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RESEARCH ARTICLE

Europe-wide survey of estrogenicity in wastewater treatment plant effluents: the need for the effect-based monitoring

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Abstract A pan-European monitoring campaign of the wastewater treatment plant (WWTP) effluents was conducted to obtain a concise picture on a broad range of pollutants including estrogenic compounds. Snapshot samples from 75 WWTP effluents were collected and analysed for concentrations of 150 polar organic and 20 inorganic compounds as well as estrogenicity using the MVLN reporter gene assay. The effect-based assessment determined estrogenicity in 27 of 75 samples tested with the concentrations ranging from 0.53 to 17.9 ng/L of 17-beta-estradiol equivalents (EEQ). Approximately one third of municipal WWTP effluents contained EEQ greater than 0.5 ng/L EEQ, which confirmed the importance of cities as the major contamination source. Beside municipal WWTPs, some treated industrial wastewaters also exhibited detectable EEQ, indicating the importance to investigate phytoestrogens released from plant processing factories. No steroid estrogens were detected in any of the samples by instrumental methods above their limits of quantification of 10 ng/L, and none of the other analysed classes of chemicals showed correlation with detected EEQs. The study demonstrates the need of effect-based monitoring to assess certain classes of contaminants such as estrogens, which are known to occur at low concentrations being of serious toxicological concern for aquatic biota.

Keywords In vitro bioassay · Monitoring · Sewage · Rivers · Hormones · EDCs · Endocrine disruptors

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Abbreviations

E1	Estrone
E2	17β-Estradiol
E2max	Maximal response of standard ligand - E2
EE2	17α-Ethynylestradiol
EEQ	17β-Estradiol equivalents
HDPE	High-density polyethylene
LOD	Limit of detection
LOQ	Limit of quantification
NP	Nonylphenol
OP	Octylphenol
PNECs	Predicted no-effect concentrations
PPCPs	Pharmaceuticals and personal care products
PFASs	Perfluoroalkyl substances
WWTP	Wastewater treatment plant
YES	Yeast estrogen screen

Introduction

Estrogenic compounds present in treated wastewaters have been shown to mainly contribute to adverse reproductive effects in aquatic biota. Feminisation of male fishes living downstream from wastewater treatment plants (WWTPs) has been observed worldwide (e.g. Sumpter and Johnson 2008; Wang et al. 2013). Steroid estrogens, particularly natural hormones such as estrone (E1) and 17β-estradiol (E2) and

the synthetic hormone 17α-ethynylestradiol (EE2) used in many contraceptives, have been identified as the major causative agents in treated, domestic wastewaters (Arditsoglou and Voutsa 2008; Jarosova et al. 2014). Feminisation of fishes has been also observed at several locations downstream from industrial WWTP discharges near places with textile and tannery industries, where greater concentrations of alkylphenols have been detected (Sumpter and Johnson 2008; Keith et al. 2001). The most potent estrogenic alkylphenols are 4-tertiary isomers of nonylphenol (NP) and to lesser extend also octylphenol (OP). NP and OP have been reported to be the primary cause of adverse effects downstream of the industrial WWTPs (Sumpter and Johnson 2008; Sole et al. 2000). Compared to steroid estrogens, alkylphenols are at least a thousand times less potent estrogens (Environment Agency 2004; Leusch et al. 2010), but their concentrations detected near textile industry may exceed 100 μg/L (e.g. Sole et al. 2000). That is approximately 100,000 times more than the common environmental concentrations of steroid estrogens (Runnalls et al. 2010).

The observation that steroid estrogens in surface waters can cause adverse effects on reproduction to sensitive organisms, such as fish at low nanogram per litre concentrations, stimulated efforts to improve analytical techniques for environmental samples (Sumpter and Johnson 2008). Despite these efforts, reliable quantification of steroid estrogens in environmental mixtures such as wastewaters remains a problem (Caldwell et al. 2012). In addition, even reliable detection of a few selected estrogens does not guarantee identification of actual estrogenic potential in environmental samples (Villeneuve et al. 1998). Some unexpected molecules or interactions increasing or inhibiting the overall estrogenicity have been observed in several studies (e.g. Cargouet et al. 2004; Pawlowski et al. 2003). Therefore, there was a need to complement the targeted chemical instrumental methods by biological approaches (Leusch et al. 2010). Naturally, in situ and in vivo bioassays would be the most relevant to detect adverse effects, but they are expensive and time and animal consuming which limits their application for broader monitoring of water quality. Alternatively, in vitro bioassays can serve as rapid and cost-effective screening methods to estimate total estrogenic activity of all compounds that act through the same mode of action (i.e. binding to estrogenic receptor) present in the mixtures, and they are currently being considered to be used in the tiered monitoring of estrogenicity of environmental waters (Leusch et al. 2010). The need for the effect-based monitoring and trigger values was recently highlighted also by other authors (Escher et al. 2013; Tang et al. 2013).

In past decades, several polar organic compound classes including estrogens were found to be discharged via WWTP effluents into receiving waters (Reemtsma et al. 2006). Majority of information about these so-called emerging chemicals and their eventual effects (such as estrogenic activity) in wastewaters is available from scattered national or local studies. Such studies are often narrow, focussing in detail on a specific chemical group and using different methodologies in the sample preparation and analysis. Therefore, it has been complicated to compare results across studies and to draw general estimates of probable concentrations or biological activities at a broader scale. In 2010, effluents from 90 WWTPs were collected within 16 European countries and analysed in order to obtain a large data set on many so far only locally investigated "emerging" compounds (Gawlik et al. 2012). The study was designed to provide the first concise overview of concentrations of emerging pollutants occurring in WWTP effluents across Europe including countries for which only limited information was publically available before such as Cyprus, Czech Republic or Lithuania. The study focussed on a range of pharmaceuticals and personal care products (PPCPs), veterinary (antibiotic) drugs, perfluoroalkyl substances (PFASs), organophosphate ester flame retardants, pesticides and their metabolites, industrial chemicals such as corrosion inhibitors benzotriazoles, polycyclic musk fragrances, X-ray contrast agents, gadolinium compounds, and siloxanes (Loos et al. 2012). Targeted chemical analyses were complemented by the effect-based monitoring approaches aiming at estrogenicity, dioxin-like activity, and yeast and diatom culture acute toxicity (Loos et al. 2013). In the present paper, we discuss in detail the results of estrogenicity detected using the reporter gene bioassay in the extracts of 75 WWTP effluents and compare the bioassay responses with the chemical analyses of emerging pollutants. Environmental risks of detected estrogenicity (expressed as nanograms per litre 17β-estradiol equivalents (EEQ)) are discussed by comparing the detected concentrations of EEQ with effective in vivo concentrations of major estrogens to aquatic biota such as fish.

