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Annual Fluctuations of Endocrine-Disrupting Compounds at the Lower End of the Lima River, Portugal, and in Adjacent Coastal Waters

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Abstract The Lima River is a Spanish–Portuguese water body. Notwithstanding the fact that the river incorporates protected natural areas, levels of endocrine-disrupting compounds (EDCs) within its waters have never been measured; such EDCs include the following: natural and pharmaceutical oestrogens (17β-estradiol, E1, and 17α-ethynylestradiol), industrial and household pollutants (4-octylphenol, 4-nonylphenol, and their monoethoxylates and diethoxylates, and bisphenol A), phytoestrogens (formononetin, biochanin A, daidzein, genistein), and phytosterols (namely, sitosterol). To obtain an understanding of levels of EDCs, water samples were taken from eight sampling sites along the river every 2 months during a 1-year period (2011). The water samples were preconcentrated (Oasis HLB cartridges), cleaned (silica cartridges), and analysed using gas chromatography. Results showed that levels of oestrogens and industrial and household pollutants were higher in summer than in other seasons. Although oestrogens were more abundant (approximately 40 ng/L) on the southern margin of the river, levels of other pollutants were higher (approximately 124 ng/L) in the north. Phytoestrogens and sitosterol showed clear seasonal fluctuations with higher amounts of formononetin (approximately 389 ng/L), biochanin A (approximately 160 ng/L), and sitosterol (≥5 μg/L) measured in summer. The overall oestrogenic load, expressed in ethynylestradiol equivalents, was 18 ng/L for oestrogens, 0.5 ng/L for industrial and household pollutants, and 13 ng/L for phytoestrogens. Water physicochemical parameters indicate anthropogenic pollution because $\Sigma_{\text{nitrites,nitrates}}$ (>1 mg/L) and phosphates (approximately 0.4 mg/L) were high. The study showed that the waters of the Lima River are subject to impacts and that levels of EDCs pose risks to the river's biota.

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The Lima River originates in the Talariño Mountain of Spain and flows east to west across the Costa Verde (Green Coast) region of northern Portugal. Across its 108-km course, 67 km of which flow through Portugal, the river runs mainly through wild protected areas (namely, the Peneda-Gerês National Park) and through several agricultural zones. The river ultimately reaches the Atlantic Ocean near the city of Viana do Castelo, which hosts an important harbour, one marina, several metallurgic industries, and the second largest shipyard of Portugal. This infrastructure supported the appearance of many related industries along with intense urban activities (Rocha et al. 2012). The Lima River, including its estuary and the nearby coast, also comprise an important touristic region. It is possible that the Lima River is being subjected to inputs of endocrine-disruptor compounds (EDCs), which, even in small amounts (from ng/L to μg/L), may exert an important influence on local fauna (Mills and Chichester 2005). In fact, recent ecological studies concluded that the Lima River estuary is moderately disturbed (Ramos et al. 2006; Oliveira et al. 2009; Costa-Dias et al. 2010). However, to

date there is no information specific to the Lima river, estuary or nearby Atlantic coastline concerning levels of various important EDCs, which have been found to occur at physiologically/toxicologically significant levels elsewhere in Portugal and in ecosystems worldwide (Atkinson et al. 2012; Oketola and Fagbemigun 2013; Kostich et al. 2013; Xu et al. 2012; Rocha et al. 2013a, b). EDCs of concern include the following: potent natural and pharmaceutical oestrogens, such as estradiol (E2) and 17α-ethynylestradiol, along with synthetic compounds used in industry, such as alkylphenols (APs) and alkylphenol polyethoxylates (APEOs)—chemicals included as "priority substances in the field of water policy" (Commission Decision 2455/2001/EC). Less studied, but not necessarily less significant EDCs, are other natural phytoestrogens and phytosterols; these compounds are known to act as EDCs when present in high amounts despite their originating from natural/vegetal sources (Hoerger et al. 2009; Clotfelter et al. 2010).

In view of the above, we selected 6 sites at the Lima estuary and on the nearby coast, in addition to 2 upstream sites, to monitor, during a 1-year period, levels of 12 anthropogenic/industrial EDCs: estrone (E1), E2, ethynylestradiol (EE2), 4-*n*-octylphenol (4-*n*-OP), 4-*t*-octylphenol (4-*t*-OP), nonylphenol (4-*n*-NP and NP), and several polyethoxylates (4-octylphenol monoethoxylate [OP1EO], 4-octylphenol diethoxylate [OP2EO], 4-nonylphenol monoethoxylate [NP1EO], 4-nonylphenol diethoxylate [NP2EO], and bisphenol A [BPA]). In parallel, we monitored four phytoestrogens (formononetin [FORM], biochanin A [BIO-A], daidzein [DAID], genistein [GEN]) and one phytosterol (sitosterol [SITO]). Analyses were performed using gas chromatography coupled with mass detection (GC-MS). To complement this analysis, physicochemical water-quality parameters linked to the presence of anthropogenic contamination were measured: dissolved oxygen (DO), pH, nitrates, nitrites, ammonia, and phosphates. The acquired new data are not only relevant *per se*, but they are also important for the purposes of correlation with ecological data from the selected ecosystem (Azevedo et al. 2013).

Materials and Methods

Study Area

The Lima River exhibits a semidiurnal and mesotidal regime (3.7 m) with an average flushing rate of 0.40 m^3/s, a flow rate of 70 m^3/s, and a hydraulic residence time of 9 days (Ramos et al. 2006). The river drains into the Atlantic Ocean at 41°40′N and 8°50′W at a location close to the city of Viana do Castelo, which has 32,000 inhabitants (Fig. 1). The estuary is a small open area with approximately 6 km^2 comprising the river mouth, which is partially obstructed by a 2 km-long jetty, which deflects river flow to the south. The studied area is located in the lower stretch of the river and includes the estuary, which has an initial deep navigation channel and an upstream shallow salt marsh zone, with many longitudinal sandy islands (Fig. 1). The sampling sites were selected to include coverage of several important/different habitats (Fig. 1). At the northern margin, we sampled the Forte beach (S1), the shipyard industrial area (S2), the marina and the salt marsh zone (S3), and the border of the city of Ponte de Lima (S4). At the southern margin, we investigated the other border of Ponte de Lima (S5), a saltmarsh area (S6), a small sea port zone (S7), and the well-known beach of Cabedelo (S8).

Water Collection and In Situ Measurements

Water samples were collected in 2011 from eight sampling sites (Fig. 1) using a peristaltic pump (Model: WS300, Global Water, Gold River, CA, USA). Samples were collected at low tide at 1-m depth during the four seasons: winter (08 January and 08 March), spring (05 May), summer (27 July), and autumn (29 September and 26 November). All bottles were prerinsed with local water before collection of the sample. Temperature, pH, DO, and conductivity were measured *in situ* using portable instruments (pH 330i/Set WTW, OXi 330i/ Set WTW, and LF 330/ Set WTW; Weilheim, Germany). During transport to the laboratory, all flasks were stored at approximately 5 °C. Samples were then immediately filtered using 0.45-μm glass fibre filters (Millipore, Cork, Ireland) to eliminate particulate matter and other suspended solids. All filtrates were acidified to pH 2 with H_2SO_4 95 %–97 % p.a. (Sigma-Aldrich) and then subjected to solid-phase extraction (SPE) within 48 h.

