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CHARACTERIZATION OF QUALITY OF SEDIMENTS FROM PARANAGUÁ BAY (BRAZIL) BY COMBINED IN VITRO BIOASSAYS AND CHEMICAL ANALYSES

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Abstract: The present study characterizes the quality of sediments from the Paranaguá Estuarine Complex (South Brazil). Polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), and organochlorine pesticides (OCPs) were determined in sediment samples together with a series of different in vitro bioassays. The fish hepatoma cell line (PLHC-1) was used to determine the presence of cytotoxic compounds and CYP1A- and oxidative stress-inducing agents in sediment extracts. Ovarian microsomal fractions from sea bass (*Dicentrarchus labrax*) were used to detect the presence of endocrine disruptors that interfered with the synthesis of estrogens (ovarian CYP19). Despite the relatively low levels of pollutants and no evidence of negative effects based on guideline levels, sediments collected close to harbors were enriched with CYP1A-inducing agents and they showed higher cytotoxicity. In contrast, sediments from internal areas inhibited CYP19 activity, which suggests the presence of endocrine disruptors at these sites. Overall, the selected bioassays and the chemistry data led to the identification of potentially impacted areas along the Paranaguá Estuarine Complex that would require further action to improve their environmental quality. *Environ Toxicol Chem* 2017;36:1811–1819. © 2016 SETAC

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INTRODUCTION

Estuaries are highly vulnerable ecosystems that deserve special attention because of their high ecological significance. Because they also provide excellent opportunities for development of socioeconomic activities, estuaries are among the aquatic ecosystems most susceptible to the continuous influx of heterogeneous contaminant mixtures [1]. The distribution and fate of pollutants is often associated with the proximity of anthropogenic activities. However, transport processes, physicochemical properties of the compounds, and environmental conditions will also significantly affect the environmental behavior of pollutants [2].

Sediments are widely recognized as environmental sinks and reservoirs capable of long-term storing of pollutants. Certain characteristics, such as grain size and total content of carbon (TOC), may affect the accumulation of those pollutants: for instance, organic carbon acts as an accumulator of chemical compounds and constitutes a measure of sorption capacity of the sediment, while fine sediments have greater cohesive properties [3]. Therefore, the health of aquatic ecosystems is often assessed by measuring sediment characteristics and the pollutants trapped in sediments. It is not feasible, however, to identify and measure the concentrations of all potential toxicants in the sediment and the synergistic and antagonistic interactions among them by using chemical analysis alone [4]. Indeed, there are many challenges in establishing a safety limit of pollutant levels [5].

Endocrine disruptors are exogenous agents that interfere with the production, transport, metabolism, action, or secretion of natural hormones or hormone receptors that are responsible for maintaining homeostasis and the development of regulatory processes [6,7]. Estrogenic activity can be detected by various methods such as ligand-binding assays or reporter-gene or yeast assays, among others, based on the molecular and cellular mechanisms of estrogen action [7].

In vitro mechanism-based cellular bioassays have proved to be valuable tools for the detection and quantification of biologically active chemicals in environmental mixtures [8]. The use of in vitro tests reduces the need for whole-animal testing and provides powerful and predictive tools for risk assessment [9]. Such tests allow the evaluation of alterations in key toxicological pathways in response to chemicals. However, they have a limited value for risk assessment, because they do not take into account all toxicity pathways (which are not completely known) and do not consider the complexity of humans and animals [8,9]. Therefore, because sediments have to be processed to provide a form suitable for in vitro exposure (e.g., concentrated organic sediment extracts), mechanisms such as bioavailability, bioaccessibility, and water solubility of chemicals contained in the sediment are not considered.

The use of fish cells offers some advantages and disadvantages. For instance, the loss of realism and biological relevance is compensated for by other aspects, such as reduced experimental noise, reduced costs, and the relative ease of assaying a wide range of compounds and concentrations, as well as ethical issues [10]. Among them, PLHC-1 is a permanent fish cell line that retains the capability to induce cytochrome P450 (CYP)1A through activation of the aryl hydrocarbon receptor by numerous pollutants such as pharmaceuticals, polycyclic aromatic hydrocarbons (PAHs), and dioxins. The PLHC-1

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Figure 1. Map of the sampling sites.

line has also been used successfully to assess cytotoxicity and oxidative stress of different compounds, including sediments extracts [11].

The CYP enzymes also play a key role in the synthesis of sex steroids in fish gonads. In fish ovaries, androgens are converted into estrogens (estrone and estradiol) by cytochrome P450 aromatase (CYP19). The interference of pollutants with this enzymatic pathway may alter endogenous levels of active hormones within the organism. It may become a potential mechanism of endocrine disruption by affecting sexual differentiation and gamete growth in fish [6,10]. Bioassays based on the use of fish gonad subcellular fractions have been successfully applied to detect endocrine disrupters in sediment extracts [4,12].

