Water Research 88 (2016) 740–749

Contents lists available at ScienceDirect

Water Research

journal homepage: www.elsevier.com/locate/watres

Estrogenic activity in Finnish municipal wastewater effluents

Pia Välitalo [a, b, *], Noora Perkola [a], Thomas-Benjamin Seiler [c], Markus Sillanpää [a], Jochen Kuckelkorn [c], Anna Mikola [b], Henner Hollert [c], Eija Schultz [a]

[a] Finnish Environment Institute, Laboratory Centre, Hakuninmaantie 6, 00430 Helsinki, Finland
[b] Aalto University, Department of Civil and Environmental Engineering, Tietotie 1E, 02150 Espoo, Finland
[c] RWTH Aachen University, Department of Ecosystem Analyses, Institute for Environmental Research, Worringerweg 1, 52074 Aachen, Germany

ARTICLE INFO

Article history:
Received 2 September 2015
Received in revised form
16 October 2015
Accepted 28 October 2015
Available online 4 November 2015

Keywords:
Wastewater effluent
Estrogenic activity
In vitro
Chemical analysis

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

* Corresponding author. Finnish Environment Institute, Laboratory Centre, Hakuninmaantie 6, 00430 Helsinki, Finland.
E-mail address: pia.valitalo@ymparisto.fi (P. Välitalo).

http://dx.doi.org/10.1016/j.watres.2015.10.056
0043-1354/

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.

2. Methods and materials

2.1. Chemicals

17α-ethinylestradiol EE2 (≥98%), estrone E1 (≥99%), estriol E3 (≥97%), 17β-estradiol E2 (≥98%), 17α-estradiol (≥98%), progesterone (≥99%) and bisphenol A BPA (≥99%), and the LC-MS Chromasolv® methanol used in the standard solutions, extraction and as an LC eluent were purchased from Sigma Aldrich (Saint Louis, MO, USA). A 25% ammonia solution used in LC eluents was produced by Merck (Darmstadt, Germany).

Stock solutions (1 mg/mL) of each solid standard were prepared in methanol. The spike solution (1 μg/mL) and calibration standards for the chemical analysis were prepared from the stocks in methanol-water.

Cell culture media were obtained from Sigma Aldrich (Schnelldorf, Germany), Invitrogen (Darmstadt, Germany) and Otto Nordwald (Hamburg, Germany). Standards for the ERα-CALUX® were purchased from Sigma Aldrich (Schnelldorf, Germany).

2.2. Effluent sample collection

Effluent samples were collected from eight WWTPs in Finland treating municipal wastewater and different shares of industrial wastewater. The selected WWTPs mostly represent typical treatment plants in Finland, where the treatment process is activated sludge with DN configuration and simultaneous phosphorus precipitation. Some treatment plants with a significant industrial load were selected as well. A detailed description of the WWTPs is given in Table 1. The samples (10 L) were collected as 24-h composite samples in February 2014 and transferred immediately to the lab for further sample treatment. Upon the day of arrival the samples were divided into 1 L portions in HDPE plastic bottles and stored in freezer (−20 °C) until extraction.

2.3. Sample extraction

Solid-phase extraction was used to separate and concentrate organic compounds from the wastewater effluents. Samples were centrifuged prior to extraction to remove the solid particles. The collected effluent samples were divided in to four sets for the different analysis as shown in Fig. 1.

For ERα-CALUX®, 2 L of each sample was extracted with Atlantic HLB-L (47 mm) extraction discs (Horizon Technology Inc., Salem, NH, USA). The discs were prewashed with 10 mL of methanol and 10 mL of deionized water after which 2 L of sample was introduced through each disc. After loading of the samples, the disc was washed with 5% methanol in water. The compounds retained in the cartridge were eluted with 10 mL of methanol. The extract was evaporated to dryness with EZ-Envi centrifugal evaporator (Genevac Ltd, Ipswich, UK). The extract was re-dissolved in 1 mL of the solvent dimethylsulfoxide (DMSO) and stored at 4 °C for the ERα-CALUX®.

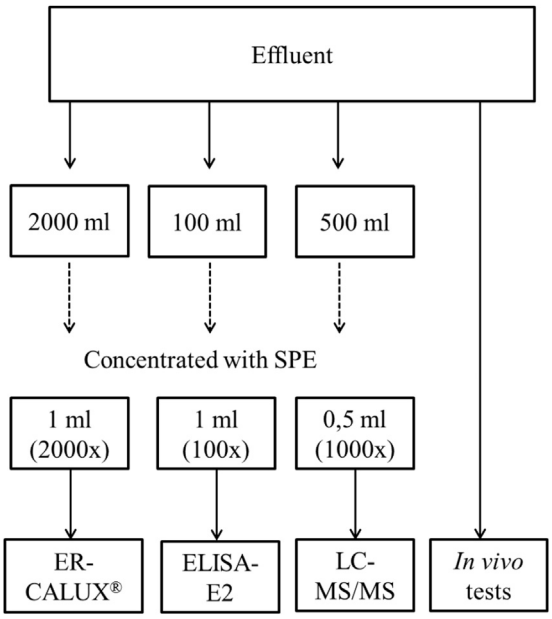

Fig. 1. A scheme of the sample treatment process. For each analysis the samples were extracted with the same SPE method, but from different volumes of effluent with different concentrations factors. Raw effluent sample was used for *in vivo* tests.

For the chemical analysis, 500 mL of the effluent was extracted with the same method as above. After bringing the extract to dryness it was re-dissolved in 0.5 mL of 50% methanol in water (v/v). In addition, a replicate of each wastewater was spiked with 100 μL of 1 μg/mL hormone solution (E1, E2, E3, EE2 α-estradiol, progesterone and BPA) and 500 mL of deionized water spiked with the same solution was extracted at the same time with the sample for quality control and to calculate recoveries.

For the ELISA-E2 assay 100 mL samples were extracted with Oasis HLB cartridges (6 cc, 200 mg, Waters, Milford, MA, USA). The estrogens were eluted with 6 mL of methanol, after which the extract was evaporated to dryness and re-dissolved in 1 mL of the extraction buffer supplied with the ELISA assay kit.

2.4. LC-MS/MS

The compounds selected for the analysis were E1, E2, E3, EE2, 17α-estradiol, progesterone and BPA. For the chemical analysis, 7.5 μL of each extract was injected into an Acquity ultra performance liquid chromatograph coupled to a Xevo TQ mass spectrometer (Waters, Milford, MA, USA) with an electrospray ionization (ESI) source. The analytes were separated in an Acquity BEH C18 analytical column (1.7 μm, 2.1 × 50.0 mm) with a gradient consisting of 2% NH_3 in water and methanol. The MS/MS quantifier

Table 1
Information on the eight WWTPs in Finland selected for sampling of effluent.

Location	Population equivalent	Average flow (m3/d)	Industrial influent % of the total and type	Secondary treatment	Tertiary treatment	Receiving water
WWTP 1	43,200	2000	30%, food processing	Trickling filter + activated sludge	No	River
WWTP 2	40,000	12,500	4%, miscellaneous	Activated sludge	No	Baltic Sea
WWTP 3	330,000	83,000	7%, miscellaneous	Activated sludge	Sand filtration	Baltic Sea
WWTP 4	13,000	7400	0%	Activated sludge	Sand filtration	Baltic Sea
WWTP 5	1,100,000	264,000	17%, miscellaneous	Activated sludge	Denitrifying filters	Baltic Sea
WWTP 6	94,000	2500	18%, dairy	MBBR + activated sludge	No	River
WWTP 7	16,000	2700	0%	Activated sludge	No	River
WWTP 8	50,000	8000	85%, paper mill and meat processing	MBBR + flotation	No	River

(and qualifier) reactions were following: progesterone 315 > 97 (315 < 109), EE2 295 > 198.9 (295 > 269), E1 269 > 145, E2 271 > 145 (271 < 183), 17α-estradiol 271 > 145 (271 > 239), E3 287 > 171 (287 > 145), BPA 227.19 > 133.05 (227.19 > 92.96). The concentrations were calculated by external calibration. The results are recovery-corrected based on spiked samples. The chemical analysis was performed at the Finnish Environment Institute, which is accredited by the Finnish Accreditation Service (FINAS) as an environmental testing laboratory T003 following the standard SFS-EN ISO/IEC 17025.

