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Liver damage in two neotropical fish species from a polluted estuarine area

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Abstract

A multi-biomarker approach was used to evaluate the liver health of two Neotropical fish species (*Cathorops spixii* and *Atherinella brasiliensis*) that inhabit two different sites of the Paranaguá bay (Paraná – Brazil) and a reference site in the Garatuba bay. Fish were sampled during summer and winter, so the variation of the responses can reflect the actual conditions of sampling sites. Data showed that fish from both sites of the Paranaguá bay are affected by the presence of pollutants from different sources. Both fish species presented adverse biomarker responses mainly in the summer, probably due to the increased human population during the period. Chronic effects in the liver related with the contamination. Thus, the results demonstrated that pollution in Paranaguá bay induce liver damage in fish that inhabit this area.

Key words: Multi-biomarker; Paranaguá; Metal; Polycyclic aromatic hydrocarbon; Seasonal

INTRODUCTION

Biomarkers are measurements of alterations in different levels of biological organization of a bioindicator (such as genetic, biochemical, and histopathological) and indicate early signs of exposure to toxic agents that result from anthropogenic activities in impacted environments (Van der Oost *et al.*, 2003). Genetic biomarkers such as the comet assay and DNA diffusion assay accounts for the DNA integrity and its function (Singh *et al.*, 1988; Singh, 2000). Biochemical alterations are usually the first responses detected as result of environmental variation and are mostly related to biotransformation and oxidative stress (Van der Oost *et al.*, 2003). Histopathology, when in target organs or tissues, is able to detect acute and chronic exposure of individuals to xenobiotics (Hinton *et al.*, 1992). It is possible to measure all these biomarkers of fish health in the liver, which is the target organ for contaminants as it performs biotransformation, bioaccumulation and excretion of toxicants (Schlenk, 2005).

The Paranaguá bay, nursery of a high diversity of fish species, is near to the biggest preserved area of Atlantic Rain Forest in Brazil. Nonetheless, anthropogenic actions from different sources historically affected this area, e.g., domestic and industrial sewage, agricultural and harbor activities (Marone *et al.*, 2005). Fishing activities, tourism, industries, fuel and grain terminals (the largest in Latin America) drive the economy of this region (Martins *et al.*, 2010). The Paranaguá harbor is the largest in southern Brazil, handling more than 44 tons of cargo in 2012 (APPA, 2015). The loading and unloading of containers and grains frequently requires removal of a large amount of sediment that may result in resuspension of several compounds, including toxic ones (Marone *et al.*, 2005). High concentrations of DDT, PAHs, metals and metalloids (mainly As, Hg, Cr, Cu, Ni and Pb),

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fecal steroids, and organotin compounds have been found in the sediment biota of the estuary (Sá *et al.*, 2006; Choueri *et al.*, 2009; Santos *et al.*, 2009; Martins *et al.*, 2010; Santos *et al.*, 2014). The region is home of several marinas, docks, and shipyards that receive numerous leisure boats, especially during summer, as well as fishing boats that represent sources of livelihood for many coastal communities. Guaratuba bay, used in this study as reference site, is a touristic area and commonly known for low-scale fishing, angling, banana and citrus cultivation, and mariculture (Mizerkowski *et al.*, 2012). Even so, the levels of contamination found are acceptable for the activities aforementioned (Combi *et al.*, 2013; Rodrigues *et al.*, 2013; Madi *et al.*, 2015; Prodócimo *et al.*, 2015).

In the Paranaguá bay, fishing, an important source of income for the population, has had troubles arising from the contamination of the region, mainly related to port activity. Among the species that inhabit this area, the yellow catfish (*Cathorops spixii)* is an abundant and economically important resource. Another relevant species of the bay is the Brazilian silverside (*Atherinella brasiliensis)*, which is a typical estuarine resident species, also described as one of the most abundant fish in shallow estuarine areas of southeastern Brazil (Pessanha & Araújo, 2001). Due to their characteristics, both species are frequently used as bioindicators in ecological and environmental monitoring studies (Azevedo *et al.*, 2009; Katsumiti *et al.*, 2009; Valdez Domingos *et al.*, 2009, Fernandez *et al.*, 2011; Souza-Bastos & Freire, 2011, Oliveira Ribeiro *et al.*, 2013, Santos *et al.*, 2014).

The aim of the current study was to perform a multi-biomarker approach in the liver of two estuarine fish species (*Cathorops spixii* and *Atherinella brasiliensis*) to assess the effects of the pollution from Paranaguá bay. For this purpose, we assessed the genotoxicity, oxidative stress, metabolic processes (phase I and II enzyme activities), metallothioneins expression, and histopathological findings. Additionally, we obtained the biometric indexes and chemical analysis of metals and As in target organs and PAHs metabolites quantification in bile.

MATERIAL AND METHODS

Fish sampling

Adult *C. spixii* (16.6 ± 1.85 cm body length, 35.09 ± 12.05 g body mass) were sampled using a bottom otter trawl, and adult *A. brasiliensis* (10.13 ± 0.57 cm body length, 5.65 ± 1.03 g body mass) using a beach trawl. We sampled 100 individuals from each species (20 per site and season) in the summer (February/ 2012) and winter (August/ 2012) in the same two sites of the Paranaguá bay and during winter (August/ 2013) in the reference site (Figure 1). The first site (HB) is near the Paranaguá harbor. The second site (CO) is around the city of Pontal do Paraná, next to the coast of Paraná. These two sites (HB and CO) of Paranaguá bay are known to be impacted by harbor activities and human occupation (Sá *et al.*, 2006; Choueri *et al.*, 2009; Santos *et al.*, 2009; Martins *et al.*, 2010; Santos *et al.*, 2014). The reference site (RF) is on the Guaratuba bay, a remarkably non-impacted bay, as described by several recent studies (Combi *et al.*, 2013, Rodrigues *et al.*, 2013, Madi *et al.*, 2015, Prodócimo *et al.*, 2015). We transported the fish to a research station right after the sampling, and obtained the total weight (TW) and length (TL). The euthanasia of fish occurred by immersion in a lethal concentration of benzocaine, then the liver, biliary vesicle and muscle were removed. We maintained a liver subsample in fetal bovine serum for 24 h to perform the comet assay, and another subsample in liquid nitrogen to perform the biochemical and chemical analyzes. The Ethics Committee on Animal Use at the Federal University of Paraná approved this study (n ° 23075.000756/2012-22).