Chemicals and Materials

E1(CAS no. 53-16-7), E2 (CAS no. 50-28-2), EE2 (CAS no. 57-63-6), 17β-estradiol-d_2 (E2-d_2; CAS no. 53866-33-4), 4-t-OP (CAS no. 140-66-9), 4-*n*-OP (CAS no. 1806-26-4), BPA (CAS no. 80-05-7), bisphenol A-d_{16} (BPA-d_{16}, CAS no. 96210-87-6), Igepal CA-210 (OP1EO, OP2EO, CAS no. 9036-19-5), and Igepal CO-630 (NP1EO and NP2EO, CAS no. 68412-54-4), FORM (CAS no. 485-72-3), BIO-A (CAS no. 491-80-5), DAID (CAS no. 207-635-4), GEN (CAS no. 446-72-0), and SITO (CAS no. 83-46-5) were obtained from Sigma-Aldrich. 4-Nonylphenol (4-*n*-NP; CAS no. 104-40-5) and nonylphenol isomers (NP; CAS no. 25154-52-3) were supplied by Riedel-deHaën (Seelze-Hannover, Germany). Stock solutions of individual

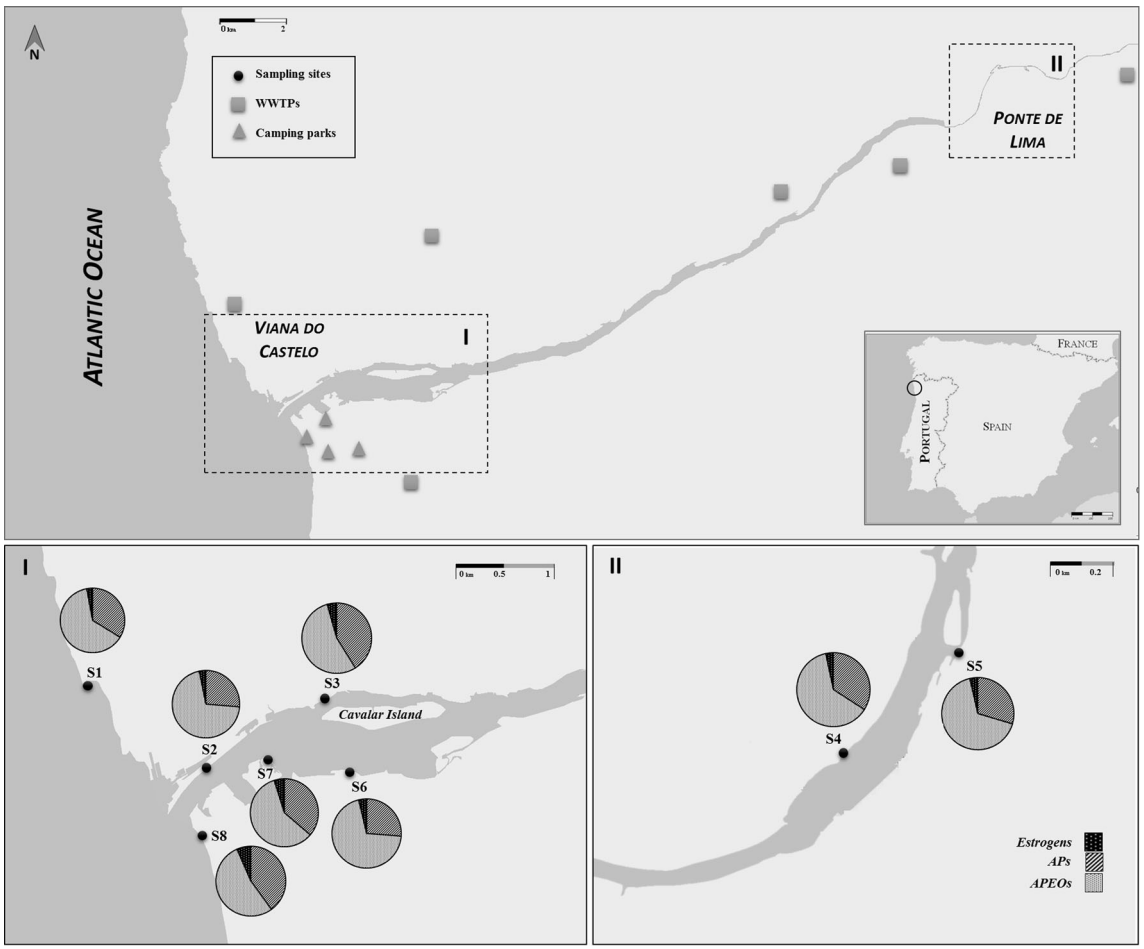

Fig. 1 Location of sampling sites within the Lima River (*S1–S8*) and at the nearby coast, Portugal (Microsoft MapPoint 2010). *I* sampling sites at the estuary and coastline; *II* sampling sites at the river. A pie chart is shown for each sampling site indicating the percentage of oestrogens, APs and APEOs

standards (100 mg/L) were prepared in methanol, transferred to amber bottles, and stored in the dark at −20 °C. Anhydrous pyridine, *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) added with 1 % (w/v) trimethylchlorosilane (TMCS), and hexane were supplied by Sigma-Aldrich. Dichloromethane and methanol were acquired from Romil Ltd. (Cambridge, UK). SPE cartridges, 200 mg Oasis HLB (hydrophilic-lipophlic balance), 6 mL, were acquired from Waters Corporation (Milford, MA, USA), and 1,000 mg silica cartridges, 6 mL, were purchased from Teknokroma (Barcelona, Spain). Ultrapure water was supplied by a Milli-Q water system (conductivity 0.054 µS/cm at 25 °C).

Sample Preparation

All 17 targeted EDCs were extracted by SPE (OASIS HLB) per published protocols (Rocha et al. 2013c). In brief, the cartridges were conditioned with 10 mL CH_2Cl_2:CH_3OH (50:50 [v/v]) followed by 6 mL CH_3OH and 13 mL ultrapure water at a flow rate of 1 mL/min. Water samples (1 L) at pH 2, spiked with E_2–d_2 and BPA–d_{16} (deuterated surrogate; herein used also as internal standards), were loaded on top of SPE cartridges at a constant flow rate of 5 mL/min; this was 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]). Given that the extracts were sticky and dark, they were cleaned using silica cartridges (1 g). The resulting extracts were evaporated to dryness in a water bath at 36 °C under a gentle N_2 stream and reconstituted with 250 µL of anhydrous methanol; the sample concentration factor was 4000-fold, and recoveries of all assayed EDCs surpassed 80 %. Samples were then derivatised using BSTFA added with 1 % (w/v) TMCS; this proved to be the best agent for the diversity of chemicals in this study (Rocha et al. 2013c).