2.5. Determination of estrogenic potency with ERα-CALUX®

The human U2OS osteosarcoma1 cells used in the ERα-CALUX® assays were provided and licenced by BioDetection Systems b. v. (BDS), Amsterdam, Netherlands. The assay was performed according to the BDS (2007) protocol. Briefly, cells were cultivated in "growth medium" Dulbecco's modified Eagle's medium (D-MEM)/F12 with GlutaMAX™ containing a phenol red as pH indicator and supplemented with 7.5% fetal bovine serum (FBS), 1% minimum essential medium (MEM) nonessential amino acids and a 0.2% penicillin/streptomycin solution (5000 U/mL penicillin and 5000 U/mL streptomycin). Cells were grown at 37 °C in 5% CO_2.

For the assay "growth medium" was replaced by "assay medium" containing D-MEM/F12 medium with L-glutamine without phenol red and supplemented with 5% stripped FBS, 1% nonessential amino acids (MEM) and 0.2% penicillin/streptomycin solution (5000 U/mL penicillin and 5000 U/mL streptomycin). Cells were seeded at a density of 10,000 cells/well in 96-well microtiter plates in assay medium. The plates were incubated for 24 h at 37 °C in 5% CO_2. After incubation, the cells were exposed to the samples and standards in assay medium for 24 h. The final DMSO solvent concentration was 0.1% in each well. Subsequently, the cells were lysed with 30 μl of lysis reagent. After addition of 100 μl/well of glowmix, the luciferase activity was measured with a luminometer (Infinite® M 200, Tecan, Switzerland). Readings as relative luminescent unit (RLU)/well were processed using a sigmoidal calibration curve formulated with an MS Excel template provided by BDS together with an add-in "solver". Estrogenic potential as E2 equivalents (EEQs) were calculated as described by Legler et al. (1999) and BDS (2007). The limit of detection (LOD) is 0.035 ng/L.

The contributions of selected individual compounds to the observed effects in ERα-CALUX® were calculated as equivalents for the substance specific chemical analysis (*chemEEQs*) according to Maletz et al. (2013). The *chemEEQs* are based on compound-specific relative estrogenic potencies (REP) with ERα-CALUX® determined in previous studies (Table 2).

$$chemEEQ = \text{Relative estrogenic potency(REP)} \times \text{concentration(ng/L)}.$$

Cytotoxicity of the extracted effluent samples to the U2OS cells was tested with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay to make sure that the effects observed with ERα-CALUX® are not affected by possible cytotoxic effects of the samples. In the assay, the conversion of MTT to insoluble formazan by dehygrogenase enzymes of undamaged mitochondria of viable cells is measured. The U2OS cells were exposed to the extracted effluent samples in the same way as in the ERα-CALUX® assay, but after 24 h MTT solution was added. Cells were incubated for 30 min during which the formazan crystals were solubilized with DMSO. The absorbance (492 nm) was measured with Autoreader (Absorbance reader, Cayman Chemicals). The absorbance directly correlates with the number of viable cells.

Table 2
Relative estrogenic potencies (REP) for selected endocrine disrupting compounds based on the ERα-CALUX®.

Compound[d]	E1	E2	E3	EE2	BPA
REP ER-CALUX	1.6E-02[a]	1	3.5E-02[b]	1.8[a]	5.9E-05[c]

[a] (Murk et al., 2002).
[b] (Sonneveld et al., 2006).
[c] (Legler et al., 1999).
[d] Data not available for 17α-estradiol or progesterone.

2.6. Measurement of 17β-estradiol with an enzyme-linked immunosorbent assays (ELISA-E2)

The ELISA-E2 kit was purchased from Neogen corporation (Product no. 402110, Lansing, MI, USA). The kit is designed for measuring E2 from biological fluids but is potentially suitable for wastewater effluent. The assay was performed according to the protocol provided with the kit with minor modifications. Briefly, samples and standard solutions were first added to the 96-well antibody coated microplate. Each effluent sample was tested in four dilutions (undiluted, 1:2, 1:4, 1:8) in duplicate. Extraction buffer included in the kit was used in eight wells for negative control and a dilution series of E2 standard solution was measured in duplicate. After adding the samples and standard solution on the plate, the enzyme conjugate was added and the plates were shaken and incubated at room temperature for 60 min. After incubation the plate was washed to remove all the material that was not bound to the binding sites. For the detection of bound enzyme conjugates K-Blue substrate was added to the wells and the plate was incubated in room temperature for 30 min. The absorbance was measured with Victor^3 (Perkin Elmer, Singapore) at 620 nm. The intensity of blue color development is inversely proportional to the amount of E2 in the sample or the standard.

2.7. Acute and chronic long term toxicity of effluent samples

Acute toxicity of the samples was measured from raw wastewater effluent samples. Samples were tested for acute toxicity with *D. magna* based on the ISO 6341:2012 standard with minor modifications. The water fleas were exposed to different dilutions of the effluent and their mobility was recorded after 24 h and after 48 h. Each dilution was tested as four replicates with 5 fleas/10 mL of test solution in each one.

In addition, samples were tested for acute toxicity with the *V. fischeri* test which was performed according to the ISO 11348-3 standard. *V. fischeri* produces light as a result of its normal metabolism and in case of a disturbance by harmful substances the light production is inhibited. Briefly, bacteria were exposed to effluent samples for 30 min and after which their light production was measured. The luminescence of the exposed bacteria was compared to the control sample.

Additional samples were available for WWTP 1, 4, 6, 7 and 8 and they were furthermore tested for long term toxicity to *D. magna* based on the ISO 1706:2000 standard with minor modifications. In short, *D. magna* females were exposed to different dilutions of the effluent for a period of 21 days. Each dilution was tested with 5 replicates. The survival of the adult water fleas was recorded together with the number of offspring produced per parent at the end of the test. In addition, the survival of the offspring was recorded.

3. Results and discussion

3.1. Detection of estrogenic compounds with chemical analysis (LC-MS/MS)

The occurrence of seven natural and synthetic EDCs E1, E2, E3, EE2, 17α-estradiol, progesterone and BPA was measured with LC-MS/MS. The recoveries for the target compounds were measured by comparing spiked samples with unspiked effluent samples. The recoveries were between 30 and 45% for E1, 36–54% for E2, 38–62% for E3, 38–55% for EE2, 37–61% for 17α-estradiol, 16–28% for progesterone and 25–46% for BPA (Fig. 2). Recoveries were also tested with spiked deionized water. The results showed that recoveries in spiked deionized water were generally a bit higher for all of the compounds than in spiked effluent samples indicating the effect of the matrix on the recovery. Recoveries reported in previous studies have varied between 31 and 119% for estrogens, which is in line with the results of this study (Huang and Sedlak, 2001, Chang et al., 2011, Guo et al., 2013; Guedes-Alonso et al., 2015). In addition to matrix effects, the recovery results can be affected by extracted sample volume and the amount of spiked compound. Higher spiking concentrations may result in greater ion suppression. Interestingly enough, it is not uncommon that studies do not report any information on recoveries or results are calculated based on recoveries determined with another sample matrix (e.g. deionized water) or just one wastewater effluent. It was evident that there are clear differences between the samples, even though the overall trend for each compound was similar. Due to the variation in recoveries, the use of sample-specific recoveries gives more reliable results especially when mass-labeled standards cannot be applied. Mass-labeled standards are not applicable when biotests are used, because the standards can contribute to the effects. Progesterone was not excluded from further analysis because of the poor recoveries.

