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Research article

Estrogenic activity in wastewater treatment plants through *in vitro* effect-based assays: Insights into extraction phase

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ABSTRACT

Effluents of wastewater treatment plants can abundantly spread endocrine disrupting chemicals in the environment. To improve water quality monitoring, the use of effect-based tools that measure estrogenic activity has been suggested, however their results could be influenced by different factors.

This study compared the estrogenic activity of wastewater samples extracted with two stationary phases and tested with two *in vitro* effect-based assays to investigate whether and how stationary phases and assays could influence biomonitoring data.

During four seasonal periods, the effluents of six WWTPs located in northern Italy were sampled. After the extraction using two different stationary phases (HLB, C18), the samples ($n = 72$) were tested using two effect-based assays: a gene reporter luciferase assay on mammalian cells (MELN) and yeast estrogen screen assay (YES).

The results showed that estrogenic activity of HLB extracts was significantly different from the activity of C18 extracts, suggesting that extraction phase can influence biomonitoring data. Moreover, the estrogenic activity was overall higher using gene reporter MELN assay than using YES assay, suggesting that, due to difference in cell membrane permeability and metabolic activation, the applied cell model can affect the biomonitoring results. Finally, from the comparison between the activity of the final effluent and the environmentally safe estrogenic levels in surface waters, MELN data suggested that the activity of this effluent may pose an environmental risk, while YES data showed that it should not be considered a threat to the receiving surface waters.

This study pointed out that a standardized approach is needed to assess the estrogenic activity of waters; it reported important data to select the most suitable stationary phase for samples extraction (samples extracted with C18 sorbent showed higher estradiol equivalent concentration values) and the most appropriate bioassay (gene reporter luciferase MELN assay was more sensitive than YES assay) to assess the environmental risk, thus protecting human health.

1. Introduction

Endocrine disrupting chemicals (EDCs) are environmental contaminants able to interfere with the function of the endocrine system causing negative health effects, both in humans and animals (Gogoi et al., 2018; Vieira et al., 2021). EDCs can affect health at very low doses, at a cellular level, and they may disrupt hormone functions through the direct bind with hormone receptors (Pamplona-Silva et al., 2018). In humans, the suspected effects of EDCs include cancer of hormone sensitive organs (e.g. breast, prostate, testis), early puberty, cryptorchidism, hypospadias and reduced fertility (Kabir et al., 2015; Pamplona-Silva et al., 2018); moreover, exposure to EDCs can be particularly harmful during childhood or adolescence, inducing effects that are evident after years (Kabir et al., 2015).

EDCs are ubiquitous because they are contained in many products used in residential, industrial and agricultural applications (Metcalfe et al., 2022). They may be classified by origin in natural compounds (sexual steroids, phytoestrogens, mycotoxins) and synthetic compounds,

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such as those employed in industry (phthalates, phenols, polycyclic aromatic hydrocarbons), agriculture (DDT, atrazine, glyphosate) and in the pharmaceutical field (17α-ethynilestradiol, diethilstilbestrol) (Kabir et al., 2015; Chen et al., 2022).

The discharge of effluents from wastewater treatment plants (WWTPs) into receiving water bodies is considered one of the main causes of EDCs release in the aquatic environment. Indeed, although EDCs are subjected to numerous treatments in WWTPs (such as coagulation, flocculation, precipitation, and biological oxidation), they are often detected in wastewater effluents of both municipal and industrial origin because they are only partially removed by these treatments (Alygizakis et al., 2023; Bicchi et al., 2009; Gogoi et al., 2018; Kabir et al., 2015; Pamplona-Silva et al., 2018; Ting and Praveena, 2017). Therefore, the monitoring of EDCs occurrence in WWTP effluents is of particular interest in order to protect both humans and environment. EDCs monitoring can be carried out through chemical and effect-based biological analyses. Chemical analysis allows identification and quantification of specific compounds contained in wastewaters, so it cannot detect all EDCs including their metabolites. Instead, biological analysis allows the assessment of the biological effect induced by all compounds with a similar action mechanism. For example, through biological analysis the overall activity on estrogen receptor can be quantified considering all estrogenic molecules (including unknown compounds and those that cannot be detected through analytical methods) and the possible additive, synergic and antagonistic effects among them (Gea et al., 2020; Jarošová et al., 2014; Kunz et al., 2015). In this contest effect-based monitoring can serves as a complementary tool to the chemical analysis approach for WWTP effluents (Alygizakis et al., 2023; Escher et al., 2020).