Methods

Description of the campaign, WWTPs, sampling and sample storing

The selection of WWTP was not done by researchers. Instead, the selection of the WWTPs was done by voluntarily participating European Union Member States (and Switzerland), and no criteria were required by the coordinator of the project (Loos et al. 2013). Participants were, however, aware of the aims of the study and therefore wastewaters from WWTPs of different capacities and diverse sources (domestic with or without storm water; and also some with larger proportions of industrial effluents were collected from the participating countries). Table 1 gives a list of the 75 WWTPs from 16 different countries investigated in the present study. This table

Table 1 Characterisation of sampled wastewater treatment plants (WWTPs) and the detected estrogenic activity

Label in this article	Country	Location/WWTR name	Composition of wastewater	Plant capacity (thousands of m^3/d)	Capacity population equivalent (thousands)	Type of secondary (and tertiary if applied) treatment	Detected EEQ (ng/L)
WWTP A1	Italy	Roma nord ACEA	Dom. Ind. Rain	354	780	biological, not specified, final disinfection step	12.2
WWTP A2	Czech Rep.	Not displayed	Dom. Ind. Rain	>200	>500	AS, DN, N, CHP	2.1
WWTP A3	Czech Rep.	Not displayed	Dom. Ind. Rain	>100	>500	AS, DN, N, CHP	1.3
WWTP A4	Finland	Helsinki	Dom. Ind. probably Rain	30[a]	825[a]	AS, DN, N, CHP	<0.5
WWTP A5	Germany	Bremen	Dom. Ind. Rain	94	1 000	AS, D/N, CHP	<0.5
WWTP A6	Germany	Klärwerk Gut Marienhof	Dom. Ind. Rain	493	1 500	AS, DN, N, CHP	<0.5
WWTP A7	Ireland	Dublin		400	1 900	AS (sequencing batch reactor) with DN/N, UV Light Treatment	<0.5
WWTP A8	Netherlands	Harnaschpolder	Dom. Ind. Rain	150	1 400	AS, DN/N, BP	<0.5
WWTP A9	Netherlands	Rotterdam Dokhaven	Mainly Dom.	117	500	AS, D/N - SHARON® and ANAMMOX®, CHP	<0.5
WWTP A10	Switzerland	Zürich Werdhölzli	Dom. Ind. Rain		640	AS, DN, N, BP, CHP	<0.5
WWTP B1	Slovenia	Ljubljana	Dom. (62 %), Ind. (11 %), Rain (21 %)	103	360	AS not further specified	4.1
WWTP B2	Czech Rep.	Not displayed	Dom. Ind. Rain	52	170	AS, DN, N, CHP	1.7
WWTP B3	Lithuania	Kaunas		82	370	AS, DN/N, CHP	1.0
WWTP B4	Netherlands	Venlo		71[b]		AS, DN/N, BP	0.9
WWTP B5	Netherlands	Almere	Dom., Hospital, no Rain		330	not specified	0.6
WWTP B6	Austria	Wiener Neustadt - Sud	Dom. (90 %), Paper Ind.	37	260	AS, DN/N, P removal not specified,	0.5
WWTP B7	Austria	AWV Hall i. Tirol-Fritzens	Dom. Ind. (Rain was not further specified)	16	120	AS not further specified	<0.5
WWTP B8	Belgium	Deurne	Waste water from Antwerp	50[a]	325	AS not further specified	<0.5
WWTP B9	Finland	Espoo	Dom. Ind. Rain not specified	110	250	AS, DN, N, P removal not specified	<0.5
WWTP B10	Netherlands	Amstelveen	Dom.		125	AS not further specified	<0.5 ∇
WWTP B11	Netherlands	Nieuwgraaf	Dom. Ind. (30-40 %), Hospital		395	AS not further specified	<0.5 ∇
WWTP B12	Netherlands	Garmerwold (Noorderzijlvest)	Dom.		300	AS, DN/N - SHARON®, P removal not specified	<0.5
WWTP B13	Netherlands	Zaandam Oost	Dom. Urban runoff, Ind. Craft Industry		150	AS, DN/N, P removal not specified	<0.5
WWTP B14	Lithuania	Klaipedo vanduo	Dom. Ind. (Rain was not further specified)	95	200[a]	AS, DN/N, P removal not specified	<0.5
WWTP B15	Lithuania	Panevezys regional	Dom. Ind. Rain	70		not specified	<0.5
WWTP C1	Cyprus	Larnaka	Dom.	6	27.5	AS, no DN, N and P removal not specified, sand filtration, chlorination	3.6
WWTP C2	Spain	Ulldecona		1.6	13.5	not specified	3.3
WWTP C3	Czech Rep.	Not displayed	Dom. Rain	3	15	AS, N, DN, CHP	1.2
WWTP C4	Austria	Eisenstadt eisbachtal		12[b]	42[b]	AS, DN/N not specified, CHP	<0.5
WWTP C5	Austria	Feldkirchen		6.6	50	AS, N, DN, BP	<0.5
WWTP C6	Belgium	Hasselt	Dom.	12	65	AS, (DN/N and P removal not specified)	<0.5
WWTP C7	Cyprus	Limassol	Dom. Ind.	15	70	AS, N, DN, no BP (CHP not specified), sand filtration, chlorination	<0.5
WWTP C8	Czech Rep.	Not displayed	Dom. Rain	19	75	AS, N, DN, CHP	<0.5
WWTP C9	Ireland	Oberstown			80	cyclic AS, N, DN, CHP	<0.5
WWTP C10	Netherlands	Leek (Noorderzijlvest)	Dom.		34	not specified	<0.5 ∇
WWTP C11	Netherlands	Simpelveld	Dom., Health Care Unit		20.5	not specified	<0.5
WWTP C12	Netherlands	Winterswijk	Dom. Ind. (30-40 %). Hospital		83.5	not specified	<0.5
WWTP C13	Spain	Tortosa		10	46.8	not specified	<0.5
WWTP C14	Switzerland	Affoltern a.A.	Dom. Ind. Rain		14	AS, DN/N not specified, CHP	<0.5

