. The wastewater lagoon was also effective at improving wastewater quality before discharge into receiving waters (Fig. 1).

### Concentrations of Indicator Compounds

The apparent recoveries for the 16 indicator compounds were first evaluated using Milli-Q water. Of the 16 indicator compounds analyzed, 12 were found to have mean recoveries > 80%, including the estrogens, estrone (113 ± 47%), 17β-estradiol (113 ± 48%) and 17α-ethinylestradiol (121 ± 47%). There were lower recoveries for DEET and sulfamethoxazole of 57 ± 30% and 57 ± 28%, respectively. A high apparent recovery of 604 ± 426% for acesulfame K indicates that there was signal enhancement for this compound; probably because a constituent of the sample matrix increased the ionization efficiency. The responses to the internal standards spiked into all samples of water and wastewater were used for quantitation of all indicator compounds.

The analytical data for wastewater samples collected at WWTPs 1 and 2 are summarized in Table 2 and the data for WL and receiving waters are summarized in Table 3. The estrogens, 17β-estradiol and 17α-ethinylestradiol, the pharmaceutical, gemfibrozil, and the herbicides, atrazine, bentazon and MCPA were not detected in any of the samples. Acesulfame K and carbamazepine were detected in all influent and effluent samples from WWTP 1 and were widely detected in samples collected from WWTP 2 (Table 2) and WL (Table 3). Carbamazepine is known to be poorly removed in WWTPs (Blair et al. 2013), and the artificial sweetener, acesulfame K is also poorly removed by wastewater treatment (Subedi and Kannan 2014). The antibiotics, sulfamethoxazole and trimethoprim were widely detected

in both influent and effluent samples from WWTP 1 and WWTP 2 (Table 2) but these compounds were detected less frequently in grab samples from WL (Table 3). The non-prescription analgesic, ibuprofen and the antibacterial compound, triclosan were frequently detected in influent samples collected in WWTP1, but not in effluent samples, which is consistent with the high removals usually reported for these compounds in WWTPs (Blair et al. 2013).

It is notable that androstenedione, which is an intermediate in the biosynthesis of testosterone, was detected at concentrations  $> 100 \text{ ng L}^{-1}$  in several influent samples from the WWTPs (Table 2). There are only limited monitoring data for androstenedione in the literature. However, Baalbaki et al. (2017) detected androstenedione in influent samples but not in effluent samples collected from WWTPs, indicating that this compound is effectively removed by wastewater treatment. Estrone was detected in selected influent samples collected in June and August from WWTP 1 (Table 2) and from influent samples collected in May and September in WL (Table 3). The concentrations of estrone were lower in the effluent. This intermediate in the biosynthesis of  $17\beta$ -estradiol was present at concentrations  $< \text{LOQ}$  in selected influent and effluent samples collected from WWTP 2 in June and August (Table 2).

For DEET, the active ingredient in some insect repellents, concentrations were highest in the months of June and July at WWTP 1 and WWTP 2 (Table 2), presumably because of the higher numbers of biting insects in the summer. However, at WL, DEET levels peaked in May (Table 3). Overall, DEET appears to be partially removed by wastewater treatment, with generally lower levels detected in effluent samples. The herbicide, 2,4-D was detected in wastewater samples collected in June and July at WWTP 1 and occasionally detected in samples collected from WWTP 2 (Table 2) and WL (Table 3). We assume that this herbicide made its way into domestic wastewater from storm water overflows. However, since 2,4-D has been banned since 2009 in Ontario for cosmetic weed control, it is difficult to speculate on the sources of this herbicide. Acesulfame K, DEET and ibuprofen were frequently detected in the surface water samples collected from a river 2.0 km downstream of the discharge from the wastewater lagoon (Table 3). The river sub-watershed is approximately 31% agricultural, 17% urban, 3% roads, 3% golf courses, and 3% industrial with the remainder being natural heritage features.

Caution should be used in interpreting the data on the relative concentrations of the target compounds in influent and effluent samples as an indicator of the removals of contaminants by wastewater treatment. The hydraulic retention times for wastewater of 1–3 days in WWTPs and even longer in some wastewater lagoons means that influent and effluent samples collected on the same day are not synchronized in terms of the composition of the wastewater (Ort et al. 2010). This is especially problematic when interpreting the analytical results from the grab samples of influent and effluent collected at the wastewater lagoon. To overcome this problem, we recently used a modelling approach to estimate the removals of contaminants of emerging concern in WWTPs (Baalbaki et al. 2017).

Table 2

Mean concentrations (ng L<sup>-1</sup>; ± %SD) of microcontaminants in wastewater sampled in 2014 from influent and effluent of WWTP 1 and WWTP 2. ND = Not detected at concentrations > LOD; P = Present at concentrations < LOQ; NA = Not analyzed.

