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Review

What level of estrogenic activity determined by *in vitro* assays in municipal waste waters can be considered as safe?

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ABSTRACT

In vitro assays are broadly used tools to evaluate the estrogenic activity in Waste Water Treatment Plant (WWTP) effluents and their receiving rivers. Since potencies of individual estrogens to induce *in vitro* and *in vivo* responses can differ it is not possible to directly evaluate risks based on *in vitro* measures of estrogenic activity. Estrone, 17beta-estradiol, 17alfa-ethinylestradiol and to some extent, estriol have been shown to be responsible for the majority of *in vitro* estrogenic activity of municipal WWTP effluents. Therefore, in the present study safe concentrations of Estrogenic Equivalents (EEQs-SSE) in municipal WWTP effluents were derived based on simplified assumption that the steroid estrogens are responsible for all estrogenicity determined with particular *in vitro* assays. EEQs-SSEs were derived using the bioassay and testing protocol-specific *in vitro* potencies of steroid estrogens, *in vivo* predicted no effect concentration (PNECs) of these compounds, and their relative contributions to the overall estrogenicity detected in municipal WWTP effluents. EEQs-SSEs for 15 individual bioassays varied from 0.1 to 0.4 ng EEQ/L. The EEQs-SSEs are supposed to be increased by use of location-specific dilution factors of WWTP effluents entering receiving rivers. They are applicable to municipal wastewater and rivers close to their discharges, but not to industrial waste waters.

Contents

Abbreviations: cEEQ, calculated E2-Equivalents; E1, Estrone; E2, 17β-estradiol; E3, Estriol; EE2, 17α-ethinylestradiol; EEF, Estrogenic Equivalency Factor; EEQ, 17β-estradiol equivalent; EEQ-SSE, concentration of EEQ which is safe regarding major Steroid Estrogens; Ei, E1, E2, E3 or EE2; EQS, Environmental Quality Standard; ER, Estrogenic Receptor; NP, Nonylphenol; OP, Octylphenol; *P*, Percentage of total cEEQ; PNEC, Predicted No Effect Concentration; TIE, Toxicity Identification and Evaluation; VTG, Vitellogenin; WWTP, Waste Water Treatment Plant; YES, Yeast Estrogenicity Screening Assay.

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1. Introduction

Municipal waste waters are one of the main sources of estrogenic compounds in aquatic environments (*e.g.* Bolong et al., 2009). Feminization of fish downstream of Waste Water Treatment Plants (WWTPs) discharges has been observed worldwide (Sumpter and Johnson, 2008). Some estrogenic chemicals, particularly steroid estrogens, are known to cause disruption of the endocrine system of fishes and abnormalities of the reproductive tract (*e.g.* Bolong et al., 2009; Petrovic et al., 2004) in ng/L concentrations, which commonly occur in aquatic environment worldwide.

Several approaches exist to monitor the presence of estrogenic compounds in surface waters. Traditional assessment of water contamination has been based on identifying and quantifying individual chemicals, but this approach has some limitations. It is expensive because it requires sophisticated equipment and highly trained personnel (Caldwell et al., 2012). Furthermore, the individual constituents of complex mixtures occurring in the environment might not be known or there might not be methods or standards for them. In addition, the methods might not be sufficiently sensitive to measure the individual constituents or there might be matrix interferences affecting the quantification (Caldwell et al., 2012; Korner et al., 2000). Finally, chemical analyses of selected individual micropollutants cannot always identify total estrogenic potential present in environmental samples because some antagonistic or synergistic interactions can occur (Leusch et al., 2005). Therefore, biological monitoring approaches are needed. *In situ* and *in vivo* bioassays are the most relevant tools for the detection of adverse effects but they are also expensive and time and animals consuming.

In vitro bioassays can serve as a rapid, sensitive and relatively inexpensive integrative screening method to estimate total estrogenic activity of all compounds in the mixtures that act through the same mode of action (Hilscherova et al., 2000). The most frequently used *in vitro* assays for detection of estrogenicity are transactivation assays (Kinnberg, 2003) which evaluate the ability of samples/chemicals to stimulate estrogen receptor and upregulate subsequent expression of a reporter gene (hereinafter *in vitro* estrogenicity assays). Moreover, *in vitro* estrogenicity assays are currently being considered to be used in tiered monitoring of environmental waters (Leusch et al., 2010). Several studies comparing estrogenic activity detected in environmental samples by different *in vitro* assays have been conducted showing that the assays are useful for environmental monitoring (Leusch et al., 2010; Murk et al., 2002). However, the *in vitro* potency of individual estrogens can be significantly different from their *in vivo* potencies (Environmental Agency, 2004). This was demonstrated *e.g.* in a study by Wehmas et al. (2011) who observed *in vivo* responses in male fathead minnows (*Pimephales promelas*) such as elevated levels of hepatic vitellogenin (VTG) and estrogen receptor α subunit transcripts after exposure to WWTP effluent containing 1–2 ng/L EEQ determined by T47D-KBluc assay. In contrast, isolated E2 induced *in vivo* responses at much greater concentrations (10–100 ng/L) (Wehmas et al., 2011). Therefore more work is needed to better understand what can be learned from the results of these *in vitro* assays towards *in vivo* situation; and to identify trigger levels of estrogenic activity which would allow prioritization of samples for further investigation (Leusch et al., 2010).

Concentrations greater than 1 ng/L EEQ from *in vitro* assays are often considered to be associated with adverse effects on individuals *in vivo*. This could be based on observation that the standard reference compound E2 causes adverse *in vivo* effects at concentrations greater than 1 ng/L (Environmental Agency, 2004). However, such direct comparison is not relevant because other compounds also contribute to estrogenicity detected by *in vitro* assays. For example, in a study by Vethaak et al. (2005) elevated levels of VTG in male bream (*Abramis brama*) were found in a river with EEQ levels as low as 0.17 ng/L determined by *in vitro* ER-CALUX assay. Another reason why 1 ng/L EEQ might be considered is that UK Environmental Agency (2004) derived 1 ng/L E2 equivalent as a predicted-no-effect concentration (PNEC) for instrumental analyses of total steroid oestrogens. However, this instrumental PNEC accounted for concentrations of individual steroids and their *in vivo* potencies which are, as the authors of the derivation clearly stated, significantly different from their *in vitro* potencies (Environmental Agency, 2004). Therefore this PNEC of total steroid oestrogens should not be misinterpreted as a safe concentration for *in vitro* bioassays.

The goal of this paper was the derivation of safe concentrations of total EEQ measured by *in vitro* bioassays in municipal effluents that are expected to cause no adverse effects. The main purpose of their derivation was to improve the interpretation of *in vitro* results towards *in vivo* situation. The safe EEQ concentrations were derived by: i) comparing estrogenic potencies of major known estrogens among different *in vitro* assays; ii) considering *in vivo* potencies of major steroid estrogens; and iii) taking into account relative contributions of steroid estrogens to the overall *in vitro* estrogenic activities detected in municipal WWTP effluents. The applicability of derived safe EEQ concentrations is discussed in detail.

2. Methods

2.1. Selection of the most relevant compounds responsible for estrogenic activity in municipal waste waters

A variety of diverse chemicals present in the environment have been shown to interfere with regulation of endogenous estrogens. Despite their relatively great concentrations in the environment, their potency is mostly too small to significantly contribute to observed overall estrogenic activity in complex samples (Sumpter and Johnson, 2008). There is a strong evidence from both *in vivo* and *in vitro* studies that both endogenous and synthetic steroid estrogens, including estrone (E1), 17β-estradiol (E2), 17α-ethinyl estradiol (EE2), and for most *in vitro* assays also estriol (E3) are usually responsible for most of the estrogenic activity in municipal waste waters and their receiving waters (*e.g.* Aerni et al., 2004; Korner et al., 2001). The first researchers who described these compounds as the causative estrogens were Desborow et al. (1998) in UK WWTP effluents. They used a Toxicity Identification and Evaluation (TIE) approach combining fractionation procedures with biological screening to separate the active extract until a sample is clean enough for efficient chemical analyses. Purdom et al. (1994) and Routledge et al. (1998) demonstrated that concentrations of steroid estrogens present in the effluents (ng/L range) could cause the effects (such as elevated levels of plasma VTG) observed in wild fish living downstream of some WWTPs. Other studies (reviewed in Caldwell et al., 2012; Environmental Agency, 2004) demonstrated that environmentally relevant concentrations of steroid estrogens can cause effects like impaired reproduction, disrupted gonadal development or altered development of sexual characteristics. Another piece of evidence that human-excreted chemicals are most probably responsible for feminization of fish is that there was no correlation between feminization of fish and amounts of industrial waste waters in UK Rivers (Jobling et al., 2006). On the other hand, the same study demonstrated clear links between the degree of endocrine disruption in wild fish and the proportion of sewage effluent in the river, and showed that predicted exposures to steroid estrogens in UK rivers correlated well with widespread sexual disruption in wild fish populations (Jobling et al., 2006).

A similar situation was observed also in other countries. For example, Snyder et al. (2001) concluded by the use of a TIE approach that E2 and EE2 were the dominant estrogens (contributed 88–99% to the total EEQ) in water samples from 3 municipal WWTPs in Michigan and Nevada, USA. Also in vicinity of Paris, France and Tamagawa River in Tokyo, Japan, steroid estrogens were identified to cause most observed estrogenicity in WWTPs effluents and their receiving waters (Cargouet et al., 2004; Nakada et al., 2004). A bioassay-directed

fractionation method was also developed and applied on male fish bile, since estrogens are mainly excreted *via* bile into the intestine in fish (Houtman et al., 2004). The natural hormones E2, E1, and E3 accounted for the majority of estrogenic activity in male bream bile at all 3 tested locations in the Netherlands (Houtman et al., 2004).

Other studies which have focused on identifying and quantifying causative estrogens in municipal WWTP effluents used comparison of chemical analyses of known estrogenic compounds with *in vitro* assessment of estrogenicity. Concentrations of detected compounds were multiplied by their relative potencies compared to E2 (derived using the *in vitro* assay); and summed using concentration additivity. The calculated E2-equivalents (cEEQ) were compared to the overall estrogenic activity determined for the whole sample extract by the *in vitro* assay (EEQ). Authors of these studies mostly concluded that steroid estrogens contributed more than 90% of the measured estrogenic activity (*e.g.* Aerni et al., 2004; Korner et al., 2001; Rutishauser et al., 2004). However, at some locations concentrations of cEEQ were significantly different from the EEQs determined by the bioassays (*e.g.* Aerni et al., 2004; Thorpe et al., 2006; Vermeirssen et al., 2005). Authors of these studies often stated that it was not clear whether the difference was caused by the combination of uncertainties in the accuracy of analytical and bio-analytical methods or by unknown estrogenic compounds or their interactions (Aerni et al., 2004; Thorpe et al., 2006; Vermeirssen et al., 2005). To address the methodological uncertainties, Avbersek et al. (2011) developed a protocol for determining steroid estrogens in environmental samples which unified the sample preparation for chemical and biological analyses. The authors obtained strong correlations ($r^2 > 0.92$) between calculated concentrations of cEEQ based on steroid estrogens and EEQ measured *in vitro* for both spiked and environmental waste water samples. However, until now their approach had not been applied to a sufficient number of waste waters to make a general conclusion.

Beside steroid estrogens, alkylphenols particularly 4-tertiary isomers of nonylphenol (NP) and to lesser extend also octylphenol (OP) have been reported to be responsible for adverse effects on fish at several hot spots associated with certain industries (Sole et al., 2000; Sumpter and Johnson, 2008). In these rivers, concentrations of NP exceeded 100 μg/L whereas their common environmental concentrations occur in the low μg/L units or less (Johnson and Jurgens, 2003; Sole et al., 2000). NP and OP are transformation products of two of the most important alkylphenol polyethoxylates which have been economically important as nonionic surfactants for decades and used in a variety of industrial and household applications and therefore are ubiquitous (Johnson et al., 2005). Despite their ubiquity, their contributions to *in vitro* estrogenicity in rivers and municipal WWTPs effluents, contrary to WWTP effluents from textile industries, is usually small and corresponds with their small *in vitro* potencies in nearly all *in vitro* assays (Table 1). In the European Union (EU), in contrast to the USA, use of nonylphenol ethoxylates as surfactants has been restricted (Directive 2003/53/EC) and consequently, their concentrations in the environment and relative contributions to estrogenicity have been decreasing in the EU in recent years. Correspondingly, the reduction of adverse estrogenic effects to fish as a result of decrease in the concentration of alkylphenol polyethoxylates and NP has been described *e.g.* in Aire river, England (Sheahan et al., 2002). In the EU, NP and OP are considered priority pollutants and their concentrations in surface waters should be reduced to less than the Environmental Quality Standards (EQSs) which are 0.3 μg NP/L and 0.1 μg OP/L as annual averages of all detected concentrations (Directive 2008/105/EC). In a recent British study of more than 160 WWTP effluents, the median concentration of NP was 0.22 μg/L, while the median concentration in streams of the USA was reported to be 0.8 μg NP/L (Gardner et al., 2012; Kolpin et al., 2002). Although the median concentration of NP reported for the study of streams in the USA was influenced by a greater focus on more polluted locations (Kolpin et al., 2002), these results indicate that different legislative regulation could result in different environmental concentrations of estrogens in various countries.

In a few studies, natural estrogenic compounds, such as phytoestrogens, have been reported to contribute significant proportions of estrogenicity in municipal WWTP effluents or their receiving waters (Liu et al., 2010). In one river in Japan, genistein was identified as the compound responsible for most of the estrogenic activity (Kawanishi et al., 2004). Genistein is one of the most abundant phytoestrogens present in soya, flour and many vegetables and it was also identified in substantial concentrations (around 10 μg/L) in treated effluents from wood pulp mills (Kiparissis et al., 2001). Some other flavonoids have been identified in WWTP effluents or rivers but their concentrations and/or estrogenic potencies were much lower (Kawanishi et al., 2004; Lagana et al., 2004; Pawlowski et al., 2003). Compounds with relatively high estrogenic potency are also mycoestrogens, such as zearalenol, although few studies (Lagana et al., 2004; Pawlowski et al., 2003) document their occurrence. A few other studies have investigated estrogenicity in surface water at localities with minimal sources from human activities and detected some estrogenic activity which might have been caused by phytoestrogens (Jarosova et al., 2012; Nadzialek et al., 2010) but these studies were not designed to identify the responsible compounds. Overall, it seems that the wide variety of phytoestrogens present in WWTP effluents and/or in surface waters could contribute to measured estrogenic activity, even though the examples of their identification are rare. Phytoestrogens should be considered as possible significant contributors to estrogenicity detected in samples from places in the vicinity of plant–product manufactures or places with greater consumption of soya (Liu et al., 2010).

Although there is always the possibility that some unexpected compounds could contribute to estrogenicity of municipal WWTP effluents at specific places, the information in literature document that steroid estrogens, particularly E1, E2, EE2 and occasionally also E3 (when *in vitro* assays responsive to E3 are used) are usually responsible for majority of estrogenic activity of municipal WWTP effluents entering rivers (Sumpter and Johnson, 2008). Therefore, the present study further focused in detail on these compounds.

2.2. In vitro potency of model estrogens

Estrogenic potencies of various compounds relative to that of E2 in different *in vitro* assays, expressed as Estrogenic Equivalency Factors (EEF), have been reviewed and the results are summarized in Table 1. The EEFs were obtained by dividing EC50 of E2 as a reference by the EC50 of corresponding compound. According to the reviewed data, EEFs of estrogens can differ by orders of magnitude, not only among different *in vitro* models but also for the same model among laboratories using different testing protocols. For example, Gutendorf and Westendorf (2001) used 48 h exposure in the MVLN assay and reported EEF of E1 to be 0.01 whereas Van den Belt et al. (2004) used 20 h exposure in the same assay and reported the EEF of E1 to be 0.2. The largest differences in EEFs of steroid estrogens among different assays can be seen for E3 (Table 1). In the YES assay, the EEF of E3 was lower by a factor of 15–416 compared to other assays. Since there can be relatively large differences in EEFs even for the same models depending on the testing procedure, the most accurate determination of the safe EEQs would be with the EEFs for the major estrogens derived in the same *in vitro* model with the same testing protocol as used for the assessment of samples. In our approach, specific sets of EEFs reported for each model and testing approach in literature and also the set determined in the model used in our laboratory (MVLN) were used to derive the safe EEQs concentrations to see potential differences among assays with various potencies of the standard estrogens. Thus, further in the text when we write about bioassays it refers not only to the used model but also to the specific testing protocol used in each laboratory that derived the EEFs, which is described in detail in the references listed in Tables 1 and SD1–SD7.