The purpose of the present study was to characterize the quality of sediments from the Paranaguá Estuarine Complex by integrating results from the analysis of priority pollutants and different *in vitro* bioassays to detect cytotoxic compounds and endocrine disrupters trapped in the sediments. The PLHC-1 cell line was used to evaluate the cytotoxicity, generation of reactive oxygen species (ROS), and CYP1A-inducing agents (activity of ethoxyresorufin-O-deethylase [EROD]) in sediment-trapped pollutants. Ovarian microsomal fractions from female sea bass (*Dicentrarchus labrax*) were used to detect the presence of bioactive chemicals acting as endocrine disrupters by altering the synthesis of estrogens (ovarian CYP19).

MATERIALS AND METHODS

Study area

The Paranaguá Estuarine Complex has an extension of approximately 612 km² characterized by the presence of mangroves, islands, and inlets. The largest port for grain export in South America, the Paranaguá Port, is located on the east–west axis, where fertilizers, minerals, and petroleum derivatives are

also handled [13]; the navigation channel of the Port (Galheta Channel) and a channel for discharging and small boat traffic (DNOS) are also located there. The urban population of the city of Paranaguá is 150 000 [14]. In contrast, the North–South axis is an Environmental Protected Area with no anthropogenic activities or industrial pressures in the vicinity.

Organic compounds such as PAHs, polychlorinated biphenyls (PCBs), and organochlorinated pesticides (OCPs) have already been reported at this estuary [15–17] and may represent an environmental problem, considering the possible negative effects on aquatic organisms even at low concentrations.

Sediment sampling

Sediments were collected with a Van Veen grab sampler at 8 stations, including areas influenced by harbor activities, navigation, urban effluents, and potential reference sites (Figure 1). The contents of multiple grabs were composited at each station and homogenized. Physical and chemical parameters (coordinates, depth, temperature, salinity, sigma-t, pH, and turbidity) from each site were obtained with a Multi-Parameter Water Quality Meter Probe, U-50 series from Horiba (Table 1). In the laboratory, samples were lyophilized and stored at 20 °C.

Chemical analysis

Total organic carbon. The TOC content was measured in all samples after acid digestion to remove the carbonate fraction with hydrochloric acid 16% (v/v). The organic carbon was converted to CO₂ and quantified (TOC-VCPH; Shimadzu).

Extraction, cleanup, and chromatographic analysis of PAHs. To quantify 16 PAHs listed as priority by the US Environmental Protection Agency, the procedure described by Machado et al. [18] was followed with minor modifications. Sediment (10 g) was extracted using an accelerated solvent extractor (ASE; Dionex) in 5-min static cycles (100 °C; 4 MPa)

Table 1. Geographic coordinates and physicochemical parameters of sampling points (temperature 26.4–27.2 °C and pH 7.6–8)

Sample	Longitude	Latitude	Depth (m)	Salinity (%)	Turbidity (NTU)	Description of the area	Pressure/impact
P1	733642	7182601	4.75	22.9	25.1	Antonina Bay	Agricultural activities upstream/harbor activities
P4	748298	7177168	5.6	27.3	8.6	Paranaguá Harbor	Industrial/harbor activities
P5	751753	7175715	3.3	25.5	4.8	Paranaguá City/Itibere River	Urban/industrial activities/river discharge
P9	754571	7176497	5.7	29.2	5.7	Cotinga Island	Runoff from urban effluents
P10	757924	7178675	7.95	27.6	6.6	Cobras Island/Galheta Channel	Intense ship navigation
P12	765632	7169797	9.2	29.5	10.8	Pontal do Paraná/DNOS channel	Intense traffic of tourist boats
P16	759967	7189170	3.85	27.9	22.0	Rasa Island/Laranjeiras Bay	Agricultural activities upstream/protected area
P17	766806	7188513	4.45	28.0	170.8	Pecas Island/Laranjeiras Bay	Low traffic of tourist boats/preserved area

with a mixture of dichloromethane/acetone (1:1, v/v). Sulfur was removed from the extracts using activated copper. The PAHs were isolated from other compounds using columns (7-mm inner diameter) filled with silica gel (~1.3 g) and sodium sulfate (~0.2 g) and eluted with toluene:hexane (1:3, v/v) (3 mL $\times$ 4 mL). This fraction was dried under gentle nitrogen flow and redissolved with hexane. A solution with the 16 priority PAHs (PAH Mix Z-014G; AccuStandard) was used as a standard solution to obtain a calibration curve for quantification. Deuterated PAHs (fluorine-d10 and acenaftilene-d8) were added as internal standards to monitor the efficiency of the analytical procedure. The recovery rates were higher than 75% for most of the PAHs. Quantification was conducted by gas chromatography–mass spectrometry (GC-MS) coupled to a Varian 430 Quadrupole mass spectrometer with electron-impact ionization. The GC was equipped with a factor 4 VF 5MS fused silica capillary column (60 m, 0.25-mm inner diameter, 0.25- μ m film thickness) and helium as a carrier gas with a constant flow of 1.2 mL min⁻¹. Both full scan and selected ion monitoring modes were applied.

