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Spatial and seasonal distribution of 17 endocrine disruptor compounds in an urban estuary (Mondego River, Portugal): evaluation of the estrogenic load of the area

Maria João Rocha · Catarina Cruzeiro · Mário Reis · Miguel Ângelo Pardal · Eduardo Rocha

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Abstract The Mondego River estuary demonstrates signs of pollution, but the levels of endocrine disrupting compounds (EDCs), such as the natural (17β-estradiol and estrone) and pharmaceutical (17α-ethynylestradiol) estrogens, xenoestrogenic industrial pollutants (4-octylphenol, 4-nonylphenol, and their mono- and diethoxylates and bisphenol A), phytoestrogens (formononetin, biochanin A, daidzein, and genistein), and sitosterol were either poorly or never measured in this area. Thus, to conclude about the influx of EDCs in this estuary, water samples were taken every 2 months, during 1 year (2010) in low tide, at eight sites distributed along the estuary. Water samples (1 L) were preconcentrated in the Oasis HLB cartridges and cleaned in silica cartridges before their analysis by GC-MS. In summer, potentially hazardous amounts of estrogens (≈26 ng L^{-1}), alkylphenols (≈11.5 μg L^{-1}), alkylphenolethoxylates (≈13 μg L^{-1}), and phytoestrogens (≈5.6 μg L^{-1}) were measured. These data suggest that changes in the hydrodynamics of the estuary coupled with the increase of water temperatures interfere with the amount of EDCs in the water. Complementary physicochemical parameters also point to high levels of anthropogenic pollution in this area. Globally, the estrogenic load, expressed in ethynylestradiol equivalents, attained 71.8 ng L^{-1} demonstrating that, all together, the measured EDCs pose important health risks for both biota and humans.

Keywords Estrogens · Alkylphenols · Alkylphenol ethoxylates · Phytoestrogens · Sitosterol

M. J. Rocha (✉) · C. Cruzeiro · E. Rocha
Laboratory of Cellular, Molecular and Analytical Studies, Interdisciplinary Centre for Marine and Environmental Research (CIIMAR), CIMAR Associate Laboratory (CIMAR LA), University of Porto (UPorto), Oporto, Portugal
e-mail: mjsrocha@netcabo.pt

M. J. Rocha
CESPU, Superior Institute of Health Sciences-North (ISCS-N), Gandra (Paredes), Portugal

M. J. Rocha · E. Rocha
Laboratory of Histology and Embryology, Institute of Biomedical Sciences Abel Salazar (ICBAS), UPorto, Porto, Portugal

M. Reis · M. Â. Pardal
CFE-Centre for Functional Ecology, Department of Life Sciences, University of Coimbra, Apartado 3046, Coimbra 3001-401, Portugal

Introduction

The Mondego River flows from the highest continental mountain of Portugal (Serra da Estrela) to the Atlantic Ocean, at the Figueira da Foz city which is located at the well-known Portuguese Silver Coast. During its course of 227 km, the Mondego River runs through agricultural areas (about 15,000 ha of cultivated land) producing mainly rice and maize draining nutrient-rich waters into the estuary (Lillebø et al. 2012). By contrast, the surroundings of the estuary are industrialized holding several factories (mostly cellulose- and paper-related factories) and aquaculture systems (semi-intensive farming, mainly raising seabass *Dicentrarchus labrax* and

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gilthead seabream *Sparus aurata*). The estuary also holds the city of Figueira da Foz, an important harbor and a marina. Recently, signs of endocrine disruption in fish from this estuary were reported (Carrola et al. 2012). Other warning signal of anthropogenic pressure observed in this area has been the eutrophication of the estuary, which become so severe in the last 15 years that is being object of mitigation measures (Dolbeth et al. 2011). Thus, it is quite possible that the estuary can be subjected to the presence of endocrine disrupting compounds (EDCs), which even in low concentrations (nanograms per liter to micrograms per liter) may exert an important impact on local fauna (Mills and Chichester 2005). However, until now, scarce information exists about the levels of important EDCs such as the natural and pharmaceutical estrogens, alkylphenols (APs) and alkylphenol polyethoxylates (APEOs)—chemicals included in the group of "priority substances in the field of water policy." In addition, despite the eutrophication of this aquatic system, few data exist on the seasonal variation of either phytoestrogens or sitosterol. These last compounds, despite being of natural/vegetal sources, are known to act also as EDCs when present in high amounts (Hoerger et al. 2009; Clotfelter et al. 2010), as it is possible to occur in eutrophic areas. Thus, the present study aimed to provide recent and new insights about the levels of several of the most concerning worldwide EDCs such as estrone (E1), estradiol (E2), ethynylestradiol (EE2), 4-n-octylphenol (4-n-OP), 4-t-octylphenol (4-t-OP), nonylphenol (4-n-NP and NP), several polyethoxylates (4-octylphenol monoethoxylate (OP1EO), 4-octylphenol diethoxylate (OP2EO), 4-nonylphenol monoethoxylate (NP1EO), 4-nonylphenol diethoxylate (NP2EO), and bisphenol A (BPA) in the Mondego estuary. Moreover, considering the endocrine activity of phytoestrogens (formononetin (FORM), biochanin A (BIO-A), daidzein (DAID), genistein (GEN), and sitosterol (SITO), these EDCs were also monitored and their seasonal trends explored by gas chromatography coupled to mass detection (GC-MS). Additionally, several physicochemical water quality parameters, linked with the presence of fecal contamination and eutrophication, such as dissolved oxygen (DO), pH, nitrates, nitrites, ammonia, and phosphates were measured. The data are relevant not only locally, but also globally as this area is an important touristic destination with beautiful nearby sandy coastal areas that attract thousands of tourists every year, which number increases every year, as referred in local press. Ultimately, it is also of public interest to be able to investigate the agreement between the biomonitoring data (namely ovotestis rates in fish and growth of green macroalgae blooms) with chemical data.

