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Effect-directed analysis of a hospital effluent sample using A-YES for the identification of endocrine disrupting compounds

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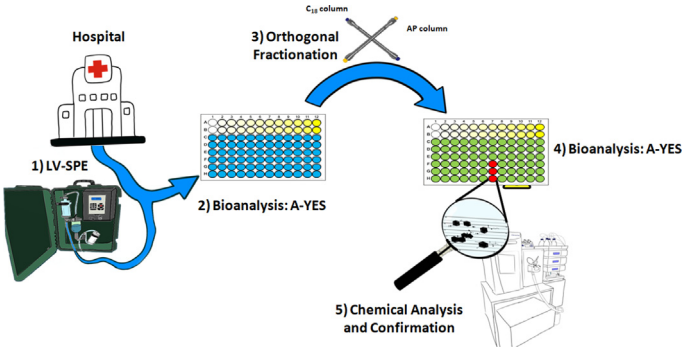
HIGHLIGHTS

- An effect-directed analysis methodology was developed for the identification of EDCs present in a hospital effluent.
- Recoveries of different steps of the methodology were studied for 184 compounds, detecting major losses during evaporation.
- Four compounds were identified as estrogenicity drivers, explaining ~50 % of the total activity of the sample.
- Results support the need of assessing endocrine disruption as a prioritization criterion for water quality monitoring.

GRAPHICAL ABSTRACT



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ABSTRACT

An effect-directed analysis (EDA) approach was used to identify the compounds responsible for endocrine disruption in a hospital effluent (Basque Country). In order to facilitate the identification of the potentially toxic substances, a sample was collected using an automated onsite large volume solid phase extraction (LV-SPE) system. Then, it was fractionated with a two-step orthogonal chromatographic separation and tested for estrogenic effects with a recombinant yeast (A-YES) in-vitro bioassay. The fractionation method was optimized and validated for 184 compounds, and its application to the hospital effluent sample allowed reducing the number of unknowns from 292 in the raw sample to 35 after suspect analysis of the bioactive fractions. Among those, 7 of them were confirmed with chemical standards. In addition, target analysis of the raw sample confirmed the presence of mestranol, estrone and dodemorph in the fractions showing estrogenic activity. Predictive estrogenic activity modelling using quantitative structure-activity relationships indicated that the hormones mestranol (5840 ng/L) and estrone (128 ng/L), the plasticiser bisphenol A (9219 ng/L) and the preservative butylparaben (1224 ng/L) were the main contributors of the potential toxicity. Derived bioanalytical equivalents (BEQs) pointed mestranol and estrone as the main contributors (56 % and 43 %, respectively) of the 50 % of the sample's explained total estrogenic activity.

1. Introduction

It is already well-known that urban wastewater constitutes one of the main pathways for contaminants of emerging concern (CECs) into the aquatic ecosystems since their complete removal in wastewater treatment

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plants (WWTPs) is not achieved (Lopez-Herguedas et al., 2021; Rodil et al., 2012; Hug et al., 2014). Among the different types of wastewaters discharged into the WWTPs (urban, industrial, agricultural), hospital wastewater deserves special attention. The amount of wastewater discharged from hospitals can reach 400 L/bed/day (Chartier, 2014), which can contain micropollutants up to 150 times more concentrated than in urban effluents (Chartier, 2014; Verlicchi et al., 2010; Santos et al., 2013; Orias and Perrodin, 2013), potentially exhibiting effects, such as pharmaceuticals, cosmetics, additives, surfactants, disinfectants and all their metabolites. In the specific case of the Basque Country, the analyses carried out in the effluent of Galdakao hospital confirmed the presence of antibiotics (e.g., ciprofloxacin, 0.6–267 μg/L), nonsteroidal anti-inflammatory drugs (e.g., ibuprofen, <0.10–148 μg/L; acetaminophen, < 0.10–37 μg/L) and synthetic opioid agonists (e.g., methadone, <0.10–3.8 μg/L) (). Moreover, these compounds were also detected in Galindo WWTP effluents (Basque Country) (Adecuación de Vertido Hospitalario a Red de Saneamiento Mediante Tratameintos Terciarios Específicos, 2012, González Canal et al., 2018; Mijangos et al., 2018). Galindo WWTP is responsible for the treatment of waters from the metropolitan area of Bilbao and neighbouring (hospital 0.4 %, industrial 3.7 % and urban 92 %) including Galdakao hospital.

The chemicals that occur in hospital effluents need to be deeply studied in order to avoid overlooking their potential long-term ecotoxicological effects, particularly regarding disturbances in hormonal homeostasis (endocrine disruption) (Fent et al., 2006). Undesired endocrine effects are a matter of concern since the 90s (Colborn and Clement, 1992), even if it has been overlooked in traditional ecotoxicology studies (Kabir et al., 2015) compared with other evaluated endpoints. That is why an updated methodology and revised applications are of need in order to fully understand the effects on reproduction, development and growth the endocrine disrupting compounds (EDCs) may have in wildlife and humans. Moreover, it is known that many of EDCs, may exhibit, even at low concentration levels, synergic and cumulative effects (Santos et al., 2013; Tiwari et al., 2017; Schmidt, 2018), therefore, their assessment in complex mixtures is needed.