In the evaluation of biological activity, environmental matrices cannot be analysed directly both because endogenous chemical substances would disturb the analysis and because pollutants are present in very low concentrations. For these reasons, extraction is necessary to isolate, concentrate and enrich pollutants (Escher et al., 2020). As regards wastewater samples, solid phase extraction (SPE) is one of the most applied extraction techniques capable of capturing a large fraction of organic chemicals (Escher et al., 2020; Neale et al., 2018; Robitaille et al., 2022; Wangmo et al., 2018).

Since this method is based on affinity between the sorbent solid phase and molecules contained in the environmental sample, the use of different sorbent phases could lead to the retention of different molecules depending on their chemical characteristics (Neale et al., 2018; Sadutto and Picó, 2020; Schäfer et al., 2011).

Samples extraction could significantly alter biomonitoring data (Robitaille et al., 2022) and only few studies compared biological effects (estrogenic activity) of wastewater samples extracted using different sorbent phases (Abbas et al., 2019; Escher et al., 2008; Körner et al., 1999; Leusch et al., 2006; Wagner and Oehlmann, 2011).

Therefore, the aim of the present study was to compare the estrogenic activity of wastewater samples extracted with two different sorbent solid phases (HLB and C18 sorbents), to establish whether and how the choice of the extraction phase may affect the results of biological assays. The effluents of six WWTPs located in Northern Italy were collected during four seasonal sampling campaigns and were extracted using two different solid phases (HLB and C18 sorbents). After extraction, the estrogenic activity of the extracts was tested using two assays: a gene reporter luciferase assay on mammalian cells (MELN) and a yeast estrogen screen assay (YES assay).

The estrogenic activity was compared among effluent samples extracted with different phases, among effluent samples from different water treatment plants treating both municipal and industrial wastewaters and among effluent samples collected in different seasons. Moreover, the results obtained with the two biological assays were compared with each other. Finally, the estrogenic activity of the final cumulative effluent was discussed for its potential to pose a risk for the environment.

2. Materials and methods

2.1. WWTPs

The effluents of six WWTPs located in Northern Italy were analysed. These WWTPs belong to a unique system whose schematic representation is reported in Fig. 1. Each WWTP includes water and sludge treatment lines, the treatment technology of water line is composed by grit removal, oil separation, sedimentation, de-nitrification, nitrification, oxidation, flocculation, disinfection (using sodium hypochlorite or peracetic acid). WWTP 1 treats both municipal and industrial (tannery) wastewaters. WWTP 2 treats only industrial (pharmaceutical) wastewaters and its effluent (OUT 2) is discharged in the untreated water of WWTP 3 that deals with both municipal and industrial (galvanic) wastewaters. WWTP 4 treats both urban and industrial (tanning and textile) wastewaters and WWTP 5 treats both urban and industrial (food, tanning, textile) wastewaters. WWTP 1, 3, 4, 5 collect and treat together municipal and industrial wastewaters, while WWTP 6 treats separately municipal and industrial (tannery) wastewaters; after the wastewater treatments, the municipal effluent and the industrial effluent of WWTP 6 (OUT 6M and OUT 6I, respectively) are mixed together in a final effluent (OUT 6 = 33 % OUT 6M + 67 % OUT 6I). The final effluents of the WWTPs 1, 3 (which collect and treat the final effluent of WWTP 2), 4, 5, 6 are mixed together in a cumulative effluent which is discharged in a river (OUT 7 = OUT 1 + OUT 3 + OUT 4 + OUT 5 + OUT 6). OUT 7 is a flow proportional composite pool of the other effluents and it is highly influenced by OUT 6 (i.e. the effluent of WWTP 6) since OUT 6 represents approximately 45 % of the total volume of OUT 7 (Table S1 of Supplementary Materials shows the flow rates of all WWTPs and the relative percentage of volume to total WWTP 7).

2.2. Sampling

The effluents of the six WWTPs were collected as 24 h composite samples (3 L) during four seasonal sampling campaigns (winter, spring, summer, autumn). In each sampling campaign, one sample was collected at the end of WWTP 1, 2, 3, 4, 5 (OUT 1, 2, 3, 4, 5), while three samples were collected at the end of WWTP 6 (municipal effluent - OUT 6M, industrial effluent – OUT 6I, and final effluent – OUT 6). The cumulative effluent of the six WWTPs was also sampled (OUT 7). Therefore, 9 effluent samples were collected for each sampling campaign (n = 36).