Biomarkers

We used liver tissue for the genotoxic assessment of hepatocytes through the comet assay (CA) and DNA diffusion assay (DA), to measure activities of ethoxyresorufin-O-deethylase (EROD) and glutathione S-transferase

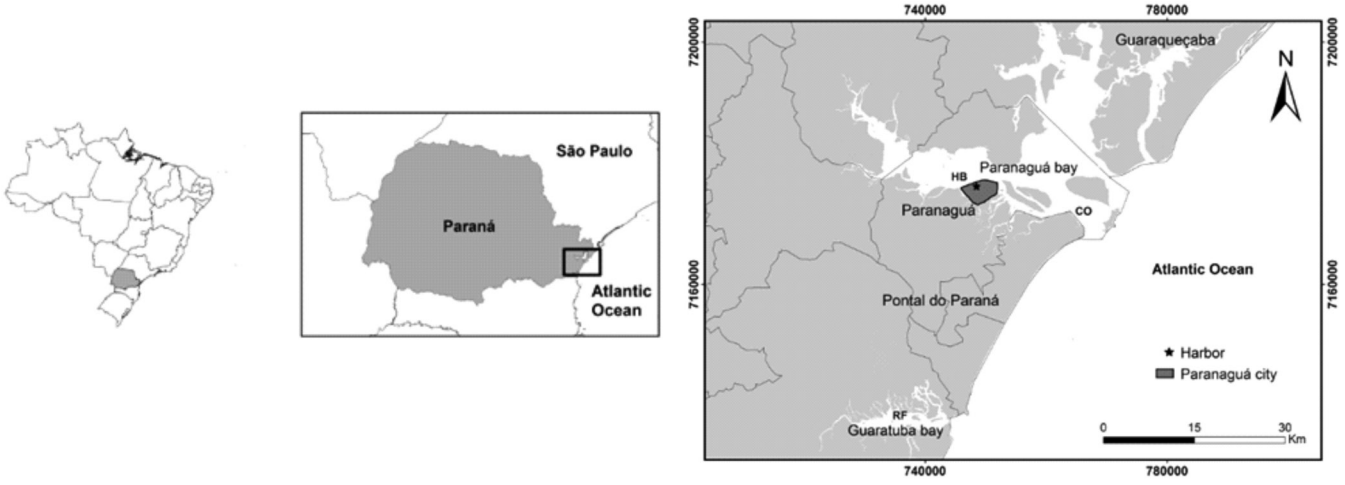

Fig. 1 Map of Paranaguá and Guaratuba bays showing sites. Harbor (HB), Pontal do Paraná coast (CO), and Reference site (RF).

(GST), for the quantification of peroxidized lipids (LPO), metallothioneins (MT) and for histopathological analyzes.

Genetic biomarkers

First, we preserved and homogenized the tissue in fetal bovine serum, diluted 10 µL of this homogenate in LMP agarose gel spread on the base layer of a slide and covered with ultrapure agarose (Ramsdorf *et al.* 2009). We place the slides in a lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 0.8% NaOH, 1% N-lauryl-sarcosinate, 1% Triton X-100 and 10% DMSO) for 72 h at 4°C. After this period, they were immersed in a NaOH (10 M) and EDTA (200 mM), pH > 13 solution for 25 minutes for DNA denaturation and subjected to electrophoresis at 300 mA and 25 V for 25 minutes. We neutralized the slides with 0.4 M Tris, pH 7.5, fixed with ethanol for 10 minutes and stained with ethidium bromide 0.02 g mL^{-1} (Singh *et al.*, 1988). We scored 100 comets per individual using a Leica epifluorescence microscope DMLS2 into one of five damage classes.

DNA diffusion assay assessed frequency of apoptotic cells in liver cells based on the same protocol for comet assay with the omission of electrophoresis (Singh, 2000). We scored 100 nucleoids per individual into normal and apoptotic cells.

Biochemical biomarkers

We homogenized the liver samples in phosphate buffer with pH 7.0 (liver), pH 7.5 (muscle). The centrifugation of liver was at 15000 x *g* for 30 min at 4 °C, and muscle at 10000 x *g* for 20 min at 4 °C. We stored the supernatants at -80 °C. We combined liver from two individuals after preparation to test the activity of EROD, GST, levels of LPO and MT. We determined the protein concentration with bovine serum albumin as standard (Bradford, 1976).

EROD activity: we kept 50 µl of sample and 200 µl of reaction solution (0.1 M Tris / pH 7.5, 0.1 M NaCl, 3 mM 7-ethoxy-resorufin) in a microplate and incubated for 5 min. After incubation, we added 10 µL of NADPH (3 mM). We used a wavelength of 530 nm (excitation) and 590 nm (emission) for 10 min at 27 ° C in the analysis (Webb *et al.*, 2008).

GST activity: we kept 20 µl of supernatant and 180 µL of reaction medium (3.0 mM GSH, 3.0 mM CDNB, 0.1 M potassium phosphate buffer, pH 6.5) in a 96-well microplate. Absorbance increase measurement was at 340 nm at intervals of 15 s, and we used the extinction coefficient for CDNB of 9.6 mM^{-1} cm^{-1} to calculate the enzymatic activity (Keen *et al.*, 1976)

Levels of LPO: we kept 30 µl of supernatant in a microplate, with four replica. Then, each microplate received 270 µl of reaction solution (Xylenol Orange at 100 mM, 25 mM H_2SO_4, BHT (Butylated hydroxytoluene) at 4 mM, $FeSO_4NH_4$ (Ammonium Ferrous Sulfate) at 250 mM, added to methanol 90%). We analyzed the samples in a Synergy HT spectrophotometer at 570 nm. Expression of the results were in concentration of hydroperoxide (µmol mg $protein^{-1}$). We evaluate the levels of LPO by assessing the concentration of hydroperoxides in the FOX assay (Ferrous Oxidation/ Xylenol Orange Method) (Jiang *et al.*, 1992).

Metallothioneins-like protein (MT): we homogenized the liver samples in buffer (0.5 mM PMSF (phenylmethylsulphonyl fluoride), 0.01% β-mercaptoethanol, 20 mM Tris-HCl, 500 mM sucrose, pH 8.6) and centrifuged at 15000 x g for 30 min at 4 °C. We mixed 300 µl of supernatant and 342 µL of ethanol-chloroform and centrifuged at 6000 x g for 10 min at 4 °C. Then, we mixed 490 µl of the new supernatant with 1502 µl of ethanol-HCl and maintained the mix at -20 °C for 1 h. After this period, we centrifuged the tubes (6000 x g for 10 min, 4 °C) and suspended pelleted proteins in 50 µl of NaCl at 250 mM. We added 50 µl of EDTA solution (4 mM EDTA-2Na 4mM, 1M HCl) and 1000 µl of Ellman's solution (2 mM DTNB, 0,2 M phosphate sodium, 2 M NaCl) to the tubes and centrifuged (3000 x g for 5 min) to determine the absorbance at 412 nm. The determination of sulfhydryl content was through comparison with a glutathione curve and metallothionein-like protein concentration considering the concentration of cysteine-metallothionein of 30%, described for mussels (Viarengo *et al.*, 1997).