COMPOUND	MEAN CONCENTRATIONS (± %SD)									
	(ng L <sup>-1</sup> )									
	Influent	Effluent	Influent	Effluent	Influent	Effluent	Influent	Effluent	Influent	Effluent
	April		May		June		July		August	
<b>WWTP 1</b>										
Acesulfame K	42 ± 6	84 ± 5	115 ± 13	154 ± 5	121 ± 8	182 ± 18	111 ± 4	97 ± 15	81 ± 13	106 ± 16
Sulfamethoxazole	944 ± 4	391 ± 3	ND	ND	558 ± 17	211 ± 11	644 ± 13	< LOD	984 ± 4	ND
Trimethoprim	634 ± 4	235 ± 2	412 ± 6	ND	241 ± 7	ND	242 ± 12	115 ± 15	537 ± 5	ND
Carbamazepine	434 ± 13	429 ± 16	303 ± 13	465 ± 6	270 ± 7	321 ± 11	338 ± 12	154 ± 7	333 ± 10	343 ± 3
Ibuprofen	P	ND	P	ND	18223 ± 12	ND	19345 ± 14	ND	29022 ± 23	ND
Gemfibrozil	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Triclosan	ND	ND	ND	ND	3469 ± 5	ND	3534 ± 12	ND	ND	ND
Atrazine	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Bentazon	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
2,4-D	ND	ND	ND	ND	247 ± 6	102 ± 7	65 ± 14	ND	ND	ND
MCPA	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
DEET	291 ± 2	172 ± 5	5653 ± 5	1582 ± 11	10173 ± 8	146 ± 9	11908 ± 14	ND	3107 ± 14	ND
Androstenedione	564 ± 4	ND	ND	ND	ND	ND	193 ± 16	ND	508 ± 13	ND
17β-Estradiol	NA	NA	ND	ND	ND	ND	ND	ND	ND	ND
Estrone	NA	NA	NA	NA	26 ± 12	P	NA	NA	13 ± 40	5 ± 20
17α-Ethinylestradiol	NA	NA	NA	NA	ND	ND	NA	NA	ND	ND
<b>WWTP 2</b>										
Acesulfame K	<LOD	<LOD	88 ± 16	83 ± 10	<LOD	74 ± 9	106 ± 12	92 ± 8	49 ± 6	41 ± 6
Sulfamethoxazole	< LOD	< LOD	515 ± 8	< LOD	1002 ± 18	544 ± 10	< LOD	< LOD	576 ± 11	432 ± 10
Trimethoprim	307 ± 9	239 ± 7	324 ± 22	249 ± 4	384 ± 12	193 ± 11	287 ± 9	112 ± 9	236 ± 7	209 ± 9
Carbamazepine	477 ± 6	436 ± 5	488 ± 14	529 ± 11	619 ± 10	334 ± 15	340 ± 12	212 ± 8	445 ± 8	367 ± 13
Ibuprofen	P	ND	P	P	P	3322 ± 8	P	315 ± 14	P	ND
Gemfibrozil	P	ND	ND	ND	ND	ND	ND	ND	ND	ND
Triclosan	P	ND	ND	ND	1203 ± 19	321 ± 11	ND	ND	ND	63 ± 12

COMPOUND	MEAN CONCENTRATIONS ( $\pm$ %SD)									
	(ng L <sup>-1</sup> )									
	Influent	Effluent	Influent	Effluent	Influent	Effluent	Influent	Effluent	Influent	Effluent
	April		May		June		July		August	
Atrazine	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Bentazon	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
MCPA	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
2,4-D	ND	ND	ND	ND	ND	34 $\pm$ 12	ND	ND	ND	ND
DEET	708 $\pm$ 14	593 $\pm$ 1	2554 $\pm$ 12	1974 $\pm$ 6	10092 $\pm$ 7	2440 $\pm$ 10	14274 $\pm$ 9	389 $\pm$ 9	4277 $\pm$ 3	146 $\pm$ 11
Androstenedione	ND	ND	ND	ND	407 $\pm$ 36	ND	ND	ND	419 $\pm$ 24	ND
17 $\beta$ -Estradiol	NA	NA	ND	ND	ND	ND	ND	ND	ND	ND
Estrone	NA	NA	NA	NA	P	P	NA	NA	P	P
17 $\alpha$ -Ethinylestradiol	NA	NA	NA	NA	ND	ND	NA	NA	ND	ND

Table 3

Mean concentrations (ng L<sup>-1</sup>; ± %SD) of microcontaminants in wastewater sampled in 2014 from influent (INF) and effluent (EFFL) of WL and in a river surface water 2.0 km downstream of the lagoon discharge. ND = Not detected at concentrations > LOD; P = Present at concentrations < LOQ; NA = Not analyzed.