Materials and methods

Study area

The Mondego River estuary is small (8.6 km^2 area) and locates in a warm temperate region on the Atlantic coast of Portugal (Fig. 1). The river flows into the Atlantic Ocean, at the Figueira da Foz city (40°09′ N, 8°52′ W), where two sampling sites are still located inside the estuary but close to the Atlantic Ocean (sampling sites S1 and S2; Fig. 1). The estuary is constituted by two arms (north and south) with different hydrologic characteristics, separated by the Morraceira Island, which then converges again near the mouth (Fig. 1). The north arm is deeper (4–8 m during high tide and tidal range 1–3 m) and highly hydrodynamic constituting the main navigation channel and the location of the Figueira da Foz harbor (sampling site S3; Fig. 1). The south arm is shallower (2–4 m during high tide, tidal range 1–3 m) and is characterized by large areas of exposed intertidal flats during low tide (sampling site S4; Fig. 1). Circulation in the southern arm is dependent on the tides and on the freshwater input from the Mondego River and from the Pranto River, a small tributary (sampling site S5; Fig. 1). Sampling points S6 to S8 were equidistantly located from each other, about 3 km. Presently, the Mondego River estuary has two main sources of disturbance: dredging and shipping activity in the north arm and raw sewage and nutrient inputs from agricultural and fish farms in the upstream areas of the south arm (Clotfelter et al. 2010).

Water sampling and water quality measurements

Throughout 2010 and using a peristaltic pump (Model: WS300, Global Water, Gold River, CA, USA), the water samples were collected at low tide, at 1 m depth, in winter (13th January), spring (24th March and 19th May), summer (19th June and 17th September), and autumn (16th November) at eight sampling sites (Fig. 1). Before the collection of water samples, each bottle was rinsed three times with local water. Temperature, pH, DO, and conductivity were measured in situ

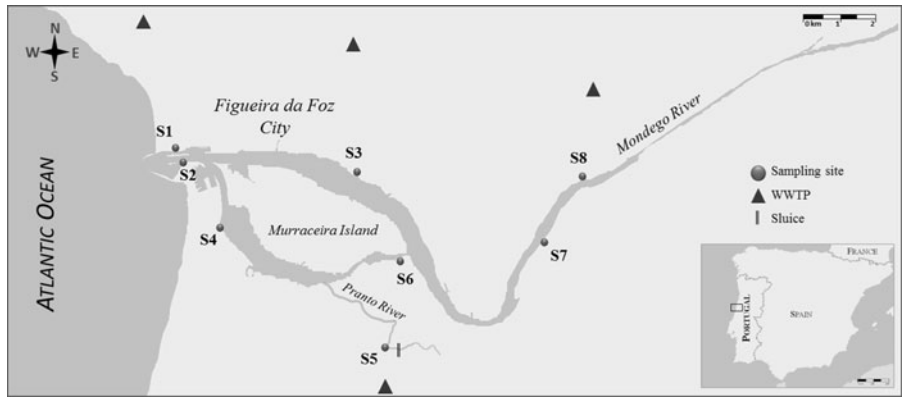

Fig. 1 Location of the sampling sites within the Mondego estuary (S1 to S8), Portugal (Microsoft MapPoint 2010)

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 ca. 5 °C. Then, samples were immediately filtered using 0.45 μm glass fiber filters (Millipore, Cork, Ireland) to eliminate particulate matter and other suspended solids. All filtrates were further acidified to pH 2 with H_2SO_4 95–97 % p.a. (Sigma-Aldrich, Steinheim, Germany), and then subjected to solid phase extraction (SPE) within 48 h.

Chemicals and materials

E1, E2, EE2, 17β-estradiol-d2 (E2-d_2), 4-t-OP, 4-OP, BPA, bisphenol A-d_{16} (BPA-d_{16}), Igepal CA-210 (OP1EO and OP2EO) and Igepal CO-210 (NP1EO and NP2EO), FORM, BIO-A, DAID, GEN, and SITO were obtained from Sigma-Aldrich (Steinheim, Germany). 4-Nonylphenol (4-n-NP) and nonylphenol isomers (NP) were supplied by Riedel-de-Haën (Seelze-Hannover, Germany). Stock solutions of individual standards (100 mg L^{-1}) 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 (Steinheim, Germany). Dichloromethane and methanol were acquired from Romil Ltd. (Cambridge, UK). The 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^{-1}, at 25 °C).

Sample preparation

All 17 EDCs were extracted by SPE using OASIS HLB cartridges fitted onto an off-line SPE vacuum extraction manifold device (Waters) and using previously published protocols (Rocha et al. 2013b). Briefly, 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^{-1}. Water samples (1 L) at pH 2 spiked with E_2-d_2 and BPA-d_{16} (deuterated surrogates, herein used also as internal standards, IS), were loaded on top of SPE cartridges at a constant flow rate of 5 mL min^{-1} 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 36 °C under a gentle N_2 stream and reconstituted with 250 μL of anhydrous methanol – the sample concentration factor was 4,000 fold and the recoveries of the assayed EDCs surpass 80 %. Then samples were derivatized using BSTFA added with 1 % (w/v) TMCS that was proved to be the best agent for the present diversity of chemicals (Rocha et al. 2013b).

Quantification by GC-MS

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^{-1}. Column oven temperatures were programmed using several ramps: a) from 100 °C (initial equilibrium time 1 min) to 200 °C at 10 °C min^{-1}; b) from 200 to 260 °C at 6 °C $min^{-1}$and, finally c) from 260 to 290 °C at 1 °C min^{-1}, 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 PTV liner ranged from 35 to 250 °C via a ramp of 10 °C s^{-1}. 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 (SIM) using external calibration. Working solutions were prepared diluting the stock solution with methanol at six calibration levels ranging from 10 to 375 ng L^{-1} for all 17 EDCs and 50 ng L^{-1} for E2-d_2 and for BPA-d_{16} (deuterated surrogates, herein used also as internal standards). The analytic parameters about the used GC-MS method are summarized in Table 1. As the current EDCs were measured in nanograms per liter, method blanks were used to ensure the absence of contamination by laboratory material. Beyond this, unbiased water samples were spiked with all assayed EDCs at an intermediate concentration (150 ng L^{-1}) of the calibration curve and then submitted to usual analysis.

