Development and validation of a GC–MS method for the evaluation of 17 endocrine disruptor compounds, including phytoestrogens and sitosterol, in coastal waters – their spatial and seasonal levels in Porto costal region (Portugal)

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ABSTRACT

A gas chromatography with mass spectrometric detection (GC–MS) method was developed and optimized for the determination of 17 endocrine disrupting compounds (EDCs) in coastal water samples. The evaluated EDCs were from different origins and included estrogens, bisphenol A, alkylphenolethoxylates, alkylphenols, phytoestrogens and sitosterol (SITO). The EDCs were extracted from samples using Oasis HLB (Hydrophilic–Lipophilic Balance) cartridges and derivatized with *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) added with 1% trimethylchlorosilane (TMCS). The validation parameters revealed that this method was highly specific for all assayed compounds (>99%) and the linearity of the calibration curves showed a correlation higher than 0.99. The detection limits were at low ng/L level and the recovery rates were higher than 70%. The performance of the method was checked using coastal water samples taken every 2 months during 2009–2010 from the Douro River estuary and the Porto coastline (Portugal). These data revealed that approximately 98.0% of the analyzed compounds showed levels above their limits of detection (LODs). The measured estrogens (2–20 ng/L) and industrial pollutants (up to 1.1 μg/L) were in biologic hazardous concentrations. Besides, a clear seasonal pattern of fluctuation was established for phytoestrogens and SITO. The physicochemical data, namely the amounts of nitrates, nitrites and phosphorous, confirmed the low water quality of this area.

Key words | alkylphenol ethoxylates, alkylphenols, estrogens, method validation, phytoestrogens, sitosterol

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INTRODUCTION

In the aquatic environment, there is a multiplicity of compounds able to act as endocrine disrupting compounds (EDCs) in both fish and mammals, due to their ability to either mimic or counteract endogenous hormones (Langston *et al.* 2005). This occurrence has been demonstrated for natural and synthetic estrogens, for alkylphenols (APs) (Langston *et al.* 2005) and phytoestrogens (Kiparissis *et al.* 2003) which can interfere negatively with the endocrine system of wild animals. The source of these compounds is diverse since these can be ensuing of discharges from wastewater treatment plants (WWTPs), from industrial effluents, untreated sewage or agricultural runoff (Rocha *et al.* 2011, 2012). Also, their persistence is sometimes high enough to produce deleterious effects in the biota, namely in marine environments where they persist more often than was initially expected (Rocha *et al.* 2012). Amongst the most active EDCs, those that are usually present in higher amounts in polluted aquatic environments, are several kinds of estrogens, such as estrone (E1), 17β-estradiol (E2), and ethynylestradiol (EE2) (Mills & Chichester 2005).

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Besides this, industrial or household cleaning products easily reach aquatic environments. The most important compounds of the last category able to act as EDCs are bisphenol A (BPA), alkylphenol polyethoxylates (APEOs) (chemicals included in the group of 'priority substances in the field of water policy' (2455/2001/EC) due to their potential toxicity and persistence), APs such as 4-nonylphenol (4-*n*-NP) and nonylphenol isomers (NP), 4-octylphenol (4-OP) and 4-nonylphenol isomers (4-NP). Besides, although it is known that polluted waters may hold high amounts of organic matter of vegetal origin (Azevedo *et al.* 2006; Ribeiro *et al.* 2009), there is a global scarcity of information regarding the environmental amounts of phytoestrogens, such as daidzein (DAID), genistein (GEN), biochanin A (BIO-A) or sitosterol (SITO) (Hoerger *et al.* 2009), some of which are already known as EDCs (Kiparissis *et al.* 2003). Because valid interpretation of environmental data needs validated methods, this study describes the development and the validation of an analytical protocol which allows the evaluation of the global amounts of the above referred EDCs in coastal matrices – marine and estuarine environments, by gas chromatography coupled to mass spectrometric detection (GC–MS). The extraction method, in spite of being based in a previous study done by our group (Ribeiro *et al.* 2007), was enlarged to embrace a further eight EDCs – namely the phytoestrogens and SITO that are highly difficult to extract together with APs and APEOs. To evaluate the efficacy of this technique for monitoring purposes, water samples were collected during 1 year from the Douro River estuary and Porto city coastline (Portugal). The chosen river is an international watercourse that originates in north-central Spain and flows down 772 km (480 miles) along the Spanish-Portuguese border before its entrance in the Atlantic Ocean, at the city of Porto. The city area is densely inhabited having more than 700,000 residents in the last 8 km of the estuary (Bordalo & Vieira 2005). Due to this, Porto coastal waters are highly prone to be impacted by chemicals released either upstream or inside the estuary, where they have a residence time that may range from 8 h to more than 2 weeks, depending on river flow (Vieira & Bordalo 2000).

Thus, this study aimed to provide (1) the validation of a robust analytical protocol to evaluate anthropogenic pollutants, phytoestrogens and SITO in polluted surface coastal waters; (2) the results of an annual monitorization of the Douro River estuary and Porto coastline waters; (3) the values of several physicochemical quality parameters, linked with the presence of fecal contamination and eutrophication, such as pH, dissolved oxygen (DO), nitrates, nitrites, ammonia, un-ionized ammonia, and phosphates. The new data are not only of local relevancy but also of global interest since they integrate our continuous efforts to characterize the presence and impacts of EDCs in the surface waters of the Iberian peninsula.

MATERIALS AND METHODS

Chemicals and materials

Analytical grade solvents including hexane, anhydrous pyridine, *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) added with 1% (w/v) trimethylchlorosilane (TMCS) were supplied by Sigma-Aldrich (Steinheim, Germany). Dichloromethane and methanol were acquired from Romil Ltd (Cambridge, UK). Ultrapure water was supplied by a Milli-Q water system (conductivity = 0.054 μS/cm, at 25 °C). The solid phase extraction (SPE) cartridges, 200 mg Oasis HLB (Hydrophilic–Lipophilic Balance), 6 cc, were acquired from Waters Corporation (Milford, MA, USA) and 1,000 mg silica cartridges, 6 cc, were purchased from Teknokroma (Barcelona, Spain).

Reference standards

E1, E2, EE2, 17β-estradiol-d_2 (E2-d_2), 4-*t*-OP, 4-OP, BPA, bisphenol A-d_{16} (BPA-d_{16}), Igepal CA-210 (OP1EO and OP2EO), Igepal CO-210 (NP1EO and NP2EO), FORM, BIO-A, DAID, GEN and SITO were obtained from Sigma-Aldrich (Steinheim, Germany). 4-*n*-NP and 4-NP were supplied from Riedel-de-Haën (Seelze-Hannover, Germany). Stock solutions of individual standards (100 mg/L) were prepared in methanol, transferred to amber bottles and stored in the dark at −20 °C. All standard solutions were stable and evidence of decomposition was never observed during the 1 year period. Working solutions were prepared by diluting the stock solution with methanol. From the stock solutions, six nominal calibration standard mixtures

were prepared and spiked in clean water artificial matrices containing 15–20‰ (w/v) salinity (coastal matrices) (Eaton *et al.* 1995). These fortified matrices were used as calibration standards and to demonstrate the applicability of the method. The final range of concentrations in spiked water samples were 10–300 ng/L for all 17 EDCs and 50 ng/L for E2-d_2 and for BPA-d_{16} (deuterated surrogates, herein used also as internal standards, IS). For the evaluation of precision, accuracy and recovery assays three quality control (QC) standard solutions, containing all EDCs and both IS, were prepared and added to coastal water samples: 5, 25, 100 ng/L, 12.5, 25, 100 ng/L or 10, 500, 200 ng/L depending on the analyzed compound. Calibration curves were produced using standard/IS ratios versus the above referred standard concentrations (ng/L).

