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Baseline

Baseline levels of oxidative stress biomarkers in species from a subtropical estuarine system (Paranaguá Bay, southern Brazil)

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ABSTRACT

Offshore petroleum exploration has increased the risks of oil spills in coastal tropical and subtropical habitats. Monitoring tools are needed to assess and protect environmental health. We determined baseline values of antioxidant biomarkers (CAT, SOD, GPx, GST, MDA) for five ecologically relevant species in a subtropical system in southern Brazil. Regional baseline levels are compared with literature data as a basis to eventually test their efficacy as post-spill monitoring tools. Differences in the antioxidant response among species, contamination, and seasons were tested using univariate and multivariate analyses. The bivalves *Anomalocardia flexuosa* and *Crassostrea rhizophorae* and the catfish *Genidens genidens* emerge as suitable sentinel species. Seasonality is the main factor accounting for biomarkers variability, and not background contamination level. However, interactions between season and contamination level are also significant, indicating that biomarkers respond to complex environmental settings, a fact that needs to be fully understood for designing proper monitoring programs.

Biomarkers are biochemical, cellular or physiological measurable endpoints used as early and sensitive indicators of sublethal effects of contaminants in exposed organisms (Nahrgang et al., 2010). Despite their potential usefulness, conceptual and methodological issues still need to be addressed before their implementation as tools for monitoring programs. The first is related to the function of different biomarkers, which is usually in maintaining homeostasis in the organism and, consequently, affected by reproductive cycles, food availability, and temporal variation in environmental drivers. The second issue concerns the often equivocal selection of the so-called sentinel species, mainly based on practical and economic criteria (Viarengo et al., 2007), rather than on their ecological adequacy as proxies for communities or ecosystems. As a result, ecotoxicological inferences are too frequently extrapolated from a single species to indicate ecosystem health. Lastly, since no single biomarker can unequivocally measure environmental degradation alone, multi-biomarker and multivariate approaches need to be implemented (Galloway et al., 2004).

Pollution monitoring in tropical and subtropical regions is often based on models originally developed for temperate regions. There is an urgent need to increase information on biomarker levels of key tropical and subtropical species both in contaminated and uncontaminated conditions, at different spatial and temporal scales. The risk of oil pollution, either by dramatic disasters or (primarily) by diffuse sources, has increased as the world's economy has expanded. Oil production in Brazil has risen 5% on average since 2000 (Rapoza, 2015). Shipboard transport of petroleum products has also increased, and, consequently, so has the risk of oil spills in Brazilian coastal waters. Among the most vulnerable continental coastal systems, the Paranaguá Estuarine System (PES), in southern Brazil, hosts the third largest harbor in the country (Martins et al., 2010). PES covers a total area of 612 km^2 and presents a great diversity of pristine or preserved habitats, and sustains small-scale fisheries, incipient aquaculture, and urban touristic areas (Combi et al., 2013). Oil refining, storing and transporting, which may be seen as potential risks for sustaining multipurpose activities, are currently carried out in the Transportation Terminal of Paranaguá (TEPAR) at Paranaguá Harbor, located within the confined sector of the bay (Egres et al., 2012).

In this paper, we determine baseline levels for four major antioxidant enzymes and a biomarker of oxidative stress in five tropical and subtropical species from PES. Besides species-specific variations, two other potential sources of biomarker variation are evaluated, one related to seasonal changes and the other to background contamination conditions. Baseline levels are also compared with literature data summarized from other estuaries along the Brazilian coast, as a basis to eventually test their efficacy as post-spill monitoring tools. With