Table 1 (continued)

Label in this article	Country	Location/WWTR name	Composition of wastewater	Plant capacity (thousands of m^3/d)	Capacity population equivalent (thousands)	Type of secondary (and tertiary if applied) treatment	Detected EEQ (ng/L)
WWTP D1	Czech Rep.	Not displayed	Dom. Ind. no Rain	0.3	2.5	AS, N, DN, CHP	1.9
WWTP D2	Germany	AZV Hungerbachtal			7[a]	AS not further specified	0.8
WWTP D3	Hungary	Alattyán	Mainly Dom.		0.25	not specified	0.8
WWTP D4	Switzerland	Wenslingen	Dom. Rain		0.7	AS (DN/N and P removal not specified)	0.6
WWTP D5	Czech Rep.	Not displayed	Dom. Ind. no Rain	0.7	5	AS, N, DN, CHP	<0.5
WWTP D6	Finland	Nummi-Pusula		1[b]	6[a]	Fe coag., As (no DN/N)	<0.5
WWTP D7	Spain	Godall		0.15	0.9	not specified	<0.5
WWTP D8	Switzerland	Konolfingen	Dom. Ind. Rain		7.9	AS, CHP (DN/N not specified)	<0.5
WWTP D9	Switzerland	Seuzach	Dom. Rain	4	6.5	AS, CHP (DN/N not specified)	<0.5 ∇
WWTP E1	Belgium	Agristo	Food industry (potato products)				3.4
WWTP E2	Belgium	TWZ Evergem	Tank cleaning and various ind. activities				1.8
WWTP E3	Belgium	Bayer Antwerpen	Chemical industry (e.g. pesticide production)				1.2
WWTP E4	Belgium	3M	Different industrial branches				0.8
WWTP E5	Belgium	Janssen Pharmaceuticals	Pharmaceutical industry				0.6
WWTP E6	Austria	WV Hofsteig	Dom. (25 %). Ind. (75 %) (Metal, food, textile)	138	216	AS not further specified	<0.5
WWTP E7	Belgium	Ajjinomoto Omnichem	Herbal extracts, polyphenols production				<0.5 ∇
WWTP E8	Belgium	Ardo	Food industry (frozen vegetable)				<0.5
WWTP E9	Belgium	Colortex	Textile industry (dyeing)				<0.5 ∇
WWTP E10	Belgium	EOC Oudenaarde	Chemical industry (e.g. adhesives, surfactants)				<0.5
WWTP E11	Belgium	Tack Oostrozebeke	Tank cleaning and various industrial activities				<0.5 ∇
WWTP E12	Belgium	Taminco	Chemical industry (Amine company)				<0.5 ∇
WWTP F1	Hungary	Martfű	Dom. or soya or brewery production?	1			17.9
WWTP F2	Portugal	Parada				AS, DN, N, no BP	6.0
WWTP F3	Austria	AWV Region Feldkirch		380		AS not further specified	1.2
WWTP F4	Portugal	Viana do Castelo			90[a]	AS not further specified	0.7
WWTP F5	Greece	Thessaloniki (EELTH)	Dom. Ind. probably Rain				0.7
WWTP F6	Italy	Depuratore 'Jugendwerk Brebbia'					0.6
WWTP F7	Belgium	Geel				trickling filter, AS (INVENT®), sand filtration	<0.5
WWTP F8	Belgium	Ronse					<0.5
WWTP F9	Belgium	Waregem	Region with textile industry				<0.5 ∇
WWTP F10	Finland	Lohja					<0.5
WWTP F11	Finland	Mäntsälä					<0.5
WWTP F12	Finland	Vihti					<0.5
WWTP F13	Greece	Thessaloniki (EEL AINEIA)	Waste water from Thermaikos city				<0.5
WWTP F14	Belgium	Claerebout					<0.5
WWTP F15	Belgium	Shanks lokeren					<0.5

Dom. domestic, *Ind*. industrial, *AS* reservoirs with activated sludge, *DN* denitrification, *N* nitrification, *DN/N* biological treatment of nitrogen (not specified if N, DN or both are used), *BP* biological removal of phosphorus, *CHP* chemical precipitation of phosphorus

[a] Approximate number

[b] Average daily discharge or currently connected equivalent citizens and not maximal capacity of WWTP

∇ ∇ Cytotoxic/antiestrogenic samples (open and full symbols indicate less and more pronounced effects, respectively)

contains (besides results of the estrogenicity of samples) information on the type of discharges treated in the plant (domestic or industrial), plant capacity (m^3/d), capacity in population equivalents, type of secondary treatment, and, if applicable, type of tertiary treatment applied. Unfortunately, not all participants of the campaign (owners of the WWTPs) provided all the information requested. Information was collected for 48 municipal and 12 industrial WWTPs, whereas no available metadata were available for 15 tested WWTPs (information not provided by the owners, neither found at other information sources nor on the internet). With the exception of a few small WWTPs (capacity of equivalent population, CEP<10,000) and possibly also some of the WWTPs for which there was no information, all investigated municipal WWTPs included activated sludge processes with nitrification and/or denitrification and chemical precipitation of phosphorus, which represent the most common WWTP technology in Europe. Only four municipal WWTPs reported use of biological phosphorus removal technology and other four municipal WWTPs utilised tertiary treatment step (filtration and chlorination or UV light). Some of the smaller WWTPs (CEP<10,000) utilised activated sludge and chemical precipitation of organics and phosphorus without denitrification/nitrification (Table 1).

With respect to the objective and broad character of the study, i.e. 'snapshot' screening of the European situation, both grab and 24-h composite samples were provided by WWTP owners. Eight 1-L aliquots of water were collected from each WWTP, stored in high-density polyethylene (HDPE) plastic bottles, then shipped to the coordinator (Joint Research Centre (JRC), Ispra, Italy) by fast courier in polystyrene boxes with cooling elements. Samples were stored at ~4 °C and further distributed as fast as possible to the other expert laboratories for analyses (Loos et al. 2013).