COMPOUND	MEAN CONCENTRATIONS (± %SD)											
	(ng L <sup>-1</sup> )											
	INFL	EFFL	SURF WATER	INFL	EFFL	SURF WATER	INFL	EFFL	SURF WATER	INFL	EFFL	SURF WATER
	May			June			September (Set 1)			September (Set 2)		
Acesulfame K	154 ± 4	79 ± 15	39 ± 11	106 ± 6	23 ± 10	ND	33 ± 6	36 ± 5	3 ± 19	74 ± 12	ND	ND
Sulfamethoxazole	ND	ND	ND	ND	ND	ND	ND	ND	ND	1125 ± 13	ND	ND
Trimethoprim	ND	ND	ND	ND	ND	ND	184 ± 6	87 ± 4	20 ± 11	119 ± 13	ND	ND
Carbamazepine	74 ± 2	ND	41 ± 4	152 ± 14	ND	ND	196 ± 15	111 ± 13	ND	793 ± 11	ND	ND
Ibuprofen	P	P	261 ± 9	ND	209 ± 22	ND	P	325 ± 10	118 ± 7	P	ND	160 ± 13
Gemfibrozil	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Triclosan	ND	ND	ND	ND	ND	3599 ± 14	ND	ND	ND	ND	ND	ND
Atrazine	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Bentazon	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
2,4-D	ND	ND	39 ± 4	ND	ND	ND	ND	ND	ND	ND	ND	ND
MCPA	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
DEET	19849 ± 11	173 ± 3	ND	3124 ± 6	ND	ND	931 ± 5	175 ± 7	45 ± 12	2326 ± 12	ND	218 ± 12
Androstenedione	231 ± 7	ND	ND	241 ± 7	NA	NA	359 ± 3	ND	ND	298 ± 15	ND	NA
17β-Estradiol	ND	ND	ND	NA	NA	NA	ND	ND	ND	NA	NA	NA
Estrone	7	P	P	NA	NA	NA	P	P	P	NA	NA	NA
17α-Ethinylestradiol	ND	ND	ND	NA	NA	NA	ND	ND	ND	NA	NA	NA

## Results of MTS Assay

In working with human cell lines, the endocrine endpoint generally exhibits greater sensitivity than endpoints of toxicity (Kolkman et al. 2013). Nonetheless, it is important to evaluate whether a reduced response *in vitro* assays is caused by cytotoxicity (*i.e.*, decreased cell viability). The MTS assay indicated that cell viability was not affected by exposure to the sample extracts at all dilutions examined (data not presented). Note that all cell lines employed (*i.e.*, MCF7, Qiagen transfected MCF7, U2OS, Nrf2 cells) were tested for cell viability using the MTS assay. Thus, any change in response using these cell lines can be attributed to pathway-specific impacts.

## Results of 10-Pathway Reporter Array

The results of the 10-Pathway Reporter Array were used to select which cellular responses should be investigated more closely. Table 4 summarizes the mean IR values (n = 3) for the various receptors following exposure to selected wastewater extracts. All sample extracts induced upregulation of the estrogen receptor and liver X receptor (Table 4). IRs of 12 to 47 were recorded for the estrogen receptor, while IRs ranged from 10 to 45 for the liver X receptor. There were no obvious reductions to upregulation of these receptors in treatments with effluent samples relative to treatments with influent samples. There was also upregulation of the vitamin D and retinoid X receptors in some treatments, with IRs ranging from 2 to 16 and from 3 to 19, respectively (Table 4). For the progesterone receptor, the IR values were

≥ 5 in treatments with only four influent samples, namely WWTP1 in August 2014, WWTP2 in June 2014 and August 2014, and WL in September 2014. Only minimal upregulation of the glucocorticoid and retinoic acid receptors was observed. Likewise, upregulation of the peroxisome-proliferator activation (PPAR) receptor was only observed in treatments with the June 2014 and September 2014 influent samples from WWTP1 and WL, respectively (Table 4).

Table 4

Mean (± SD) induction ratios for upregulation of cellular receptors in the 10-Pathway Reporter Array in treatments with extracts prepared from wastewater collected from WWTP 1, WWTP 2 and WL, and surface water downstream of WL.