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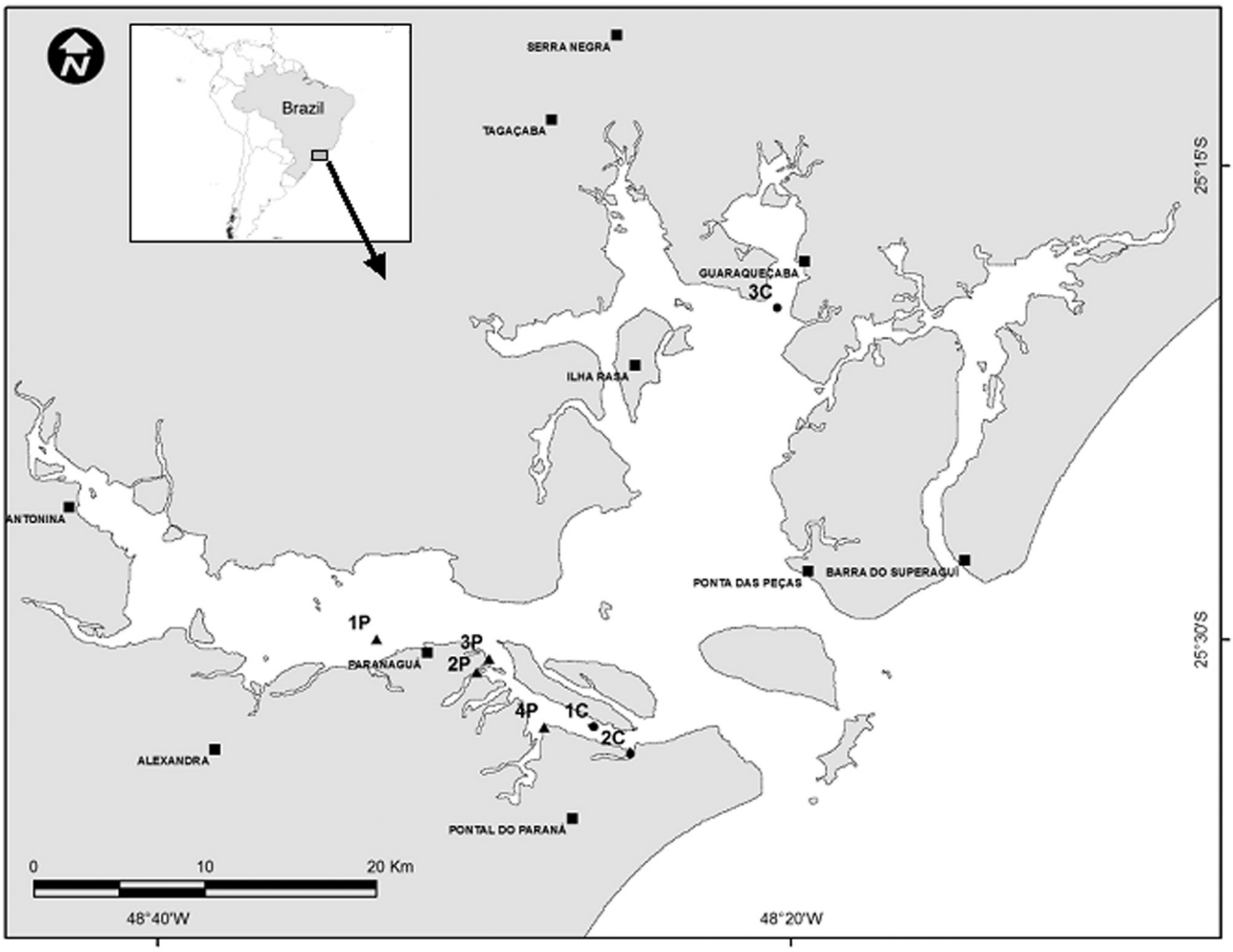

Fig. 1. Sampling locations at the Paranaguá Estuarine System southern Brazil. Points labeled with C correspond to locations considered as control or reference, while points labeled with P refer to polluted locations. *Anomalocardia flexuosa* control 1C, polluted 3P; *Crassostrea rhizophorae* control 1C, polluted 2P; *Neritina virginea* control 1C, polluted 3P; *Uca maracoani* control 2C, polluted 4P and *Genidens genidens* control 3C, polluted 1P.

that, we aim to identify potential sentinel species to monitor oil pollution in subtropical estuarine systems of the Southwestern Atlantic.