Sample collection and preparation

Surface water samples were collected from the Douro River estuary and from two popular sandy beaches situated about 1 km north (Foz) and 1 km south (Lavadores) of the estuary mouth (Sites S1 and S8, Figure 1). Sampling sites S2 and S3 are located at Porto city margin, near the discharge points of effluents from three WWTPs (Figure 1). The other experimental areas are located near the dam of Crestuma (S4 and S5) and at the Vila Nova de Gaia city (S6 and S7), where there are several effluents of small rivers and three WWTPs (Figure 1). Water samples (1 L) were always collected at low tide during the months of November 2009 and January, March, May, July and September 2010 ($n = 48$) into 2.5 L amber glass bottles, which were previously rinsed in the laboratory with ultrapure water and later, on site, with water sample. The sampling occurred at 1 m depth using a peristaltic pump (Global Water: WS 3000, California, USA).

Water quality measurement

All physicochemical parameters such as temperature, pH, DO and salinity were evaluated *in situ*, using an electrochemical analyzer (Hach Lange, HQD-Electrochemistry multi meter). During transport to the laboratory all flasks were stored at *ca.* 5 °C. At the laboratory, the amounts of nitrites, nitrates, ammonia and phosphates were measured using the Palintest 7000 Interface Photometer.

Sample preparation

Water samples were immediately filtrated, to eliminate particulate matter and other suspended solids, through a

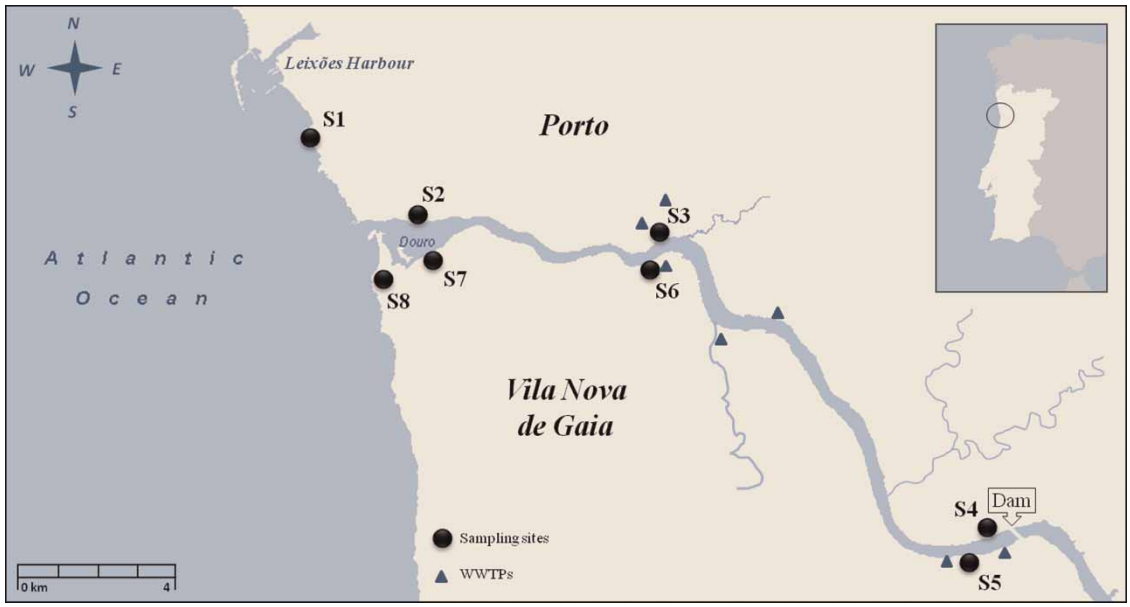

Figure 1 | Location of the sampling sites at the Porto coastline (S1 and S8) and at the Douro River estuary (S2 to S7), Portugal.

0.45 μm glass fiber filter (Millipore, Ireland). The filtrates were acidified with H_2SO_4 to pH 2 and then subjected to SPE within a maximum period of 48 h – during this phase all samples were maintained at ±4 °C in darkness until extraction. All target EDCs were extracted, from the fortified water matrices and real water samples, by SPE using Oasis HLB cartridges adapted in an off-line SPE vacuum extraction device (Waters). In this protocol, the condition step was carried out with 10 mL of CH_2Cl_2:CH_3OH (50:50, *v/v*), to remove residual bonding agents, followed by 6 mL of CH_3OH and 13 mL of ultrapure water, at a flow rate of 1 mL/min. Spiked water samples and surface water matrices (1 L) with added E2-d_2 and BPA-d_{16} (deuterated surrogates, herein used also as internal standards, IS) were loaded onto SPE cartridges at a constant flow rate of 5 mL/min followed by a washing step with 13 mL of ultrapure water and 1 mL of CH_3OH. Cartridges were dried under vacuum for 30 min and then eluted with 10 mL of CH_2Cl_2:CH_3OH (50:50, *v/v*). Because these extracts were sticky and dark, they were submitted to a cleaning step through silica cartridges (1 g). The resulting extracts were evaporated to dryness in a heating block at 40 °C under a gentle N_2 stream and reconstituted with 250 μL of anhydrous methanol – the sample concentration factor was 4,000 fold.

Due to the low volatility of the majority of the present compounds, which gave rise to poor chromatographic peaks, derivatization was essential (Rocha *et al.* 2011). In this step, 50 μL of each of the SPE extracted fractions, containing the studied compounds in mixture, were transferred into GC vials and evaporated to dryness under a gentle N_2 stream. Fifty microliters of pyridine were added to the dry residues which were derivatized by the addition of 50 μL of BSTFA (1% TMCS). BSTFA (1% TMCS) was tested together with the *n*-methyl-*n*-(trimethylsilyl) trifluoroacetamide, however the former was more efficient to derivatize all assayed EDCs, mainly the APs and APEOs. Finally, after the addition of BSTFA (1% TMCS) the vials were mixed using a vortex system and heated, in a heating block, for 30 min at 70 °C.

GC–MS conditions

The trimethylsilyl (TMS)-derivatives were further evaporated to dryness under a gentle nitrogen stream, reconstituted with 100 μL of hexane and subjected to GC–MS analysis. GC–MS analysis was performed using a gas chromatograph (Trace GC ultra, Thermo Finnigan Electron Corporation) coupled with an ion trap mass spectrometer (Thermo Scientific ITQ™ 1100 GC-MS^n), an autosampler (Thermo Scientific TriPlus™) and a TR5MS capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness). Helium carrier gas (99.9999% purity) was maintained at a constant flow rate of 1.0 mL/min. Column oven temperatures were programed using several ramps: (a) from 100 °C (initial equilibrium time 1 min) to 200 °C at 10 °C/min; (b) from 200 to 260 °C at 6 °C/min; and, finally (c) from 260 to 290 °C at 1 °C/min, at this point the GC oven was maintained at 290 °C for 5 min. The mass spectrum (MS) was achieved by electron impact ionization and operated, for quantitative analysis, the selected ion monitoring system (SIM) was preferred. A solvent delay time of 5 min was used to protect the ion multiplier of the MS instrument from saturation. Temperatures of the programmable temperature vaporization (PTV) liner ranged from 35 to 250 °C via a ramp of 10 °C/s. Both the MS transfer line and ion source were at 280 °C. Sample injection (3 μL) was programed in splitless mode using an 80 mm injection needle.