Extraction, cleanup, and chromatographic analysis of PCBs and OCPs. The analytical procedure for PCBs and OCPs was based on the methodology described in Combi et al. [16] with minor modifications. The sediment (10 g) was extracted using ASE (Dionex) in 7-min static cycles (130 °C; 1500 psi) with a mixture of n-hexane/acetone (1:1, v/v). Activated copper was added to remove sulfur. The extracts were concentrated and then purified using a flash column (7-mm inner diameter) filled with alumina (~1.5 g). Elution was performed with n-hexane/dichloromethane (70:30) and the eluate was concentrated under nitrogen. Tetrachloro-m-xylene (M-8082-SS-10X; AccuStandard) was added as internal standard. The determination of PCBs (sum of 51 congeners) and OCPs (sum of 24 compounds including DDT, hexachlorocyclohexane isomers [HCHs], and chlordane-related compounds [CHLs]) was performed by using an Agilent 6890 GC with electron capture detection. The GC column was an HP-5 fused silica column (length, 30-m; inner diameter, 250 μ m; film thickness, 0.25 μ m). The quantification of the analytes was based on a mixture of surrogate standards of PCB 103 (C-103N) and PCB 198 (C-198N) (both from AccuStandard), which was added before sample extraction.

Toxicological analysis

For toxicological tests, 3 g of sediment (125 μ m size) were extracted with a mixture of dichloromethane/hexane (1:1, v/v) and dichloromethane/acetone (1:1, v/v), as described in Fernandes et al. [4]. The extracts were combined, evaporated, and reconstituted in dimethyl sulfoxide (DMSO) and serially diluted to the desired concentrations. The DMSO never exceeded 0.5% in the exposure medium.

PLHC-1 bioassays. The PLHC-1 cells were obtained from ATCC (CRL-2406) and routinely cultured in Eagle's Minimum Essential Medium supplemented with 5% fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 1.5 g/L sodium bicarbonate, 50 U/mL penicillin G, and 50 μ g/mL streptomycin, and incubated at 30 °C, 5% CO₂. When 90% of confluence was reached, cells were dissociated with trypsin–ethylenediamine tetraacetic acid and resuspended in culture medium for further bioassays.

Cell viability was evaluated with Alamar Blue and 5-carboxyfluorescein diacetate acetoxyethyl ester (CFDA-AM) [19,20]. Cells, seeded at a density of 75 000 cells at 200 μ L per well in a 96-well plate, were incubated for 24 h and then exposed to sediment extracts at concentrations ranging from 2 mg dry weight sediment equivalent extract (eQsed)/mL to 60 mg eQsed/mL for 24 h. The medium was removed, and cells were washed with 200 μ L phosphate-buffered saline (PBS; pH 7.4) before addition of a solution containing Alamar Blue and CFDA. Fluorescence was measured after 1 h of incubation at the excitation/emission (ex/em) wavelengths pairs of 530/590 nm for Alamar Blue, and 485/530 nm for CFDA-AM in a fluorescent plate reader (Varioskan, Thermo Electron). Results are expressed as percentage of relative fluorescent units (RFUs) of control cells.

Generation of ROS was measured in PLHC-1 cells exposed to sediment extracts as described in LeBel et al. [21], with slight modifications. Cells were seeded at a density of 10⁵ cells at 200 μ L per well; after 24 h of incubation, the culture medium was removed, cells were washed with PBS, and a solution of 20 μ M 2',7'-dichlorofluorescein (H₂DCF-DA) was added. After 30 min of incubation, cells were washed with PBS and exposed to sediment extracts to stimulate ROS generation. The fluorescence emitted by the oxidized H₂DCF-DA was measured at the ex/em wavelength pairs of 485/528 nm in a fluorescent plate reader (Varioskan; Thermo Electron) after 15 min, 30 min, and 60 min. As positive control, 5 μ M of SIN-1 was used. Results are expressed as fold induction of the basal fluorescence in control cells (% DMSO). Treatments were carried out in triplicate.

