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Annual Evaluation of 17 Oestrogenic Endocrine Disruptors and Hazard Indexes in the Douro River Estuary—The Atlantic Discharge of the Highest-Flow River of Southwestern Europe

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Abstract: The concentrations of seventeen endocrine disruptor compounds (EDCs) that included oestrogens, phytoestrogens, sitosterol, and banned industrial pollutants were investigated at ten sites of the Douro River estuary. Surface waters were collected during 2019. After evaluating the physicochemical data (ammonia, nitrates, nitrites and phosphates), the waters were filtrated and submitted to solid-phase extraction (SPE) to extract and pre-concentrate (4000-fold) the EDCs. The extracts were derivatized with BSTFA + 1% TMS and analysed by gas chromatography-mass spectrometry (GC-MS). All EDCs showed a high detection rate (97%, on average), exhibiting ubiquity in this estuary. The finding of biologically relevant amounts of oestrogens (up to 8.5 ng/L for oestradiol, E2), phytoestrogens (up to 827 ng/L for biochanin A, BIO-A) and industrial pollutants (up to 2.7 µg/L for nonylphenol di-ethoxylated, NP2EO) strongly support ongoing risks of endocrine disruption for the local aquatic wildlife. Globally, there was an E2-equivalents (E2-EQs) concentration of 25 ng/L and a hazard index (HI) of 26, which further indicates considerable potential for adverse effects on local biota. Moreover, the physicochemical data suggest direct sewage discharges. Beyond possible toxicological effects on fauna, the detected contaminants may pose risks to humans via direct contact (bathing at local fluvial beaches) or by ingestion (local fish).

Keywords: alkylphenols; EDCs; oestradiol-equivalents; hazard quotients; oestrogens; phytoestrogens; surface waters

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1. Introduction

Potable water has been a critical issue in the European Union (EU) due to its scarcity and quality assurance, improper usage, and the threat of climate change (www.unwater.org, accessed on 14 June 2022). Since most rivers in the EU flow amongst different countries, legislation has tried to standardise their quality by creating the Water Frame Directive (WFD) [1]. Despite being updated over the years, implementing the WFD has been challenging, as not all the EU member states comply well with reaching a good quality surface water status, which includes ecological and chemical issues [2].

The Douro River is one example where the application of the WFD has been complex due to its international nature. This river runs for 897 km (557 miles) from north-central Spain to Oporto (Portugal), flowing out into the Atlantic Ocean. Although the river is a significant source of potable water (www.portoprotocol.com/case-studies/livingplantit/, accessed on 14 June 2022), during its course, it passes through agricultural and industrial areas. Its last 22 km is surrounded by the urban and densely populated (over 1,700,000 permanent inhabitants) agglomerates of the so-called "Great Oporto". Therefore, this area is highly

prone to be impacted by chemicals released either upstream or inside the estuary. Previous studies demonstrated toxicologically relevant (and often high) amounts of endocrine disruptor compounds (EDCs), such as pharmaceutical and industrial oestrogens, and pesticides in this area [3,4]. The origin of these compounds was attributed to discharges from wastewater treatment plants (WWTPs), untreated sewages, agricultural run-offs, and even summer tourism [3,5]. Moreover, the jetties built-in 2008 at the mouth of the Douro River, planned to stabilize the banks of the estuary and improve the navigability for some boats, decreased the contact between the estuarine and the ocean waters, increasing the residence time, as well as the concentrations of pollutants in the estuary [3,5].

The adequate application of the WFD to transitional waters, such as the Douro River estuary, implicates chemical monitoring for specific synthetic pollutants, including, but not exclusively, the WFD priority list substances [6]. Toxicants that have been grabbing the attention of researchers, governing bodies and even the public, in general, are the EDCs, and particularly those that are xenoestrogens. These include natural and manufactured compounds. Examples of such EDCs are oestrone (E1), 17-beta-estradiol (E2) and 17-alpha-ethinylestradiol (EE2), phytoestrogens, and the industrial pollutants bisphenol A (BPA), alkylphenol polyethoxylates (APEOs), octylphenols (OPs) and nonylphenols (NPs) [3]. Due to their potential toxicity and persistence, some of those chemicals are "priority substances in the field of water policy" [6]. The natural E2 and synthetic EE2 hormones entered the first watch list in 2013 (European Parliament and Council) [7] due to their ability to induce an endocrine disruption in fish [8].

To estimate the levels of the above-referred EDCs, and sitosterol (SITO) in surface waters, several analytical methods can be considered, such as those validated by Rocha et al. [3]. The latter implies extracting the EDCs from the estuarine matrix by solid-phase extraction (SPE), followed by their quantification by gas chromatography coupled to mass spectroscopy (GC–MS). Other parameters, such as physico-chemical ones, have been measured in parallel as broad quality elements for transitional waters [3,6].

Considering the goals of the WFD [7], this study intends to update the water quality status of the Douro River estuary concerning the levels and hazards of a range of diverse EDCs, for which there has been advances in the technologies aimed for their removal from wastewaters [9,10]. The results could justify, or not, further monitoring efforts, namely targeting biological quality elements. The specific objective of this study was to investigate the concentrations of 17 EDCs for one year at ten sampling sites distributed between the two estuary margins. Selected statistical and mathematical tools were used to interpret the data and study the eventual non-compliance with the WFD directive [7]. The new data gives novel scientific references, against which the success of the execution of interventions recommended to eliminate EDCs in the water [9,10] can be measured, not only in the Douro but also in other streams across the world with similar anthropogenic pressures.

2. Materials and Methods

2.1. Chemicals, Materials and Standards

All chemicals were analytical grade. Hexane and anhydrous pyridine, N,O-bis(trimethylsilyl) trifluoroacetamide (BSTFA) added with 1% (*w*/*v*) trimethylchlorosilane (TMCS) were supplied by Sigma-Aldrich (Steinheim, Germany). Dichloromethane and methanol were from Romil Ltd. (Cambridge, UK). A Milli-Q water system provided ultrapure water (conductivity = 0.054 µS/cm, at 25 °C). The 200 mg Oasis HLB (Hydrophilic-Lipophilic Balance) SPE cartridges were supplied by Waters Corporation (Milford, MA, USA). The 1 g silica cartridges were acquired from Teknokroma (Barcelona, Spain).

Oestrogens (E1, E2, EE2), phytoestrogens (BIO-A, DAID, FORM, GEN), SITO, BPA, OPs (4-n-octylphenol (4-n-OP) and 4-t-octylphenol (4-t-OP)), octylphenol ethoxylates (OPEOs) (Igepal CA-210 (octylphenol monoethoxylate (OP1EO) and 4-octylphenol diethoxylate (OP2EO)), nonylphenol ethoxylates (NPEOs) (Igepal CO-210 (4-nonylphenol monoethoxylate (NP1EO), 4-nonylphenol diethoxylate (NP2EO)) and internal standards (IS), i.e., 17β-estradiol-d2 (E2-d2) and bisphenol A-d16 (BPA-d16) were supplied by Sigma-

Aldrich (Steinheim, Germany). 4-Nonylphenol (4-n-NP) and nonylphenol isomers (NP) were provided by Riedel-de-Haën (Seelze-Hannover, Germany).

Stock solutions (100 mg/L in methanol of all EDCs) were stored in the dark at −20 °C, without decay for less than one year. Calibration curves used six concentrations, ranging from 10 to 300 ng/L, and 50 ng/L of E2-d2 and BPA-d16 (IS, deuterated surrogates) [3].

2.2. Sample Collection and Physico-Chemical Parameters

Surface water was collected (at 1 m depth) at low tide from ten areas of the Douro River estuary (Figure 1). Sampling occurred in February (winter), May (spring), July (summer) and November (autumn) 2019. Each sample (2 L) was placed into a 2.5 L amber glass bottle. This was previously rinsed in the laboratory with ultrapure water and later, on-site, with the water sampled on the place. The sampling spots S1 to S5 and S6 to S7 are located, respectively, at the north and south margins of the Douro River estuary.