Validation studies and matrix effect

The validation protocol followed harmonized guidelines (Thompson *et al.* 2002) which included the evaluation of linearity, accuracy, precision, limits of detection (LODs) and quantification (LOQs). For this purpose, artificial coastal water samples were used as blank matrices (free of all target EDCs) and accuracy and precision (intra and inter batch) were evaluated analyzing three replicates of each QC sample at three levels of concentration (low, medium and high). Precision was expressed in terms of relative standard deviation (% RSD) of the replicate measurements. Accuracy was estimated as the percentage of agreement between the method results and the nominal amount of added compound. Blank matrices of coastal water, fortified at three QC concentrations allowed the calculation of recovery and the effectiveness of the extraction step. The LODs and LOQs were determined evaluating the signal/noise ratio of blanks (S/N 3 for LODs and S/N 10 for LOQs). Moreover, since the current

compounds were measured in ng/L, method blanks and controls, constituted by unbiased estuarine or sea water samples spiked with all assayed EDCs, including phytoestrogens and SITO, at an intermediate concentration of the calibration curve (300 ng/L), were always applied during the monitoring process to guarantee the quality of data. The matrix effect was evaluated fortifying real water samples from the estuary and sea with standards at three different levels, added with 50 ng/L of both IS, and injected in triplicate. The ratio areas and MS spectra of standards spiked in real samples were compared with those of artificial fortified matrices and those acquired using methanol standards.

Quantification parameters

Quantitative analysis was performed in a SIM mode using external calibration. Working solutions were prepared diluting the stock solution with methanol at six calibration levels ranging from 10 to 375 ng/L for all 17 EDCs and 50 ng/L for E2-d_2 and for BPA-d_{16}. The analytic parameters for the used GC–MS method are summarized in Table 1 and typical chromatograms of all EDCs in solvents and added to the matrix are shown in Figure 2. Since the current EDCs were measured in ng/L, method blanks were used to ensure the absence of contamination by laboratory material. Beyond this, random water samples were spiked with all assayed EDCs at an intermediate concentration (150 ng/L) of the calibration curve and then submitted to the usual analysis.

Statistical analysis

Results are expressed as mean ± standard error of the mean (SEM) and data on Figures 3 and 4 were subjected to statistical analysis using the software SigmaStat for Windows (version 3.5). After checking assumptions of normality and homogeneity of variances, data sets were analyzed by one-way analysis of variance (ANOVA). Normality and homogeneity of variances were evaluated

Table 1 | Quantification and diagnostic ions used in GC–MS analysis. Inside brackets refers to the relative abundance of ions (*m/z*) for each target EDC

Compound	t_R (min)	Molecular mass	Quantification ions (*m/z*)	Diagnostic ions (*m/z*)	Segment [time (min)]
4-*t*-OP	10.8	206.3	207 (100)	–	8.5–17.2
4-NP	11.5–12.5	220.4	207 (100)	179 (84.9), 193 (31.9), 221 (31.9)	8.5–17.2
4-OP	12.9	206.3	179 (100)	180 (17.7)	8.5–17.2
4-*n*-NP	14.2	220.4	179 (100)	292 (35.9)	8.5–17.2
OP1EO	14.8	250.4	251 (100)	207 (97.2), 135 (68.9)	8.5–17.2
NP1EO	15.8–16.6	264.4	251 (100)	265 (64.4), 207 (59.5), 135 (45.5)	8.5–17.2
BPA-d_{16}	17.9	244.3	368 (100)	369 (34.7), 386 (9.1)	17.2–22.5
BPA	18.1	228.3	357 (100)	358 (30.8)	17.2–22.5
OP2EO	18.6	294.4	295 (100)	207 (76.5), 115 (55.2)	17.2–22.5
NP2EO	19.6–19.9	308.5	295 (100)	207 (74.9)	17.2–22.5
E1	24.7	270.4	342 (100)	357 (55.1)	22.5–25.3
E2-d_2	24.9	272.4	287 (100)	418 (75.2), 328 (72.8)	22.5–25.3
E2	25.0	274.4	285 (100)	416 (85.2), 326 (48.4)	22.5–25.3
EE2	27.3	296.4	425 (100)	285 (48.0), 426 (34.7)	25.3–29.0
FORM	28.6	268.3	340 (100)	339 (76.0), 355 (22.6)	25.3–29.0
BIO-A	30.5	284.3	356 (100)	341 (34.3)	29.0–35.0
DAID	30.5	254.2	398 (100)	383 (76.0), 355 (22.6)	29.0–35.0
GEN	30.8	270.2	471 (100)	473 (19.9)	29.0–35.0
SITO	43.4	414.7	396 (100)	486 (53.4), 255 (49.4)	35.0–45.0