LOCATION	DATE	SAMPLE	Induction Ratio (± SD)								
			Estrogen	Androgen	PPAR	Retinoic Acid	Vitamin D	Glucocorticoid	Progesterone	Retinoid X	Liver X
WWTP1	June	Influent	13 ± 1	0.6 ± 0.1	3 ± 1	1 ± 0.1	9 ± 3	1 ± 0.2	3 ± 1	5 ± 2	10 ± 5
		Effluent	14 ± 4	0.8 ± 0.3	2 ± 1	1 ± 1	2 ± 0.2	1 ± 0.2	1 ± 0.2	4 ± 2	12 ± 5
	August	Influent	28 ± 2	3 ± 1	11 ± 7	2 ± 0.2	16 ± 8	3 ± 1	9 ± 3	19 ± 6	31 ± 9
		Effluent	18 ± 3	2 ± 1	4 ± 2	1 ± 0.2	3 ± 1	1 ± 0.3	2 ± 0.1	7 ± 3	45 ± 8
WWTP2	June	Influent	22 ± 3	1 ± 0.1	4 ± 1	2 ± 1	5 ± 0.4	1 ± 0.1	8 ± 2	7 ± 1	18 ± 4
		Effluent	31 ± 14	1 ± 0.1	2 ± 0.1	2 ± 0.1	5 ± 2	1 ± 0.4	3 ± 1	7 ± 3	27 ± 2
	August	Influent	22 ± 8	2 ± 1	4 ± 0.2	3 ± 1	11 ± 1	2 ± 1	6 ± 2	14 ± 5	27 ± 5
		Effluent	22 ± 6	1 ± 0.3	3 ± 1	1 ± 0.4	5 ± 2	1 ± 0.2	4 ± 1	4 ± 2	30 ± 2
WL	May	Influent	12 ± 2	1 ± 0.2	4 ± 3	1 ± 0.3	5 ± 1	2 ± 1	1 ± 1	7 ± 4	18 ± 3
		Effluent	47 ± 25	1 ± 0.04	3 ± 1	2 ± 1	5 ± 4	3 ± 1	3 ± 2	5 ± 4	31 ± 12
		Surface Water	20 ± 10	1 ± 1	2 ± 0.2	4 ± 4	5 ± 0.3	2 ± 2	2 ± 1	7 ± 6	17 ± 2
	Sept (Set 1)	Influent	30 ± 16	2 ± 1	7 ± 3	3 ± 1	11 ± 6	5 ± 2	8 ± 2	9 ± 5	17 ± 6
		Effluent	28 ± 8	1 ± 1	2 ± 1	1 ± 0.3	5 ± 4	1 ± 0.2	4 ± 1	3 ± 1	11 ± 4
Surface Water	23 ± 8	1 ± 0.2	1 ± 0.4	1 ± 0.3	2 ± 1	1 ± 1	3 ± 1	3 ± 1	12 ± 3		

While there was some evidence that the influent extracts were more potent than the effluent extracts in upregulating some of the receptors, it was not possible to determine if the assay is sensitive enough to reproducibly detect differences between treatments with influent versus effluent extracts. There was no significant upregulation of the androgen receptor or the retinoic acid receptor, with maximum IR values of 3 and 4, respectively (Table 4). The lack of a significant response for upregulation of the androgen receptor is worth noting, since androstenedione was detected in several influent samples.

In a study of *in vitro* bioassays to assess wastewater treatment, Escher et al. (2014) observed that 5 out of 25 nuclear receptors were activated when exposed to effluent extracts; including the pregnane X, PPAR $\gamma$ , liver X and glucocorticoid receptors. Based on the results from the 10-Pathway Reporter Array described in the current study, it is apparent that the estrogen and liver X receptors showed the greatest upregulation in treatments with wastewater extracts. Note that upregulation of these pathways occurred in treatments with samples of influent, effluent and surface water. Significant upregulation was also observed for the retinoid X receptor.

Upregulation of liver X and retinoid X pathways is of particular interest as the two receptors form heterodimers that can then regulate genes associated with a range of cellular processes, such as lipid metabolism and inflammation. (Gage et al. 2016). The regulation of

PPAR receptors by wastewater extracts is also of interest as these receptors are targeted by cholesterol-regulating drugs (Roberts et al. 2015), including the gemfibrozil drug selected for analysis in this study. Metcalfe et al. (2013) detected PPAR-agonists in extracts prepared from wastewater using an *in vitro* assay, but these responses were not correlated with the concentrations of cholesterol-reducing drugs targeted for analysis. A variety of other compounds that can be present in wastewaters have the capacity to bind with PPARs, including anti-inflammatory drugs (Gijsbers et al. 2011), and phthalates, perfluorinated compounds and bisphenol-based compounds (Desvergne et al. 2009, Riu et al. 2011, Chamorro-Garcia et al. 2012). Synthetic glucocorticoids such as prednisone and hydrocortisone are drugs that are widely prescribed for suppression of inflammation. Synthetic progestins are the active ingredients for hormone therapies (e.g., for endometrial hyperplasia) and in many birth-control formulations. Future monitoring of wastewater using analytical techniques could include analysis for glucocorticoid and progesterone agonists used for therapy (Schriks et al. 2010, Wu et al. 2019).