Activity of EROD was measured according to a procedure previously described [4]. Cells were seeded in a 48-well plate at a density of 6.5 $\times$ 10⁵ cells at 500 μ L per well and incubated for 24 h. Then the culture medium was replaced by a culture medium containing the sediment extracts, 0.5% DMSO (solvent control), or 1 μ M of β -naphthoflavone (BNF; positive control), and incubated for 24 h. After the exposure period, the medium was aspirated and the cells were rinsed with 500 μ L PBS and immediately incubated with 2 μ M 7-ethoxyresorufin in 50 mM Na-phosphate buffer, pH 8.0, at 30 °C. After 15 min of

incubation in the microplate reader (Varioskan; Thermo Electron), the fluorescence was directly read at the ex/em wavelength pairs of 537/583 nm. Quantification was made by calibration with 7-hydroxyresorufin. Fluorescamine was used to measure the protein content of cells, using bovine serum albumin (BSA) as standard [22]. Results are expressed as mean of pmol of resorufin generated per minute per milligram of protein (pmol/min/mg prot) for at least 3 independent replicates.

Ovarian P450 aromatase activity (CYP19): Microsomal fraction and *in vitro* incubation. Sea bass (*Dicentrarchus labrax*) were supplied by an aquaculture facility located in the Northeast of Spain. Immediately after collection, animals were euthanized and gonads were dissected. A subsample of the central part of the gonad was fixed in 10% buffered formalin for histological examination to determine the sexual maturation stage (SMS). The rest of the gonad was deep-frozen in liquid nitrogen and stored at 80 °C until preparation of subcellular fractions as described by Fernandes et al. [4].

The interaction with sea bass steroidogenic enzymes was investigated *in vitro* by preincubating the microsomes in the presence of the sediment extracts (at concentrations of 60 mg eQsed/mL, 20 mg eQsed/mL, 10 mg eQsed/mL, and 2 mg eQsed/mL) for 10 min at 25 °C, prior to the addition of the reaction substrate and cofactor. The solvent was removed from the assay by evaporation under a gentle flow of nitrogen before starting the preincubation. Control reactions consisted of incubation of the corresponding subcellular fraction in the absence of sediments. Blanks that consisted of reactions stopped at time 0 were also analyzed. Assays were carried out using individual gonads from 4 to 5 different individuals.

Aromatase activity was determined by the tritiated-water release method as previously described in Lavado et al. [23]. For the assay, 0.1 mg of ovarian microsomal proteins were incubated for 1 h at 25 °C in the presence of 100 mM Tris-HCl, pH 7.6, 0.2 mM nicotinamide adenine dinucleotide phosphate, reduced (NADPH), and [1β - 3 H] androstenedione (40 pmol, 1 μ Ci). The reaction was stopped using 3 mL of cold methylene chloride, and the excess substrate was further eliminated from the aqueous phase by extraction with methylene chloride ($\times 3$). The remaining tritiated steroids were further eliminated by the addition of a suspension of 2.5% (w/v) activated charcoal and 0.25% dextran in Milli-Q water. The solution was centrifuged for 60 min at 3600 rpm, and the amount of tritiated water formed was counted in 2 aliquots of the aqueous phase (Tri-Carb 2100TR, Packard).

Statistical analysis

Statistical differences were detected by analysis of variance (ANOVA) with 1 factor with Dunnett's test, using the software SPSS 19.0. Dose-response curves and concentrations which caused 50% of effect and inhibition (EC50 and IC50) were calculated by using Sigmaplot 13.0. A *p* value of <0.05 was considered statistically significant. To summarize the obtained information and to estimate the degree of anthropogenic impact in the different stations, a principal component analysis was carried out with PLS Toolbox 8.0 and MatLab software.

RESULTS

Chemical concentrations and composition of sediments

Sediments from inner parts of estuary P1 (east-west axis) and P17 and P16 (north-south axis) had the highest TOC contents (85 mg C/g sediment, 70 mg C/g sediment, and 52.3 mg C/g sediment, respectively; Table 2). In terms of sediment grain size distribution, P1 was mostly muddy, with high silt (<62 μ m) and fine sand (250–62 μ m) contents, 46% and 56%, respectively. In general, fine and medium sand (63–500 μ m) and silt (4–63 μ m) fractions dominated most of the sample compositions, whereas clay particles (<4 μ m) were absent.

Total concentration of PAHs in sediments ranged from 36.9 ng/g to 89.1 ng/g dry weight. The site near Paranaguá city (P5) had the highest concentration, and sites P16 (36.9 ng/g) and P17 (37.3 ng/g), both located in the north-south axis of the estuary, had the lowest.

The values of PCBs ranged from 0.1 ng/g to 7.6 ng/g dry weight and those of HCHs from 0.2 ng/g to 13.0 ng/g dry weight. Site P5 had the highest concentration of both groups of compounds (Table 2), whereas sediments from internal parts of the estuary had the highest concentrations of DDT (P1 = 1.89 ng/g and P17 = 1.15 ng/g).