The role of bioanalytical tools is especially relevant when we want to tie the observed toxicological effects caused by the contaminants present in a given sample (Brack et al., 2016), and effect-directed analysis (EDA) integrates toxicity testing and chemical analysis of a sample in the same workflow in order to get the maximum information. The key elements in the workflow are (i) the use of methods to reduce the complexity of chemical mixture by chromatographic fractionation; (ii) the use of biotests that allow the effect of chemicals on organisms to be detected and, (iii) the use of multi-target and/or suspect chemical analysis to identify the chemicals potentially responsible of the observed effects (Brack et al., 2016; Mijangos et al., 2020). During the last ten years, several effluents (i.e., municipal wastewaters (Hashmi et al., 2018; Smital et al., 2011; Sonavane et al., 2018; Houtman et al., 2020; Zwart et al., 2020; Hashmi et al., 2020; Zwart et al., 2018; Ma et al., 2017), surface waters closed to urban settlements (Houtman et al., 2020; Zwart et al., 2018; Tousova et al., 2017; Muschket et al., 2018; Chen et al., 2016) or even offshore produced water discharges (Thomas et al., 2009)) have been assessed by EDA approach trying to identify endocrine disruption causative compounds present in ecosystems. As far as we know, there is only one study in the literature that reported (Itzel et al., 2018) EDA focused on hospital effluents. According to the literature, the Yeast Estrogen Screening (YES) assay is robust, fast, sensitive and inexpensive, and it is one of the easiest bioassays to handle (Rutishauser et al., 2004). This is why the YES assay has been suggested as a suitable screening tool for the assessment of estrogenic activity in a variety of environmental samples (Rutishauser et al., 2004) and is also used in EDA approaches (Smital et al., 2011; Chen et al., 2016; Thomas et al., 2009). Although most of the mentioned YES studies are based on the yeast strain *Saccharomyces cerevisiae,* recently the A-YES based on the strain *Arxula adeninivorans* was validated (Hettwer et al., 2018) and showed to be ideal to evaluate highly matrix-loaded samples (Gehrmann et al., 2016) as hospital wastewater effluents (Itzel et al., 2018).

Within this context, the main aim of this work was to identify potential EDCs present in a hospital effluent (Biscay, Basque Country) using the A-YES as a toxicological in-vitro bioassay in an EDA workflow. The sample was gathered using an automated onsite large volume solid phase extraction (LV-SPE), subsequently fractionated on a two-step orthogonal chromatographic separation and analysed by liquid chromatography coupled to both qOrbitrap and triple quadrupole tandem mass spectrometer (LC-QqQ-MS/MS) to facilitate the analytical and toxicological identification of the concerning substances. The method was optimized and validated for 184 compounds, and further applied to the extracted sample and fractions.

2. Experimental section

2.1. Reagents and materials

All chemicals and laboratory materials used in the study are provided in Section S1 of the Supporting Information (SI). Compounds names, chemical family, CAS numbers, molecular formulas, InChIKey and the mass analyser parameters used in their analysis are summarized in Table S1 of the SI.

2.2. Sampling and sample treatment

A composite sample (45 L) was collected during 24 h in March 2020, directly from the main discharging sewage drain of the hospital. The sample was collected using an onsite LV-SPE (MAXX Mess-u. Probenahmetechnik GmbH, Rangendingen, Germany) sampling system designed by Schulze et al. (Schulze et al., 2017) following the protocol of Välitalio et al. (Välitalo et al., 2017) with some modifications, as described elsewhere (González-Gaya et al., 2021) (see Section S2 of the SI). Moreover, 5 L of UHPLC-MS grade water containing 0.1 % sodium chloride was circulated 9 times through the LV-SPE sampling system to get the corresponding procedural blank (B). Both the raw water sample and blank sample were treated equally following the steps described by Schulze et al. (Schulze et al., 2017), obtaining extracts with an enrichment factor of 250 (EF 250).

2.3. Effect-Directed Analysis (EDA)

The scheme of the EDA process performed in this work can be found in the SI (Fig. S1). Briefly, the previously obtained preconcentrated raw and procedural blank samples (EF 250) were subjected to a two-step fractionation (see Section 2.3.1). At each fractionation step, a recombined sample of all the fractions (named RS and ΣF-RS, for first fractionation and second fractionation, respectively) was prepared to test whether bioactivity losses occurred during the fractioning. Based on the bioactivity observations for the first fractions, a pool of the estrogenic and neighbouring fractions (ΣF) was prepared to include all the estrogenic compounds in a single extract. The A-YES was applied to the raw sample, procedural (B) and recombined blank (RB), all the fractions (18 for first fractionation and 16 for second fractionation), and finally to the pooled (ΣF) and recombined samples (RS and ΣF-RS) obtained at both fractionations (see Section 2.3.2). Chemical analysis was restricted to the raw sample, B and RB, and the RS, ΣF and ΣF-RS (see Section 2.3.3). Dose range values are given in terms of relative enrichment factor (REF), which is the product of the concentration factor of the LV-SPE process and the dilution of the extract in the bioassay test media (Escher et al., 2021).