Physico-chemical parameters of the OUT 7 samples (pH, suspended solids, chemical oxygen demand, ammoniacal nitrogen, nitrites, nitrates, chlorides, sulphates, total phosphorus) were measured and compared with the regulatory limits imposed by the Italian legislation for the effluents discharged in surface waters. All the samples were stored in brown glass bottles at 4 °C until extraction.

2.3. Extraction

Wastewater samples were extracted as described by Schilirò et al. (2009). Briefly, each sample (3 L) was brought to room temperature, then 5 mL of methanol were added, the pH was acidified to 2.5 with H_2SO_4 and conductivity was adjusted to 8500 μS/cm with NaCl. The solid phase extraction was performed using two different sorbents: a volume of 1.5 L was extracted using Oasis® HLB sorbent (HLB) and a volume of 1.5 L was extracted using Sep-Pak® Vac (C18) sorbent. Each sorbent was conditioned consecutively with acetone, methanol and deionized water (pH 2), then each sample was extracted. The sorbent phase was dried and eluted with acetone. Each extract was then evaporated to 1 mL under nitrogen flow, 1000 μL of dimethylsulfoxide (DMSO) were added and acetone was completely removed under nitrogen flow. The extracts were stored in glass vials at −20 °C until testing.

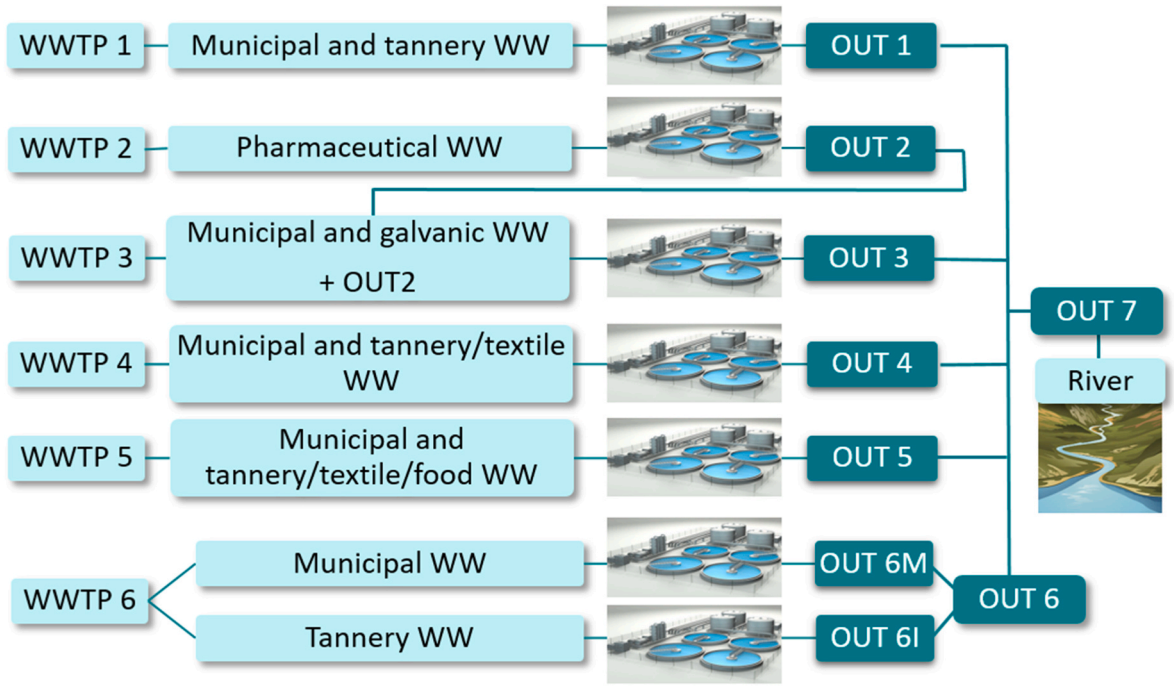

Fig. 1. Schematic representation of the wastewater treatment plants (WWTPs). WW = wastewaters; OUT = wastewater effluent.

2.4. Estrogenic activity

The estrogenic activity of the WWTP effluents was measured with two assays: a gene reporter assay on mammalian cells (MELN) and the XenoScreen XL YES Assay kit on the genetically modified yeast *Saccharomyces cerevisiae*. The first assay was performed using the protocols reported by Balaguer et al. (1999), further modified by (Schilirò et al., 2012; Gea et al., 2022), while the YES assay was performed according to the manufacturer protocol (Xenometrix, Instructions for Use Version 3.08). Estrogenic activity was expressed as 17β-estradiol equivalent concentration (EEQ). The detailed methodologies are reported in **paragraphs S1** and **S2** of Supplementary Materials.