Cytotoxicity

Sediments collected from P1, P4, and P10 had the highest cytotoxicity (EC50 between 53 mg eQsed/mL and 88 mg eQsed/mL), whereas no significant cytotoxicity was recorded in sediments collected from other areas. The highest concentration tested was 60 mg eQsed/mL (Table 3).

Table 2. Concentrations of organic compounds in sediments (ng/g dry sediment) and total organic carbon (mg C/g sed)

Sample	Σ PAHs ^a	Σ PCBs ^b	Σ 7 PCBs ^c	Σ HCHs ^d	Σ DDTs ^e	Σ CHLs ^f	Σ OCPs ^g	TOC
P1	73.98	0.34	0.31	0.64	1.89	0.16	2.81	84.95
P4	69.71	2.31	1.47	0.76	1.29	0.35	2.66	52.28
P5	89.14	7.59	5.92	12.99	nd	0.74	13.73	23.66
P7	83.11	0.13	0.12	0.45	0.62	0.30	1.38	25.62
P10	73.88	1.26	0.08	0.42	0.92	0.02	2.27	47.69
P12	50.47	2.95	nd	0.24	0.57	0.06	1.06	16.15
P16	36.92	0.87	nd	0.34	0.71	0.22	1.93	46.49
P17	37.27	0.37	nd	0.40	1.15	0.36	2.07	70.03

^aSum of 16 priority polycyclic aromatic hydrocarbons (PAHs).

^bSum of 51 congeners of polychlorinated biphenyls (PCBs).

^cSum of PCBs 28, 52, 101, 118, 138, 153, and 180.

^dSum of α -, β -, δ -, and γ -hexachlorocyclohexane isomers (HCHs).

^eSum of dichlorodiphenyldichloroethylenes, dichlorodiphenyldichloroethanes, and dichlorodiphenyltrichloroethanes.

^fSum of chlordane-related compounds (CHLs).

^gSum of organochlorinated pesticides (OCPs).

TOC = total organic carbon; nd = not detected.

Table 3. Contaminants measured in the sediments^a against classical sediment quality guidelines^b

	Average	Maximum	ERL	ERM	TEL	PEL
$\sum$ 7 PCBs	0.99 $\pm$ 1.4	1.47	22.7	180	21.6	189
$\sum$ DDT	0.89 $\pm$ 0.4	1.89	1.58	46.1	3.89	51.7
$\sum$ CHLs	0.28 $\pm$ 0.2	0.74	0.50	6.0	2.26	4.79
Dieldrin	nd	nd	0.02	8.0	0.72	4.30
γ -HCH	0.11 $\pm$ 0.1	0.23	nd	nd	0.32	0.99
Acenafthene	0.36 $\pm$ 0.1	0.63	16	500	6.71	88.9
Acenaphthylene	0.21 $\pm$ 0.1	0.68	44	640	5.87	128
Anthracene	0.37 $\pm$ 0.2	1.08	85.3	1100	46.9	245
Fluorene	0.15 $\pm$ 0.0	0.29	19	540	21.2	144
Naphthalene	2.60 $\pm$ 2.2	3.80	160	2100	34.5	391
Phenanthrene	0.82 $\pm$ 0.3	1.46	240	1500	86.7	544
Benzo[a]anthracene	0.48 $\pm$ 0.3	1.22	261	1600	74.8	693
Benzo[a]pyrene	2.30 $\pm$ 2.5	12.42	430	1600	88.8	763
Chrysene	1.60 $\pm$ 1.4	6.56	384	2800	108	846
Dibenzo[a, h]anthracene	0.11 $\pm$ 0.2	0.76	63.4	260	6.22	135
Fluoranthene	0.51 $\pm$ 0.2	0.96	600	5100	113	1494
Pyrene	0.50 $\pm$ 0.4	1.22	665	2600	153	1398

^aAveraged $\pm$ relative standard deviation and maximum values in ng/g dry sediment.

^bValues described by Macdonald et al. [24] and National Oceanic and Atmospheric Administration [25] in ng/g dry sediment.

ERL = effects range-low; ERM = effects range-median; TEL = threshold-effect level; PEL = probable effects level; PCBs = polychlorinated biphenyls; CHLs = chlordane-related compounds; HCH = hexachlorocyclohexane isomer; nd = not detected.

Generation of ROS

Production of ROS was recorded over 60-min exposure to sediment extracts. Sediments from all stations were able to generate ROS in PLHC-1 cells in the range of 1.5-fold to 2-fold induction (Figure 2). Cells exposed to the positive control (SIN-1) induced ROS up to 1.9-fold, 2.4-fold, and 4.2-fold, after 15 min, 30 min, and 60 min, respectively (data not shown).