## Results of ER $\alpha$ CALUX Assay

The activation of the estrogen receptor observed in all samples tested with the 10-Pathway Reporter Array highlighted the need for additional tests of estrogenicity using the ER $\alpha$  CALUX assay. The results from the ER $\alpha$  CALUX assay in the present study, expressed as ng L<sup>-1</sup>E2 equivalents, demonstrated differences in the estrogenic potency of influent and effluent extracts, as there was a decrease in the estrogenicity in all effluent samples collected following wastewater treatment (Table 5). The mean estrogenic response to extracts from WWTP 1 influent ranged from 27 to 72 ng L<sup>-1</sup> E2 equivalents, while the mean estrogenic response to extracts from WWTP 1 effluent ranged from 1 to 10 ng L<sup>-1</sup> E2 equivalents (Table 5). The highest estrogenic activity was observed in samples collected in the months of June and August (Table 5). For samples collected from WWTP2, the mean estrogenic response to influent samples ranged from 34 to 59 ng L<sup>-1</sup> E2 equivalents, while the mean estrogenic potency of effluent samples ranged from 2 to 14 ng L<sup>-1</sup> E2 equivalents (Table 5). Similarly to WWTP 1, the highest estrogenic responses from WWTP 2 were observed in treatments with samples collected in May, June and August (Table 5).

No significant difference in estrogenic activity was observed between effluent samples from WWTP1 and WWTP2. Previously, it was suggested that nitrification may enhance the degradation of steroid estrogens (Servos et al. 2005, Khanal et al. 2006). The treatment train for WWTP2 includes a nitrification step, but since the mean concentration of nitrate plus nitrite in the effluent of WWTP 2 (i.e. 16.1 mg L<sup>-1</sup>) over the monitoring period was only marginally higher than the mean concentration of nitrate and nitrite in the effluent of WWTP 1 (i.e. 14.8 mg L<sup>-1</sup>), it is difficult to speculate on whether nitrification is an important parameter for reducing estrogenic activity. The treatment train in WWTP 2 includes tertiary treatment by filtration, whereas there is only secondary treatment at WWTP 1, but the additional treatment step in WWTP 2 did not seem to enhance the reduction in estrogenicity. For grab samples collected from the wastewater treatment lagoon (WL), the mean estrogenic responses to extracts from influent ranging from 56 to 215 ng L<sup>-1</sup> E2 equivalents were higher than the estrogenic responses observed in treatments with influent from WWTPs 1 and 2 (Table 6). Nonetheless, the estrogenicity of effluents from WL was comparable to the estrogenicity of the effluents from the WWTPs, with mean values ranging from 4 to 13 ng L<sup>-1</sup> E2 equivalents. These values are higher than the 1-216 pg/L E2 equivalent values reported previous for wastewater (Kase et al. 2018). This indicates that treatment of wastewater in lagoons that are properly managed can be equally efficient as conventional WWTPs. Finally, surface water samples collected downstream of the WL discharge were also estrogenic, with mean potencies of 5 to 17 ng L<sup>-1</sup> E2 equivalents (Table 6). CALUX assays have been used previously to evaluate the biological potency of wastewater samples (Roberts et al. 2015, Kase et al. 2018, Könemann et al. 2018). In a study investigating endocrine activity in a large Australian sewage treatment plant, estrogenic and anti-androgenic potency in CALUX assays was reduced following both primary and secondary treatment (Roberts et al. 2015). Könemann et al. (2018) tested wastewater samples from 17 sites in European countries in Central and Southern Europe and concluded that results from the ER-CALUX assay were comparable to other methods, including the luciferase-transfected human breast cancer cell line (MELN) gene reporter assay, the ER-GeneBLazer assay, the stably transfected human estrogen receptor-alpha transcriptional activation Assay using hER $\alpha$ -HeLa-9903 cells (HeLa-9903 assay) and the planar Yeast Estrogen Screen (pYES).

Often, *in vitro* assays are more sensitive than analytical methods for detecting the presence of agonists (Escher et al. 2012, Kase et al. 2018). This could have been the case in the present study, where significant upregulation of the estrogen receptor and positive responses in the ER $\alpha$  CALUX assay were detected, even though the concentrations of two of the most potent estrogens were below the limits of detection (i.e. 17 $\alpha$ -ethinylestradiol, 17 $\beta$ -estradiol). Analytical methods used for the monitoring of priority substances needs a LOQ equal or below a value of 30% of the EQS (Könemann et al. 2018), which for estrogens can only be achieved using state-of-the-art instruments dedicated to these analysis, which was not the case here. However, estrone was detected in samples collected from WWTP 1 and WL and was present at concentrations < LOQ in samples from WWTP 2. It is important to remember that there are a variety of estrogenic compounds that could contribute to the estrogenic potency in extracts of wastewater, including alkylphenols, bisphenol A and

phytoestrogens. However, Brand et al. (2014) reported that steroid estrogens (e.g. 17 $\beta$ -estradiol) were by far the most potent agonists in the ER $\alpha$  CALUX assay.

Different values have been proposed as effect-based trigger (EBT) values for wastewater, 100–500 pg/L E2-equivalent (EEQ), and based on the discussion presented in Kase et al. (2018), the use of an EBT of 400 pg /L EEQ seems justified. Considering that the values measured in the treated wastewater (effluent) were much higher, in the range of 1–14 ng/L E2 equivalents, the results indicate a potential ecological risk associated with the discharge of the effluent. In the EU, an EBT value for the ER $\alpha$  CALUX assay of 3.8 ng L<sup>-1</sup> E2 equivalents has been proposed for drinking water and source waters (Brand et al. 2013, Brand et al. 2014). Since estrogenic activity was detected in surface waters downstream of the lagoon discharge (5 to 17 ng L<sup>-1</sup> E2 equivalents), it may be advisable to monitor drinking water for estrogenic activity using the ER $\alpha$  CALUX assay or another sensitive *in vitro* assay.