The LOQs calculated for the target compounds in this study were on a similar level compared to previous studies (Table 3). Some of the analyzed compounds were present at concentration below the LOQ, implying that there is a need to further develop methods to achieve lower LOQs for these compounds. This is crucial for compounds such as EE2. The estrogenic potential of the compound is high, but the LOQs are typically also high making the reliable detection of EE2 challenging (Ingrand et al., 2003; Nelson et al., 2007; Vieno, 2014).

E1 was detected in all of the samples with concentrations ranging between 2.7 and 27.2 ng/L. The E1 concentrations were notably higher (>15 ng/L) in effluent samples from WWTP 5, 6 and 8 compared to the other effluent samples but similar to concentrations reported in previous studies (Table 3).

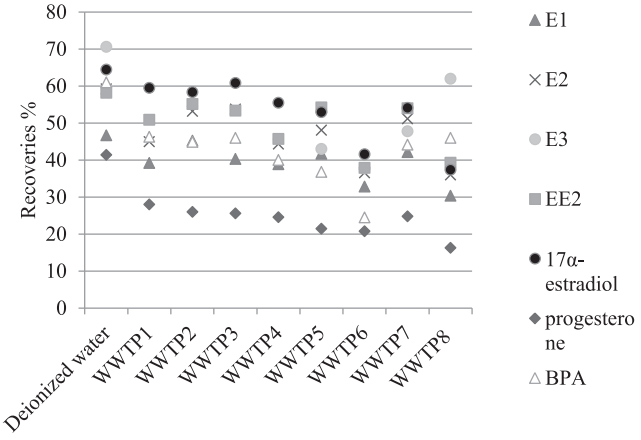

Fig. 2. Sample specific recoveries for target EDCs.

E2 was detected in two effluent samples, WWTP 6 (6.5 ng/L) and WWTP 8 (6.8 ng/L). E3 (0.8 ng/L) and 17α-estradiol (4 ng/L) was detected only in effluent sample from WWTP 8. There is not much data available on concentrations of 17α-estradiol in effluent or aquatic samples, since it has not been analyzed as often as the other compounds selected for this study. Whereas E2 originates primarily from human endocrine systems, 17α-estradiol is the predominant estrogen in cattle feces (Isobe et al., 2006). WWTP8 receives influent from a food and a fodder production plant, which could explain the presence of 17α-estradiol in the effluent sample. 17α-estradiol is used for medical purposes in humans for hair loss and hormone replacement therapy, but not in such large quantities that it would explain why the compound was detected. EE2 and progesterone were not detected in any of the samples. Recoveries for progesterone were likely too low for the detection (<30%). The LOQ (10 ng/L) for EE2 was clearly too high for the detection of the compound and concentrations higher than 10 ng/L are not expected according to the calculations based on consumption (Vieno, 2014).

Comparison of estrogen concentrations reported here with previously published studies indicate that the levels of compounds are generally in the same range as those found throughout Europe and elsewhere in the world (Table 3). Here the highest concentrations were found for E1, followed by E2 and E3, which is consistent with previously published studies. This trend can be primarily explained by the human urinary excretion of these compounds in addition to transformation of these compounds during the wastewater treatment process (Salvador et al., 2007; Liu et al., 2009).

The BPA concentrations ranged between 130 and 690 ng/L in six of the samples, but concentrations were higher in WWTP1 effluent (1345 ng/L) and notably higher in WWTP 8, where the concentration was 962,008 ng/L. There is not much data available on BPA concentrations in municipal wastewater effluents in Europe. To our knowledge, in Finland there has been only one monitoring study where BPA concentrations have been measured from municipal wastewater effluent and only from three treatment plants (Huhtala et al., 2011). The results reported in this study for the six plants with lower concentrations are comparable to those reported in the monitoring study of 2011 (240–440 ng/L). This study included two of the same treatment plants that were sampled also in the monitoring study and the concentrations for BPA were on a similar level. The concentration of BPA in WWTP3 was 137 ng/L in this study and 240 ng/L in the report from 2011, and for WWTP 5 the concentrations were 685 ng/L and 440 ng/L, suggesting that the loading of BPA has not changed considerably over the last few years. Overall, looking on a more global scale the concentrations were on the higher side compared to results from previous studies, though concentrations as high as 17 μg/L have been reported (Table 3). The exceptionally high amount of BPA in WWTP 8 effluent can likely be traced back to two paper production factories that discharge into the WWTP. In the EU, only a small portion of BPA is used for paper production (Oehlmann et al., 2008). However, locally the paper industry can have significant contribution to the loading of BPA to the aquatic environment (Fürhacker et al., 2000; Fühacker, 2003). In this case, the factory discharging most of the BPA has reported a discharge of 2.2% of BPA from the overall amount of BPA used in their process. This means that more than 11 t of BPA in a year or approximately 30 kg/day is discharged from the factory to the treatment plant. Removal efficiencies between 77 and 92% have been reported for conventional activated sludge treatment plants (Nakada et al., 2006; Stasinakis et al., 2008; Guerra et al., 2015). Based on the removal rates reported, with a 90% removal efficiency, the load to receiving waters would be approximately 3 kg/day in this case. The loading from the paper production plant would

Table 3
Occurrence of estrogens in WWTP effluent.