EROD activity

Sediments from P4 had the highest ability to induce EROD activity (IC₅₀, 12 mg eQsed/mL), followed by those from P10 and P1 (IC₅₀ equal to 55 mg eQsed/mL and 61 mg eQsed/mL, respectively; Table 2 and Figure 3). In contrast, low EROD activity was recorded in cells exposed to sediments from P7, P12, P16, and P17 (<11 pmol/min/mg prot), which indicates the presence of very low concentrations of CYP1A inducers.

Ovarian CYP19 activity

All organic extracts strongly inhibited P450 aromatase enzyme activity (by 68–97%) when tested at a concentration of 60 mg eQsed/mL (Figure 4). The sites P17, P1, P16, P12, P7,

Figure 2. Generation of ROS after 15 min, 30 min, and 60 min of exposure to extracts of sediments at a concentration of 60 mg dry weight sediment equivalent extract/mL. Results are expressed as fold induction with respect to control cells (mean $\pm$ standard error of the mean, $n = 3$ assays). An asterisk (*) indicates significant difference from control cells.

and P4 had to the highest inhibition of enzyme activity (IC₅₀s ranged from 4.7 mg eQsed/mL to 7 mg eQsed/mL), whereas P10 and P5 had IC₅₀s of 16 mg eQsed/mL and 24 mg eQsed/mL, respectively.

Factorial analysis

The principal component analysis rearranged the set of data into 2 factors, which together explained 79% of the total variance. The predominant factor accounted for 50% of the variance and grouped the sediments from P1 and P4 on the positive end, whereas sediments from P5 were located on the extreme negative side of the axis. The second factor explained 29% of the variance and grouped the sediments from P7, P10, P12, P16, and P17 on the negative side, whereas it isolated P5 on the positive end (Figure 5). Overall, among the sediments tested, those collected from P1 and P4 (Antonina and Paranaguá harbors) showed the highest anthropogenic impact. An association among CYP1A, CYP19, and CFDA assays, as well as the presence of PAHs and CHLs in samples, was detected (Figure 5).

DISCUSSION

The chemical concentration data were compared with values indicated by sediment quality guidelines, which define critical

Figure 3. Ethoxyresorufin-O-deethylase (EROD) activity in PLHC-1 cells after 24 h of exposure to sediment extracts. Results are expressed in pmol of resorufin/min/mg protein as mean $\pm$ standard error of the mean of at least 3 assays. Dotted line represents positive control (β -naphthoflavone [BNF]).

Figure 4. In vitro interference of sediment extracts from the Paranaguá Estuary on microsomal ovarian P450 aromatase activity (2 dry weight sediment equivalent extract [eQsed]/mL, 10 mg eQsed/mL, 20 mg eQsed/mL, and 60 mg eQsed/mL) in sea bass tests. Values are mean $\pm$ SEM ($n = 3$). An asterisk (*) indicates significant difference relative to control ($p < 0.05$).

levels of organic compounds and classify them according to expected effects on organisms such as effects range-low, effects range-median, threshold-effects level, and probable-effects level [24,25]. All values were below the limits, and therefore, there was no indication of any effect for biota according to the limits defined by the sediment quality guidelines for PAHs, PCBs (sum of 7 congeners: 28, 52, 101, 118, 138, 153, and 180), DDTs, CHLs, and dieldrin (Table 3). In terms of organic carbon concentration, sediments from P1 were the only ones close to the alert value of 10% (TOC = 8.5%).

Some compounds were positively correlated, indicating the same origin or source in the environment. The HCHs were

positively correlated with PCBs (0.91, $p < 0.05$) and CHLs (0.84, $p < 0.05$). Site P5, located near the Itibere River, had the highest levels of these compounds, demonstrating its negative influence on sediment quality. Nevertheless, DDTs did not show any correlation with the other pollutants. They were mainly detected at the inner parts of the estuary (Antonina and Laranjeiras Bay), where it might receive the influence of upstream agricultural activities.

The bioassays selected allowed assessment of sediment quality from another perspective: whether the mixture of organic contaminants trapped in the toxic sediments contained significant amounts of cytotoxic and/or endocrine disrupter compounds. The highest cytotoxicity was associated with areas influenced by harbor activities, namely, sediments next to Antonina and Paranaguá harbors (P1 and P4, respectively), followed by sediment extracts from P10, an area that is affected by commercial shipping. Interestingly, sediments from P10 showed higher cytotoxicity with Alamar Blue than with CFDA-AM, suggesting a higher ability of the toxicants to impair mitochondrial activity than membrane stability of PLHC-1 cells (Table 4).