Due to high number of cooperating subjects, the time from sampling to extraction differed from days to 2 and occasionally even 3 months. Possible transformation of estrogenic compounds during shipping of samples was considered, and samples collected in the country where the bioassays were performed (Czech Republic) were divided into two aliquots and tested within 2 days after sampling as well as after the shipping procedure (45 days later). The samples originated from seven different WWTPs and represented municipal WWTPs with wide range of capacities from <10,000 to more than 1,000,000 equivalent citizens. Differences in the estrogenicity between the samples extracted immediately after sampling and with delay were studied to investigate stability of the samples during storage and shipping.

Sample preparation by solid-phase extraction

Water samples were extracted by solid-phase extraction with Oasis HLB cartridges (6 mL, 500 mg, Waters, CZ). Samples were filtered through glass fibre filters (2 μm, Fisher Scientific, CZ) prior to extraction. Each cartridge was activated by 6 mL of methanol (MeOH) and equilibrated by 8 mL of distilled water without vacuum. The water samples (500 mL) were passed through the wet cartridges at a flow rate of about 5 mL min^{-1}, then the columns were left to dry for 10 min, and consequently eluted by 6 mL of MeOH. Eluates were concentrated by nitrogen stream at laboratory temperature to final volumes which corresponded to 1,200 times of concentrated original effluents. This equivalent was selected as a maximal concentration which was not cytotoxic to the cells in our previous studies and enabled detecting estrogenic activity with the limits of detection (LOD) for estrogenicity of 0.5 ng EEQ per litre. Sample extracts were stored at −18 °C until analyses.

In vitro bioassays

To determine total estrogenicity of the sample extracts as well as specific potencies of individual estrogens (E1, E2, estriol (E3) and EE2), human breast carcinoma MVLN cells stably transfected under the control of estrogen receptor with firefly luciferase gene were used (Demirpence et al. 1993). Cells were grown in DMEM-F12 without phenol red (Sigma Aldrich, USA) containing 10 % foetal calf serum at 5 % CO_2 and 37 °C. Once the cells reached about 80 % confluence, they were trypsinised and seeded into a sterile 96-well plate at density 25,000 cells/well. For experiments, cells were grown in medium containing foetal calf serum treated with dextran-coated charcoal (strongly reduces concentrations of natural steroids in the calf serum). After 24 h, cells were exposed to the reference estrogen, 17β-estradiol (dilution series 1–500 pM E2), or the dilution series of other steroid estrogens (1–10,000 pM for E1 or E3; 0.1–500 pM for EE2), to the dilution series of the tested samples (at least five different concentrations), and blank and solvent controls (0.5 %*v*/*v* methanol). Exposures were conducted in three replicates for 24 h at 37 °C. After the exposure, intensity of luminescence was measured using Promega Steady Glo Kit (Promega, Mannheim, Germany). Analyses of the estrogenic potency of E1, E3 and EE2 were repeated and compared to E2 independently at least three times. Assessment of in vitro activity of the first 25 extracts of wastewaters was performed at least two times. The median standard deviation was 18 % (maximum 46 %) which was in a good agreement with our long-term results. The remaining 50 wastewaters were then analysed in a single experiment in three replicates.

Quantification of estrogenicity

Results of the estrogenicity bioassay were expressed as EEQ with respect to the standard estrogen, E2. After subtraction of the response in the solvent control, detected induction of

luminescence was related to the maximal response of standard ligand (E2max) and converted into percentages of E2max. Since most extracts did not reach 50 % of E2max (i.e. EC_{50}), the results were determined as EC_{25}. The EC_{25} values were based on relating the amount of E2 causing 25 % of the E2 response (EC_{25}) to the amount of sample causing the same level of response (Villeneuve et al. 2000). Values were determined from the nonlinear logarithmic regression of dose-response curve of calibration standard and samples using the GraphPad Prism Software (GraphPad Software, San Diego, USA).

Determination of the MVLN-cell-line-specific potencies of E1, E3 and EE2 relative to E2

After converting the results into percentages of E2max (as described in this section), EC_{50} values of dose-response curves of E2, E1, E3 and EE2 were determined from the nonlinear logarithmic regression in GraphPad Prism (GraphPad Software, San Diego, USA). The specific potencies were then determined as the ratio of EC_{50} of the model compound (E1, E3 or EE2) and EC_{50} of the reference E2. The EC_{50} of each of the model compound was always divided by EC_{50} of E2 which was obtained from measurements of cells exposed on the same microwell plate. The final specific potency relative to E2 was the mean of three independent experiments.

Statistical analyses

Nonparametric Wilcoxon match pairs test was used to assess the significance of differences of estrogenicity detected in samples extracted within 48 h and after delivery from the coordinator 45 d later. Differences in estrogenicity among the samples from six groups of WWTP effluents (four categories of municipal WWTP effluents divided according to the plant capacities, industrial WWTP effluents and 'unidentified' WWTP effluents) were tested by the nonparametric Kruskal-Wallis test. Spearman correlation was used to investigate the relationship between the results of chemical and biological analyses. All statistical analyses were performed with Statistica for Windows® 10.0 (StatSoft, Tulsa, OK, USA). For the statistical analyses, the concentrations below the LOD were replaced by one half of LOD.

Results and discussion

Verification of stability of selected samples during storage and shipping

Estrogenicities of the seven effluents extracted within 48 h after sampling were not significantly different from effects of the same samples extracted after delivery from the coordinator 45 d later (Wilcoxon match pairs test, $P=0.4$). Coefficients of variation between the freshly and later extracted samples were lower or comparable to the standard error of the used bioassay (Table 2). In two of the samples, greater concentrations of EEQs were detected in extracts prepared after longer storage (Table 2). These results demonstrate that, at least in the case of samples from the Czech Republic, there was no significant change in the estrogenic activity during prolonged storage and shipping.