2.3.1. Fractionation procedure

The fractionation of the extract was performed by semi-preparative reverse-phase high-pressure liquid chromatography (RP-HPLC). The HPLC system consisted of an Agilent 1100 series HPLC chromatographic system equipped with an autosampler and an automatic fraction collector (Agilent Technologies, series 1100, Avondale, PA, USA). The whole system was controlled using Chemstation B.04.03 software. The sequential fractionation was performed based on previous experience of the research group (Mijangos et al., 2020) combining two different columns with an

orthogonal selectivity: a reverse phase C_{18} column (250 × 10 mm, 5 μm, Macherey-Nagel Nucleodur, Düren, Germany) (F1-18) and an aminopropyl (AP) column (150 × 10 mm, 3 μm, Imtakt, Portland, OR, USA) (ΣF-1-16) using gradient elution with water and MeOH, both containing 0.1 % of HCOOH, at a flow rate of 2.36 mL/min (see the methodology detailed in Section S3 and Table S2 in SI). All the fractions were concentrated by a rotavap evaporator system (Büchi, Flawil, Switzerland) at 40 °C and 200 rpm to have <5 % of MeOH (Hashmi et al., 2018), then diluted with Milli-Q water and submitted to the SPE extraction according to Lopez-Herguedas et al. (2021), in order to get a suitable medium for the chemical analysis and A-YES (see methodologies detailed in Sections S3 and S4 in SI, respectively).

2.3.2. Biological assay: A. adeninivorans Yeast Estrogenic Screen (A-YES)

The estrogenic activity of the aqueous samples was determined by the A-YES, based on the genetically modified recombinant yeast strain *A. adeninivorans* G1214 expressing the human estrogen receptor alpha (hERα) gene. Tested samples and concentrations can be found in Table S3. The bioassay was performed (details in SI, Section S4) following the supplier protocol (New Diagnostics, München, Germany, http://bioval.new-diagnostics.com/). A seven-point calibration curve for the reference compound 17β-estradiol (E2) was prepared between 1 ng/L and 80 ng/L in Milli-Q water. The measured phytase activity of the tested samples was adjusted to the calibration curve concentrations, and results are provided in E2 equivalents (EEQ, ng/L) (Fig. S2). For testing purposes, 96 deep-well plates were prepared adding aliquots of 400 μL of each sample or calibration solution and 100 μL of activated yeast in each well. Each sample was tested in triplicate. Each plate included two replicates of each level of the calibration curve and negative controls with yeast (i.e., Milli-Q water). Furthermore, two replicates of controls without yeast (negative controls (i.e., Milli-Q water), positive controls (i.e., highest E2 calibration level) and two samples selected randomly) were included in each plate to measure background absorbance. Loaded plates were covered with porous adhesive foil and incubated at 30 °C while shaking at maximum speed (420 rpm) for 48 h on a shaker with an incubator hood (KS-15 Edmund Bühler GmbH, Bodelshausen, Germany).

The bioactivity of samples was measured with an Eon™ High-Performance Microplate Spectrophotometer (BioTek Instruments, Winooski, VT, USA) at a wavelength of 405 nm. For the determination of yeast cell growth, the yeast pellets in the deep-well plate were re-suspended by vigorous shaking on a vortex shaker and the absorbance was measured in the spectrophotometer at 600, 620 and 630 nm wavelengths. Growth and phytase activation variability between the calibration curve replicates and between sample replicates and with the calibration curve were assessed to enhance the QA/QC and to evaluate cytotoxicity (see details in SI, Section S4). The estrogenic response was obtained in the form of sigmoidal concentration-response curves ranging from 0 to 100 % effect and for all samples, the effect concentrations (ECs) were calculated using the vendor-provided software (New Diagnostics, http://bioval.new-diagnostics.com/) and expressed as REF.

Statistical data treatment and the dose-response curves fitted with the PROBIT model were performed using SPSS Statistics software (SPSS, Version 26, IBM SPSS, Armonk, NY, USA).

2.3.3. Chemical analysis and data treatment

2.3.3.1. LC-q-Orbitrap analysis. The analysis of the B, raw sample and fractions showing estrogenic activity was carried out by a Thermo Scientific Dionex UltiMate 3000 UHPLC coupled to a Thermo Scientific Q Exactive Focus quadrupole-Orbitrap mass spectrometer (UHPLC-q-Orbitrap, Thermo Fisher Scientific, MA, USA) operated in full scan-data dependant MS2 (Full MS-ddMS2) discovery acquisition mode based on a previously developed method (Lopez-Herguedas et al., 2021; González-Gaya et al., 2021) detailed in Section S5-1 of the SI.

Suspect screening analysis was carried out using the Compound Discoverer 3.1 (CD, Thermo Fisher Scientific) software and mzCloud (Thermo Fischer Scientific) library. The workflow used for the identification of tentative candidates following Schymanski's identification level system (Schymanski et al., 2014) is described elsewhere (González-Gaya et al., 2021). Briefly, only peaks following a Lorentzian shape and included in the SusDat NORMAN database (40,059 compounds, http://www.norman-network.net/, DOI: https://doi.org/10.5281/zenodo.2664077) were considered for peak-picking. An error <5 ppm for the *m/z* values, values higher than 30 % for the intensity tolerance, a minimum peak intensity of 1e6 and a sample/procedural blank ratio of 10 were set to consider a compound as a feasible candidate. Only those peaks with available MS2 spectra and relative standard deviation (RSD %) lower than 30 % in the three injection replicates were considered. In addition, regarding the proposed CD molecular formulas, they were chosen the ones which explain MS1 spectra satisfactory (Sfit >30 % and pattern coverage >80 %). Structural assignments were carried out based on ddMS2 fragments annotated by CD. Afterwards, characteristics including the exact mass, the isotopic pattern, MS2 fragmentation and abundances of the selected features were compared with those available in the mzCloud (best match >70 %) library (https://www.mzcloud.org/). Fragmentation was also evaluated with the mzLogic tool (Thermo-Fisher Scientific). When the features were not available in mzCloud, fragmentation spectra explaining ≥50 % of the main fragments were considered as feasible candidates. When the standard was available a ± 0.1 min was set for positive confirmation whereas, an estimated retention time (tR) from Retention Time Indices Platform (RTI, National and Kapodistrian University of Athens, Greece, http://rti.chem.uoa.gr/) (Aalizadeh et al., 2021) was calculated for the candidates with no available standard. The SMILES of the features were obtained from United States Environmental Protection Agency (USEPA, https://comptox.epa.gov/dashboard/batch-search) in order to estimate the tRs.

