Environ Monit Assess (2009) 159:415–427
DOI 10.1007/s10661-008-0639-1

Spatial distribution and quantification of endocrine-disrupting chemicals in Sado River estuary, Portugal

Cláudia Ribeiro · Miguel Ângelo Pardal · Maria Elizabeth Tiritan · Eduardo Rocha · Rui Miguel Margalho · Maria João Rocha

Received: 24 March 2008 / Accepted: 5 November 2008 / Published online: 24 December 2008

Abstract The important Portuguese Sado River estuary has never been investigated for the presence of potentially endocrine-disrupting chemicals (EDCs), such as natural estrogens (estradiol, estrone), pharmaceutical estrogens (17α-ethynylestradiol), phytoestrogens (daidzein, genistein and biochanin A), or industrial chemicals (4-octylphenol, 4-nonylphenol, and bisphenol A). Thus, the main objective of this study was to evaluate their presence at 13 sampling points distributed between both the industrial and the natural reserve areas of the estuary, zones 1 and 2, respectively. For that, water samples collected in summer and winter were processed by solid phase extraction and analyzed by high-performance liquid chromatography with photodiode array detection and gas chromatography–mass spectroscopy. Results showed that estrone, ethynylestradiol, all the aforementioned phytoestrogens as well as bisphenol A and 4-octylphenol were found in zone 1. In zone 2, neither estrogens nor 4-OP were found. However, in the same zone, daidzein (500 ng/L) and genistein (320 ng/L) attained their highest levels in summer, whereas biochanin A peaked in winter (170 ng/L). Furthermore, bisphenol A was also found in some areas of zone 2, but showed similar concentrations in both surveys (about 220 ng/L). This study demonstrated that the Sado River estuary had low EDCs levels, suggesting that the Sado's high hydrodynamic activity may be involved in the dilution of local pollution. It was suggested that at the current levels of concentrations, all assayed EDCs are unlikely to individually cause endocrine disruption in local

Electronic supplementary material The online version of this article (doi:10.1007/s10661-008-0639-1) contains supplementary material, which is available to authorized users.

C. Ribeiro · M. E. Tiritan · M. J. Rocha (✉)
Department of Pharmaceutical Sciences, Superior Institute of Health Sciences–North (ISCS-N), Rua Central de Gandra, 1317, 4585-116 Gandra PRD, Portugal
e-mail: mjsrocha@netcabo.pt

C. Ribeiro · E. Rocha · M. J. Rocha
Interdisciplinary Centre for Marine and Environmental Research (CIIMAR), CIMAR Associate Laboratory (CIMAR LA), University of Porto (UPorto), Porto, Portugal

C. Ribeiro · M. E. Tiritan
Centre of Studies of Organic Chemistry, Phytochemistry and Pharmacology of the University of Porto (CEQOFFUP), Porto, Portugal

C. Ribeiro · E. Rocha
Institute of Biomedical Sciences Abel Salazar (ICBAS), University of Porto (UPorto), Porto, Portugal

M. Â. Pardal · R. M. Margalho
IMAR—Institute of Marine Research, Department of Zoology, University of Coimbra, Coimbra, Portugal

animals. However, under a continuous exposure scenario, an additive and/or synergistic action of the estrogenic chemicals load can not be excluded, and so, continuous monitoring is advisable.

Keywords Biochanin-A · Bisphenol-A · Daidzein · Endocrine-disrupting chemicals (EDCs) · Genistein · Phytoestrogens · Sado River estuary

Introduction

The Sado River, which is located in the West in the Iberian Peninsula, Portugal, flows from the Serra do Caldeirão to the Atlantic Ocean. It is one of the few European rivers flowing South to North. During its course of 175 km, it runs mainly through rural areas before reaching its estuary, which is commonly divided in two zones (Ferreira et al. 2002). Zone 1 is located near the city of Setúbal and the Atlantic Ocean and is characterized by a large bay that receives effluents of sewage treatment plants. The most polluting industries of this zone are those involving the production of pulp and paper, pesticides, fertilizers, processed food, and shipyards (Catarino et al. 1987). Zone 2 is a natural reserve park of high environmental importance (approximately 23,160 ha) which includes many shallow sandy shores surrounded by typical Mediterranean forests (Ferreira et al. 2006). The aquatic fauna of this area includes many species of fish, mussels, cephalopods and mammals such as the dolphins, *Tursiops truncatus*, whose population has been reduced to just 25–30 animals. Recent studies showed that during the last decade, pollution in this region increased, confirmed by the rising levels of nitrates, eutrophication, pesticides and biocides, heavy metals, organotins, polychlorinated biphenyls, and polycyclic aromatic hydrocarbons (Quevauviller et al. 1989; Ferreira and Vale 2001; Caeiro et al. 2005; Silva et al. 2006; Almeida et al. 2007). However, to our best knowledge, estrogens or estrogen mimics (phytoestrogens, alkylphenols, etc.), which can contribute to diverse fish disorders (Jobling et al. 2002; Mills and Chichester 2005), were never investigated in this estuary. These chemicals, referred to as endocrine-disrupting compounds (EDCs), include natural estrogens [estrone (E1), 17β-estradiol (E2)], pharmaceutical estrogens [ethynylestradiol (EE2)], phytoestrogens [daidzein (DAID), genistein (GEN), biochanin A (BIO-A)], and industrial pollutants [bisphenol A [BPA], 4-octylphenol (4-OP), 4-nonylphenol (4-NP)]. It is known that the above EDCs can exhibit, even in a few nanograms per liter, additive or synergic toxic effects and further strongly suspected to be responsible for the appearance of ovotestis in aquatic animals (Vethaak et al. 2005). Since such a condition was reported in this estuary for the segment worm (*Hediste diversicolor*, Polychaete) and the snail (*Hinia reticulata*, Gastropoda) (Pessoa et al. 2001; Sousa et al. 2005; Moreira et al. 2006), the main objectives of this study were to (1) identify and quantify the above referred EDCs in this area, (2) report possible differences among the patterns of pollution between the industrial area and the natural reserve zone, and (3) verify if any of the studied compounds exist in hazardous amounts. Moreover, this study is part of a comparative screening we are making in main Portuguese estuaries, which already proved the presence of EDCs in the estuaries of Douro and Mondego (Ribeiro et al. 2008a, b).

Materials and methods

Study area

The Sado River estuary is located on the west coast of Portugal (38°35′ N, 8°55′ W; Fig. 1).