Liver histopathology (LHP)

We chemically preserved a liver subsample in ALFAC (85% ethanol, 10% formalin, and 5% acetic acid) for 16 h and kept it in 70% ethanol until embedding at 4 °C. We dehydrated the samples in a graded series of alcohol concentrations (80%, 90%, 95% and 100%) and embedded in Paraplast Plus (Sigma/ Aldrich®). We obtained 5 µm thickness sections in a Leica microtome and stained with hematoxylin and eosin. Image records were taken on a digital camera attached to a Leica light photomicroscope. Main findings evaluated in liver: circulatory disturbances (hemorrhage), regressive changes (necrosis), inflammation (leukocyte infiltration), progressive changes (hypertrophy and hyperplasia), tumor (benign and malign tumor), presence of melanomacrophage centers (MMC), and parasites (Oliveira Ribeiro & Narciso, 2013).

Bile polycyclic aromatic hydrocarbons metabolites

We pooled bile of 3 individuals and stored in amber glass vials at -80 °C. Dilutions of bile samples were diluted (1:1000) in 48% methanol. They were then added onto 96-well black microplate for PAHs detection through different excitation/ emission wavelengths (288/330 naphthalene-type, 334/376 pyrene-type, 364/406 benzo(a)pyrene-type, and 380/422 benzo(ghi)perylene-type), corresponding to the number of rings of each compound (2, 4, 5, and 6 rings, respectively) in spectrofluorimeter Shimadzu RF-6000. For quantification, we used the PAH mix (Cod. 47930-U, SUPELCO) to establish a PAHs standard curve (Hanson *et al.*, 2009). Given the small size and fragility of the biliary vesicle of these fish, this analysis was only qualitative.

Metal analysis

We performed the metals and As analysis only in fish sampled from Paranaguá and Guaratuba during the summer. We determined total Arsenic (As), Lead (Pb), Tin (Sn), and Mercury (Hg) using inductively coupled argon plasma optical emission spectrometry (ICP OES - Varian 720-ES). We homogenized about 500 mg of fish liver and muscle and digested with a mixture of nitric acid (65 %) and hydrogen peroxide (5 %) for 20 minutes. Analytical lines were 193.696 nm for arsenic (As), 220.353 nm for lead (Pb), 189.925 nm for tin (Sn), and 253.652 nm for mercury (Hg) with the following limits of quantification: As (0.02 mg kg^{-1}), Pb (0.002 mg kg^{-1}), Sn (0.002 mg kg^{-1}), and Hg (0.01 g kg^{-1}). We established the detection limit and reference value based on Decree No. 55 871 (Brazil, 1965), and reported all metal and As concentrations in fish tissues in mg kg^{-1} wet weight.

Statistical analysis

We checked all data for normality and homoscedasticity before performing two-way analysis of variance. When we did not achieve these parameters, we performed the nonparametric Kruskal–Wallis analysis followed by Dunn's post-hoc test with a level of significance of 0.05.

For a multivariate statistical approach, we conducted a log (x+1) transformation to let all biological data with equal importance. We made a principal component analysis (PCA) to verify the interdependence among sites, seasons, and biomarkers responses in both fish species. For this, we used only the individuals that had all data for biomarker responses. We determined the number of interpretable components of PCA through the Kaiser-Guttman criterion.

RESULTS

Biomarkers responses in Cathorops spixii

Regarding the genetic analysis, scores of CA of fishes from the reference site were significant different from CO (summer) and both sites in the winter. Fish from both sites presented higher values for the DA than the RF site in the summer; in the seasonal comparison, we observed higher frequency of apoptosis in fish from HB in the summer (Figure 2 A-B).

Regarding the metabolic enzymes, EROD activity did not differ among sites or seasons, while the activity of GST was significant higher in fish from RF than the ones sampled in