Quantification by GC-MS

All analyses were performed using a gas chromatograph (Trace GC ultra; Thermo Finnigan Electron Corporation) coupled with an ion trap mass spectrometer (ITQ 1100 GC-MSn; Thermo Scientific) and an autosampler (Thermo Scientific TriPlus). A Trace TR-5MS column (length 30 m, ID 0.25 mm, film thickness 0.25 μm) was used. Helium carrier gas (99.9999 % purity) was maintained at a constant flow rate of 1.0 mL/min. Oven temperatures were programmed as follows: (1) from 100 (initial equilibrium time 1 min) to 200 °C at 10 °C/min; (2) from 200 to 260 °C at 6 °C/min, and (3) from 260 to 290 °C at 1 °C/min; at this point, the GC oven was maintained at 290 °C for 5 min. A solvent delay time of 8.5 min was used to protect the ion multiplier of the MS instrument from saturation. Temperatures of the programmable temperature vaporization inlet liner ranged from 35 to 250 °C by way of a ramp of 10 °C/s. Both MS transfer line and ion source were at 280 °C. Sample injection (3 μL) was programmed in splitless mode using an 80-mm injection needle. Quantitative analysis was performed in a selected ion monitoring mode using external calibration. Working solutions were prepared by diluting the stock solution with methanol at six calibration levels ranging from 10 to 375 ng/L for the 17 EDCs to 50 ng/L for E2–d_2 and BPA–d_{16}. The analytic parameters of the GC-MS method used are listed in Table 1. Because the current EDCs were measured in ng/L, method blanks were used to ensure the absence of contamination by laboratory material. In addition, unbiased water samples were spiked with all assayed EDCs at an intermediate concentration (150 ng/L) of the calibration curve and then submitted to usual analysis.

Ethynylestradiol Equivalents

The oestrogenic potency of a compound can be related to that of EE2 and be expressed as EE2 equivalents (EE2eq). The EE2eq calculus for the present oestrogenic compounds varied according to their EE2eq factor (F) as obtained from different E-Screen assays. EE2eq values were calculated using potencies derived from several results of such studies as shown in the following equation:

$$\mathrm{EE2eq} = C \times F$$

where C is the environmental measured concentration of a given compound, and F is its respective EE2eq value. The values of F followed published values (Coldham et al. 1997; Urbatzka et al. 2012).

Statistical Analysis

All data were subjected to statistical analysis using the software STATISTICA 8 (StatSoft). After checking

Table 1 Quantification, diagnostic ions, and LOQ for each compound analysed by GC-MS

EDCs	Retention Time (tr) min	Quantification ions (m/z)	Diagnostic ions (m/z)	LOQ (ng/L)
4-t-OP	10.54	207 (100)	–	4.8
4-NP	11.0–12.2	207 (100)	179 (84.9), 193 (31.9), 221 (31.9)	18.1
4-OP	12.7	179 (100)	180 (17.7)	11.6
4-n-NP	14.9	179 (100)	292 (35.9)	2.0
OP1EO	14.5	251 (100)	207 (97.2), 135 (68.9)	17.5
NP1EO	15.8–16.3	251 (100)	265 (64.4), 207 (59.5), 135 (45.5)	6.1
BPA-d_{16}	17.6	368 (100)	369 (34.7), 386 (9.1)	–
BPA	17.8	357 (100)	358 (30.8)	2.4
OP2EO	18.3	295 (100)	207 (76.5), 115 (55.2)	3.1
NP2EO	19.4–20.4	295 (100)	207 (74.9)	6.8
E1	24.3	342 (100)	357 (55.1)	3.2
E2-d_2	24.4	287 (100)	418 (75.2), 328 (72.8)	–
E2	24.4	285 (100)	416 (85.2), 326 (48.4)	2.8
EE2	27.2	425 (100)	285 (48.0), 426 (34.7)	4.4
FORM	28.5	340 (100)	339 (76.0), 355 (22.6)	8.6
BIO-A	29.2	356 (100)	341 (34.3)	4.6
DAID	29.9	398 (100)	383 (76.0), 355 (22.6)	4.1
GEN	31.7	471 (100)	473 (19.9)	3.8
SITO	42.1	396 (100)	486 (53.4), 255 (49.4)	6.6

Relative abundance of ions (m/z) for each target EDC is indicated in parentheses

LOQ limit of quantification

assumptions of normality (Shapiro–Wilk W test) and homogeneity of variances (Levene's test), data sets were analysed by one-way analysis of variance. To evaluate significant differences between factors, *post hoc* Tukey test was used. Results were considered statistically significant at $p < 0.05$ (two-tailed analysis).

Results

Oestrogens

The frequency of occurrence of E1, E2, and EE2 in all analysed samples was approximately 100 %. Figure 1 (I and II) shows several pie graphs in which the proportion of oestrogens ($\Sigma_{E1,E2,EE2}$) is related with global amounts of industrial/household products at each sampling site. Figure 2 shows annual seasonal fluctuation patterns of E1, E2, and EE2, whereas Table 2 refers to individual amounts measured at each sampling site per season. The graphics in Fig. 1 and the data in Table 2 confirm that, in general terms, lower amounts of oestrogens were recorded from the northern margins than from the southern one. This was clearly observed in summer when global oestrogen amounts totalled 22 ng/L at the north margin (average of values recorded at sites S1–S4) and 40 ng/L at the south one (average of values recorded at sites S5–S8). In parallel, water taken from Cabedelo beach (S8) at the Green Coast contained approximately 4-fold higher amounts of E1, E2, and EE2 (approximately 77 ng/L) than samples collected from the beach of Forte (S1, Table 2). Furthermore, Fig. 2 shows that levels of E1 increase significantly in summer ($p < 0.05$).

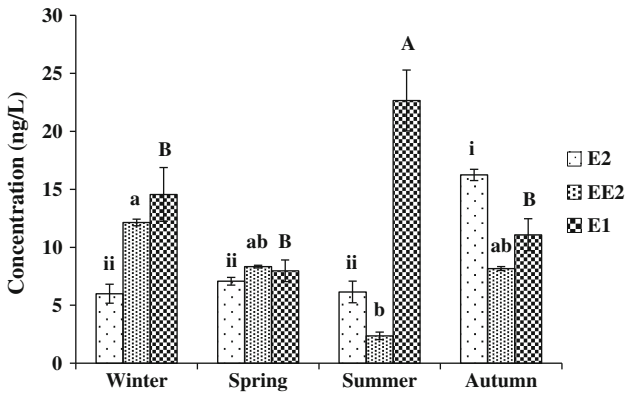

Fig. 2 Spatial and seasonal fluctuations of E1, E2, and EE2 at the Lima River as well as its estuary and coastline. Data are shown as mean ± SEM (SE); $n = 16$ in winter and autumn, and $n = 8$ in spring and summer. *Different letters* refer to statistical differences per Tukey test results ($p < 0.05$)

Industrial and Household Compounds

The incidence of occurrence of APEOs (octylphenol ethoxylates (OPEOs) and nonylphenol ethoxylates (NPEOs), APs (4-*n*-OP, 4-*t*-OP, 4-*n*-NP and 4-NP), and BPA was approximately 100 % in all analysed samples. Figures 3 and 4 show seasonal fluctuation patterns of APEOs (Fig. 3a, b), APs (Fig. 4a, b), and BPA (Fig. 4c). Table 2 lists the amounts of these latter compounds at each sampling site and during each season. The pie graphs provided in Fig. 1 (I and II) show that the north margin had higher amounts of these EDCs than the south margin ($p < 0.05$). This fact is more evident in summer when the global total of these compounds ($\Sigma_{APs,APEOs,BPA}$) was 124 ng/L at the north margin (average of values recorded at sites S1–S4) and 75 ng/L at the south one (average of values recorded at sites S5–S8). At the coastline, it was observed that the concentration of these pollutants was approximately 3-fold higher (approximately 525 ng/L) at S1 (Forte) than at S8 (Cabedelo) (Table 2).