Estrogenic potency

The estrogenic potency of a compound can be related to that of EE2 and be expressed as EE2 equivalents (EE2eq). The EE2eq calculation for the present estrogenic compounds varied according to their EE2 equivalent factor (*F*) obtained from different E-Screen assays. EE2eq values were calculated using potencies derived from several results of those 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 EE2

Table 1 Quantification, diagnostic ions and limits of quantification (LOQ) of each compound analyzed by GC-MS

EDCs	tr (min)	Quantification ions (*m*/*z*)	Diagnostic ions (*m*/*z*)	LOQ (ng L^{-1})
4-t-OP	10.54	207 (100)	–	4.8
4-NP	11.0–12.2	207 (100)	79 (84.9), 193 (31.9), and 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) and 135 (68.9)	17.5
NP1EO	15.8–16.3	251 (100)	65 (64.4), 207 (59.5), and 135 (45.5)	6.1
BPA-d_{16}	17.6	368 (100)	369 (34.7) and 386 (9.1)	–
BPA	17.8	357 (100)	358 (30.8)	2.4
OP2EO	18.3	295 (100)	207 (76.5) and 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) and 328 (72.8)	–
E2	24.4	285 (100)	416 (85.2) and 326 (48.4)	2.8
EE2	27.2	425 (100)	285 (48.0) and 426 (34.7)	4.4
FORM	28.5	340 (100)	339 (76.0) and 355 (22.6)	8.6
BIO-A	29.2	356 (100)	341 (34.3)	4.6
DAID	29.9	398 (100)	383 (76.0) and 355 (22.6)	4.1
GEN	31.7	471 (100)	473 (19.9)	3.8
SITO	42.1	396 (100)	486 (53.4) and 255 (49.4)	6.6

Values inside brackets are referred to the relative abundance of ions (mass-to-charge ratio (*m*/*z*)) for each target EDC

equivalent factor. The values of F followed published values (Coldham et al. 1997; Urbatzka et al. 2012).

Statistical analysis

All data were subjected to a statistical analysis using the software STATISTICA 8 (StatSoft 2007). After checking assumptions of normality (Shapiro–Wilk's W test) and homogeneity of variances (Levene's test), data sets were analyzed by one-way analysis of variance. To evaluate significant differences between factors, a post-hoc Tukey's test was used. Results were considered statistically significant for $p<0.05$ (two-tailed analysis).

Results

Estrogens

In all analyzed samples, the frequency of occurrence for E1, E2, and EE2 was ≈100 %. Figure 2 shows the seasonal fluctuation patterns of these compounds, and Table 2 reports the mean levels, per site and season, of E1, E2, and EE2. Considering the data in Fig. 2, it is observed that all estrogens show their highest amounts in winter and summer ($p<0.05$), occasions when E1 and E2 attained, respectively, ≈9 and 11 ng L^{-1} (Fig. 2; Table 2). The amounts observed in spring and autumn were ≈3 ng L^{-1} for E1, ≈4 ng L^{-1} for E2, and ≈2 ng L^{-1} for EE2 (Fig. 2; Table 2). Inside each season and sampling site, the levels of E1, E2, and EE2 were not significantly different ($p>0.05$; Table 2 (A)). However, when the $\sum_{Estrogens}$ measured at each sampling site was considered, the highest amounts of these EDCs were found in winter and summer (≈26 ng L^{-1}; $p<0.001$). In addition, considering the annual middling amounts of each estrogen, it was possible to verify that the levels of EE2 (≈4 ng L^{-1}) were lower than those of E1 (≈6 ng L^{-1}) and these lower than to those of E2 (≈8 ng L^{-1}) ($p<0.05$).

Industrial/household compounds

In all analyzed samples, the incidence of occurrence for APEOs, APs, and BPA was ≈100 %. Figures 3 and 4 show, respectively, the annual fluctuation patterns of APEOs (the octylphenol ethoxylates (OPEOs) and the nonylphenol ethoxylates (NPEOs), APs (specifically 4-n-OP, 4-t-OP, 4-n-NP, and 4-NP), and the BPA. Table 2 (B and C) reports the mean levels, per site and season, of these nine compounds. Considering the data in Fig. 3, both the OPEOs (Fig. 3a) and the NPEOs (Fig. 3b) showed higher amounts in summer than in other seasons ($p<0.05$). The same was verified for the APs, namely the 4-n-OP, 4-t-OP (Fig. 4a), and 4-n-NP (Fig. 4b), which also showed higher amounts in summer than in other seasons ($p<0.05$). In this class of EDCs, the exception was the 4-NP (Fig. 4b) which amounts were constant among samplings. Likely to the majority of the APEOs and the APs, the BPA was always ubiquitous in the studied sites (Table 2); however, in opposition to the

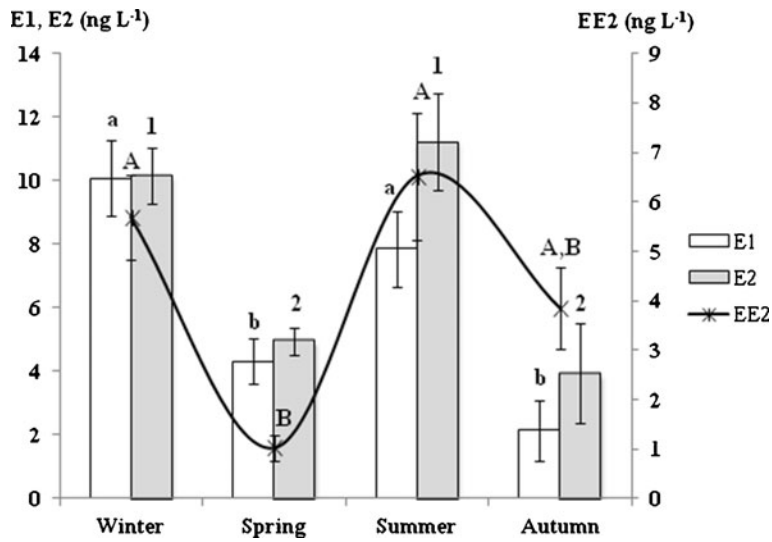

Fig. 2 Spatial and seasonal fluctuations of E1, E2, and EE2 at the Mondego estuary. Data are shown as mean±standard error of the mean (SE); $n=8$ in winter and autumn and $n=16$ in spring and summer. *Different letters or numbers* refer to statistical differences ($p<0.05$)