2.3. Predicted-no-effect concentrations of steroid estrogens

Steroid estrogens are known to be the most potent estrogens in *in vivo* assays, all having potencies more than a thousand-fold greater in the most sensitive organism (fish) than other estrogenic xenobiotics (Caldwell et al., 2012; Environmental Agency, 2004). Data from studies of effects on reproduction of fishes were used to develop a species sensitivity distribution and PNECs of 0.1 and 2 ng/L for EE2 and E2, respectively, were derived (Caldwell et al., 2012). These PNECs were derived from long-term studies of reproduction used as the most sensitive endpoint in fishes, and should be sufficient for protection of reproductive health in fish exposed continuously for several life stages or multiple generations. PNECs for shorter-term exposure of less than 60 d, were also derived at 0.5 and 5 ng/L for EE2 and E2, respectively (Caldwell et al., 2012). Insufficient data were available to use the same methods to derive PNECs for E1 and E3, and therefore, PNECs were based on *in vivo* VTG induction studies and *in vitro* estrogenicity study accompanied with application of safety factors and the assumption that the relative ability to induce VTG by each of the steroid estrogens corresponds with the relative effects on reproductive endpoints (Caldwell et al., 2012). Resulting PNECs were 6 ng/L for E1 and 60 ng/L for E3 during longer-term exposures, and 20 and 200 ng/L for E1 and E3 in shorter-term exposures, respectively (Caldwell et al., 2012).

2.4. Derivation of safe concentrations of EEQ

Considering that E1, E2, E3 and EE2 are usually responsible for more than 90% of *in vitro* estrogenicity of treated municipal waste waters and that these compounds are highly potent *in vivo* (especially EE2), we derived safe concentrations of EEQ for municipal WWTP effluents based on the simplified assumption that steroid estrogens are responsible for all estrogenicity determined with the *in vitro* assays. The safe concentration of EEQ is hereinafter called EEQ Safe regarding Steroid Estrogens (EEQ-SSE) to reflect how they were derived. To determine EEQ-SSE knowledge of maximal contributions of the individual steroids to total estrogenic activity of municipal WWTP effluents was needed. Therefore the literature on occurrence of E1, E2, E3 and EE2 in municipal wastewaters was reviewed. Consequently, the maximal contributions of the individual steroids to total estrogenic activity were calculated.

2.4.1. Occurrence of steroid estrogens in municipal WWTP effluents

Concentrations of all four major estrogens were analyzed in 112 samples from 51 WWTP effluents (Table 2). In total about 150 papers investigating concentrations of estrogens in WWTP effluents were reviewed but most studies either reported only summarized results or did not investigate the presence of E3 because of its relatively small potencies to cause endocrine disruption compared to E1, E2 and EE2 (Caldwell et al., 2012). However, E3 can occur in significant amounts in WWTP effluents (Table 2) and it is quite potent estrogen in some *in vitro* systems (Table 1) and therefore it might be important for interpretation of the overall results. Forty seven out of the 51 WWTP listed in Table 2 included activated sludge treatment, which is the most common technology in municipal WWTPs. Most WWTPs also employed a nitrification step, which is known to enhance degradation of steroid estrogens (*e.g.* Khanal et al., 2006). Three WWTPs utilized nitrifying and denitrifying bacteria supported by solid filters and one WWTP was a system of lagoons without any artificial biological or chemical treatment. Authors of some studies reported concentrations of steroid estrogens as means of multiple samples collected at particular WWTP. Results of these studies were also included in the dataset (Table 2, samples with $N > 1$).

Estrone was the most frequently detected steroid estrogen with the greatest concentrations in most WWTP effluents (Table 2). There are two main reasons for this. First, E1 was the second most abundant steroid estrogen in WWTP influents (*e.g.* Anderson et al., 2012; Liu et al., 2009), but the most abundant one—E3 is known to be quickly degraded in the treatment processes (Anderson et al., 2012; Jin et al., 2008). Second, besides degradation of E1 during treatment, E1 can also be newly formed as a degradation product of E2 (Johnson and Sumpter, 2001). Based on the published reviewed studies (supplementary materials in Anderson et al., 2012), it can be generally concluded that conventional WWTPs, utilizing activated sludge systems without de/nitrification steps, are efficient at removal of E2 (median removal 85%) and E3 (median 97%), but removal of E1 is lower with median of 67%. Some studies found E2 to occur at the greatest concentrations in WWTP effluents (Table 2), which indicates the importance of operational conditions and technology of the specific WWTPs. Comparable or greater concentrations of E2 than E1 are typically detected at municipal WWTPs with solid supported bacteria or at conventional WWTPs with shorter retention time of solids, which does not support development of diverse microbial community, particularly nitrifiers (Kirk et al., 2002; Svenson et al., 2003). Due to its relatively lower potency, E3 is rarely investigated compared to E1, E2, and EE2. E3 has been reported to be rather rapidly degraded in conventional WWTPs (Anderson et al., 2012). However, in effluents of some municipal WWTPs E3 was detected at concentrations that were greater than E1, E2 or EE2. E3 which has been reported to be the most polar estrogen, might be lost during some procedures in analytical laboratories especially cleanup of samples by use of silica (Aerni et al., 2004; Fernandez et al., 2007). The lowest concentrations and frequency of detection were reported for synthetic steroid EE2 (Table 2). Since the primary route of entry of EE2 into the aquatic environment is through excretion by women using contraceptives, the initial load of this chemical is lower than E1, E2 or E3 (Environmental Agency, 2004). EE2 is the least abundant steroid estrogen in effluents of municipal WWTPs (Table 2 and 3), but its potency to cause ED, especially in fish, is high. Moreover, its limits of detection are mostly greater than concentrations considered to be biologically potent (Table 2, Environmental Agency, 2004).

To confirm the representativeness of concentrations of steroid estrogens included in this study, their median and maximal concentrations were compared with previously reported comprehensive data sets on occurrence of estrogens in treated waste waters (Gardner et al., 2012; Miege et al., 2009). Miege et al. (2009) compiled data about concentrations of emerging pollutants including E1, E2, E3 and EE2 in WWTP influents and effluents but this compilation was not limited to the studies where all four compounds were analyzed simultaneously as in our study. Gardner et al. (2012) reported recent results of a British national study of more than 160 different municipal WWTP effluents. The medians of all three investigations are similar (Table 3). The maximal observed concentration of E1 was greater in present study compared to study by Miege at al. (2009). However, the 95%ile of concentration of E1 in this study was much lower compared to the maximal value and the 95%ile was also comparable to 95%ile reported by Gardner et al. (2012). Similarly, the maximal observed concentration of E2 was 158 ng/L in the present study and 30 ng/L in a previous study by Miege et al. (2009). However, this difference was caused by one outlier value detected in the sample from a Canadian lagoon system and the 95%ile concentration of E2 was similar to that reported by others (Table 3). The 95%ile of EE2 in the British study by Gardner et al. (2012) was lower than those reported in the present study or by Miege et al. (2009). The data in the study by Gardner et al. (2012) were more consistent with predictions by Hannah et al. (2009) who calculated concentrations of EE2 based on estimates of per capita use of EE2, water use of 200 L/capita/day, loss of EE2 *via* metabolism, and loss *via* removal in secondary treatment step in Europe and the USA to range from 0.4 to 1.2 ng/L. However, higher concentrations of EE2 reported in the present study as well as in the database presented by Miege et al. (2009) largely originate from the study of 4 WWTPs around Paris, France, where greater concentrations could be explained by greater consumption of EE2 compared to other cities (Cargouet et al., 2004).

2.4.2. Determination of percentage contribution of steroid estrogens to total cEEQ

Based on known concentrations of E1, E2, E3 and EE2 ([E1], [E2], [E3] and [EE2]) in municipal WWTP effluents and *in vitro* potencies of individual compounds relative to E2 (EEF); the cEEQ for each WWTP effluent and each bioassay were calculated (Eq. (1)).

$$cEEQ = ([E1] \times EEF_{E1}) + ([E2] \times EEF_{E2}) + ([E3] \times EEF_{E3}) + ([EE2] \times EEF_{EE2}) \quad (1)$$

As demonstrated above, the relative potencies of these four major estrogens can vary widely among different bioassays (Table 1) and this can affect the detection power of the specific assay for each estrogen. Thus, the percentage contribution of each of these four estrogens to total EEQ was derived specifically for each set of relative potencies, this means for every bioassay. Fifteen sets of EEFs for all four major steroid estrogens in estrogenicity bioassays were available in literature. MVLN assay, used at laboratory where the authors mainly work, was chosen as an example (Table 2). Calculated EEQ for the other 14 assays are available in supplementary data (Table SD 1–7).

Consequently, the percentage of total cEEQ for each steroid estrogen and each *in vitro* bioassay was determined (Eq. (2)).

$$P_{Ei} = ([Ei] \times EEF_{Ei}/cEEQ) \times 100\% \quad (2)$$

Where: P_{Ei} is percentage of total cEEQ for Ei, where Ei is E1, E2, E3 or EE2, [Ei] is concentration of Ei.

Within the extensive dataset (Table 2) we had to deal with the important issue if and how to take into consideration the concentrations bellow limits of detection (LOD) to make sure that it would not lead to underestimation or overestimation of the actual proportions of contribution of each estrogen to total EEQ. Thus, to obtain the most realistic proportions we have compared two different approaches of calculations regarding LOD to assess how much the values below LOD influence the maximal P_{Ei} values ($P_{Ei\text{-}max}$). The first approach included all samples where at least two steroid estrogens were detected (N = 78) and 1/2 of LOD was taken into account when some estrogen was not detected at concentrations greater than LOD. The second approach included only those samples in which concentrations of all 4 steroids were detected above LODs (N = 32), thus there was no influence of LOD at all. The summary of these two approaches are listed in the bottom lines of Table 2 and Tables SD 1–7 in Supplementary materials. $P_{Ei\text{-}max}$

Table 1
Estrogenic potencies of model compounds relative to 17β-estradiol (Estrogenic Equivalency Factors—EEFs) determined in different *in vitro* assays.

Chemical	YES	ER-CALUX	MELN	T47D-KBluc	E-screen	MVLN
Estrone	0.19 [a]	0.06 [b]	0.03 [c]	1.4 [d]	0.01 [c]	0.01 [e]
	0.40 [f]	0.02 [g]	0.25 [c]	0.02 [c]	0.01 [c]	0.19 [h]
	0.38 [i]	0.15 [j]			0.13 [k]	0.2 [f]
	0.10 [c]	0.4 [l]			0.10 [m]	0.13 [n]
	0.25 [c]	0.12 [o]			0.04 [c]	
	0.10 [c]				0.01 [c]	
	0.50 [p]					
	0.33 [q]					
	0.10 [q]					
	0.68 [r]					
Estriol	3.50E−03 [a]	1.00 [c]	0.18 [c]	0.23 [d]	0.07 [c]	0.083 [e]
	6.31E−03 [c]	0.04 [g]	0.08 [c]	0.05 [c]	0.30 [k]	0.11 [n]
	2.40E−03 [i]	0.14 [l]			0.25 [c]	
	3.00E−03 [q]	0.13 [o]			0.09 [c]	
	3.70E−03 [q]					
17α-ethinylestradiol	2.20 [a]	1.20 [b]	2.45 [c]	7.23 [d]	1.26 [c]	1.25 [e]
	0.89 [f]	1.86 [g]	1.15 [c]	0.35 [c]	1.07 [c]	1.6[f]
	1.19 [s]	1.2 [j]			0.17 [c]	0.10 [t]
	2.29 [c]	1.68 [l]			1.35 [k]	1.09 [n]
	0.95 [c]	1.12 [o]			1.91 [c]	
	0.71 [c]				0.91 [m]	
	0.89 [c]				1.12 [c]	
	1.23 [c]				0.68 [c]	
	1.20 [c]					
	1.14 [p]					
	1.00 [q]					
	0.50 [q]					
	1.8 [r]					
4-Nonylphenol	2.19E−05 [c]	2.29E−05 [b]	1.58E−06 [c]	3.72E−05 [c]	1.29E−05 [c]	1.3E−05 [e]
	5.75E−04 [c]	2.29E−05 [c]	9.55E−06 [c]		2.88E−05 [c]	2.8E−06 [h]
	1.00E−04 [f]	1.20E−04 [c]			7.76E−05 [c]	1.3E−05 [t]
	2.51E−05 [s]	2.30E−05 [j]			2.34E−07 [c]	3.30E−05 [f]
	7.24E−07 [c]	3.70E−05 [o]			5.75E−05 [k]	
	2.69E−04 [c]				7.59E−05 [m]	
	1.10E−03 [c]				3.89E−05 [c]	
	4.7E−04 [r]				6.92E−05 [c]	
4-tert-Octylphenol	4.79E−04 [c]	Cytotoxic [uc]	4.79E−06 [c]	1.91E−05 [c]	6.46E−05 [v]	8.3E−05 [e]
	3.63E−06 [c]	7.30E−05 [o]			9.77E−05 [k]	6.7E−06 [h]
	2.14E−03 [c]				7.59E−05 [m]	1.90E−05 [t]
	1.70E−03 [c]				6.03E−04 [c]	
	7.80E−06 [s]				4.17E−04 [c]	
Genistein	2.45E−04 [c]	6.03E−05 [c]	6.46E−04 [c]	3.02E−05 [w]	1.29E−05 [e]	1.32E−04 [e]
	4.90E−04 [c]				2.82E−04 [m]	
	4.50E−05 [x]				1.41E−04 [c]	
	3.00E−03 [yz]				8.91E−05 [c]	

values calculated by both approaches were in very good agreement for E2 and E3, and the values from more conservative second approach were used for these two compounds for further calculations. There were greater differences in case of E1 and EE2. E1 was quite often the dominant steroid detected in WWTPs effluents at high concentrations many fold greater than the LODs of other compounds (see Table 2), the determination of its $P_{E1\text{-}max}$ was not affected by LOD. Therefore $P_{E1\text{-}max}$ calculated from the measurements including LOD (91% in case of MVLN assay, see bottom of Table 2) is more realistic and relevant. On the other hand, different situation can be seen for EE2. $P_{EE2\text{-}max}$ could be more influenced by use of 1/2 of LOD, since it was much more often bellow limit of detection (more than 60% of samples) and the limits of detection varied greatly among studies (Table 2). Hence for this compound, the way of LOD calculation could have stronger effect and lead to overestimation of the actual proportions of EE2. Thus, in case of EE2 the maximal relative contributions derived from the samples where all 4 estrogens are detected is more realistic and precise. These values were also in very good agreement with 95%ile of $P_{EE2\text{-}max}$ determined by the approach including 1/2 of LOD across all assays. In summary, derivation of the most realistic EEQ-SSE_{Ei} was thus based on $P_{E2\text{-}max}$, $P_{E3\text{-}max}$ and $P_{EE2\text{-}max}$ from measurements with all values above LOD and on $P_{E1\text{-}max}$ derived from all measurements where least two steroid estrogens were detected. When less than two steroids were detected at concentrations greater than the LOD in some WWTP effluents, the percentage of total cEEQs was not determined for any steroid in this effluent, because the values would rather be indicative of the LOD than the actual contribution of cEEQ.

Percentages of contributions to total cEEQ which were derived by use of EEFs specific for the MVLN *in vitro* assay are presented in Table 2 as an example. Percentages of contributions to total cEEQ calculated for the other 14 bioassays are presented in Supplementary data (Table SD 1–7). In case of the MVLN *in vitro* assay, the ranges of percentages of total cEEQ for E1 and E2 among individual WWTPs of total cEEQ were very wide (from <10 to >90%, Table 2). The maximal percentages of total cEEQ for E3 and EE2 were 40 and 39%, respectively. Similar patterns were obtained when other *in vitro* assays were used. The maximal contribution of E1 to total cEEQ was 97% in case of YES assays and also ER-CALUX assays, 95% in case of MELN assays and 91% in case of E-screen assays (Supplementary materials—Table SD 1–7). Maximal percentage of contribution to cEEQ for E2 was more than 90% in all assays. E3 was responsible maximally for 4% of the cEEQ in the assessment on YES assays but the maximal contribution to total cEEQ by E3 was 69% when assessed by other bioassays. EE2 was usually responsible for 8–34% of total cEEQ (medians of percentage of cEEQ), but the maximal value from all of the assays was 77% (Table SD 1–7).

2.4.3. Derivation of EEQ-SSE for municipal waste waters

After determination of maximal percentage of total cEEQ contributed by each considered estrogen by use of each bioassay, EEQ Safe regarding each Steroid Estrogen (EEQ-SSE_{Ei}) was derived (Eq. (3)). It is defined as the concentration of EEQ in every bioassay below which PNECs of the steroids would not be exceeded.

$$\text{EEQ-SSE}_{Ei} = \text{EEF}_{Ei} \times \text{PNEC}_{Ei} / (P_{Ei\text{-}max}/100\%) \quad (3)$$

Where: Ei is E1, E2, E3 or EE2, EEF_{Ei} is estrogenic potency of a compound (Ei) relative to 17β-estradiol determined in specific *in vitro* assay, $PNEC_{Ei}$ is *in vivo* derived PNEC for individual Ei, and $P_{Ei\text{-}max}$ is maximal percentage of total cEEQ for each Ei determined for specific bioassay.

Here a final EEQ-SSE *i.e.* concentration of total measured EEQ in municipal effluents that is expected to cause no adverse effects is derived and represents *in vitro* EEQ at which none of the PNECs for individual estrogens, E1, E2, E3 or EE2 is exceeded. When EEQ-SSE_{Ei} were calculated for all four of these compounds, the lowest concentration was reported as the proposed EEQ-SSE.

