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Estrogenic activity in Finnish municipal wastewater effluents

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ABSTRACT

Effluents from wastewater treatment plants (WWTPs) are a major source of estrogenic compounds to the aquatic environment. In the present work, estrogenic activities of effluents from eight municipal WWTPs in Finland were studied. The main objectives of the study were to quantify the concentrations of selected estrogenic compounds, to evaluate their contribution to estrogenic potency and to test the feasibility of the commercial bioassays for wastewater analysis. The effluent samples were analyzed by two *in vitro* tests, i.e. ERα-CALUX® and ELISA-E2, and by liquid chromatography mass spectrometry for six estrogenic compounds: estrone (E1), 17β-estradiol (E2), estriol (E3), 17α-ethinylestradiol (EE2), 17α-estradiol and bisphenol A (BPA). Estrogenic effects were found in all of the effluent samples with both of the bioassays. The concentrations measured with ELISA-E2 (8.6–61.6 ng/L) were clearly higher but exhibited a similar pattern than those with chemical analysis (E2 <limit of quantification – 6.8 ng/L) and ERα-CALUX® (0.8–29.7 ng E2 EEQ/L). Due to the concentrations under limit of quantification, the evaluation of the chemical contribution to estrogenic potency was possible only for E1 and BPA, which contributed less than 10% to the observed effects, except in one sample with a high BPA contribution (17%). The contribution of E2 was significant in two samples where it was detected (28% and 67%). The results demonstrated that more comprehensive information on potential estrogenic activity of wastewater effluents can be achieved by using *in vitro* biotests in addition to chemical analysis and their use would be beneficial in monitoring and screening purposes.

1. Introduction

Endocrine disrupting compounds (EDCs) can interfere with hormone action and major physiological systems, and in doing so they can have adverse effects on human and wildlife health (Colborn, 1995; Roig et al., 2012). Chemicals with estrogenic activities have been under special focus and hundreds of chemicals have been newly identified as having estrogenic activities (Lintelmann et al., 2003; Nakada et al., 2004; Vethaak et al., 2005).

Municipal wastewater effluent is considered to be one of the major sources of EDCs to the aquatic environment (Aerni et al., 2004). Primary reason for the presence of estrogenic compounds in wastewater effluent is natural and synthetic estrogens excreted by humans. However, traces of household products such as pharmaceuticals, personal care products, plasticizers and fire retardants are also major sources of estrogenic compounds in municipal wastewater effluent. Some municipal WWTPs also have loading from industrial sources. Typically, estrone (E1) is the most frequently detected estrogen in municipal wastewater effluents, which can be explained by the human urinary excretion rates (Liu et al., 2009). In addition, E1 can be formed during the treatment process because it is an oxidation product of 17β-estradiol (E2) (Salvador et al., 2007). Other estrogenic compounds that have been regularly detected in municipal wastewater effluents are E2, 17β-ethinylestradiol (EE2), estriol (E3) and bisphenol-A (BPA). According to calculations based on consumption, excretion and population, it has been evaluated that 0.55 kg/year of EE2 and 15.3 kg/year of E2 are discharged to the Finnish WWTPs (Vieno, 2014). In the EU, BPA is widely used for industrial purposes, such as polycarbonate production (71%) and epoxy resins (25%) and as a consequence it is ubiquitous in the environment (Oehlmann et al., 2008). It has been shown to be one of the more potent man-made ER agonist and

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many aquatic organisms such as fish (Metcalfe et al., 2001; Honkanen et al., 2004; Birceanu et al., 2015) and mollusks (Oehlmann et al., 2000) have been shown to be sensitive to BPA. Nevertheless, information on BPA levels in treated wastewater effluent in Finland is scarce.

To this day the majority of wastewater treatment plants (WWTPs) have been designed for the removal of pathogens, phosphorus and nitrogen, with no particular consideration of organic micropollutants such as estrogens. Finland has more than 500 municipal WWTPs of which 20% serve more than 10,000 people. The primary technique used is activated sludge. Many WWTPs have a tertiary treatment step in their process. Typical tertiary treatment steps are different filtration processes (e.g. sand filtration) or dissolved air flotation. The removal of natural and synthetic estrogens is incomplete in the activated sludge process and such substances have been regularly detected in WWTP effluent globally (Bolong et al., 2009; Jarošová et al., 2014b). Conventional biological treatment removes only a portion of the different types of EDCs, and the removed compounds are mainly polar ones (Petrović et al., 2003). Some advanced biological treatment techniques, such as membrane bioreactor (MBR), have been shown to successfully reduce estrogenicity of effluents (Maletz et al., 2013).