Table 2 Environmental levels of all EDCs measured in Mondego estuary from January to November 2010

EDCs/season		Sampling sites (ng L^{-1})							
		S1	S2	S3	S4	S5	S6	S7	S8
A - Estrogens									
E1	Winter	15	12	8.4	13	8.8	13	6.6	4.9
	Spring	6.2	3.7	4.3	2.7	6.5	7.0	1.5	2.7
	Summer	3.5	6.5	3.4	7.7	11	12	11	7.6
	Autumn	7.8	1.6	1.2	1.5	9.6	1.0	1.0	2.1
E2	Winter	12	15	12	8.6	8.2	8.7	8.0	8.9
	Spring	6.9	4.2	5.5	3.3	5.2	4.3	6.3	4.1
	Summer	7.1	11	10	16	17	18	11	8.0
	Autumn	13	1.5	2.4	5.0	1.8	1.7	2.1	3.9
EE2	Winter	4.9	5.1	4.3	3.8	3.7	8.6	4.7	10
	Spring	2.5	1.4	0.9	0.3	0.8	1.2	0.4	0.6
	Summer	0.9	9.0	1.7	11	8.8	6.7	7.8	5.9
	Autumn	1.2	3.8	7.2	1.0	5.0	3.7	5.0	3.8
B - Alkylphenols (APs) and Bisphenol A									
4-t-OP	Winter	242	549	631	832	209	275	372	307
	Spring	115	391	86	86	8,033	2,499	30	58
	Summer	282	17,806	27,502	6,373	10,526	12,002	2,404	799
	Autumn	853	519	651	1,812	1,228	838	920	974
4-NP	Winter	87	330	481	823	134	81	255	232
	Spring	108	214	135	193	1,003	787	415	373
	Summer	284	325	398	314	322	351	357	303
	Autumn	148	122	164	514	202	215	270	233
4-n-OP	Winter	52	104	87	126	48	37	24	29
	Spring	7.1	1.8	2.1	1.9	1.5	7.0	0.7	1.7
	Summer	88	810	1,279	230	505	500	145	160
	Autumn	146	120	145	111	115	164	128	133
4-n-NP	Winter	83	182	135	201	65	75	40	56
	Spring	83	90	98	56	113	90	21	195
	Summer	283	1,727	2,770	475	948	1,102	281	357
	Autumn	91	73	104	82	80	100	114	92
BPA	Winter	17	161	52	185	42	8.5	175	182
	Spring	69	39	47	44	45	73	16	51
	Summer	90	57	45	78	50	115	115	56
	Autumn	72	30	44	42	28	28	77	46
C- Alkylphenol ethoxylates (APEOs)									
OP1EO	Winter	170	320	264	462	149	183	89	108
	Spring	31	44	32	14	11	16	16	282
	Summer	82	1,450	2,337	409	531	796	260	183
	Autumn	309	166	260	233	211	327	315	260
OP2EO	Winter	37	37	73	65	55	47	25	22
	Spring	96	63	217	37	86	31	32	154
	Summer	99	2,330	833	1,147	1,242	1,965	344	113
	Autumn	79	61	179	144	124	134	148	124

Table 2 (continued)

EDCs/season		Sampling sites (ng L^{-1})							
		S1	S2	S3	S4	S5	S6	S7	S8
NP1EO	Winter	135	467	371	578	122	110	154	177
	Spring	157	194	444	130	1,027	1,083	131	820
	Summer	481	5,322	6,643	3,460	5,556	7,794	859	344
	Autumn	124	95	224	321	297	168	252	212
NP2EO	Winter	207	702	464	750	192	119	198	124
	Spring	816	805	1,207	384	18,327	12,671	540	1,084
	Summer	579	15,435	17,312	3,173	9,686	11,539	736	584
	Autumn	289	207	659	892	587	570	558	538
D - Phytoestrogens and Sitosterol									
FORM	Winter	26	173	359	90	72	34	581	36
	Spring	388	478	115	84	481	484	135	136
	Summer	735	3,281	189	4,579	181	209	149	502
	Autumn	2,867	1,308	1,085	5,494	1,000	675	355	1,826
BIO-A	Winter	143	50	484	180	141	88	289	94
	Spring	417	393	590	275	459	575	424	300
	Summer	198	191	238	335	101	156	408	580
	Autumn	224	129	72	93	195	143	89	135
DAID	Winter	270	1,024	3,756	1,227	464	661	2,392	858
	Spring	134	413	209	75	434	328	103	134
	Summer	550	5,620	137	8,881	357	53	1,193	1,309
	Autumn	11,945	4,928	3,913	6,826	4,950	4,212	3,679	5,779
GEN	Winter	427	826	2,519	827	570	445	1,119	708
	Spring	1,231	1,699	1,465	1,006	2,072	2,631	1,089	1,301
	Summer	1,402	5,093	4,705	542	386	259	999	1,438
	Autumn	825	218	128	1,343	550	503	335	557
SITO	Winter	1,382	987	1,200	1,251	843	307	699	267
	Spring	751	1,760	859	836	625	1,366	891	794
	Summer	385	181	97	424	150	136	198	853
	Autumn	266	306	170	181	172	96	604	256