Due to the extension of this estuary (235 km^2), 13 sampling stations (S1 to S13) were selected from the river mouth, near the Atlantic Ocean, to Alcácer do Sal. The sampling stations S1 to S3 were located near the city of Setúbal (zone 1; Fig. 1), which is a highly industrialized zone with about 100,000 inhabitants. The other sampling stations, S4 to S13, were distributed inside the natural reserve of the Sado River estuary (zone 2; Fig. 1). Due to the vast area of the latter zone (approximately 23,160 ha), it is usually divided into three main regions: the Comporta channel (S6 to S9; Fig. 1), the Marateca channel (S10 and S11; Fig. 1), and the Alcácer do Sal channel (S12 and

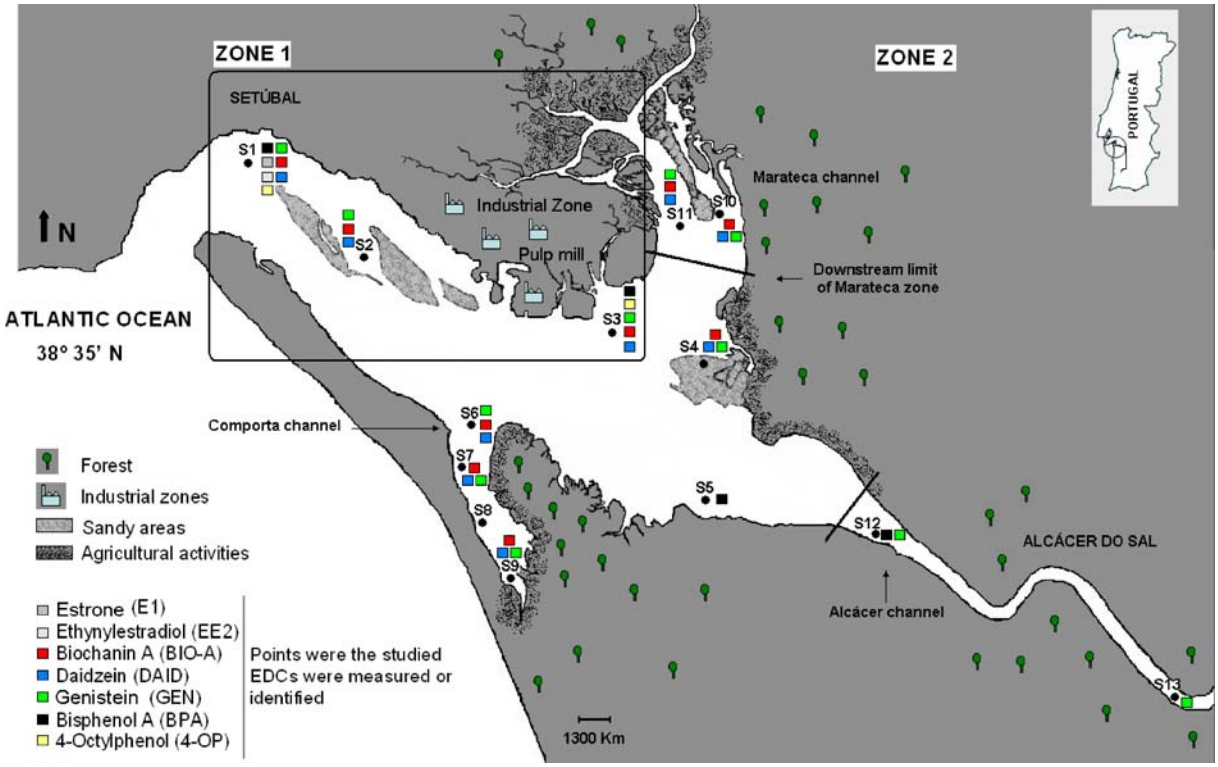


Fig. 1 Map of the Sado River estuary showing the locations of the thirteen sampling areas (S1 to S13), the main industrial poles, forest and agricultural areas, and the location where the studied EDCs were found

S13; Fig. 1). Sampling stations S4 and S5 (Fig. 1) represent the middle estuary.

Sampling collection

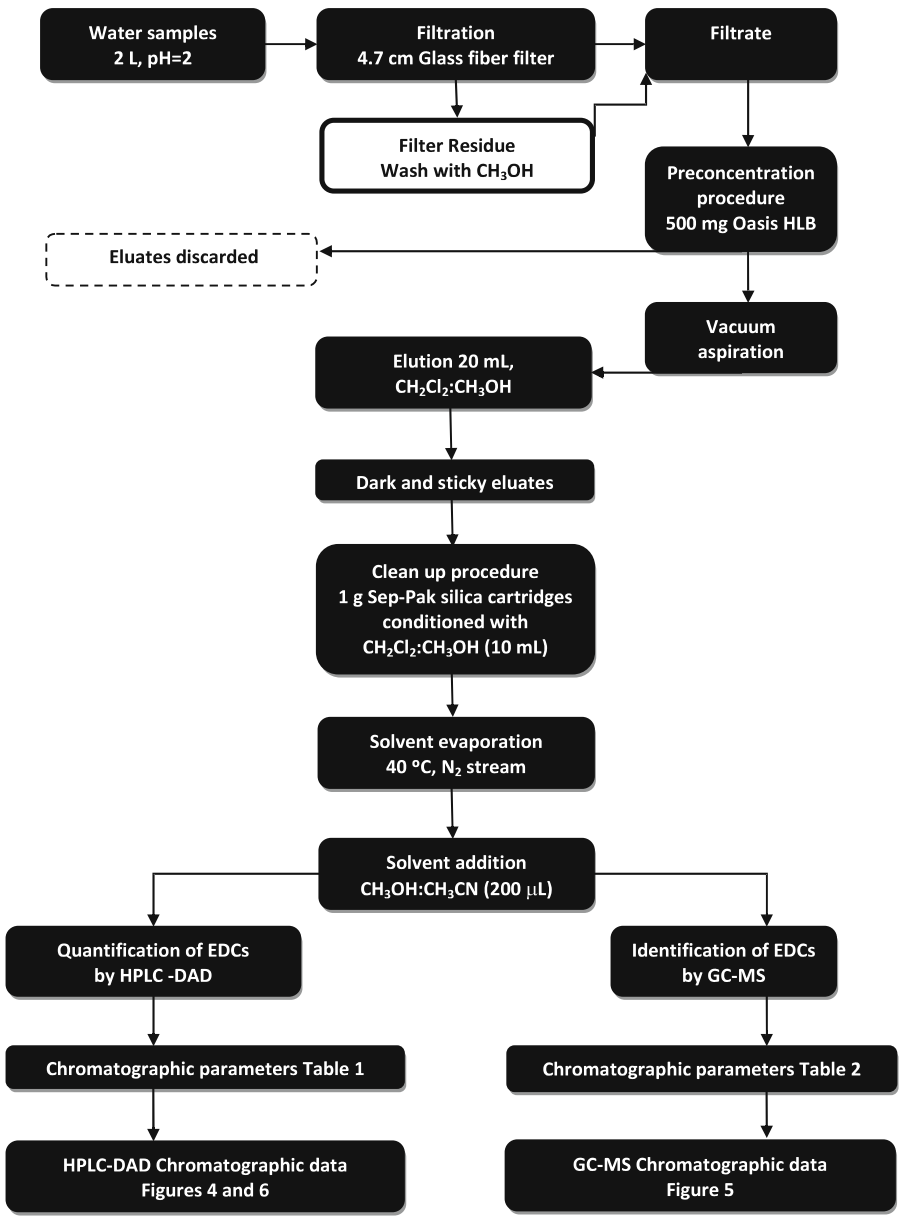
In summer (July 2006) and winter (December 2006), 2 L of estuarine water samples were collected at a depth of 1 m using a peristaltic sampler pump (Global Water, model WS300). Sampling occurred at both high and low tides and at 13 sampling stations (Fig. 1). Water temperature, salinity, pH, and dissolved oxygen values were evaluated by the portable meters LF 330/ Set WTW, pH 330i/ Set WTW, and OXi 330i/ Set WTW, respectively. All bottles were rinsed two or three times before collecting the water samples, which were immediately filtrated through Millipore glass fiber filters type 3 (retention 1.2 μm, circles size 4.7 cm) to eliminate particulate matter and any other suspended solids. Following this procedure, each filter was washed several times with small amounts of CH_3OH that were added to the latter filtrate. Finally, all samples were acidified with H_2SO_4 to pH 2 to prevent biodegradation and kept at approximately 5°C during transport to the laboratory.