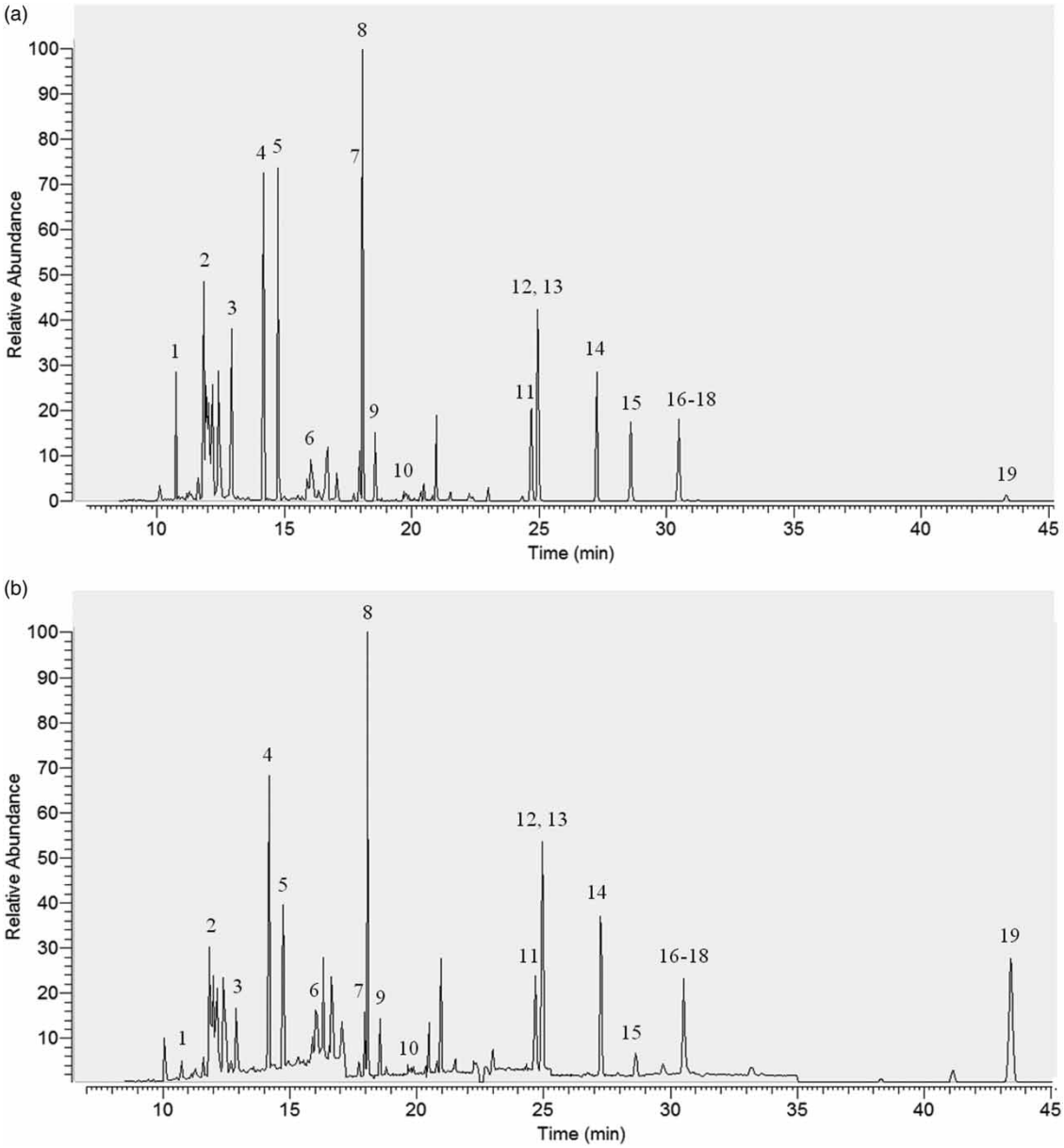

Figure 2 | Chromatograms of a standard mixture of the target 17 EDCs (1,000 ng/L) (a) and of a coastal water sample spiked with the 17 EDCs (500 ng/L) assayed in this study (b) in selected ion monitoring (SIM) mode. The numbers above each chromatographic peak correspond to: (1) 4-*t*-OP; (2) 4-NP; (3) 4-OP; (4) 4-*n*-NP; (5) OP1EO; (6) NP1EO; (7) BPA; (8) BPA-d_{16}; (9) OP2EO; (10) NP2EO; (11) E1; (12) E2-d_2; (13) E2; (14) EE2; (15) FORM; (16) BIO-A; (17) DAID; (18) GEN; (19) SITO.

using the Kolmogorov–Smirnov and the Bartlett tests, respectively. When, normalization was not possible the ANOVA on Ranks–Student–Keuls Method was used. For the evaluation of temporal differences, an ANOVA was used followed by the *post-hoc* Holm-Sidak test. Results were considered statistically significant for $p < 0.05$ (two-tailed analysis).

RESULTS

Solid-phase extraction

The sample pre-treatment was successfully optimized for the simultaneous extraction of 17 EDCs since the recovery rates reported in Table 2 ranged from 70 to 120% demonstrating

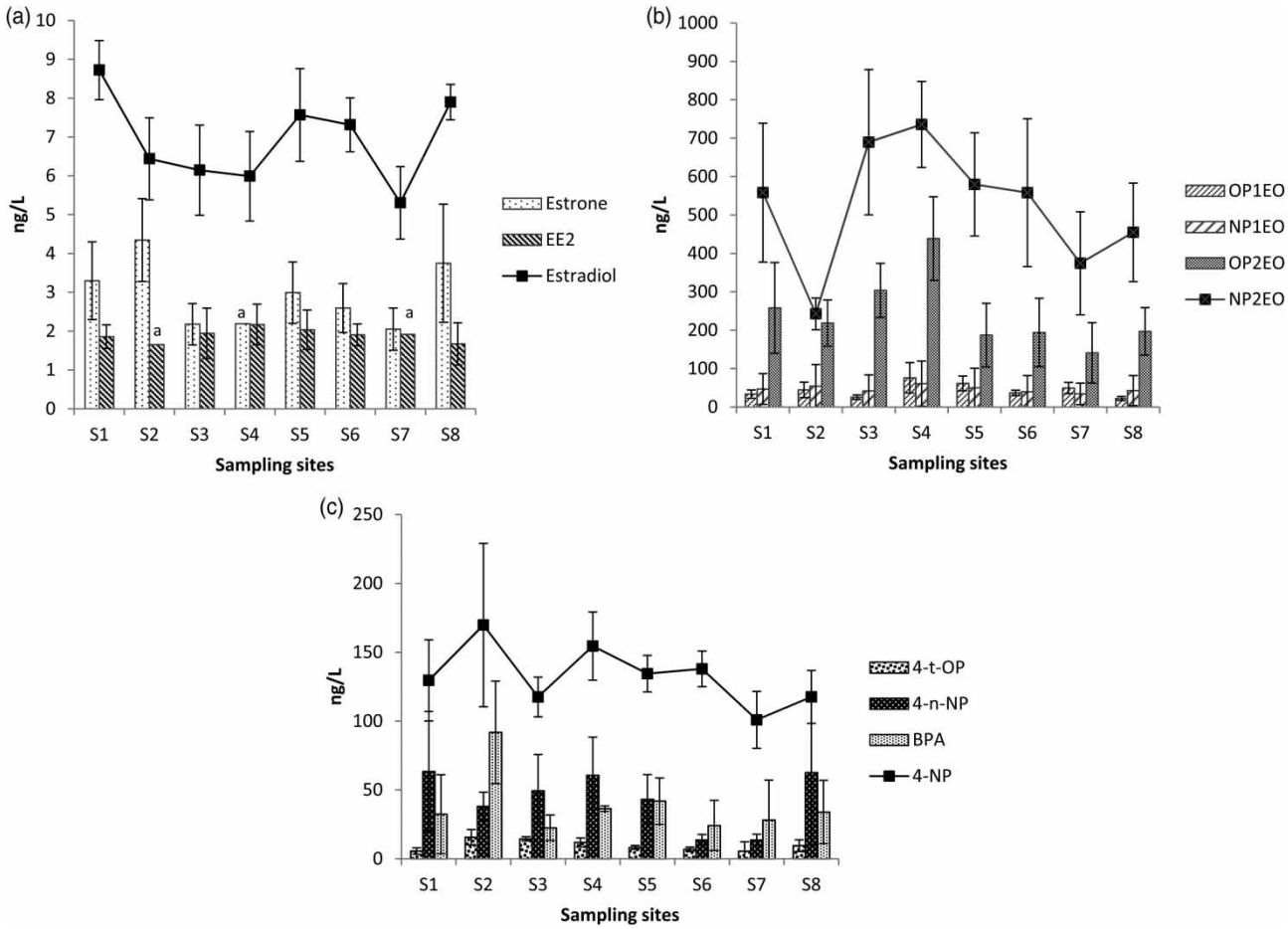

Figure 3 | Environmental levels (ng/L) of E1, E2, EE2 (a), NP1EO, NP2EO, OP1EO, OP2EO (b), and NPs, OPs, BPA (c) at each sampling site located in the Douro River estuary and Porto coastline on each sampling occasion. Data are presented as mean ± standard error of the mean (SEM).

that this SPE method can be used for the extraction of the selected EDCs. This observation was confirmed by the analysis of a blind sample, prepared in triplicate, containing all analyzed EDCs at concentrations which were not known by the analyst (150 ng/L for all EDCs). These results produced average accuracies of 97% and precision of 9.8% RSD.