Figure 3 shows that levels of both alkylphenol diethoxylates (OP2EO and NP2EO) decreased in summer ($p < 0.05$) when levels of the monoethoxylate NP1EO were higher ($p < 0.05$). In parallel, and as shown in Fig. 4, amounts of 4-*t*-OP and 4-NP decreased in summer ($p < 0.05$, Fig. 4a, b) when amounts of 4-*n*-OP attained their maximum values ($p < 0.05$). The same trend was observed for BPA as for 4-*t*-OP and 4-NP with the lowest amounts recorded in summer ($p < 0.05$, Fig. 4c).

Phytoestrogens and SITO

The occurrence of all analysed phytoestrogens and SITO was approximately 100 % at all sampling sites. Figure 5 shows the annual/seasonal fluctuation patterns of these EDCs, whereas Table 2 lists the amounts of these latter compounds at each sampling site and during each season. Considering first the data shown in Fig. 5a, one can observe that the level of DAID (that is, the demethylated form of FORM) was at its lowest concentration of approximately 6 ng/L in summer ($p < 0.05$; Fig. 5a and Table 2). GEN (that is, the demethylated metabolite of BIO-A) was also at its lowest in summer (approximately 36 ng/L; $p < 0.05$). In contrast, levels of FORM (approximately 389 ng/L) and BIO-A (approximately 160 ng/L) were higher in autumn than in spring ($p < 0.05$). Figure 5c shows the fluctuation pattern of SITO, for which the highest levels were recorded in summer ($p < 0.05$), when values surpassed 5 µg/L (Table 2). Site S5 is an exception because the highest value was found in autumn. SITO levels showed a particular behaviour characterized by high values during all of the year with the exception of the winter season when its levels decreased even 50 times lower.

Table 2 Environmental levels of all EDCs (ng/L) measured at the Lima River as well as its estuary and coastline from January to November 2010

EDCs (ng/L)/season		Sampling sites							
		S1	S2	S3	S4	S5	S6	S7	S8
A									
E1	Winter	6.80	12.9	22.7	13.8	9.5	24.3	8.19	18.3
	Spring	7.21	13.0	7.62	7.68	6.2	10.6	6.85	4.59
	Summer	16.8	16.0	28.7	13.7	24.6	23.2	21.9	36.3
	Autumn	4.82	17.4	14.0	10.9	8.49	13.7	10.5	8.89
E2	Winter	4.66	5.68	6.62	6.55	5.68	5.26	5.92	7.60
	Spring	3.88	7.16	7.56	9.00	8.19	5.29	7.51	7.99
	Summer	2.49	2.39	3.74	2.53	4.41	4.30	4.95	24.4
	Autumn	9.56	21.4	20.4	16.6	19.8	10.3	16.9	15.2
EE2	Winter	12.6	15.1	15.1	8.86	10.0	5.38	16.8	13.3
	Spring	4.83	7.52	19.4	4.71	4.45	4.78	14.9	6.08
	Summer	0.40	0.46	0.36	0.54	0.27	0.53	0.25	16.1
	Autumn	2.30	8.51	10.0	13.7	9.41	5.11	8.88	7.34
B									
4-t-OP	Winter	38.2	57.0	105	16.5	40.8	48.9	13.0	14.4
	Spring	32.5	43.5	37.9	30.1	31.6	34.0	37.5	27.8
	Summer	6.50	5.71	20.0	22.4	14.6	23.0	7.98	9.37
	Autumn	15.6	46.9	26.2	22.2	70.4	38.9	17.8	75.1
4-NP	Winter	168	190.9	649.8	78.8	112.3	102.8	128	181
	Spring	258	255.0	246.3	367.5	242.5	159.6	376.8	285
	Summer	14.2	21.3	6.2	22.8	11.7	12.0	8.2	3.90
	Autumn	132	142.3	147	218	168.6	179.3	136.1	212
4-n-OP	Winter	23.8	13.1	46.5	19.8	13.3	24.9	16.1	22.7
	Spring	17.8	33.9	17.6	35.1	35.6	17.7	25.6	42.1
	Summer	53.9	81.1	23.6	86.5	44.6	45.4	31.1	15.0
	Autumn	18.1	24.8	19.0	34.3	26.1	6.24	8.8	30.8
4-n-NP	Winter	13.4	31.6	28.4	20.6	10.4	8.6	11.8	3.01
	Spring	6.72	8.9	17.6	12.4	15.0	11.1	10.5	8.30
	Summer	22.1	33.2	9.71	35.4	18.3	18.6	12.8	6.14
	Autumn	22.6	18.4	19.4	29.7	17.7	18.6	11.4	7.05
BPA	Winter	17.2	11.1	-	5.50	-	20.3	10.5	24.5
	Spring	22.7	11.6	18.0	14.2	17.2	7.92	21.8	21.4
	Summer	6.90	10.3	3.01	11.0	5.68	5.82	4.01	1.91
	Autumn	6.91	9.8	20.1	26.4	17.4	12.9	35.7	19.2
C									
OP1EO	Winter	8.80	66.3	–	27.0	22.2	92.5	125	34.2
	Spring	14.6	13.9	18.8	21.6	17.9	21.9	25.6	19.0
	Summer	12.6	10.8	52.2	46.8	95.3	28.1	16.2	11.8
	Autumn	19.5	24.5	32.9	27.5	26.1	29.3	18.6	22.3
NP1EO	Winter	45.4	86.7	–	54.9	112.3	104.5	163.1	89.7
	Spring	45.2	79.0	86.9	64.7	55.3	46.3	79.1	54.5
	Summer	161.5	243	70.8	259.1	133.6	136.0	93.2	44.9
	Autumn	58.4	162	203.6	158.5	144.9	166.7	92.7	77.4