Quantification of the identified compounds was performed using a homemade database included in the quantification software (TraceFinder 4.1, Thermo Fischer Scientific) which takes into account the exact mass, isotopic pattern, tR and the most characteristic MS2 fragments of the annotated compounds. Only peaks with a mass error lower than 5 ppm, a 70 % of isotopic pattern fitting and tR window of 60-s were accepted to be quantified.

2.3.3.2. LC-QqQ-MS/MS analysis. Although some pharmaceuticals, pesticides, personal care products and additives are considered EDCs, reproductive hormones interacting specifically with estrogen receptors (oestrogens) are the ones that have a large contribution to estrogenicity. The analysis of such compounds is often performed by LC and using basic pH mobile phases that are not commonly used in standard suspect screening approaches (Vega-Morales et al., 2012; Välitalo et al., 2016). In this work, in order to avoid overlooking such compounds, the target analysis of 16 compounds was performed in the active fractions as well as neighbouring fractions by LC-QqQ-MS/MS. The analysis method was based on a previously developed method (Mijangos et al., 2015) with slight modifications (see the methodology description included in SI in Section S5-2). Instrumental operations, data acquisition and peak integration were performed with the Masshunter Workstation Software (Version B.06.00, Agilent Technologies).

2.3.4. Chemical and effect confirmation

Confirmation of the estrogenic activity determined by A-YES induced by chemically detected compounds was performed using specifically designed chemical standard mixtures. In order to reduce the number of possible estrogenicity contributors detected in the active fractions, predictive estrogenic activity modelling by means of quantitative structure-activity relationships (QSARs) was performed. The SMILES codes of the determined compounds were entered into the VEGA-QSAR model (version 1.1.5, downloaded from www.vegahub.eu), and modelled for Estrogen Receptor-Mediated Effect (ERME, EPA-CERAPP model) and Estrogen Receptor Binding Affinity (ERBA, IRFMN model). In that way, only compounds that were predicted to be active on one or both of those pathways were further considered as estrogenicity drivers (Black et al., 2021).

The quantitative contribution of the predicted estrogen-receptor (ER) active contaminants to the mixture effect was confirmed on the basis of the bioanalytical equivalent concentration (BEQ) concept, being a common application when measuring estrogenic activity (Escher et al., 2021; Escher et al., 2008). A sigmoidal dose-response curve was fitted through the individual compounds tested concentrations to derive the effective concentration at 25 % of the total measured effect (EC_{25}) in order to calculate individual relative effect potencies (REP_i) in comparison to the reference compound (E2, 17 ng/L) (Eq. (1)):

$$REP_i = \frac{EC_{25}(reference\ compound)}{EC_{25}(compound\ i)} \quad (1)$$

In that way, BEQ expected from the quantified compounds present on the sample (BEQ_{chem}) can be calculated (Eq. (2)) for comparison with biologically derived BEQ_{bio} (Eq. (3)). An artificial mixture containing the identified chemicals at the same concentrations found in the original sample was also prepared to elucidate synergic or antagonistic effects according to $BEQ_{bio\_mixture}$ (Eq. (4)).

$$BEQ_{chem}(ng/mL) = \Sigma_{i=1}^{n} C_i \times REP_i \quad (2)$$

$$BEQ_{bio}(ng/mL) = \frac{EC_{25}(reference\ compound)}{EC_{25}(sample)} \quad (3)$$

$$BEQ_{bio\_mixture}(ng/mL) = \frac{EC_{25}(reference\ compound)}{EC_{25}(mixture)} \quad (4)$$

Stock solutions were prepared in Milli-Q water approximately 1 h before the beginning of the experiment. Dose ranges (5–5 × 10^4 ng/mL) of the identified candidates were chosen considering concentrations at EC_{25} of identified potentially estrogenic chemicals in the raw sample.

3. Results and discussion

3.1. Method validation

Calibration curve linearity, instrumental and procedural identification limits (LOI) and quantification limits (LOQs), absolute recoveries and precision for the different steps involved in the EDA protocol were determined using a list of 184 compounds. All the details are included in SI (see Section S6 and Table S4).

3.2. Hospital effluent estrogenic activity

All blanks (B and RB) were tested with the A-YES and no estrogenic activity was observed below the maximum concentration level tested for the raw sample (REF 10).

The toxicity of the hospital effluent (raw sample) was tested at 10 different REF values (ranging from REF 0.001–10) (see Fig. S4 in SI). The hospital effluent showed estrogenic activity in all tested concentration levels above REF 0.1, showing an increasing trend up to REF 5 and REF 10 where no phytase activity was observed. It is noteworthy to mention that even if no estrogenic effects were observed in the two highest concentration REFs, no significant differences were found in yeast growth (measured as optical density). Therefore, higher concentrations of chemicals may result in a null activity not connected to yeast survival, but maybe to other sublethal effects such as cytostatic effects not evaluated in this study.