Even though the concentrations of estrogens and estrogen-like compounds in municipal WWTP effluent are typically in the low ng/L range, there is room for improvement in the removal efficiency of estrogenic compounds (Laganà et al., 2004; Sim et al., 2011). Many examples show that EDCs can cause adverse effects at low concentrations (Colborn, 1995; Roepke et al., 2005). The detection of such low concentrations of estrogenic compounds from a complex matrix like WWTP effluent is challenging and sensitive methods are required. There are several approaches for monitoring the presence of estrogenic compounds in wastewater effluents, however monitoring of these compounds is traditionally based on chemical approaches alone. With chemical analysis, only a limited number of compounds can be measured from wastewaters with only little information on potential effects in the environment. Furthermore, it is not uncommon that chemical analyses, such as LC-MS/MS or GC–MS/MS, have quantification or detection limits that are higher than the concentrations in treated effluent samples for estrogenic compounds (Ingrand et al., 2003; Carballa et al., 2004; Nelson et al., 2007; Vieno, 2014). Therefore, biological tests are needed. If toxicity tests are required in wastewater analysis, often tests such as acute and long term toxicity to *Daphnia magna* are applied. In many cases *in vivo*/*in situ* tests with fish would be the most relevant tools for the detection of adverse environmental effects. However, these types of test have their limitations, they are usually time and sample consuming, and there is always the ethical aspect of using animals. Another downside of using acute toxicity tests is that specific endpoints such as estrogenic effects can remain undiscovered.

An alternative to using chemical analysis or acute toxicity tests for effluent analysis is to apply different combinations of *in vitro* bioassays and chemical analysis (Aerni et al., 2004; Pessala, 2008). Some studies have also added multiple endpoints and *in situ* biological monitoring (Leusch et al., 2014; Ihara et al., 2015). *In vitro* bioassays are generally thought to be relatively cost-effective, rapid and sensitive methods for estimating the estrogenic activity of samples. The results observed in the biotests can be used to direct the more expensive chemical analysis toward those chemicals that actually cause harmful effects. Sum parameter based *in vitro* bioassays are not compound specific but they measure the potential effects of the whole mixture including compounds that might be missed by chemical analysis. The disadvantage of using only these types of assays is that it is hard, if not impossible, to tell which compounds actually cause the observed effects. In addition, the tests represent a simplified system and the results are not directly comparable to *in vivo* effects (Jarošová et al., 2014a).

A number of different *in vitro* bioassays have been used to determine the estrogenic potential of environmental samples. These include yeast-based screens (Ma et al., 2007; Sun et al., 2008; Brix et al., 2010), cell proliferation assays (Körner et al., 1999) and competitive ligand binding assays (Murk et al., 2002; Bain et al., 2014). Estrogenic compounds, including natural estrogens and man-made chemicals, have many different pathways or mechanisms through which they can interfere with the endocrine system of humans and wildlife and each assay measures different aspects of being exposed to estrogens. One of these mechanisms is to act through high affinity receptors and there are bioassays that focus on the estrogen receptor (ER) mediated effects. By using an Estrogen Receptor-mediated, Chemical-Activated LUciferase reporter gene-eXpression (ERα-CALUX®) assay, estrogenic potency can be measured as a sum parameter of all compounds present in the effluent that can interfere with the estrogen receptor. The assay takes into consideration also mixture effects, even if the concentrations of individual estrogenic compounds are below the no-effect concentration (Legler et al., 1999). The ER-CALUX test have been used for single substance studies as well, thus information on the affinity of natural estrogens and industrial compounds, such as BPA, for the ER receptor are available. They can be used for estimating the contribution of target compounds to estrogenic effects in a complex sample matrix like wastewater effluent (Maletz et al., 2013).

To achieve comprehensive information on the estrogenic potency of wastewater effluent, it would be beneficial to use several bioassays that give information on the same endpoint, but are based on a different molecular mechanism. In addition to ER mediated tests, *in vitro* assays such as enzyme-linked immunosorbent assays (ELISA) can be used to identify individual or multiple compounds depending on the type of antibody. They are based on the selectivity and affinity of an antibody for its antigen. The benefits of ELISA are that it requires small sample volumes, is rapid, highly sensitive and specific, and provides the possibility of analyzing a large number of samples simultaneously (Caron et al., 2010). There are several types of ELISAs available for various toxicological endpoints, however, only a few studies have utilized the assay for the detection of estrogenic compounds (Allinson et al., 2010; Manickum and John, 2014).

To our knowledge, this is the first study that employs a biochemical assay (ELISA-E2), a reporter gene assay (ERα-CALUX®), acute and chronic *in vivo* tests and chemical analytical methods (LC-MS/MS) for the analysis of estrogenic activity in different types wastewater effluents. The results from chemical analysis were corrected with sample- and compound-specific recoveries, and their contributions to the observed estrogenic activity were estimated. In this study, results with exceptionally high estrogenic activity were obtained, especially related to wastewater effluents with extremely high BPA loading. The objectives of this study were: (a) To determine the estrogenic potencies of effluents and identify their contributors in eight municipal WWTPs in Finland by combining chemical analysis (LC-MS/MS) with two *in vitro* bioassays (ERα-CALUX® and ELISA-E2). (b) To test the suitability of the ELISA-E2 test for wastewater effluent analysis and compare it with chemical analysis and ERα-CALUX®. (c) To analyze the quality of the effluents using conventional toxicity tests (*Vibrio fischeri* and *D. magna* acute toxicity and *D. magna* reproduction) (d) To analyze the significance of sample and compounds specific recoveries on the interpretation of the final results.