Table 2 continued

EDCs (ng/L)/season		Sampling sites							
		S1	S2	S3	S4	S5	S6	S7	S8
OP2EO	Winter	223	374	334	101	74.2	357	64.7	110
	Spring	134	86.3	103	80.5	81.3	82.1	85.7	76.5
	Summer	77.3	116	33.9	124	64.0	65.1	44.6	21.5
	Autumn	60.0	248	111	94.6	221	85.1	52.6	103
NP2EO	Winter	263	348	259	211	161	336	169	186
	Spring	230	235	237	206	263	195	190	181
	Summer	170	256	74.6	273	141	143	98.2	47.3
	Autumn	158	467	316	285	449	240	185	271
D									
FORM	Winter	490	783	184	200	202	490	193	146
	Spring	165	157	161	144	269	145	141	149
	Summer	358	578	447	90	377	279	122	239
	Autumn	95.5	801	292	225	632	471	378	219
BIO-A	Winter	81.6	71.2	79.0	57.0	51.6	23.5	65.6	84.7
	Spring	55.6	57.9	79.0	88.7	63.1	72.7	61.7	78.1
	Summer	187	93.7	144	234	164	110	137	143
	Autumn	350	89.1	242	148	121	132	153	91.9
DAID	Winter	67.1	71.9	78.5	46.0	42.6	48.2	50.2	43.0
	Spring	29.2	38.9	35.6	38.9	38.5	27.5	48.9	43.4
	Summer	2.86	4.44	7.06	5.24	4.17	10.6	8.37	4.89
	Autumn	9.16	3.93	14.2	13.1	39.2	10.8	14.5	21.4
GEN	Winter	46.4	98.2	120.3	117.9	93.5	109.3	67.0	77.8
	Spring	59.8	77.6	60.1	60.8	89.5	64.0	69.3	49.4
	Summer	25.8	28.7	20.7	19.4	19.8	24.2	18.5	20.7
	Autumn	51.4	82.4	43.2	40.5	51.1	35.9	52.7	43.1
SITO	Winter	62.4	138	249	92.2	115.1	82.9	89.1	57.4
	Spring	1334	1415	2923	3849	5748	1677	1291	2670
	Summer	3389	4632	6960	6083	5919	8074	7420	2710
	Autumn	1660	1684	4080	4178	6110	2858	3783	1796

Data are presented as mean ± SEM (SE) ($n = 6$ for each sampling site). *Note*: sites at north are S1–S3, south are S6–S8, and inland are S4, S5

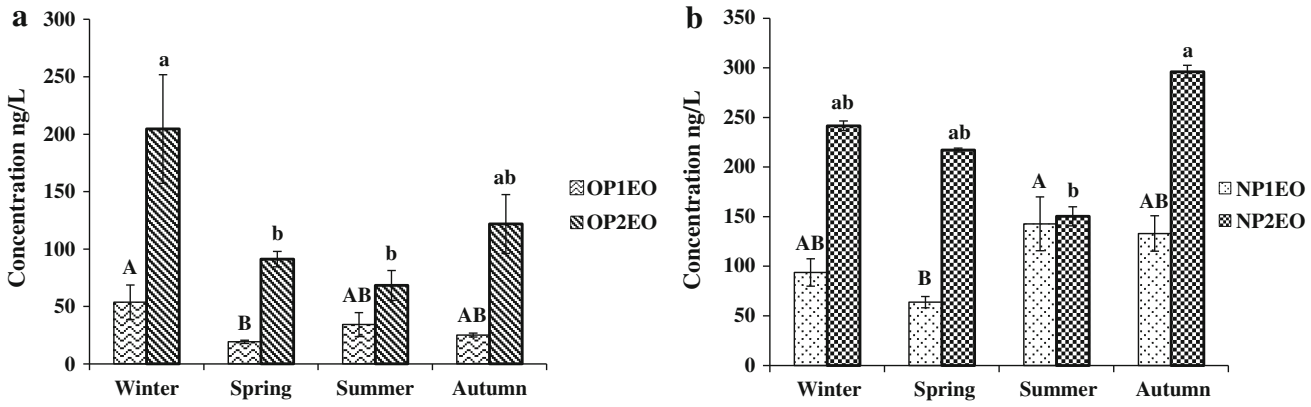

Fig. 3 Spatial and seasonal fluctuations of **a** APs and **b** APEOs at the Lima River as well as its estuary and coastline. Data are shown as mean ± SEM (SE); $n = 16$ in winter and autumn, and $n = 8$ in spring and summer. *Different letters* refer to statistical differences per Tukey test results ($p < 0.05$)

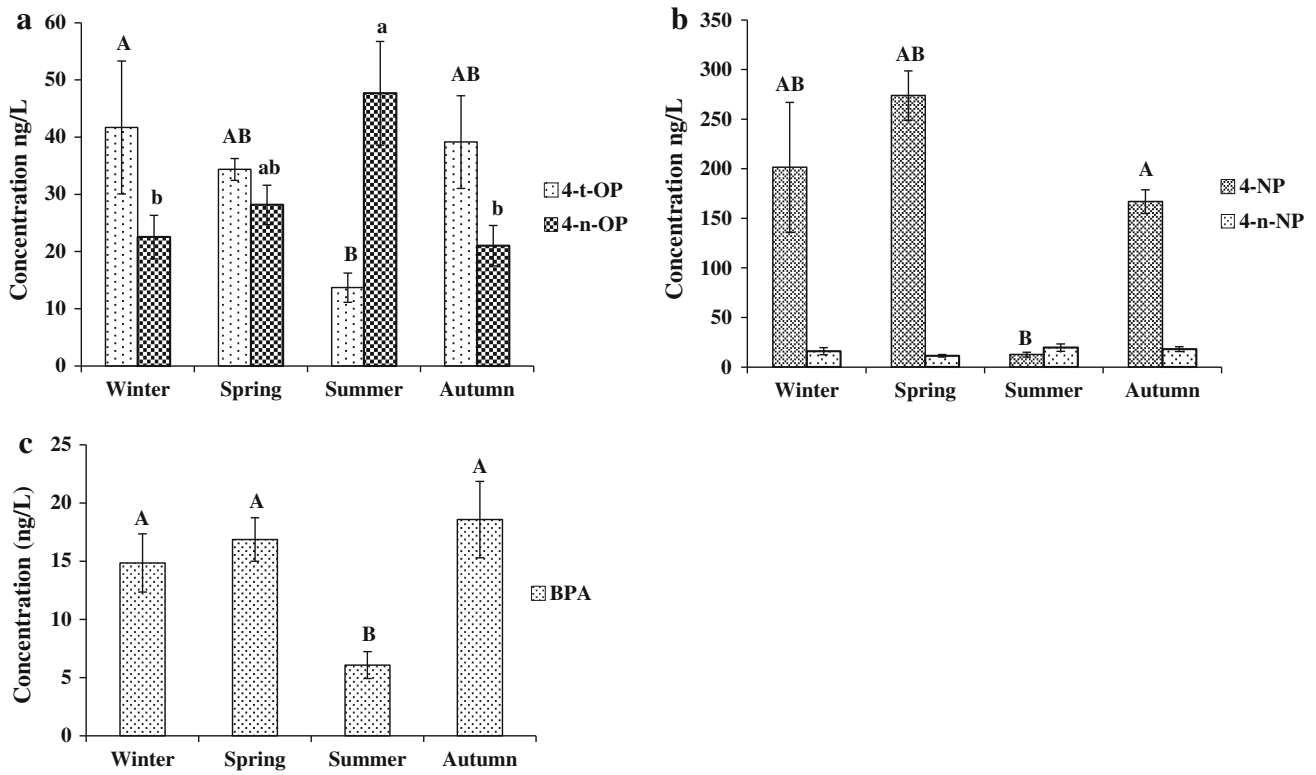

Fig. 4 Spatial and seasonal fluctuations of **a** 4-n-OP and 4-t-OP; **b** 4-n-NP and 4-NP; and **c** BPA at the Lima River as well as its estuary and coastline. Data are shown as mean ± SEM (SE); $n = 16$ in winter and autumn, and $n = 8$ in spring and summer. *Different letters* refer to statistical differences ($p < 0.05$)

