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A multi-assay screening approach for assessment of endocrine-active contaminants in wastewater effluent samples

Chris D. Metcalfe [a,*], Sonya Kleywegt [b], Robert J. Letcher [c], Edward Topp [d], Purva Wagh [e], Vance L. Trudeau [e], Thomas W. Moon [e]

[a] Environmental and Resource Studies, Trent University, Peterborough, ON, K9J 7B8, Canada
[b] Standards Development Branch, Ontario Ministry of the Environment, 40 St. Clair Ave. West, Toronto, ON, M4V 1M2, Canada
[c] Ecotoxicology and Wildlife Health Division, Science and Technology Branch, Environment Canada, National Wildlife Research Centre, Carleton University, Ottawa, ON, K1A 0H3, Canada
[d] Agriculture and Agri-Food Canada, Southern Crop Protection and Food Research Centre, London, ON, N5V 7T3, Canada
[e] Department of Biology and Centre for Advanced Research in Environmental Genomics, University of Ottawa, Ottawa, ON, K1N 6N5, Canada

HIGHLIGHTS

- Tested wastewater for in vitro activity for comparison to analytical measurements of EDCs.
- Estrogenic activity was detected only in extracts that contained estradiol or estrone.
- Positive relationship observed for binding to thyroid transport protein and 4-OH-PBDE17.
- Wastewater showed response in peroxisome proliferator receptor assay, but no relationship observed with levels of contaminants.
- Feasible to use in vitro testing as a screening tool before instrumental analysis of contaminants.

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ABSTRACT

Environmental agencies must monitor an ever increasing range of contaminants of emerging concern, including endocrine disrupting compounds (EDCs). An alternative to using ultra-trace chemical analysis of samples for EDCs is to test for biological activity using *in vitro* screening assays, then use these assay results to direct analytical chemistry approaches. In this study, we used both analytical approaches and *in vitro* bioassays to characterize the EDCs present in treated wastewater from four wastewater treatment plants (WWTPs) in Ontario, Canada. Estrogen-mediated activity was assessed using a yeast estrogenicity screening (YES) assay. An *in vitro* competitive binding assay was used to assess capacity to interfere with binding of the thyroid hormone, thyroxine (T4) to the recombinant human thyroid hormone transport protein, transthyretin (i.e. hTTR). An *in vitro* binding assay with a rat peroxisome proliferator responsive element transfected into a rainbow trout gill cell line was used to evaluate binding and subsequent gene expression via the peroxisome proliferator activated receptor (PPAR). Analyses of a suite of contaminants known to be EDCs in extracts from treated wastewater were conducted using either gas chromatography with mass spectrometry (GC-MS) or liquid chromatography with tandem mass spectrometry (LC-MS/MS). Estrogenic activity was detected in the YES assay only in those extracts that contained detectable amounts of estradiol (E2). There was a positive relationship between the degree of response in the T4-hTTR assay and the amounts of polybrominated diphenyl ether (PBDE) congeners 47 and 99, triclosan and the PBDE metabolite, 4-OH-BDE17. Several wastewater extracts gave a positive response in the PPAR assay, but these responses were not correlated with the amounts of any of the EDCs analyzed by LC-MS/MS. Overall, these data indicate that a step-wise approach is feasible using a combination of *in vitro* testing and instrumental analysis to monitor for EDCs in wastewater and other environmental matrixes.

1. Introduction

Modulation or disruption of the homeostatic function of endocrine systems by xenobiotic compounds continues to be a concern in humans and in wildlife (Hotchkiss et al., 2008). The aquatic environment is the ultimate sink for natural and man-made chemicals. One of the primary sources for the release of endocrine disrupting compounds (EDCs) into the aquatic environment is municipal wastewater. In Canada, there are a number of classes of contaminants that have been designated as EDCs that are discharged into surface waters from wastewater treatment plants (WWTPs), including natural estrogen hormones, alkylphenol

* Corresponding author. Tel.: +1 705 748 1011x7272; fax: +1 705 748 1569.
E-mail address: cmetcalfe@trentu.ca (C.D. Metcalfe).

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surfactants, estrogenic plasticizers, prescription and non-prescription pharmaceuticals, personal care products and brominated flame retardants. There is compelling evidence that EDCs released into the aquatic environment are impacting aquatic organisms, including populations of fish (Mills and Chichester, 2005; Fent et al., 2006; Tetreault et al., 2011).

The proliferation in the number and diversity of EDCs, and the need to detect these compounds at part per trillion concentrations has taxed the capacity of institutions that rely on analytical chemistry approaches to monitor wastewater and surface water (Richardson and Ternes, 2011). In addition, there may be endocrine disrupting activity in wastewater because of the presence of metabolites of microcontaminants (Miao et al., 2005; Metcalfe et al., 2010; Trudeau et al., 2011) or the production of disinfection by-products (McDowell et al., 2005; Bedner and McCrehan, 2006; Larcher et al., 2012). However, analytical capacity is presently lacking to monitor WWTPs and surface waters for the presence of these compounds.

An alternative to using ultra-trace chemical analysis to monitor for EDCs is to test for biological activity using a multi-tiered screening approach. The advantage of this approach is that extracts prepared from wastewater or surface water can be tested using sensitive, relatively rapid and inexpensive *in vitro* assays to determine if EDCs are present in the extracts. Then, the results of these assays can direct and prioritize the more expensive and time-consuming work of ultra-trace chemical analysis. For example, a lack of endocrine disrupting activity would indicate that additional chemical analysis may not be necessary. Conversely, when a sample is biologically active, the nature of the endpoint and the intensity of the response may direct the analytical effort to contaminants of specific interest.