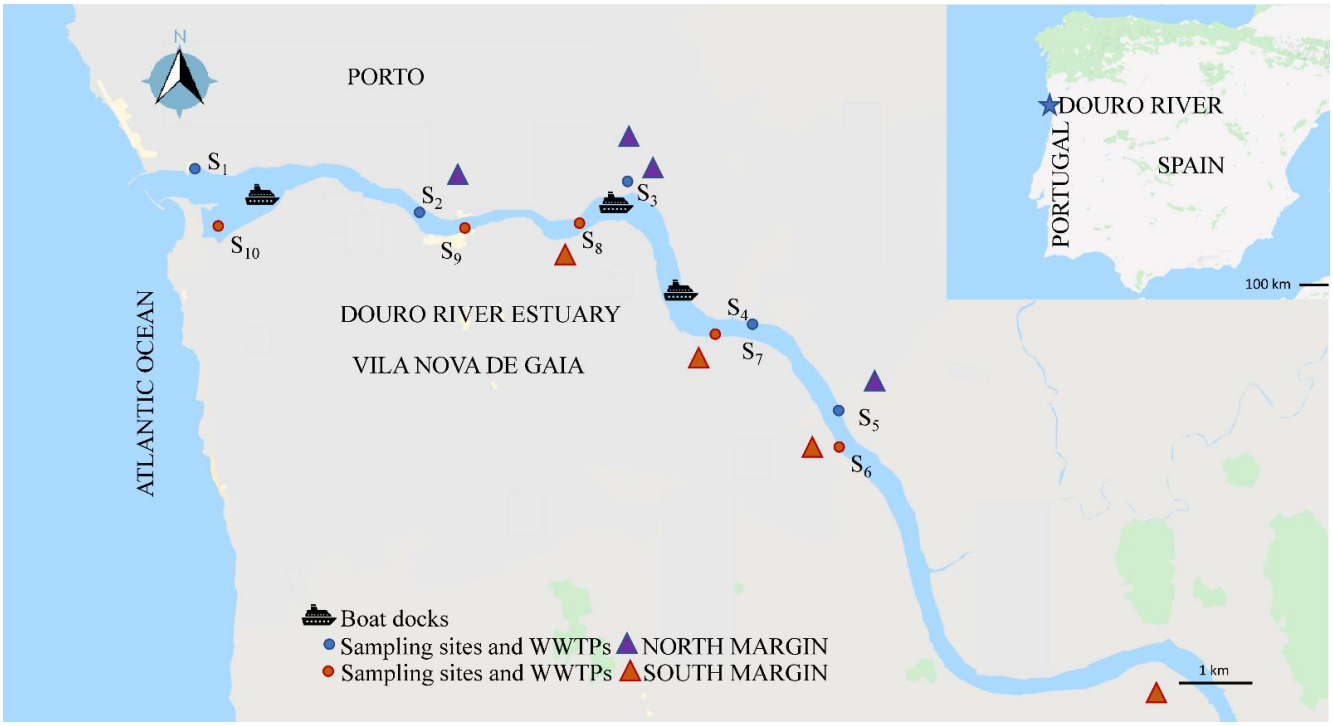

Figure 1. Location of the sampling sites within the Douro River estuary (S1 to S10), Portugal. For easier visualization, sampling areas at the north margin are marked in blue, and those at the south are in red. Anthropogenic sources that may contribute to higher amounts of EDCs, such as the WWTPs effluents and boat docks, are referred in this figure (adapted from KML Map).

2.2.1. Sample Collection at the North Margin of the Estuary (n = 5)

S1 (41°08′50.5″ N, 8°40′24.1″ W) is located at the mouth of the river. S2 (41°08′52.0″ N, 8°39′12.7″ W) is placed at the Bird Observatory close to the Sobreiras WWTP, which treats urban effluents of ca. 200,000 inhabitants (www.aguasdoporto.pt, accessed on 14 June 2022). S3 (41°08′24.5″ N, 8°36′58.8″ W) is equidistant from the above-referred WWTP and the Freixo WWTP. S4 (41°08′37.6″ N, 8°34′47.0″ W) is located near the entrance of the last WWTP, which was designed to deal with the swages of ca. 170,000 inhabitants (www.aguasdoporto.pt/etar/etar-freixo, accessed on 14 June 2022). S5 (41°08′13.6″ N, 8°34′18.7″ W) is at the inner part of the estuary, approximately 15 km from the Atlantic Ocean.

2.2.2. Sample Collection at the South Margin of the Estuary (n = 5)

S6 (41°07′18.8″ N, 8°33′27.2″ W) is located at the fluvial beach of (Areínho de Avintes). S7 (41°08′23.4″ N, 8°34′56.5″ W) is also at fluvial, which potentially receives the influence of the Febros WWTP (www.simdouro.pt, accessed on 14 June 2022), which treats the urban

discharges of ca. 80,000 inhabitants. S8 (41°08′23.4″ N, 8°34′56.5″ W) is placed south of other WWTP effluent, which treats the swages of ca. 31,000 inhabitants (www.simdouro.pt, accessed on 14 June 2022). S9 (41°08′20.3″ N, 8°36′36.9″ W) is a tourist area with many small boats and several docks. Finally, S10 (41°08′12.0″ N, 8°39′39.8″ W) is located close to a Natural Reserve with restricted access.

2.2.3. Physico-Chemical Parameters

After sampling, temperature (°C) and salinity were measured in situ. The waters were transported to the laboratory at 4 °C, and the levels of nitrites (NO_2^-), nitrates (NO_3^-), ammonium (NH_4^+), and phosphates (PO_4^{2-}) were measured by photometry (pHotoFlex STD Colorimeter, WTW, Troistedt, Germany). For this, certificated evaluation kits were used, and all samples and blanks were analysed in triplicate to ensure analytical accuracy. The calculation of toxic (unionized) ammonia (NH_3), dependent on temperature (°C) and pH, used the NH_4^+ data and the conversion model described by Emmerson et al. [11].

2.3. Sample Preparation

For quantifying the EDCs, water samples were filtrated through a 0.45 µm glass fibre filter (Millipore, Ireland) to eliminate particulate matter and other suspended solids. The filtrates were acidified with H_2SO_4 to pH 2 and subjected to SPE within a period of 24 h. During this phase, all samples were maintained at ±4 °C in the dark until extraction.

The SPE used OASIS HLB cartridges adapted to an off-line SPE vacuum extraction device (Waters, Milford, MA, USA) following the protocol of Rocha et al. [3]. The first step involved conditioning the cartridges with 10 mL of CH_2Cl_2:CH_3OH (50:50, *v*/*v*), followed by 6 mL of CH_3OH and 13 mL of ultrapure water, at a flow rate of 1 mL/min. The second step consisted in loading the SPE cartridges with water samples (1 L) added with 50 ng/L of E2-d_2 and BPA-d_{16} at a constant flow rate of 5 mL/min. The third step required a washing process with 13 mL of ultrapure water and 1 mL of CH_3OH and the drying of the cartridges under vacuum for 30 min. Then, the EDCs were eluted with 10 mL of CH_2Cl_2:CH_3OH (50:50, *v*/*v*). Due to their dark and sticky appearance, the extracts were cleaned using silica cartridges (1 g) [3]. The cleaned extracts were evaporated to dryness in a heating block (40 °C), under a gentle N_2 stream, for ≈5 min, and reconstituted with 250 µL of anhydrous methanol, leading to a concentration factor of 4000-fold.

2.4. Quantification by GC-MS

Due to the low volatility of the targeted EDCs, their derivatization was essential [3]. In this step, 50 µL of the referred reconstituted extracts were evaporated to dryness under a gentle N2 stream, added 50 µL of pyridine + 50 µL of BSTFA (1% TMCS) and heated (30 min at 70 °C). The TMS derivatives were further evaporated and reconstituted with 100 µL of hexane before GC-MS analysis (Table S1 in supplement) [3].