Levels of detected estrogenic activity

The present study shows an overview of the pan-European situation regarding the estrogenic compounds, and for countries such as Cyprus, Lithuania or the Czech Republic, it brings some of the first publically available data on estrogenic potential in their WWTP effluents. Of the 75 WWTP effluents, 27 extracts showed estrogenic activity higher than the detection limit (>0.5 ng/L EEQ). Estrogenic activity in the 27 samples ranged from 0.53 to 17.9 ng/L EEQ with median and arithmetic mean being 1.2 and 2.7 ng/L EEQ, respectively (Fig. 1). Median and arithmetic mean of all 75 tested samples were <0.5 and 0.9 ng/L EEQ, respectively. The levels of detected EEQs are well comparable to the results of previous studies evaluating estrogenic activity of European WWTP

Table 2 Estrogenic activity in extracts of seven wastewater treatment plant (WWTP) effluents prepared directly after sampling (at 48 h) and after longer storage (45 d)

Number of WWTP	Extraction 48 h after sampling (ng/L EEQ)	Extraction 45 d after sampling (ng/L EEQ)	Coefficient of variation between samples extracted at 48 h and 45 d (%)
WWTP A2	2.0±0.4	2.1±0.5	2
WWTP A3	1.0±0.3	1.3±0.2	13
WWTP B2	0.7±0.2	1.7±0.7	40
WWTP C3	2.0±0.2	1.2±0.2	25
WWTP C8	0.8±0.2	<0.5	23[a]
WWTP D1	1.0±0.3	2.0±0.4	32
WWTP D5	<0.5	<0.5	0

Estrogenicity is expressed as 17β-estradiol equivalents (EEQ)

[a] Coefficient of variation was calculated as if the value <0.5 was 0.5

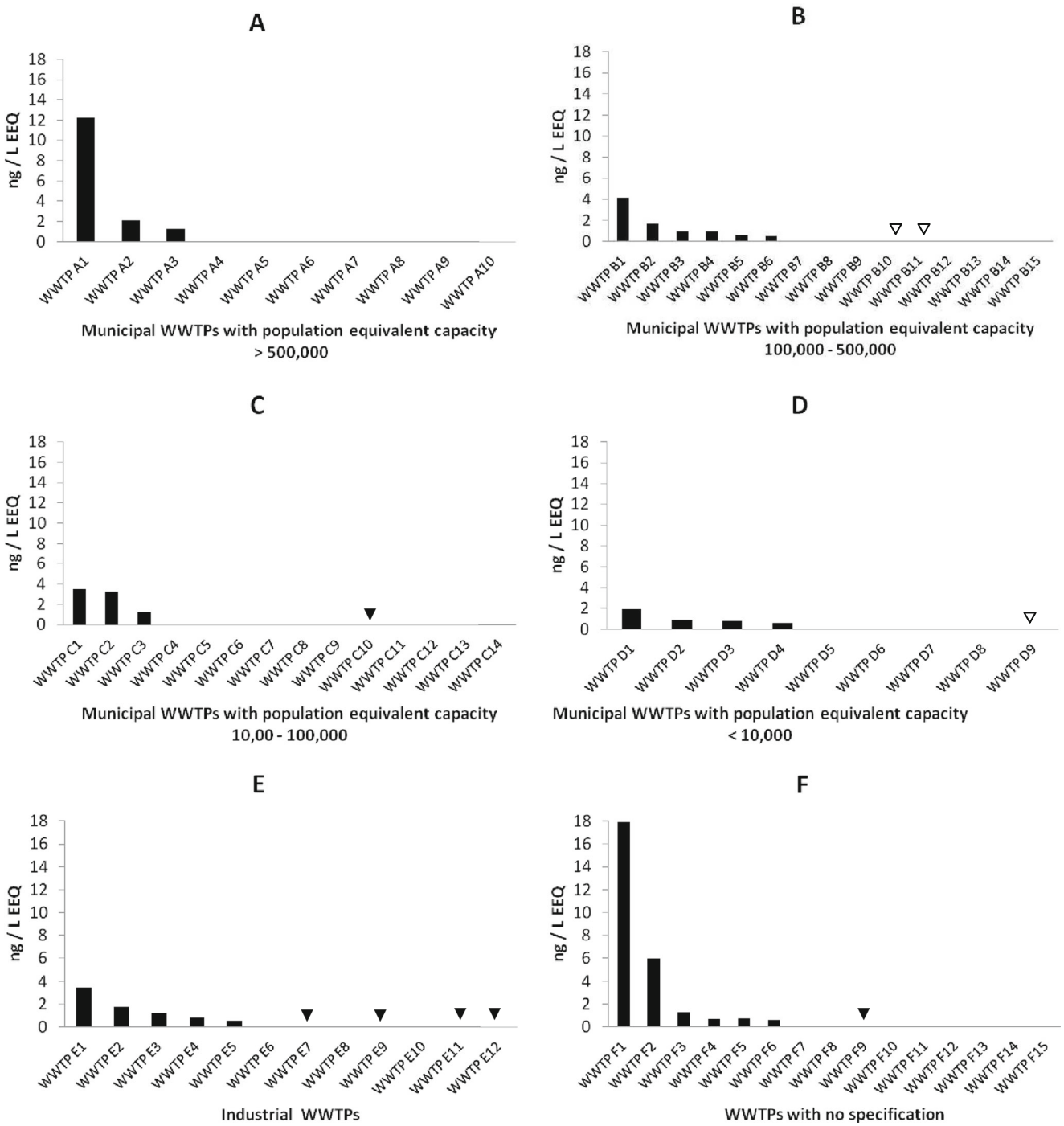

Fig. 1 Estrogenic activity expressed as 17β-estradiol equivalents (EEQ) of 75 extracts of European wastewater treatment plant (WWTP) effluents determined by MVLN in vitro assay. If no value is presented, the concentration of EEQ was less than the LOD (<0.5 ng/L). *Triangles* show cytotoxic/antiestrogenic samples (*open* and *full symbols* indicate less and more pronounced effects, respectively). **a**–**c** Municipal WWTPs, with domestic and some industrial wastewaters; **d** smaller WWTPs with most wastewaters of domestic origin; **e** industrial WWTPs; and **f** WWTPs for which no detailed information was available