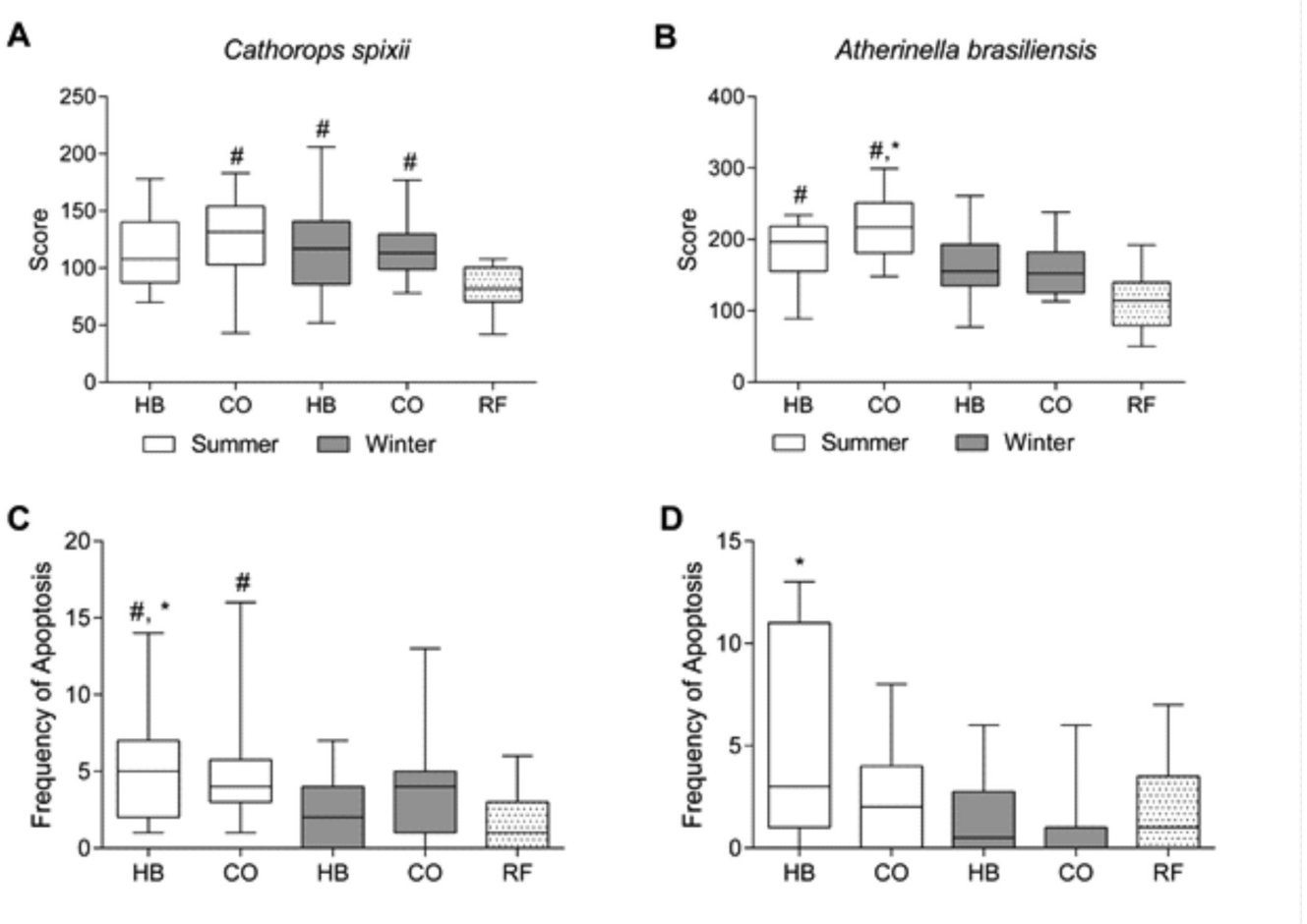

Fig. 2 Genetic biomarkers in *C. spixii* and *A. brasiliensis*. A and B – Comet assay in liver (H = 14.4 and 41.41); C and D – Diffusion assay in liver (H = 21.91 and 14.59). * indicate seasonal differences between sites; # indicate differences to the reference site.

both sites during winter, as well as higher in CO (summer) in comparison to CO (winter) (Figure 3 A-C). We did not observe significant differences in concentrations of peroxidized lipids (Figure 3 E). Finally, fish from CO (summer) had significant higher concentrations of metallothioneins than fish from CO (winter) (Fig. 3 G).

Fish from HB (summer) had higher histopathological index than fish from RF. Individuals from all sites and seasons presented hemorrhages, leukocyte infiltrations (Figure 4 B), melanomacrophage centers (Figure 4 D), parasites (Figure 4 E), and necrosis. In the summer sampling, fish from HB presented high incidence of vacuolization (Figure 4 C), while fish from CO had hemorrhages, MMC, parasites, necrosis (Figure 4 F-G), and parasites (Figure 4 H). On the other hand, fish from the winter sampling presented high incidence of leukocyte infiltrations and necrosis in HB, while fish from CO had extensive MMC and necrosis (Table 1).

Biomarkers responses in Atherinella brasiliensis

CA scores of fish from both sites in the summer were significant different from RF (Figure 2 C). We observed significant higher CA scores in fish from CO (summer) in comparison to fish from CO (winter). Finally, DA showed a higher level of apoptosis in fish from HB (summer) in comparison to fish from HB (winter) (Figure 2 D).

Metabolic biomarkers showed that the activity of EROD was significant higher in fish from HB (summer) when compared to fish from CO (summer) and HB (winter) (Figure 3 B). The GST activity of fish sampled during summer from both sites and RF were significant higher than the fish from both sites sampled during winter (Figure 3 D). Concentrations of peroxidized lipids in HB (summer) were significant higher than RF (Figure 3 F). The concentration of metallothioneins was significant higher in fish from HB (summer) than in fish from HB (winter) and RF (Figure 3 H).

Histopathological index was significant higher in CO (winter) than RF. In general, hepatic parenchyma of *A. brasiliensis* was more basophilic in comparison to *C. spixii* (Figure 4 A, Figure 5 A). Individuals from all sites and seasons presented hemorrhages (Figure 5 B), vacuolization (Figure 5 C), and necrosis. In the summer sampling, fish from HB presented high incidence of hemorrhages and vacuolization, while fish from CO presented vast necrotic areas. Nevertheless, during the winter sampling, fish from HB presented high incidence of MMC (Figure 5 D) and neoplastic cells, while fish from CO showed MMC, parasites (Figure 5 E), and necrosis (Figure 5 F) (Table 1).

Bile polycyclic aromatic hydrocarbons metabolites

It was possible to verify the presence of PAHs metabolites in all sites and seasons, especially those of two and four rings (Table 2). Two-ringed PAH concentration was higher in all Paranaguá samples in comparison with RF for both species. For *C. spixii,* four and five ringed PAHs concentration was higher in HB (summer and winter) than RF. We observed six-ringed PAHs in both fish species from HB site.

Metals analysis

Concentrations of As in liver and muscle of both fish species from Paranaguá sites were higher than the values observed in fish from RF. Pb was not detected in any fish during this study. Sn was observed mainly in the liver of *A. brasiliensis* from HB, although it was observed in liver of both fish species from CO. Small concentrations of Hg in liver and muscle from *C. spixii* from all sites were observed, whereas this toxicant was detected in muscle from *A. brasiliensis* from RF and HB (Table 3).

Multivariate analysis

We made the principal component analysis (PCA), based on individual variables and biomarkers (CA, DA, EROD, GST, LPO, MT, and LHP) to acquire an unified view of the responses of both species. Three principal components were necessary to explain *C. spixii* data (Figure 6, left). Principal component 1 (PC1) accounted for 30.6% of the variability in data. This PC was negatively loaded by CA, DA, GST, MT, and LHP and positively loaded by EROD. PC2 accounted for less variability in data (27.3%), being negatively loaded by DA, EROD, and LHP and positively loaded by GST, LPO and MT. PC3 accounted for 16.9% of the variability in data, being negatively loaded by CA, DA, GST, and LHP and positively loaded for EROD and MT.

Three principal components were necessary to explain the PCA data for *A. brasiliensis* (Figure 6, right). PC1 accounted for 40.2% of the variability. This PC was only positively loaded by DA, EROD, and GST. PC2 accounted for 21.4% of the variability in data being most negatively loaded by CA, DA, and LHP and positively loaded by EROD. PC3 accounted for 17.6% of the variability in data, being negatively loaded by DA and GST and positively loaded for CA, EROD, LPO, and LHP.