Compound	Country	WWTP type (*n*)	Analysis method	LOD or (LOQ) ng/L	Effluent concentration (ng/L)	Reference
E1	Finland	Advanced nutrient removal (8)	LC-MS/MS	(1)	3–27	This study
	France	n.d. (3)	LC-MS/MS	(5)	<LOQ–5	(Ingrand et al., 2003)
	Netherlands	Activated sludge (5)	GC–MS/MS	0.3–1	<LOD–47	(Belfroid et al., 1999)
	Korea	Biological treatment (5)	HPLC-MS/MS	(0.8)	<LOQ–24	(Behera et al., 2011)
	USA	Different types (12)	GC–MS/MS	0.2 (0.4)	<LOD–11	(Kolodziej et al., 2003)
	Canada	Different types (5)	GC-HRMS	5.4	<LOD–27	(Nelson et al., 2007)
	Spain	Activated sludge (1)	GC–MS/MS	0.5 (1)	<LOQ–4	(Carballa et al., 2004)
	Australia	Different types (4)	HPLC-MS/MS	5	<LOD–110	Leusch et al., 2014
	Japan	Biological treatment (3)	UPLC-MS/MS	0.3	2–62	Ihara et al., 2014
	France	Biological treatment (3)	LC-MS/MS	(0.3–2.7)	2–20	Gabet-Giraud et al., 2014
	Spain	Different types (4)	UPLC-MS/MS	4.1	<LOD–19	Guedes-Alonso et al., 2015
E2	Finland	Advanced nutrient removal (8)	LC-MS/MS	(5)	<LOQ–7	This study
	Spain	Activated sludge	GC–MS/MS	0.5 (1.0)	<LOQ–3	(Carballa et al., 2004)
	Canada	Different types (5)	GC-HRMS	4.9	<LOD–11	(Nelson et al., 2007)
	USA	Different types (12)	GC–MS/MS	0.1 (0.3)	<LOD–4	(Kolodziej et al., 2003)
	Australia	Different types (4)	HPLC-MS/MS	5	<LOD	Leusch et al., 2014
	Japan	Biological treatment (3)	UPLC-MS/MS	0.5	<LOD–4	Ihara et al., 2014
	France	Biological treatment (3)	LC-MS/MS	(0.3–2.7)	<LOQ	Gabet-Giraud et al., 2014
17α-etsradiol	Finland	Advanced nutrient removal (8)	LC-MS/MS	(4)	>LOQ–4	This study
	Portugal	Different types (3)	LC-MS/MS	0.4–4.8	<LOD	(Sousa et al., 2010)
	Spain	Activated sludge (2)	LC-MS/MS	70	<LOD	(Pedrouzo et al., 2011)
	Australia	Different types (4)	HPLC-MS/MS	5	<LOD	Leusch et al., 2014
E3	Finland	Advanced nutrient removal (8)	LC-MS/MS	(3)	<LOQ	This study
	Austria	Activated sludge (4)	LC-MS/MS	1	<LOD–275	(Clara et al., 2005)
	Canada	Different types (5)	GC-HRMS	5.9	≤LOD–9	(Nelson et al., 2007)
	Australia	Different types (4)	HPLC-MS/MS	50	<LOD-170	Leusch et al., 2014
	Japan	Biological treatment (3)	UPLC-MS/MS	0.5	<LOD	Ihara et al., 2014
	Spain	Different types (4)	UPLC-MS/MS	4.5	<LOD	Guedes-Alonso et al., 2015
EE2	Finland	Advanced nutrient removal (8)	LC-MS/MS	(10)	<LOQ	This study
	France	n.d. (3)	LC-MS/MS	10	<LOD	(Ingrand et al., 2003)
	Netherlands	Activated sludge (5)	GC–MS/MS	0.3–1.8	<LOD–8	(Belfroid et al., 1999)
	Canada	Different types (5)	GC-HRMS	6.9	<LOD	(Nelson et al., 2007)
	Spain	Activated sludge (1)	GC–MS/MS	0.5 (1)	<LOQ	(Carballa et al., 2004)
	Australia	Different types (4)	HPLC-MS/MS	5	<LOD	Leusch et al., 2014
	Japan	Biological treatment (3)	UPLC-MS/MS	0.5	<LOD	Ihara et al., 2014
	France	Biological treatment (3)	LC-MS/MS	(0.3–9.0)	<LOQ	Gabet-Giraud et al., 2014
BPA	Finland	Advanced nutrient removal (8)	LC-MS/MS	(0.7)	131–956 000	This study
	Austria	Activated sludge (1)	GC–MS	500	<LOD–2500	(Fürhacker et al., 2000)
	Japan	Activated sludge (1)	GC–MS	(6)	<LOQ–39	(Nakada et al., 2004)
	Greece	Activated sludge (1)	GC–MS	n.d.	20–48	(Pothitou and Voutsa, 2008)
	Australia	Activated sludge (5)	GC–MS	n.d.	104–2847	(Tan et al., 2007)
	Canada	Activated sludge (4)	GC–MS	n.d.	10–17 300	(Lee et al., 2004)
	Canada	Different types (25)	GC–MS and LC-MS/MS	4.2–33	5–7400	(Guerra et al., 2015)

n: number of WWTPs; LOD:limit of detection; LOQ: limit of quantification; n.d: no data available.

explain approximately 40% of the observed concentrations based on the values presented above, but the discharge of BPA to the treatment plant is more likely to be an irregular phenomenon as opposed to an equally constant flow. A more intensive sampling campaign should be performed to make better conclusions on the risks related to BPA at this treatment plant.

3.2. Estrogenic potency of wastewater effluents measured with ERα-CALUX® and the compounds contributing to the effects

Due to the complex nature of wastewater effluents the samples may contain factors that cause cytotoxic effects. Thus the cytotoxicity of all samples was tested with the methyl tetrazolium (MTT) assay to exclude cytotoxic effects. None of the samples were toxic to the BDS U2OS cells (data not shown). The ERα-CALUX® was able to detect estrogenic activity in all of the effluent samples. The estrogenic potencies measured with ERα-CALUX® were 0.8–29.7 E2 EEQ ng/L (Table 4 and Fig. 3). The E2 EEQ values were lowest in WWTP 7 (0.8 ng/L) and highest in samples from WWTP 6 (9.6 ng/L) and WWTP 8 (23.8 ng/L) which is consistent with the results from the chemical analysis. ERα-CALUX® has been previously used for measuring the estrogenic activity in municipal wastewater effluents in Europe and the E2 EEQ concentrations reported have varied between 0.03 and 16.1 ng E2 EEQ/L (Murk et al., 2002; van der Linden et al., 2008; Mendonça et al., 2009). Higher E2 EEQ values have been reported from hospital effluents (24 ± 2 ng/L) (van der Linden et al., 2008). The E2 EEQ values of the municipal wastewater effluent samples measured in this study with ERα-CALUX® were generally slightly higher compared to the results from the previous studies. It cannot be concluded that the results are higher due to actual higher amounts of estrogenic compounds or less efficient treatment processes, since results might be affected by the differences in sample recoveries and sample treatment. ERα-CALUX® has not previously been used to analyze wastewater effluents in Finland, however Pessala et al. (2004) investigated the estrogenic activity of Finnish wastewater effluents by analyzing vitellogenin induction in fish hepatocytes. The results from this study support the previous findings of linking estrogenic potential with effluents, but also provide a good addition with a more specific endpoint for the comparison of different effluent samples as opposed to a yes or no result. The use of multiple assays based on different mechanisms and test organisms would be most beneficial to get a comprehensive view on the risks related to waters receiving effluents that are potentially estrogenic, which have been concluded in other studies as well (Smital et al., 2011, Leusch et al., 2014).

Table 4
Calculated[a] *chemEEQs* for effluent samples from eight WWTPs in Finland.

Treatment plant	E1	E2	BPA	Total[b]	ERα-CALUX® (ng/L)	% explained[c]
WWTP1	0.18	–	0.048	0.23	1.95	12
WWTP2	0.11	–	<0.01	0.11	1.58	7.2
WWTP3	0.071	–	<0.01	0.076	1.24	6.1
WWTP4	0.086	–	<0.01	0.088	1.17	7.5
WWTP5	0.30	–	0.025	0.33	2.82	12
WWTP6	0.30	6.5	0.011	6.8	9.63	70
WWTP7	0.043	–	<0.01	0.048	0.925	5.2
WWTP8	0.046	6.8	4.2	11	23.8	48

[a] The calculations were done according to Maletz et al. (2013); Calculated concentrations of EEQ = Relative estrogenic potency × concentration (ng/L). The REP values were determined in previous studies (Legler et al., 1999; Murk et al., 2002; Sonneveld et al., 2006).
[b] For E3 and EE2 the concentrations were under the LOQ in all of the samples, thus the contribution cannot be calculated. 17α-estradiol and progesterone were not included in the analysis because there is no data available on their affinity for the ER-receptor.
[c] "% Explained" = (Total/ER-CALUX® EEQ (ng/L))*100.