The Cotinga channel (Fig. 1), within the PES, is in direct contact with Paranaguá Harbor and Paranaguá city, the largest human settlement in the area (150,000 inhabitants). Paranaguá city discharges up to 50% of domestic sewage directly to the waters of the Cotinga sub-estuary, significantly contributing to the increase in organic pollution and fecal steroids (Martins et al., 2010; Souza et al., 2013; Brauko et al., 2016). The current health status at the Cotinga sub-estuary is considered good, with low levels of hydrocarbon contamination in most of the sampled locations. Total polyaromatic hydrocarbon contamination (16 priority PAH USEPA) in sediments is in overall much lower in PES than in other regions of the world (Cardoso et al., 2016). The area near Paranaguá City usually presents the higher values of total PAH in sediments of the area (28.7–232.74 ng g^{-1}) (Froehner et al., 2011; Cardoso et al., 2016; Rizzi et al., 2016).

Five numerically dominant species with diverse life strategies and at different trophic levels were selected. The edible clam *Anomalocardia flexuosa* (also identified as *Anomalocardia brasiliana*) is an abundant, infaunal suspension feeder commonly found in unvegetated tidal mudflats and responds to hydrocarbon pollution (Sandrini-Neto et al., 2016; Sardi et al., 2016). The mangrove oyster *Crassostrea rhizophorae* is an euryhaline sessile filtering species, usually associated with the trunks and roots of mangrove trees. The grazing snail *Neritina virginea* is numerically dominant in local salt marshes. The omnivorous crab *Uca maracoani* lives in burrows in the sediment of intertidal mudflats where it can reach relatively high abundances. The catfish *Genidens genidens* has local economic and nutritional value, demersal behavior and alternates between detritivorous and carnivorous feeding habits. Little data indicating detoxification capacity in *G. genidens* is currently available, though some work has been done with the closely related catfish *Cathorops spixii* (Azevedo et al., 2009; Katsumiti et al., 2009; Azevedo et al., 2013).

Specimens were collected during the austral winter, characterized by low precipitation rates, and austral summer, also denoted as the rainy season. Adult individuals were collected at two different locations with varying levels of contamination. Reference and polluted precise locations were not the same for all species since they live in different habitats (Fig. 1). Sites were selected based on chemical data available in the literature for the contamination gradient along the Cotinga channel as also by availability of the selected species (Table S1).

After collection, animals were transported in ice-cooled estuarine water to the lab. Once in the lab, animals were dissected, and target tissues (Table S1) were immediately frozen by immersion in liquid nitrogen. Fish dissection was done on the boat right after capture. All tissues were dissected and transported in dry ice. Once at the lab, all samples were stored at −80 °C until further analysis.

Fragments of tissue (between 100 and 200 mg) were placed in tubes containing glass beads and cold 0.1 mol L^{-1} phosphate, 2.5% NaCl buffer pH 7.6 (1:10 *w*/*v*) and homogenized using a Precellys 24 Lysis and Homogenizer (Bertin Technologies). Samples were then centrifuged at 12,500 g for 30 min at 4 °C in a Hermile z233 MK-2 microcentrifuge.

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Aliquots were prepared on ice for enzymatic analyses and stored at −80 °C until further analysis. For liver and digestive glands samples, the cytoplasmic was recuperated after a second centrifugation at 21,500 g for 120 min at 4 °C. Activity of GST was assessed within the cytoplasmic fraction.

Total protein concentration was quantified using the Quick Start™ Bradford Protein Assay (BioRad) (Bradford, 1976). The reaction was measured spectrophotometrically at 595 nm using a Perkin Elmer Multilabel counter 1420 VICTOR 3 microplate reader.