The gas chromatograph (Trace GC Ultra, Thermo Finnigan Electron Corporation, Waltham, MA, USA) was coupled with an ion trap mass spectrometer (Thermo Scientific ITQ™ 1100 GC-MSn), 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. The oven temperatures went up from 100 °C (initial equilibrium time 1 min) to 200 °C at 10 °C/min, from 200 °C to 260 °C at 6 °C/min, from 260 °C to 290 °C at 1 °C/min and finally 290 °C for 5 min [3]. For quantitative analysis, the mass spectrum (MS) was achieved by electron impact ionization and operated in the selected ion monitoring system (SIM). The liner temperatures ranged from 35 °C to 250 °C via a ramp of 10 °C/s [3]. The MS transfer line and the ion source were set at 280 °C. Samples were injected (3 µL) in the splitless mode using an 80 mm injection needle. As the selected EDCs were measured in ng/L, method blanks ensured that contamination by laboratory material never occurred [3]. Beyond this, random replicates of water samples were spiked with a mix containing all assayed EDCs (standards) at an intermediate concentration (150 ng/L) to guarantee continuous data quality. All glassware

was washed by a washing machine, which works accordingly to EPA Method 200.7, 524.2, 525.1 and 8720, which test for common post-wash residues.

2.5. Calculation of Oestrogenic Equivalents and Hazard Quotients

The oestrogenic equivalence of each EDC was calculated considering the potency of each compound in relation to that of E2 (E2-EQ) (1). Here, F is the E2 equivalent factor obtained from in vitro assays [12,13].

$$\text{E2} - \text{EQ} = \text{Environmental concentration of a specific EDC} \times \text{F} \quad (1)$$

The potential ecotoxicological risk for fish when exposed to EDCs was calculated based on the hazard quotient (HQ) (2). Accordingly to Wentsel et al. [14], it was considered the following scale: HQ < 1.0, no significant risk; 1.0 ≤ HQ < 10, minor potential for adverse effects; 10 ≤ HQ < 100, considerable potential for adverse effects; HQ ≥ 100, potential adverse effects should be expected. The sum of several HQs is called hazard index (HI).

$$\text{HQ} = \text{MEC} \div \text{PNEC} \quad (2)$$

Here, MEC refers to the measured environmental concentration and PNEC to the predicted no-effect concentration of each analysed EDC. According to previous studies, which used the sensitivity distribution (SSD) model, for aquatic organisms the PNECs were 1 ng/L and 2 ng/L for EE2 and E2, respectively [15]. Furthermore, considering relative differences between in vivo VTG induction, the last authors derived a PNEC of 6 ng/L for E1 [13]. The PNEC for BPA was 1.5 µg/L [16]. For 4-t-OP the PNEC was 1.0 µg/L [17], whereas for 4-n-OP and OPEOs was 1.22 µg/L [18]. Finally, the PNECs for both NPs and NPEOs was 0.8 µg/L [19].

As there is no known value for phytoestrogens' PNEC, this study estimated their HQs using the E2-EQs found for each compound.

2.6. Data Presentation and Statistical Analyses

Descriptive and inferential statistics were performed by PAST 4.02 [20] and GraphPad Prism software's (6.01, GraphPad Software, Inc., San Diego, CA, USA). In Tables, the data are shown as means followed by the standard deviations (SD). In the Figures, the graphs display boxplots (with median, minimum, maximum, and 1st and 3rd quartiles). When the concentrations of the EDCs were below the limits of detection (LODs) of the GC–MS method, they were treated as proposed by EPA [21], i.e., data = LOD ÷ ($\sqrt{2}$). Comparisons between independent sites and groups of compounds were investigated through unidirectional analysis of variance (ANOVA). Tukey's test evaluated post hoc comparisons. The Shapiro–Wilk W and the Levine tests checked the ANOVA assumptions of normality and homogeneity of variances. Whenever the parametric assumptions and subsequent data transformation failed, the non-parametric Kruskal–Wallis test was used, followed by Dunn's post hoc test, or the inferential statistics, the significance level (α) was set at 0.05. Principal component analysis (PCA) was made to visualize the similarity of EDCs sources amongst sampling sites and sampling occasions. From the correlation matrix, the principal components (PCs) were extracted considering both the Kaiser (i.e., eigenvalue > 1) and the Scree Plot criteria [22].

3. Results

Data referring to the seasonal mean amounts of the 17 EDCs are in Table 1.

Figure 2 displays boxplots concerning the annual concentrations of (a) oestrogens, (b) phytoestrogens and SITO, (c) OPs, OPEOs and BPA, (d) NPs and NPEOs in Douro estuary.

Table 1. Limits of detection (LOD), detection rate (DR), and levels of the seventeen EDCs analysed in surface waters of the Douro River estuary, presented as mean ± standard deviation.

EDCs	LOD (ng/L)	DR (%)	Winter (ng/L)	Spring (ng/L)	Summer (ng/L)	Autumn (ng/L)	Annual Average (ng/L)
Oestrogens							
E_1	0.9	100	3.0 ± 1.1	3.1 ± 1.9	3.6 ± 2.1	3.7 ± 1.7	3.4 ± 1.7
E_2	0.9	100	6.1 ± 5.6	7 ± 5.2	11.5 ± 7	9.3 ± 5.7	8.5 ± 6.1
EE_2	1.3	95	2.9 ± 1.4	2.2 ± 0.8	4 ± 3	3.8 ± 2.2	3.2 ± 2.1
Phytoestrogens and SITO							
BIO-A	1.3	100	881 ± 951	711 ± 517.4	945.9 ± 776.7	769.9 ± 558	827.1 ± 657.8
DAID	1.4	100	3.9 ± 2.8	4.5 ± 3.3	2.6 ± 1.8	3.2 ± 3.1	3.6 ± 2.8
FORM	2.6	100	12.2 ± 5.1	11.4 ± 3.0	13.5 ± 7.3	12.9 ± 8.8	12.5 ± 6.2
GEN	1.1	100	9.1 ± 5.5	8.3 ± 6.7	4.4 ± 2.7	9.3 ± 12.6	7.8 ± 7.8
SITO	2.0	100	118 ± 69.7	152.8 ± 78.4	104.6 ± 94.7	155.8 ± 180.4	132.7 ± 112.4
Industrial Pollutants							
BPA	0.7	100	53.9 ± 44	55.1 ± 41.2	45.5 ± 35	38.5 ± 28.3	48.3 ± 36.8
4-n-OP	3.5	100	1.8 ± 1.2	1.6 ± 0.7	2.3 ± 1.7	2.1 ± 2.1	2.0 ± 1.5
4-t-OP	1.5	55	19.7 ± 27.6	12.7 ± 6.9	15.8 ± 19.8	18.2 ± 29	16.6 ± 21.9
OP_1EO	5.3	100	95.7 ± 55.5	86 ± 34	135.1 ± 121	70.2 ± 44.8	96.8 ± 73.5
OP_2EO	0.9	100	965 ± 816.2	1093 ± 1070.1	1104 ± 494.8	770.2 ± 420.5	983.1 ± 730.7
4-NP	5.5	100	802 ± 594	825.5 ± 721	829.7 ± 544.1	575.3 ± 345.7	758.1 ± 555.7
4-n-NP	0.6	100	25.5 ± 11.2	20.7 ± 8.7	30.3 ± 18.9	19.1 ± 12.7	23.9 ± 13.6
NP_1EO	1.8	100	538 ± 704.4	449.4 ± 598.3	551.2 ± 612.1	391.6 ± 319.5	482.6 ± 558.2
NP_2EO	2.1	100	2501 ± 1878	1963 ± 1757.4	4220 ± 4344.1	2067 ± 1728.2	2687.7 ± 2723.0