DISCUSSION

We conducted the study to investigate liver damages in two fish species sampled in two distinct sites of the Paranaguá bay in southern Brazil, in comparison with a reference site. It also sought to understand how the anthropogenic occupation affects these organisms. For that, we performed a multi-biomarker approach and chemical analysis. Regarding the anthropogenic occupation of the area, the HB site represented by the Paranaguá harbor, has a large turnover of ships and shipments, while the CO site is represented by the city of Pontal do Paraná, which is a seaside town that, in the summer, becomes home of many transient inhabitants (more than 300 000). In general, data showed that the presence of chemicals in this area affected the fish, as observed in previous studies (Valdez Domingos *et al.*, 2009; Katsumiti *et al.*, 2009; Oliveira Ribeiro *et al.*, 2013; Santos *et al.*, 2014). The analysis

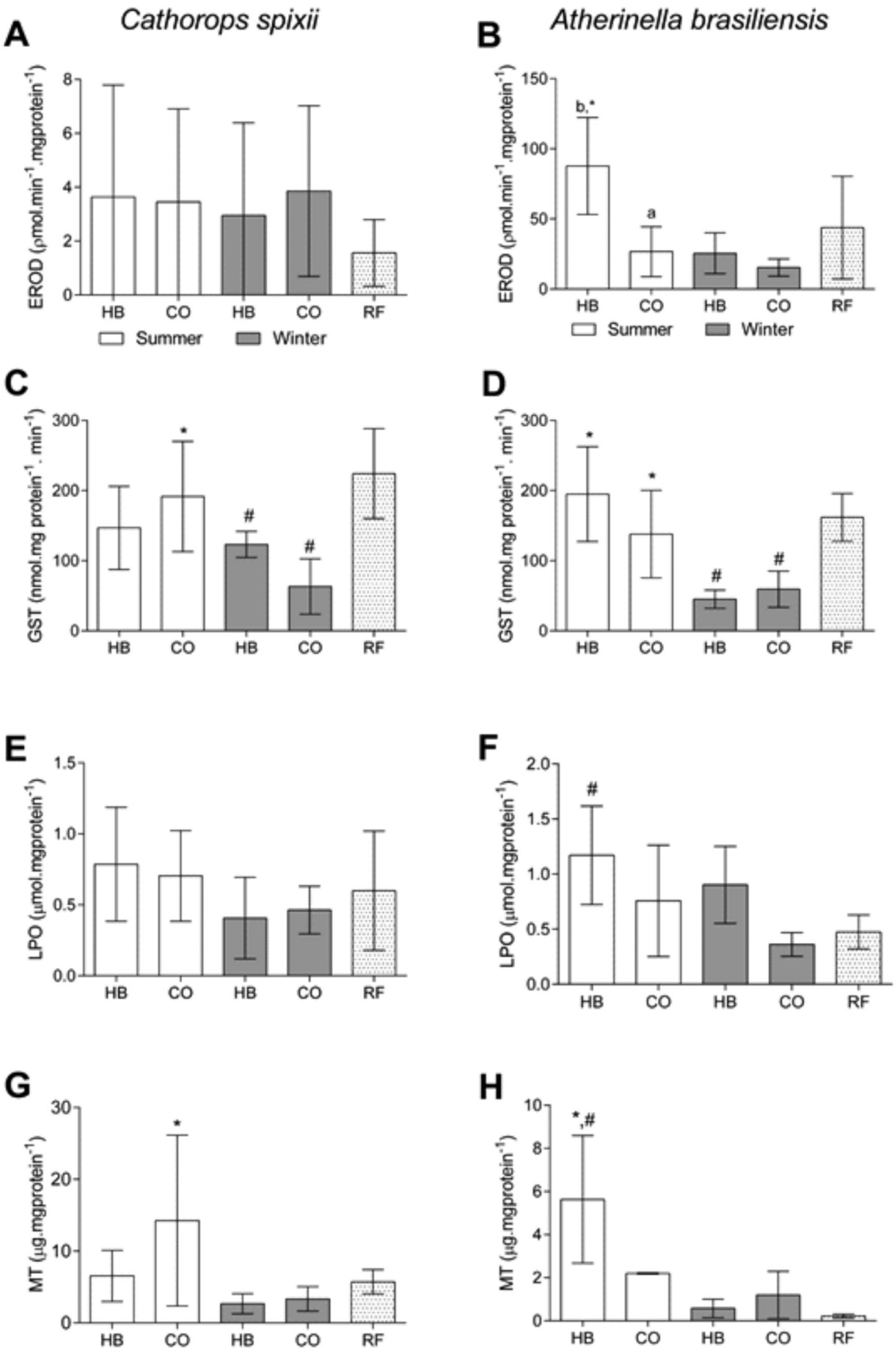

Fig. 3 Biochemical biomarkers in *C. spixii* and *A. brasiliensis*. A and B – EROD activity (H = 2.799 and 22.18); C and D – GST activity (H = 27.53 and 33.23); E and F – LPO (H = 7.289 and F = 5.401); G and H – MT (H = 16.27 and 14.77). Letters indicate significant differences between sites in the summer (a, b); * indicate seasonal differences between sites; # indicate differences to the reference site.