2.5. Statistical analysis

Data were statistically analysed using SPSS 26.0 (IBM Statistics). The normality of the data distribution was assessed using the Shapiro test. The EEQs of samples extracted with the HLB sorbent was compared with the EEQs of samples extracted with the C18 sorbent using the Wilcoxon test, while the Kruskal-Wallis test followed by Tukey *post-hoc* test was applied to compare the EEQs among the different sites. Finally, the correlation between the EEQs of samples extracted with the HLB sorbent and the EEQs of samples extracted with the C18 sorbent was assessed with the Spearman correlation coefficient. Comparisons and correlations were considered significant for $p < 0.05$.

3. Results

3.1. Physico-chemical parameters of effluents

Table 1 reports the physico-chemical parameters of the cumulative effluent (OUT 7) and the Italian limit values for effluents discharged into surface waters (Italian Legislative Decrees n. 152/2006 and 4/2008). Table S2 of Supplementary Materials reports the physico-chemical parameters of effluents (OUT 1, OUT 3, OUT 4, OUT 5, OUT 6) that merge together into the OUT 7 which is lately discharged into the river; being the only one that actually reach the environment, only OUT 7 must comply with the threshold limits. Despite the flow rates varied among seasons (101,212 m^3/day in winter, 95,068 m^3/day in spring, 90,747 m^3/day in summer and 88,698 m^3/day in autumn), the physico-chemical parameters of OUT 7 samples were always below the Italian limit values.

Table 1
Physico-chemical parameters of the cumulative effluent (OUT 7) compared with the Italian limit values for effluents discharged into surface waters (Italian Legislative Decrees n. 152/2006 and 4/2008), during the four seasonal sampling campaigns (winter, spring, summer, autumn).

Physico-chemical parameters	OUT 7 winter	OUT 7 spring	OUT 7 summer	OUT 7 autumn	Limit values[b]
pH	8.1	8.1	8.2	8.1	≥5.5; ≤9.5
Suspended solids (mg/L)	15	15	32	5	≤35
COD[a] (mg/L)	71	86	110	130	≤160
Ammoniacal nitrogen (mg/L)	<0.5	<0.5	<0.5	<0.5	≤15
Nitrites (mg/L)	0.03	0.06	0.03	<0.02	≤0.6
Nitrates (mg/L)	11.4	12.9	10.6	11.3	≤20
Chlorides (mg/L)	890	990	985	1005	≤1200
Sulphates (mg/L)	700	815	855	875	≤1000
Total phosphorus (mg/L)	1.7	0.8	1.3	1.1	≤10

[a] COD (Chemical Oxygen Demand).
[b] Italian Legislative Decrees n. 152/2006 and April 2008.

3.2. Estrogenic activity (gene reporter luciferase assay)

The results of the gene reporter luciferase assay are reported in Fig. 2a.

The EEQ values ranged from 0.12 ng/L to 22 ng/L for samples extracted with HLB sorbent and from below the LOD to 24 ng/L for samples extracted with C18 sorbent. All the samples induced an estrogenic activity except for the sample OUT 6I collected in autumn and extracted with C18 sorbent. The EEQ of this sample was considered equal to half of the LOD for the statistical analyses (LOD = 0.034 ng/L; autumn OUT 6I C18 = 0.017 ng/L). A significant correlation was found