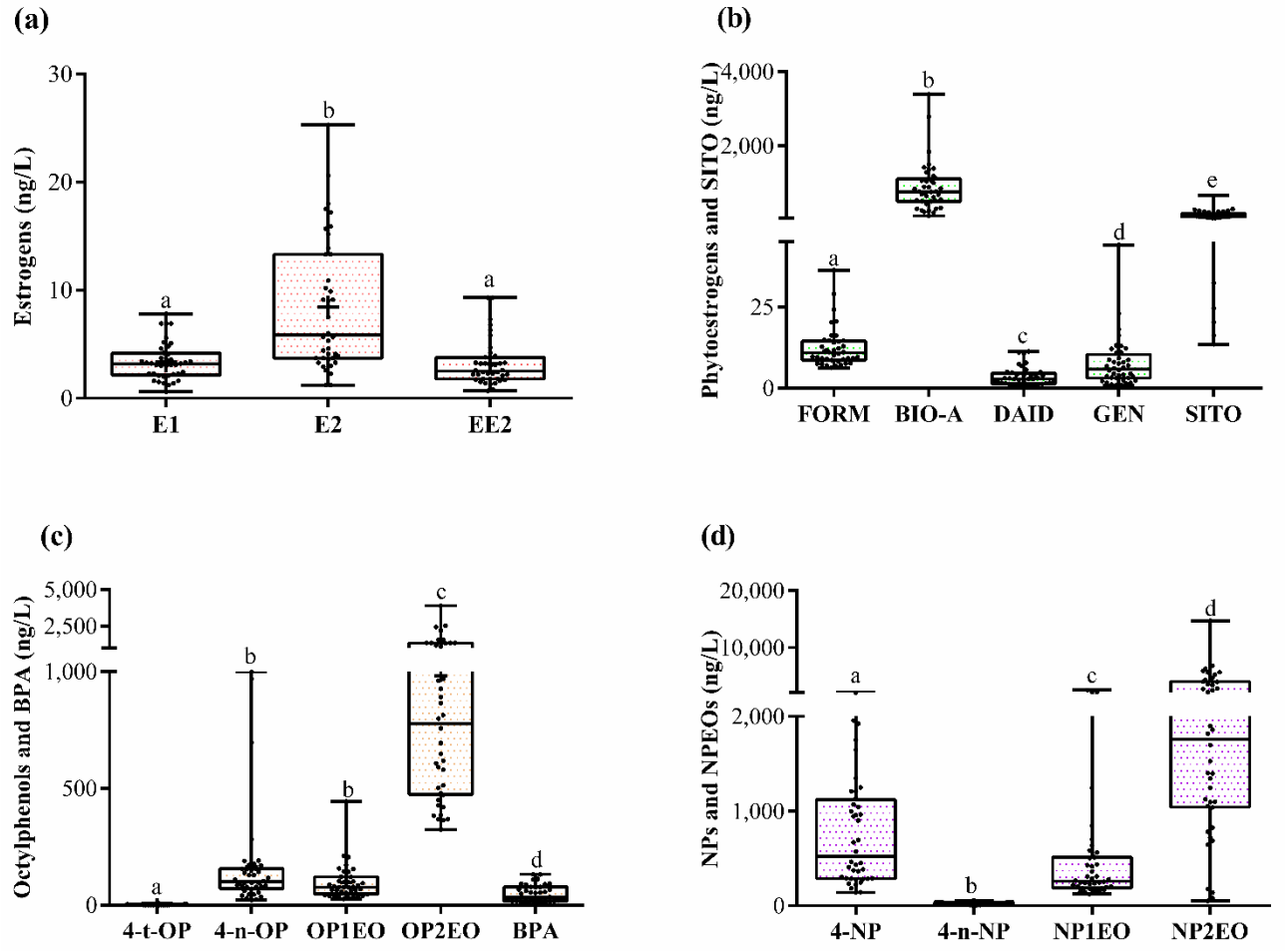

Figure 2. Global levels (ng/L) of: (**a**) oestrogens; (**b**) phytoestrogens and SITO; (**c**) OPs, OPEOs and BPA; (**d**) NPs and NPEOs. Data are expressed in boxplots with the minimum, median, maximum, average (+), and interquartile ranges Q1–Q3. Dots represent average individual values measured in ten areas (n = 40). Different low case letters indicate significant differences amongst sampling sites ($p < 0.05$).

Figure 3 shows boxplots concerning the annual distribution patterns of (a) ∑oestrogens, (b) ∑phytoestrogens, SITO, (c) BPA, (d) $\sum_{OPs,OPEOs}$ and (e) $\sum_{NPs,NPEOs}$, by sampling site.

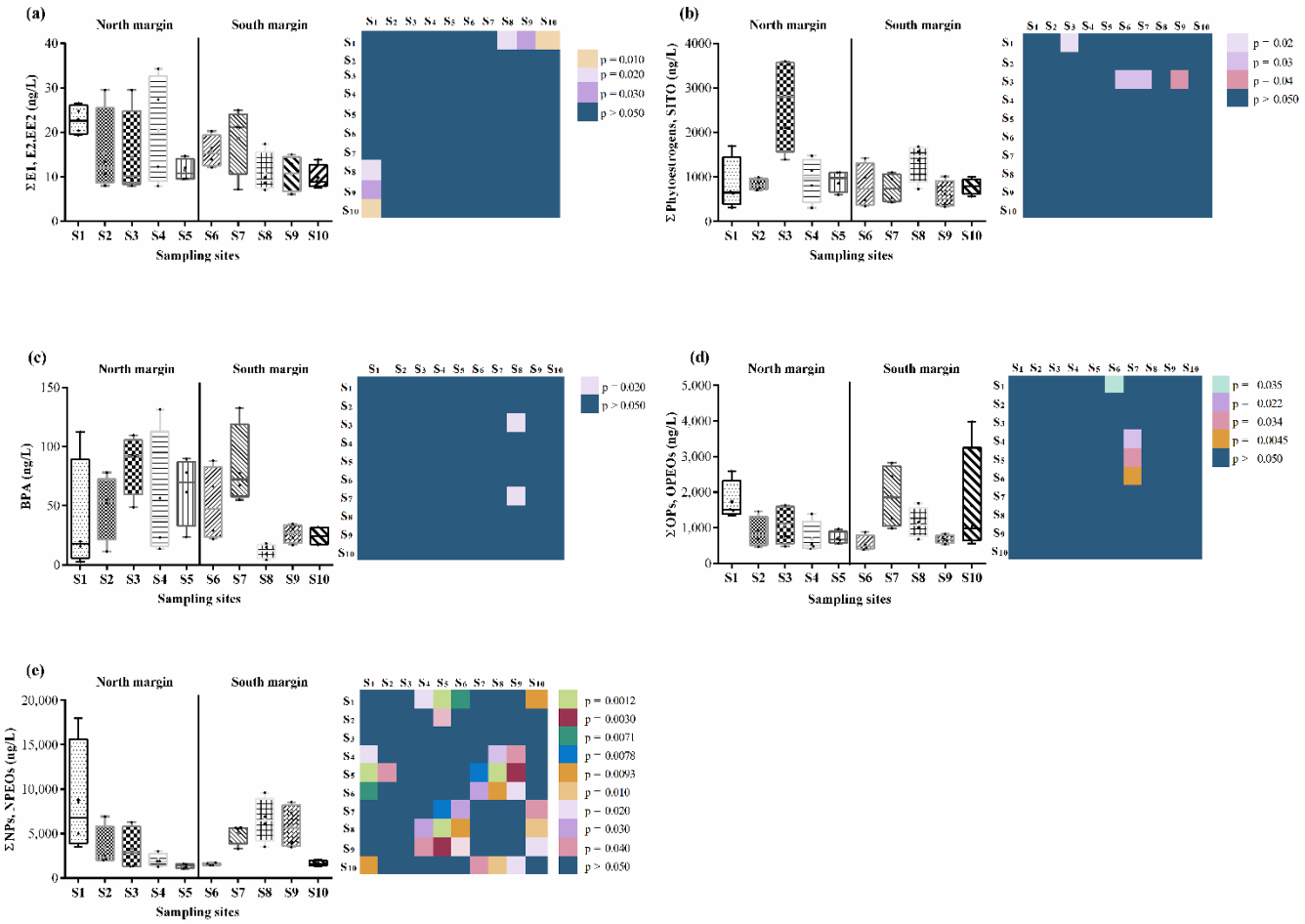

Figure 3. Levels (ng/L) of: (**a**) ∑oestrogens; (**b**) ∑phytoestrogens and SITO; (**c**) BPA; (**d**) ∑OPs and OPEOs; (**e**) ∑NPs and NPEOs per sampling site (n = 4). Data are expressed in boxplots with the minimum, median, maximum, average (+), and interquartile range Q1–Q3. Dots represent average individual values measured in different sampling occasions. Significant differences are reported in the colour chart.