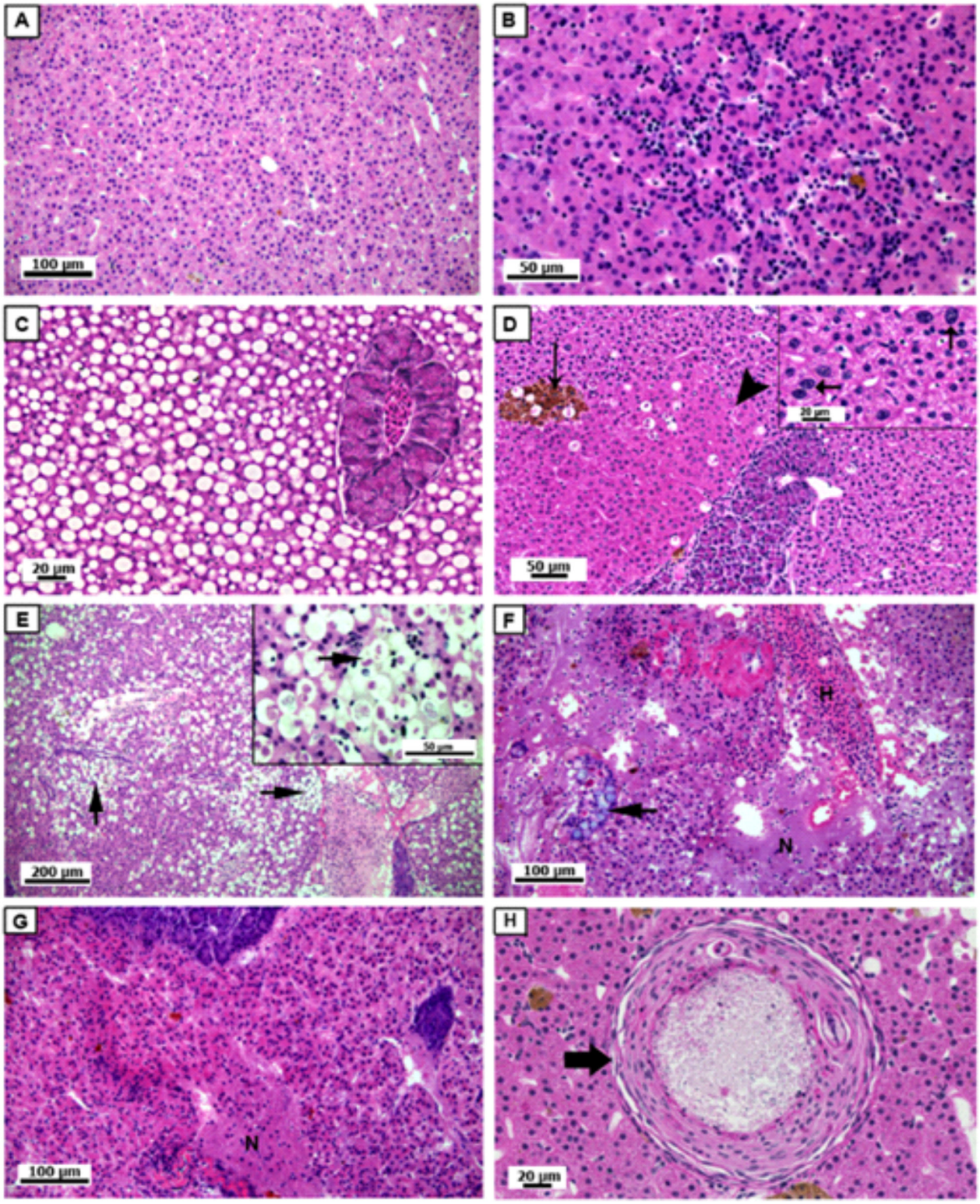

Fig. 4 Liver cross section from *C. spixii* sampled in Paranaguá Bay. A – Hepatic parenchyma without significant abnormalities. B – Leucocyte infiltrations. C – Steatosis. D – Eosinophilic foci (arrowhead) with hypertrophied hepatocytes, and presence of melamomacrophage centers (arrow) associated with unicellular parasites. Insert – Detailed pleomorphic nucleus (arrow) from hypertrophied hepatocytes. E – High incidence of unicellular parasites in hepatic parenchyma. Insert – Detail of parasites, note the difference between parasites and steatosis. F, G – Large area of necrosis (N). H – Neoplastic cells with architectural and structural alterations in hepatocytes.

suggests that the presence of pollutants in these sites are related to human activities which, in turn, have a close relation to the seasonal variation, since these activities are remarkably pronounced during the summer.

Therefore, in the summer, we observed a distinct response for both sites concerning the species under analysis. Most of the discharge occurs through irregular sewage outfalls that flow right next to the substrate, from the coast to the

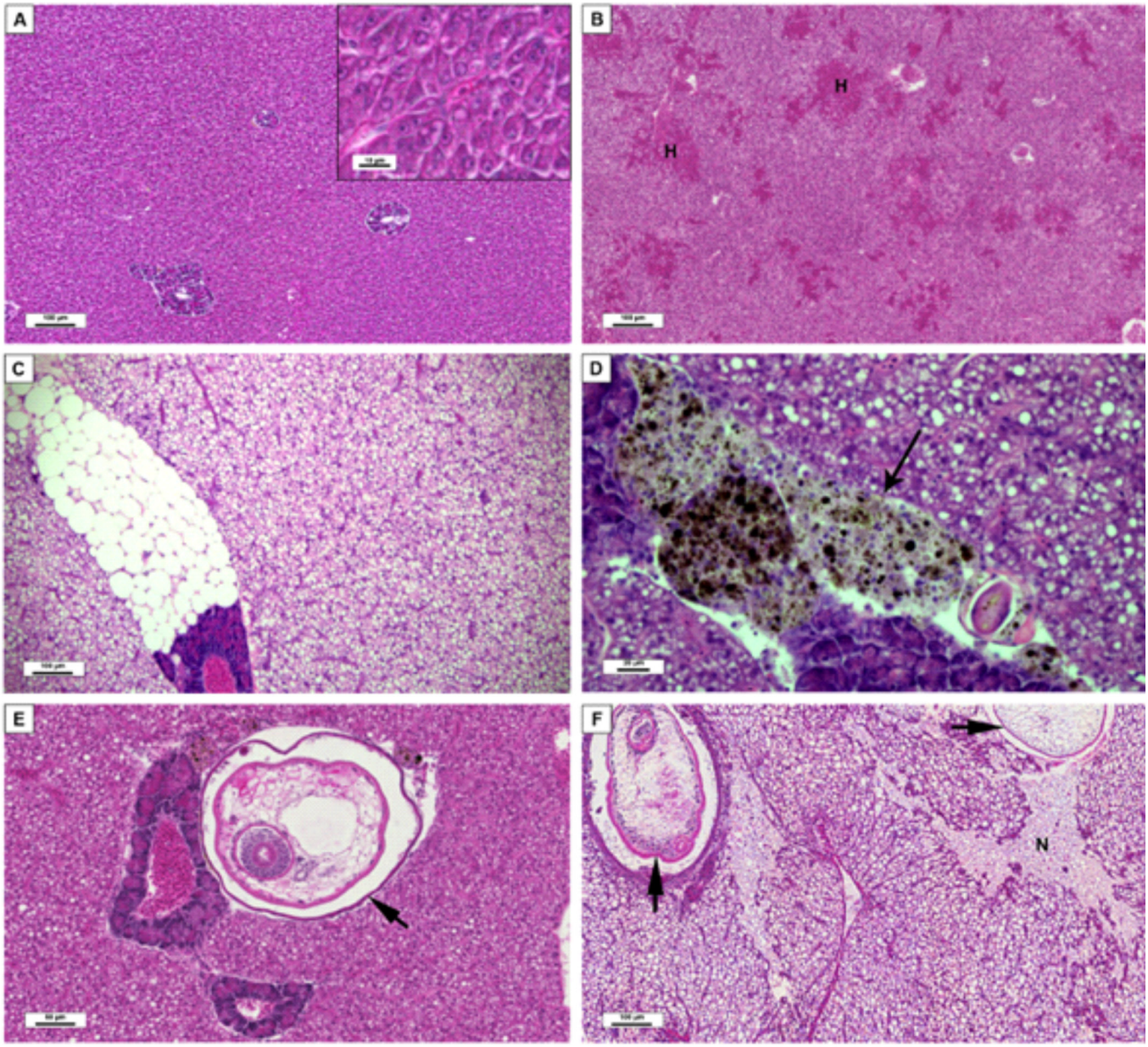

Fig. 5 Liver cross section from *A. brasiliensis* sampled in Paranaguá Bay. A – Basophilic Hepatic parenchyma without significant abnormalities. B – Hemorrhage (H). C – Steatosis, adipocytes. D –Presence of melanomacrophage centers (arrow). E – Detail of parasite. F – Large area of necrosis (N), and parasites (arrow).