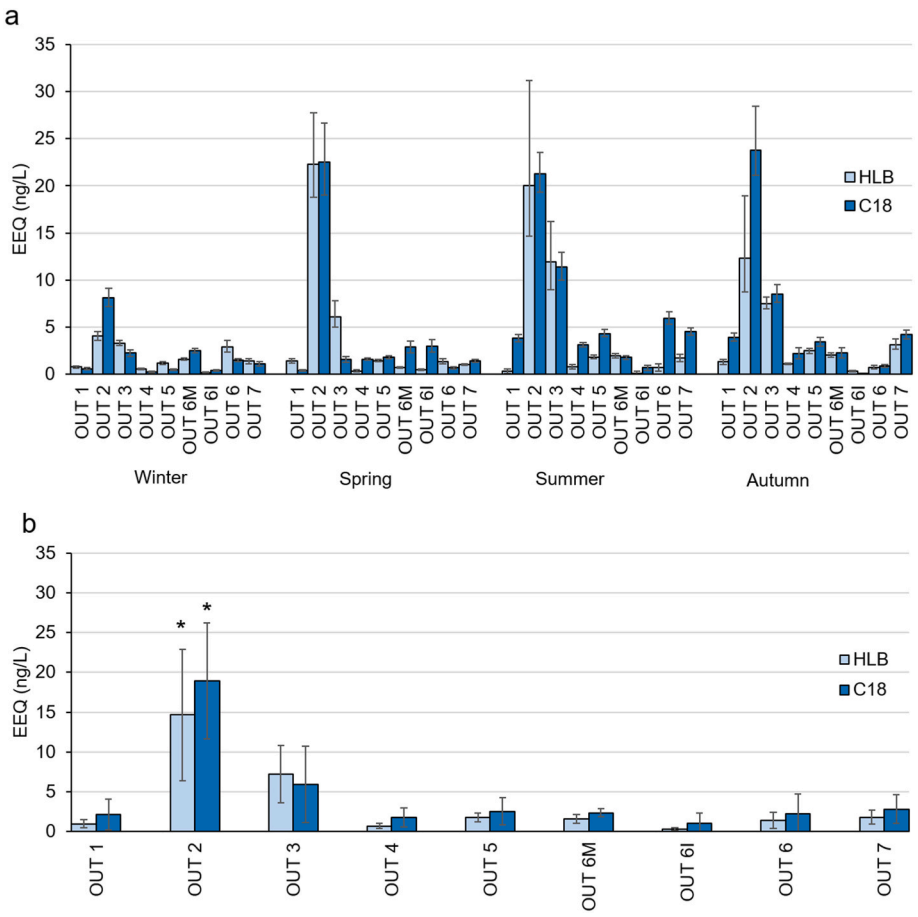

Fig. 2. Estrogenic activity of the nine effluents extracted using two different sorbents (HLB and C18) assessed using the gene reporter luciferase assay. a) Estrogenic activity of all the seasonal samples (mean EEQ ± confidence intervals 95%). b) Mean annual estrogenic activity of the samples (mean EEQ ± standard deviation). * = $p < 0.05$ Kruskal-Wallis test followed by Tukey post-hoc test.

between the EEQ of the samples extracted using C18 sorbent and the EEQ of the samples extracted using HLB sorbent (Spearman's Rho = 0.584, $p < 0.001$).

Fig. 2b shows the annual mean EEQ of the nine effluents extracted using both the sorbents. Among the effluents extracted using the HLB sorbent, the OUT 2 samples induced the highest estrogenic activity (Kruskal-Wallis test followed by Tukey post-hoc test, $p < 0.001$). The same result was obtained comparing the estrogenic activity of the effluents extracted using the C18 sorbent (Kruskal-Wallis test followed by Tukey post-hoc test, $p < 0.001$). No statistically significant difference was detected comparing the EEQs of the other samples (Kruskal-Wallis test followed by Tukey post-hoc test, $p > 0.05$).

The Wilcoxon test was applied to compare the EEQs of the samples extracted using the two different sorbents. The EEQs of samples extracted with the HLB sorbent (mean EEQ = 3.4 ng/L) were significantly different from the EEQs of samples extracted with the C18 sorbent (mean EEQ = 4.6 ng/L) (Wilcoxon test, $p < 0.05$).

3.3. Estrogenic activity (yeast estrogen screen – YES assay)

The estrogenic activity measured using the YES assay was lower than the activity measured using the gene reporter luciferase assay. In the YES assay, only 10 out of 36 samples extracted with the HLB sorbent and 5 out of 36 samples extracted with the C18 sorbent induced a detectable estrogenic activity (28% and 14%, respectively). The EEQs ranged from below the LOD to 3.8 ng/L for samples extracted with HLB sorbent and from below the LOD to 3.4 ng/L for samples extracted with C18 sorbent (Supplementary Materials Fig. S1). Since using the YES assay the estrogenic activity was generally low and it was detected only for few samples, the statistical analyses were not performed on the results of this assay.

4. Discussion

The monitoring of WWTP effluents plays a crucial role since they are also among the main sources of EDCs dispersion in the environment. In this context, it is important to study the factors that could influence the monitoring results (Robitaille et al., 2022).