GC–MS instrumental data

GC separation was achieved evaluating different ranges of temperatures. Initially full-scan mass spectra of individual silylated EDCs (EDCs–TMS) were analyzed. Then, all standards were injected in separated groups (estrogens, phenols, APEOs and phytoestrogens) and finally all EDCs–TMS in the mixture. The SIM segments were established containing for each compound the specific ion mass-to-charge ratio (m/z) (Table 1). The selection of high mass fragments associated with high percentages of m/z quantification ions is widely considered of great interest when complex matrices are analyzed. A typical chromatogram, obtained using a standard mixture of the 17 EDCs, is illustrated in Figure 2(a). Figure 2(b) shows a typical chromatogram of an environmental coastal water sample spiked with all EDCs referred in this study.

Validation data

Selectivity

In this study, it was observed that when standard solutions of all EDCs were spiked in real seawater and estuarine

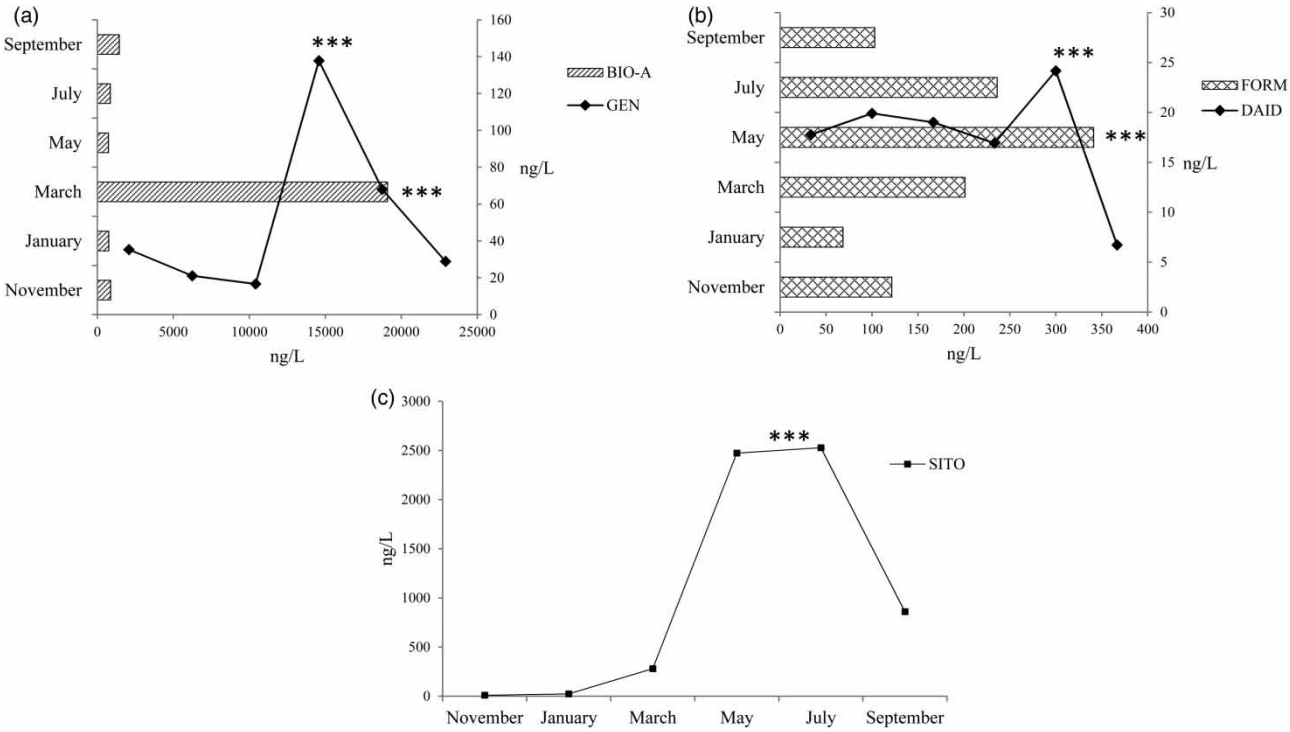

Figure 4 | Seasonal fluctuation of phytoestrogens and SITO (ng/L) at the Douro River estuary and the Porto coastline. Significance was considered at $p < 0.001$ (***). BIO-A and GEN (a); FORM and DAID (b); SITO (c).

water samples (QC) both the retention times and the mass spectra were maintained between standards and fortified matrices (RSD < 5%). Therefore, it was concluded that this chromatographic procedure is a selective method for the quantification of the 17 EDCs presented in Table 1. Furthermore, the absence of matrix interferences, even in highly polluted matrices, guarantees the suitability of this method for monitoring purposes in polluted coastal water samples (Figure 2(b)).

Linearity and range

The range, slope, interception points and correlation levels ($r^2 > 0.99$) of each analytical curve are presented in Table 3.

Precision and accuracy

The precision of this method was based on the determination of the repeatability (intra-day assays) and the intermediary precision (inter-day assays) (Table 2). In this method, precision and accuracy mean values were, for all calibration concentrations, in the range of 100% ± 1.0 and 9.8% ± 0.4, respectively. Instrumental precision (%RSD), calculated automatically by the GC–MS software, was 20% for E2-d_2 and 19% for BPA-d_{16} ($n = 50$ injections).

Limits of detection and quantification

The LODs and LOQs ranged from 0.60 to 5.47 ng/L and from 1.98 to 18.05 ng/L, respectively (Table 3).

Matrix effects

To confirm that our matrix did not affect the SPE protocol, QC standard solutions were spiked in real seawater and estuarine water samples. The analysis of these data confirmed that both retention times (RSD < 5%) and ion fragmentation were not affected, i.e., the tolerances were ±10% for ions with a relative intensity >50% of the base peak, ±15% for ions with a relative intensity of 20–50%, ±20% for ions with a relative intensity of 10–20% and ±50% for ions with a relative intensity of <10%. Also, the

Table 2 | Intra- and inter-day precision, accuracy and recovery data for the 17 EDCs assayed in this study