Figure 4 reports PCA results, considering the seasonal distribution of variances for (a) oestrogens, (b) phytoestrogens and SITO (c) OPs, OPEOs and BPA, and (d) NPs and NPEOs.

The PCA assessment of octylphenols defined three principal components, with a variance of PC1 (40.5%), PC2 (24.3%), PC3 (20.3%), and eigenvalues ≥ 1. The most important contributors were 4-n-OP (0.59) for PC1, 4-t-OP for PC2 (0.92), and OP1EO for PC3 (0.80) (Table S2). The PCA score plot (95% ellipses) demonstrates similar distribution profiles between autumn/winter and spring/summer, Figure 4c.

3.1. Oestrogens

The oestrogens analysis showed a 98.3 % of detection rate (DR). On average, the annual amounts of E1, E2 and EE2 were 3.4 ng/L, 8.5 ng/L and 3.2 ng/L, respectively (Table 1). The concentrations of E2 were higher than those of E1 ($p = 9 \times 10^{-7}$) and EE2 ($p = 4 \times 10^{-8}$), Figure 2a. The levels of E1 and EE2 were similar ($p > 0.05$). Considering the sum of all oestrogens (∑E1, E2, EE2), no significant differences were registered amongst sampling sites (Figure 3a) and sampling seasons.

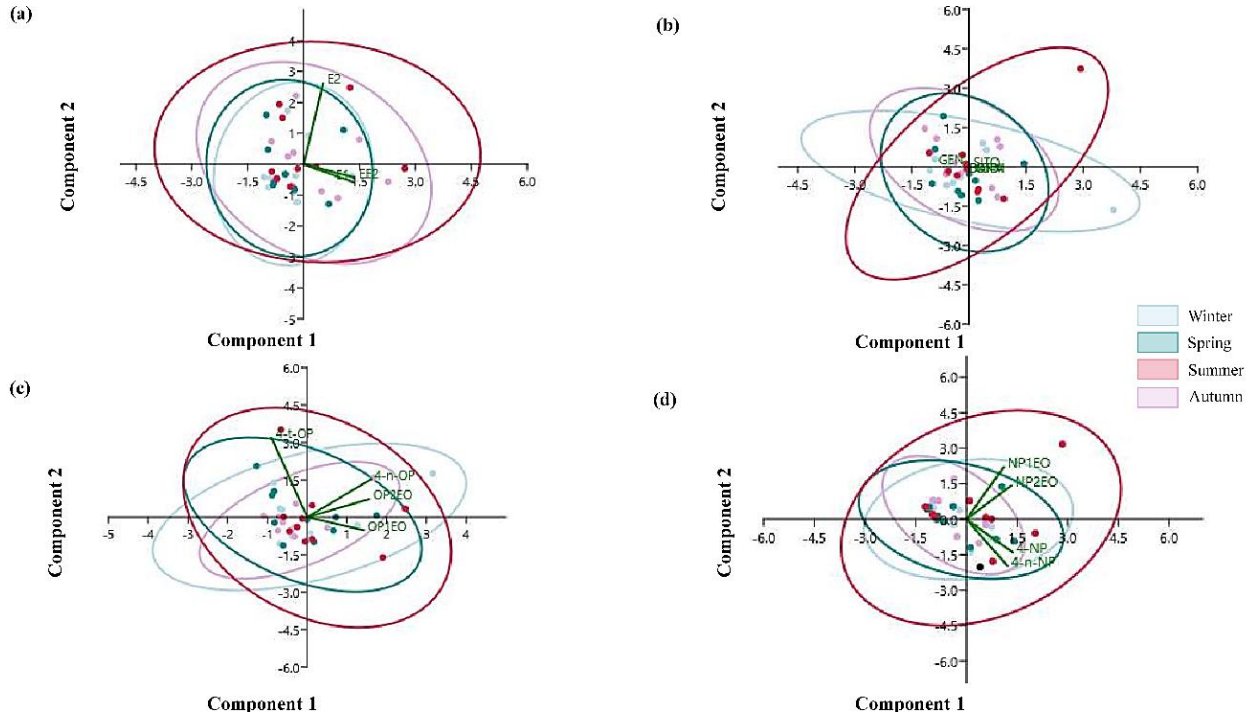

Figure 4. Principal Component Analysis (PCA) score plots of PC1 vs PC2 illustrating the distribution of individual EDCs by category, sampling occasion (n = 4) and sites (S1 to S10, n = 10), i.e., (**a**) oestrogens; (**b**) phytoestrogens and SITO; (**c**) OPs and OPEOs; and (**d**) NPs and NPEOs.

The PCA defined two principal components for oestrogens with a variance of 58.7% (PC1) and 31.5% (PC2), respectively, and eigenvalues ≥ 1. The main contributors of PC1 were both E1 (0.68) and EE2 (0.69), and that of PC2 was E2 (0.96) (data in Supplement, Table S2). The 95% ellipses reveal that the sampling occasion with higher data variability occurred in summer, Figure 4a.

Altogether, the oestrogens reached an E2-EQ value of 13.7 ng/L and showed a HI = 8 (Table 2). Globally, oestrogens are 55.4% of the E2-EQs of this estuary and pose 31.0% of HI.

Table 2. Annual average concentrations of the seventeen EDCs converted in E2-EQs. Here, the HQ posed by these compounds is also shown, considering the PNEC values for aquatic organisms, and the HI. For phytoestrogens, the HQs were calculated considering their E2-EQs.

EDCs	Annual Average Concentration (ng/L)	Relative Potency to E_2	E_2-EQs (ng/L)	Totals ($\sum_{E2\text{-}EQ}$) (ng/L)	PNEC (ng/L)	HQs	HI ($\sum_{HQs}$)	Ref.
	Oestrogens							
E1	3.4	3.0×10^{-1}	1.0		6.0	0.6		[15]
E2	8.5	1.0	8.5		2.0	4.2		[15]
EE2	3.2	1.3	4.2	13.7	1.0	3.2	8.0	[15]
	Phytoestrogens and SITO							
BIO-A	827.1	9.1×10^{-3}	7.5		-	3.8		-
DAID	3.6	1.3×10^{-3}	4.6×10^{-3}		-	0.0		-
FORM	12.5	5.6×10^{-3}	7.0×10^{-2}		-	0.0		-
GEN	7.8	4.9×10^{-2}	3.8×10^{-1}	8.0	-	0.2	4.0	-
SITO	827.1	9.1×10^{-3}	7.5		-	3.8		-
	Industrial Pollutants							
BPA	48.3	1.0×10^{-4}	4.8×10^{-3}		1.5×10^{3}	0.03		[16]
4-n-OP	2.0	4.0×10^{-4}	7.8×10^{-4}		1.2×10^{2}	0.02		[18]
4-t-OP	16.6	1.3×10^{-5}	2.2×10^{-4}		1.0×10^{3}	0.02		[16]
OP_1EO	96.8	5.0×10^{-6}	4.8×10^{-4}		1.2×10^{2}	0.79		[16]
OP_2EO	983.1	5.0×10^{-6}	4.9×10^{-3}		1.2×10^{2}	8.06		[18]
4-NP	758.1	4.0×10^{-3}	3.0		8.0×10^{2}	0.95		[17]
4-n-NP	23.9	5.0×10^{-5}	1.2×10^{-3}		8.0×10^{2}	0.03		[19]
NP_1EO	482.7	6.3×10^{-7}	3.0×10^{-4}		8.0×10^{2}	0.60		[19]
NP_2EO	2687.7	6.3×10^{-7}	1.7×10^{-3}	3.0	8.0×10^{2}	3.36	13.9	[19]
Totals			E_2-EQs =	24.7		HI =	25.9	

Data describing the levels of E2-EQs, HQs and HIs are in Table 2.