In this study, 36 effluent samples collected from six WWTPs were extracted using two different sorbents (C18 and HLB) and the estrogenic activity of the extracts ($n = 72$) was evaluated using two different effect-based assays on mammalian cells (gene reporter luciferase assay) and on yeasts (YES assay).

Overall, in the gene reporter luciferase assay, the estrogenic activity expressed as EEQ was equal to 0.12–22 ng/L for samples extracted using HLB sorbent and <LOD – 24 ng/L for samples extracted using C18 sorbent, while in the YES assay the estrogenic activity was equal to < LOD - 3.8 ng/L and <LOD ng/L - 3.4 ng/L for HLB and C18 sorbent respectively. These results are in accordance with previous studies in which the estrogenic activity of wastewater samples was detected using similar sorbents for sample extraction (Allinson et al., 2010; Archer et al., 2020; Fernandez et al., 2009; Guo et al., 2022; Huggett et al., 2003; Jugan et al., 2009; Kibambe et al., 2020; Li et al., 2010; Liang et al., 2021; Mispagel et al., 2009; Muller et al., 2008; Salste et al., 2007; Smital et al., 2011; Sun et al., 2008; Välitalo et al., 2016; Wang et al., 2015; see Supplementary Materials Table S3).

The estrogenic activity induced by the samples was different depending on the biological assay applied; in particular, using the gene reporter luciferase assay EEQ values were generally higher than using the YES assay. Moreover, using the first assay an estrogenic activity was detected for a higher frequency of samples than using the YES assay (gene reporter luciferase assay = 71/72 samples, 99%; YES assay = 15/72 samples, 21%), so the gene reporter luciferase assay was more responsive to these samples than the YES assay. This result could be due to the different characteristics of the two assays. Indeed, yeasts are characterized by different membrane permeability, transport and metabolic activity with respect to mammalian cells, generally showing a lower sensitivity to E2 (Gea et al., 2020; Gómez et al., 2021; Robitaille et al., 2022). Moreover, as reported also by other studies, the two assay types are characterized also by a different LOD (generally higher for the YES assay than for gene reporter luciferase assay based on mammalian cells) (Gea et al., 2020; Gehrmann et al., 2018; Simon et al., 2022a), which could explain the higher number of estrogenic samples detected using mammalian cells with respect to yeasts.

Comparing the EEQs measured with the gene reporter luciferase assay among the different effluents, the highest EEQ values were detected for the effluent of the WWTP 2 (OUT2) using both sorbents. This result is not surprising; indeed, it should be considered that this effluent is only subjected to preliminary treatments performed in WWTP 2, which probably are not able to reduce the concentrations of pollutants, resulting in a high estrogenic activity. However, this effluent is not discharged directly in the cumulative effluent and in the river, but it is mixed with the influent of the WWTP 3 and, in this WWTP, it is subjected to additional treatments. Moreover, the high estrogenic activity of this effluent could be due to its chemical composition: OUT2 is composed by industrial wastewaters of pharmaceutical industries. These wastewaters could contain pharmaceutical compounds, which have an estrogenic effect such as furosemide and progesterone (Ballaré et al., 2006; Fent et al., 2006; Isidori et al., 2009; Kiyama et al., 201).