Data are presented as mean±standard error of the mean

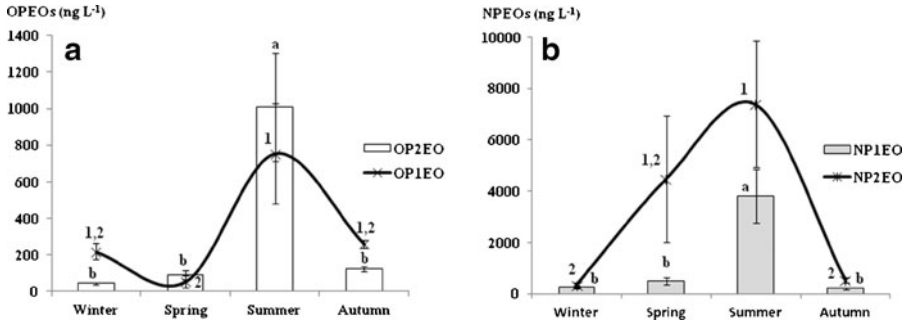

Fig. 3 Spatial and seasonal fluctuations of **a** APs and **b** APEOs at the Mondego estuary. Data are shown as mean±SE; n=8 in winter and autumn and n=16 in spring and summer. *Different letters or numbers* refer to statistical differences (p<0.05)

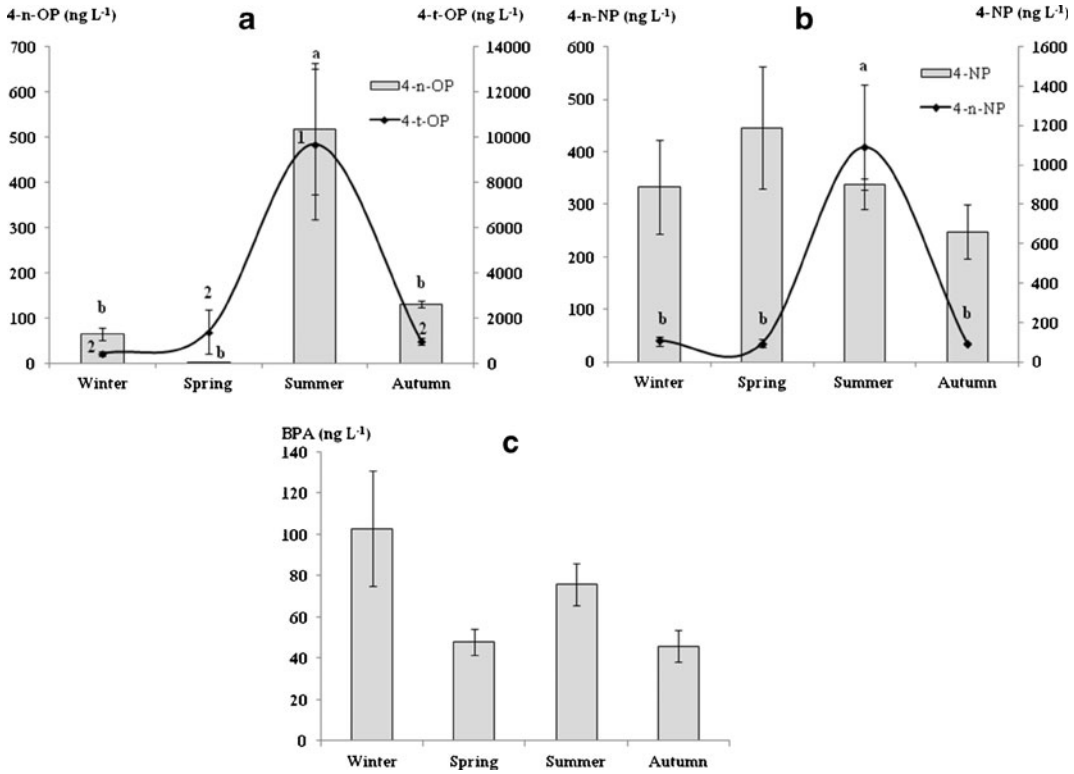

Fig. 4 Spatial and seasonal fluctuations of **a** 4-n-OP and 4-t-OP, **b** 4-n-NP and 4-NP, and **c** BPA at the Mondego estuary. Data are shown as mean±SE; $n=8$ in winter and autumn and $n=16$ in spring and summer. *Different letters or numbers* refer to statistical differences ($p<0.05$)

last referred compounds, the BPA did not show any particular fluctuation pattern either among zones or seasons (Fig. 4c; $p>0.05$). The sampling site that showed higher amounts of APEOs and APs in summer was S3 where the global amounts of these compounds exceeded 27 and 31 μg L^{-1}, respectively (Table 2 (B and C)).

Phytoestrogens and SITO

All phytoestrogens showed seasonal patterns of fluctuation (Fig. 5). Considering the FORM and its demethylated metabolite, the DAID, it is observed in Fig. 5a that both compounds showed their lowest amounts in winter and spring, and then begin to rise in summer and peak in autumn ($p<0.05$). In this occasion, FORM attained more than 1.8 μg L^{-1} and DAID exceeded 5 μg L^{-1}. Concerning the other two phytoestrogens, it is observed in Fig. 5b that BIO-A attained their maximal amounts in spring (up to 478 ng L^{-1}; $p<0.05$) and summer (up to 292 ng L^{-1}; $p<0.05$), whereas its demethylated metabolite, the GEN, showed its highest amounts in both spring (≈1.6 μg L^{-1}; $p<0.05$) and summer (≈1.8 μg L^{-1}; $p<0.05$). Considering the phytoestrogens as a whole, it is observed on Table 2 (D) that the levels of these compounds were extremely high in summer and autumn at S4 ($\Sigma_{Phytoestrogens}$≈14 μg L^{-1}). Figure 5c shows the fluctuation pattern of SITO which levels were higher in winter and spring ($p<0.05$; Fig. 5c) than in other seasons. In Table 2 (D), it is observed that the highest concentrations were measured in sites S1 to S6. The occurrence of all measured phytoestrogens and SITO in the analyzed samples was 100 %.

Physicochemical parameters

All analyzed physicochemical parameters are shown in Table 3. Concerning the annual average levels of nitrites (≈2 μmol L^{-1}), nitrates (≈21 μmol L^{-1}), ammonia (≈15 μmol L^{-1}), and phosphates (≈7 μmol L^{-1}), no differences were found among the studied zones. The annual average amounts of dissolved oxygen (DO) were ≈9 mg L^{-1}, and signs of hypoxia were never observed at any site or occasion. Table 3 also referred the annual values of pH, salinity, and temperature.