An estrogenic activity of 11 ng EEQ/L was measured at REF 1 (i.e., no-preconcentrated sample) of the raw hospital sample. Considering the Safe Estrogenic Equivalents (SEE) for long-term exposure for 15 in-vitro bioassays (Jarošová et al., 2014), estimated to be on a 0.1 to 0.4 ng EEQ/L range, the EDCs delivered at the hospital effluent are two orders of magnitude higher, confirming the need for effective treatments to avoid the release of this chemical mixture into the environment. The results of the A-YES, as E2 equivalent concentration (EEQ, ng/L) at the different concentration levels (REF 0.001-2), were modelled in a sigmoidal dose-response curve (Fig. 1) with its respective 95 % confidence limits using the PROBIT model up to an equivalent 100 % EEQs estrogenicity. The cease of the enzymatic activity of the yeast at the highest concentration levels tested, prevented (Hashmi et al., 2018) from getting the complete full concentration-effect curve. Based on that observation, EC_{25} values were chosen for toxicity comparison (Escher et al., 2021), exhibiting a 0.011 BEQ_{bio} ng/mL.

The E2 EEQ concentration levels reported in other European hospital effluents varied between 2.8 and 26 ng EEQ/L (ERα-CALUX® bioassay) (Gehrmann et al., 2016; van der Linden et al., 2008). The estrogenic activity of the hospital effluent analysed in this study with A-YES was in line with the activity measured in previous studies. Although generally lower values were measured in municipal wastewater effluents (van der Linden et al., 2008), similar estrogenic potencies (10.8 ng EEQ/L for a WWTP in Zagreb) (Smital et al., 2011) and even higher (29.7 ng EEQ/L for a WWTP in Finland) (Välitalo et al., 2016) have been reported. Hashmi et al. reported the estrogenic activity of the Danube River (Serbia) impacted by untreated municipal wastewater, exhibiting a BEQ_{bio} exceeding 1 ng/L (Hashmi et al., 2018). Itzel et al. suggested ozonation as an efficient treatment in estrogenicity removal since a high reduction in the activity was observed after ozonation (> 90 % of the activity) (Itzel et al., 2018). In the case of the effluent evaluated here, the receiving WWTP does not count with an ozonation treatment. Therefore, more specific and effective treatments should be implemented to avoid the confirmed risk of these waters, either in hospital waters for a more site-specific treatment or either in the receiving WWTPs.

3.3. Identification of active fractions by A-YES

The toxicity of the recombined sample (RS) was tested at 7 concentration levels between REF 0.001 and REF 2 to evaluate toxicity losses during the first fractionation. Higher concentrations were not tested considering the lack of yeast activity at REF 5 and REF 10 of the raw sample. In this case, the RS showed a BEQ_{bio} = 0.007 ng/mL for EC_{25}, thus, raw and RS showed matching bioactivity values due to overlapping curves with a 95 % confidence level (Fig. 1 and Fig. S4 in SI). Therefore, no remarkable estrogenicity losses were appreciable during the first fractionation.

Fractions (n = 18) from the first fractionation were individually tested at a REF 2, and fraction F12 was majorly responsible for estrogenic activity ($\rho_{value} < 0.05$, Kruskal-Wallis). In comparison with the raw sample (15 ng EEQ/L at REF 2) (Fig. S4), higher E2 EEQ concentration levels were measured for the F12 (37 ng EEQ/L) (Fig. 2A). Although fractions F6, F16, F17 and F18 showed some bioactivity, they were not further considered

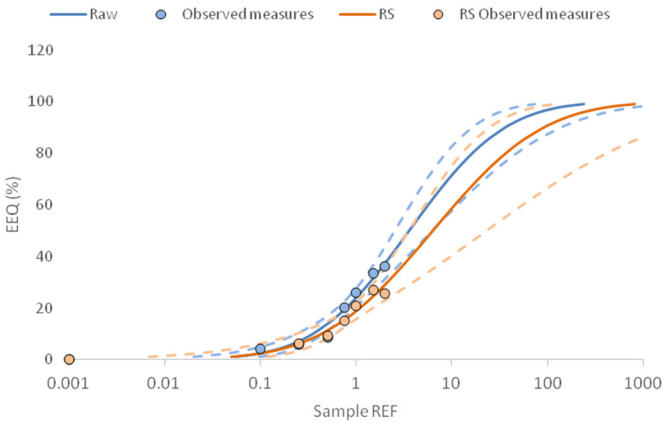

Fig. 1. The dose-response curve of raw (hospital effluent) and RS samples obtained with the measured E2 equivalent concentration (EEQ, %). Continuous lines show the effect concentration (EC) fit values and dashed lines indicate the confidence level (95 %).