Table 5

Mean ( $\pm$  %SD) responses in the ER $\alpha$  CALUX assay (ng L<sup>-1</sup> E2 equivalents) and Nrf2 assay (mg L<sup>-1</sup> tBHQ equivalents) in treatments with extracts prepared from wastewater collected from WWTP 1 and WWTP 2.

DATE	SAMPLE	WWTP 1		WWTP 2	
		ER $\alpha$ CALUX	Nrf2	ER $\alpha$ CALUX	Nrf2
		ng L <sup>-1</sup> E2 EQUIVALENTS $\pm$ %SD	mg L <sup>-1</sup> tBHQ EQUIVALENTS $\pm$ %SD	ng L <sup>-1</sup> E2 EQUIVALENTS $\pm$ %SD	mg L <sup>-1</sup> tBHQ EQUIVALENTS $\pm$ %SD
April	Influent	28.4 $\pm$ 6.3	0.31 $\pm$ 12	34.1 $\pm$ 13.7	0.41 $\pm$ 10
	Effluent	1.3 $\pm$ 8.6	0.07 $\pm$ 14	13.7 $\pm$ 10.6	0.17 $\pm$ 29
May	Influent	37.8 $\pm$ 14.7	0.29 $\pm$ 10	52.2 $\pm$ 2.2	0.43 $\pm$ 7
	Effluent	9.7 $\pm$ 9.7	3.5 $\times$ 10 <sup>-3</sup> $\pm$ 50	3.1 $\pm$ 14.7	0.24 $\pm$ 17
June	Influent	71.6 $\pm$ 6.2	0.34 $\pm$ 15	50.2 $\pm$ 4.4	0.52 $\pm$ 2
	Effluent	3.8 $\pm$ 5.2	0.10 $\pm$ 10	2.6 $\pm$ 11.7	0.21 $\pm$ 10
July	Influent	26.6 $\pm$ 12.6	0.28 $\pm$ 4	36.4 $\pm$ 8.3	0.42 $\pm$ 10
	Effluent	2.5 $\pm$ 4.0	0.10 $\pm$ 20	1.5 $\pm$ 5.6	0.19 $\pm$ 5
August	Influent	46.7 $\pm$ 11.9	0.26 $\pm$ 4	58.6 $\pm$ 3.7	0.56 $\pm$ 17
	Effluent	1.6 $\pm$ 9.5	0.12 $\pm$ 17	12.8 $\pm$ 10.0	0.17 $\pm$ 35

Table 6

Mean ( $\pm$  %SD) responses in the ER $\alpha$  CALUX (ng L<sup>-1</sup> E2 equivalents) and Nrf2 (mg L<sup>-1</sup> tBHQ) in treatments with extracts prepared from wastewater collected from WL and surface water downstream of the discharge from the lagoon.

DATE	SAMPLE	ER $\alpha$ CALUX	Nrf2
		ng L <sup>-1</sup> E2 EQUIVALENTS $\pm$ %SD	mg L <sup>-1</sup> tBHQ EQUIVALENTS $\pm$ %SD
May	Influent	172.9 $\pm$ 7	0.50 $\pm$ 10
	Effluent	4.5 $\pm$ 3	0.25 $\pm$ 12
	Surface Water	4.9 $\pm$ 12	0.14 $\pm$ 28
June	Influent	56.3 $\pm$ 8	0.38 $\pm$ 21
	Effluent	3.7 $\pm$ 8	0.12 $\pm$ 8
	Surface Water	15.4 $\pm$ 6	0.28 $\pm$ 4
September (Set 1)	Influent	214.7 $\pm$ 8	0.48 $\pm$ 8
	Effluent	13.4 $\pm$ 20	0.27 $\pm$ 11
	Surface Water	17.1 $\pm$ 10	0.25 $\pm$ 8
September (Set 2)	Influent	137.5 $\pm$ 13	0.49 $\pm$ 8
	Effluent	8.6 $\pm$ 13	0.14 $\pm$ 14
	Surface Water	4.9 $\pm$ 15	0.13 $\pm$ 3

## Results of Nrf2 Assay

The Nrf2 assay is an indicator of oxidative stress in cells. More specifically, the bioassay measures induction of the Nrf2-Keap-ARE pathway, which protects cells against oxidative damage resulting from spontaneous cellular processes or exposure to contaminants. (Jia et al. 2015) observed that the Nrf2-Keap-ARE pathway responds to “a very wide range of chemicals” but did not specify which classes of compounds were active. Martin et al. (2010) reported that 165 of the 309 chemicals tested in the Phase I ToxCast survey conducted by the US EPA induced oxidative stress, as detected by Nrf2 activation. After testing 19 compounds for responses in various *in vitro* assays, van der Linden et al. (2014) reported that 2,4-dichlorophenol, curcumin, ethyl acrylate, p-nitrophenol and propyl gallate, in addition to tBHQ, gave a positive response in an Nrf2 bioassay. Tang et al. (2013) detected significant Nrf2 activation in treatments with extracts from urban storm water and commented that, “further chemical analysis is required to identify the causative agents for the underlying toxicity”.