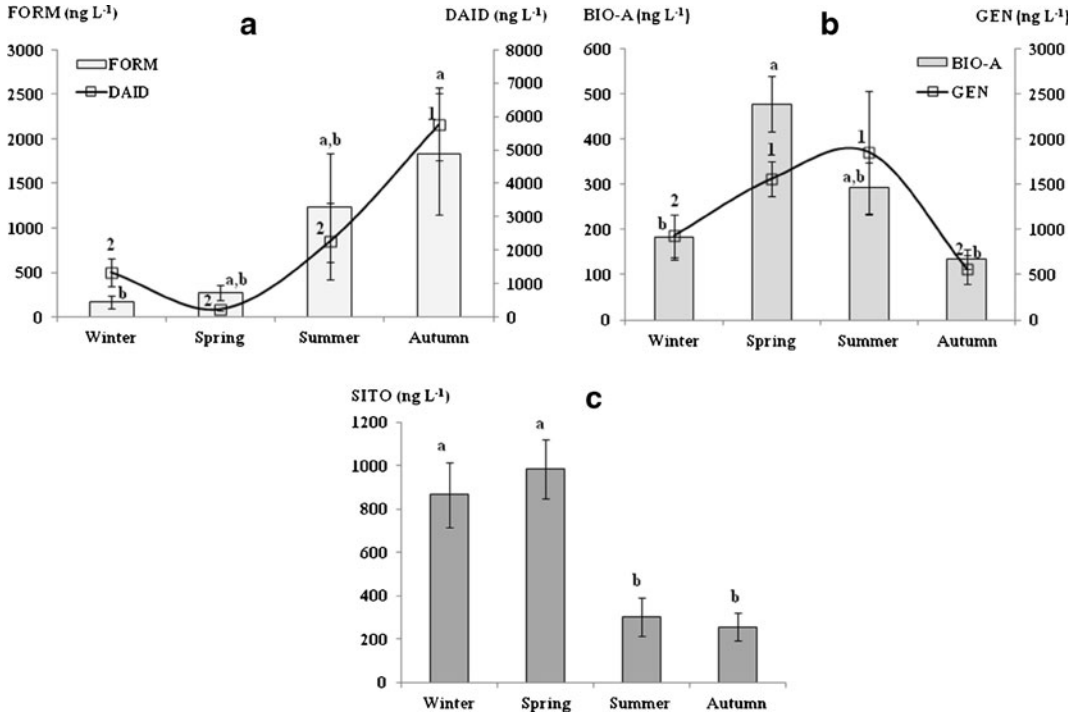

Fig. 5 Spatial and seasonal fluctuations of **a** FORM and DAID, **b** BIO-A and GEN; and **c** SITO. Data are shown as mean±SE; $n=8$ in winter and autumn and $n=16$ in spring and summer. *Different letters or numbers* refer to statistical differences ($p<0.05$)

Ethynylestradiol equivalents

The average annual values found for each estrogenic EDC were converted in EE2eq and are shown in Table 4.

Discussion

Natural and pharmaceutical estrogens

E1, E2, and EE2 when found in aquatic environments usually from human and animal excretion, being the waste water treatment plants (WWTPs) effluents their main source (Ying et al. 2002). Herein, despite the existence of several WWTPs in the estuary, bibliography refer that the sewage of about 90 % of the population (ca. 60,000 inhabitants) is discharged into this estuary without treatment (Ferreira et al. 2003). Nevertheless, we believe that this situation has already been surpassed due to construction, in 2001–2003, of other WWTP near S3 (Fig. 1) (Associação Portuguesa de distribuição e drenagem de águas, A 2005). However, it is also of public knowledge that some of the sewages coming from several poultry and livestock farms are directly discharged into the estuary or directly into the Atlantic Ocean. Given this fact, and in opposition to other Portuguese estuaries (Rocha et al. 2013b, 2013c) where

Table 3 Physicochemical parameters evaluated locally in Mondego estuary from January to November 2010

Season	pH	Temperature (°C)	Salinity (‰)	Dissolved O_2 (mg L^{-1})	NO_2^- (μmol L^{-1})	NO_3^- (μmol L^{-1})	NH_4^+ (μmol L^{-1})	PO_4^{3-} (μmol L^{-1})
Winter	7.9±0.3	12.4±0.8	0.9±1.3	10.2±0.9	1.1±0.6	26.0±10.2	11.8±8.1	9.2±3.6
Spring	7.9±0.2	17.5±0.9	8.0±6.8	8.7±1.0	1.8±0.6	11.8±3.9	9.3±3.4	7.7±2.6
Summer	8.1±0.2	20.6±4.7	13.4±11.6	7.7±0.6	2.6±0.8	27.7±13.6	9.9±5.0	8.9±2.5
Autumn	8.4±0.0	14.6±0.4	9.0±7.8	7.7±0.7	2.6±1.3	20.0±8.4	29.7±17.3	3.1±1.0

Data are presented as mean±standard error of the mean

Table 4 Average concentration levels of the estrogenic compounds, their relative potencies to E2, and calculated EE2-equivalents (EE2eq) concentrations