Catalase (CAT) activity was assayed according to Aebi (1984). Final concentrations in a volume of 1500 μL were 0.1 mol L^{-1} phosphate buffer pH7.6, and 20 mmol L^{-1} H_2O_2. Molar extinction coefficient employed was 40 mol L^{-1} cm^{-1}. Specific activity as expressed as mmol H_2O_2 degraded min^{-1} mg^{-1} protein.

Glutathione peroxidase (GPx) activity was assayed according to Hafeman et al. (1974). Final concentrations in a volume of 200 μL were 0.1 mmol L^{-1} potassium phosphate buffer pH 7.6, 2 mmol L^{-1} sodium azide, 1 mmol L^{-1} EDTA, 0.2 mmol L^{-1} NADPH, 2 mmol L^{-1} GSH, 1 U/ml glutathione reductase and 1.5 mmol L^{-1} H_2O_2. Specific activity was expressed as μmol min^{-1} mg^{-1} protein and determined using the molar extinction coefficient of 0.622 mmol L^{-1} cm^{-1}.

Glutathione S-transferase (GST) activity was measured following increases in absorbance at 340 nm as described by Keen et al. (1976). Final concentrations in a volume of 200 μL were 3 mmol L^{-1} GSH, 3 mmol L^{-1} CDNB and 70 mmol L^{-1} potassium phosphate buffer, pH 7.6. Extinction coefficient of GS-DNB conjugate was 9.6 mol L^{-1} cm^{-1} and activity expressed as μmol min^{-1} mg^{-1} protein.

Superoxide dismutase (SOD) activity was determined following the inhibition of pyrogallol autoxidation as described by Gao et al. (1998) with modifications specified in Sardi et al. (2016). Activity was expressed in activity units, where one unit of SOD corresponds to the SOD concentration that inhibits pyrogallol oxidation in 50%. Malondialdehyde (MDA), a by-product of lipid peroxidation, was measured as described by Shaw et al. (2004).

Log transformed data were analyzed using R (R Development Core Team, 2009). Differences in mean activity between treatments were tested with an analysis of variance (ANOVA) for each species (alpha = 0.05). The testing design consisted of 2 factors orthogonal to each other, season (fixed, two levels, winter, and summer), contamination condition (fixed, two levels, reference and polluted), and their interaction. The effect size of each factor was calculated by dividing the sum of squares for the significant factor by the total sum of squares. Results are presented as confidence plots, where points indicate the mean, and whiskers extend from it to the upper 97.5-th and lower 2.5-th percentile; providing the 95% confidence distribution of the mean. Also, 50% of the mean distribution is enclosed in rectangles (Greenacre, 2016).

We carried out a Redundancy analysis (RDA) for each species using enzymes activities as predictors, and factors as explanatory or constraining variables to assess if biomarker responses differ between seasons or depending on levels of contamination. Significant differences among treatments were assessed with a PERMANOVA, run using a factorial design that included season and contamination condition as fixed orthogonal factors. A reduced RDA model including species and the interaction between season and contamination condition factors was employed (See Table S2 for details). All multivariate procedures were carried out using package *vegan* (Oksanen et al., 2013).

Baseline levels of activity for CAT, GPx, SOD and GST and MDA are summarized in Table 1. For most studied species the activity of GST and GPx was higher during summer while CAT and SOD activity was higher in winter (Table 1).