In the gene reporter luciferase assay, the EEQs of the samples extracted with the HLB sorbent were significantly different from the EEQs of the samples extracted with C18 sorbent, showing that the type of sorbent used for the extraction can significantly affect the *in vitro* results. The higher mean EEQ of the samples extracted with the C18 sorbent than the samples extracted with the HLB sorbent, could be explained hypothesizing that the C18 sorbent may have a higher extraction efficiency for the estrogenic compounds than the HLB sorbent. Moreover, this difference could also be explained hypothesizing that C18 may extract less anti-estrogenic compounds. These considerations are in accordance with a previous study in which the endocrine activity of wastewaters extracted using three sorbents (Oasis HLB, Telos C18/ENV and Supelco ENVI-Carb +) was assessed using the YES assay and other *in vitro* assays (Abbas et al., 2019). The Telos C18/ENV sorbent followed by the Oasis HLB sorbent were the most effective sorbents for the extraction of estrogenic compounds, while samples extracted with Supelco ENVI-Carb + showed a lower estrogenic activity. From the results obtained, Abbas et al. (2019) concluded that for most of the *in vitro* assays, including the YES assay, the Telos C18/ENV sorbent seemed to be the best extraction sorbent. Moreover, a similar result was found by another study (Wagner and Oehlmann, 2011). In this study, Wagner and Oehlmann tested the estrogenic activity of bottle water extracted with six different sorbents (C18, Carb, ENV+, HLB, SDB, SDBxc) using the E-screen assay. The sample extracted with C18 sorbent induced the highest estrogenic activity among the samples extracted with the six sorbents. A third study compared the estrogenic activity of wastewater samples extracted with different sorbents using the E-screen assay (Körner et al., 1999). However, the sorbents compared by Körner et al. (1999) were the C18 and the ENV+, while the HLB sorbent was not considered. Therefore, this study cannot be compared with the present one. Finally, the study of Leusch et al. (2006) applied receptor binding assays in order to compare the estrogenic activity of spiked water samples (12 ng/L of 17β-estradiol) and influent wastewaters extracted with three different sorbents (Oasis HLB, Supelclean C18 and Isolute C2/C18). In contrast with the present study, Leusch et al. (2006) did not found a statistically significant difference in EEQs among the different sorbents. The different results of this study with respect to the present study could be due to the different estrogenic activity assays applied. In accordance with Leusch et al. (2006), also the study of Escher et al. (2008) concluded that there is no preferential extraction sorbent for environmental samples and that different sorbents have a comparable extraction efficiency (samples: wastewaters, river waters; tested sorbents: LiChrolut EN/C18, Empore SDB-RPS, Empore C18; assay: YES assay). However, Escher et al. (2008) did not compare directly HLB and C18 sorbents, so these results could not be directly compared with the results of the present article.

Overall, the EEQs measured with the gene reporter luciferase assay did not show a seasonal trend, except for the samples of OUT 2 and OUT 3. Indeed, OUT2 samples induced a lower estrogenic activity in winter than in the other seasons, while OUT3 samples induced a lower estrogenic activity in winter and spring with respect to summer and autumn. Moreover, using the YES assay, the estrogenic activity was detectable mainly in the sampling campaigns characterized by a warmer temperature (summer and spring). The lower estrogenic activity in winter than in the other seasons could be partially due to a higher flow rate of the effluents in this season. Indeed, the flow rates of the cumulative effluent (which is the sum of the flow rates of all the other effluents) were equal to 101,212 m^3/day in winter, 95,068 m^3/day in spring, 90,747 m^3/day in summer and 88,698 m^3/day in autumn, so in winter the higher flow rate could have diluted the EDC concentrations.

The estimation of the risk posed by WWTP effluents to the receiving waters is challenging because it is influenced by many factors, such as EEQ levels in the effluents but also amounts of wastewater discharged, extent of dilution and flow rate of the receiving water (Jarošová et al., 2014; Välitalo et al., 2017). To assess whether the estrogenic activity measured with assays poses acceptable or unacceptable risk, the measured EEQs are usually compared with effect-based trigger values (EBTs) reported in literature (Leusch et al., 2017). The EBT is a threshold value that indicate acceptable risk for environmental complex mixtures (Escher et al., 2018) and, in estrogenic activity assessment, it is the EEQ that can be considered safe, as it causes no adverse effects (Jarošová et al., 2014). Numerous authors have calculated EBTs for different matrices (Escher et al., 2018; Leusch et al., 2017), the EBTs proposed for WWTP effluent using the gene reporter luciferase assay and the YES assay are 0.80–1.6 ng/L and 1.2–2.0 ng/L, respectively, for short-term exposure and 0.2–0.3 ng/L and 0.2–0.4 ng/L, respectively, for long-term exposure (Jarošová et al., 2014), while the EBTs proposed for surface waters are 0.37 ng/L for the gene reporter luciferase assay (MELN cells) and 0.88 ng/Lfor the YES assay (according to Routledge and Sumpter, 1996; Escher et al., 2018). In addition, in 2019, the NORMAN association proposed an EBT value for WWTP effluents for estrogenic activity (gene reporter luciferase assay CALUX) indicating a potential ecological risk of 0.1 ng/L.

The EEQs of OUT 7 (cumulative effluent discharged in surface water) measured with the gene reporter luciferase assay ranged from 1.0 to 3.1 ng/L (mean = 1.4 ng/L) for samples extracted with HLB sorbent and from 1.1 to 4.5 ng/L (mean = 2.3 ng/L) for samples extracted with C18 sorbent. The higher values of the range were above the proposed EBTs, so the presence of estrogenic compounds in OUT 7 may pose a risk to the receiving waters. At this regards the NORMAN association proposed that exceedance should trigger appropriate actions by WWTP operators such quantify specific target compounds which are known to cause the effects observed in the respective bioassay (*toxicity drivers*) and continue to monitor until the EBT <0.1 ng/L (NORMAN Association N° W604002510, 2019).