Overall, the responses to wastewater extracts in the Nrf2 assay used in the present study indicate that there was a significant decrease in the capacity to induce oxidative stress in effluent extracts relative to influent extracts from samples collected at WWTP 1 and WWTP 2 (Table 5) and in WL (Table 6). In treatments with extracts from WWTP 1, exposure to untreated wastewater samples induced mean responses of tBHQ equivalents ranging from 0.26 to 0.34 mg L<sup>-1</sup>, while mean responses to effluent samples did not exceed 0.12 mg L<sup>-1</sup> and were as low as 3.5 $\times$ 10<sup>-3</sup> mg L<sup>-1</sup> (Table 5). Likewise for samples from WWTP 2, exposures to extracts from influent samples resulted in mean tBHQ equivalents ranging from 0.41 to 0.56 mg L<sup>-1</sup>, while exposure to effluent samples resulted in significantly lower responses, with mean tBHQ equivalents ranging from 0.17 to 0.24 mg L<sup>-1</sup> (Table 5). Jia et al. (2015) also reported that activity was reduced in extracts from samples collected after wastewater treatment in an Nrf2 Luciferase Luminescence Assay with a different cell line.

The data for WL showed similar trends, with responses to treated effluent samples indicating reduced capacity to induce oxidative stress (Table 6). The responses to extracts from surface water samples collected downstream of WL indicated that there were compounds present that induce oxidative stress, but the responses to extracts from surface water showed no apparent correlation with the activity in the corresponding lagoon effluent samples. For example, while mean tBHQ equivalent values for effluent and surface water samples collected in May of 2014 were 0.25 and 0.14 mg L<sup>-1</sup>, respectively, treatments with extracts from June 2014 effluent and surface water samples showed mean responses of 0.12 and 0.28  $\pm$  mg L<sup>-1</sup> tBHQ equivalents, respectively (Table 6). However, exposures to extracts from effluent and surface water samples collected in September resulted in very similar responses (Table 6).

## Conclusions

Analysis of extracts of wastewater using the 10-Pathway Reporter Array showed that there was widespread ER upregulation, but also upregulation of liver X, vitamin D and retinoid X receptors. Also, exposure to certain samples resulted in upregulation of the glucocorticoid, peroxisome and PPAR receptors. Future work should include evaluations of the activity of wastewater extracts for induction of these receptors. For instance, there are CALUX reporter gene assays that can test for agonistic activity for the glucocorticoid and progesterone receptors (Brand et al. 2013) and PPAR receptors (Gijsbers et al. 2011), as well as oxidative stress through the Nrf2 pathway (van der Linden et al. 2014). Previous studies have associated endocrine disruption (e.g., estrogenicity) to sediments, regardless of the level of endocrine activity in the surface water (Peck et al. 2004, Koyama et al. 2013). Thus, future work could include evaluations of the activity of extracts prepared from sediments in receiving waters or sludge/biosolids from wastewater treatment plants.

The present study demonstrated that there were reductions in the estrogenic activity and capacity to induce oxidative stress in treated wastewater relative to untreated wastewater collected from both conventional WWTPs and a sewage lagoon, however values of E2 equivalent were higher than previous reported values for treated wastewater (Kase et al. 2018), suggesting that further investigation are required to minimize the ecological risks associated with the discharge of the treated effluent. The reductions in cellular responses were consistent with improvements in wastewater quality after treatment that were measured using standard wastewater monitoring methods (e.g. TSS, cBOD, total ammonia). However, analysis of targeted contaminants in wastewater from the classes of pharmaceuticals and personal care products, pesticides and steroid hormones indicated that several of these compounds were below detection limits (E2 & EE2) or not removed effectively by wastewater treatment (e.g. carbamazepine, trimethoprim, acesulfame K, DEET).