Physicochemical Parameters

All analysed physicochemical parameters are listed in Table 3. With reference to annual average levels of nitrites (approximately 0.0 at the river and estuary and 0.1 mg/L at the coastline), nitrates (approximately 1.2 at the river, 2.0 at the estuary, and 2.9 mg/L at the coastline) and ammonia (approximately 0.4 at the river, 0.0 at the estuary, and 0.4 mg/L at the coastline), similar amounts were observed across sampling sites. The highest levels of phosphates were recorded in the river (approximately 0.4 mg/L) with these being almost undetectable in the estuary and at the coastline. Annual average amounts of DO were similar across sampling sites (approximately 10 at the river and estuary and 12.3 mg/L at the coastline), and no signs of hypoxia were observed at any site on any occasion. Table 3 lists values for pH (7–8.5), salinity (0–31 ‰), and temperature (9–27 °C) recorded during sampling.

EE2eq

Average annual values obtained for each oestrogenic EDC were converted to EE2eq and are listed in Table 4.

Discussion

Natural and Pharmaceutical Oestrogens

It is well established that the main environmental sources of E1 through EE2 are usually waste water treatment plant (WWTP) effluents or untreated domestic discharges (Ying et al. 2002a). There are five WWTPs in the Lima River estuary (Fig. 1) with the area also receiving effluent from another four WWTPs. Four of the last WWTPs, located in the Lima watershed, were designed to service 65,000 inhabitants; (Ferreira et al. 2003) however it is suspected, on the basis of criticisms voiced in local newspapers, that sewage from several sources is still being discharged directly into the estuary or into the sea at the coast without any form of treatment. Probably due to this fact, the annual average amounts of E1, E2, and EE2 recorded in this study were similar across sites at the coastline, estuary, and river. The likely occurrence of direct discharges is also supported by the higher amounts of oestrogens recorded at sampling sites located near four camping parks within this important touristic zone (Fig. 1, S6–S8) with higher values being recorded mainly in summer. During the last tourist season, the number of campers increased, totalling >50 % of the

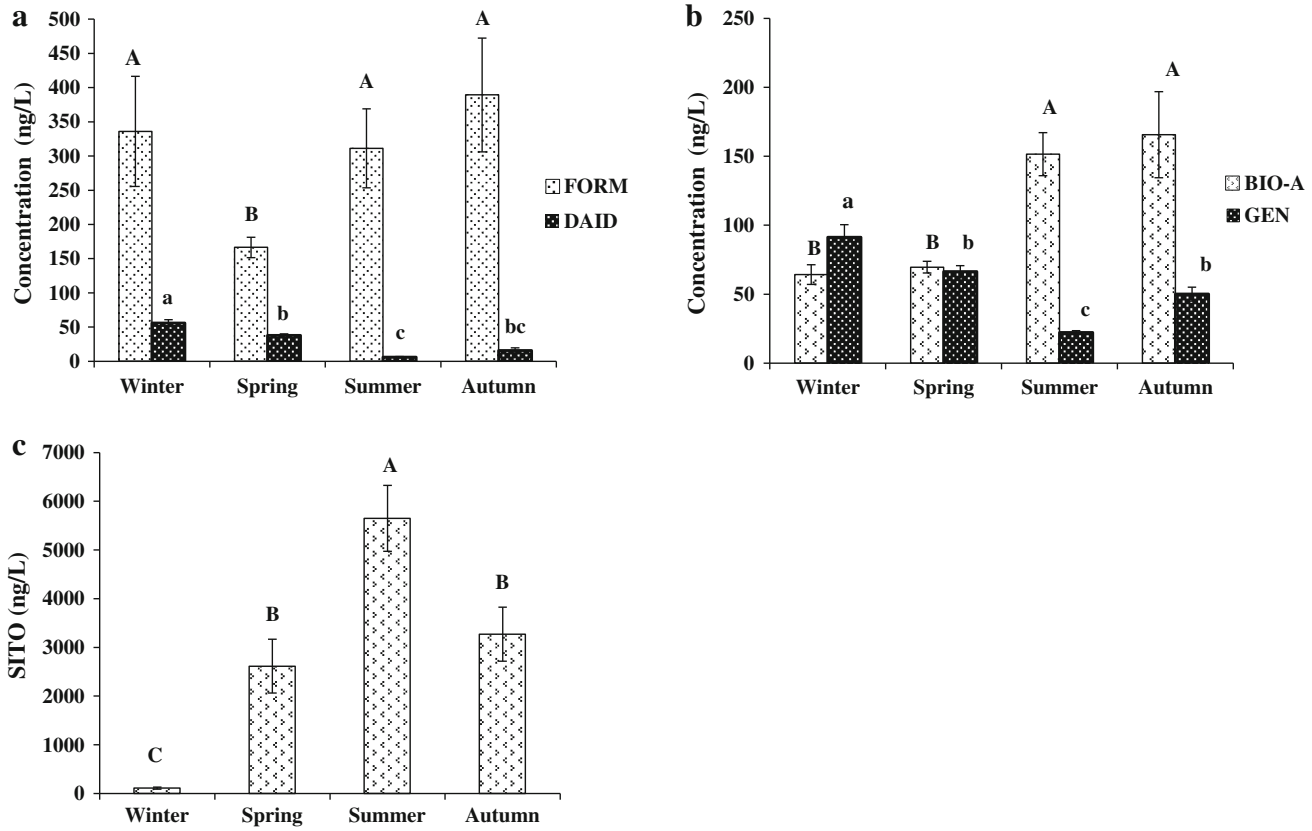

Fig. 5 Spatial and seasonal fluctuations of **a** FORM and DAID; **b** BIO-A and GEN; and **c** SITO at the Lima River as well as its estuary and coastline. Data are shown as mean ± SEM (SE); $n = 16$ in winter and autumn and $n = 8$ in spring and summer. *Different letters* refer to statistical differences per Tukey test results ($p < 0.05$)

Table 3 Physicochemical parameters evaluated locally at the Lima River as well as its estuary and coastline from January to November 2010