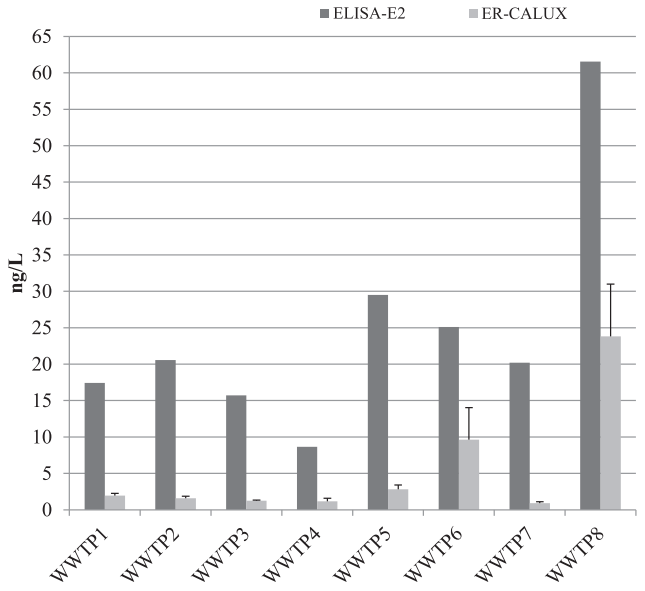

Fig. 3. Results from ELISA-E2 (E2 ng/L) and ERα-CALUX® (E2 EEQ ng/L) assays for eight WWTPs. The error bars represent standard deviation of three replicates in ERα-CALUX® tests.

The contribution of the compounds selected for the chemical analysis to the estrogenic activity observed with ERα-CALUX® was presented as *chemEEQs* (Table 4), which consider the affinity of the selected substances for the ER receptor. The *chemEEQs* are calculated based on REP values determined from previous single substance studies (Table 2) with ERα-CALUX® and the results from the chemical analysis from this study. The results showed that E1 was the main substance contributing to the *chemEEQs* out of the compounds that were detected (Table 4). E2 was also a contributing substance with samples from WWTP 6 and 8. E2 concentrations were under the LOQ in the other samples, due to which the contribution of E2 cannot be evaluated in those cases. E2 could have a significant contribution to effects at lower concentrations than 5 ng/L, because of the high REP value of E2. A comprehensive analysis on the contribution of BPA to the estrogenic effects was possible, because it was detected in all of the samples. Due to the low REP value of BPA, it only contributed little to the estrogenic activity measured with ERα-CALUX® in all of the samples except WWTP8, where the BPA concentration was more than three orders of magnitude higher. In that sample BPA was a significant contributor to the observed effects (37%). Concentrations of E2, EE2, 17α-estradiol and E3 were mainly under the LOQ, thus their contributions to the *chemEEQs* were left undiscovered stressing the importance of developing more sensitive chemical analytical methods for the detection of compounds that are present at very low concentrations. Some of these compounds (e.g. EE2) have high REP values, indicating that the contribution to the observed effects could be significant even at concentrations which are below the LOQs. There was no data available concerning REP values for 17α-estradiol. However, the estrogen equivalency factor for 17α-estradiol in E-Screen cell proliferation assay is 0.1 (1.0 for E2), suggesting that the estrogenic potency of E2 might be on the lower side in human cell based assays (Tan et al., 2007). In many respects, the LOQs in chemical analysis proved to be a limiting factor for calculating the contributions.

3.3. Concentration of E2 measured with ELISA-E2

The E2 concentrations measured with a commercially available ELISA-E2 assay varied between 8.6 and 61.6 ng/L. These concentrations were approximately 10 fold higher than those determined with chemical analysis or ERα-CALUX®(Fig. 3). However, the results obtained from the ELISA-E2 assay follow a similar pattern as results from the ERα-CALUX® and the chemical analysis. The measured concentrations were highest in WWTP 6 (25.1 ng/L) and 8 (61.6 ng/L), and in this case also a high concentration in WWTP 5 effluent (29.5 ng/L). Variations between different assays measuring estrogenic activity have been described previously (Maletz et al., 2013). One theory for the lower values determined with ERα-CALUX®, is that the samples can contain antagonists for the ER receptor, due to which also inhibiting actions can take place during the exposure resulting in lower estrogenic activity. However, there are no previous studies comparing the performance of ERα-CALUX® and ELISA-E2 assay, so any further conclusions are difficult to make. A few studies have applied ELISA tests for the analysis of estrogens in wastewater effluents. In those studies the authors reported also quite high concentrations for E2 (<0.05–170 ng/L) (Allinson et al., 2010; Manickum and John, 2014). However, the results were not compared to chemical analytical methods. Thus is it impossible to say whether the concentrations measured with ELISA would also be higher than concentrations determined by chemical analysis in those cases. The ELISA-E2 assay performed well when looking at the percentage of maximal binding of the antibodies (% B/B0) in relation to the dilutions of the samples resulting in a reliable dose–response relationship (Fig. 4). The cross reactivity of several hormones and other compounds have been tested by the manufacturer, and they are in the <0.05% range. However, this information is not available for all compounds, for example BPA. Thus, the high concentration may be due to some unknown compound/compounds cross reacting with the monoclonal antibodies.

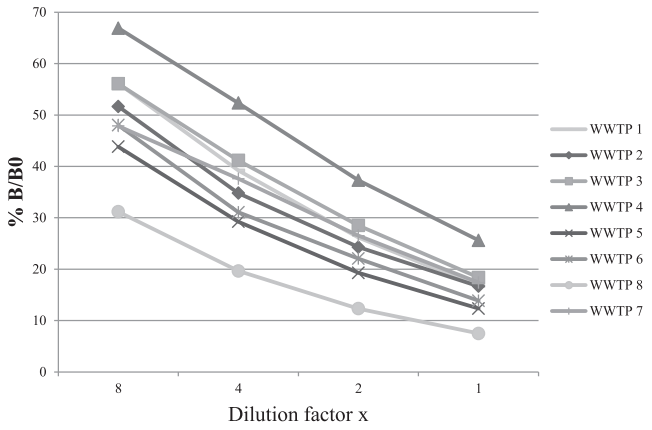

Fig. 4. The percent of maximal binding (% B/B0) in the effluent samples in four dilutions.

Dissolved organic matter and matrix interferences might be factors that could potentially interfere with the analysis of E2 (Hanselman et al., 2004; Silva et al., 2013).

3.4. Acute toxicity tests and chronic long term toxicity to D.magna

None of the effluent samples showed acute toxicity in tests with *D. magna* or *V. fischeri* (data not shown). This can be expected, since treated effluents are not usually found to be acutely toxic (Pessala et al., 2004; Hernando et al., 2005). Five of the samples were furthermore tested for long term toxicity to *D. magna*. There was no significant mortality or reproductive effects in any of the samples compared to the control (data not shown), however there was significant mortality among the offspring in two of the strongest dilutions from WWTP 8 effluent samples (Fig. 5). Effluent from WWTP 8 contained the highest estrogenic activity measured with ERα-CALUX®, ELISA-E2 and chemical analysis and a notably high concentration of BPA. The high concentration of BPA alone does not explain the observed effects, since BPA is not highly toxic to daphnids ($LOEC_{number\ of\ juveniles\ per\ adult}$ 1.73 mg/L, $EC_{50\ immobility\ of\ juveniles}$ >20 mg/L for BPA tested with the long term toxicity to *D. magna*) (Jemec et al., 2012). The available data on chronic exposure of BPA vary between different species and taxa, and many aquatic organisms have been shown to be sensitive to BPA (Oehlmann et al., 2008). Effect concentrations as low as 48 ng/L for the ramshorn snail *Marisa cornuarietis* and 10 μg/L for medaka have been reported (Oehlmann et al., 2000; Metcalfe et al., 2001). Some of the BPA concentrations measured in this study well exceed the toxicity values mentioned above, especially in WWTP 8 sample. This indicates that further studies are needed to estimate the risk of effluents, especially those with high concentrations of BPA, to the receiving waters in question.