		1st Day		2nd Day		3rd Day			
EDCs spiked in estuarine matrix (ng/L)		Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)	Recovery (%)	RSD (%)
4-*t*-OP	5	99	11	104	14	96	4	83	(20)
	25	82	45	98	4	119	5	102	(8)
	100	113	2	101	3	104	1	120	(1)
4-OP	12.5	117	13	88	15	120	6	120	(7)
	25	89	8	82	4	102	13	100	(18)
	100	102	7	93	8	116	7	99	(8)
4-NP	12.5	111	20	77	8	77	11	111	(4)
	25	98	18	101	14	94	20	103	(5)
	100	117	13	89	11	114	9	94	(5)
4-*n*-NP	10	120	6	95	16	120	12	91	(9)
	50	82	6	99	14	91	12	75	(16)
	200	97	8	91	19	99	12	90	(5)
OP1EO	12.5	99	9	75	10	83	12	104	(9)
	25	102	5	82	5	82	15	98	(8)
	100	101	17	94	5	106	13	98	(14)
NP1EO	5	116	17	107	5	113	7	114	(11)
	25	73	14	114	7	109	11	94	(13)
	100	104	7	106	5	116	2	78	(5)
BPA	5	113	10	94	10	95	14	100	(20)
	25	103	12	98	6	90	20	96	(8)
	100	105	8	90	5	101	3	104	(8)
OP2EO	5	117	13	93	7	106	15	119	(16)
	25	74	10	81	11	94	10	118	(9)
	100	110	8	105	4	98	15	102	(7)
NP2EO	5	105	7	99	10	90	17	81	(5)
	25	10	16	98	15	84	11	87	(15)
	100	10	9	100	4	80	9	97	(18)
E1	5	104	23	120	2	107	6	114	(6)
	25	111	13	98	6	104	8	100	(20)
	100	108	8	111	8	101	9	117	(9)
E2	5	119	2	105	19	100	7	101	(19)
	25	110	2	95	6	104	8	94	(1)
	100	100	10	105	3	99	4	98	(9)
EE2	5	114	13	114	10	106	6	70	(5)
	25	114	19	106	2	98	6	100	(20)
	100	96	15	79	10	114	7	111	(15)
FORM	5	117	3	100	12	102	6	112	(11)
	25	117	17	105	11	102	5	108	(24)
	100	69	18	106	3	102	7	87	(10)
BIO-A	5	82	12	108	3	95	19	96	(3)
	25	103	9	117	2	120	17	112	(11)
	100	93	24	100	4	96	2	87	(12)
DAID	5	97	21	111	20	120	6	89	(18)
	25	96	13	91	13	97	7	96	(15)
	100	109	16	92	11	111	6	93	(14)

(*continued*)

Table 2 | continued

EDCs spiked in estuarine matrix (ng/L)		1st Day		2nd Day		3rd Day		Recovery (%) RSD (%)	
		Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)		
GEN	5	101	2	96	4	109	11	93	(9)
	25	120	5	84	13	110	7	117	(4)
	100	93	14	97	6	108	16	95	(13)
SITO	5	99	10	101	9	93	9	120	(2)
	25	78	17	101	18	98	16	104	(14)
	100	94	23	85	3	84	3	83	(19)

Table 3 | Calibration parameters of the method including the calibration curves, limits of detection (LOD) and quantification (LOQ) of the 17 EDCs analyzed by GC–MS

Compound	Linearity parameters			
	Calibration Equation	r^2	LOD (ng/L)	LOQ (ng/L)
4-*t*-OP	$y = 0.004x$	0.99	1.46	4.82
4-*n*-OP	$y = 0.004x$	0.99	3.50	11.55
4-NP	$y = 0.008x + 0.8$	0.99	5.47	18.05
4-*n*-NP	$y = 0.007x$	0.99	0.60	1.98
OP1EO	$y = 0.004x$	0.99	5.29	17.46
NP1EO	$y = 0.0005x$	0.99	1.84	6.07
BPA	$y = 0.006x$	0.99	0.73	2.41
OP2EO	$y = 0.0007x + 0.1$	0.99	0.94	3.10
NP2EO	$y = 0.0004x + 0.2$	0.99	2.07	6.83
E1	$y = 0.003x$	0.99	0.97	3.20
E2	$y = 0.005x$	0.99	0.86	2.84
EE2	$y = 0.002x$	0.99	1.34	4.42
FORM	$y = 0.001x - 0.05$	0.99	2.61	8.61
BIO-A	$y = 0.00001x$	0.99	1.38	4.55
DAID	$y = 0.003x - 0.02$	0.99	1.25	4.13
GEN	$y = 0.0005x$	0.99	1.14	3.76
SITO	$y = 0.0005x$	0.99	2.01	6.63

peak areas were similar when comparing QC standards spiked in real water samples or in coastal water artificial matrices.

EDCs in water samples from Douro River estuary and Atlantic Ocean

Out of the 816 measurements made (48 samples from eight sites × 17 compounds) less than 3% refer to values below the LODs of the method (Table 3). In Table 4, the average amounts of all proposed EDCs are reported.

Estrogens

In all analyzed samples, the detection frequency of E1 and E2 were both close to 100%. In Figure 3 the spatial fluctuation patterns of E1, E2 and EE2 are shown, and in Table 4 their mean monthly amounts are reported, considering that there are no significant differences among the sampling sites ($p > 0.05$). In spite of this, occasionally extremely high amounts of estrogens were measured at sites S2 and S5 that are located near the WWTPs effluents, and also at site S8 where direct sewage discharges are suspected to occur (Figures 1 and 3(a)).

Industrial and household cleaning compounds

Figure 3 shows spatial distributions of the nine industrial compounds (APEOs and APs) and Table 4 refers to their annual amounts, similarly to estrogens no differences were found either among sampling sites or in a particular occasion of the year ($p > 0.05$). With the exception of 4-*n*-OP, the industrial compounds showed a frequency of 100% in the analyzed samples (Figure 3(b) and 3(c)). APEOs attained in some unexpected/random occasions individual values of 2,700 and 1,230 ng/L for NPEOs and OPEOs, respectively, near the WWTPs of the Porto city margin (S3 and S4; Figure 3(b)). Similarly, both APs and BPA peaked somewhat randomly, nonetheless, no significant differences were found for their levels among the

Table 4 | Environmental levels of all analyzed EDCs measured at the Douro River and the Porto coastline from November 2009 to September 2010. Data are presented as mean ± standard error of the mean (SEM)

	Mean (ng/L) ± SE [% *f*]					
EDCs	**23-Nov-09 (*n* = 8)**	**18-Jan-10 (*n* = 8)**	**16-Mar-10 (*n* = 8)**	**17-May-10 (*n* = 8)**	**19-Jul-10 (*n* = 8)**	**22-Sep-10 (*n* = 8)**
4-*t*-OP	30.6 ± 9.3	10.1 ± 1.5	15.2 ± 4.9	7.1 ± 1.7	6.4 ± 1.3	5.1 ± 1.1
4-NP	137.0 ± 18.0	110.3 ± 11.0	160.5 ± 50.9	147.4 ± 16.4	170.0 ± 15.6	88.2 ± 12.9
4-*n*-OP	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
4- *n*-NP	40.1 ± 13.2	3.3 ± 0.6	9.9 ± 6.4	25.1 ± 12.6	115.7 ± 46.6	64.3 ± 27.8
4-OP1EO	60.4 ± 32.7	41.8 ± 8.7	33.3 ± 17.0	49.4 ± 17.3	41.4 ± 13.7	33.0 ± 5.6
4-NP1EO	141.3 ± 19.5	161.2 ± 39.2	100.7 ± 24.3	354.4 ± 62.4	284.2 ± 52.0	321.0 ± 99.6
BPA	313.7 ± 69.6	36.7 ± 7.1	32.1 ± 9.4	20.4 ± 4.2	36.3 ± 6.0	69.2 ± 26.9
4-OP2EO	424.0 ± 110.5	187.7 ± 63.1	100.4 ± 34.5	394.1 ± 62.8	198.4 ± 66.6	274.0 ± 84.4
4-NP2EO	1,147.5 ± 232.8	505.3 ± 160.2	211.7 ± 66.5	631.4 ± 190.6	552.5 ± 109.1	477.8 ± 180.2
E1	2.7 ± 0.8	1.8 ± 0.4	1.5 ± 0.3	3.3 ± 0.6	4.6 ± 1.2	3.6 ± 0.9
E2	8.5 ± 1.9	7.6 ± 0.9	5.9 ± 1.2	7.0 ± 1.0	7.5 ± 0.5	5.4 ± 0.6
EE2	<LOQ	<LOD	<LOD	<LOQ	4.5 ± 0.3	<LOQ
FORM	121.5 ± 23.0	68.4 ± 9.7	201.2 ± 23.5	341.0 ± 63.9	236.1 ± 33.1	102.9 ± 24.3
BIO-A	869.8 ± 330.8	745.9 ± 274.0	19,091.1 ± 1,040.0	728.4 ± 187.8	849.5 ± 126.6	1439.1 ± 356.7
DAID	17.8 ± 1.7	19.9 ± 3.1	19.0 ± 2.4	16.9 ± 3.3	24.2 ± 4.1	6.7 ± 0.8
GEN	35.3 ± 5.0	21.1 ± 3.5	16.6 ± 4.0	137.8 ± 39.0	68.0 ± 9.9	28.9 ± 5.9
SITO	8.7 ± 1.3	22.7 ± 13.2	279.7 ± 107.3	2,472.8 ± 546.6	2,527.5 ± 264.3	858.4 ± 122.0