As it was mentioned in Section 2.4.2 EEQ-SSEs were derived specifically for the 15 bioassays for which the data on EEFs of all 4 estrogens were available. For nine of the 15 included bioassays (Table 4) the lowest EEQ-SSE_{Ei} was found for EE2 (EEQ-SSE_{EE2}) despite the fact that EE2 occurred at the lowest concentrations of the investigated compounds (Table SD 8). The reason for this is the greater *in vivo* estrogenic potency

Notes to Table 1:
YES—yeast estrogenicity screening assay (Routledge and Sumpter, 1996).
ER-CALUX—Estrogen Receptor mediated Chemical Activated LUciferase gene eXpression assay (Van der Burg et al., 2010).
MELN—MCF-7 cells stably transfected with the estrogen responsive gene ERE-betaGlob-Luc-SVNeo (Balaguer et al., 2000).
T47D-KBluc—T47D human breast cancer cells stably transfected with a triplet estrogen-responsive elements–promoter–luciferase reporter gene construct (Wilson et al., 2004).
E-SCREEN—the MCF7 cell proliferation assay (Soto et al., 1998).
MVLN—MCF-7 cells stably transfected with luciferase gene under the control of estrogen receptor (Demirpence et al., 1993).
[a] Svenson et al. (2003).
[b] Murk et al. (2002).
[c] Leusch et al. (2010).
[d] Bermudez et al. (2012).
[e] Gutendorf and Westendorf (2001).
[f] Van den Belt et al. (2004).
[g] Sonneveld et al. (2006).
[h] Furuichi et al. (2004).
[i] Aerni et al. (2004).
[j] Legler et al. (2002).
[k] Drewes et al. (2005).
[l] Avbersek et al. (2011).
[m] Korner et al. (2001).
[n] Original unpublished data—*in vitro* potencies determined by the authors of the present study by comparing the EC_{50} values from dose–response curves of E_2 and other estrogens.
[o] Houtman et al. (2004).
[p] Pawlowski et al. (2004).
[q] Caldwell et al. (2012).
[r] Thorpe et al. (2006).
[s] Rutishauser et al. (2004).
[t] Snyder et al. (2001).
[u] 4-tert-Octylphenol was cytotoxic to the cells at concentrations lower than EC_{50}.
[v] Leusch et al. (2006).
[w] Wilson et al. (2004).
[x] Breinholt and Larsen (1998).
[y] Value based on EC_{10}, not EC_{50}.
[z] Nishihara et al. (2000).

Table 2
Concentrations of four main steroid estrogens (E1, E2, E3 and EE2) and their relative percentage contribution (*P*) to total calculated estrogenic equivalents (cEEQ) if assessed by MVLN assay in municipal WWTP effluents.

Country	WWTP name or code	Equiv. citizens (thousands)	N	Concentration (ng/L)				cEEQ MVLN[a]	*P*-Percentage of total cEEQ for MVLN assay[a]			
				E1	E2	E3	EE2	(ng/L)	E1	E2	E3	EE2
Austria (Clara et al., 2005)	WWTP 1	2 500	1	72	30.0	275	5.0	73.6	12	41	40	7
	WWTP 2	167	1	8.0	<5	17.0	3.0	8.6	12	29[b]	21	38
	WWTP 3	135	1	<1	<5	<1	<1	–	–	–	–	–
	WWTP 4	6	1	4.0	<5	<1	4.0	7.4	7	34[b]	1[b]	59
			1	<1	8.0	1.0	<1	8.7	1[b]	92	1	6[b]
			1	2.0	4.0	<1	<1	4.8	5	83	1[b]	11[b]
California (Drewes et al., 2005)	WWTP 1	>100	1	0.6	<1	<2	<0.7	–	–	–	–	–
	WWTP 2	>100	1	<1	<1	<1	<0.7	–	–	–	–	–
	WWTP 3	>100	1	17.7	4.4	4.0	4.1	11.5	19	38	4	39
	WWTP 4	>500	1	50.4	1.5	<4.7	<0.7	8.4	75	18	3[b]	5[b]
	WWTP 5	>100	1	11.1	6.0	4.9	<0.7	8.3	17	72	6	5
	WWTP 6	>100	1	27.5	<0.6	<3.3	<0.7	–	–	–	–	–
	WWTP 7	>500	1	16.4	1.8	<3.3	<0.7	4.4	47	41	3[b]	9[b]
Canada (Fernandez et al., 2007)	WWTP B[TF]	740	1	69.0	5.0	8.0	1.0	15.6	55	32	5	7
			1	147.0	2.0	<1.5	<7.1	24.3	76	8	0[b]	16[b]
			1	<7.6	10.0	<1.5	1.0	11.6	4[b]	86	1[b]	9
			1	<7.6	1.0	<1.5	<7.1	–	–	–	–	–
			1	<7.6	3.0	<1.5	1.0	4.6	10[b]	65	2[b]	23
			1	25.0	6.0	<1.5	<7.1	13.1	24	46	1[b]	30[b]
			1[c]	85.0	6.0	1.0	<7.1	20.6	52	29	1	19[b]
	WWTP C	195	1	10.0	<7.1	<1.5	<7.1	–	–	–	–	–
	WWTP D	720	1	18.0	<7.1	<1.5	<7.1	–	–	–	–	–
	WWTP E[W]	20	1	28.0	57.0	<1.5	<7.1	64.4	5	88	0[b]	6[b]
			1	39.0	72.0	4.0	<7.1	81.2	6	89	1	5[b]
			1	56.0	158	23.0	5.0	172.9	4	91	1	3
China, Chongqing (Ye et al., 2012)	WWTP A	117	1[d]	4.7	<1.5	<2.5	<2.5	–	–	–	–	–
	WWTP B	214	1[d]	30.4	1.9	<2.5	<2.5	7.2	53	26	2[b]	19[b]
	WWTP C	330	1[d]	4.9	<1.5	<2.5	<2.5	–	–	–	–	–
	WWTP D	59	1[d]	8.6	<1.5	8.4	<2.5	4.1	26	18[b]	22	33[b]
	WWTP E	144	1[d]	3.8	<1.5	7.7	<2.5	3.4	14	22[b]	24	40[b]
	WWTP F	150	1[d]	4.0	<1.5	<2.5	<2.5	–	–	–	–	–
	WWTP G	160	1[d]	10.6	<1.5	<2.5	<2.5	–	–	–	–	–
	WWTP H	88	1[d]	8.1	<1.5	11.0	<2.5	4.3	24	17[b]	27	32[b]
	WWTP I	n.a.	1[d]	8.4	<1.5	<2.5	<2.5	–	–	–	–	–
	WWTP J	170	1[d]	4.0	<1.5	<2.5	<2.5	–	–	–	–	–
Finland (Bjorkblom et al., 2008)	Turku	160	1[d]	65.5	0.7	<0.6	<0.2	9.0	91	8	0[b]	1[b]
France, Boredeaux (Labadie and Budzinski, 2005a)	Eysines	50	1	71.4	<2	<1	<4	–	–	–	–	–
			1[d]	57.8	4.4	2.9	<2	13.0	56	34	2	8[b]
			1[d]	17.2	<1.0	<1.0	<1.0	–	–	–	–	–
France, Saine (Labadie and Budzinski, 2005b)	Elbeuf	110	1	<2.0	<1.9	<4.5	<3.0	–	–	–	–	–
			1	4.3	<3.8	<8.0	<5.3	–	–	–	–	–
			1	<3.5	<0.6	<4.9	<0.8	–	–	–	–	–
			1	<0.5	<0.4	<0.8	<0.8	–	–	–	–	–
			1	<4.3	<2.4	<5.6	<1.1	–	–	–	–	–
	Rouen	450	1	<1.8	<1.9	<4.0	<2.9	–	–	–	–	–
			1	<3.0	<3.8	<8.0	<5.3	–	–	–	–	–
			1[d]	<3.3	<0.5	3.5	<1.1	–	–	–	–	–
			1	<0.5	<0.4	<2.1	<1.0	–	–	–	–	–
			1	<3.4	<2.5	<7.3	<1.2	–	–	–	–	–
	Tancarville	n.a.	1	<2.8	<2.5	<3.0	<2.5	–	–	–	–	–
			1[d]	4.2	<0.8	<1.8	<0.7	–	–	–	–	–
			1[d]	1.8	<0.3	<3.6	<1.0	–	–	–	–	–
			1[d]	8.3	<0.3	<1.9	<0.7	–	–	–	–	–
			1[d]	4.9	<1.4	<5.0	<1.0	–	–	–	–	–
Italy, Roma (Baronti et al., 2000), (Johnson et al., 2000)	Cobis	40	1	<0.5	<0.5	0.7	<0.5	–	–	–	–	–
			1	13.0	2.9	3.3	1.0	6.0	27	49	6	18
			1	17.0	2.2	7.3	<0.3	5.3	40	42	15	3[b]
			1	6.9	0.7	5.7	0.5	2.7	32	27	22	19
			1	5.8	0.6	1.3	<0.3	1.6	46	35	9	10[b]
			1	5.4	1.0	1.1	0.4	2.3	30	44	5	21
	Fregene	120	1	2.0	4.0	4.0	<0.5	4.9	5	81	9	5[b]
			1	3.0	7.0	5.0	2.2	10.3	4	68	5	23
			1	6.5	2.1	1.6	1.7	4.9	17	43	3	37
			1	2.5	0.6	2.2	<0.3	1.3	25	44	18	13[b]
			1	3.7	0.4	0.6	0.3	1.2	39	29	5	27
			1	4.3	0.4	0.4	0.3	1.3	40	31	3	25
			1	3.3	1.2	0.9	0.4	2.2	19	55	5	21
	Ostia	350	1	31.0	3.0	<0.5	0.6	7.6	51	40	0[b]	9
			1	54.0	6.0	18.0	<0.5	14.9	45	40	13	2[b]
			1	82.1	3.3	1.4	1.1	14.9	69	22	1	8
			1	13.0	0.7	0.6	<0.3	2.6	63	28	3	6[b]
			1	46.0	3.0	1.5	0.5	9.4	61	32	2	5

Table 2 (*continued*)

Country	WWTP name or code	Equiv. citizens (thousands)	N	Concentration (ng/L)				cEEQ MVLN[a]	*P*-Percentage of total cEEQ for MVLN assay[a]			
				E1	E2	E3	EE2	(ng/L)	E1	E2	E3	EE2
			1	35.0	1.7	0.7	0.8	7.0	62	24	1	12
Italy, Roma (Baronti et al., 2000),			1	47.0	3.5	1.1	<0.3	9.7	61	36	1	2[b]
(Johnson et al., 2000)	Roma Sud	1200	1	20.0	3.0	7.0	<0.5	6.5	38	46	11	4[b]
			1	52.0	4.0	20.0	<0.5	12.9	50	31	16	2[b]
			1	51.0	3.1	11.0	1.2	12.0	53	26	10	11
			1	30.0	1.9	6.7	<0.3	6.5	58	29	11	2[b]
			1	22.0	1.6	5.8	0.5	5.5	50	29	11	10
			1	8.7	0.5	1.8	0.5	2.4	46	22	8	24
			1	4.0	2.3	18.0	0.4	5.1	10	45	37	8
	Roma Est	800	1	9.7	0.8	0.6	0.4	2.5	49	33	3	16
			1	8.0	0.7	0.4	<0.3	1.9	52	37	2	8[b]
			1	3.7	0.6	0.8	0.4	1.6	30	40	6	25
			1	6.9	0.8	0.8	0.7	2.6	34	32	3	31
			1	10.0	0.8	1.4	0.3	2.5	49	32	6	13
	Roma Nord	800	1	11.0	3.0	11.0	<0.5	5.8	24	52	20	5[b]
			1	19.0	2.0	28.0	<0.5	7.6	31	26	39	4[b]
			1	10.0	0.9	1.1	0.3	2.7	47	35	4	14
			1	6.4	0.4	0.7	<0.3	1.5	54	30	5	11[b]
			1	6.4	0.9	1.7	0.6	2.5	32	36	7	24
			1	6.6	0.7	1.0	0.5	2.2	37	33	5	26
			1	40.0	1.9	8.4	0.5	8.3	60	23	11	7
Slovenia (Avbersek et al., 2011)	WWTP 1	50	1	4.0	1.5	12.5	<2.0	4.4	11	34	30	25[b]
			1	1.7	2.9	18.4	<2.0	6.1	3	47	32	18[b]
	WWTP 2	360	1	16.5	2.1	<1.4	<2.0	5.3	39	39	1[b]	20[b]
	WWTP 3	100	1	61.8	8.1	<1.4	<2.0	17.0	46	48	0[b]	6[b]
			1	51.1	9.0	45.7	<2.0	21.3	30	42	23	5[b]
			1	5.2	<0.4	<1.4	<2.0	–	–	–	–	–
France (Cargouet et al., 2004)	Evry	250	6	7.2	4.5	7.3	3.1	9.5	9	47	8	35
	Valenton	1200	6	6.5	7.2	5.0	4.4	13.3	6	54	4	36
	Colombes [TF]	800	6	4.3	6.6	5.7	2.7	10.7	5	62	6	28
	Aheres	8000	6	6.2	8.6	6.8	4.5	15.0	5	57	5	33
France (Muller et al., 2008)	WWTP 1	120	3[d]	5.0	1.0	<1.0	2.0	3.9	16	26	1[b]	56
			3[d]	2.0	3.0	<1.25	<2.5	4.7	5	64	1[b]	29[b]
Grees (Pothitou and Voutsa, 2008)	WWTP 1	n.a.	5	<3	<2	<3	<2.0	–	–	–	–	–
Norway (Thomas et al., 2007)	Oslo	610	6	4.0	<3	<3	<0.3	–	–	–	–	–
Switzerland (Aerni et al., 2004)[e]	Glatt	88	7	11.9	0.7	7.2	<(0.7–1)	3.4	44	20	22	14[b]
	Rontal	27	4	27.3	3.4	9.9	1.6	9.6	35	36	11	18
	Surental	38	5	4.0	2.0	<(1–1.5)	<(0.7–1)	3.0	16	66	2[b]	15[b]
France (Aerni et al., 2004)[e]	Fr. 1	30	5	5.3	2.7	<(1–1.5)	<(0.7–1)	3.8	17	69	2[b]	12[b]
	Fr. 2	28	4	4.2	6.5	<(1–1.5)	<(0.7–1)	7.6	7	86	1[b]	6[b]
Values below LOD included as ½ LOD (n = 78)	Average			17.6	5.1	6.6	1.2	11.9	32	42	8	17
	Median			6.8	1.7	1.4	0.6	6.3	31	37	5	13
	95%ile			67.1	8.8	18.2	3.8	30.4	64	86	30	38
	Max			147	158	275	5.0	173	91	92	40	59
Measurements with all values above LOD (n = 32)	Average			20.7	7.1	11.0	1.5	13.9	33	40	8	20
	Median			8.7	2.5	4.0	0.9	5.7	33	35	5	20
	95%ile			69.8	17.0	23.0	4.6	41.7	62	65	29	37
	Max			147	158	275	5.0	173	69	91	40	39

cEEQ—calculated Estrogenic Equivalent (Eq. (1)).
N—number of samples. If N > 1, only the averaged concentrations for N samples were available.
n—number of causes (measurements) included in this calculations.
n.a.—not available.
[TF]—trickling filter technology.
[W]—wetland lagoons without any other treatment steps (17d hydraulic retention time).
[a] EEF_{E1} was 0.13; EEF_{E2} was 1; EEF_{E3} was 0.11; and EEF_{EE2} was 1.09 as determined by the authors of the present study by comparing the EC50 values from dose–response curves of E2 and other estrogens in MVLN assay.
[b] ½ of LOD was taken into account.
[c] one measurement was excluded from displayed data as outlier value.
[d] N samples were measured in triplicates. Mean concentrations from repeated measurements are displayed.
[e] Only minimal and maximal values were reported in this study, therefore the averages were calculated from these values.

of EE2. The PNEC of EE2 was lower than PNECs of E1, E2 or E3 by factors ranging 10–600. For six of the 15 bioassays the EEQ-SSE_{E1} was the lowest EEQ-$SSE_{Ei.}$ These 6 bioassays had EEF_{E1} values ranging from 0.01 to 0.03, which is approximately an order of magnitude less than the EEF_{E1} derived by use of most bioassays (Table 1). In all investigated bioassays EEQ-SSE_{E2} and especially EEQ-SSE_{E3} were much greater (by factors 3–15 in the case of EEQ-SSE_{E2} and 20–95 in the case of EEQ-SSE_{E3}), than the final EEQ-SSEs, which is indicative of the lower risks posed by E3 and to a lesser extent E2 compared to E1 and EE2. This result is consistent with previous assumptions as discussed *e.g.* by Johnson and Sumpter (2001).

3. Results and discussion

3.1. Derived concentrations of EEQ-SSEs

Since *in vivo* PNECs for steroids have been determined for longer-term exposures (multi-generation studies, more than 60 d) and shorter-term

Table 3
Comparison of medians and maximal concentrations of steroid estrogens in municipal waste water treatment plant effluents among different data sets.