However, it is important to highlight that, due to dilution processes, the estrogenic activity of OUT 7 is not equal to the estrogenic activity of the receiving surface water. Therefore, in the surface water the EEQ could be lower than the EBT, thus causing no adverse effects on the

environment (Jarošová et al., 2014). For example, some authors considered an EBT for wastewaters tenfold higher than the EBT for surface waters assuming a fixed tenfold dilution of the effluents in the receiving surface water (Simon et al., 2022b). Moreover, it should be considered that the EBTs proposed by Escher et al. (2018) are intended for surface waters and not for wastewaters. In addition, the EBTs proposed by Jarošová et al. (2014) are applicable to municipal wastewater but not to industrial wastewaters; so, since the OUT 7 is the cumulative effluent of six WWTPs that treats both municipal and industrial wastewaters, these EBTs may not be suitable to these wastewaters. Indeed, the EBT were estimated considering the predicted-no-effect concentrations of steroidal estrogens which are predominant in municipal wastewaters, while industrial wastewaters could contain other compounds characterized by other the predicted-no-effect concentrations.

Finally, the EEQs of OUT 7 measured with the YES assay using both the sorbents were always below the LOD except for the spring sample extracted with the HLB sorbent, which induced an estrogenic activity equal to 0.31 ng/L. Therefore, the values measured using this assay were below or similar to the EBTs, suggesting that the estrogenic activity of the cumulative effluent measured with YES assay is safe for the environment.

Currently, the water quality is monitored using targeted chemical analysis also in the context of the European Water Framework Directive (European Parliament and European Council, 2000); however, there is an increasing awareness about some limitations of this approach. Indeed, it is not possible to quantify all chemicals, data about the biological effect of each chemical are not always available, and there is a lack of information about the cumulative effects of multiple known and unknown chemicals. Therefore we cannot expect a small subset of compounds identified and quantified through regulation to contain all the risk-determining factors of the mixture as a whole (Escher et al., 2020); in this regards, there is interest in finding new approaches to monitor water quality.

In addition to the targeted chemical analysis, the use of effect-based tools, such as *in vitro* cell-based assays, seems to be promising to assess water quality since it can provide data about the biological effect of all chemicals and transformation products in the environmental matrices (Escher et al., 2018, 2020; Simon et al., 2022a). Although effect-based methods may become a key tool for water quality management and monitoring (Neale et al., 2023), detailed methods for biomonitoring should be defined before implementation of future assays in regulations.

5. Conclusions

This study once again highlights the importance of biomonitoring alongside traditional monitoring, in particular it demonstrated how the methods of extracting water samples from WWTPs and the types of biological tests could influence the results of biomonitoring. Indeed, it showed that estrogenic activity of HLB extracts was significantly different from the activity of C18 extracts, suggesting that extraction stationary phase can influence biomonitoring data. Moreover, it also highlighted that biomonitoring results can be affected by the assay selection. Indeed, the estrogenic activity was overall higher using MELN assay than using YES, suggesting that, due to difference in cell membrane permeability and metabolic activation, the applied cell model can affect the results. Finally, from the comparison between the activity of the cumulative effluent and the environmentally safe estrogenic levels, MELN data revealed that the activity of this effluent may pose an environmental risk, while YES data showed that it should not be considered a threat to the receiving surface waters.

This study pointed out that a standardized approach is needed to assess the endocrine disrupting potential of waters and it reported data that can be important to select the most suitable stationary phase for extraction and the most appropriate bioassay to assess the environmental risk, thus protecting human health.

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CRediT authorship contribution statement

Marta Gea: Writing – original draft, Visualization, Validation, Investigation, Formal analysis, Data curation. **Federica Spina:** Writing – review & editing, Validation, Investigation, Data curation, Conceptualization. **Roberta Revello:** Investigation. **Elisabetta Fea:** Supervision, Resources. **Giorgio Gilli:** Supervision. **Giovanna Cristina Varese:** Supervision, Resources, Project administration, Conceptualization. **Tiziana Schilirò:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Conceptualization.

Declaration of Generative AI and AI-assisted technologies in the writing process

In the writing process Generative AI and AI-assisted technologies were not used.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jenvman.2024.120412.

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