Extracts from P1, P4, and P10 also had the highest content of CYP1A inducers, with EC50s in the range of 12 mg to 61 mg eQsed/mL. In contrast, P5, P7, P12, P16, and P17 contained a rather small amount of CYP1A inducers. Chemicals such as PAHs and PCBs can induce EROD activity in fish [26]. However, in the natural environment, fish are exposed to complex mixtures of pollutants that can act as both inducers and inhibitors [23,27]. Estrogens such as estradiol and octylphenol, for example, have a modulating effect on the expression of CYP1A in fish, inhibiting EROD activity [28].

Figure 5. Factor analysis with principal component extraction: principal component 1 (PC1) versus principal component 2 (PC2) biplot. Blue triangles represent loadings for biological and chemical variables; red diamonds represent site scores: Paranaguá Estuary (P1, P4, P5, P7, P10, P12, P16, and P17). HCHs = hexachlorocyclohexane isomers; TOC = total organic carbon; PAHs = polycyclic aromatic hydrocarbons; CHLs = chlordane-related compounds; PCBs = polychlorinated biphenyls.

Table 4. Summarized toxicological data for sediments collected from Paranaguá Estuarine Complex^a

Site	AB ^b	CFDA ^b	EROD ^c	CYP 19 ^d	ROS ^e
P1	53.0 ± 5.4	74.5 ± 4.4	61.1 ± 1.4	5.5 ± 1.5	1.66 ± 0.0
P4	64.6 ± 3.8	87.0 ± 2.0	12.0 ± 1.3	7.10 ± 1.3	1.47 ± 0.1
P5	nd	nd	nd	23.66 ± 1.8	1.87 ± 0.1
P7	nd	nd	nd	6.58 ± 1.9	1.62 ± 0.1
P10	87.5 ± 6.2	nd	54.6 ± 1.4	16.30 ± 2.2	1.93 ± 0.2
P12	nd	nd	nd	5.65 ± 1.9	1.73 ± 0.1
P16	nd	nd	nd	5.62 ± 1.7	1.64 ± 0.1
P17	nd	nd	nd	4.69 ± 1.8	1.85 ± 0.0

^aValues are mean ± standard error of the mean of at least 3 independent experiments.

^bMedian effective concentration (EC50) expressed in mg eQsed/mL in the cytotoxicity assay (AB, CFDA).

^cConcentration of sediment required to induce a response equivalent to 1 µM β-naphthoflavone.

^dMedian inhibitory concentration (IC50) for ovarian CYP19 assay.

^eReactive oxygen species (ROS) generation after 15-min exposure to 60 mg eQsed/mL.

AB = Alamar Blue; CFDA = 5-carboxyfluorescein diacetate acetoxy-methyl ester; nd = not detected.

The production of ROS may be increased in the presence of natural or synthetic compounds derived from urban, industrial, or agricultural activities [29]. The potential anthropogenic compounds capable of promoting ROS generation are those that undergo redox cycles (quinones, aromatic compounds, nitroamines, bipyridyls herbicides), PAHs (benzene, PAH oxidation products), halogenated hydrocarbons (bromobenzene, dibromomethane, PCBs, lindane), dioxin, pentachlorophenol, and metal pollutants [30]. In general, ROS generation in exposed cells was similar in all other sediments (<2-fold induction), suggesting similar exposure to ROS-generating compounds. Nonetheless, sites P1, P5, and P12 showed slightly higher values. These sites are located near Antonina, Paranaguá, and Pontal do Paraná cities, respectively. Sediments from P5 were collected near the mouth of the Itibere River, which flows through Paranaguá City. In turn, P12 sediments were collected next to the discharge of an artificial drainage channel (DNOS), which received sewage entering from Pontal do Sul City. The external sector of the channel is dominated by marinas and boat traffic. It has been determined that both places suffer from the impact of untreated urban sewage [31,32].

Various anthropogenic pollutants act as endocrine disruptors (e.g., PCBs, pesticides, pharmaceuticals, personal care products) by altering the expression and biosynthesis of steroids. Bioassays using microsomal fractions of ovarian sea bass have been previously used to detect the presence of endocrine disruptors in marine sediments [4,12]. This test detects agents that modulate aromatase activity (CYP19), which is involved in processes of sexual differentiation, embryo growth, and maturation. Inhibition of CYP19 activity detected in sediments from this estuary indicates the presence of potential endocrine-disrupting chemicals (68–83% at 60 mg eQsed/mL). A similar range was reported for sediments from the Arade (65–92% at 60 mg eQsed/mL) and Guardiana (22–91% at 60 mg eQsed/mL) estuaries [4]. Pérez-Albadalejo et al. [12] also reported inhibition of CYP19 activity by sediment extracts from the Adriatic and Black Sea, with inhibition slightly greater in most places (up to 54% by 2 mg eQsed/mL).