	E1 (ng/L)				E2 (ng/L)				E3 (ng/L)				EE2 (ng/L)			
	N	Med	Max	95%ile	N	Med	Max	95%ile	N	Med	Max	95%ile	N	Med	Max	95%ile
This study[a]	112	7	147	67	112	1.7	158	8.8	112	1.4	275	18	112	0.6	5	3.8
Miege et al. (2009)	79	10	95	n.a.	63	1.5	30	n.a.	33	1.4	275	n.a.	33	0.5	5	n.a.
Gardner et al. (2012)	162	12	n.a.	80	162	1.3	n.a.	9.5	0	–	–	–	162	0.47	n.a.	1.36

med—median.
n.a.—not available.
N—number of investigated WWTP effluents.
[a] Values below LOD included as ½ LOD.

situations (less than 60 d), EEQ-SSEs were also calculated for both exposure scenarios. Calculated *in vitro* EEQ-SSEs for longer-term exposures ranged among individual bioassays from 0.1 to 0.4 ng/L EEQ with a median of 0.3 ng/L EEQ, while EEQ-SSEs for shorter-term exposures ranged from 0.5 to 2 ng/L EEQ with a median of 1.4 ng/L EEQ (Table 4). The smaller values for the EEQ-SSEs are near LOD of most bioassays (Leusch et al., 2010). However, it is important to emphasize that WWTP effluents are usually diluted by recipients so EEQ-SSEs should further be divided by appropriate dilution factor. For example if the contribution of WWTP effluent to the river flow was 10%, the EEQ-SSEs would vary from 1 to 4 ng EEQ/L and 5 to 20 ng/L EEQ for longer-term and shorter-term exposures, respectively. Use of EEFs for individual steroid hormones and knowledge of dilution factors for specific points in space and time enable comparison of LODs of the bioassays with the EEQ-SSEs. This allows qualified decisions *e.g.* whether less expensive assays (with greater LODs) can be used for specific WWTP.

Under environmental conditions concentrations of the steroids in rivers receiving WWTP effluents vary depending on EEQ concentrations in the effluents, on amounts of waste waters discharged and on river flow, hence the dilution factor of the effluent in the river (Anderson et al., 2012). EEQ-SSEs derived for longer-term exposure scenarios are more protective and should be generally used. The EEQ-SSEs for shorter-term exposures can be used in specific cases when the samples are collected during short periods of highest concentrations of EEQ (*e.g.* during sewage over-flows or during short periods of low flows of rivers receiving concentrated WWTP effluents). In some rivers, river flow can be much lower during rainless days and/or dryer seasons and since there is less dilution, concentrations of estrogens in rivers can be greater. Increasing concentrations will increase the risk to fish health especially if this occurs during critical windows of development. However, such conditions can be of relatively short duration, lasting only several days (Anderson et al., 2012). Therefore if samples of WWTP effluents are collected during these short periods of greatest EEQ concentrations, shorter-term derived EEQ-SSE might be more accurate limit than the longer-term EEQ-SSE.

The EEQ-SSEs recalculated for the dilution factor are more relevant than the previously suggested 1 ng/L. The Table 4 demonstrates that EEQ of 1 ng/L would be protective for shorter-term exposures in 67% of the bioassays. However, for longer term exposure it would not be protective enough for any of the bioassays. As demonstrated in Section 2.2 there can be relatively great differences in the potencies of the individual estrogens among bioassays and thus the same sample can cause different levels of responses in various bioassays. The differences in EEFs among laboratories using the same model actually demonstrate the need of standardized protocols (including media, serum, cell density, exposure time etc.) for each model to be able to apply the specific set of EEFs in calculations relative to environmental samples. Certainly, the most precise EEQ-SSE derivation is based on EEFs for the major estrogens determined in the same model with the same procedure as used for the samples. On the other hand, there are at maximum 4fold differences in the overall EEQ-SSE among assays (Table 4). If some general EEQ-SSE should be derived, it should be based on the bioassays with the lowest EEFs.

3.2. EEQ-SSEs for untreated waste waters and rivers receiving municipal WWTP effluents

When untreated waste waters are considered as a possible source of estrogenic contamination, the percentage of total cEEQ for EE2 would be lower due to the presence of greater concentrations of natural estrogens (Anderson et al., 2012; Liu et al., 2009; Miege et al., 2009; Muller et al., 2008). Therefore, EEQ-SSEs derived for municipal WWTP effluents are likely to be protective enough also for untreated municipal waste waters.

EEQ-SSEs developed to assess municipal WWTP effluents might be directly applicable for the reaches of rivers that are influenced primarily by municipal WWTP effluents. The values presented in Table 4 are protective regarding all 4 considered estrogens. With increasing distance from discharges, proportions of total cEEQ might change due to differential weathering in rivers. For E1 and E2 similar ranges of half-lives at 20 °C in river water were reported to be 5 and 3 d, respectively, whereas EE2 was more persistent (Jurgens et al., 2002). Photodegradation is the primary mechanism of transformation of EE2 with a half-life in water of approximately 17 d (Jurgens et al., 2002; Sumpter et al., 2006). Greater proportions of EE2 to cEEQ were observed in river water compared to WWTP discharge (Cargouet et al., 2004). Information about compounds responsible for estrogenicity as well as for other specific modes of actions in rivers is limited compared to what is available for WWTP effluents or rivers close to their discharges. Therefore, more research is needed to enable derivation of safe concentrations of EEQ for parts of rivers which are not in close vicinity of WWTP discharges.

3.3. Applicability of derived EEQ-SSEs and future research

The derived *in vitro* EEQ-SSEs are applicable for municipal WWTP effluents and parts of rivers close to their discharges where E1, E2, E3 and EE2 are expected to be responsible for the majority of the estrogenicity. Most information on the occurrence of steroid estrogens in waste waters presented here originate from European countries, therefore the best applicability of the EEQ-SSEs should be for the situation in Europe. Different patterns might occur in other regions of the world which could change the proportion of occurrence of estrogenic compounds in waters. For instance, in Japan, there is little use of the contraceptives and therefore the contribution of EE2 to the estrogenicity would be expected to be less than in EU countries (Sumpter and Johnson, 2008). This demonstrates the possibility of different $P_{EE2\text{-}max}$ compared to those reported in dataset used in this study. Most WWTP effluents investigated in this study employed primary treatment followed by activated sludge treatment, which represent the most common type of municipal WWTPs. However, different types of treatment could also result in different ratios of steroid estrogens. Once the proposed EEQ-SSE approach is applied, the datasets used for $P_{Ei\text{-}max}$ derivation can be enlarged or modified according to relevant available information *e.g.* from national reports.

It is also necessary to point out the limited ability of *in vitro* estrogenicity assays to detect some compounds with lower *in vitro*

Table 4
Safe estrogenic equivalents regarding steroid estrogens (EEQ-SSE) as calculated for *in vitro* bioassays and municipal waste water treatment plant effluents and/or rivers close to their discharges. The EEQs-SSEs are supposed to be increased by use of location-specific dilution factors of WWTP effluents entering receiving rivers.

Assay	EEQ-SSE (ng/L EEQ)	
	Longer-term exposures	Shorter-term exposures
YES (Aerni et al., 2004), (Rutishauser et al., 2004)	0.3	1.7
YES (Svenson et al., 2003)	0.4	2.0
YES (Caldwell et al., 2012)	0.3	1.6
YES (Leusch et al., 2010)	0.2	1.2
ER-CALUX (Sonneveld et al., 2006)	0.2	0.6
ER-CALUX (Avbersek et al., 2011)	0.4	2.0
ER-CALUX (Houtman et al., 2004)	0.3	1.4
MELN (Leusch et al., 2010)	0.2	0.8
MELN (Leusch et al., 2010)	0.3	1.6
E-screen (Gutendorf and Westendorf, 2001)	0.1	0.5
E-screen (Drewes et al., 2005)	0.3	1.6
E-screen (Leusch et al., 2010)	0.3	1.1
E-screen (Leusch et al., 2010)	0.1	0.5
MVLN[a]	0.3	1.4
MVLN (Gutendorf and Westendorf, 2001)	0.1	0.5
Min	0.1	0.5
Max	0.4	2.0
Median	0.3	1.4

YES—yeast estrogenicity screening assay (Routledge and Sumpter, 1996).
ER-CALUX—Estrogen Receptor mediated Chemical Activated LUciferase gene eXpression assay (Van der Burg et al., 2010).
MELN—MCF-7 cells stably transfected with the estrogen responsive gene ERE-betaGlob-Luc-SVNeo (Balaguer et al., 2000).
E-SCREEN—the MCF7 cell proliferation assay (Soto et al., 1998).
MVLN—MCF-7 cells stably transfected with luciferase gene under the control of estrogen receptor (Demirpence et al., 1993).
[a] Unpublished data—*in vitro* potencies were determined by the authors of the present study by comparing the EC50 values from dose–response curves of E2 and other estrogens.

potencies such as NP and OP, which might lead to underestimation of their potential estrogenic effects *in vivo*. *In vivo* PNECs have not been determined yet for many estrogenic compounds and therefore more research is needed to evaluate the applicability for the samples where the steroid estrogens cannot be expected as the dominant estrogens. It should be always kept in mind that all mentioned *in vitro* estrogenicity assays evaluate one specific mechanism of action (activation of estrogen receptor, ER) and that there are usually compounds with different modes of actions in environmental matrices which might induce similar effects (*i.e.* reproduction disorders) *in vivo*.

With respect to the issue of direct modulation of ER, one should also consider potential interference of anti-estrogenic compounds, which could be present in the sample along with the steroid estrogens (Johnson and Jurgens, 2003; Preuss et al., 2010). However, several lines of evidence indicate that antiestrogens are not a major issue in common municipal waste waters. First, steroid estrogens addressed in the present study are strong activators of ER, and their presence in the complex mixture is likely to overweigh potential effect of, generally weaker, antiestrogens. There is little information on antiestrogenic potency of effluents of municipal WWTPs, whereas numerous studies have found estrogenicity (*e.g.* Aerni et al., 2004; Vethaak et al., 2005). Nevertheless, in the samples containing eventual antiestrogens, the effect of the whole mixture determined in the *in vitro* assay would probably underestimate the actual content of estrogens. Antiestrogens could partially mask the effect of estrogenic compounds. Further research is needed to quantify the possible influence of antiestrogens.

The main purpose of derivation of EEQ-SSEs was not to derive any guideline value but to better understand what can be learned from the results of *in vitro* bioassays towards *in vivo* situation. According to our opinion, adoption of such limits into legislation needs further consideration. Traditional guideline limits are derived from PNECs of particular compounds and multiplied by factors of uncertainties. When such limits for E2 and EE2 were proposed for consideration under EU Water Framework Directive, the suggested EQSs for surface waters were as low as 0.4 ng/L for E2 and 0.035 ng/L for EE2, respectively (European Commission, 2012). Correspondingly, values of EEQ-SSEs are relatively low (yet higher than mentioned EQSs), since they are derived from the low PNEC values. EEQ-SSEs based on PNEC were however derived to protect individuals not populations, which will be most probably affected at higher concentrations of estrogens (Harris et al., 2011).

4. Conclusions

Safe levels of estrogenic equivalents (EEQ-SSE) in municipal WWTP effluents were derived considering bioassay specific *in vitro* potencies of major steroidal estrogens, *in vivo* derived PNECs of these compounds, and their relative contributions to the overall estrogenic activity detected in common municipal WWTP effluents. Since the *in vivo* PNECs for the steroids have been determined for longer-term (more than 60 d) and shorter-term (less than 60 d) exposures, also the EEQ-SSEs have been calculated for shorter-term and longer-term exposure scenarios. The derived EEQ-SSEs for 15 individual bioassays varied from 0.1 to 0.4 ng/L EEQ for longer-term exposures and from 0.5 to 2 ng/L EEQ for shorter-term exposures, respectively. The EEQs-SSEs are supposed to be increased by dilution factors of WWTP effluents in receiving rivers. The best applicability of the derived EEQ-SSEs is for areas, where steroidal estrogens have been confirmed or suspected as being responsible for fish feminization downstream municipal WWTPs.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.envint.2013.12.009.

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Supplementary data

What level of estrogenic activity determined by in vitro assays in municipal waste waters can be considered as safe?

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1. Relative percentage contributions of four main steroid estrogens to the total calculated estrogenic equivalents in municipal WWTP effluents determined for different in vitro bioassays

Table SD 1. Relative percentage contribution of four main steroid estrogens (E1, E2, E3 and EE2) to total concentrations of calculated estrogenic equivalents (cEEQ) in municipal WWTP effluents determined for YES bioassay using 2 sets of EEFs reported in Aerny et al. (2004) and Rutishauer et al. (2004) and Svenson et al. (2003)

Country	WWTP Name or Code	Equiv. Citizens (thousands)	N	YES assay (Aerni et al. 2004, Rutishauser et al. 2004)					YES assay (Svenson et al. 2003)				
				cEEQ[#] ng/L	*P*-Percentage of total cEEQ				cEEQ[##] ng/L	*P*-Percentage of total cEEQ			
					E1	E2	E3	EE2		E1	E2	E3	EE2
Austria (Clara et al. 2005)	WWTP 1	2,500	1	64.0	43	47	1	9	55.6	25	54	2	20
	WWTP 2	167	1	9.2	33	27*	0	39	10.7	14	23*	1	62
	WWTP 4	6	1	8.8	17	28*	0*	54	12.1	6	21*	0*	73
			1	8.8	2*	91	0	7*	9.2	1*	87	0	12*
			1	5.4	14	75	0*	11*	5.5	7	73	0*	20*
California (Drewes et al. 2005)	WWTP 3	> 100	1	16.0	42	27	0	30	16.8	20	26	0	54
	WWTP 4	> 500	1	21.1	91	7	0*	2*	11.9	81	13	0*	6*
	WWTP 5	> 100	1	10.6	40	56	0	4	8.9	24	67	0	9
	WWTP 7	> 500	1	8.5	74	21	0*	5*	5.7	55	32	0*	14*
Canada (Fernandez et al. 2007)	WWTPB[TF]	740	1	32.4	81	15	0	4	20.3	64	25	0	11
			1	62.1	90	3	0*	7*	37.7	74	5	0*	21*
			1	12.6	11*	79	0*	9	12.9	6*	77	0*	17
			1	5.6	26*	53	0*	21	5.9	12*	51	0*	37
			1	19.7	48	30	0*	21*	18.6	26	32	0*	42*
			1[a]	42.5	76	14	0	10*	30.0	54	20	0	26*
	WWTP E[w]	20	1	71.9	15	79	0*	6*	70.1	8	81	0*	11*
			1	91.1	16	79	0	5*	87.2	8	83	0	9*
			1	185.3	11	85	0	3	179.7	6	88	0	6
China, Chongqing (Ye et al. 2012)	WWTP B	214	1[b]	14.9	77	13	0*	10*	10.4	55	18	0*	26*
	WWTP D	59	1[b]	5.5	59	14*	0	27*	5.2	32	15*	1	53*
	WWTP E	144	1[b]	3.7	39	20*	0	40*	4.2	17	18*	1	65*
	WWTP H	88	1[b]	5.3	58	14*	0	28*	5.1	30	15*	1	54*
Finland (Bjorkblom et al. 2008)	Turku	160	1[b]	25.7	97	3	0*	0*	13.4	93	5	0*	2*
France (Labadie & Budzinski 2005a)	Eysenes	50	1[b]	27.6	80	16	0	4*	17.6	62	25	0	13*
Italy, Roma (Baronti et al. 2000), (Johnson et al. 2000)	Cobis	40	1	9.0	55	32	0	13	7.6	33	38	0	29
			1	8.9	73	25	0	2*	5.8	56	38	0	6*
			1	4.0	66	19	0	15	3.1	42	23	1	34
			1	2.9	75	19	0	6*	2.0	55	28	0	17*
			1	3.6	57	28	0	15	3.0	34	33	0	32
	Fregene	120	1	5.1	15	79	0	6*	4.9	8	81	0	11*
			1	10.8	11	65	0	24	12.4	5	56	0	39