Table 1 Occurrence of liver findings in both species *C. spixii* and *A. brasiliensis* sampled around Paranaguá bay and the reference site (Data as mean ± standard deviation; * indicate differences to the reference site).

	Summer						Winter			
	C. spixii	*A. brasiliensis*	C. spixii		A. brasiliensis		*C. spixii*		*A. brasiliensis*	
	RF	RF	HB	CO	HB	CO	HB	CO	HB	CO
Histopathological index	11±8.8	6.4±3.7	26.3±13.2*	21.8±9.9	15.5±8.1	17.5±8*	20±7.9	20±7.9	14.9±5.3	17.2±10.9
Hemorrhage (%)	22.2	18.2	28.6	50	90.9	50	44.4	44.4	33.3	25
Leukocyte Infiltration (%)	11.1		57.1	50	36.4	25	66.6	66.6	44.4	25
Vacuolization (%)		36.4	42.8		90.9	75	11.1	11.1	22.2	62.5
MMC (%)	77.8	27.3	57.1	100		12.5	55.5	55.5	100	50
Parasite (%)	55.5	9	28.6	50		12.5	44.4	44.4	44.4	37.5
Necrosis (%)	77.7	63.6	85.7	100	81.8	87.5	100	100	100	75
Neoplasia (%)				14.3						33.3

Table 2 - Concentration of PAHs metabolites (mean ± SD, mg mL^{-1}) in the bile of fish species sampled in the Paranaguá bay and reference site (Data as mean ± standard deviation; nd = not determined).

	2 rings	4 rings	5 rings	6 rings
Cathorops spixii				
RF	2.83±0.89	1.98±0.44	0.7±0.3	0.08±0.02
Summer				
HB	9.93±1.8	2.68±0.45	1.2±0.08	0.18±0.02
CO	8.8±0.94	2.19±0.29	0.8±0.13	0.14±0.02
Winter				
HB	10.27±0	5.3±0	2.03±0	0.3±0
CO	6.73±1.42	1.32±0.32	0.6±0.09	0.08±0
Atherinella brasiliensis				
RF	4.19±0.05	0.7±0.05	0.17±0.04	0.02±0
Summer				
HB	5.04±1.13	0.67±0.06	0.35±0.05	0.06±0
CO	10.46±0	0.77±0	0.42±0	0.08±0
Winter				
HB	8.94±0	1.96±0	0.72±0	0.13±0
CO	nd	nd	nd	nd

Table 3 - Metals and As analysis (mg kg^{-1}) in muscle and liver of fish species sampled in the Paranaguá bay and reference site.

	As (mg kg^{-1})		Pb (mg kg^{-1})		Sn (mg kg^{-1})		Hg (mg kg^{-1})	
	Liver	Muscle	Liver	Muscle	Liver	Muscle	Liver	Muscle
Cathorops spixii								
RF	<0.10	<0.10	<0.10	<0.10	<0.10	<0.10	0.01	0.015
HB	1.83	2.93	<0.10	<0.10	<0.10	<0.10	0.02	0.03
CO	2.74	8.54	<0.10	<0.10	0.32	<0.10	0.07	0.038
Atherinella brasiliensis								
RF	<0.10	<0.10	<0.10	<0.10	<0.10	<0.10	<0.001	0.012
HB	1.53	1.19	<0.10	<0.10	3.96	<0.10	<0.001	0.022
CO	3.14	1.94	<0.10	<0.10	0.32	<0.10	<0.001	<0.001

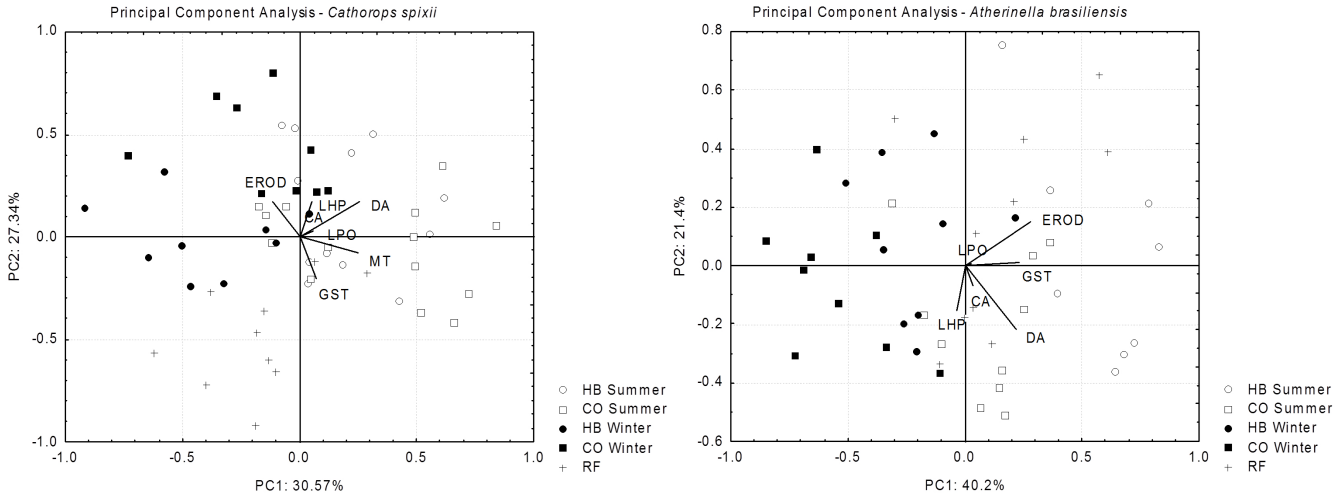

Fig. 6 Principal component analysis (PCA) involving biomarkers in *Cathorops spixii* (left) and *Atherinella brasiliensis* (right).

inner continental shelf (Cunha *et al.*, 2011). *C. spixii* is an omnivore-scavenger species and has a benthonic habit, where there is a wide variety of sewage disposal. Arsenic is one pollutant present in the coast and detected in this study. We found concentrations up to eight times the ones allowed by Brazilian environmental agencies, reaching almost 9 mg/kg in *C. spixii* from the CO. Although arsenic, in the Paranaguá bay, is naturally released through weathering of rocks in which this element is abundant (Sá *et al.*, 2006), the anthropogenic factor seems to have an important contribution to the increased influx during the summer. In addition, when we made an intrasite comparison (CO summer x CO winter), we observed an increased level of metallothioneins expression and an increased activity of GST during the summer. MTs

concentrations were higher when compared to results of similar studies who observed concentrations in *C. spixii* from Santos bay ranging from 0.49 mg g^{-1} to 3.1 mg g^{-1} (Azevedo *et al.*, 2009), while MT levels up to 33.83 mg g^{-1} were found in this study.