Chemicals

E1, E2, EE2, DAID, GEN, BIO-A, BPA, 4-OP, and the derivatizing reagent *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) were obtained from Sigma-Aldrich (Steinhein, Germany), whereas 4-NP was obtained from Riedel-de-Haën (Seelze-Hannover, Germany). Stock solutions of individual standards were prepared by dissolving known amounts of each compound in CH_3OH/CH_3CN (50:50, *v*/*v*) high-performance liquid chromatography (HPLC) grade, acquired from Sigma-Aldrich, to obtain final concentrations of 500 mg/L. All other solvents were of analytical grade from Sigma-Aldrich. Ultrapure water was supplied by a Milli-Q water system.

Sample preparation

All the previously filtered and acidified water samples (2 L) were pre-concentrated by a solid phase extraction through a 500-mg Oasis HLB cartridge purchased from Waters Corporation (Milford, MA, USA). Firstly, the Oasis cartridges (polymer of *N*-vinylpyrrolidone and divinylbenzene) were sequentially washed with 25 mL of CH_2Cl_2/CH_3OH (50:50, *v*/*v*), 12 mL of CH_3OH, and 25 mL of ultrapure Milli-Q water (Ribeiro et al. 2007). Secondly, all samples were applied to cartridges under vacuum at a constant flow rate of 5–7 mL/min and washed with 25 mL of ultrapure Milli-Q water followed by 1 mL of CH_3OH; in this step, all eluates were discarded. Finally, the cartridges were kept under vacuum aspiration for 30 min to dry out residual water, and the



Scheme 1 Derivatization with MSTFA and analysis by GC

Table 1 Chromatographic and calibration data obtained for the Sado River estuary

Chemicals (EDCs)	Retention time (t_r, min)	Linear dynamic range (μg/mL)	Intercept	Slope	R^2	Recoveries (%)	LOD (ng/L)	LOQ (ng/L)
DAID	8.1	0.4–20.0	1463.3	57,644.7	0.99	98	8.3	25.0
GEN	10.6	0.4–20.0	3325.2	53,156.1	0.99	95	8.6	26.0
BPA	12.6	0.9–2.7	−19.2	7,947.3	0.99	99	4.0	12.0
E2	3.6	0.8–2.4	150.4	3,975.2	0.99	99	18.0	55.0
EE2	14.4	1.0–3.0	429.2	3,658.8	0.99	99	18.0	57.0
E1	15.2	0.8–2.4	910.8	3,813.5	0.99	97	10.0	29.0
BIO-A	15.7	0.1–1.2	−4866.0	45,175.5	0.99	91	10.0	21.0
4-OP	25.9	2.0–6.0	507.2	5,355.4	0.99	70	9.0	28.0
4-NP	27.8	2.0–6.0	−746.2	4,694.0	0.99	61	13.0	39.0

LOD limit of detection, *LOQ* limit of quantification

elution process was performed with 20 mL of CH_2Cl_2/CH_3OH (50:50, *v*/*v*). It was noted that the extracts from the estuarine water samples had a dark and sticky appearance, so all extracts were quantitatively transferred to the pre-washed 1-g Sep-Pak silica cartridges (CH_2Cl_2/CH_3OH, 50:50, *v*/*v* from Waters Corporation). Seven milliliters of the same solvent composition was used to elute samples into a round-bottomed tube and evaporated to dryness in a thermostatic bath at 40°C under N_2 and then further dissolved in 200 μL of CH_3OH/CH_3CN (50:50, *v*/*v*). Twenty microliters of each sample was injected in triplicate into the HPLC with photodiode array detection (HPLC-DAD) system for quantitative analysis (Ribeiro et al. 2007). To reconfirm the identity of the measured EDCs, 50 μL of each sample was evaporated to dryness before derivatization with MSTFA and analysis by gas chromatography (GC-MS; Scheme 1). The recovery rates, calculated after the pre-concentration and cleanup steps of all samples in both OASIS and silica cartridges, showed for the majority of the EDCs higher than 94%, with the exception of BIO-A (91%), 4-OP (70%), and 4-NP (61%; Table 1).

Chromatographic system and main methodological characteristics

The high-pressure liquid chromatography system consisted of a Merck Hitachi HPLC apparatus equipped with a LaChrom diode array detector L-7455 (HPLC-DAD) and a LiChroCART C_{18} reverse-phase analytical column (250 × 4-mm i.d., 5-μm particle size, Merck, Darmstadt, Germany). Data acquisition was performed by the HPLC System Manager HSM D-7000, version 3.0. All analyses in this system followed a previously validated method developed for the present estuarine water samples (Ribeiro et al. 2007). Briefly, the chromatographic analysis was performed at room temperature using a gradient solvent program with a flow rate of 1 mL/min. The initial composition of the mobile phase was CH_3CN/H_2O (25:75, *v*/*v*) acidified with CF_3CO_2H, pH 2. The gradient was programmed to increase linearly the amount of organic solvent from 0 to 5 min (25–40%), 5 to 14 min (40–55%), 14 to 17 min (55–57%), and 17 to 30 min (57–90%). After each chromatographic run, the amount of CH_3CN increased up to 100% and maintained isocratically during 5 min before a new injection. The wavelengths used for detection of the studied EDCs were 246 nm for DAID, 260 nm for GEN and BIO-A, 278 nm for BPA, 4-OP and 4-NP, and 280 nm for E2, E1, and EE2. All working calibration curves used for the calculation of the levels of the latest EDCs showed correlation factors higher than 0.99, and peak areas were used for the quantitative analysis as shown in Table 1 and previously reported (Ribeiro et al. 2007). During the application of this method, several quality control parameters were performed taking into consideration the International Conference of Harmonization rules (ICH 1996), i.e., the robustness of the method was constantly checked. For that, the instrumental precision and accuracy were monitored regularly by the measurement of the peak areas of injections

containing both standard mixture and fortified matrix (relative standard deviation, RSD < 1%). Furthermore, the maintenance of the retention times among samples and standards was always verified as each analyzed sample was injected both individually ($n=2$) and spiked with all standards ($n=1$). Finally, only the areas of peaks showing purity tests higher than 99% (this value was calculated automatically by the HPLC-DAD software) were considered for the quantitative analysis (Fig. 6).