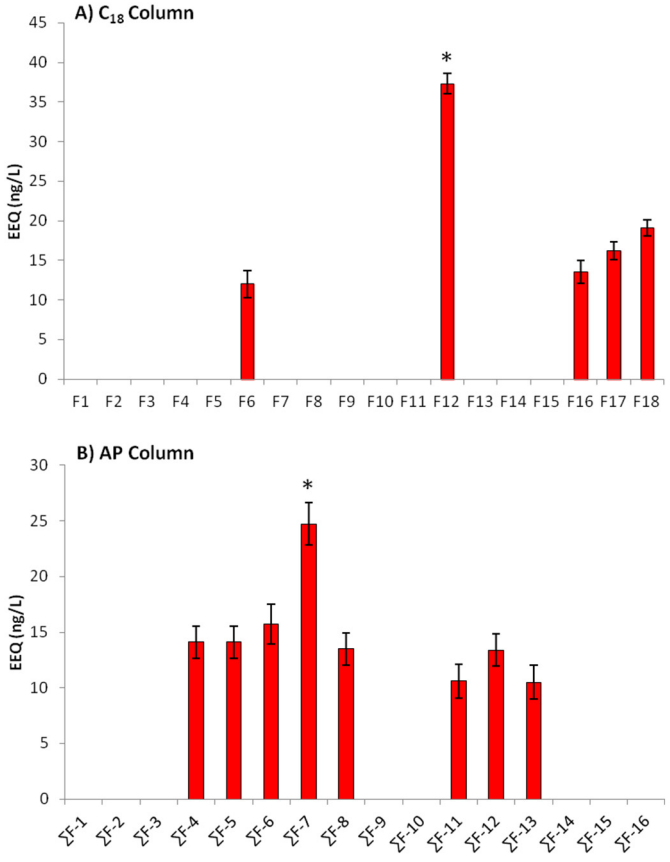

Fig. 2. Measured E2 equivalent concentration (EEQ, ng/L) ± standard deviation (SD) of fractions from first (A) C_{18} Column, (REF 2) and second (B) AP Column (REF 5) fractionations. Asterisks represent significant differences according to the Kruskal-Wallis probe ($\rho_{value} < 0.05$).

since no significant estrogenicity ($\rho_{value} > 0.05$) difference was observed between those and no effect fractions, according to the Kruskal-Wallis test (see Fig. 2A). Nonetheless, their potential environmental risk cannot be neglected (Jarošová et al., 2014).

In order to further reduce chemical complexity, a pooled sample (ΣF) constituted by the active (F12) and neighbouring (F11 and F13) fractions was once again fractionated into new 16 fractions (see Section 2.3.1) and them tested at REF 5. A second recombinant sample (ΣF-RS) which included the pool of the 16 fractions (second fractionation) was also collected in order to confirm the recovery of the estrogenic potential during the second fractionation procedure. As can be observed in Fig. 2B, ΣF-7 was the fraction showing the highest estrogenic response (24.7 EEQ ng/L), but even other seven fractions maintained a detectable estrogenic activity, between 10 and 15 EEQs (ng/L). This result can be interpreted as a spread of estrogenic compounds on the AP column over different fractions. The AP column was selected for fractionation considering its orthogonality to separation on C_{18} column and its ability to isolate steroids (Shao et al., 2005; Yang et al., 2009). Although, in this study, the isolation of the estrogenic compounds in a single fraction was not achieved, only the fraction showing a significant estrogenic activity ($\rho_{value} < 0.05$, Kruskal-Wallis) (BEQ_{bio} = 0.015 ng/mL) was further studied. Dose-response curves of the samples and fractions which exhibited estrogenic activity (ΣF and ΣF-7) fitted following a PROBIT model (see Section 2.3.2) can be seen in Fig. 3. Out of them, the EC_{25} and BEQ_{bio} values were calculated, and are given in Table S8 in the SI. Considering the overlapping intervals of raw and ΣF curves, it can be assured that the significant estrogenic activity of the full sample is recovered in the combined active fractions, and in consequence, the proper isolation of potentially estrogenic contributors has been achieved. Moreover, the estrogenic response was further isolated in ΣF-7 since its curve also overlaps with the raw and ΣF curves (95 % confidence level). It must be outlined the high variability observed in the upper part of the models, reflecting a wide variability in the effects caused at higher concentrations. This can be attributed to a lower number of tested concentrations (with a high concentration difference between them) and to a higher intrinsic variability in the flat area of the sigmoidal curve. The EC_{25} in the lower area of the model, ensure concentrations at which the present chemicals produce 25 % EEQs, and correspond to a REF of 1.1, 1.7 and 0.7 for the raw, ΣF and ΣF-7 extracts. Taking into account the 95 % confidence levels, all of them can be assumed as non-significantly different. Knowing that REF 1 corresponds to the water sample as it was in the effluent, it can be concluded that this water can cause at a 25 % EEQs level estrogenic effects if released into the environment without further treatment.

3.4. Identification of potentially estrogenic compounds and toxicity assessment

Suspect analysis of the bioactive fractions (ΣF and ΣF-7), both recombined samples (RS and ΣF-RS) and the raw sample, was carried out

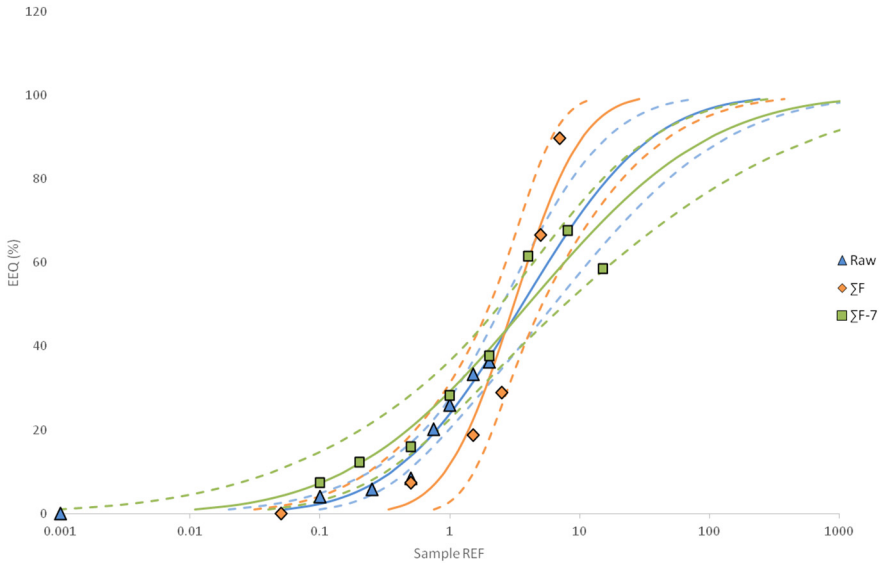

Fig. 3. The dose-response curves of the estrogenic active samples and fractions (Raw, ΣF and ΣF-7) obtained with the measured E2 equivalent concentration (EEQ, %). Continuous lines show the EC fit values and dashed lines indicate the confidence level (95 %).