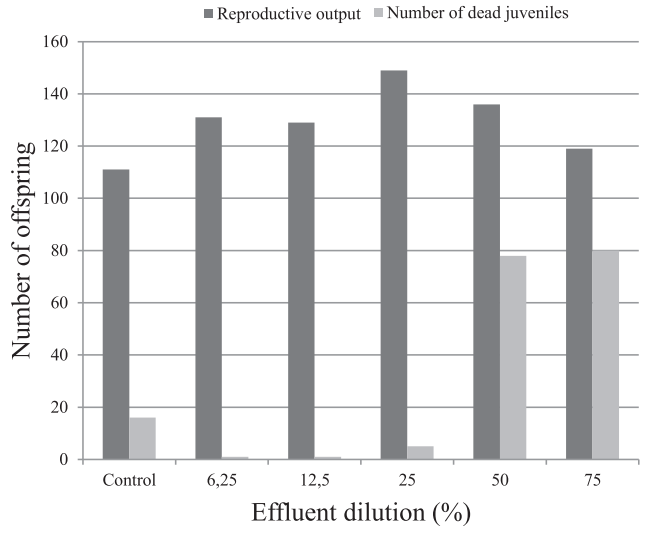

Fig. 5. The reproductive output (number of juveniles per adult) and the number of dead juveniles in different dilutions of effluent from WWTP 8.

4. Conclusions

Effluent samples taken from eight Finnish WWTPs showed estrogenic activity in both the ER-mediated assay (ERα-CALUX®) and the immunosorbent assay (ELISA-E2). ELISA-E2 proved to be sensitive, easy to use and suitable for screening purposes. E1 and E2 were significant contributors to the estrogenic potency observed with ERα-CALUX®, and BPA was a significant contributor to the effects in one sample taken from WWTP receiving industrial wastewater. In chemical analyses (LC-MS/MS), E1 was detected in all of the samples (range 2.7–27.2 ng/L), E2 was detected in two samples, and E3 and 17α-estradiol were detected only in one effluent sample. The LOQs in chemical analysis were a limiting factor in evaluating the contribution of individual compounds to the biological response detected in the bioassays. Thus, the estrogenic activity of wastewater effluents may be underestimated if only chemical methods are used. The results also emphasized the importance of using sample and compound specific recoveries when chemical and biological methods are combined for investigation of samples with a complex matrix. The results also show that, in addition to chemical analyses, *in vitro* assays and *in vivo* toxicity tests are complementary to each other for assessment of the wastewater effluent quality and potential environmental risks. Finally, the results indicate that that there is a need to advance the effluent treatment process, especially for municipal WWTPs with significant industrial loading, to minimize the risks related to estrogenic compounds to the receiving waters.

Acknowledgments

The authors would like to thank Maj ja Tor Nessling foundation, Maa-ja vesitekniikan tuki ry., Ekokem and the Finnish Ministry of the Environment for funding this research. We thank all of the WWTPs for participating in the sampling. We also thank Ilse Heiskanen, Kaisa Heinonen, Minna Sepponen and Kirsi Rosendahl for technical assistance at the Finnish Environment Institute. We also thank Timon Heide, Beat Thalman, Simone Hotz, Hongxia Xiao and Zsolt Tarcai for their skillful laboratory assistance at RWTH Aachen University and Harrie Besselink from BDS for advice on the ERα-CALUX® analysis.

References

Aerni, H.-R., Kobler, B., Rutishauser, B., Wettstein, F., Fischer, R., Giger, W., Hungerbühler, A., Marazuela, M.D., Peter, A., Schönenberger, R., Vögeli, A.C., Suter, M.F., Eggen, R.L., 2004. Combined biological and chemical assessment of estrogenic activities in wastewater treatment plant effluents. Anal. Bioanal. Chem. 378 (3), 688–696.

Allinson, M., Shiraishi, F., Salzman, S.A., Allinson, G., 2010. Vitro and immunological assessment of the estrogenic activity and concentrations of 17β-estradiol, estrone, and ethinyl estradiol in treated effluent from 45 wastewater treatment plants in Victoria, Australia. Arch. Environ. Contam. Toxicol. 58 (3), 576–586.

Bain, P.A., Williams, M., Kumar, A., 2014. Assessment of multiple hormonal activities in wastewater at different stages of treatment. Environ. Toxicol. Chem. 33 (10), 2297–2307.

BDS, 2007. Analysis of Estrogen Receptor Mediated Luciferase Activity in ER CALUX Cells. P-BDS-011(H).