The presence of the compounds of interest in the samples was confirmed by GC-MS on derivatized samples. The GC-MS analytical procedure used for the current estuarine water samples was based in published protocols, with adjustments (Lee et al. 2004; Ballesteros et al. 2006; Shareef et al. 2006; Silveira 2007). Briefly, The GC-MS consisted in a Varian CP 3800 apparatus equipped with a VF-5ms type capillary column (30-m × 0.25-mm i.d; *df*, 0.25 μm) connected to an ion trap mass spectrometer (Varian Saturn 2200). The helium carrier gas (99.99% purity) was maintained at a constant flow rate of 1.2 mL/min, and the GC column temperature was programmed from 50°C (initial equilibrium time 1 min) to 150°C via a ramp of 20°C/min, 150–270°C via a ramp of 10°C/min and maintained at 270°C for 10 min, 270–280°C via a ramp of 10°C/min and held for 10 min. Sample injection (1 μL) was in splitless mode. The MS conditions were as follows: electron impact ionization energy was 70 eV, and the ion source and transfer line temperature were maintained at 280°C. As the majority of the an-

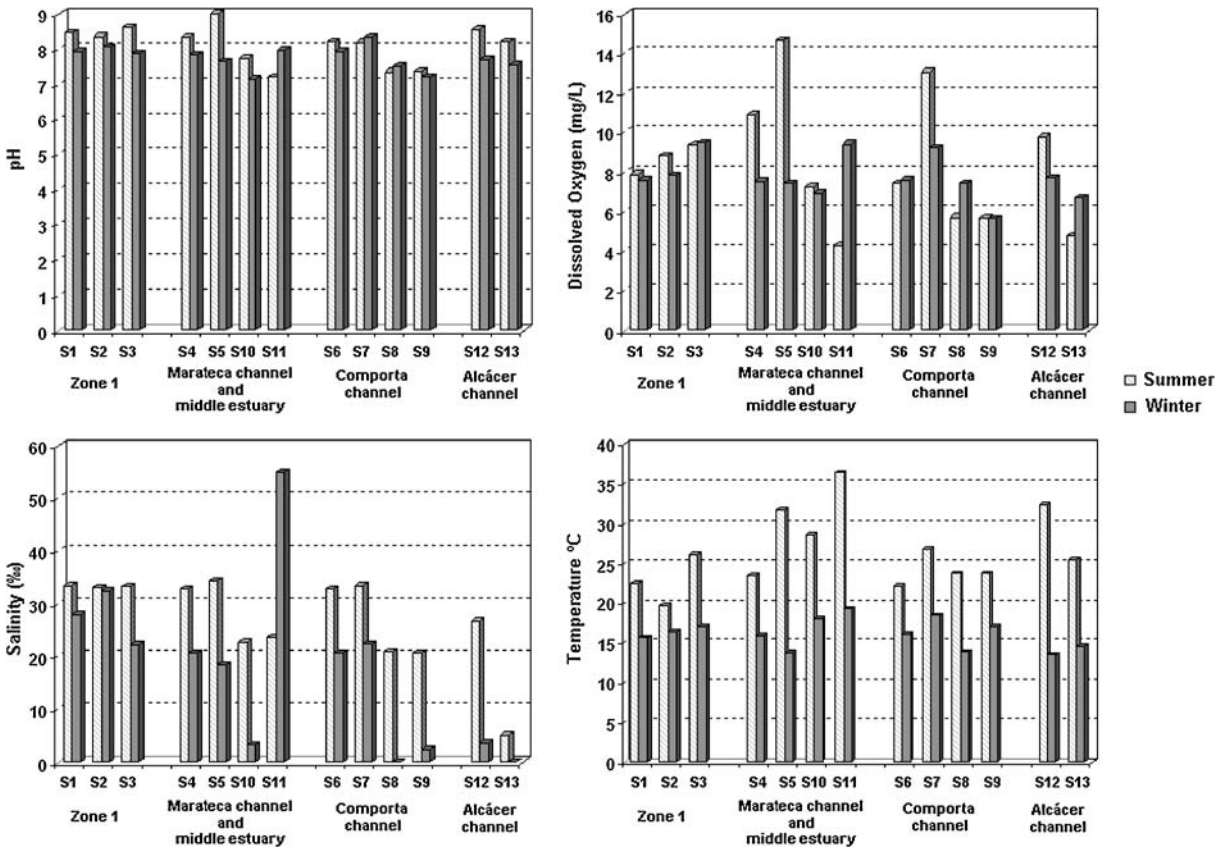


Fig. 2 Ranges of pH, dissolved oxygen (mg/L), salinities (‰), and temperature (°C) measured in the industrial area (zone 1) and in the natural reserve region (zone 2 Marateca and middle estuary, Comporta and Alcácer channels) in summer and winter

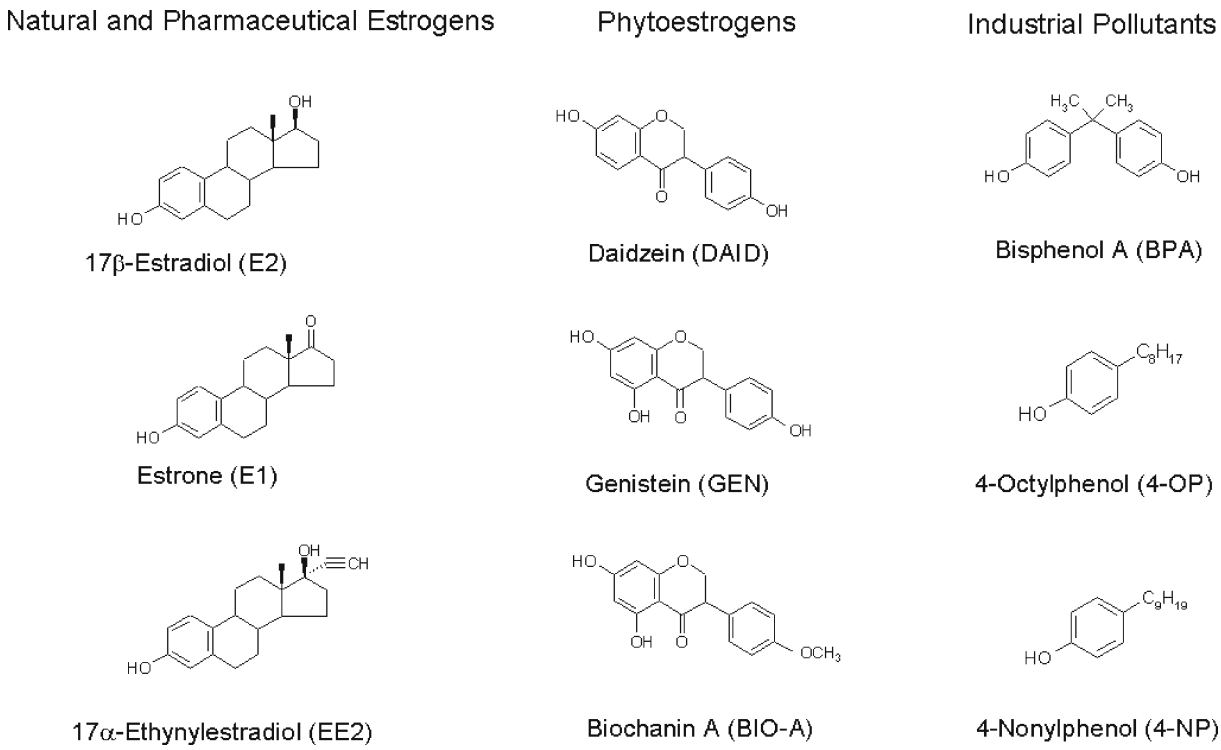


Fig. 3 Chemical structure of the EDCs investigated in the Sado River estuary

alyzed EDCs (Fig. 3) contain several hydroxyl groups, the MSTFA was the preferred derivatization reagent for preparing the trimethylsilyl derivatives (Lee et al. 2004; Shareef et al. 2006). Data acquisition was obtained using the selected ion monitoring mode. The identification of each chromatographic peak was achieved by comparing the retention times with co-injected standards and matching the characteristic ions of standards and samples (Table 2).