assayed estuary sites and those in the coastline ($p > 0.05$; Figure 3(c)).

Phytoestrogens and sitosterol

Phytoestrogens showed a clear seasonal pattern of fluctuation (Figure 4). In spring (March), the levels of BIO-A rose significantly in both the estuary and coastline ($p < 0.001$) and then decreased in May, the occasion when its demethylated metabolite, the GEN, rose sharply ($p < 0.001$; Figure 4(a)). Similar behavior was observed for FORM which peaked in May (up to 340 ng/L) ($p < 0.001$) and its metabolite, the DAID, was highest in July (up to 25 ng/L) ($p < 0.001$; Figure 4(b)). Later on, in both autumn and winter, the values of all phytoestrogens were minimal, even when compared with summer ($p < 0.05$). SITO increased almost 1,000 fold in May–July ($p < 0.001$; Figure 4(c)) resembling the behavior of the above referred isoflavones, the levels of which were maximal in spring.

Physicochemical parameters

All analyzed physicochemical parameters were very similar among sampling sites and no specific variation was observed during sampling (Table 5).

Table 5 | Physicochemical data evaluated locally at each sampling site from November 2009 to September 2010. Data are shown as mean ± standard deviation (SD)

Physicochemical parameters	**Sea (*n* = 12)**	**Estuary (*n* = 36)**
Dissolved O_2 (mg L^{-1})	9.9 ± 0.9	9.3 ± 1.9
T (°C)	16.7 ± 3.0	17.3 ± 5.8
Salinity (‰)	30.2 ± 2.7	2.2 ± 2.4
Conductivity (mS cm^{-1})	44.0 ± 2.3	10.5 ± 16.2
pH	8.3	7.8
Nitrites (mg L^{-1})	0.0 ± 0.0	0.2 ± 0.2
Nitrates (mg L^{-1})	1.0 ± 0.5	2.4 ± 1.3
Ammonia (mg L^{-1})	0.4 ± 0.4	1.2 ± 0.9
Un-ionized ammonia (mg L^{-1})	0.0 ± 0.0	0.1 ± 0.1
Phosphates (mg L^{-1})	0.6 ± 0.2	0.9 ± 0.2

DISCUSSION

The validated method allows the evaluation of the global amounts of the 17 proposed EDCs in coastal matrices since the SPE step shows high recoveries and precision for all assayed compounds (Thompson *et al.* 2002) and the quantification step has the specificity, linearity, precision, accuracy, sensitivity and recoveries required by the International Union of Pure and Applied Chemistry (IUPAC) validation guidelines (Thompson *et al.* 2002). The low LOQs (ng/L levels) of this method associated with its fast application and moderate costs makes it excellent for monitoring programs of complex coastal matrices where extremely low amounts of contaminants are usually present. Another advantage of this chromatographic method, compared to others (Ribeiro *et al.* 2009; Rocha *et al.* 2011), is its feasibility for analyzing 17 EDCs, with different origins and physical chemical properties, in one single chromagraphic run. The inclusion of both phytoestrogens and SITO was considered by our group an important technical improvement due to the scarcity of information concerning these compounds (Hoerger *et al.* 2009) and their increasing importance as environmental endocrine disruptors (Kiparissis *et al.* 2003). Finally, the application of the validated protocol allowed the evaluation of all assayed EDCs in the Douro River estuary and at the Porto coastline.

Natural and synthetic estrogens

In the estuary, the measurement of extremely high amounts of E1 and E2 (up to 20 ng/L) at the sites S2, S5 and S6 demonstrates that the major route of entrance of these estrogens are the effluents coming from the WWTPs located upstream of the last referred sampling sites (Figure 1). These observations are in accordance with the knowledge that points to the WWTP effluents as the main source of these compounds because these facilities have difficulty in achieving the eradication of these compounds (2002/657/EC). In the coastline, the direct discharges are the most probable source of these EDCs as their amounts were similar to those found at the estuary (Figure 3; $p > 0.05$). In spite of the concentrations measured in this study being at low ng/L levels, they are capable of inducing estrogenic effects such as vitellogenesis (Kolpin *et al.* 2002), intersex (Allen *et al.* 1999) and even impact on the long term with the sustainability of wild fish populations (Jobling *et al.* 2006; Kidd *et al.* 2007). In the Iberian peninsula, there are few studies reporting these phenomena, however, some describe the occurrence of intersex in fish caught in this Douro River estuary (Ferreira *et al.* 2005) and imposex along the northwest Portuguese coast, i.e., the development of male accessory sex organs in several female gastropods such as the Atlantic dogwinkle (*Nucella lapillus*) and the European sea snail (*Nassarius reticulatus*) (Coelho *et al.* 2006; Castro *et al.* 2007); the latter studies also agree with the current data since they demonstrate that the presence of EDCs is not restricted to freshwater systems and estuaries, but can also be found in coastal and other marine ecosystems. This observation is quite worrying since both assayed beaches are considered clean areas according to the European and the Portuguese legislation (76/160/EEC; Decreto-Lei n° 236/98) and are used by local inhabitants for recreational proposes (fisheries, baths, surfing) and/or as an important source of seafood.