Behera, S.K., Kim, H.W., Oh, J.E., Park, H.S., 2011. Occurrence and removal of antibiotics, hormones and several other pharmaceuticals in wastewater treatment plants of the largest industrial city of Korea. Sci. Total Environ. 409 (20), 4351–4360.
Belfroid, A.C., Van der Horst, A., Vethaak, A.D., Schäfer, A.J., Rijs, G.B.J., Wegener, J., Cofino, W.P., 1999. Analysis and occurrence of estrogenic hormones and their glucuronides in surface water and waste water in The Netherlands. Sci. Total Environ. 225 (1–2), 101–108.
Birceanu, O., Servos, M.R., Vijayan, M.M., 2015. Bisphenol A accumulation in eggs disrupts the endocrine regulation of growth in rainbow trout larvae. Aquat. Toxicol. 161 (0), 51–60.
Bolong, N., Ismail, A.F., Salim, M.R., Matsuura, T., 2009. A review of the effects of emerging contaminants in wastewater and options for their removal. Desalination 239 (1–3), 229–246.
Brix, R., Noguerol, T.-N., Piña, B., Balaam, J., Nilsen, A.J., Tollefsen, K.-E., Levy, W., Schramm, K.-W., Barceló, D., 2010. Evaluation of the suitability of recombinant yeast-based estrogenicity assays as a pre-screening tool in environmental samples. Environ. Int. 36 (4), 361–367.
Carballa, M., Omil, F., Lema, J.M., Llompart, M.a., Garcıa-Jares, C., Rodrıguez, I., Gómez, M., Ternes, T., 2004. Behavior of pharmaceuticals, cosmetics and hormones in a sewage treatment plant. Water Res. 38 (12), 2918–2926.
Caron, E., Sheedy, C., Farenhorst, A., 2010. Development of competitive ELISAs for 17beta-estradiol and 17beta-estradiol +estrone+estriol using rabbit polyclonal antibodies. J. Environ. Sci. Health B 45 (2), 145–151.
Chang, H., Wan, Y., Wu, S., Fan, Z., Hu, J., 2011. Occurrence of androgens and progestogens in wastewater treatment plants and receiving river waters: comparison to estrogens. Water Res. 45 (2), 732–740.
Clara, M., Kreuzinger, N., Strenn, B., Gans, O., Kroiss, H., 2005. The solids retention time—a suitable design parameter to evaluate the capacity of wastewater treatment plants to remove micropollutants. Water Res. 39 (1), 97–106.
Colborn, T., 1995. Environmental estrogens: health implications for humans and wildlife. Environ. Health Perspect. 103 (Suppl. 7), 135–136.
Fühacker, M., 2003. Bisphenol A emission factors from industrial sources and elimination rates in a sewage treatment plant. Water Sci. Technol. 47 (10), 117–122.
Fürhacker, M., Scharf, S., Weber, H., 2000. Bisphenol A: emissions from point sources. Chemosphere 41 (5), 751–756.
Gabet-Giraud, V., Miège, C., Jacquet, R., Coquery, M., 2014. Impact of wastewater treatment plants on receiving surface waters and a tentative risk evaluation: the case of estrogens and beta blockers. Environ. Sci. Pollut. Res. 21 (3), 1708–1722.
Guedes-Alonso, R., Santana-Viera, S., Sosa-Ferrera, Z., Santana-Rodríguez, J.J., 2015. Molecularly imprinted solid-phase extraction coupled with ultra high performance liquid chromatography and fluorescence detection for the determination of estrogens and their metabolites in wastewater. J. Sep. Sci. http://dx.doi.org/10.1002/jssc.201500764.
Guerra, P., Kim, M., Teslic, S., Alaee, M., Smyth, S.A., 2015. Bisphenol-A removal in various wastewater treatment processes: operational conditions, mass balance, and optimization. J. Environ. Manag. 152, 192–200.
Guo, F., Liu, Q., Qu, G.-b., Song, S.-j., Sun, J.-t., Shi, J.-b., Jiang, G.-b., 2013. Simultaneous determination of five estrogens and four androgens in water samples by online solid-phase extraction coupled with high-performance liquid chromatography–tandem mass spectrometry. J. Chromatogr. A 1281, 9–18.
Hanselman, T.A., Graetz, D.A., Wilkie, A.C., 2004. Comparison of three enzyme immunoassays for measuring 17β-Estradiol in flushed dairy manure wastewater. J. Environ. Qual. 33 (5), 1919–1923.
Hernando, M.D., Fernández-Alba, A.R., Tauler, R., Barceló, D., 2005. Toxicity assays applied to wastewater treatment. Talanta 65 (2), 358–366.
Honkanen, J.O., Holopainen, I.J., Kukkonen, J.V.K., 2004. Bisphenol A induces yolk-sac oedema and other adverse effects in landlocked salmon (Salmo salar m. sebago) yolk-sac fry. Chemosphere 55 (2), 187–196.
Huang, C.-H., Sedlak, D.L., 2001. Analysis of estrogenic hormones in municipal wastewater effluent and surface water using enzyme-linked immunosorbent assay and gas chromatography/tandem mass spectrometry. Environ. Toxicol. Chem. 20 (1), 133–139.
Huhtala, S., Munne, P., Nakari, T., Nuutinen, J., Perkola, N., Sainio, P., Schultz, E., Schultz, L., 2011. WP3 Innovative Approaches to Chemical Controls of Hazardous Substances. Country Report of Finland. COHIBA, Control of Hazardous Substances in the Baltic Sea Region.
Ihara, M., Ihara, M.O., Kumar, V., Narumiya, M., Hanamoto, S., Nakada, N., Yamashita, N., Miyagawa, S., Iguchi, T., Tanaka, H., 2014. Co-occurrence of estrogenic and antiestrogenic activities in wastewater: quantitative evaluation of balance by in vitro ERα reporter gene assay and chemical analysis. Environ. Sci. Technol. 48 (11), 6366–6373.
Ihara, M., Kitamura, T., Kumar, V., Park, C.-B., Ihara, M.O., Lee, S.-J., Yamashita, N., Miyagawa, S., Iguchi, T., Okamoto, S., Suzuki, Y., Tanaka, H., 2015. Evaluation of estrogenic activity of wastewater: comparison among in vitro ERα reporter gene assay, in vivo vitellogenin induction, and chemical analysis. Environ. Sci. Technol. 49 (10), 6319–6326.
Ingrand, V., Herry, G., Beausse, J., de Roubin, M.-R., 2003. Analysis of steroid hormones in effluents of wastewater treatment plants by liquid chromatoraphy–tandem mass spectrometry. J. Chromatogr. A 1020 (1), 99–104.
Isobe, T., Serizawa, S., Horiguchi, T., Shibata, Y., Managaki, S., Takada, H., Morita, M., Shiraishi, H., 2006. Horizontal distribution of steroid estrogens in surface sediments in Tokyo Bay. Environ. Pollut. 144 (2), 632–638.
Jarošová, B., Bláha, L., Giesy, J.P., Hilscherová, K., 2014a. What level of estrogenic activity determined by in vitro assays in municipal waste waters can be considered as safe? Environ. Int. 64 (0), 98–109.
Jarošová, B., Erseková, A., Hilscherová, K., Loos, R., Gawlik, B., Giesy, J., Bláha, L., 2014b. Europe-wide survey of estrogenicity in wastewater treatment plant effluents: the need for the effect-based monitoring. Environ. Sci. Pollut. Res. 21 (18), 10970–10982.
Jemec, A., Tišler, T., Erjavec, B., Pintar, A., 2012. Antioxidant responses and whole-organism changes in Daphnia magna acutely and chronically exposed to endocrine disruptor bisphenol A. Ecotoxicol. Environ. Saf. 86 (0), 213–218.
Kolodziej, E.P., Gray, J.L., Sedlak, D.L., 2003. Quantification of steroid hormones with pheromonal properties in municipal wastewater effluent. Environ. Toxicol. Chem. 22 (11), 2622–2629.
Körner, W., Hanf, V., Schuller, W., Kempter, C., Metzger, J., Hagenmaier, H., 1999. Development of a sensitive E-screen assay for quantitative analysis of estrogenic activity in municipal sewage plant effluents. Sci. Total Environ. 225 (1–2), 33–48.
Laganà, A., Bacaloni, A., De Leva, I., Faberi, A., Fago, G., Marino, A., 2004. Analytical methodologies for determining the occurrence of endocrine disrupting chemicals in sewage treatment plants and natural waters. Anal. Chim. Acta 501 (1), 79–88.
Lee, H.-B., Peart, T.E., Chan, J., Gris, G., 2004. Occurrence of endocrine-disrupting chemicals in sewage and sludge samples in Toronto, Canada. Water Qual. Res. J. Can. 39 (1), 57–63.
Legler, J., van den Brink, C.E., Brouwer, A., Murk, A.J., van der Saag, P.T., Vethaak, A.D., van der Burg, B., 1999. Development of a stably transfected estrogen receptor-mediated luciferase reporter gene assay in the human T47D breast cancer cell line. Toxicol. Sci. 48 (1), 55–66.
Leusch, F.D.L., Khan, S.J., Gagnon, M.M., Quayle, P., Trinh, T., Coleman, H., Rawson, C., Chapman, H.F., Blair, P., Nice, H., Reitsema, T., 2014. Assessment of wastewater and recycled water quality: a comparison of lines of evidence from in vitro, in vivo and chemical analyses. Water Res. 50, 420–431.
Lintelmann, J., Katayama, A., Kurihara, N., Shore, L., Wenzel, A., 2003. Endocrine disruptors in the environment (IUPAC Technical Report). Pure Appl. Chem. 75 (5), 631–681.
Liu, Z.-h., Kanjo, Y., Mizutani, S., 2009. Urinary excretion rates of natural estrogens and androgens from humans, and their occurrence and fate in the environment: a review. Sci. Total Environ. 407 (18), 4975–4985.
Ma, M., Rao, K., Wang, Z., 2007. Occurrence of estrogenic effects in sewage and industrial wastewaters in Beijing, China. Environ. Pollut. 147 (2), 331–336.
Maletz, S., Floehr, T., Beier, S., Klümper, C., Brouwer, A., Behnisch, P., Higley, E., Giesy, J.P., Hecker, M., Gebhardt, W., Linnemann, V., Pinnekamp, J., Hollert, H., 2013. In vitro characterization of the effectiveness of enhanced sewage treatment processes to eliminate endocrine activity of hospital effluents. Water Res. 47 (4), 1545–1557.
Manickum, T., John, W., 2014. Occurrence, fate and environmental risk assessment of endocrine disrupting compounds at the wastewater treatment works in Pietermaritzburg (South Africa). Sci. Total Environ. 468–469, 584–597.
Mendonça, E., Picado, A., Paixão, S.M., Silva, L., Cunha, M.A., Leitão, S., Moura, I., Cortez, C., Brito, F., 2009. Ecotoxicity tests in the environmental analysis of wastewater treatment plants: case study in Portugal. J. Hazard. Mater. 163 (2–3), 665–670.
Metcalfe, C.D., Metcalfe, T.L., Kiparissis, Y., Koenig, B.G., Khan, C., Hughes, R.J., Croley, T.R., March, R.E., Potter, T., 2001. Estrogenic potency of chemicals detected in sewage treatment plant effluents as determined by in vivo assays with Japanese medaka (Oryzias latipes). Environ. Toxicol. Chem. 20 (2), 297–308.
Murk, A.J., Legler, J., van Lipzig, M.M.H., Meerman, J.H.N., Belfroid, A.C., Spenkelink, A., van der Burg, B., Rijs, G.B.J., Vethaak, D., 2002. Detection of estrogenic potency in wastewater and surface water with three in vitro bioassays. Environ. Toxicol. Chem. 21 (1), 16–23.
Nakada, N., Nyunoya, H., Nakamura, M., Hara, A., Iguchi, T., Takada, H., 2004. Identification of estrogenic compounds in wastewater effluent. Environ. Toxicol. Chem. 23 (12), 2807–2815.
Nakada, N., Tanishima, T., Shinohara, H., Kiri, K., Takada, H., 2006. Pharmaceutical chemicals and endocrine disrupters in municipal wastewater in Tokyo and their removal during activated sludge treatment. Water Res. 40 (17), 3297–3303.
Nelson, J., Bishay, F., van Roodselaar, A., Ikonomou, M., Law, F.C.P., 2007. The use of in vitro bioassays to quantify endocrine disrupting chemicals in municipal wastewater treatment plant effluents. Sci. Total Environ. 374 (1), 80–90.
Oehlmann, J., Oetken, M., Schulte-Oehlmann, U., 2008. A critical evaluation of the environmental risk assessment for plasticizers in the freshwater environment in Europe, with special emphasis on bisphenol A and endocrine disruption. Environ. Res. 108 (2), 140–149.
Oehlmann, J., Schulte-Oehlmann, U., Tillmann, M., Markert, B., 2000. Effects of endocrine disruptors on Prosobranch snails (Mollusca: Gastropoda) in the laboratory. Part I: bisphenol a and octylphenol as xeno-estrogens. Ecotoxicology 9 (6), 383–397.
Pedrouzo, M., Borrull, F., Pocurull, E., Marcé, R., 2011. Presence of pharmaceuticals and hormones in waters from sewage treatment plants. Water Air Soil Pollut. 217 (1–4), 267–281.
Pessala, P., 2008. Tracking and Identifying Wastewater Toxicants and Assessing Their Biodegradation Products. Doctoral thesis. University of Helsinki, Helsinki.
Pessala, P., Schultz, E., Nakari, T., Joutti, A., Herve, S., 2004. Evaluation of wastewater effluents by small-scale biotests and a fractionation procedure. Ecotoxicol.