in order to identify potentially estrogenic candidates. All the features present in the procedural blanks (B and RB), included in the suspect workflow, were discarded (see Section 2.3.3.1). More than 230,000 features (in both positive and negative ionization modes) were detected in the raw sample. The list of feasible features present in the raw sample was drastically reduced to 292 candidates when only the features that fulfil the criteria described in Section 2.3.3.1. were considered. From the 292 candidates found in the raw sample, only 35 features were also detected in the estrogenic fractions (Tables S8a and S8b), and thus, considered as estrogenicity contributor candidates. According to Schymanski classification (Schymanski et al., 2014), seven compounds were annotated at level 1, including the plasticiser bisphenol A (BPA), the food preservative butylparaben, the herbicide diuron, the antihypertensive irbesartan, the hormones progesterone and testosterone, and the antineoplastic bicalutamide. Besides, 16 compounds were annotated at level 2a or 2b. Several other compounds were also detected but it was not possible to differentiate between isomers (level 3) or confirm their MS2 (level 4). In addition, target analysis performed under basic conditions allowed the detection of three more compounds in the ER-active fractions; the hormones estrone and mestranol and the fungicide dodemorph.

The number of potentially estrogenic compounds (26 candidates, considering only compounds with levels 1, 2a or 2b and the ones identified by target analysis) provided by the chemical analysis was narrowed down to 6 candidates (BPA, butylparaben, estrone, mestranol, bicalutamide and testosterone) according to the VEGA-QSAR model (see Section 2.3.4). BPA, butylparaben, estrone and mestranolwere predicted to have estrogenic activity on at least one of the estrogen end points studied in the VEGA-QSAR model. In the case of the other two, bicalutamide and testosterone, non-estrogenic results were obtained categorized as non-reliable, but they were considered for toxicity assessment in order to evaluate the possible estrogenic, synergistic or antagonistic effects. The contribution of the identified substances to the total estrogenic activity was estimated by measuring dose-response relationships of all individual compounds (Fig. S5 in SI) and a mixture containing those 6 compounds at the concentrations found in the raw sample (Fig. S6 in SI). As predicted, estrone and mestranol showed to be potent ER agonists while BPA and butylparaben showed weaker estrogenicity. On the other hand, bicalutamide and testosterone showed to be ER inactive and since no synergic or antagonistic effects were induced with their presence, they were excluded from further studies. Concentrations of estrogenic compounds (expressed as ng/L concentration level) in the raw sample and fractions are summarized in Table 1. Considering the mentioned 4 compounds, we were able to explain almost the 50 % of estrogenic activity in the raw sample (BEQ_{bio} = 0.011 and BEQ_{chem} = 0.005, Fig. 4) where the hormones mestranol and estrone were the predominant contributors to the total explained estrogenic response (56 % and 43 %, respectively), followed in a much lesser extent by the plasticiser BPA (explaining 1 %). The preservative butylparaben was the one with the lowest contribution to the total estrogenicity, explaining only 0.01 %. Contributions of these compounds and their specific mode of action (MoA), can be found in Table 1. The hormones estrone and mestranol (Uraipong et al., 2017; Ngamniyom et al., n.d.) and BPA (Schmitt et al., 2012) are already reported as ER agonists. Estrone is a naturally occurring female sex hormone, which has been used as a medication for bone density conservation or as an antineoplastic agent (Wilson, 2009), while mestranol is widely used as the estrogen component in several oral contraceptives (Uraipong et al., 2017). BPA is a compound widely

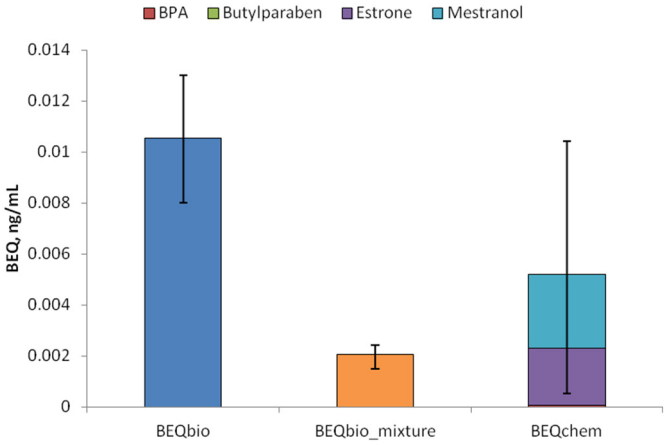

Fig. 4. Bioanalytical equivalent concentrations (BEQ) determined for the raw sample (BEQ_{bio}), the artificial mixture ($BEQ_{bio\_mixture}$) containing the four estrogenic compounds at the same concentration as they have been detected in the raw sample (see Table 1), and the relative contribution of each chemical (BEQ_{chem}) tested individually (mestranol, estrone, BPA and butylparaben). Error bars refer to standard deviation (SD).

distributed in the environment, used as a raw material in several industrial applications (i.e. additive modifier in plastic products and food packaging materials), which can mimic estrogen-like activity after exposure (Valcárcel et al., 2018). The estrogenic effects of butylparaben, widely used as a food additive and in cosmetics preservation, has also been reported in the literature, although its estrogenic potency are 10,000-fold lower compared to 17β-estradiol (Routledge et al., 1998).