Results and discussion

The results are displayed in Figs. 1, 2, 4, 5, and 6. To better understand the data, it is important to stress that zone 1 is located around the city of Setúbal (Fig. 1), which is an area characterized by significant human and industrial impact (Ferreira et al. 2006; Vasconcelos et al. 2007). This is in accordance with findings, in both winter and summer, of natural (E1) and pharmaceutical estrogens

Table 2 Ions and fragment ratios used for identification of the nine proposed analytes by GC-MS

EDCs	Main source[a]	MW (molecular weight g/mol)	Retention times (min)	Ions selected in standard mixtures (*m*/*z*) (% relative abundance)
4-OP	Industrial and municipal effluents	206	12.0	278 (30), 179 (100)
4-NP	Industrial and municipal effluents	220	13.1	292 (34), 179 (100)
BPA	Industrial effluents	228	15.7	372 (6), 357 (100)
E1	Urban and municipal effluents	270	19.7	342 (100), 257 (45)
E2	Urban and municipal effluents	272	19.9	416 (100), 285 (75)
EE2	Municipal effluents	296	21.4	425 (100), 285 (40)
BIO-A	Agricultural runoff	284	22.4	428 (8), 413 (100)
DAID	Agricultural runoff	254	23.4	398 (100), 383 (85)
GEN	Agricultural runoff	270	23.7	471 (1), 399 (100)

[a]From Lintelmann et al. (2003) and Lagana et al. (2004)

(EE2) in this area (Figs. 1 and 5). However, comparison studies done in other Portuguese estuaries, such as the Mondego (Ribeiro et al. 2008b) in Sado, the levels of both E1 and EE2 were also under the quantification limits of the current HPLC-DAD method in spite of being in this environment (GC-MS data, Fig. 5). Since it is well established that these estrogens are able to produce disrupting effects in wildlife, even in very low nanograms per liter concentrations (Mills and Chichester 2005), it is possible that E1 and EE2, in conjunction, may additively contribute to reproductive disturbances, which include the induction of intersex in aquatic species of this estuary (Pessoa et al. 2001; Sousa et al. 2005; Moreira et al. 2006). Concerning E2, the lack of detection of this estrogen in Sado estuary is in accordance with our previous studies in Douro and Mondego Rivers (Ribeiro et al. 2008a, b). This occurrence agrees with the fact that humans excrete lower levels of E2 than E1 and that there is a rapid degradation of the former to E1 (Johnson et al. 2000).

Beyond estrogens, an interesting issue of this study was the measurement of phytoestrogens. This comes from the knowledge that zone 1 contains several paper and pulp mill industries, fertilizer industrial units, and food processing plants (tomato paste and olive oil production, among others; Ferreira et al. 2002), which may be important sources of these compounds (Liggins et al.

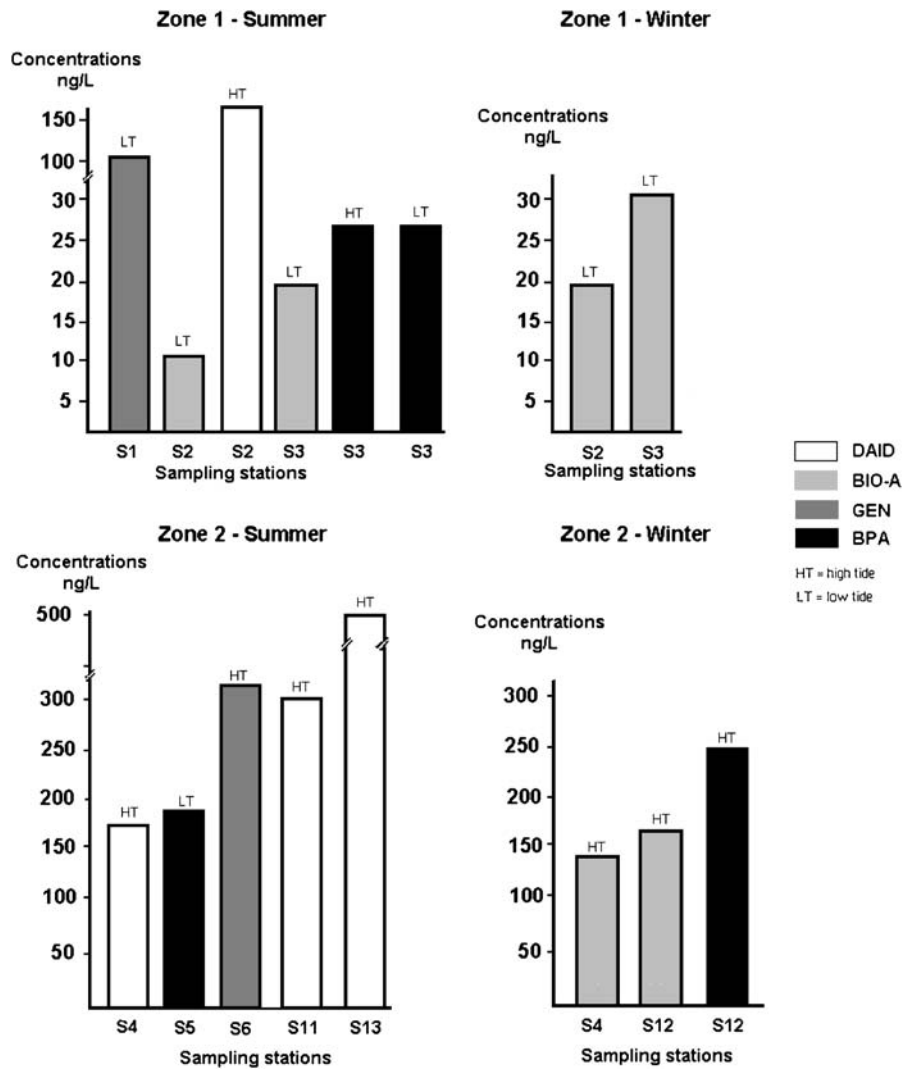


Fig. 4 Concentration of the EDCs measured in each of the sampling sites (S1 to S13) of the Sado River estuary at high and low tides