Compound	Relative potency to E2 (*F* factor)	Average annual concentrations of each estrogenic compound measured in the Mondego River estuary (ng L^{-1})	EE2 equivalents (EE2eq, ng L^{-1})
EE2	1.3	4.3	4.3
E2	1.0	7.9	6.3
E1	3.0^{E-01}	6.4	1.5
4-t-OP	4.0^{E-04}	3.1^{E+03}	1.0
4-n-OP	1.3^{E-05}	1.7^{E+02}	2.0^{E-03}
OP2EO	5.0^{E-06}	2.8^{E+02}	1.0^{E-03}
OP1EO	5.0^{E-06}	3.2^{E+02}	1.0^{E-03}
4-n-NP	5.0^{E-05}	3.2^{E+02}	1.3^{E-02}
NP2EO	6.0^{E-07}	3.3^{E+03}	2.0^{E-03}
NP1EO	6.0^{E-07}	1.3^{E+03}	1.0^{E-03}
BPA	1.0^{E-04}	6.8^{E+01}	5.0^{E-03}
NP	4.0^{E-03}	3.2^{E+02}	1.0
GEN	4.9^{E-02}	8.8^{E+02}	3.4^{E+01}
DAID	1.3^{E-03}	2.6^{E+02}	2.7^{E-01}
FORM	5.6^{E-03}	1.2^{E+03}	5.5
BIO-A	9.1^{E-03}	2.4^{E+03}	1.8^{E+01}
SITO	–	–	–
∑EE2eq in the estuary			71.8

The relative potencies of the estrogenic compounds vary depending on the cellular assays and consequently, approximate values were based on data reported in the following studies (Coldham et al. 1997; Urbatzka et al. 2012)

WWTPs are more effective in the treatment of the urban wastes, herein the annual average amounts of E1 (≈6 ng L^{-1}) and E2 (≈8 ng L^{-1}) were about 1.5- to 2-fold higher than those measured in the Ave or in the Douro River estuaries (Rocha et al. 2013b, 2013c). By contrast, the concentration of EE2 (≈4 ng L^{-1}), an estrogen commonly used in contraceptive pills, was about threefold lower than those measured in the last referred estuaries (Rocha et al. 2012; Rocha et al. 2013b). This fact agrees with the knowledge that a high percentage of the population in the area of the Mondego River estuary is older than 41 years (≈60 %) or it is male (≈53 %) (Medeiros et al. 2012).

Overall, the estrogenic load in this area was similar to other coastal areas reported in Spain (Rodriguez-Mozaz et al. 2004), Italy (Laganà et al. 2004), and North America (Sellin et al. 2009). To better understand the effect of these estrogenic compounds, the current data were normalized in EE2 equivalents (EE2eq; Table 4). In this sense, comparing the values calculated for the Douro River estuary (≈20 ng L^{-1}) (Urbatzka et al. 2012) that supports the so-called Great Porto city (ca. 1,700,000 inhabitants), with those observed here, it is observed that, in average, the Mondego River estuary contained lower EE2eq (≈12 ng L^{-1}; Table 4). However, as these values rose during summer, occasion when they reached ≈27 ng L^{-1} EE2eq mitigation measures should be undertaken as these data strongly support that the presence of ovotestis in fish caught in this estuary (Carrola et al. 2012). The increase of estrogens in summer may be explained by the rise in the number of tourists that double the quantity of local inhabitants coupled with the decrease of the freshwater flow of the river. Moreover, as there is a likely link between exposure to these chemicals and several human endocrine disorders (Safe 2000), the consumption of local fish and shellfish should be carefully monitored, due to its potential risk for human health.

Industrial pollutants

In the past, APEOs were used to produce nonionic detergents, however, given their ability to promote estrogenic effects on both wild fauna and humans (Safe 2000; Zoller 2006), they were banned from Europe in 2003 (2003/53/EC). Thus, it was not clear to understand the origin of APEOs in the Mondego River estuary. However, it is possible to accept that the sources are mainly industrial and agricultural as several facilities often associated to the usage of these chemicals, such as pulp and paper production and textile manufacturing are present in the Mondego River basin (Rocha et al. 2013b). The use of APEOs in the formulation of crop protection chemicals can also be an important source of contamination. Herein, and with the exception of NP2EO, all other APEOs reached maximal values in summer, occasion when their levels increased significantly in all sampling stations attaining similar amounts to those found in other Portuguese, Spanish, or Greek coastal waters (Arditsoglou and Voutsa 2008; David et al. 2009; Rocha et al. 2012). This observation suggests that these compounds are still being used, and thus

constitute a wide global problem of several coastal areas (Soares et al. 2008). The increase levels of APEOs in summer is probably due to the decrease of rain and thus reduction of water flow from several tributaries that usually supply the estuary with fresh water; nonetheless further studies are needed to confirm this hypothesis. Once in the environment, the APEOs degrade within 1 or 2 days, deriving APs (NPs and OPs), which are much more harmful as EDCs and persistent than their parents (Safe 2000). Herein, the fluctuation patterns of the NPs and OPs were consistently close related to those of the NPEOs and OPEOs, and thus their levels were also higher in summer, occasion when the concentrations of the Σ_{NPs} reached 0.64 μg L^{-1} and those of Σ_{OPs} attained 3.3 μg L^{-1}. As the Directive 2000/60/EC of the European Parliament and of the Council established 0.3 μg L^{-1} as the maximal annual average value allowed in surface waters for APs, it was concluded that both unambiguous identification of the sources and mitigation actions should be considered in the Mondego River estuary. Both APEOs and APs are much less estrogenic than E1, E2, and EE2, but as they exist in huge amounts they contribute, as estimated to a whole, with 1.0 ng L^{-1} of EE2eq (Table 4). In the Douro River estuary, the estrogenic contribution of these pollutants was much smaller (0.007 ng L^{-1}; (Urbatzka et al. 2012), suggesting that direct discharges still occur in the Mondego River estuary. In addition, BPA, systematically measured at all sites (≈68 ng L^{-1}), was demonstrated to be a ubiquitous compound in this estuary. BPA amounts were higher than those refereed in other surface waters, either in Portugal (Urbatzka et al. 2012), Spain (Céspedes et al. 2005), or The Netherlands (Vethaak et al. 2005). Nevertheless, the values presently estimated are lower than the concentrations measured in 2006 in this aquatic system (Ribeiro et al. 2009). At that occasion the levels of BPA attained 589.5 ng L^{-1}. These observations suggest a chronic action of this compound over the local fauna, which may also trigger serious endocrine dysfunctions (Crain et al. 2007).