No correlations could be established between the results of chemical analyses and the cellular activity detected in *in vitro* assays. Similarly, Tang et al. (2014) used four *in vitro* cell-based assays to evaluate biological responses to extracts from wastewater and recycled water and also analyzed these extracts for 299 organic compounds and observed that the concentrations of known chemicals explained less than 3% of the cytotoxicity and less than 1% of the oxidative stress responses to the extracts. In addition, some *in vitro* assays, such as the CALUX assays can be more sensitive at detecting cellular responses from treatments with wastewater extracts than the analytical methods used to detect the target compounds that may induce these responses (e.g. steroid hormones) (Könemann et al. 2018). There are currently no regulatory applications of *in vitro* bioassays (Snyder and Leusch 2018) and the present study contributes further evidence for the implementation of bioassays in regulatory frameworks, such as the one proposed in Europe (Könemann et al. 2018, Snyder and Leusch 2018). Overall, the present study showed that *in vitro* assay systems can be valuable additions to evaluating treatment performances and monitoring programs for water and wastewater. It can also be used as screening methods to focus analytical methods on candidate compounds that are a threat to the aquatic environment and/or to human health.

## Declarations

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**Code availability** – Not applicable

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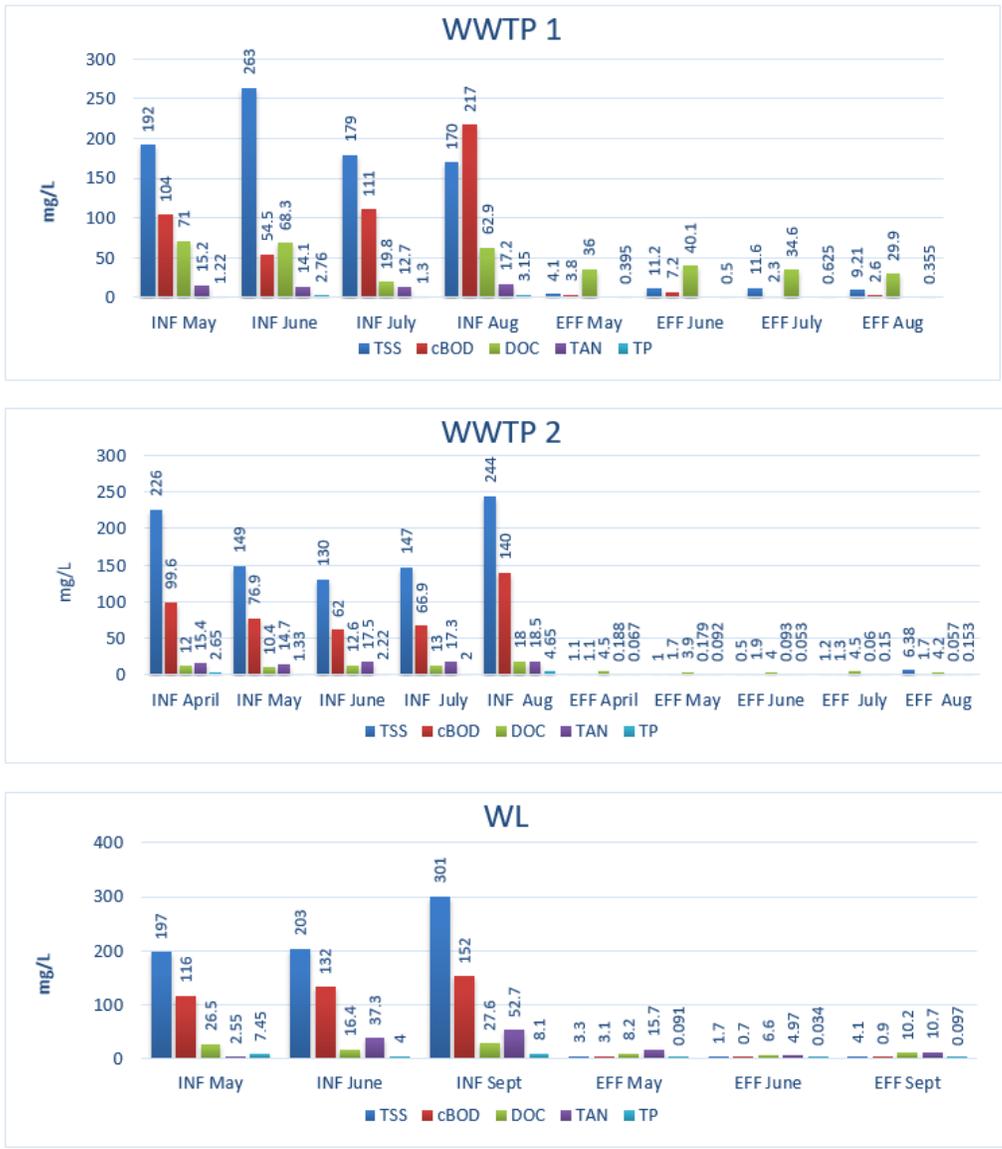
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## Figures



**Figure 1**

Concentrations (mg L<sup>-1</sup>) of total suspended solids (TSS), carbonaceous biological oxygen demand (cBOD), dissolved organic carbon (DOC), total ammonia nitrogen (TAN) and total phosphorus (TP) in influent (INF) and effluent (EFF) samples collected from WWTP 1, WWTP 2 and WL during the sampling period in 2014. NA = Not analyzed.

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