effluents by different in vitro bioassays. For example, Svenson et al. (2003) used human estrogen receptor, hosted in a yeast strain, to quantify estrogenicity in samples of effluents from 20 Swedish municipal WWTPs. In this Swedish study, the treatment plants were selected to represent different treatment processes regarding chemical precipitation (coagulation and precipitation by Al or Fe to remove phosphorus and coagulate dissolved organic material) and microbial processes. The EEQs detected in Swedish WWTP effluents ranged from less than 0.1 to 15 ng/L. The other larger studies evaluating

estrogenicity of European WWTP effluents were for example those performed by Korner et al. (2001) in Germany, Vethaak et al. (2005) in the Netherlands, Aerni et al. (2004) in Switzerland or Cargouet et al. (2004) in France. After the exclusion of the one outlying value (53 ng/L EEQ) reported by Aerni et al. (2004), the levels of measured EEQ in all these studies varied from less than 0.03 to 24 ng/L, which is also in a good agreement with the results determined in the present study.

However, all the other studies reported higher frequencies of detection of positive samples in comparison to our survey. Several reasons could be considered. First, we have done no further concentrations of the initially negative samples. The detection limit of 0.5 ng/L EEQ in the present study was thus slightly higher in comparison to previous investigations (0.03–0.1 ng/L EEQ), which resulted in lower number of positive 'estrogenic' samples. Second, higher levels of EEQ in previously published studies were often detected at municipal WWTPs with other treatment technologies then activated sludge with nitrification, which was the most frequent in our study. For example, in the Dutch study by Vethaak et al. (2005) where most treatment plants consisted of an activated sludge system with nitrification step (similar to the present pan-European campaign), the frequency of positively estrogenic samples with EEQ>0.5 ng/L would be only 10 % (in contrast to reported 95 % with lower LOD of 0.1 ng/L EEQ). Second, nine samples showed decrease in estrogenic response compared to the solvent control, which may indicate either nonspecific cytotoxicity or antiestrogenicity. These two endpoints cannot be reliably distinguished by the used bioassay, and therefore, the samples were marked as antiestrogenic or cytotoxic. However, the cytotoxicity is much more common than the antiestrogenicicity in effluents, especially in municipal wastewaters. These usually contain highly potent estrogenic compounds like steroid estrogens having strong affinities to estrogenic receptors and overweighting thus the potencies of antiestrogenic compounds (e.g. Preuss et al. 2010; Johnson and Jurgens 2003). Many authors have reported estrogenic potential of WWTPs effluents (e.g. Vethaak et al. 2005; Aerni et al. 2004), but antiestrogenicity in this type of waters has only rarely been reported (Jalova et al. 2013). The nonspecific cytotoxicity can also mask the estrogenic potential, especially in highly contaminated samples. For example, Sole et al. (2000) reported fish feminisation downstream of a WWTP from textile industry where only cytotoxic (but not estrogenic) effects were detected using the in vitro system. In that case, high concentrations of contaminants (specifically the estrogenic alkylphenols) masked the actual estrogenic effect of these compounds (Sole et al. 2000). Therefore, samples found to be cytotoxic in the bioassay should be considered potentially estrogenic (or with lower probability potentially antiestrogenic).

Also, cytotoxicity in these nine specific samples could mask the effects of estrogens present in the complex mixtures. This would correspond to the study of Sole et al. (2000) who reported fish feminisation downstream of a WWTP from textile industry, where only cytotoxic (but not estrogenic) effects were detected using the in vitro system. The described arguments may explain the lower frequency of detection of estrogenic samples in the present study compared to other investigations. It should, however, be pointed out that although we have found good stability of estrogenic responses in seven of the studied samples, eventual degradation of the active compounds in other effluents cannot be fully excluded. Nevertheless, the detected ranges of EEQs provide a concise pan-European picture and correspond very well to the values reported in previous local studies from some of the countries.

Estrogenicity of different categories of WWTPs

Approximately one third of the 48 effluents that originated from municipal WWTPs displayed estrogenicity greater than the LOD of 0.5 ng/L EEQ, and 4 samples were cytotoxic/antiestrogenic (Fig. 1). The EEQ of positive extracts varied from 0.53 to 12.2 ng/L, and the greatest value was detected at a WWTP of one of the major cities with one of the highest capacities. However, statistical comparisons in estrogenicity among the groups of municipal WWTPs of different capacities showed no significant differences. Although the quantitative information on proportion of industrial and domestic wastewaters was available for a limited number of WWTPs (Table 1), the larger WWTPs with CEP of more than 100,000 typically contained not only domestic but also significant proportions of industrial wastewaters (about 11–40 %). The proportion of industrial wastewaters in effluents of municipal WWTPs had been reported to have little effect on observed rates of feminisation of fish (Jobling et al. 2006). There was no correlation between feminisation of fish and amounts of industrial wastewaters in rivers in the UK. In the same study, there was an association between the proportion of the municipal sewage effluent in the river and the incidence and magnitude of endocrine disruption in wild fishes (Jobling et al. 2006).

In the present study, there was no significant difference between estrogenicities of municipal and 'purely' industrial WWTP effluents where 9 out of 12 industrial WWTP effluents were either estrogenic (5 extracts with EEQ ranging from 0.6 to 3.4 ng/L) or cytotoxic/antiestrogenic (4 extracts). The sample with the greatest estrogenic activity among the industrial effluents originated from the WWTP of a factory that processes potatoes. The second most potent sample was from a WWTP treating wastewaters from tank vehicles, silo vehicles and cleaning of rail cars. Other samples exhibiting estrogenicity originated from a pharmaceutical factory and a company producing pesticides, whereas the cytotoxic/antiestrogenic extracts were treated wastewaters from companies processing plants in order to produce polyphenols, dyeing

textiles, cleaning tanks and vehicles (the company also accepts wastewaters from different industrial branches) or synthesizing amines. It should be pointed out that the majority of the industrial samples originated from a single country, Belgium (Table 1), so they cannot represent the Europe-wide situation for the industrial WWTP effluents.