			1	6.6	37	32	0	31	7.1	17	30	0	53
			1	1.7	56	33	0	11*	1.4	35	41	1	24*
			1	2.1	66	17	0	17	1.7	41	20	0	38
			1	2.4	68	17	0	15	1.9	43	21	0	36
			1	3.0	42	41	0	17	2.8	23	44	0	34
Italy, Roma (Baronti et al. 2000), (Johnson et al. 2000)	Ostia	350	1	15.5	76	19	0*	5	10.2	58	29	0*	13
			1	26.9	76	22	0	1*	16.9	61	36	0	3*
			1	35.8	87	9	0	4	21.3	73	15	0	11
			1	5.8	85	12	0	3*	3.5	70	20	0	9*
			1	21.0	83	14	0	3	12.8	68	23	0	8
			1	16.0	83	11	0	6	10.1	66	17	0	17
			1	21.5	83	16	0	1*	12.8	70	27	0	3*
	Roma Sud	1,200	1	10.9	70	27	0	3*	7.4	52	41	0	7*
			1	24.1	82	17	0	1*	14.5	68	28	0	4*
			1	23.9	81	13	0	6	15.5	63	20	0	17
			1	13.5	84	14	0	1*	8.0	72	24	0	4*
			1	10.6	79	15	0	6	6.9	60	23	0	16
			1	4.5	74	12	0	14	3.3	50	16	0	34
			1	4.3	35	53	1	11	4.0	19	57	2	22
	Roma Est	800	1	4.9	75	17	0	9	3.5	53	24	0	23
			1	3.9	77	18	0	5*	2.6	59	28	0	13*
			1	2.4	58	25	0	17	2.1	34	30	0	37
			1	4.3	61	19	0	20	3.7	35	22	0	43
			1	5.0	76	16	0	7	3.4	56	24	0	20
	Roma Nord	800	1	7.5	56	40	0	4*	5.7	37	53	1	10*
			1	9.6	75	21	1	3*	6.3	58	32	2	9*
			1	5.1	74	18	0	8	3.6	53	26	0	20
			1	3.1	80	14	0	6*	2.0	61	22	0	17*
			1	4.0	61	23	0	17	3.4	36	27	0	37
			1	3.9	65	19	0	16	3.2	40	23	0	37
			1	17.7	86	11	0	3	10.6	71	18	0	10
Slovenia (Avbersek et al. 2011)	WWTP 1	50	1	4.2	36	35	1	28*	4.5	17	33	1	49*
			1	4.8	14	61	1	25*	5.5	6	53	1	40*
	WWTP 2	360	1	9.6	66	22	0*	12*	7.4	42	28	0*	30*
	WWTP 3	100	1	32.8	72	25	0*	4*	22.0	53	37	0*	10*
			1	29.7	65	30	0	4*	21.1	46	43	1	10*
France (Cargouet et al. 2004)	Evry	250	6	10.9	25	41	0	34	12.7	11	35	0	54
	Valenton	1,200	6	14.9	17	48	0	35	18.1	7	40	0	53
	Colombes	800	6	11.5	14	58	0	28	13.4	6	49	0	44
	Aheres	8,000	6	16.3	14	53	0	33	19.7	6	44	0	50
France (Muller et al. 2008)	WWTP 1	120	3[b]	5.3	36	19	0*	45	6.4	15	16	0*	69
			3[b]	5.2	14	57	0*	28*	6.1	6	49	0*	45*

Switzerland (Aerni et al. 2004)c	Glatt	88	7	5.7	79	12	0	9*	3.9	58	17	1	24*
	Rontal	27	4	15.7	66	22	0	12	12.2	42	28	0	29
	Surental	38	5	4.0	37	50	0*	13*	3.7	20	54	0*	25*
France (Aerni et al. 2004)c	Fr. 1	30	5	5.2	39	51	0*	10*	4.6	22	58	0*	20*
	Fr. 2	28	4	8.6	19	76	0*	6*	8.2	10	79	0*	11*
values below LOD included as ½ LOD (n=78)	average			17	55	32	0	13	14	38	36	0	26
	median			8.9	63	22	0	9	7.4	38	28	0	21
	95%ile			62	86	79	1	36	40	72	81	1	55
	max			185	97	91	1	54	180	93	88	2	73
measurements with all values above LOD (n=32)	average			18	56	29	0	15	15	38	32	0	30
	median			9.8	63	20	0	14	7.3	38	26	0	33
	95%ile			48	84	61	1	33	37	70	57	1	54
	max			185	87	85	1	35	180	73	88	2	54

cEEQ - calculated Estrogenic Equivalent; cEEQ = [E1] × EEF_{E1} + [E2] × EEF_{E2} + [E3] × EEF_{E3} + [EE2] × EEF_{EE2}
where [E1], [E2], [E3] and [EE2] are concentrations of estrogens displayed in Table 2 and EEF_s are *in vitro* potencies determined for individual bioassays

[#] EEF_{E1} was 0.38; EEF_{E2} was 1; EEF_{E3} was 0.0024; and EEF_{EE2} was 1.19 as determined by Aerny et al. (2004) and Rutishauer et al. (2004)

[##] EEF_{E1} was 0.19; EEF_{E2} was 1; EEF_{E3} was 0.0035; and EEF_{EE2} was 2.2 as determined by Svenson et al. (2003)

* value derived from ½ of LOD

[a] one measurement was excluded from displayed data as outlier value

[b] N samples were measured in triplicates. Mean concentrations from repeated measurements are displayed

[c] averages were calculated from minimal and maximal values reported in the study

N - number of samples. If N>1, only the averaged concentrations for N samples were available.

n - number of causes (measurements) included in this calculations

[TF] trickling filter technology

[W] wetland lagoons without any other treatment steps (17d hydraulic retention time)

Table SD 2. Relative percentage contribution of four main steroid estrogens (E1, E2, E3 and EE2) to the total calculated estrogenic equivalents (cEEQ) in municipal WWTP effluents determined for YES bioassay using 2 sets of EEFs reported by Routledge et al. in Caldwell et al. (2012) and Fang et al. in Leusch et al. (2010)

Country	WWTP Name or Code	Equiv. Citizens (thousands)	N	YES assay (Caldwell et al. 2012) *P*-Percentage of total cEEQ					YES assay (Leusch et al. 2010) *P*-Percentage of total cEEQ				
				cEEQ[#] ng/L	E1	E2	E3	EE2	cEEQ[##] ng/L	E1	E2	E3	EE2
Austria (Clara et al. 2005)	WWTP 1	2,500	1	59.6	40	50	1	8	43.1	16	70	4	10
	WWTP 2	167	1	8.2	32	31*	1	37	6.0	13	41*	2	44
	WWTP 4	6	1	7.8	17	32*	0*	51	6.5	6	39*	0*	55
			1	8.7	2*	92	0	6*	8.5	1*	94	0	5*
			1	5.2	13	77	0*	10*	4.6	4	86	0*	10*
California (Drewes et al. 2005)	WWTP 3	> 100	1	14.4	41	31	0	29	9.8	17	45	0	37
	WWTP 4	> 500	1	18.5	90	8	0*	2*	6.6	72	23	0*	5*
	WWTP 5	> 100	1	10.0	37	60	0	3*	7.4	14	81	0	4*
	WWTP 7	> 500	1	7.6	72	24	0*	5*	3.7	42	49	0*	8*
Canada (Fernandez et al. 2007)	WWTPB[TF]	740	1	28.8	79	17	0	3	12.5	53	40	0	7
			1	54.1	90	4	0*	7*	19.2	73	10	0*	16*
			1	12.3	10*	82	0*	8	11.3	3*	89	0*	8
			1	5.3	24*	57	0*	19	4.3	9*	70	0*	21
			1	17.8	46	34	0*	20*	11.6	21	52	0*	27*
			1[a]	37.6	75	16	0	9*	17.3	47	35	0	18*
	WWTP E[w]	20	1	69.8	13	82	0*	5*	62.8	4	91	0*	5*
			1	88.4	15	81	0	4*	78.9	5	91	0	4*
			1	182	10	87	0	3	168	3	94	0	3
China, Chongqing (Ye et al. 2012)	WWTP B	214	1[b]	13.2	76	14	0*	9*	5.9	49	32	0*	19*
	WWTP D	59	1[b]	4.9	58	15*	1	26*	2.7	30	27*	2	41*
	WWTP E	144	1[b]	3.3	38	23*	1	38*	2.3	16	33*	2	49*
	WWTP H	88	1[b]	4.7	57	16*	1	27*	2.7	29	28*	3	41*
Finland (Bjorkblom et al. 2008)	Turku	160	1[b]	22.4	96	3	0*	0*	7.0	89	10	0*	1*
France (Labadie & Budzinski 2005a)	Eysenes	50	1[b]	24.5	78	18	0	4*	10.8	51	41	0	8*
Italy, Roma (Baronti et al. 2000), (Johnson et al. 2000)	Cobis	40	1	8.2	52	35	0	12	5.1	25	57	0	18
			1	8.0	70	28	0	2*	4.0	41	55	1	3*
			1	3.5	65	21	0	14	1.9	35	40	2	23
			1	2.6	73	21	0	6*	1.2	44	44	1	11*
			1	3.2	55	31	0	14	1.9	27	52	0	20
	Fregene	120	1	4.9	13	81	0	5*	4.4	4	90	1	5*
			1	10.2	10	69	0	22	9.3	3	75	0	21
			1	5.9	36	35	0	29	4.2	15	49	0	36
			1	1.5	54	36	0	10*	0.9	25	59	1	14*
			1	1.9	65	19	0	16	1.0	36	36	0	27
			1	2.1	66	19	0	14	1.1	37	37	0	25
			1	2.7	40	44	0	15	1.9	17	63	0	20
Italy, Roma	Ostia	350	1	13.8	74	22	0*	4	6.5	46	46	0*	8

Country	WWTP Name or Code	Equiv. Citizens (thousands)	N	YES assay (Caldwell et al. 2012) *P*-Percentage of total cEEQ					YES assay (Leusch et al. 2010) *P*-Percentage of total cEEQ				
				cEEQ# ng/L	E1	E2	E3	EE2	cEEQ## ng/L	E1	E2	E3	EE2
(Baronti et al. 2000), (Johnson et al. 2000)			1	24.1	74	25	0	1*	11.5	45	52	1	2*
			1	31.5	86	10	0	3	12.1	65	27	0	8
			1	5.2	83	14	0	3*	2.1	59	34	0	6*
			1	18.7	81	16	0	3	7.8	56	38	0	5
			1	14.1	82	12	0	6	5.8	58	30	0	12
			1	19.2	81	18	0	1*	8.1	55	43	0	2*
	Roma	1,200	1	9.9	67	30	0	3*	5.2	37	58	1	4*
	Sud		1	21.5	80	19	0	1*	9.3	53	43	1	2*
			1	21.2	80	15	0	6	9.1	53	34	1	12
			1	12.0	83	16	0	1*	4.9	58	38	1	3*
			1	9.4	77	17	0	5	4.2	50	38	1	11
			1	3.9	73	13	0	13	1.8	45	29	1	25
			1	4.1	32	56	1	10	3.2	12	73	4	11
	Roma Est	800	1	4.4	73	19	0	8	2.1	45	40	0	15
			1	3.5	75	21	0	4*	1.6	47	44	0	8*
			1	2.2	56	28	0	16	1.3	27	48	0	24
			1	3.8	60	21	0	19	2.1	31	38	0	31
			1	4.4	75	18	0	7	2.0	47	39	0	14
	Roma Nord	800	1	6.9	53	43	0	4*	4.3	24	69	2	5*
			1	8.6	73	23	1	3*	4.2	43	47	4	5*
			1	4.6	72	20	0	7	2.2	44	43	0	13
			1	2.7	78	16	0	6*	1.2	51	37	0	11*
			1	3.6	59	25	0	16	2.0	30	45	1	25
			1	3.5	63	21	0	15	1.8	34	40	0	26
			1	15.6	84	12	0	3	6.2	61	31	1	7
Slovenia (Avbersek et al. 2011)	WWTP 1	50	1	3.9	34	39	1	26*	2.9	13	53	3	31*
			1	4.5	12	64	1	22*	4.1	4	71	3	22*
	WWTP 2	360	1	8.5	64	25	0*	12*	4.6	34	46	0*	19*
	WWTP 3	100	1	29.5	69	27	0*	3*	14.9	40	54	0*	6*
			1	27.0	62	33	1	4*	15.1	32	60	2	6*
France (Cargouet et al. 2004)	Evry	250	6	10.0	24	45	0	31	8.0	9	56	1	35
	Valenton	1,200	6	13.8	16	52	0	32	11.8	5	61	0	33
	Colombe -	800	6	10.7	13	61	0	25	9.5	4	70	0	25
	Aheres	8,000	6	15.2	13	57	0	30	13.2	4	65	0	30
France (Muller et al. 2008)	WWTP 1	120	3[b]	4.7	35	21	0*	43	3.3	15	31	0*	55
			3[b]	4.9	13	61	0*	25*	4.3	4	70	0*	26*
Switzerland (Aerni et al. 2004)c	Glatt	88	7	5.0	78	13	0	8*	2.2	51	30	2	17*
	Rontal	27	4	14.1	64	24	0	11	7.5	35	46	1	19
	Surental	38	5	3.7	35	54	0*	11*	2.8	14	72	0*	14*
France (Aerni et al. 2004)c	Fr. 1	30	5	4.8	36	55	0*	9*	3.5	14	75	0*	11*
	Fr. 2	28	4	8.3	17	78	0*	5*	7.3	6	89	0*	5*
values below LOD included as ½ LOD			average	15	53	34	0	12	10	31	51	1	17
			median	8	61	25	0	8	5	32	46	0	13

Country	WWTP Name or Code	Equiv. Citizens (thousands)	N	YES assay (Caldwell et al. 2012) cEEQ[#] ng/L	*P*-Percentage of total cEEQ E1	E2	E3	EE2	YES assay (Leusch et al. 2010) cEEQ[##] ng/L	*P*-Percentage of total cEEQ E1	E2	E3	EE2
(n=78)			95%ile	55	85	81	1	33	23	62	90	3	42
			max	182	96	92	1	51	168	89	94	4	55
measurements with all values above LOD (n=32)			average	17	54	31	0	14	12	31	48	1	20
			median	9	61	23	0	13	5	33	44	0	20
			95%ile	44	83	65	1	30	27	60	74	3	35
			max	182	86	87	1	32	168	65	94	4	37

cEEQ - calculated Estrogenic Equivalent; cEEQ = [E1] × EEF_{E1} + [E2] × EEF_{E2} + [E3] × EEF_{E3} + [EE2] × EEF_{EE2}
where [E1], [E2], [E3] and [EE2] are concentrations of estrogens displayed in Table 2 and EEF_s are *in vitro* potencies determined for individual bioassays

[#] EEF_{E1} was 0.33; EEF_{E2} was 1; EEF_{E3} was 0.003; and EEF_{EE2} was 1 as determined by Routledge et al. in Caldwell et al. (2012)

[##] EEF_{E1} was 0.096; EEF_{E2} was 1; EEF_{E3} was 0.006; and EEF_{EE2} was 0.89 as determined by and Fang et al. in Leusch et al. (2010)

* value derived from ½ of LOD

[a] one measurement was excluded from displayed data as outlier value

[b] N samples were measured in triplicates. Mean concentrations from repeated measurements are displayed

[c] averages were calculated from minimal and maximal values reported in the study

N - number of samples. If N>1, only the averaged concentrations for N samples were available.

n - number of causes (measurements) included in this calculations

[TF] trickling filter technology

[W] wetland lagoons without any other treatment steps (17d hydraulic retention time)

Table SD 3. Relative percentage contribution of four main steroid estrogens (E1, E2, E3 and EE2) to the total calculated concentrations of estrogenic equivalents (cEEQ) in municipal WWTP effluents determined for ER-CALUX bioassay using 2 sets of EEFs reported in Sonneveld et al. (2006) and Avbersek et al. (2011)

Country	WWTP Name or Code	Equiv. Citizens (thousands)	N	ER-CALUX assay (Sonneveld et al. 2006) *P*-Percentage of total cEEQ					ER-CALUX assay (Avbersek et al. 2011) *P*-Percentage of total cEEQ				
				cEEQ# ng/L	E1	E2	E3	EE2	cEEQ## ng/L	E1	E2	E3	EE2
Austria (Clara et al. 2005)	WWTP 1	2,500	1	50.2	2	60	19	19	105.7	27	28	36	8
	WWTP 2	167	1	8.8	1	28*	7	63	13.1	24	19*	18	38
	WWTP 4	6	1	10.0	1	25*	0*	74	10.9	15	23*	1*	62
			1	9.0	0*	89	0	10*	9.2	2*	87	2	9*
			1	5.0	1	80	0*	19*	5.7	14	70	1*	15*
California (Drewes et al. 2005)	WWTP 3	> 100	1	12.4	2	35	1	61	18.9	37	23	3	36
	WWTP 4	> 500	1	3.0	26	49	3*	21*	22.6	89	7	1*	3*
	WWTP 5	> 100	1	7.0	3	86	2	9*	11.7	38	51	6	5*
	WWTP 7	> 500	1	2.8	9	65	2*	24*	9.1	72	20	2*	6*
Canada (Fernandez et al. 2007)	WWTPB[TF]	740	1	8.2	13	61	3	23	35.4	78	14	3	5
			1	11.0	21	18	0*	60*	66.9	88	3	0*	9*
			1	11.9	1*	84	0*	16	13.3	11*	75	1*	13
			1	4.9	1*	61	1*	38	6.3	24*	48	2*	27
			1	13.0	3	46	0*	51*	22.1	45	27	0*	27*
			1[a]	14.0	10	43	0	47*	46.1	74	13	0	13*
	WWTP E[w]	20	1	64.1	1	89	0*	10*	74.3	15	77	0*	8*
			1	79.4	1	91	0	8*	94.1	17	76	1	6*
			1	169	1	93	0	6	192	12	82	2	4
China, Chongqing (Ye et al. 2012)	WWTP B	214	1[b]	4.8	10	40	1*	49*	16.3	74	12	1*	13*
	WWTP D	59	1[b]	3.5	4	21*	8	66*	7.5	46	10*	16	28*
	WWTP E	144	1[b]	3.4	2	22*	8	68*	5.4	28	14*	20	39*
	WWTP H	88	1[b]	3.6	4	21*	11	65*	7.6	42	10*	20	28*
Finland (Bjorkblom et al. 2008)	Turku	160	1[b]	1.9	54	36	1*	10*	27.1	97	3	0*	1*
France (Labadie & Budzinski 2005a)	Eysenes	50	1[b]	7.3	13	60	1	26*	29.6	78	15	1	6*
Italy, Roma (Baronti et al. 2000), (Johnson et al. 2000)	Cobis	40	1	5.1	4	57	2	37	10.2	51	28	5	16
			1	3.0	9	73	9	9*	10.3	66	21	10	2*
			1	2.0	6	38	10	46	5.1	54	14	16	16
			1	1.0	10	57	5	29*	3.3	70	17	6	8*
			1	1.9	4	51	2	42	4.1	53	25	4	18
	Fregene	120	1	4.6	1	86	3	10*	5.8	14	69	10	7*
			1	11.3	0	62	2	36	12.6	10	56	6	29
			1	5.4	2	39	1	58	7.8	33	27	3	37
			1	1.0	4	59	8	29*	2.1	47	26	15	12*
			1	1.0	6	35	2	57	2.4	61	14	3	21
			1	1.1	6	38	1	54	2.7	63	15	2	19
			1	2.1	3	58	2	38	3.4	39	36	4	21
Italy, Roma	Ostia	350	1	4.6	11	65	0*	24	16.4	75	18	0*	6