3.2. Phytoestrogens and SITO

Phytoestrogens and SITO were measured in 100% of the surface water samples (Table 1). The annual average concentrations of BIO-A, DAID, FORM, GEN and SITO were, respectively, 827.1 ng/L, 3.6 ng/L, 12.5 ng/L, 7.8 ng/L and 132.7 ng/L. BIO-A values were higher than those of SITO (p = 0.00318), FORM ($p = 6.86 \times 10^{-12}$), GEN ($p = 1.41 \times 10^{-20}$) and DAID ($p = 1.91 \times 10^{-29}$). Other differences also occur amongst the last referred compounds, as shown in Figure 2b. Furthermore, amongst sampling sites, there are significant differences concerning the evaluated compounds, with lower amounts predominating in the south margin (Figure 3b). The mean levels of these EDCs were higher at S3 than those measured at S6 (p = 0.02574), S7 (p = 0.02735) and S9 (p = 0.003993). On the contrary, no seasonal fluctuations were found for these EDCs.

The PCA for phytoestrogens and SITO defined two principal components with a variance of 51.9% (PC1) and 19.9% (PC2), and eigenvalues ≥ 1. FORM (0.53) and BIO-A (0.53) were the most important contributors to PC1, whereas GEN (0.96) was the phytoestrogen that most contributed to PC2. The 95% ellipses reveal that while the winter and spring had similar patterns of distribution as shown in Figure 4b, the summer was closer to PC1 and autumn to PC2 (data in Supplement, Table S2).

The phytoestrogens summed an E2-EQ value of 8.0 ng/L, being BIO-A responsible for 94% of this value. Altogether, the phytoestrogens showed a HI = 4 (Table 2).

Globally, phytoestrogens contribute 32.3% to the overall oestrogenic load (E2-EQ) and 15.4% of the HI value.

3.3. Industrial Compounds

3.3.1. BPA

Table 1 shows that BPA was measured in 100% of the water samples, attaining an average concentration of 48.3 ng/L. Differences were found for BPA amongst sampling sites Figure 3c, but not on sampling occasions. The lowest concentrations were found at S8 when compared to S3 (p = 0.01605) and S7 (p = 0.01979).

BPA contribute 0.03% to the overall oestrogenic load (E2-EQ) and with 0.12% of the HI value (Table 2).

3.3.2. OPs and OPEOs

Concerning these industrial EDCs, except for 4-t-OP (DR = 55%), they were quantified in 100% of the water samples (Table 1). The global concentrations of 4-n-OP, 4-t-OP, OP1EO and OP2EO were 2.0 ng/L, 16.6 ng/L, 96.8 ng/L, and 983.1 ng/L (Table 1). The levels of OP2EO were higher than those of 4-t-OP ($p = 6.46 \times 10^{-5}$), 4-n-OP ($p = 2.08 \times 10^{-14}$), and OP1EO ($p = 6.49 \times 10^{-5}$), as shown in Figure 2c.

Furthermore, significant differences existed amongst sampling sites. Globally, the concentrations measured at the inner part of the estuary (S4 to S6) were lower than those measured at S7 (see Figure 3d). In contrast, no differences were found amongst sampling seasons.

The PCA assessment of octylphenols defined three principal components, with a variance of PC1 (40.5%), PC2 (24.3%), PC3 (20.3%), and eigenvalues ≥ 1. The most important contributors were 4-n-OP (0.59) for PC1, 4-t-OP for PC2 (0.92), and OP1EO for PC3 (0.80) (Table S2). The PCA score plot (95% ellipses) demonstrates similar distribution profiles between autumn/winter and spring/summer (Figure 4c).

This category of compounds summed an E2-EQ value of 0.006 ng/L, being OP2EO, as the one that that most contributed to this estuary's oestrogenic load E2-EQ = 0.005 ng/L and to HQ = 8.9 (Table 2). OPs and OPEOs contribute 0.03% to the overall oestrogenic load (E2-EQ) and 34.5% of the HI.

3.3.3. NPs and NPEOs

The concentrations of 4-NP, 4-n-NP, NP1EO and NP2EO were 758.1 ng/L, 23.9 ng/L, 482.7 ng/L and 2687.7 ng/L (Table 1). The levels of NP2EO were higher than those of 4-n-NP ($p = 2.91 \times 10^{-24}$), NP1EO ($p = 0.67 \times 10^{-6}$) and 4-n-NP (0.0111). Other differences amongst these EDCs are shown in Figure 2d.

Differences were also detected amongst sampling sites, with those with lower concentrations being located at the inner part of the estuary mainly when comparing the concentrations between S1 and S4 ($p = 0.02154$), S1 and S5 ($p = 0.001212$), S1 and S6 ($p = 0.007111$), and that close to the Natural Reserve, i.e., between S1 and S10 ($p = 0.009298$). Other significant differences are reported in Figure 3e.

The PCA calculation revealed that two principal components characterize nonylphenols. Here, the most important contributors for PC1 (52.2%) were both 4-NP (0.54) and NP2EO (0.53) and, for PC2 (26.0%), the most important was NP1EO (0.62) (data in Supplement, Table S2). The PCA score plots (95% ellipses) demonstrated that summer was the occasion when higher variability occurred.

Within this class of compounds, 4-NP was the one that most contributed to this estuary's oestrogenic load E2-EQ = 3.0 ng/L and NP2EO to HQ = 3.4 (Table 2). NPs and NPEOs contribute with 12.3% of the global E2-EQs and represent 19.1% of HI.

3.4. Physicochemical Data

Table 3 shows the physicochemical data by season. Temperature levels ranged from 11.9 °C in winter to 22.3 °C in summer, while salinity varied from ≈9 PSU (S1 and S8) to 1.8 at S6 (Table 3). The pH (7.5) was almost constant all-year-round (Table 3). The average concentrations of NO_2^-, NO_3^-, NH_4^+ and PO_4^{3-} were, respectively, 0.05 mg/L, 1.5 mg/L, 1.9 mg/L and 0.14 mg/L (Table 3).

Table 3. Physico-chemical data evaluated in surface waters of the Douro River estuary, presented as mean ± standard deviation.

Seasons	Winter	Spring	Summer	Autumn
T (°C)	11.9 ± 0.7	17.0 ± 0.9	22.3 ± 1.4	15.4 ± 0.7
Salinity (PSU)	2.4 ± 2.1	3.3 ± 2.8	10.5 ± 3.4	2.9 ± 3.4
pH	7.5 ± 0.2	7.5 ± 0.3	7.5 ± 0.3	7.1 ± 0.3
NO_3^- (mg/L)	1.9 ± 0.4	1.5 ± 0.5	1.2 ± 0.1	1.3 ± 0.3
NO_2^- (mg/L)	0.09 ± 0.05	0.03 ± 0.04	0.04 ± 0.01	0.03 ± 0.01
NH_4^+ (mg/L)	0.6 ± 1.2	2.5 ± 0.9	2.3 ± 0.0	2.2 ± 0.2
Unionized ammonia (mg/L)	0.004 ± 0.01	0.031 ± 0.02	0.052 ± 0.04	0.010 ± 0.01
NH_3 (mg/L)	0.004 ± 0.01	0.031 ± 0.02	0.052 ± 0.04	0.010 ± 0.01
PO_4^{3-} (mg/L)	0.14 ± 0.2	0.04 ± 0.0	0.16 ± 0.1	0.23 ±0.2

NO_3^- and NH_4^+ attained the highest concentrations amongst this class of chemicals. All significant differences are in Figure 5a. Globally, the location S4 showed the highest global amounts of NO_2^-, NO_3^-, NH_4^+, and PO_4^{3-} (Figure 5b). The mathematical evaluation of unionized ammonia per sampling occasion revealed that summer attained the highest amounts of this parameter (0.054 mg/L; Table 3).