Season	pH	Temperature (°C)	Salinity (‰)	Dissolved O_2 (mg/L)	NO_2^- (mg/L)	NO_3^- (mg/L)	NH_4^+ (mg/L)	PO_4^{2+} (mg/L)
Winter								
River	6.4 ± 0.1	9.9 ± 0.7	0.1 ± 1.1	11.4 ± 0.6	0.0 ± 0.0		0.2 ± 0.2	
Estuary	7.1 ± 0.8	10.8 ± 1.2	3.4 ± 13.8	11.1 ± 0.3	0.0 ± 0.0	–	0.0 ± 0.0	–
Coastline	8.4 ± 0.6	12.6 ± 0.3	29.2 ± 5.0	12.6 ± 2.5	0.1 ± 0.0		0.4 ± 0.0	
Spring								
River	7.1 ± 0.0	16.5 ± 0.4	0.0 ± 0.0	10.6 ± 0.0	0.0 ± 0.0		0.1 ± 0.0	0.8 ± 0.4
Estuary	8.4 ± 0.7	17.8 ± 0.9	10.6 ± 1.2	10.3 ± 0.4	0.0 ± 0.0	–	0.0 ± 0.0	0.8 ± 0.5
Coastline	8.6 ± 0.4	17.5 ± 1.1	36.6 ± 4.2	13.6 ± 5.6	0.1 ± 0.0		0.2 ± 0.1	0.4 ± 0.0
Summer								
River	7.9 ± 0.3	26.3 ± 0.4	0.1 ± 0.0	10.4 ± 0.3	0.0 ± 0.0	0.4 ± 0.1	0.9 ± 0.1	0.2 ± 0.1
Estuary	8.8 ± 0.1	20.4 ± 0.8	26.3 ± 1.5	10.6 ± 2.3	0.0 ± 0.0	1.6 ± 1.2	0.0 ± 0.0	0.3 ± 0.0
Coastline	8.8 ± 0.1	19.2 ± 2.0	27.4 ± 3.9	13.2 ± 4.0	0.1 ± 0.1	2.5 ± 0.7	0.7 ± 0.3	0.2 ± 0.1
Autumn								
River	7.0 ± 0.5	16.2 ± 3.7	0.0 ± 0.0	7.8 ± 2.1	–	1.9 ± 0.6	0.2 ± 0.1	0.5 ± 0.2
Estuary	7.8 ± 0.6	16.0 ± 2.7	15.8 ± 11.0	7.8 ± 1.7	0.1 ± 0.0	2.3 ± 2.2	0.0 ± 0.0	2.5 ± 0.4
Coastline	8.4 ± 0.3	16.9 ± 1.9	32.6 ± 3.3	9.7 ± 5.0	0.1 ± 0.1	3.2 ± 0.7	0.2 ± 0.1	0.7 ± 0.0

Data are presented as mean ± SD (SD) ($n = 12$ at the river, $n = 24$ at the estuary, and $n = 12$ at the coastline)

total number of local inhabitants (Ferreira et al. 2003) Similar seasonal observations were performed in recent studies in other touristic areas (Rocha et al. 2013a, b, c) and also suggest that these type of seasonal oestrogen peaks seem to frequently occur in coastal areas, which are subject to abrupt increases in the number of inhabitants. The oestrogenic load of this area is actually similar to that recorded in other noteworthy coastal areas in Spain (Rodriguez-Mozaz et al. 2004), Italy (Laganà et al. 2004), and North America (Sellin et al. 2009). When normalising obtained data in terms of EE2eq, the contribution of all oestrogens measured in the Lima coastal area was found to be 18.1 ng/L EE2eq; this amount that is able to induce endocrine disorders in aquatic fauna (Mills and Chichester 2005) and is well above the recently predicted no-effect concentration of 0.1 ng/L EE2 eq for long-term exposures in surface waters (Caldwell et al. 2012). These aspects are quite worrisome because such high amounts of oestrogens have potential impacts not only on local marine ecosystems (Mills and Chichester 2005) but also on humans (Safe 2000); it is important to stress that S1 and S8 are very popular beaches and are consequently used by locals as well as by a significant number of tourists. Notwithstanding the above-mentioned concerns, the EE2eq values calculated for these sites were lower than those obtained for other Portuguese coastal areas (approximately 21 ng/L EE2eq) (Urbatzka et al. 2012).

Industrial and Household Compounds

APEOs were banned from Europe in 2003 (2003/53/EC) due to their ability to promote oestrogenic effects in both wild fauna and humans (Safe 2000; Zoller 2006). However, recent studies showed that these EDCs are still being used, not only in Portugal, but also in other countries (Zoller et al. 2004, 2006; Micić and Hofmann 2009; Rocha et al. 2013a, b). This study provides recent/unique data about current amounts of APEOs in the Lima River coastal area. Although the source of APEOs in this aquatic habitat could not be clearly ascertained, it is believed that these may be the result of (1) industrial discharges because several industries often associated with use of these chemicals (metallurgy and shipyards) (Kim et al. 2007) are present in the Rio Lima estuary (Fig. 1), (2) lixiviation of pesticides used in agriculture, and (3) urban direct discharges containing detergents and other household products (Ying et al. 2002b). Once in the environment, APEOs degrade within 1 or 2 days, leading to the formation of APs (NPs and OPs), which are much more potent and persistent than their parents in inducing oestrogenic disorders (Safe 2000). In this study, the fluctuation patterns of some APs (4-*t*-OP, 4-*n*-NP) seemed to be dependent on those of APEOs (OPEOs and NP2EO), which is understandable because these are derived in summer when temperature increases under aerobic conditions (Ying et al. 2002b). Interestingly, and in total accordance with the presence of intense industrial activities on the north margin of the river, site S2 showed the highest annual average levels of APEOs (704 ng/L), whereas the highest annual average values of APs were recorded at site S3 (365 ng/L). These findings may also be due to local currents, which are described as flowing southwest due to the existence of jetties (Ribeiro et al. 2013) as well as the presence of Cavalar Island, which decreases water circulation/renovation (Fig. 1). When considering all studied sites, the annual average concentration of APs was found to be 0.26 μg/L indicating that preventive measures are needed not only at site S3 but across all studied areas to avoid reaching the maximum permitted concentration of 0.3 μg/L (European Commission 2000, Directive 2000/60/EC of the European Parliament and of the Council). Despite these findings, it would appear that the Lima area is not as polluted by these EDCs as other coastal areas (Ying et al. 2002b; Arditsoglou and Voutsa 2008; David et al. 2009). When converting data to EE2eq, it was observed that the oestrogenic contribution of these EDCs was 0.5 ng/L; although this value is low, it may still be toxicologically relevant, particularly considering the fact that these EDCs are not the only xenoestrogens present in the monitored aquatic environment.