Environ. Saf. 59 (2), 263–272.
Petrović, M., Gonzalez, S., Barceló, D., 2003. Analysis and removal of emerging contaminants in wastewater and drinking water. TrAC Trends Anal. Chem. 22 (10), 685–696.
Pothitou, P., Voutsa, D., 2008. Endocrine disrupting compounds in municipal and industrial wastewater treatment plants in Northern Greece. Chemosphere 73 (11), 1716–1723.
Roepke, T.A., Snyder, M.J., Cherr, G.N., 2005. Estradiol and endocrine disrupting compounds adversely affect development of sea urchin embryos at environmentally relevant concentrations. Aquat. Toxicol. 71 (2), 155–173.
Roig, B., Mnif, W., Hadj Hassine, A.I., Zidi, I., Bayle, S., Bartegi, A., Thomas, O., 2012. Endocrine disrupting chemicals and human health risk assessment: a critical review. Crit. Rev. Environ. Sci. Technol. 43 (21), 2297–2351.
Salvador, A., Moretton, C., Piram, A., Faure, R., 2007. On-line solid-phase extraction with on-support derivatization for high-sensitivity liquid chromatography tandem mass spectrometry of estrogens in influent/effluent of wastewater treatment plants. J. Chromatogr. A 1145 (1–2), 102–109.
Silva, C.P., Lima, D.L.D., Schneider, R.J., Otero, M., Esteves, V.I., 2013. Development of ELISA methodologies for the direct determination of 17β-estradiol and 17α-ethinylestradiol in complex aqueous matrices. J. Environ. Manag. 124 (0), 121–127.
Sim, W.-J., Lee, J.-W., Shin, S.-K., Song, K.-B., Oh, J.-E., 2011. Assessment of fates of estrogens in wastewater and sludge from various types of wastewater treatment plants. Chemosphere 82 (10), 1448–1453.
Smital, T., Terzic, S., Zaja, R., Senta, I., Pivcevic, B., Popovic, M., Mikac, I., Tollefsen, K.E., Thomas, K.V., Ahel, M., 2011. Assessment of toxicological profiles of the municipal wastewater effluents using chemical analyses and bioassays. Ecotoxicol. Environ Saf. 74 (4), 844–851.
Sonneveld, E., Riteco, J.A.C., Jansen, H.J., Pieterse, B., Brouwer, A., Schoonen, W.G., van der Burg, B., 2006. Comparison of in vitro and in vivo screening models for androgenic and estrogenic activities. Toxicol. Sci. 89 (1), 173–187.
Sousa, A., Schönenberger, R., Jonkers, N., Suter, M.F., Tanabe, S., Barroso, C., 2010. Chemical and Biological Characterization of Estrogenicity in Effluents from WWTPs in Ria de Aveiro (NW Portugal). Arch. Environ. Contam. Toxicol. 58 (1), 1–8.
Stasinakis, A.S., Gatidou, G., Mamais, D., Thomaidis, N.S., Lekkas, T.D., 2008. Occurrence and fate of endocrine disrupters in Greek sewage treatment plants. Water Res. 42 (6–7), 1796–1804.
Sun, Q., Deng, S., Huang, J., Shen, G., Yu, G., 2008. Contributors to estrogenic activity in wastewater from a large wastewater treatment plant in Beijing, China. Environ. Toxicol. Pharmacol. 25 (1), 20–26.
Tan, B.L.L., Hawker, D.W., Müller, J.F., Leusch, F.D.L., Tremblay, L.A., Chapman, H.F., 2007. Comprehensive study of endocrine disrupting compounds using grab and passive sampling at selected wastewater treatment plants in South East Queensland, Australia. Environ. Int. 33 (5), 654–669.
van der Linden, S.C., Heringa, M.B., Man, H.-Y., Sonneveld, E., Puijker, L.M., Brouwer, A., van der Burg, B., 2008. Detection of multiple hormonal activities in wastewater effluents and surface water, using a panel of steroid receptor CALUX bioassays. Environ. Sci. Technol. 42 (15), 5814–5820.
Vethaak, A.D., Lahr, J., Schrap, S.M., Belfroid, A.C., Rijs, G.B.J., Gerritsen, A., de Boer, J., Bulder, A.S., Grinwis, G.C.M., Kuiper, R.V., Legler, J., Murk, T.A.J., Peijnenburg, W., Verhaar, H.J.M., de Voogt, P., 2005. An integrated assessment of estrogenic contamination and biological effects in the aquatic environment of The Netherlands. Chemosphere 59 (4), 511–524.
Vieno, N., 2014. Haitalliset Aineet Jätevedenpuhdistamoilla – Hankkeen Loppuraportti. Vesilaitosyhdistys, Helsinki, p. 273.