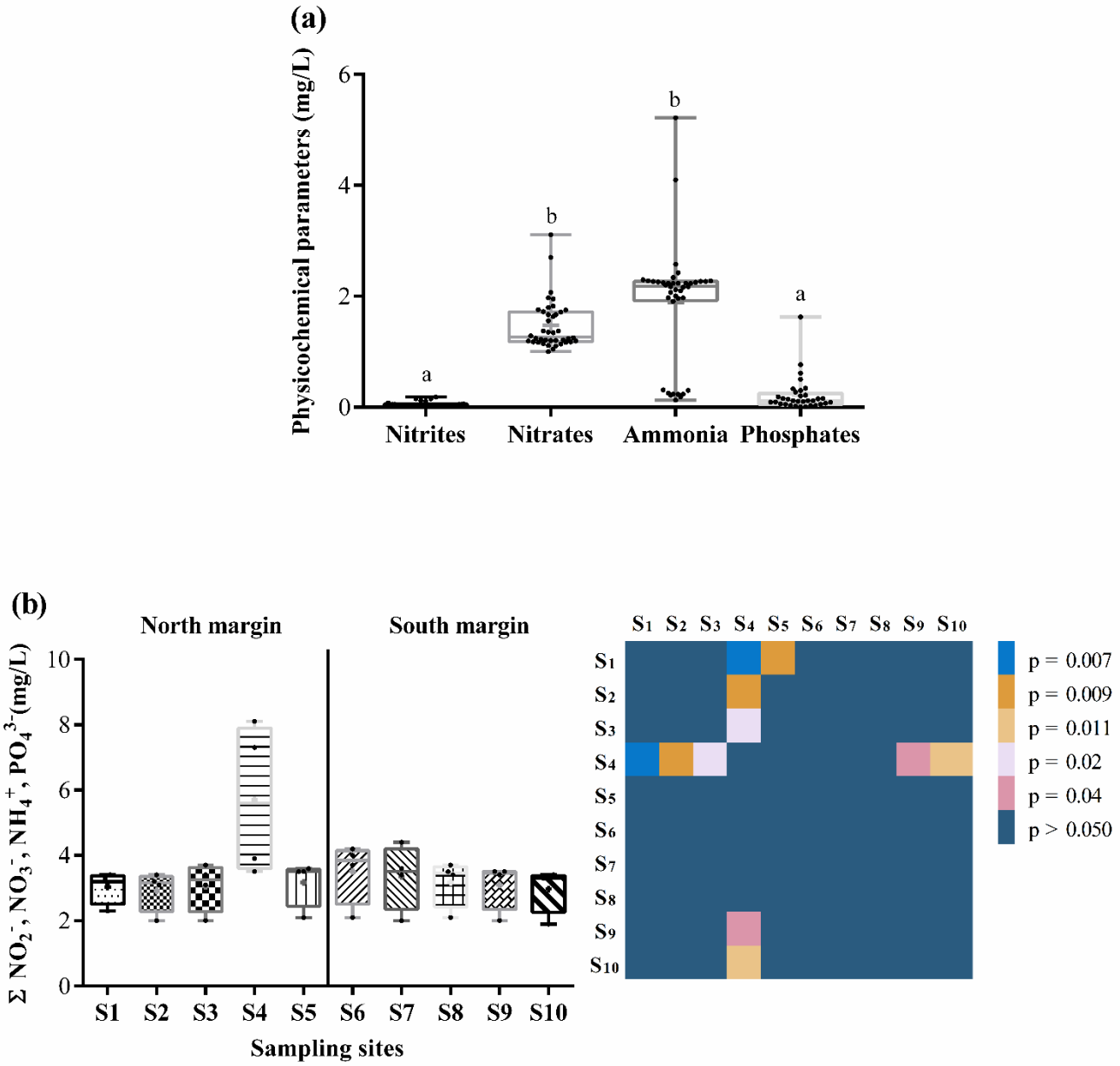

Figure 5. (**a**) Global levels (mg/L) of nitrites, nitrates, ammonia, and phosphates (n = 40). (**b**) Levels (mg/L) of ∑nitrites, nitrates, ammonia, and phosphates per sampling site (n = 4). Data are expressed in boxplots with the minimum, median, maximum, average (+), and interquartile ranges Q1–Q3. Significant differences are reported in the colour chart. Different low case letters indicate significant differences amongst physiochemical parameters ($p < 0.05$).

4. Discussion

4.1. Oestrogens

In fish and other aquatic animals, waterborne oestrogens cause hormonal disruption, even at concentrations as low as one or a few ng/L of E2 [23,24]. Several Portuguese and worldwide aquatic environments show considerable amounts of E1, E2 and EE2 [25]. In the past, the Douro River estuary was one of the areas where these EDCs reached concentrations that induced endocrine disruption in fish, i.e., E1, E2, and EE2 were, respectively, 2.9 ng/L, 7.0 ng/L and 4.5 ng/L [3]. On that occasion, those levels were correlated with the presence of a high percentage of local male mullets (*Mugil cephalus*) with intersex (ovotestis), a well-known impact of oestrogenic exposure [26]. Since these EDCs may be very deleterious

for aquatic organisms, they are currently in the UE watch list regulation [8], and thus their environmental levels should not be neglected.

In this vein, to assess the application of the last directive E1, E2 and EE2 were quantified in water samples collected during 2019 in the Douro River estuary. The current mean values of these EDCs (Table 1) surprisingly matched those found in samples from 2010 [3], suggesting that the pollution by these EDCs did not meliorate in one decade. One likely cause can be no improvement in the efficiency of local WWTPs for these EDCs. This hypothesis is supported both by the similarity between present and past concentrations as well as by the relative amounts of these compounds, that is, E2 > E1 = EE2 (Figure 2a).

Since there are no differences amongst sampling sites oestrogens are ubiquitous in this estuary (Figure 3a). The current data also revealed no seasonal trends for oestrogens. Despite this, the PCA unveiled that the variability of these EDCs is greater in the summer (Figure 4a). This suggests that the decrease in rainfall together with the increase in the number of inhabitants in this area, due to touristic inflow, can change the distribution of these EDCs in the estuary in summer.

Considering the values of PNECs, our data supports that at least E2 and EE2 should be capable of inducing endocrine disruption in the local fish (Table 2). Moreover, looking at the oestrogens concentrations after their conversion to E2-EQ, it is concluded that they contribute to 31% of the oestrogenic load of this estuary (Table 2). Besides, their HQs show that oestrogens provide 59% of the HI, which suggests the maintenance of gonadal disruption conditions previously reported for fish from this area [26].

Therefore, as the oestrogenic conditions recorded in 2010 were maintained in 2019, it is possible that the treatment of urban sewages that reach the WWTPs located across the estuary remains inadequate (Figure 1).

Moreover, since the concentrations of oestrogens in the Porto River estuary are higher than most of the latest reports in Europe [25,27], it is suggested that the application of the WFD [7] is failing in this area.

4.2. Phytoestrogens and SITO

Phytoestrogens and SITO are compounds of plant origin that structurally and/or functionally resemble oestrogens or their active metabolites [28]. Among them, the isoflavones BIO-A, GEN, FORM and DAID, show affinity to oestrogen receptors [28]. Despite being less active than E2, they can exert similar actions when their concentrations are in the µg/L range [29]. Endocrine disruption can also be induced by SITO, a phytosterol structurally like cholesterol [28]. This EDC is an essential component of vascular plants. In mammals, SITO can decrease the availability of cholesterol to P450scc (an enzyme involved in converting SITO into pregnenolone) or reduce this enzyme's activity [30,31].

It was shown that phytoestrogens and SITO could be very abundant in Portuguese aquatic environments, including the Douro River estuary [25]. The latter study found their concentrations were often very high and well within the range capable of triggering endocrine disruption phenomena in aquatic organisms.