Industrial and household cleaning compounds

The occurrence of the industrial and household cleaner compounds is a consequence of anthropogenic activity. Due to their wide use, these pollutants have been detected in surface waters, in estuaries, rivers, streams and lakes, at concentrations up to μg/L (Zoller 2006; Arditsoglou & Voutsa 2008). The present data show that APEOs, known to promote unexpected estrogenic effects on both wild fauna and humans (Arditsoglou & Voutsa 2008; David *et al.* 2009), were the most abundant EDCs in both Douro River estuary and Porto coastline, they were measured, frequently, in amounts that surpass 500 ng/L, reaching sometimes μg/L at both estuary and coastline. However, as these concentrations are similar to others measured in other coastal environments of Spain and Greece (Arditsoglou & Voutsa 2008; David *et al.* 2009) it is possible that this is a wide global problem regarding coastal areas, and not a particular occurrence of our study area. In addition to the weak baseline action of these compounds as endocrine disruptors, it is known that in the environment APEOs degrade within 1 or 2 days deriving NPs and OPs,

which are much more harmful and persistent than their parents (2455/2001/EC). Unfortunately, herein the levels of both NPs (ca. 200 ng/L) and OPs (ca. 15 ng/L) were systematically higher than 10 ng/L which, according to European regulations, is the maximal value accepted for these compounds in surface waters (Erickson 2002). In the past, other aquatic environments with similar amounts of these compounds were submitted to remediation programs and risk assessment studies for local populations, e.g., River Elbe, recently submitted to a cleaning process, showed estuarine and coastal amounts for 4-*t*-OP of 0.8–1.3 and 0.1–16 ng/L and for NP of 9.5–13 and 0.3–63 ng/L, respectively (Erickson 2002). BPA is one of the most controversial EDCs because safety values for its presence in the environment have been difficult to establish when its occurrence is a chronic problem (Crain *et al.* 2007). In this study, BPA was considered a persistent EDC because its presence was systematically demonstrated not only during the current sampling surveys, but also in previous studies performed in this area (Ribeiro *et al.* 2009; Rocha *et al.* 2011, 2012). Compared to other aquatic environments, the global BPA amounts found here were similar to those referred in other surface waters either in Portugal (Azevedo *et al.* 2001; Quiros *et al.* 2005), Spain (Cespedes *et al.* 2005), or the Netherlands (Vethaak *et al.* 2005). Importantly, no differences were found between the BPA levels measured in the coastline and those in the estuary.

Phytoestrogens and sitosterol

Phytoestrogens are nonsteroidal compounds naturally occurring in plants and known to exert estrogenic activities (Benassayag *et al.* 2002; Kiparissis *et al.* 2003). Among phytoestrogens the isoflavones, such as BIO-A, GEN, FORM and DAID, share a similar mechanism of action of endogenous estrogens through their affinity to the estrogenic receptors. Fortunately, since the last referred compounds show EC_{50} values that are three to four orders of magnitudes higher than that of E2 (Hoerger *et al.* 2009) the (total) concentration of these compounds must be at least 1,000-fold higher than that of E2, i.e., in the μg/L range, to produce an estrogenic effect equivalent to that of E2 (Hoerger *et al.* 2009). In the environment, the sources of phytoestrogens are complex, however it is known that both DAID and GEN are among the most important components of fruits, cereals, and vegetables (Liggins *et al.* 2000a, 2000b, 2002), which are abundantly produced in this region, and BIO-A and FORM are present in considerable amounts in red clover (*Trifolium pratense* L., Fabaceae) and papilonaceous (Papilionaceae) that flourish in the studied area (Ribeiro *et al.* 2009). Besides, since the aquatic flora of the studied area is abundant in meadows of seagrass, *Zostera noltii*, which are rich sources of these compounds (Volkman *et al.* 2008), it is possible that they strongly contribute to the values found in either the Douro River estuary or Porto coastline. Besides, all along the Douro River there are several food industries the wastes of which may contribute to the actual load of these compounds in the water, however since this study reports an indubitable seasonal trend for the assayed phytoestrogens, which in a global manner increased their levels significantly in spring and early summer (March to July), it is feasible that the main origin of these compounds are natural, rather than industrial. Individually, BIO-A was the one that rose significantly in March (up to 2,000 ng/L). In May, the environmental concentrations of BIO-A were beginning to decline whereas those of its demethylated metabolite, GEN, increased significantly in both the estuary and coastline (up to 300 ng/L). In May, the levels of FORM also attained a maximum (up to 600 ng/L) but later on, in July, these amounts decreased while its demethylated metabolite, DAID, peaked (up to 40 ng/L). These data are consistent with both the vast vineyards and orchards (mainly of apple, orange, and almond) located in both margins of this river that flourish in March and rest during the cold seasons. SITO is a phytosterol that was identified as an important component of vascular plants (Benassayag *et al.* 2002) and mentioned as an ubiquitous environmental compound (Ali *et al.* 2009). Because this compound has a similar chemical structure to cholesterol, SITO also acted as an endocrine disruptor (Santos *et al.* 2008). Because SITO is present in several plants, such as wheat, rice, pumpkin and corn, which are agricultural products of this region, its evaluation was considered herein. Thus, in parallel to phytoestrogens, SITO also showed a seasonal pattern of fluctuation, i.e., the highest amounts of this compound were measured in spring (up to 6,000 ng/L) and the lowest in winter. In spite of the fact that endocrine disruption seems to occur with

higher amounts of SITO (Santos *et al.* 2008) than those found in both estuary and coastline further studies are needed to discard this possibility in the studied area. In addition, further biological studies are needed to confirm if the total load of all these compounds has potential for synergistic activity, either with other phytoestrogens or other estrogenic chemicals able to produce endocrine disruption in aquatic species.

Physicochemical data

Some physicochemical parameters closely related to sewage and WWTPs discharges (pH, DO, ammonium, nitrites, nitrates and phosphates) were additionally evaluated herein, at both coastline and estuary. Because the effect of the recent (2008) construction of jetties at the mouth of the estuary was not yet clear, the evaluation of salinity was also considered. Concerning this latter aspect, this study clearly demonstrated that compared to other studies performed in this area before 2008 the salinity decreased significantly in the estuary (Ribeiro *et al.* 2009), i.e., the influence of the ocean was obviously diminished, logically favoring the increase of the residence time of pollutants in the estuary (certainly promoting impacts on the health of local organisms). Corroborating this thesis, the levels of nitrates, nitrites, ammonia and phosphates were also tenfold higher in this study than in 2005 (Ribeiro *et al.* 2009). These values were so critical during 2009–2010 that the amounts of toxic (un-ionized) ammonia, which is dependent of both pH and temperature, were twofold higher than that required by the Portuguese legislation (Decreto-Lei n° 236/98). Additionally, the total amounts of phosphorous, were also measured in excessive amounts, i.e., almost threefold higher than the maximal concentrations of 0.1 mg/L recommended for rivers and streams by the Water Quality Criteria (1972). Thus, our data confirmed that this coastal area was being strongly impacted by urban WWTPs and non-treated sewages, evidencing the need for remediation programs.

CONCLUSIONS

This paper describes the development and validation of a simple and fast GC–MS method which can be easily adjusted to monitoring programs of water assays. To confirm the applicability and utility of the method all 17 assayed EDCs were evaluated in the Porto coastal waters (Douro River estuary and Porto coastline) during a 1 year survey. The current data revealed the existence of EDCs within the range defined as able to induce endocrine disorders in aquatic animals, particularly in fish. Besides, it was demonstrated that both phytoestrogens and SITO have seasonal fluctuation patterns that are compatible with the growth of local flora. Finally, according to the Water Quality Criteria (1972), the physicochemical data showed alarming levels of nitrates, nitrites and phosphates, in both estuary and coastline. This fact may pose non-negligible risks for both aquatic animals and humans that use this area either for recreational purposes or as a seafood source.

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