In this study, we applied a screening assay approach to identify biological activity in extracts prepared from the effluents of four municipal WWTPs in Ontario, Canada (Table 1). We focused our experimental approach on three classes of EDCs that are active via: i) estrogen receptor binding and subsequent gene expression, ii) thyroid hormone signaling and specifically, via competitive binding with the thyroid hormone transport protein, transthyretin (TTR), and iii) peroxisome proliferator activator receptor (PPAR) binding and subsequent gene expression.

Many different agonists of the estrogen receptor have been detected in WWTP effluents, including the natural estrogens, estradiol (E2) and estrone (E1), and xeno-estrogens such as the synthetic estrogen, ethinylestradiol (EE2), and alkylphenol compounds such as nonylphenol and octylphenol (Metcalfe et al., 2001). A variety of environmental contaminants are PPAR agonists. PPARs are transcription factors that control key cellular functions, including lipid metabolism, inflammation and cell differentiation. Studies have shown that fish respond at the cellular level to PPAR agonists (Ruyter et al., 1997; Liu et al., 2005).

Thyroid system-disrupting chemicals that structurally resemble thyroxine (T4) and triiodothyronine (T3) may target multiple thyroid hormone (TH) dependent pathways. The mechanisms of interference can include competitive interactions with TH binding proteins (Miller et al., 2009; Ishihara et al., 2003). There is considerable evidence that hydroxylated (OH) and to a lesser degree methoxylated (MeO) analogues of PBDEs and PCBs alter circulating TH homeostasis (Ucan-Marin et al., 2009, 2010; Meerts et al., 2000, 2001). Several classes of brominated flame retardants including PBDEs, OH-PBDEs as well as OH-PCBs, and the anti-microbial compound triclosan have been reported in biota from the Great Lakes basin (Chen et al., 2011; Letcher and Chu, 2010; Valters et al., 2005).

We used both analytical approaches and *in vitro* assays to characterize the EDCs present in extracts prepared from WWTP effluents. Binding to the human estrogen receptor (hERα) was assessed using a yeast estrogenicity screening (YES) assay (Gaido et al., 1997). An *in vitro* competitive binding assay (Meerts et al., 2000; Ucan-Marin et al., 2010) was used to assess the capacity to interfere with binding of T4 to the human TTR. PPAR activity was evaluated using an *in vitro* binding assay with a rat peroxisome proliferator responsive element (PPRE) transfected into a rainbow trout gill cell line (Liu et al., 2005). Analyses of EDCs in extracts from WWTP effluents were conducted using either gas chromatography with mass spectrometry (GC-MS) or liquid chromatography with tandem mass spectrometry (LC-MS/MS).

2. Methods and Materials

2.1. Sample collection and preparation

Composite samples (3 L) of final treated wastewater were collected over 24 h at a rate of 125 mL per hour on each of 3 consecutive days, and refrigerated. Following the three day sampling campaign, all samples were immediately shipped from the WWTP for extraction. The samples were received within 24 h of shipment from the WWTP. Note that composite samples of treated wastewater were collected from two separate treatment streams at WWTP 1 that treat 60% and 40% of the effluent, respectively, and at two treatment streams for WWTP 4 that treat 40% and 60% of the effluent, respectively (Table 1). These separate samples were then mixed in these proportions prior to shipment for subsequent extraction.

All extractions were completed within 72 h of receipt of the shipped samples. For each day in the sampling campaign, replicate 100 mL subsamples (n = 6) of the samples were prepared with spikes of internal standards (IS) for chemical analysis, and replicate 100 mL subsamples (n = 6) were prepared without surrogate spikes for *in vitro* testing (Fig. 1). In addition, on each day, 3 blanks of

Table 1
Information on the wastewater treatment plants (WWTPs) serving four cities in Ontario, Canada that were sampled for treated wastewater. This information includes data on the population served, the treatment technologies and the hydraulic retention times (HRTs) in the aeration lagoons, and the total flow rates of the wastewater. Note that HRTs and flows are presented individually for two treatment streams in both WWTP 1 and WWTP 4.

Location	Pop	Treatment	HRT	Total flow
	Served	Technology	(hours)	(m^3/d)
WWTP 1	207,000	Non-nitrified	Day 1: 6.5; 6.8	Day 1: 48,000; 32,000
		Secondary; activated sludge	Day 2: 6.5; 6.8	Day 2: 49,800; 33,200
		Stream 1 (60%); Stream 2 (40%)	Day 3: 6.5; 6.8	Day 3: 49,800; 33,200
WWTP 2	123,000	Fully nitrified	Day 1: 7.7	Day 1: 23,497
		Secondary; activated sludge	Day 2: 7.3	Day 2: 24,691
			Day 3: 5.7	Day 3: 31,364
WWTP 3	8,523	Denitrified	Day 1: 16.32	Day 1: 3994
		Secondary; activated sludge	Day 2: 13.68	Day 2: 4764
			Day 3: 15.12	Day 3: 4327
WWTP 4	77,700	Tertiary	Day 1: 12.5; 9.8*	Day 1: 12,551; 18,826
		UV-disinfection	Day 2: 13.0; 9.8*	Day 2: 12,224; 18,337
		Stream 1 (40%); Stream 2 (60%)	Day 3: 13.0; 10.1*	Day 3: 12,141; 18,312