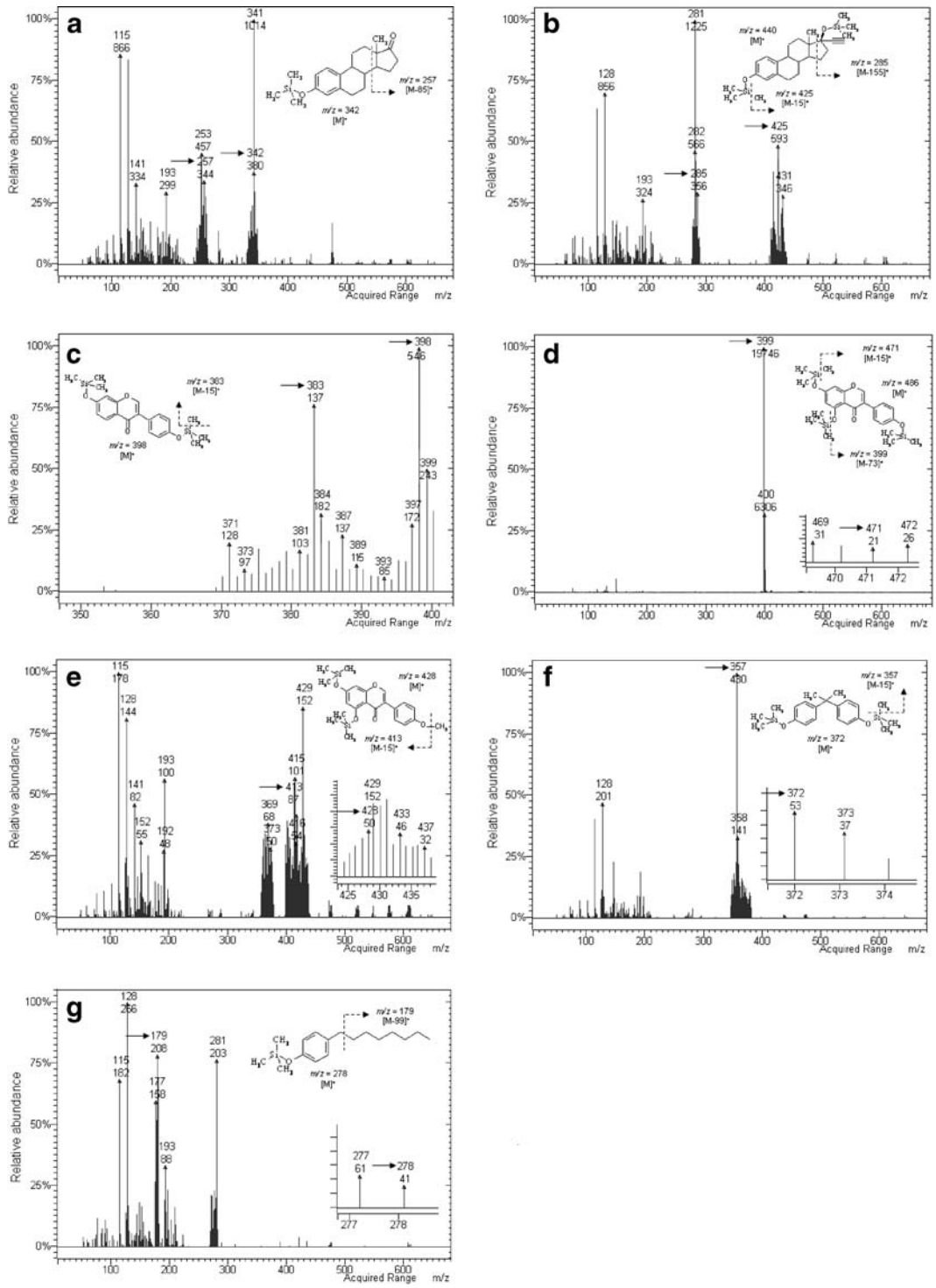


Fig. 5 MS spectra of all detected compounds in the Sado River estuary. The *arrows* point to the peaks (*m*/*z*) used for the identification of E1 (**a**), EE2 (**b**), DAID (**c**), GEN (**d**), BIO-A (**e**), BPA (**f**), and 4-OP (**g**)

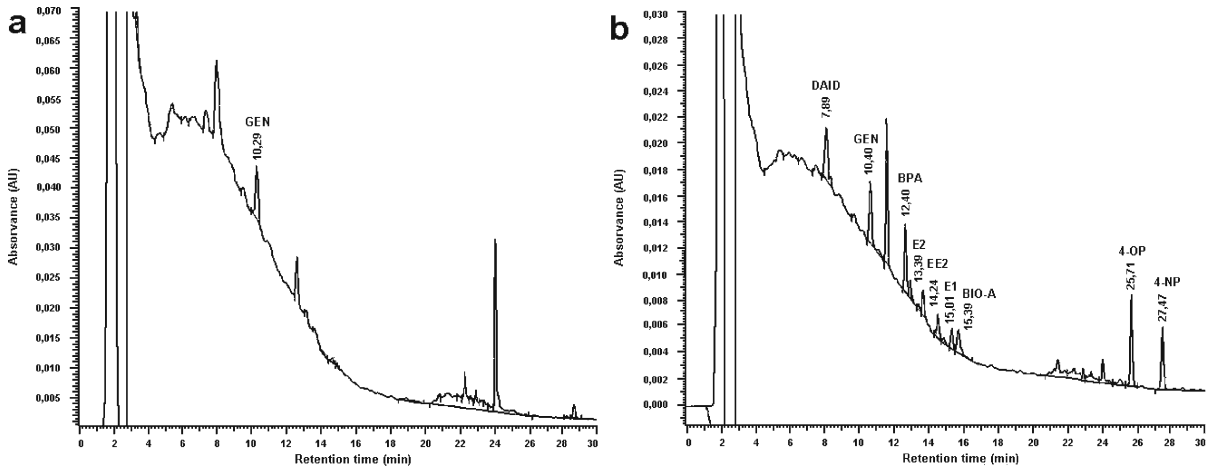


Fig. 6 Chromatograms of samples collected in summer (June 2006) at the S1 (**a**) and at the S7 (**b**) sampling points. The S7 chromatogram shows a sample spiked with standards containing 0.10 μg/L of DAID and GEN, 0.23 μg/L of BPA, 0.20 μg/L of E2 and E1, 0.25 μg/L of EE2, 0.03 μg/L of BIO-A, and 0.5 μg/L of 4-OP and 4-NP

2002). To date, these chemicals, which were never evaluated in this estuary and seldom in other estuaries, may induce endocrine disruption in wildlife (Kiparissis et al. 2003) when present in high dosages. In Sado, both DAID (up to 160 ng/L) and GEN (up to 100 ng/L) were measured in summer (Figs. 4 and 6), but in winter, both DAID and GEN were undetectable, and only BIO-A was quantified (up to 30 ng/L; Fig. 4). This occurrence seems to suggest a seasonal pattern of fluctuation among these phytoestrogens. However, further studies must be conducted to confirm this hypothesis.

Concerning the industrial pollutants, all assayed compounds were present, with the exception of 4-NP that was never found in zone 1 (Fig. 1). This may be justified by the partition coefficient of 4-NP, which associates this pollutant to organic matter in sediments (Ahel and Giger 1993). The concentrations found for BPA (up to 28 ng/L; Fig. 4) were also inferior than those expected for an area with such industrial impact, namely when comparing to other industrialized estuaries either in Portugal (10 μg/L; Ribeiro et al. 2008b) or others, such as Spain (2.9 μg/L; Cespedes et al. 2005) and Germany (50–272 ng/L; Bolz et al. 2001).

Therefore, we hypothesize that the high hydrodynamics of this area (Ferreira et al. 2002) causes an important dilution effect that may affect the concentrations of all studied EDCs. This theory is consistent with the observation that most of the quantified chemicals were measured in zone 1 in low tide when industrial pollution is visible. Later, in high tide, the strong washout of this zone promoted by the Atlantic Ocean seemed to produce a dilution of the majority of the assayed EDCs which, in spite of being present (Fig. 5), were under the quantification limits of the HPLC-DAD method. This dilution effect, more evident in winter when the atmospheric conditions are rougher than in summer, may explain the relatively low levels of EDCs in this area. This theory is in accordance with the vast diversity of fauna and flora found in this area, but do not discard the disrupting effect registered in several species (Pessoa et al. 2001; Sousa et al. 2005; Moreira et al. 2006) and the continuous decline of emblematic species, like the dolphin, *T. truncatus*, which can result from the synergy of all EDCs present in this zone together with other stressors.