On the other hand, the activity of GST is an established biomarker of phase II metabolism for a wide array of xenobiotics, many of which comes from domestic sewage (Hellou *et al.*, 2012). Therefore, both of the aforementioned biomarkers serve as evidence to support an acute exposure of fish during the summer. Other evidence that support the idea of enhanced sewage disposal is the high incidence of parasites, which are common in sewage-contaminated sites (Oliveira Ribeiro & Narciso, 2013), and necrotic areas in the liver of the fish. These injuries are associated with the high incidence of leukocytes and melanomacrophages centers, which are immune cells grouping involved in defense processes, tissue degradation, destruction, and detoxification of exogenous substances (Oliveira Ribeiro & Narciso, 2013). Finally, the presence of neoplastic areas in the liver of these organisms reflects the low quality of the water during this season, making it reasonable to believe that the set of observed responses is influenced both by the increased human population during the summer and the particular ecology of the fish.

Regarding the responses of *A. brasiliensis*, it also seems that the fish ecology and the specificity of the site during the season relates to the response of the biomarkers. As previously explained, the HB site has an intense traffic of ships and harbor activities during the summer period releasing different kinds of xenobiotics, such as PAHs. This assumption corroborates with the increased activity of EROD, which is a well-known biomarker of exposure to PAHs (Kammann *et al.*, 2014), and by the ecology of the organisms. *A. brasiliensis* is a bento-pelagic species with an omnivore feeding habit, which spend its time foraging on the euphotic zone (Contente *et al.*, 2011). Most of the contamination from the harbor happens due to the intense combustion of fossil fuels, which generates, among other residues, PAHs, as we detected in the biota in this study, and that reaches the aquatic ecosystem in different ways, e.g., direct discharge and leaching (Kammann *et al.*, 2014). In addition, due to these different ways, the pollutants tend to be primarily bioavailable in the water column, where this fish species inhabits. In a similar way, an intrasite comparison (HB summer x HB winter) also revealed increased levels of MTs, increased GST activity and apoptosis. The increased GST activity supports the increased EROD activity, since they are distinct parts of the biotransformation system (Kammann *et al.*, 2014), while MTs levels are in accordance with the presence of Arsenic. Histopathological findings showed a high incidence of hemorrhages as well as extensive areas of necrosis in *A. brasiliensis* from HB (summer) demonstrating disturbances in the hepatic system, which agrees with the results obtained from the apoptosis rate.

Still regarding the HB site in the summer sampling, we also found a high incidence of necrosis in *A. brasiliensis* from HB, although not as pronounced as in the CO site. This lesion is a possible effect of acute exposure in this site, since fish also had an increased LPO, DNA damage, and apoptosis rate in liver cells, in comparison with reference site. Yet, we can relate the high levels of LPO in liver of fish to apoptosis frequency in the same tissue (Jia *et al.*, 2014). Decomposition of unstable lipid peroxides produces highly reactive products, such as free radicals, which threaten cell integrity through a chain reaction that leads to cell death (Jia *et al.*, 2014). Previous study demonstrated the relationship among increased lipid peroxidation, DNA damage, apoptosis and necrosis caused by the presence of xenobiotics (Jia *et al.*, 2014), and are important to show that, despite the habit of *A. brasiliensis*, some sort of stress occurring in the harbor affected this species.

Regarding *C. spixii* from CO, the high incidence of necrosis and MMC could be due to a chronic exposure to arsenic. This metalloid can interact with sulfhydryl groups of proteins and enzymes, and to substitute phosphorus in a variety of biochemical reactions (Fowler *et al.*, 2007). Exposure to arsenic leads to necrosis of hepatocytes and pancreatic tissue in fish with a dose-dependent response (ATSDR, 2005). We observed an increased DNA damage and apoptosis rate in specimens from CO in comparison to the reference site, probably related with the capacity for arsenic to cause mitotic arrest, chromosomal aberrations, mutations and DNA strand breaks in fish (ATSDR, 2005).

The exposure of *C. spixii* from HB (winter), in turn, seems to have a different cause. We can explain the increased activity of GST alongside the high incidence of necrosis. The previously discussed type of contamination of the area explains this set of response, such as the activation of GST might be a reflex of the large amount of necrotic tissue, as GST is trying to metabolize the stressful agent jeopardizing the organisms. Nonetheless, we need to perform further investigations over the real causes of this stress to determine the mechanisms behind such a scenario. However, despite this uncertainty, the damages observed are associated with a chronic type of exposure (Hinton *et al.*, 1992).

By observing the biomarker responses of *A. brasiliensis*, we support the hypothesis of chronic exposure during the winter as a reflection of the summer contamination. Fish from HB (winter) presented neoplastic process in the liver, injury that was not present in the summer sampling. Neoplasia is a serious injury that takes time to build up in the tissue (Oliveira Ribeiro & Narciso, 2013). Although diminished during the winter, harbor activities are uninterrupted in this area, therefore a constant influx of pollutants from such activities is expected, and that can explain such modest carcinogenic stress.

The principal component analysis helped to understand this result. For *C. spixii*, this analysis showed clearly segregation between seasons. Through the PCA, we note that most of the biomarkers have responses related to the summer, except for the response of EROD activity. The presence of butyltins, commonly found in Paranaguá bay sediment, inhibited this enzyme (Santos *et al.*, 2014). We observed the good conditions of fish from RF through distinct biomarker responses in comparison with fish from Paranaguá bay sites.

For *A. brasiliensis*, the multivariate approach also showed segregation between seasons. For this species, the metabolic biomarkers presented congruent responses, possibly related with the detoxification process. However, apoptosis levels are corroborating with these analyses, showing that these metabolic reactions are not corroborating with the contamination of Paranaguá bay in the summer. As observed for *C. spixii*, the contamination in Paranaguá bay affected *A. brasiliensis*, mainly in the summer.

CONCLUSION

In conclusion, data showed different sources of contamination affecting both fish species, bridging a gap with different types of human activity occurring each season in the surrounding areas. These impacts have pronounced effects during the summer, probably due to the increased human population during this season. We observed chronic exposures related to damage in liver of both fish species. Finally, we demonstrated the usefulness of applying the multi-biomarker approach in liver of bioindicator species to investigate the effects of anthropogenic discharges in estuarine environments. However, determine the real causes of the stress observed are not possible without further investigation into mechanisms of these contaminants in these species.

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