Country	WWTP Name or Code	Equiv. Citizens (thousands)	N	ER-CALUX assay (Sonneveld et al. 2006) *P*-Percentage of total cEEQ					ER-CALUX assay (Avbersek et al. 2011) *P*-Percentage of total cEEQ				
				cEEQ[#] ng/L	E1	E2	E3	EE2	cEEQ[##] ng/L	E1	E2	E3	EE2
(Baronti et al. 2000), (Johnson et al. 2000)			1	8.0	11	75	8	6*	30.5	71	20	8	1*
			1	6.7	19	49	1	31	38.2	86	9	1	5
			1	1.2	17	59	2	23*	6.3	83	12	1	4*
			1	4.7	16	64	1	19	22.4	82	13	1	4
			1	3.8	15	45	1	39	17.1	82	10	1	8
			1	4.6	16	77	1	6*	22.7	83	15	1	1*
	Roma	1,200	1	4.0	8	74	6	12*	12.4	65	24	8	3*
	Sud		1	6.0	14	67	12	8*	28.0	74	14	10	1*
			1	6.5	12	47	6	34	27.1	75	11	6	7
			1	2.9	16	66	8	10*	15.1	80	13	6	2*
			1	3.1	11	52	7	31	12.1	73	13	7	7
			1	1.7	8	31	4	57	5.1	68	10	5	17
			1	3.7	2	61	17	20	7.1	23	32	36	9
	Roma Est	800	1	1.7	9	49	1	40	5.4	72	15	2	11
			1	1.1	11	63	1	24*	4.2	76	17	1	6*
			1	1.4	4	46	2	48	2.8	53	22	4	21
			1	2.3	5	35	1	59	4.9	56	16	2	25
			1	1.6	10	50	3	36	5.5	73	15	4	9
	Roma Nord	800	1	4.0	4	74	10	12*	9.4	47	32	16	4*
			1	3.8	8	53	26	12*	13.9	55	14	28	3*
			1	1.7	9	53	2	35	5.6	71	16	3	10
			1	0.8	12	52	3	33*	3.4	76	13	3	8*
			1	2.1	5	43	3	49	4.6	55	20	5	20
			1	1.9	6	40	2	53	4.4	60	17	3	20
			1	3.8	17	50	8	25	19.9	80	10	6	4
Slovenia (Avbersek et al. 2011)	WWTP 1	50	1	3.9	2	39	11	48*	6.5	25	23	27	26*
			1	5.4	0	53	12	34*	7.8	9	37	33	21*
	WWTP 2	360	1	4.2	6	49	1*	44*	10.5	63	20	1*	16*
	WWTP 3	100	1	11.0	9	74	0*	17*	34.6	71	23	0*	5*
			1	13.3	6	68	12	14*	37.5	54	24	17	4*
France (Cargouet et al. 2004)	Evry	250	6	10.6	1	42	2	54	13.6	21	33	8	38
	Valenton	1,200	6	15.7	1	46	1	52	17.9	15	40	4	41
	Colombes	800	6	11.9	1	55	2	42	13.7	13	48	6	33
	Aheres	8,000	6	17.3	1	50	1	48	19.6	13	44	5	39
France (Muller et al. 2008)	WWTP 1	120	3[b]	4.8	2	21	0*	77	6.4	31	16	1*	52
			3[b]	5.4	1	56	0*	43*	6.0	13	50	1*	35*
Switzerland (Aerni et al. 2004)c	Glatt	88	7	1.9	10	35	13	41*	7.1	66	9	14	10*
	Rontal	27	4	7.2	6	48	5	42	18.4	59	19	8	15
	Surental	38	5	2.9	2	70	1*	27*	4.4	36	46	2*	16*
France (Aerni et al. 2004)c	Fr. 1	30	5	3.5	2	75	1*	22*	5.6	38	48	2*	13*
	Fr. 2	28	4	7.4	1	88	0*	11*	9.0	19	72	1*	8*
values below LOD included as ½ LOD		average		10	7	55	4	34	19	51	27	7	15
		median		5	5	53	2	34	10	54	20	3	11

Country	WWTP Name or Code	Equiv. Citizens (thousands)	N	ER-CALUX assay (Sonneveld et al. 2006) *P*-Percentage of total cEEQ					ER-CALUX assay (Avbersek et al. 2011) *P*-Percentage of total cEEQ				
				cEEQ[#] ng/L	E1	E2	E3	EE2	cEEQ[##] ng/L	E1	E2	E3	EE2
(n=78)			95%ile	22	17	88	12	65	68	84	75	27	39
			max	169	54	93	26	77	192	97	87	36	62
measurements with all values above LOD (n=32)			average	12	6	50	4	40	21	51	24	6	18
			median	4	5	49	2	41	11	56	18	4	17
			95%ile	32	16	63	13	59	69	82	52	25	38
			max	169	19	93	19	61	192	86	82	36	41

cEEQ - calculated Estrogenic Equivalent; cEEQ = [E1] × EEF_{E1} + [E2] × EEF_{E2} + [E3] × EEF_{E3} + [EE2] × EEF_{EE2}
where [E1], [E2], [E3] and [EE2] are concentrations of estrogens displayed in Table 2 and EEF_s are *in vitro* potencies determined for individual bioassays

[#] EEF_{E1} was 0.016; EEF_{E2} was 1; EEF_{E3} was 0.036; and EEF_{EE2} was 1.86 as determined by Sonneveld et al. (2006)

[##] EEF_{E1} was 0.4; EEF_{E2} was 1; EEF_{E3} was 0.14; and EEF_{EE2} was 1.68 as determined by Avbersek et al. (2011)

* value derived from ½ of LOD

[a] one measurement was excluded from displayed data as outlier value

[b] N samples were measured in triplicates. Mean concentrations from repeated measurements are displayed

[c] averages were calculated from minimal and maximal values reported in the study

N - number of samples. If N>1, only the averaged concentrations for N samples were available.

n - number of causes (measurements) included in this calculations

[TF] trickling filter technology

[W] wetland lagoons without any other treatment steps (17d hydraulic retention time)

Table SD 4. Relative percentage contribution of four main steroid estrogens (E1, E2, E3 and EE2) to the total calculated concentrations of estrogenic equivalents (cEEQ) in municipal WWTP effluents determined for two individual bioassays using sets of EEFs reported in Houtman et al. (2004) and Pillon et al. in Leusch et al. (2010)

Country	WWTP Name or Code	Equiv. Citizens (thousands)	N	ER-CALUX assay (Houtman et al. 2004) *P*-Percentage of total cEEQ					MELN assay (Leusch et al. 2010) *P*-Percentage of total cEEQ				
				cEEQ# ng/L	E1	E2	E3	EE2	cEEQ## ng/L	E1	E2	E3	EE2
Austria (Clara et al. 2005)	WWTP 1	2,500	1	80.0	11	38	45	7	93.0	2	32	53	13
	WWTP 2	167	1	9.0	11	28*	24	37	13.1	2	19*	23	56
	WWTP 4	6	1	7.5	6	33*	1*	60	12.5	1	20*	1*	78
			1	8.8	1*	91	1	6*	9.4	0*	85	2	13*
			1	4.9	5	82	1*	12*	5.4	1	75	2*	23*
California (Drewes et al. 2005)	WWTP 3	> 100	1	11.6	18	38	4	39	15.6	3	28	5	64
	WWTP 4	> 500	1	8.2	73	18	4*	5*	4.0	31	37	10*	21*
	WWTP 5	> 100	1	8.4	16	72	8	5*	8.0	3	75	11	11*
	WWTP 7	> 500	1	4.3	45	42	4*	9*	3.3	12	54	7*	26*
Canada (Fernandez et al. 2007)	WWTP B[TF]	740	1	15.4	54	32	7	7	10.6	16	47	13	23
			1	23.7	74	8	0*	17*	14.5	25	14	1*	60*
			1	11.7	4*	86	1*	10	12.7	1*	79	1*	19
			1	4.7	10*	64	2*	24	5.7	2*	53	2*	43
			1	13.1	23	46	1*	30*	15.5	4	39	1*	56*
			1[a]	20.3	50	30	1	20*	17.0	13	35	1	51*
	WWTP E[w]	20	1	64.4	5	88	0*	6*	66.6	1	86	0*	13*
			1	81.2	6	89	1	5*	82.4	1	87	1	11*
			1	173	4	91	2	3	176	1	90	2	7
China, Chongqing (Ye et al. 2012)	WWTP B	214	1[b]	7.1	51	27	2*	20*	6.0	13	32	4*	52*
	WWTP D	59	1[b]	4.3	24	18*	26	33*	5.5	4	14*	27	56*
	WWTP E	144	1[b]	3.6	13	21*	28	39*	5.3	2	14*	26	58*
	WWTP H	88	1[b]	4.6	21	16*	31	31*	6.0	3	13*	33	51*
Finland (Bjorkblom et al. 2008)	Turku	160	1[b]	8.7	90	8	0*	1*	2.6	62	26	2*	9*
France (Labadie & Budzinski 2005a)	Eysenes	50	1[b]	12.8	54	34	3	9*	8.8	16	50	6	28*
Italy, Roma (Baronti et al. 2000), (Johnson et al. 2000)	Cobis	40	1	6.0	26	48	7	19	6.3	5	46	9	39
			1	5.4	38	41	18	3*	4.3	10	51	30	9*
			1	2.9	29	26	26	19	3.1	6	24	32	38
			1	1.6	44	35	11	11*	1.3	11	42	18	28*
			1	2.3	28	44	6	22	2.4	6	41	8	45
Italy, Roma (Baronti et al. 2000), (Johnson et al. 2000)	Fregene	120	1	5.0	5	79	10	6*	5.4	1	74	13	11*
			1	10.5	3	67	6	24	13.4	1	52	7	40
			1	5.0	16	42	4	38	6.7	2	31	4	62
			1	1.3	23	43	22	13*	1.4	5	41	28	27*
			1	1.2	37	29	6	28	1.3	7	27	8	58
			1	1.3	39	31	4	26	1.4	8	30	6	56

Country	WWTP Name or Code	Equiv. Citizens (thousands)	N	ER-CALUX assay (Houtman et al. 2004) *P*-Percentage of total cEEQ					MELN assay (Leusch et al. 2010) *P*-Percentage of total cEEQ				
				cEEQ# ng/L	E1	E2	E3	EE2	cEEQ## ng/L	E1	E2	E3	EE2
Italy, Roma (Baronti et al. 2000), (Johnson et al. 2000)	Ostia	350	1	2.2	18	55	6	21	2.5	3	48	7	42
			1	7.4	50	40	0*	9	5.3	15	57	1*	28
			1	15.1	43	40	15	2*	11.2	12	54	29	5*
			1	14.6	68	23	1	8	8.3	25	40	3	32
			1	2.5	62	28	3	7*	1.5	21	47	7	24*
			1	9.2	60	32	2	6	5.6	21	54	5	21
			1	6.9	61	25	1	13	4.7	19	36	3	42
			1	9.5	60	37	2	2*	5.2	23	67	4	7*
	Roma Sud	1,200	1	6.6	36	46	14	4*	5.4	9	56	23	11*
			1	13.1	48	30	20	2*	9.5	14	42	38	6*
			1	12.0	51	26	12	11	9.3	14	33	21	32
			1	6.5	55	29	13	3*	4.2	18	45	28	9*
			1	5.6	47	29	14	10	4.4	12	36	23	28
			1	2.4	44	22	10	24	2.3	9	23	14	54
			1	5.6	9	41	42	8	6.6	2	35	49	15
	Roma Est	800	1	2.5	47	33	3	16	2.1	12	40	6	43
			1	1.9	50	38	3	9*	1.4	15	53	6	27*
			1	1.6	28	40	7	25	1.7	5	36	9	50
			1	2.6	32	32	4	32	2.9	6	28	5	61
			1	2.5	47	32	7	14	2.1	12	39	12	37
	Roma Nord	800	1	6.0	22	50	24	5*	5.8	5	51	33	10*
			1	8.2	28	24	44	3*	8.1	6	25	62	8*
			1	2.6	45	35	5	14	2.2	11	43	9	37
			1	1.5	52	30	6	11*	1.1	15	40	12	34*
			1	2.5	30	36	9	25	2.7	6	33	11	50
			1	2.3	35	33	6	26	2.4	7	31	7	55
			1	8.4	57	23	13	7	5.6	18	34	27	22
Slovenia (Avbersek et al. 2011)	WWTP 1	50	1	4.7	10	32	34	24*	6.3	2	24	35	39*
			1	6.6	3	44	36	17*	8.7	0	33	38	28*
	WWTP 2	360	1	5.3	37	40	2*	21*	5.1	8	41	2*	48*
	WWTP 3	100	1	16.7	44	48	1*	7*	12.2	13	66	1*	20*
			1	22.2	28	41	27	5*	20.9	6	43	39	12*
France (Cargouet et al. 2004)	Evry	250	6	9.8	9	46	10	35	13.6	1	33	10	56
	Valenton	1,200	6	13.6	6	53	5	36	19.1	1	38	5	57
	Colombes	800	6	10.9	5	61	7	28	14.3	1	46	7	46
	Aheres	8,000	6	15.3	5	56	6	33	21.0	1	41	6	53
France (Muller et al. 2008)	WWTP 1	120	3[b]	3.9	15	26	2*	57	6.1	2	16	1*	80
			3[b]	4.7	5	64	2*	30*	6.2	1	48	2*	49*
Switzerland (Aerni et al. 2004)c	Glatt	88	7	3.5	41	19	27	14*	3.3	9	21	39	32
	Rontal	27	4	9.8	33	35	13	18	9.8	7	35	18	40
	Surental	38	5	3.0	16	66	3*	16*	3.3	3	61	3*	32*
France (Aerni et al. 2004)c	Fr. 1	30	5	3.8	17	69	2*	12*	3.9	3	67	3*	26*
	Fr. 2	28	4	7.6	7	86	1*	6*	7.8	1	84	1*	13*

Country	WWTP Name or Code	Equiv. Citizens (thousands)	N	ER-CALUX assay (Houtman et al. 2004)					MELN assay (Leusch et al. 2010)				
				P-Percentage of total cEEQ					*P*-Percentage of total cEEQ				
				cEEQ[#] ng/L	E1	E2	E3	EE2	cEEQ[##] ng/L	E1	E2	E3	EE2
values below LOD included as ½ LOD (n=78)			average	12	31	42	10	17	12	9	43	14	34
			median	7	29	37	6	13	6	6	40	7	32
			95%ile	30	63	86	35	38	28	23	84	39	61
			max	173	90	91	45	60	176	62	90	62	80
measurements with all values above LOD (n=32)			average	14	31	39	9	20	15	8	38	13	41
			median	6	31	35	6	20	6	6	36	8	42
			95%ile	44	60	63	33	37	53	20	53	40	62
			max	173	68	91	45	39	176	25	90	53	64

cEEQ - calculated Estrogenic Equivalent; cEEQ = [E1] × EEF_{E1} + [E2] × EEF_{E2} + [E3] × EEF_{E3} + [EE2] × EEF_{EE2}
where [E1], [E2], [E3] and [EE2] are concentrations of estrogens displayed in Table 2 and EEF_s are *in vitro* potencies determined for individual bioassays

[#] EEF_{E1} was 0.126; EEF_{E2} was 1; EEF_{E3} was 0.13; and EEF_{EE2} was 1.12 as determined by Houtman et al. (2004)

[##] EEF_{E1} was 0.025; EEF_{E2} was 1; EEF_{E3} was 0.178; and EEF_{EE2} was 2.455 as determined by Pillon et al. in Leusch et al. (2010)