In this study, we observed the presence of high amounts of these EDCs. Nonetheless, in addition to not having found any significant differences between the different sampling occasions, there was a decrease—of five to six times for BIO-A, GEN and DAID, and about 14 times for FORM and eight times for SITO—in the environmental concentrations of these compounds, in relation to previous studies [3]. Furthermore, it was found that, in general, the highest concentrations of these compounds were found in the northern margin of the estuary (Figure 2b). Therefore, the presence of these compounds in the Douro estuary waters may be currently more associated with the existing food industries' sewage (e.g., S3) rather than with the natural vegetation surrounding this area, as reported in the past [32].

The last observation seems supported by the results obtained by the PCA since there is less dispersion of these contaminants in the estuary in summer, probably due to the decrease in rainfall. Furthermore, the observed differences may be related to the seasonal production of certain types of food, i.e., winter and spring, with similar profiles that stand

out from summer and autumn (Figure 3b). It is noteworthy that in the autumn, although there was no significant increase in GEN mean levels, the PCA made it possible to observe the dominance of this compound, which is a metabolite resulting from the decomposition of BIO-A. Thus, everything seems to imply that, in addition to the possible effect of industries, there is a natural decomposition of vegetation in autumn, which promotes the emergence of this metabolite at this time. However, this reading needs further confirmatory studies.

With regards to the environmental danger of these EDCs, it was found that by presenting an oestrogenic activity equivalent to 8 ng/L of E2, phytoestrogens are capable of inducing endocrine disruption in fish [23,25]; recall that the PNEC of E2 = 2 ng/L [15]. In addition, the determination of an HI = 4 for these EDCs suggests that they also pose a risk to the health of local biota.

4.3. Industrial Compounds

BPA is released in the aquatic environment through the natural degradation of plastics [33], landfill leachates of degraded plastics [34] or even through sewage after human-ingestion of contaminated food [35]. Recently, high amounts of microplastics were measured in the Douro River estuary [36]. Thus, the presence of BPA, in quantities (48 ng/L) similar to those measured earlier in this habitat (43 ng/L) [3], confirms the ubiquitous presence of this EDC in this estuary. The BPA lowest values were found nearby the Natural Reserve, on the south bank of the estuary (Figure 3c).

APEOs are non-ionic surfactants that are commonly detected in wastewater discharges and WWTPs effluents and are present in the aquatic environment due to anthropogenic activities [25,37]. They are known to promote oestrogenic effects on wild fauna and humans, and although they have been banned from Europe since 2003 [38] they still exist in the Douro River estuary.

Since the EU and Portuguese legislations consider these EDCs non-authorized compounds [38,39], their current presence in this environment is enigmatic. One credible source of APEOs in this area is imported materials, including raw materials, as many industries (including textile) in this region [40] may use these types of compounds inadvertently. It is also possible that APEOs present in several pesticide mixtures to help their environmental dispersion reach the estuary by lixiviation [4]. Finally, urban discharges cannot be dismissed [30].

Presently the concentrations of APEOs and their derivatives (OPs and NPs) have increased significantly compared to previous reports in this estuary [3,5]; on average, the levels of 4-n-OP, OP1EO and OP2EO are, respectively, seven, two and four times higher. Furthermore, the levels of 4-NP, NP1EO and NP2EO are six, thirteen and forty times higher than those recorded in water samples collected in 2010 [3]. Since European and national regulations have defined threshold values of 10 ng/L for OPs and 300 ng/L for NPs, it is concluded that in this estuary, the concentrations of these EDCs are systematically above the recommendations of the WFD [7,39] (Directive 2013; DR 2015). It is stressed that, when degrading, APEOs originate OPs and NPs, which are much more oestrogenic and persistent [41].

Figure 3d,e suggests that the areas where there are fewer OPs and NPs are in S5 (north bank) and S6 (south bank). Both regions are less industrialized, and so it makes sense that they would hold smaller amounts of these EDCs. On the other hand, sampling site S7 receives water from the Febros River, a tributary of the Douro (Figures 1 and 3). The area downstream of Febros WWTP (Avintes) was earlier identified as ecologically problematic, and liver histopathology of gudgeon (*Gobio gobio*) and mullet (*M. cephalus*) revealed some significant impacts in animals captured downstream from that WWTP [40]. As the current study shows that this region remains a gateway for estuary pollutants, it appears that the Febros WWTP still has problems treating sewage from urban and industrial areas on the estuary's south bank. This situation may perpetuate the previously reported impacts on the local ecosystem [40]. This projection is strengthened when considering the endocrine disruptor potential of these industrial compounds. Here, our data (Table 2) exposed that

they contribute to 3 ng/L of E2-EQs and result in an HQ of 14, indicating a considerable potential to induce adverse effects on the local biota [14].

Another relevant aspect of the analysed industrial pollutants is their concentrations' stability and ubiquity at all harvesting points. In addition, the PCA revealed the close relationships between OPEOs and NPEOs and showed that summer is when the greatest variance of these EDCs occurs. The last observation is probably linked to the summer higher temperatures, as this parameter promotes faster biodegradation of APEOs in non-ethoxylated forms (OPs and NPs) [42].

When the present study results are compared to similar surveys carried out in other world regions, it becomes evident that the Douro River estuary is still far from being a clean ecosystem [27,40].

4.4. Physico-Chemical Data

Several water physicochemical parameters closely related to sewage and WWTP discharges (pH, ammonium, nitrites, nitrates, and phosphates) were evaluated here (Table 3 and Figure 5a). The levels of these parameters were within the ranges previously reported for this estuary [3]. This fact corroborates the inference that the estuary water quality has not improved over the last decade.

The levels of ammonia, nitrates, and nitrites were higher in areas closer to WWTPs (Figure 5b). However, other sources of these compounds exist in this estuary. In fact, direct discharges of human urine/faeces from touristic boats that become very active in summer have been reported, e.g., by the National Association for Nature Conservation [41]. Besides, summer is the time of the year when toxic (un-ionized) ammonia, the levels of which are dependent on both the pH and temperature of the water, may attain levels that might cause gill damage (Table 3, 0.052 mg/L) [9,43]. This knowledge fits well with the increased overload of these compounds observed in the Douro estuary in summer.

Concerning the amounts of phosphates, these presented an annual average of 0.14 mg/L, with a maximum, in summer of 0.5 mg/L at S4; a value that is well above the maximum acceptable level of 0.1 mg/L to avoid eutrophication legislation [44]. Despite this, the annual average amounts of phosphates are compatible with european legislation [44].

5. Conclusions

The existence of natural (animal and vegetal) and industrial EDCs was established in this work. The Douro River estuary has the equivalent of 25 ng/L of E2, a very high amount, unequivocally capable of causing endocrine disruption in aquatic species, notably fish. The computed HI (26) also revealed that aquatic life in this estuary is threatened.

In addition to the negative impact that the 17 EDCs evaluated in this work have on the biota, it is important to emphasize that this area has wide river beaches and is used for leisure and fishing by local inhabitants (including children). Therefore, the presence of these compounds may pose a human health issue that local authorities should consider.

The presence of ammonia (unionized), nitrates, nitrites and phosphates at levels that can be toxic for the biota is further evidence that the current WWTPs are not able to deal with the removal of damaging chemicals, which besides posing a risk for the local habitat, may also affect human health, mainly in summer, when the fluvial beaches are crowded. Another possible risk worth exploring is the one posed by eating local seafood.

In summary, this study reveals the presence of EDCs in the Douro River estuary at levels posing risks to the biota and, as a result, calls for the need to improve the presently used techniques for removing such compounds from the WWTP effluents, in line with the WFD implementation. The EDCs levels disclosed here serve as a reference for the future.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/w14132046/s1, Table S1: Quantification and diagnostic ions used in GC-MS analyses. The relative abundance of ions (m/z) is indicated between brackets (detailed information in Rocha et al., 2013); Table S2: Data referring to PCA of EDCs in Douro River estuary.

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