Two of three treated wastewaters originating from factories processing plant materials were either antiestrogenic/estrogenic or cytotoxic. So far, only the phytoestrogen genistein (abundant in soya, flour and some vegetables) has been identified as the major contributor to detected estrogenicity in environmental waters (Kawanishi et al. 2004). Many other phytoestrogens (e.g. coumestrol, zearalenone, β-sitosterol, and enterolactone) have been identified in WWTP effluents or rivers, but their concentrations and/or estrogenic potencies were lower, relative to genistein or other anthropogenic estrogens (Pawlowski et al. 2003; Kawanishi et al. 2004; Lagana et al. 2004). Phytoestrogens in the environment can be significant contributors to estrogenicity of environmental waters, especially at locations close to factories processing plant materials (Liu et al. 2010).

Two out of three treated wastewaters originating from factories processing plant materials were found to be antiestrogenic or cytotoxic. As it was already discussed, this could mask the actual estrogenic effects in these samples. It is well known that many plants contain natural compounds structurally and/or functionally similar to estrogens and their active metabolites. These so-called phytoestrogens are widely present in, for example, soybeans, fruits, and cabbages. These are commonly consumed foods, and phytoestrogens thus occur in domestic wastewaters worldwide as reviewed by Liu et al. (2010). The in vitro potencies of phytoestrogens differ among individual bioassays (Liu et al. 2010), but the concentrations of phytoestrogens detected in the European municipal WWTP effluents seem to be too low to significantly contribute to detected EEQs. However, these patterns could be different in other than European municipal waters. For example, phytoestrogen genistein (abundant in soya, flour and some vegetables) has been identified as the major contributor for estrogenicity in a river water near a Japanese town where soya is a major food constituent (Kawanishi et al. 2004). Liu et al. (2010) also concluded that phytoestrogens may significantly contribute to adverse effects in organisms living downstream from WWTPs especially in countries with high consumption of phytoestrogen-rich plants (i.e. most Asian countries). Special attention should also be paid to the waters in vicinity of plant processing factories. Also, in the present study, two out of three tested effluents from WWTP serving to factories processing plants could be potentially estrogenic, and the risks associated with the phytoestrogens in these samples should be considered.

Of the 15 WWTPs for which no or limited data on collected waters or capacities were available (Table 1), six of the effluents contained detectable estrogenic activity and one sample was cytotoxic/antiestrogenic. One of the extracts contained the greatest concentration of EEQ observed in the present study, which was 17.9 ng/L, but the owner of this WWTP provided only the information on plant discharge capacity of 1,000 m^3/d, which is one of the smallest municipal WWTPs in the present study. This WWTP is situated near a town with about 7,000 citizens with light industry and agriculture including soya production and a brewery, which hypothetically could be sources of phytoestrogens that could contribute to estrogenicity. In the other five positive samples, concentrations ranged from 0.6 to 6.0 ng/L EEQ, a range that was not significantly different from estrogenicities detected in other groups of samples (Kruskal-Wallis, $P>0.05$).

Comparison of estrogenic activity with chemical analyses

Estrone, E2 and EE2, which are known to be the most potent estrogens in wastewater effluents (Gardner et al. 2012; Anderson et al. 2012), have been investigated but not detected at concentrations that were greater than the quantification limit (LOQ) of 10 ng/L in any of the samples (Loos et al. 2013). Future studies of WWTP effluents should therefore include further development of analytical methods for estrogens with detection limits in the sub nanogram per litre concentration range.

Some of the other chemicals detected have previously been reported to be estrogenic or antiestrogenic, but their actual concentrations were too low to induce observable effects in the in vitro assay. For example, effective estrogenic concentrations of triazines, hexazinon and diazinon are greater than miligrams per liter (Danzo 1997; Vonier et al. 1996), but the greatest sum of the detected concentrations of all measured triazines and triazols (atrazine, atrazine-desethyl, simazine, terbutylazine, terbutylazine-desethyl, propazine, hexazinon and diazinon) was 1.8 μg/L in the sample WWTP B12, which did not have estrogenic activity exceeding the LOD (Fig. 1).

Concentrations of target analytes in each sample that elicited estrogenic or antiestrogenic/cytotoxic effects in the bioassay were further searched for the presence of elevated (several times higher than median) concentrations of any detected chemical. A few samples contained elevated concentrations of, for example, perfluoroalkyl substances or the pharmaceutical fluconazole, but similar or even greater concentrations of these pollutants were always present also in extracts that did not elicit measurable responses in the in vitro assay. The only sample that was positive in the in vitro assay (strongly cytotoxic/antiestrogenic) and contained much greater concentrations of some selected chemicals than other samples was the industrial WWTP effluent coded WWTP E9. This sample contained high concentration of triclosan (more than 4 μg/L), and it was also the only sample where siloxanes were detected (Loos et al. 2012). This

WWTP is run by a company which uses textile dyes, and it is the only WWTP that processes effluents from the textile industry that was investigated in the present study (Table 1, WWTP E9). Triclosan is currently used as an antimicrobial agent in various household applications or cosmetics but also in functional clothing such as shoes and underwear. The maximum concentration observed was high compared to the other WWTP effluents reviewed in Dann and Hontela (2011), and its concentration might have been even greater because HDPE bottles used in the present study may affect sampling of this compound (Loos et al. 2013). Antiestrogenic or estrogenic effects of triclosan have been observed at concentrations of 20 to 100 μg/L, which are greater than those detected in this survey. However, triclosan has been shown to disrupt thyroid hormone homeostasis and possibly the reproductive axis of tadpoles (*Rana catesbeiana*) at concentrations greater than those detected in the present study (e.g. 0.15 μg/L), and the detected concentration might also be toxic to algae (Dann and Hontela 2011; Brausch and Rand 2011). Much less information is available on the toxicity of large production volume chemicals, such as siloxanes, polymeric ingredients in the synthesis of silicone products (Warner et al. 2010). The main concerns are the possibility of their accumulation in arctic organisms and their toxicity via inhalation (Warner et al. 2010; Siddiqui et al. 2007), but recent investigations suggested rather minor risk under current emission levels (Redman et al. 2012).