Phytoestrogens and SITO

Phytoestrogens are nonsteroidal xenoestrogenic compounds produced by plants. These compounds enclose the coumestans, the isoflavones (BIO-A, GEN, FORM, and DAID), and the lignans. Structurally, phytoestrogens resemble the E2 molecule and therefore have affinity with oestrogenic receptors (Benassayag et al. 2002). However, the oestrogenic potency of phytoestrogens, particularly of isoflavones, is much lower than that of E2 (Table 4), and so their EC_{50} values are <4 orders of magnitude higher than those of that steroid (Hoerger et al. 2009). In fact, the (total) concentration of isoflavones must be at least 1000-fold higher than E2, i.e., in the μg/L range, to produce an oestrogenic effect equivalent to that of E2 (Table 4). Sources of phytoestrogens in the environment are complex, and these can either occur naturally (from local flora) or as a result of industrial activities (such as from food and paper plants). The main source of these EDCs in the study area is probably the endogenous flora, which includes species such as native (*Juncus maritimus*) and invasive *Phragmites australis* species (Costa 2001). This hypothesis is in good accordance with the life cycle of these plants and with the fluctuation patterns noted for isoflavones (Fig. 5). For example, the common reed (*P. australis*) flourishes from June to September (summer and

Table 4 Average concentration levels of oestrogenic compounds, their relative potencies to E2, and calculated EE2eq concentrations

Compound	Relative potency to E2	Mean concentrations of all estrogenic compounds measured in the Lima River and its Atlantic coastline (ng/L)	EE2 eq (ng/L)
EE2	1.3	7.8	7.8
E2	1.0	8.9	7.1
E1	3.0^{E-01}	14.1	3.4
4-t-OP	4.0^{E-04}	3.2^{E+01}	1.0^{E-02}
4-n-OP	1.3^{E-05}	3.0^{E+01}	3.2^{E-04}
OP2EO	5.0^{E-06}	1.2^{E+02}	4.9^{E-04}
OP1EO	5.0^{E-06}	3.2^{E+01}	1.3^{E-04}
4-n-NP	5.0^{E-05}	1.6^{E+01}	6.5^{E-04}
NP2EO	6.3^{E-07}	2.3^{E+02}	1.1^{E-04}
NP1EO	6.3^{E-07}	1.1^{E+02}	5.4^{E-05}
BPA	1.0^{E-04}	1.4^{E+01}	1.1^{E-03}
NP	4.0^{E-03}	1.6^{E+02}	5.2^{E-01}
GEN	4.9^{E-02}	3.0^{E+02}	1.2^{E+01}
DAID	1.3^{E-03}	2.9^{E+01}	3.0^{E-02}
FORM	5.6^{E-03}	5.7^{E+01}	2.6^{E-01}
BIO-A	9.1^{E-03}	1.1^{E+02}	8.2^{E-01}
SITO	–	–	–
		$\sum$EE2 eq in the estuary	31.7

Relative potencies of oestrogenic compounds vary depending on cellular assays; consequently, approximate values were based on data reported in Coldham et al. (1997) and Urbatzka et al. (2012)

autumn), the exact occasions when levels of FORM and BIO-A increased, also coinciding with the time when their demethylated metabolites were at a minimum (Fig. 5a, b). Later on, in late autumn and early winter, when maximal biomass occurs, levels of FORM and BIO-A decreased whereas those of their demethylated metabolites, DAID and GEN, attained their highest amounts (Fig. 5). Comparing the values from this study with those reported for other sites in Portugal, it can be noted that these are approximately 3- to 15-fold lower than those observed close to Porto (Rocha et al. 2013d), Lisbon (Rocha et al. 2013b), or at the Ria Formosa in Algarve (Rocha et al. 2013a). It is possible that the absence of seaweeds—plants described as having high concentrations of phytoestrogens (Mackova et al. 2006—in this area contributed to the lower amounts. Despite isoflavones being less active than oestrogens, converting data obtained to EE2eq showed that their oestrogenic contribution was equivalent to 12 ng/L of EE2. Although these amounts are lower than those measured in other areas, the detected quantities seem capable of contributing to local endocrine disorders in aquatic fauna. In any case, the exact synergistic or additive role of isoflavones in complex oestrogenic mixtures is not clear and definitely requires further study.

The phytosterol SITO, which is structurally similar to cholesterol, is also an endocrine disruptor because it either decreases the availability of cholesterol to the P450scc (enzyme involved in the conversion of that hormone to pregnenolone) or decreases the activity of this enzyme (Volkman et al. 2008). SITO was identified in the past as an important component of seeds and vascular plants (Benassayag et al. 2002) such as those found in the Lima River coastal area. Comparing the levels of SITO measured in this study with those found in other Portuguese coastal waters, we concluded that, as in the case of isoflavones, levels were approximately 5-fold lower in Lima than those measured in coastal waters adjacent to the Ave, Leça, and Sado rivers, and in the Ria Formosa lagoon (Rocha et al. 2012, 2013a, b, d).

Physicochemical Data

Some physicochemical parameters (pH, DO, ammonium, nitrites, nitrates, and phosphates) were also evaluated in this study (Table 3) with these being closely related to sewage and WWTP discharges. In this study, levels of DO were always >11.1 mg/L even in summer months when average temperatures were higher than in other seasons (>21 °C). Levels of salinity and pH were similar to those in other closely related aquatic systems (Rocha et al. 2012, 2013a, b, d). Data obtained for nitrites, nitrates, and ammonia indicated that the estuary and coastline were the most affected areas suggesting that direct discharges from local sewage occur; this concurs with reports from local inhabitants published by the press. When converting levels of nitrates and nitrites to nitrogen (1 mg/L as nitrate = 0.226 mg/L as nitrate–nitrogen; 1 mg/L as nitrite = 0.304 mg/L), it can be seen that the obtained values surpass 1 mg/L; the latter is the concentration above which the World Health Organization (WHO) considers the presence of nitrogen hazardous for both aquatic species and humans (WHO 2011). Furthermore, total amounts of phosphates, most commonly originating from WWTPs and organophosphorus pesticides, surpass 0.1 mg/L at all sampling sites, which is the recommended limit for total phosphorus in flowing streams to prevent eutrophication (United States Environmental Protection Agency [USEPA] 1996). This occurrence may well be explained by the agricultural area surrounding the Lima River. A programme to monitor pesticide levels in this area is thus not only opportune but also needed to prove this latter hypothesis.

Conclusion

The present study identified the ubiquitous presence of EDCs, from either anthropogenic or vegetal origins, in the

lower part and estuary of the Lima River (six sites) and in its nearby coastal area (two sites). The studied areas were similarly affected by anthropogenic EDCs (oestrogens, APs, and APEOS), the origins of which seem to be WWTP effluents and direct discharges coming from urban/industrial sewages. These EDCs attained concentrations high enough to be able to induce endocrine disruption in aquatic organisms; however, biomarkers of such potential impacts in local fish or other organisms have never been published. The chemical data undoubtedly highlight the need to monitor the Lima aquatic system because not only the biota but also humans can be contaminated through either consumption of polluted seafood or direct contact with contaminated seawater when using local beaches. The physicochemical data also corroborate the presence of anthropogenic contamination in the studied habitat. Although this ecosystem seems to be less affected by the investigated EDCs than other Portuguese aquatic systems, the toxicologically relevant amounts measured nevertheless highlight the need for measures to decrease the impact of WWTP effluents and direct urban discharges. This would be in line with European legislation (e.g., the Water Framework Directive), which provides strict parameters for the prevention, protection, and improvement of environmental quality, the protection of human health, and the rational and cautious use of natural resources.

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