Zone 2 (Fig. 1), included in the natural reserve of the Sado River estuary, is characterized by swamps, sandy areas, agricultural fields, and typical Mediterranean forests (Ferreira et al. 2006). In spite of this, all nine pollutants proposed for this study were also evaluated in this area due to its proximity to the industrial zone. However, neither estrogens, 4-OP, or 4-NP were found there, contrary to BPA that was measured in the two

sampling points located at the middle estuary (180 ng/L at S5; 248 ng/L at S12; Fig. 4). Since these levels were lower than those reported in other major Portuguese estuaries (Almeida et al. 2007, Ribeiro et al. 2008a, b) and because they are below the hazardous levels of this pollutant which regards to aquatic fauna (about 160 μg/L; Staples et al. 2002; Mills and Chichester 2005), it is suggested that industrial contamination by BPA is not critical for this region.

In zone 2, phytoestrogens were the main compounds measured in this area, supposedly due to the fact that this area is highly agricultural and highly forested (Fig. 1). Since some sampling was made among several sandy banks and shallow areas, the levels of the last compounds were apparently higher in this part of the estuary. Here, the physicochemical parameters such as temperature, pH, dissolved oxygen, and salinity show that the sampling areas with higher amounts of phytoestrogens are those where the time of water residence is longer (Fig. 2; Ferreira et al. 2006).

In summer, the main measured phytoestrogen was DAID, which was found at the Alcácer do Sal channel (500 ng/L at S13), at the Marateca channel (290 ng/L at S11), and at the middle estuary (170 ng/L at S4). In all other sampling stations, located near the Atlantic Ocean, DAID was detected by GC-MS (Figs. 1 and 5). GEN, which is an important component of cereals (such as rice; Liggins et al. 2002), was found near the rice fields located at the Comporta channel (320 ng/L at S6; Fig. 4). In winter, during the resting stage of all agricultural activities and indigenous flora, both DAID and GEN were under the limit of quantification of the current HPLC-DAD method, but continued to be present in undetectable amounts at all sampling stations (Fig. 5). In this occasion, the only phytoestrogen measured in this zone was BIO-A. Similar results were obtained in other major Portuguese estuaries (Ribeiro et al. 2008a, b), suggesting a seasonal fluctuation pattern for these compounds; however, these variations/patterns need further investigation. Thus, BIO-A was measured at the Alcácer do Sal channel (170 ng/L at S12) and at the middle of the estuary (130 ng/L at S4). All other sampling stations had levels below the detection limits of the HPLC-DAD method (10 ng/L; Table 1). Since the concentrations of all measured phytoestrogens were below the hazardous levels described in the *in vivo* experiments made by Kiparissis et al. (2003), which showed that only concentrations above 1,000 μg/L of GEN produced gonadal intersex in the Japanese medaka (*Oryzias latipes*), our current data suggest that all measured phytoestrogens may not exert biological prejudicial effects, at least for fish. This suggestion also agrees with the high biodiversity of fauna and flora found in the protected zone.

Conclusions

This study (1) showed a different pattern of pollution between the industrial and the natural reserve areas of the Sado River estuary, (2) demonstrated the presence of natural and pharmaceutical estrogens, phytoestrogens, and the industrial pollutants, BPA and 4-OP, in zone 1, (3) determined levels of DAID, GEN, and BIO-A in this estuary, allowing comparisons with other major estuaries, (4) suggested a possible seasonal variation for DAID and GEN, which were abundant in summer and, for BIO-A, apparently more abundant in winter, (5) revealed that individually, none of the measured EDCs was found in known hazardous levels, but together, they can make a relevant toxicological load, leading us to conclude that each compound is individually unlikely to exert deleterious effects in local living organisms but that their additive and/or synergistic "total estrogenic load" cannot be ignored (and should be monitored) as to investigating endocrine disruption, and finally (6) suggested that the hydrodynamic activity of this estuary is possibly involved in the dilution process of all assayed pollutants.

Acknowledgements This study was financially supported by the Fundação para a Ciência e Tecnologia (PhD Grant SFRH/BD/18231/2004/SLU) and by the Cooperativa de Ensino Superior, Politécnico e Universitário, CRL (CESPU)/ISCS-N (Research Projects 1F/13/2005/CESPU and 2F/02/2006/CESPU).

References

Ahel, M., & Giger, W. (1993). Partitioning of alkylphenols and alkylphenol polyethoxylates between water and organic-solvents. *Chemosphere, 26*, 1471–1478. doi:10.1016/0045-6535(93)90214-P.

Almeida, C., Serôdio, P., Florêncio, M. H., & Nogueira, J. M. F. (2007). New strategies to screen for endocrine-disrupting chemicals in the Portuguese marine environment utilizing large volume injection-capillary gas chromatography-mass spectrometry combined with retention time locking libraries (LVI-GC-MS-RTL). *Analytical and Bioanalytical Chemistry, 387*, 2569–2583. doi:10.1007/s00216-006-1101-2.

Ballesteros, O., Zafra, A., Navalon, A., & Vilchez, J. L. (2006). Sensitive gas chromatographic-mass spectrometric method for the determination of phthalate esters, alkylphenols, bisphenol A and their chlorinated derivatives in wastewater samples. *Journal of Chromatography. A, 1121*, 154–162. doi:10.1016/j.chroma.2006.04.014.

Bolz, U., Hagenmaier, H., & Korner, W. (2001). Phenolic xenoestrogens in surface water, sediments, and sewage sludge from Baden-Wurttemberg, south-west Germany. *Environmental Pollution, 115*, 291–301. doi:10.1016/S0269-7491(01)00100-2.

Caeiro, S., Costa, M. H., Ramos, T. B., Fernandes, F., Silveira, N., Coimbra, A., et al. (2005). Assessing heavy metal contamination in Sado Estuary sediment: An index analysis approach. *Ecological Indicators, 5*, 151–169. doi:10.1016/j.ecolind.2005.02.001.

Catarino, J., Peneda, M. C., & Santana, F. (1987). *Estudo do impacto da indústria no estuário do Rio Sado. Estimativas da poluição afluente*. Instituto Nacional de Engenharia e Tecnologia Industrial, Lisbon, Portugal: DEII, INETI.

Cespedes, R., Lacorte, S., Raldua, D., Ginebreda, A., Barceló, D., & Pina, B. (2005). Distribution of endocrine disruptors in the Llobregat River basin (Catalonia, NE Spain). *Chemosphere, 61*, 1710–1719. doi:10.1016/j.chemosphere.2005.03.082.

Ferreira, J. G., Nobre, A. M., Sirnas, T. C., Silva, M. C., Newton, A., Bricker, S. B., et al. (2006). A methodology for defining homogeneous water bodies in estuaries—application to the transitional systems of the EU Water Framework Directive. *Estuarine, Coastal and Shelf Science, 66*, 468–482. doi:10.1016/j.ecss.2005.09.016.