We also investigated possible relationships (nonparametric Spearman correlation) between the results of the in vitro assay and total concentrations of all analysed contaminants as well as with the levels of various analysed chemical classes (concentrations of pharmaceuticals, personal care products, veterinary drugs, perfluoroalkyl substances, organophosphate ester flame retardants, pesticides and their metabolites, benzotriazoles, polycyclic musk fragrances, X-ray contrast agents, gadolinium compounds, and siloxanes). None of the sums of concentrations of pollutants from each of the investigated groups was correlated with concentrations of EEQ. Some of the correlations among the chemical classes were significant (Supplementary Table SI 2). The greatest value of the correlation coefficient was found between the sums of concentrations of pharmaceuticals and sweeteners (R=0.56, Supplementary Table SI 2).

A weak correlation between concentrations of EEQ and concentrations of analytes is consistent with the fact that most investigated WWTP effluents were municipal, in which steroid hormones are most likely responsible for the estrogenicity. While most of their residues were less than the LOQ of 10 ng/L, estrogenicity was detected by the in vitro assay, demonstrating the need of complementing the chemical analyses with bioanalytical approaches. Many other studies showed the advantage of using different in vitro bioassays as monitoring tools especially in (but not limited to) wastewaters (e.g. Smital et al. 2013; Vasquez and Fatta-Kassinos 2013). The current efforts aim to utilise these bioassays within routine monitoring programmes, to harmonise and standardise the protocols and to set up proper effect-based trigger values (Escher et al. 2014; Leusch et al. 2010). Recently, bioassay-based target values for estrogenicity of municipal wastewaters were suggested by the authors of the present study (Jarosova et al. 2014; see also the next chapter). The target values for estrogenic, androgenic and other endocrine-disruptive potentials in drinking waters assessed by various bioassays were also suggested (Brand et al. 2013).

Environmental risks and specific sensitivities to E1, E3 and EE2 relative to E2

Concentrations of EEQs observed in this study (0.53–17.9 ng/L) are comparable or even greater than the lowest observable effective concentration of the most potent estrogens expected to occur in WWTP effluents. For example, complete inhibition of reproduction by Chinese rare minnows (*Gobiocypris rarus*) was caused by 0.2 ng/L of EE2 (Zha et al. 2008). Therefore, detected EEQ concentrations might be of toxicological concern even though some dilution of the effluents by recipients is considered. Unfortunately, information on the proportion of sewage effluent in the recipient river was not available for WWTPs in this study; thus, the only estimation of environmental risks could be done considering the undiluted effluents.

In other studies, estrogen-related adverse effects on aquatic organisms were observed at different concentrations of EEQ determined by use of various in vitro assays. For example, Vethaak et al. (2005) found elevated concentrations of the yolk phospholipoprotein vitellogenin in blood plasma of male bream (*Abramis brama*) in a river with 0.17 ng/L EEQ as quantified by use of the in vitro ER-CALUX assay. No in vivo response was observed in fish exposed to WWTP effluents containing 7 ng/L EEQ as quantified by use of the yeast estrogen screen, YES assay (Huggett et al. 2003). However, the same study (Huggett et al. 2003) showed elevated concentrations of vitellogenin in the blood plasma of male fish exposed to effluent from different WWTPs containing similar EEQ concentrations (around 7 ng/L EEQ) as measured by YES. These inconsistencies might be due either to different sensitivities among assays or to different compositions of specific mixtures and the fact that in vitro and in vivo sensitivities to individual compounds can be significantly different (Jarosova et al. 2014; Environment Agency 2004). Another reason might be interactions among molecules within the mixtures (Leusch et al. 2005). Nevertheless, the usefulness of in vitro assays for evaluating estrogenic activity in different types of waters has been recognised (Leusch et al. 2010; Murk et al. 2002), and bioassays are now being harmonised and standardised as a prospective tiered monitoring tool.

Although the concentration of EEQ that is of toxicological concern based on in vitro assays has not yet been determined (Leusch et al. 2010), some suggestions for municipal wastewaters have been developed recently (Jarosova et al. 2014). By combining data from the literature on the occurrence and bioassay-specific in vitro potencies of the most potent estrogens found in municipal WWTPs (i.e. E1, E2, E3 and EE2) and taking into account predicted no-effect concentrations (PNECs) for these compounds derived from fish studies (Caldwell et al. 2012; Supplementary Table SI 1), we have recently derived concentrations of EEQ less than which none of the PNECs of any of the major steroids would be exceeded (Jarosova et al. 2014). When estrogenicity of certain samples exceeds suggested PNEC for EEQ based on a specific in vitro bioassay, potential in vivo risk cannot be excluded. For the MVLN assay used in the present study, the derived estrogenic limits were 0.3 ng/L EEQ for longer-term exposures and 1.4 ng/L EEQ for shorter-term exposures (Jarosova et al. 2014). The longer-term limits were derived from PNECs of lifetime and multigeneration studies and therefore were meant to be generally used. In contrast, the shorter-term limits are relevant for events lasting only several days like sewage overflows. All samples in which estrogenicity was detected in this study (n=27) exceeded the longer-term limit, and nine of them exceeded also the shorter-term limit. This indicates that estrogens in the 'positively estrogenic samples' can cause risks to aquatic organisms unless the dilution of recipient is higher than factor of 2–60. For example, the longer-term limit in a recipient of effluent containing 17.9 ng/L EEQ would be met only if the contribution of the effluent was less than 2 % of total water volume in the recipient. For recipient of effluent with averaged estrogenicity (0.9 ng/L EEQ), the longer-term limit would be met in causes when the effluent accounts for less than about 30 % of water mass. Thus, according to the results of the bioassay, a considerable number of European WWTP effluents might pose risks to aquatic organisms living in their receiving waters.

Conclusions

This study of estrogenic potential in European WWTPs effluents clearly demonstrated how bioanalytical / bioassay tools complement the knowledge gained by traditional analytical techniques. Routine analyses of steroid estrogens were not sensitive enough to capture these compounds occurring in low concentrations, whereas bioassays revealed the overall estrogenic potential of the same samples. Furthermore, the bioanalytical results confirmed the hypothesis that a considerable number of wastewater effluents across Europe are estrogenic, and detected estrogenicity levels might be of serious toxicological concern. The study shows the importance of the effect-based monitoring approaches, which provide complementary information on potential toxicological and ecotoxicological risks of chemical mixtures.

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