* value derived from ½ of LOD

[a] one measurement was excluded from displayed data as outlier value

[b] N samples were measured in triplicates. Mean concentrations from repeated measurements are displayed

[c] averages were calculated from minimal and maximal values reported in the study

N - number of samples. If N>1, only the averaged concentrations for N samples were available.

n - number of causes (measurements) included in this calculations

[TF] trickling filter technology

[W] wetland lagoons without any other treatment steps (17d hydraulic retention time)

Table SD 5. Relative percentage contribution of four main steroid estrogens (E1, E2, E3 and EE2) to the total calculated concentrations of estrogenic equivalents (cEEQ) in municipal WWTP effluents as determined for two individual bioassays using sets of EEFs reported in Cargouet et al. in Leusch et al. (2010) and Gutendorf and Westendorf (2001)

Country	WWTP Name or Code	Equiv. Citizens (thousands)	N	MELN assay (Leusch et al. 2010) *P*-Percentage of total cEEQ					E-screen assay (Gutendorf & Westendorf 2001) *P*-Percentage of total cEEQ				
				cEEQ# ng/L	E1	E2	E3	EE2	cEEQ## ng/L	E1	E2	E3	EE2
Austria (Clara et al. 2005)	WWTP 1	2,500	1	76.2	24	39	29	8	56.5	1	53	34	11
	WWTP 2	167	1	9.3	22	27*	15	37	7.6	1	33*	16	50
	WWTP 4	6	1	8.1	12	31*	0*	56	7.6	1	33*	0*	66
			1	8.8	1*	91	1	7*	8.7	0*	92	1	7*
			1	5.1	10	78	1*	11*	4.7	0	85	1*	13*
California (Drewes et al. 2005)	WWTP 3	> 100	1	13.9	32	32	2	34	10.0	2	44	3	52
	WWTP 4	> 500	1	14.8	86	10	1*	3*	2.6	19	57	6*	17*
	WWTP 5	> 100	1	9.6	29	63	4	4*	6.9	2	87	5	6*
	WWTP 7	> 500	1	6.4	64	28	2*	6*	2.5	7	72	4*	18*
Canada (Fernandez et al. 2007)	WWTP B[TF]	740	1	24.1	72	21	3	5	7.5	9	67	8	17
			1	43.1	86	5	0*	9*	8.0	18	25	1*	56*
			1	12.2	8*	82	1*	9	11.4	0*	88	0*	11
			1	5.2	18*	58	1*	22	4.4	1*	69	1*	29
			1	16.4	38	37	0*	25*	10.8	2	56	0*	41*
			1[a]	31.5	68	19	0	13*	11.4	7	53	1	39*
	WWTP E[w]	20	1	68.2	10	84	0*	6*	61.8	0	92	0*	7*
			1	86.2	11	84	0	5*	77.1	1	93	0	6*
			1	180	8	88	1	3	166	0	95	1	4
China, Chongqing (Ye et al. 2012)	WWTP B	214	1[b]	11.1	69	17	1*	13*	3.9	8	49	2*	41*
	WWTP D	59	1[b]	5.0	43	15*	14	29*	3.0	3	25*	20	52*
	WWTP E	144	1[b]	3.8	25	20*	17	38*	2.9	1	26*	19	54*
	WWTP H	88	1[b]	5.1	40	15*	17	28*	3.2	3	24*	24	49*
Finland (Bjorkblom et al. 2008)	Turku	160	1[b]	17.3	95	4	0*	1*	1.5	44	47	1*	8*
France (Labadie & Budzinski 2005a)	Eysenes	50	1[b]	20.3	72	22	1	6*	6.4	9	68	3	20*
Italy, Roma (Baronti et al. 2000), (Johnson et al. 2000)	Cobis	40	1	7.6	43	38	4	15	4.5	3	64	5	28
			1	7.2	59	30	8	2*	3.1	6	72	17	6*
			1	3.5	50	21	13	16	1.8	4	40	22	34
			1	2.3	64	24	5	8*	0.9	7	62	10	21*
			1	3.0	46	34	3	17	1.7	3	59	5	33
	Fregene	120	1	5.1	10	78	6	6*	4.6	0	87	6	7*
			1	10.7	7	66	4	24	10.2	0	69	3	27
			1	5.8	28	36	2	34	4.4	1	48	3	48
			1	1.5	41	36	12	11*	0.9	3	60	17	20*
			1	1.7	56	21	3	21	0.8	5	43	5	47
			1	1.9	57	22	2	19	0.9	5	47	4	45

Country	WWTP Name or Code	Equiv. Citizens (thousands)	N	MELN assay (Leusch et al. 2010) *P*-Percentage of total cEEQ					E-screen assay (Gutendorf & Westendorf 2001) *P*-Percentage of total cEEQ				
				cEEQ# ng/L	E1	E2	E3	EE2	cEEQ## ng/L	E1	E2	E3	EE2
			1	2.6	32	46	3	19	1.8	2	66	4	29
Italy, Roma (Baronti et al. 2000), (Johnson et al. 2000)	Ostia	350	1	11.5	68	26	0*	6	4.1	8	73	0*	18
			1	21.3	64	28	7	1*	8.1	7	74	16	4*
			1	25.3	82	13	0	5	5.6	15	59	2	25
			1	4.2	78	17	1	4*	1.1	12	66	4	17*
			1	15.2	76	20	1	4	4.2	11	72	3	14
			1	11.5	77	15	1	8	3.1	11	55	2	32
			1	15.6	76	22	1	1*	4.2	11	83	2	4*
	Roma Sud	1,200	1	8.9	57	34	6	3*	4.0	5	75	12	8*
			1	19.0	69	21	9	2*	6.3	8	64	23	5*
			1	18.2	70	17	5	8	5.9	9	53	13	26
			1	10.2	74	19	5	2*	2.9	10	66	17	7*
			1	8.2	68	20	6	7	2.9	8	56	14	22
			1	3.5	63	15	4	17	1.4	6	38	9	47
			1	5.2	19	44	28	9	4.1	1	56	31	12
	Roma Est	800	1	3.7	65	22	1	11	1.4	7	58	3	32
			1	2.9	68	25	1	6*	1.0	8	71	3	19*
			1	2.0	46	31	3	20	1.2	3	54	5	38
			1	3.4	50	23	2	24	1.9	4	44	3	49
			1	3.8	66	21	3	9	1.4	7	58	7	28
	Roma Nord	800	1	6.9	40	43	13	4*	4.2	3	71	19	7*
			1	9.3	51	21	24	3*	4.5	4	45	44	7*
			1	3.9	64	24	2	10	1.5	7	61	5	27
			1	2.3	71	19	3	8*	0.7	9	59	7	25*
			1	3.3	49	28	4	19	1.8	4	51	7	39
			1	3.1	54	24	3	20	1.5	4	48	5	43
			1	13.2	76	14	5	4	3.5	11	54	17	18
Slovenia (Avbersek et al. 2011)	WWTP 1	50	1	4.7	22	32	22	25*	3.7	1	41	24	34*
			1	6.0	7	49	25	19*	5.5	0	53	24	23*
	WWTP 2	360	1	7.4	56	28	1*	15*	3.6	5	59	1*	35*
	WWTP 3	100	1	24.8	63	33	0*	5*	10.0	6	81	0*	13*
			1	26.7	48	34	14	4*	14.0	4	64	23	9*
France (Cargouet et al. 2004)	Evry	250	6	10.5	17	43	6	34	9.0	1	50	6	43
	Valenton	1,200	6	14.3	11	50	3	35	13.2	0	55	3	42
	Colombes	800	6	11.2	10	59	4	28	10.4	0	63	4	33
	Aheres	8,000	6	15.9	10	54	3	33	14.8	0	58	3	38
France (Muller et al. 2008)	WWTP 1	120	3[b]	4.6	27	22	1*	50	3.6	1	28	1*	70
			3[b]	5.0	10	60	1*	29*	4.6	0	65	1*	34*
Switzerland (Aerni et al. 2004)c	Glatt	88	7	4.7	63	14	12	10*	1.8	6	37	28	29*
	Rontal	27	4	12.9	53	26	6	14	6.4	4	53	11	32
	Surental	38	5	3.5	28	57	1*	14*	2.6	2	76	2*	20*
France (Aerni et al. 2004)c	Fr. 1	30	5	4.5	29	59	1*	11*	3.3	2	81	1*	16*
	Fr. 2	28	4	8.1	13	80	1*	6*	7.1	1	91	1*	8*

Country	WWTP Name or Code	Equiv. Citizens (thousands)	N	MELN assay (Leusch et al. 2010) *P*-Percentage of total cEEQ					E-screen assay (Gutendorf & Westendorf 2001) *P*-Percentage of total cEEQ				
				cEEQ[#] ng/L	E1	E2	E3	EE2	cEEQ[##] ng/L	E1	E2	E3	EE2
values below LOD accounted as ½ LOD (n=78)			average	15	45	35	5	14	9	5	60	8	27
			median	8	49	28	3	10	4	4	59	4	25
			95%ile	47	78	82	22	36	21	12	91	25	53
			max	180	95	91	29	56	166	44	95	44	70
measurements with all values above LOD (n=32)			average	16	46	32	5	17	11	5	56	8	32
			median	8	50	25	3	17	4	4	55	5	32
			95%ile	48	76	62	20	34	34	11	70	26	49
			max	180	82	88	29	35	166	15	95	34	52

cEEQ - calculated Estrogenic Equivalent; cEEQ = [E1] × EEF_{E1} + [E2] × EEF_{E2} + [E3] × EEF_{E3} + [EE2] × EEF_{EE2}
where [E1] ,[E2],[E3] and [EE2] are concentrations of estrogens displayed in Table 2 and EEF_s are *in vitro* potencies determined for individual bioassays

[#] EEF_{E1} was 0.251; EEF_{E2} was 1; EEF_{E3} was 0.081; and EEF_{EE2} was 1.148 as determined by Cargouet et al.in Leusch et al. (2010)

[##] EEF_{E1} was 0.01; EEF_{E2} was 1; EEF_{E3} was 0.071; and EEF_{EE2} was 1.259 as determined by Gutendorf and Westendorf (2001)

* value derived from ½ of LOD

[a] one measurement was excluded from displayed data as outlier value

[b] N samples were measured in triplicates. Mean concentrations from repeated measurements are displayed

[c] averages were calculated from minimal and maximal values reported in the study

N - number of samples. If N>1, only the averaged concentrations for N samples were available.

n - number of causes (measurements) included in this calculations

[TF] trickling filter technology

[W] wetland lagoons without any other treatment steps (17d hydraulic retention time)

Table SD 6. Relative percentage contribution of four main steroid estrogens (E1, E2, E3 and EE2) to the total calculated concentrations of estrogenic equivalents (cEEQ) in municipal WWTP effluents determined for E-screen assay using 2 sets of EEFs reported in Drewes et al. (2005) and Fang et al. in Leusch (2010)

Country	WWTP Name or Code	Equiv. Citizens (thousands)	N	E-screen assay (Drewes et al. 2005) *P*-Percentage of total cEEQ					E-screen assay (Leusch et al. 2010) *P*-Percentage of total cEEQ				
				cEEQ[#] ng/L	E1	E2	E3	EE2	cEEQ[##] ng/L	E1	E2	E3	EE2
Austria (Clara et al. 2005)	WWTP 1	2,500	1	127.6	8	24	64	5	107.8	3	28	64	5
	WWTP 2	167	1	12.6	9	20*	40	32	10.5	3	24*	41	32
	WWTP 4	6	1	8.6	6	29*	2*	63	7.3	2	34*	2*	62
			1	9.0	1*	89	3	7*	8.8	0*	91	3	6*
			1	5.1	5	79	3*	13*	4.8	2	84	3*	12*
California (Drewes et al. 2005)	WWTP 3	> 100	1	13.5	18	33	9	41	10.8	7	41	9	43
	WWTP 4	> 500	1	9.5	72	16	7*	5*	4.7	47	32	13*	8*
	WWTP 5	> 100	1	9.4	16	64	15	5*	8.1	6	74	15	5*
	WWTP 7	> 500	1	4.9	45	37	8*	10*	3.3	22	55	11*	12*
Canada (Fernandez et al. 2007)	WWTP B[TF]	740	1	18.0	52	28	13	7	11.1	27	45	18	10
			1	26.8	74	7	1*	18*	12.6	51	16	1*	32*
			1	12.1	4*	83	2*	11	11.5	1*	87	2*	10
			1	5.1	10*	59	4*	27	4.5	4*	67	4*	25
			1	14.4	23	42	2*	33*	11.3	10	53	2*	35*
			1[a]	22.6	51	27	1	21*	13.9	27	43	2	29*
	WWTP E[w]	20	1	65.8	6	87	0*	7*	62.4	2	91	0*	6*
			1	83.2	6	87	1	6*	78.7	2	91	1	5*
			1	179	4	88	4	4	172	1	92	3	3
China, Chongqing (Ye et al. 2012)	WWTP B	214	1[b]	8.1	51	24	5*	21*	4.9	27	38	6*	28*
	WWTP D	59	1[b]	6.1	19	12*	41	28*	4.6	8	16*	45	30*
	WWTP E	144	1[b]	5.2	10	14*	44	32*	4.3	4	18*	45	33*
	WWTP H	88	1[b]	6.8	16	11*	48	25*	5.3	7	14*	52	27*
Finland (Bjorkblom et al. 2008)	Turku	160	1[b]	9.8	91	7	1*	1*	3.7	76	19	2*	3*
France (Labadie & Budzinski 2005a)	Eysenes	50	1[b]	14.4	54	31	6	9*	8.8	29	50	8	13*
Italy, Roma (Baronti et al. 2000), (Johnson et al. 2000)	Cobis	40	1	7.0	25	42	14	19	5.4	10	54	15	21
			1	6.8	33	32	31	3*	4.9	15	44	37	3*
			1	4.0	23	18	42	16	3.0	10	24	47	18
			1	1.9	41	29	20	11*	1.3	20	42	25	13*
			1	2.6	28	38	12	22	2.0	12	50	14	25
	Fregene	120	1	5.8	5	69	20	6*	5.4	2	74	19	5*
			1	11.8	3	59	12	25	10.9	1	64	12	23
			1	5.7	15	37	8	40	4.7	6	45	9	41
			1	1.7	19	32	37	12*	1.4	8	40	40	12*
			1	1.4	35	25	12	28	1.0	16	35	14	34
			1	1.5	38	27	8	27	1.1	18	39	10	33

Country	WWTP Name or Code	Equiv. Citizens (thousands)	N	E-screen assay (Drewes et al. 2005) *P*-Percentage of total cEEQ					E-screen assay (Leusch et al. 2010) *P*-Percentage of total cEEQ				
				cEEQ# ng/L	E1	E2	E3	EE2	cEEQ## ng/L	E1	E2	E3	EE2
			1	2.5	18	48	11	23	2.1	7	58	12	23
Italy, Roma (Baronti et al. 2000), (Johnson et al. 2000)	Ostia	350	1	8.1	52	37	1*	10	5.1	27	59	1*	13
			1	18.9	38	32	28	2*	13.2	18	46	34	2*
			1	16.3	68	20	3	9	8.5	42	39	4	15
			1	2.9	61	25	6	7*	1.6	35	45	10	10*
			1	10.3	60	29	4	6	5.9	34	51	6	9
			1	7.7	61	22	3	14	4.3	35	39	4	21
			1	10.4	61	34	3	2*	6.0	34	58	5	3*
	Roma Sud	1,200	1	8.1	33	37	26	4*	5.9	15	51	30	5*
			1	17.3	41	23	34	2*	11.6	20	35	43	2*
			1	14.8	46	21	22	11	9.4	24	33	29	14
			1	8.1	50	23	24	2*	5.1	26	38	33	3*
			1	7.0	43	23	25	10	4.6	21	35	32	12
			1	2.9	40	18	18	24	1.9	20	27	23	30
			1	8.7	6	26	61	6	7.4	2	31	61	6
	Roma Est	800	1	2.8	47	29	7	17	1.8	23	45	9	22
			1	2.1	51	34	6	10*	1.3	26	54	8	13*
			1	1.8	27	34	13	26	1.4	12	45	15	28
			1	3.0	31	27	8	33	2.1	14	38	10	38
			1	3.0	45	27	14	14	1.9	23	41	18	18
	Roma Nord	800	1	8.1	18	37	40	4*	6.5	7	46	42	4*
			1	13.2	19	15	63	3*	10.1	8	20	69	3*
			1	3.0	44	31	11	15	2.0	22	46	14	18
			1	1.7	50	26	12	12*	1.1	26	41	17	16*
			1	3.0	28	30	17	25	2.2	12	41	19	28
			1	2.6	34	28	11	27	1.9	15	39	13	32
			1	10.4	52	18	24	6	6.3	28	30	33	9
Slovenia (Avbersek et al. 2011)	WWTP 1	50	1	7.1	8	21	52	19*	5.9	3	25	53	19*
			1	9.9	2	29	55	14*	8.7	1	33	53	13*
	WWTP 2	360	1	5.9	38	36	4*	23*	4.1	17	51	4*	27*
	WWTP 3	100	1	18.0	46	45	1*	7*	12.1	22	67	1*	9*
			1	30.7	22	29	44	4*	23.8	9	38	48	5*
France (Cargouet et al. 2004)	Evry	250	6	11.8	8	38	18	35	10.1	3	44	18	34
	Valenton	1,200	6	15.5	6	46	10	38	13.7	2	53	9	36
	Colombes	800	6	12.5	5	53	13	29	11.2	2	59	13	27
	Aheres	8,000	6	17.5	5	49	11	35	15.6	2	55	11	32
France (Muller et al. 2008)	WWTP 1	120	3[b]	4.5	15	22	3*	60	3.6	6	28	4*	63
			3[b]	5.1	5	58	4*	33*	4.6	2	65	3*	30*
Switzerland (Aerni et al. 2004)c	Glatt	88	7	5.0	32	14	43	12*	3.5	15	19	52	14*
	Rontal	27	4	12.2	30	28	24	18	8.9	13	38	28	20
	Surental	38	5	3.3	16	61	6*	17*	2.8	6	71	6*	17*
France (Aerni et al. 2004)c	Fr. 1	30	5	4.1	17	64	4*	14*	3.5	7	75	4*	14*
	Fr. 2	28	4	7.8	7	83	2*	7*	7.3	3	89	2*	7*