Ferreira, J. G., Simas, T., Schifferegger, K., & Lencart-Silva, J. (2002). *Identification of sensitive areas and vulnerable zones in four Portuguese Estuaries.* INAG–Instituto da Água, IMAR–Institute of Marine Research.

Ferreira, A. M., & Vale, C. (2001). Seasonal and inter-annual variations of PCB and DDT contents in the oyster *Crassostrea angulata* from the Sado Estuary (Portugal). *Ciencias Marinas, 27*, 255–268.

International Conference on Harmonisation ICH (1996). *Validation of analytical procedures: Methodology, Q. 2B (CPMP/ICH/281/96)*. London: The European Agency for the Evaluation of Medicinal Products.

Jobling, S., Beresford, N., Nolan, M., Rodgers-Gray, T., Brighty, G. C., Sumpter, J. P., et al. (2002). Altered sexual maturation and gamete production in wild roach (*Rutilus rutilus*) living in rivers that receive treated sewage effluents. *Biology of Reproduction, 66*, 272–281. doi:10.1095/biolreprod66.2.272.

Johnson, A. C., Belfroid, A., & Di Corcia, A. (2000). Estimating steroid oestrogen inputs into the activated sludge treatment works and observation on their removal from the effluent. *The Science of the Total Environment, 256*, 163–173. doi:10.1016/S0048-9697(00)00481-2.

Kiparissis, Y., Balch, G. C., Metcalfe, T. L., & Metcalfe, C. D. (2003). Effects of the isoflavones genistein and equol on the gonadal development of Japanese medaka (*Oryzias latipes*). *Environmental Health Perspectives, 111*, 1158–1163.

Lagana, A., Bacaloni, A., De Leva, I., Faberi, A., Fago, G., & Marino, A. (2004). Analytical methodologies for determining the occurrence of endocrine disrupting chemicals in sewage treatment plants and natural waters. *Analytica Chimica Acta, 501*, 79–88. doi:10.1016/j.aca.2003.09.020.

Lee, S. H., Jung, B. H., Kim, S. Y., & Chung, B. C. (2004). Determination of phytoestrogens in traditional medicinal herbs using gas chromatography–mass spectrometry. *The Journal of Nutritional Biochemistry, 15*, 452–460. doi:10.1016/j.jnutbio.2004.01.007.

Liggins, J., Mulligan, A., Runswick, S., & Bingham, S. A. (2002). Daidzein and genistein content of cereals. *European Journal of Clinical Nutrition, 56*, 961–966. doi:10.1038/sj.ejcn.1601419.

Lintelmann, J., Katayama, A., Kurihara, N., Shore, L., & Wenzel, A. (2003). Endocrine disruptors in the environment—(IUPAC Technical Report). *Pure and Applied Chemistry, 75*, 631–681. doi:10.1351/pac200375050631.

Mills, L. J., & Chichester, C. (2005). Review of evidence: Are endocrine-disrupting chemicals in the aquatic environment impacting fish populations? *The Science of the Total Environment, 343*, 1–34. doi:10.1016/j.scitotenv.2004.12.070.

Moreira, S. M., Lima, I., Ribeiro, R., & Guilhermino, L. (2006). Effects of estuarine sediment contamination on feeding and on key physiological functions of the polychaete *Hediste diversicolor*: Laboratory and in situ assays. *Aquatic Toxicology (Amsterdam, Netherlands), 78*, 186–201. doi:10.1016/j.aquatox.2006.03.001.

Pessoa, M. F., Fernando, A., & Oliveira, J. S. (2001). Use of imposex (pseudohermaphroditism) as indicator of the occurrence of organotin compounds in Portuguese coastal waters—Sado and Mira estuaries. *Environmental Toxicology, 16*, 234–241. doi:10.1002/tox.1029.

Quevauviller, P., Lavigne, R., Pinel, R., & Astruc, M. (1989). Organotins in sediments and mussels from the Sado Estuarine system (Portugal). *Environmental Pollution, 57*, 149–166. doi:10.1016/0269-7491(89)90007-9.

Ribeiro, C., Tiritan, M. E., Rocha, E., & Rocha, M. J. (2007). Development and validation of a HPLC-DAD method for determination of several endocrine disrupting compounds in estuarine water. *Journal of Liquid Chromatography & Related Technologies, 30*, 2729–2746. doi:10.1080/10826070701560652.

Ribeiro, C., Pardal, M. A., Martinho, F., Margalho, R., Tiritan, M. E., Rocha, E., et al. (2008a). Distribution

of endocrine disruptors in the Mondego River estuary, Portugal. *Environmental Monitoring and Assessment*, (in press). doi:10.1007/s10661-10008-10192-y.

Ribeiro, C., Tiritan, M. E., Rocha, E., & Rocha, M. J. (2008b). Seasonal and spatial distribution of several endocrine disrupting compounds in the Douro River estuary, Portugal. *Archives of Environmental Contamination and Toxicology*, (in press). doi:10.1007/s00244-008-9158-x.

Shareef, A., Angove, M. J., & Wells, J. D. (2006). Optimization of silylation using *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide, *N*,*O*-bis-(trimethylsilyl)-trifluoroacetamide and 4N4-(*tert*-butyldimethylsilyl)-*N*-methyltrifluoroacetamide for the determination of the estrogens estrone and 17[alpha]-ethinylestradiol by gas chromatography–mass spectrometry. *Journal of Chromatography. A, 1108*, 121–128. doi:10.1016/j.chroma.2005.12.098.

Silva, E., Batista, S., Viana, P., Antunes, P., Serôdio, L., Cardoso, A. T., et al. (2006). Pesticides and nitrates in groundwater from oriziculture areas of the 'Baixo Sado' region (Portugal). *International Journal of Environmental Analytical Chemistry, 86*, 955–972. doi:10.1080/03067310600833336.

Silveira, M. (2007). Identification of endocrine disrupters in superficial waters of Douro River estuary. Dissertation, University of Porto, Portugal.

Sousa, A., Mendo, S., & Barroso, C. (2005). Imposex and organotin contamination in *Nassarius reticulatus* (L.) along the Portuguese coast. *Applied Organometallic Chemistry, 19*, 315–323. doi:10.1002/aoc.856.

Staples, C. A., Woodburn, K., Caspers, N., Hall, A. T., & Klecka, G. M. (2002). A weight of evidence approach to the aquatic hazard assessment of bisphenol A. *Human and Ecological Risk Assessment, 8*, 1083–1105. doi:10.1080/1080-700291905837.

Vasconcelos, R. P., Reis-Santos, P., Fonseca, V., Maia, A., Ruano, M., Franca, S., et al. (2007). Assessing anthropogenic pressures on estuarine fish nurseries along the Portuguese coast: A multi-metric index and conceptual approach. *The Science of the Total Environment, 374*, 199–215. doi:10.1016/j.scitotenv.2006.12.048.

Vethaak, A. D., Lahr, J., Schrap, S. M., Belfroid, A. C., Rijs, G. B. J., Gerritsen, A., et al. (2005). An integrated assessment of estrogenic contamination and biological effects in the aquatic environment of The Netherlands. *Chemosphere, 59*, 511–524. doi:10.1016/j.chemosphere.2004.12.053.