Although none of the identified compounds have been included in any prioritization list for water quality monitoring yet (E. Commission, 2013), some progress has been made in the case of estrone and BPA. Estrone was already included in the European Water Framework Directive (WFD) Watch List for future prioritization (2015 and 2018 lists) (E. Commission, 2018), while the tolerable daily intake (TDI) in food for BPA temporarily established by the European Food Safety Authority (EFSA) has been reduced from 4 μg/kg bw/day (EFSA, 2015) to 0.04 ng/kg bw/day (Bisphenol A: EFSA draft opinion proposes lowering the tolerable daily intakeefsa.europa.eu(accessed, 2022). The results of this study support the trend of considering endocrine disruption as a criterion for water quality assessment (Colborn and Clement, 1992; Kabir et al., 2015).

In the case of the artificial mixture (see Section 2.3.4), only the linear part of the curve was considered to build the concentration-effect curve (Neale et al., 2015; Escher et al., 2014) (see Fig. S6 in SI) since experimental values did not adjust to a sigmoidal curve model providing wide confidence limits (95 %). A linear estimation was used in order to calculate $BEQ_{bio\_mixture}$ (r^2 = 0.9867). As it can be observed in Fig. 4, $BEQ_{bio\_mixture}$ value was 2.5 times lower than BEQ_{chem} and 5 times lower than BEQ_{bio}. The cocktail of chemicals present in the sample may have interacting effects as suggested in previous studies (Santos et al., 2013; Tiwari et al., 2017; Schmidt, 2018). The differences observed between the calculated BEQ values suggest the occurrence of synergic effects (Lei and Aoyama, 2010). Moreover, the identification of some other potential compounds present in the sample could be overlooked in the suspect analysis (Hashmi et al.,

Table 1
Concentrations (ng/L) of estrogenic compounds detected in the raw sample. Toxicity contribution of each compound and their respective EC_{25} (ng/mL) and mode of action (MoA) are also included.

Compound	Raw (ng/L)	EC_{25} (ng/mL)	Toxicity contribution (%)	MoA
BPA	9219	2145	1	Estrogen receptor agonist (Jarošová et al., 2014)
Butylparaben	1224	21,107	0.01	DNA and RNA synthesis inhibitor (van der Linden et al., 2008)
Estrone	128	0.64	43	Estrogen receptor agonist (Shao et al., 2005)
Mestranol	5840	23	56	Estrogen receptor agonist (Shao et al., 2005)

2018; Chen et al., 2016) and/or the spread of estrogenicity among other fractions could also explain the low $BEQ_{bio\_mixture}$ observed (see Fig. 2B). The uncertainty of EC or LC values is not often reported in literature so that the uncertainty of BEQ_{chem} value cannot be determined (Neale et al., 2015), which makes comparison between experimental data doubtful. On the contrary, the large variability observed for the BEQ_{chem} value determined in the present work can be related with the experimentally obtained EC values. The large variability of the calculations due to the intrinsic uncertainty of the measurements is in line with other studies (Mijangos et al., 2020).

4. Conclusions

An EDA procedure combining onsite sampling and extraction (LV-SPE), the in-vitro A-YES, two-step chemical fractionation and suspect and target analysis was developed and applied to reveal the potentially estrogenic compounds present in a hospital effluent water sample. In this study, four estrogenic compounds namely mestranol, estrone, BPA and butylparaben appeared as endocrine disrupting chemicals, being able to explain almost 50 % of the total estrogenic activity. The non-explained remaining activity could be attributed to the occurrence of synergic effects or overlooked estrogenic compounds present in the sample. Although the estrogenicity of those chemicals has already been reported, to the best of our knowledge, this is the first EDA study where mestranol and butylparaben appeared as estrogenicity contributors in a complex mixture. Those contaminants may induce several estrogenic effects on aquatic organisms' reproduction as well as impacts to human health. Consequently, we consider essential to further control the occurrence of such compounds by implementing broader monitoring systems which included non-directed detection techniques and bioassays for effected-oriented prioritization. Moreover, the need for efficient contaminant's removal treatments for wastewater has been highlighted, in order to avoid or decrease the ecotoxicological risk these chemicals can pose to the environment.

CRediT authorship contribution statement

Naroa Lopez-Herguedas: Investigation, Formal analysis, Writing – original draft, Visualization, Writing – review & editing. **Belén González-Gaya:** Investigation, Formal analysis, Supervision, Writing – review & editing. **Alicia Cano:** Investigation, Formal analysis, Writing – original draft. **Iker Alvarez-Mora:** Investigation, Formal analysis, Writing – review & editing. **Leire Mijangos:** Investigation, Formal analysis, Writing – review & editing. **Nestor Etxebarria:** Supervision, Resources, Funding acquisition. **Olatz Zuloaga:** Supervision, Methodology, Conceptualization, Formal analysis, Funding acquisition. **Maitane Olivares:** Supervision, Methodology, Conceptualization, Formal analysis, Data curation, Resources, Writing – review & editing. **Ailette Prieto:** Supervision, Methodology, Conceptualization, Formal analysis, Data curation, Resources, Writing – review & editing.

Data availability

Data will be made available on request.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2022.157985.

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