Country	WWTP Name or Code	Equiv. Citizens (thousands)	N	E-screen assay (Drewes et al. 2005) *P*-Percentage of total cEEQ					E-screen assay (Leusch et al. 2010) *P*-Percentage of total cEEQ				
				cEEQ[#] ng/L	E1	E2	E3	EE2	cEEQ[##] ng/L	E1	E2	E3	EE2
values below LOD included as ½ LOD (n=78)			average	14	30	36	17	17	11	15	47	19	19
			median	8	27	30	11	14	5	12	44	13	15
			95%ile	36	62	84	53	39	30	36	89	53	39
			max	179	91	89	64	63	172	76	92	69	63
measurements with all values above LOD (n=32)			average	17	30	33	16	21	14	15	44	19	23
			median	7	29	29	12	21	5	13	41	14	23
			95%ile	67	61	56	51	39	57	35	61	53	39
			max	179	68	88	64	41	172	42	92	64	43

cEEQ - calculated Estrogenic Equivalent; cEEQ = [E1] × EEF_{E1} + [E2] × EEF_{E2} + [E3] × EEF_{E3} + [EE2] × EEF_{EE2}
where [E1], [E2], [E3] and [EE2] are concentrations of estrogens displayed in Table 2 and EEF_s are *in vitro* potencies determined for individual bioassays

[#] EEF_{E1} was 0.135; EEF_{E2} was 1; EEF_{E3} was 0.295; and EEF_{EE2} was 1.349 as determined by Drewes et al. (2005)

[##] EEF_{E1} was 0.044; EEF_{E2} was 1; EEF_{E3} was 0.251; and EEF_{EE2} was 1.222 as determined by Fang et al. in Leusch (2010)

* value derived from ½ of LOD

[a] one measurement was excluded from displayed data as outlier value

[b] N samples were measured in triplicates. Mean concentrations from repeated measurements are displayed

[c] averages were calculated from minimal and maximal values reported in the study

N - number of samples. If N>1, only the averaged concentrations for N samples were available.

n - number of causes (measurements) included in this calculations

[TF] trickling filter technology

[W] wetland lagoons without any other treatment steps (17d hydraulic retention time)

Table SD 7. Relative percentage contribution of four main steroid estrogens (E1, E2, E3 and EE2) to the total calculated concentrations of estrogenic equivalents (cEEQ) in municipal WWTP effluents determined for two individual bioassays using sets of EEFs reported in Leusch et al. (2010) and Gutendorf and Westendorf (2001)

Country	WWTP Name or Code	Equiv. Citizens (thousands)	N	E-screen assay (Leusch et al. 2010) *P*-Percentage of total cEEQ					MVLN assay (Gutendorf & Westendorf 2001) *P*-Percentage of total cEEQ				
				cEEQ# ng/L	E1	E2	E3	EE2	cEEQ## ng/L	E1	E2	E3	EE2
Austria (Clara et al. 2005)	WWTP 1	2,500	1	57.6	1	52	41	6	59.8	1	50	38	10
	WWTP 2	167	1	6.1	1	41*	24	33	7.7	1	32*	18	48
	WWTP 4	6	1	5.3	1	47*	1*	51	7.6	1	33*	1*	66
			1	8.4	0*	95	1	4*	8.7	0*	92	1	7*
			1	4.4	1	91	1*	8*	4.7	0	85	1*	13*
California (Drewes et al. 2005)	WWTP 3	> 100	1	7.7	3	57	4	36	10.0	2	44	3	51
	WWTP 4	> 500	1	2.5	23	60	8*	9*	2.6	19	57	7*	17*
	WWTP 5	> 100	1	6.8	2	89	6	3*	7.0	2	86	6	6*
	WWTP 7	> 500	1	2.3	8	77	5*	10*	2.5	7	72	4*	17*
Canada (Fernandez et al. 2007)	WWTP B[TF]	740	1	7.1	11	70	10	9	7.6	9	66	9	16
			1	6.1	27	33	1*	39*	8.0	18	25	1*	56*
			1	10.8	0*	93	1*	6	11.4	0*	88	1*	11
			1	3.8	1*	79	2*	18	4.4	1*	69	1*	29
			1	8.7	3	69	1*	27*	10.7	2	56	1*	41*
			1[a]	9.4	10	64	1	25*	11.4	7	53	1	39*
	WWTP E[w]	20	1	59.8	1	95	0*	4*	61.8	0	92	0*	7*
			1	75.2	1	96	0	3*	77.2	1	93	0	6*
			1	164	0	96	1	2	167	0	95	1	4
China, Chongqing (Ye et al. 2012)	WWTP B	214	1[b]	3.2	11	60	3*	26*	3.9	8	49	3*	40*
	WWTP D	59	1[b]	2.4	4	31*	30	35*	3.1	3	24*	23	50*
	WWTP E	144	1[b]	2.3	2	33*	29	37*	3.0	1	25*	21	52*
	WWTP H	88	1[b]	2.6	3	29*	36	32*	3.3	2	23*	28	47*
Finland (Bjorkblom et al. 2008)	Turku	160	1[b]	1.5	48	46	2*	4*	1.5	44	47	2*	8*
France (Labadie & Budzinski 2005a)	Eysenes	50	1[b]	6.0	11	74	4	11*	6.5	9	68	4	19*
Italy, Roma (Baronti et al. 2000), (Johnson et al. 2000)	Cobis	40	1	4.0	4	72	7	17	4.6	3	64	6	27
			1	3.1	6	71	20	3*	3.2	5	70	19	6*
			1	1.6	5	45	30	20	1.9	4	39	25	32
			1	0.8	8	66	13	12*	0.9	6	61	12	21*
			1	1.5	4	69	6	20	1.7	3	59	5	32
	Fregene	120	1	4.5	0	88	8	4*	4.7	0	86	7	7*
			1	8.9	0	78	5	17	10.2	0	69	4	27
			1	3.5	2	61	4	33	4.4	1	47	3	48
			1	0.9	3	64	21	12*	1.0	3	59	19	20*
			1	0.6	6	54	8	32	0.8	5	43	6	46
			1	0.7	7	58	5	30	0.9	5	47	4	44

Country	WWTP Name or Code	Equiv. Citizens (thousands)	N	E-screen assay (Leusch et al. 2010) *P*-Percentage of total cEEQ					MVLN assay (Gutendorf & Westendorf 2001) *P*-Percentage of total cEEQ				
				cEEQ# ng/L	E1	E2	E3	EE2	cEEQ## ng/L	E1	E2	E3	EE2
			1	1.6	2	75	5	18	1.8	2	65	4	29
Italy, Roma (Baronti et al. 2000), (Johnson et al. 2000)	Ostia	350	1	3.8	9	79	1*	11	4.1	8	74	1*	18
			1	8.3	7	72	18	2*	8.3	6	72	18	4*
			1	5.1	18	65	2	15	5.6	15	59	2	25
			1	1.0	14	71	5	10*	1.1	12	66	5	17*
			1	4.0	13	76	3	8	4.2	11	72	3	14
			1	2.7	15	63	2	20	3.1	11	55	2	32
			1	4.2	12	83	2	2*	4.2	11	82	2	4*
	Roma Sud	1,200	1	4.0	6	75	15	4*	4.1	5	73	14	8*
			1	6.5	9	62	26	3*	6.5	8	62	26	5*
			1	5.4	11	57	17	15	6.0	8	51	15	25
			1	2.9	12	65	20	3*	2.9	10	65	19	6*
			1	2.7	9	60	18	13	2.9	7	54	16	22
			1	1.1	9	47	14	31	1.4	6	37	11	46
			1	4.1	1	55	37	7	4.3	1	53	34	12
	Roma Est	800	1	1.2	9	67	4	20	1.4	7	58	4	32
			1	0.9	9	76	4	11*	1.0	8	70	3	18*
			1	1.0	4	64	7	24	1.2	3	53	6	38
			1	1.5	5	56	5	34	1.9	4	44	4	49
			1	1.2	9	64	10	17	1.4	7	57	8	28
	Roma Nord	800	1	4.2	3	71	22	4*	4.3	3	69	21	7*
			1	4.8	4	42	50	4*	4.8	4	41	48	6*
			1	1.4	8	68	7	16	1.5	7	61	6	27
			1	0.7	11	65	9	15*	0.8	9	59	8	25*
			1	1.5	5	60	10	25	1.8	4	50	8	39
			1	1.3	6	59	7	28	1.6	4	48	5	43
			1	3.4	13	56	21	10	3.6	11	52	19	17
Slovenia (Avbersek et al. 2011)	WWTP 1	50	1	3.3	1	46	32	21*	3.8	1	39	27	33*
			1	5.2	0	56	30	13*	5.7	0	51	27	22*
	WWTP 2	360	1	3.0	6	70	2*	22*	3.6	5	59	2*	35*
	WWTP 3	100	1	9.5	7	85	1*	7*	10.0	6	81	1*	12*
			1	14.1	4	64	28	5*	14.6	4	62	26	9*
France (Cargouet et al. 2004)	Evry	250	6	7.3	1	62	9	29	9.1	1	50	7	43
	Valenton	1,200	6	10.7	1	67	4	28	13.2	0	55	3	42
	Colombes	800	6	9.0	1	74	5	20	10.5	0	63	5	32
	Aheres	8,000	6	12.3	1	70	5	25	14.9	0	58	4	38
France (Muller et al. 2008)	WWTP 1	120	3[b]	2.5	2	41	2*	55	3.6	1	28	1*	70
			3[b]	3.9	1	77	1*	22*	4.6	0	65	1*	34*
Switzerland (Aerni et al. 2004)c	Glatt	88	7	1.7	8	40	36	17*	1.9	6	35	31	28*
	Rontal	27	4	5.7	5	60	15	19	6.5	4	52	13	31
	Surental	38	5	2.4	2	84	2*	12*	2.6	2	76	2*	20*
France (Aerni et al. 2004)c	Fr. 1	30	5	3.1	2	87	2*	9*	3.3	2	81	2*	16*
	Fr. 2	28	4	6.9	1	94	1*	4*	7.1	1	91	1*	7*

Country	WWTP Name or Code	Equiv. Citizens (thousands)	N	E-screen assay (Leusch et al. 2010) *P*-Percentage of total cEEQ					MVLN assay (Gutendorf & Westendorf 2001) *P*-Percentage of total cEEQ				
				cEEQ# ng/L	E1	E2	E3	EE2	cEEQ## ng/L	E1	E2	E3	EE2
values below LOD included as ½ LOD (n=78)			average	9	6	66	11	17	9	5	59	10	26
			median	4	5	65	6	15	4	4	59	5	25
			95%ile	21	15	94	36	36	22	12	91	28	51
			max	164	48	96	50	55	167	44	95	48	70
measurements with all values above LOD (n=32)			average	11	6	64	10	20	11	5	55	9	31
			median	3	5	62	7	20	4	4	54	6	32
			95%ile	33	14	77	33	34	35	11	70	29	48
			max	164	18	96	41	36	167	15	95	38	51

cEEQ - calculated Estrogenic Equivalent; cEEQ = [E1] × EEF_{E1} + [E2] × EEF_{E2} + [E3] × EEF_{E3} + [EE2] × EEF_{EE2}
where [E1], [E2], [E3] and [EE2] are concentrations of estrogens displayed in Table 2 and EEF_s are *in vitro* potencies determined for individual bioassays

- EEF_{E1} was 0.011; EEF_{E2} was 1; EEF_{E3} was 0.085; and EEF_{EE2} was 0.676 as determined by Leusch et al.(2010)

- EEF_{E1} was 0.01; EEF_{E2} was 1; EEF_{E3} was 0.083; and EEF_{EE2} was 1.25 as determined by Gutendorf and Westendorf (2001)

* value derived from ½ of LOD

[a] one measurement was excluded from displayed data as outlier value

[b] N samples were measured in triplicates. Mean concentrations from repeated measurements are displayed

[c] averages were calculated from minimal and maximal values reported in the study

N - number of samples. If N>1, only the averaged concentrations for N samples were available.

n - number of causes (measurements) included in this calculations

[TF] trickling filter technology

[W] wetland lagoons without any other treatment steps (17d hydraulic retention time)

2. Concentrations of in vitro estrogenic activity below which the PNECs of E1, E2, E3 and EE2 would not be exceeded

Table SD 8. Concentrations of overall estrogenic activity below which the *in vivo* derived PNECs for individual steroid estrogens would not be exceeded (bold numbers represent the lowest $EEQ\text{-}SSE_{Ei}$ or in other words the EEQ-SSEs).

	longer-term exposures (ng/L EEQ)				shorter-term exposures (ng/L EEQ)			
Assay	$EEQ\text{-}SSE_{E1}$	$EEQ\text{-}SSE_{E2}$	$EEQ\text{-}SSE_{E3}$	$EEQ\text{-}SSE_{EE2}$	$EEQ\text{-}SSE_{E1}$	$EEQ\text{-}SSE_{E2}$	$EEQ\text{-}SSE_{E3}$	$EEQ\text{-}SSE_{EE2}$
YES (Aerni et al. 2004, Rutishauser et al. 2004)	2.4	2.3	14.0	**0.3**	7.9	5.9	46.5	**1.7**
YES (Svenson et al. 2003)	1.2	2.3	12.1	**0.4**	4.1	5.7	40.5	**2.0**
YES (Caldwell et al. 2012)	2.1	2.3	13.0	**0.3**	6.8	5.7	43.3	**1.6**
YES (Leusch et al. 2010)	0.6	2.1	9.4	**0.2**	2.2	5.3	31.3	**1.2**
ER-CALUX (Sonneveld et al. 2006)	**0.2**	2.1	11.0	0.3	**0.6**	5.3	36.5	1.5
ER-CALUX (Avbersek et al. 2011)	2.5	2.4	23.1	**0.4**	8.3	6.1	76.9	**2.0**
ER-CALUX (Houtman et al. 2004)	0.8	2.2	17.5	**0.3**	2.7	5.5	58.2	**1.4**
MELN (Leusch et al. 2010)	**0.2**	2.2	20.3	0.4	**0.8**	5.6	67.6	1.9
MELN (Leusch et al. 2010)	1.6	2.3	16.6	**0.3**	5.3	5.7	55.4	**1.6**
E-screen (Gutendorf & Westendorf 2001)	**0.1**	2.1	12.3	0.2	**0.5**	5.3	41.1	1.2
E-screen (Drewes et al. 2005)	0.9	2.3	27.8	**0.3**	3.0	5.7	92.8	**1.6**
E-screen (Leusch et al. 2010)	0.3	2.2	23.5	**0.3**	**1.1**	5.4	78.4	1.3
E-screen (Leusch et al. 2010)	**0.1**	2.1	12.6	0.2	**0.5**	5.2	41.9	0.9
MVLN*	0.8	2.2	16.0	**0.3**	2.8	5.5	53.5	**1.4**
MVLN (Gutendorf & Westendorf 2001)	**0.1**	2.1	13.0	0.2	**0.5**	5.3	43.5	1.2

EEQ-SSE - estrogenic equivalents - safe regarding steroid estrogens = proposed safe levels of *in vitro* determined estrogenic activity
YES - yeast estrogenicity screening assay (Routledge & Sumpter 1996)
ER-CALUX - Estrogen Receptor mediated Chemical Activated LUciferase gene eXpression assay (Van der Burg et al. 2010)
MELN - MCF-7 cells stably transfected with the estrogen responsive gene ERE-betaGlob-Luc-SVNeo (Balaguer et al. 2000)
E-SCREEN - the MCF7 cell proliferation assay (Soto et al. 1998)
MVLN - MCF-7 cells stably transfected with luciferase gene under the control of estrogen receptor (Demirpence et al. 1993)
*Unpublished data - *in vitro* potencies were determined by the authors of the present study by comparing the EC50 values from dose-response curves of E2 and other estrogens

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