Sediments from internal areas of the Paranaguá Estuary sites P1 and P17 inhibited ovarian CYP19 activity more efficiently (up to 31% inhibition by 2 mg eQsed/mL), suggesting the presence of potential endocrine disruptors at these sites. In

regard to the compounds analyzed, P1 and P17 showed the highest concentrations of DDTs (o,p'-DDT and p,p'-DDE) among all sediment samples. Site P1 also had the highest concentration of the PAH benzo[a]pyrene BaP (12.4 ng/g). The compounds DDT and BaP are estrogenic chemicals, which may interfere with the endocrine system of fish. The presence of DDT has led to reduced fecundity or larval survival. It induces vitellogenesis in male fish and adversely affects the endocrine system [10]. Also, fish exposed to BaP showed decreased androstenedione and estradiol synthesis through inhibition of cytochrome P450 17,20-lyase, 17β-hydroxysteroid dehydrogenase, and cytochrome P450 aromatase (CYP19) in different in vitro ovarian steroidogenic enzyme systems [33].

Sites P5 and P10 had the lowest ability to inhibit the activity of CYP19, even though P5 showed high concentrations of β-HCHs (12.99 ng/g), which are recognized endocrine disruptors [10]. Site P5 is greatly influenced by urban discharges. It showed the highest concentration of PCBs, although concentrations of the toxic planar congeners 77, 126, and 169 were low.

In sum, the toxicological results showed that P1 and P4 seem to be the most degraded areas at the estuary. The least contaminated area was P7. Sediments from P16 and P17, situated north of Laranjeiras Bay, were slightly contaminated according to the results from cytotoxicity, ROS generation, and EROD induction tests. In terms of CYP19 inhibition, both sites, especially P17, were highly affected by endocrine disruptor substances.

A few studies have used toxicological assays in the Paranaguá Estuary, but none included evaluation of endocrine disruptors. Morphological (liver and gill histopathology) and genetic (piscine micronucleus and DNA strand breaks) biomarkers measured in the demersal catfish *Cathorops spixii* revealed the presence of damage in fish from both reference and contaminated sites inside Paranaguá Bay [34]. Fish (*Atherinella brasiliensis*) from areas influenced by port and urban activities of the Bay showed levels of PAHs in bile, somatic indexes, activity of catalase, and genetic parameters characteristics of areas impacted by hazardous pollutants, although on a minor scale [35].

With regard to sediment toxicity, another study also found a gradient of increased toxicity toward the inner region of this estuary [13]. The area next to Antonina (equivalent to P1) was classified as highly toxic, mainly through a high concentration of metals. Also, the inner part of the north–south axis (where low toxicity was expected because of its location away from the shipping channel and ports) was only moderately impacted toxicologically, similar to what was observed in the present study (P16, P17). At sites where circulation is limited, such as internal areas, the higher residence time may promote accumulation of toxic compounds in sediments, as was reported for tributyltin (TBT) [36].

Principal component analysis was used to group sediments according to the response in the different bioassays and the levels of pollutants. The ROS assay was not included in the principal component analysis because the response was very similar at all sites (<2-fold induction). The first component further explains the variables isolating P5 on the negative side and grouping P1 and P4 on the positive side (more impacted). The analysis groups P7, P12, P16, P10, and P17 are in a central zone, suggesting that these sites are not as impacted as P1 and P4, even though they are not clean.

In addition, according to the principal component analysis, the effects shown in CYP1A, CYP19, and CFDA assays were associated with the presence of PAHs and CHLs in sediments.

This is in agreement with the fact that PAHs, mainly those of higher molecular weight, can activate CYP1A (EROD activity) by binding with the aryl hydrocarbon receptor (AhR) [26,37,38]. Moreover, other chemicals could be also responsible for EROD induction (i.e., planar chemicals such as halogenated dioxins and biphenyls) [38]. Inhibition of P450 aromatase activity by HCHs, fungicides, and PAHs has been reported in rainbow trout ovarian microsomes, human placental JEG-3 cells, and transfected human embryonic kidney 293 cells [10,39,40]. Overall, among all the sediments tested, those collected near harbors (P1 and P4) showed the highest anthropogenic impact. Sites P7, P10, P12, P16, and P17 showed less anthropogenic impact, in agreement with the fact that those sediments had fewer chemical pollutants, causing a minor response in the biological systems assayed.

CONCLUSIONS

Although chemical analysis of the sediments indicated low levels of organic compounds, and there was no evidence of negative effects based on guideline levels, the information provided by in vitro bioassays allowed the detection of significant amounts of cytotoxic compounds and endocrine disruptors in sediments from the estuary. The integrated use of both tools can be extremely useful for recognizing areas with different degrees of impact and sediment quality.

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Data availability—Data used for the present study are available on request to the corresponding author (juliane.rizzi@gmail.com).

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