Phytoestrogens and SITO

Phytoestrogens are naturally occurring plant-derived endocrine disruptors that enclose the coumestans, isoflavones (BIO-A, GEN, FORM, and DAID), and lignans. These compounds, and particularly the isoflavones, resemble the E2 molecule and so they have affinity to the estrogenic receptors (Benassayag et al. 2002). Nevertheless, their estrogenic potency is much lower than E2 (Table 4) and so their EC_{50} values are more than four orders of magnitude higher than that of E2 (Hoerger et al. 2009). In fact, the (total) concentration of the isoflavones must be at least 1,000-fold higher than that of E2, i.e., in micrograms per liter range, to produce an estrogenic effect equivalent to that of E2 (Table 4). In the environment, the sources of phytoestrogens are complex, and they can be either natural (local flora) or resultant of the activity of food industries and paper plants. In the Mondego River estuary, both kinds of situations occur, with the presence of paper plants in the south margin of the estuary and the existence of large amounts of seaweeds, which sometimes grow in excess leading to the occurrence of blooms conducting to eutrophication scenarios (Dolbeth et al. 2011). As the seaweed life cycle is coincident with the fluctuation patterns found herein for the isoflavones (Fig. 5), it is suggested that these algae are the main source of these compounds. In fact, the growing season of those primary producers start in late winter, occasion when the levels of all isoflavones are low (Fig. 5a, b). The maximal biomasses usually occur in spring (Ferreira et al. 2003; Leston et al. 2008), occasion when GEN peaks and BIO-A begin its rose. A second rise of these plants occurs in mid-summer (Ferreira et al. 2003; Leston et al. 2008), time at which BIO-A peaks (Fig. 5b). Later on, in autumn, when the environmental conditions change, the death of the green macroalgae and the decrease of the aboveground biomass of *Spartina* and *Zostera*, probably lead to the presence of high amounts of both FORM and DAID (Fig. 5a). Comparing the present values with those reported for other Portuguese aquatic habitats, it is observed that levels of FORM, DAID, and GEN were in the Mondego estuary about 3- to 15-fold higher (in summer) than those observed in the North of Portugal (Rocha et al. 2013b, 2013c). This suggests that the growth of the seaweeds might still be an important issue in this area. Furthermore, because sometimes GEN alone attained concentrations that surpass 1,000 ng L^{-1} (Table 2 (D); Fig. 5b), it is possible that estrogenic endocrine disruption may be induced locally by this phytoestrogen, as reported by others (Kiparissis et al. 2003). Considering all phytoestrogens as a whole, and using the normalized data expressed in EE2eq, it is observed that these compounds contribute with an estrogenic load of ≈58 ng L^{-1} EE2eq (Table 4). It is important to stress that these EE2eq were values based

in literature data (Coldham et al. 1997) and so, biological and toxicological assays are needed to confirm the estrogenic contribution of these EDCs in the Mondego estuarine environment to fully understand the impact of these compounds in both local fauna and humans.

Endocrine disruption can also be induced by the phytosterol SITO, which is structurally similar to cholesterol (Fig. 2). This natural compound can cause endocrine disruption either by decreasing the availability of cholesterol to the P450scc (enzyme involved in the conversion of that hormone to pregnenolone) or by decreasing the activity of this enzyme (Volkman et al. 2008). In the past, SITO was identified as an important component of vascular plants and seeds (Benassayag et al. 2002) but more recently it was also linked to the presence of seagrass (Volkman et al. 2008), which is abundant in this area. As SITO is involved in lipid metabolism (Volkman et al. 2008), it seems logical to presume that its levels are higher in the blooming season (spring) than when they are already fully grown or deceasing (summer and autumn) (Fig. 5c). However this hypothesis needs to be confirmed since, as far as we noticed, there are no studies about this issue.

Physicochemical data

Some physicochemical parameters closely related to sewage and WWTPs discharges (pH, dissolved oxygen, ammonium, nitrites, nitrates and phosphates) were additionally evaluated (Table 3). Herein, the levels of DO were always higher than 7.7 mg L^{-1}, even in summer when average temperatures are high (> 20 °C), and so the hypoxia described in previous studies (Ferreira et al. 2003) at some sites close to S4, S5 were never observed herein. In addition, the pH parameters and salinity were similar to other studies done in this area and within the same values reported for other estuaries (Rocha et al. 2013b, 2013c; Rocha et al. 2013a). Nonetheless, the data found for nitrites and nitrates reveal that their sum is higher than 13 μmol L^{-1}, which accordingly to the European Environmental Agency (EEA 1999) classifies the water quality of this area as "Bad". However, as samples were collected in low tide it is possible that in flood periods these values decrease. Also the total amounts of phosphorous, which WWTPs and organophosphorus pesticides are the most common sources, were measured in excessive amounts at all the studied sites, i.e., up to 9-fold higher (Table 3) than the maximal concentrations of 1.1 μmol L^{-1} recommended for rivers and streams by the EEA. This occurrence may well be justified by the presence of vast fields of maize, rice and some vegetable crops that use the last referred compounds to avoid plagues (Andrade and Stigter 2009).

Conclusions

The present study identified the presence of natural and pharmaceutical estrogens, APs, APEOs, phytoestrogens, and SITO at the Mondego River estuary suggesting that these compounds are ever present. The studied area, which covers the entire estuarine system, is similarly impacted by the last chemicals which in many occasions attained concentrations able to induce endocrine disruption. In fact, estrogens, industrial compounds (APs and APEOs) and phytoestrogens frequently reached in this estuary, concentrations able to trigger endocrine disruption in local fauna. This occurrence agrees with findings of endocrine disruption (ovotestis) in local aquatic species. The results highlight the need to monitor the system in flood tide situations, in order to estimate the possible risk for humans, mainly by the uptake of contaminated seafood. The physicochemical data also corroborates the present chemical EDCs monitoring, further proving that the Mondego estuary is strongly impacted by discharges coming from domestic and industrial sewages. Thus, local preventive and depollution measures are needed as the European legislation, applied in Portugal, is very strict in the prevention, protection, and improvement of the environment quality, the protection of the human health, and the rational and cautious use of natural resources.

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