Fig. 2 summarizes mean activity levels for each measured endpoint in the target species. Levels of significance are also indicated, together with the effect size, expressed as percent difference. Variation in 2 out of 5 of the measured endpoints in *A. flexuosa*, specifically in CAT and SOD (Fig. 2), was explained by the interaction between season and location condition factors. In both cases, the main factors were significant, while the level of MDA was only influenced by season, with lower levels in summer than winter. Season had a strong contribution to GST, GPx, CAT and MDA levels in *C. rhizophorae* (Fig. 2). Differences between seasons in *N. virginea* were also significant for most target biomarkers. GST and GPx activities in *U. maracoani* were higher in summer than winter. Conversely, SOD and levels of MDA were lower in summer than in winter. Seasonal variation in biomarker activity for *G. genidens* was only

Table 1
Mean enzyme activity, 2.5–97.5% quantiles of data obtained for seasons and contamination condition groups. Enzyme activity units are, CAT: $mMol.min^{-1} \cdot mg^{-1}$ of protein; GPx and GST: $\mu Mol \cdot min^{-1} \cdot mg^{-1}$ of protein; SOD: U $mg \cdot ml^{-1}$ of protein; MDA: μM g^{-1} wet weight. Abbreviations stand for AF: *Anomalocardia flexuosa*; CR: *Crassostrea rhizophorae*; NV: *Neritina virginea*; UM: *Uca maracoani*; GG: *Genidens genidens*.

		Reference winter			Polluted winter			Reference summer			Polluted summer		
		1st Qu.	Mean	3rd Qu.	1st Qu.	Mean	3rd Qu.	1st Qu.	Mean	3rd Qu.	1st Qu.	Mean	3rd Qu.
SOD	*AF*	131	220	396	70.4	108	171	261	605	2215	220	703	2532
	CR	137	350	801	240	1364	7104	30.4	146	366	204	735	1750
	NV	266	460	775	429	576	1065	79.3	354	1457	122	206	355
	UM	103	253	460	114	279	679	44.1	59.5	77.4	13.2	53.7	92.4
	GG	45.7	73.5	130.6	57.7	85.9	114	78.5	212	664	183	261	318
CAT	*AF*	1.20	10.3	25.6	0.77	3.44	6.46	0.31	0.90	2.12	0.31	1.60	3.82
	CR	2.86	14.7	30.1	1.53	11.5	55.9	1.31	5.68	14.32	0.61	4.53	9.74
	NV	0.26	3.32	10.5	0.29	1.90	3.67	0.14	0.42	1.19	0.21	0.47	0.88
	UM	0.11	2.31	8.86	0.11	0.55	0.99	0.23	2.07	4.31	0.35	2.92	8.15
	GG	2.02	10.7	45.3	0.6	4.6	9.0	2.0	4.3	8.5	2.0	7.0	18.2
GPx	*AF*	15.3	32.9	57.9	16.1	32.1	47.0	11.2	26.3	52.4	15.6	35.3	79.6
	CR	14.1	26.9	34.6	15.2	25.4	34.4	15.7	43.8	75.2	25.8	51.5	68.8
	NV	8.40	43.9	146	8.75	32.4	133	55.3	138	264	139	196	239
	UM	5.29	25.2	42.0	6.78	25.7	54.8	28.5	55.3	82.3	21.9	43.7	76.1
	GG	111	174	271	144	198	270	9.25	52.8	96.9	13.0	39.4	80.4
GST	*AF*	116	144	173	87.4	132	193	34.1	126	201	126	151	175
	CR	37.7	79.5	108	72.7	145	439	27.6	57.7	100	53.7	90.4	117
	NV	5.9	55.1	96.8	4.4	44.5	148	66.4	224	400	234	288	358
	UM	13.6	63.9	175	14.5	173	421	178	244	287	187	275	422
	GG	87.9	108	126	103	143	167	269	297	353	204	268	319
MDA	*AF*	3.42	6.74	8.85	2.41	5.16	7.61	0.35	2.42	4.75	1.01	2.87	4.47
	CR	1.05	1.84	2.89	0.65	1.72	2.96	2.58	4.70	7.14	1.60	4.93	9.93
	NV	1.49	2.41	3.40	1.98	3.14	5.02	0.96	2.11	4.35	6.01	6.86	8.21
	UM	3.92	10.3	21.8	10.7	18.9	34.5	2.86	4.59	8.48	3.71	10.0	17.0
	GG	4.84	5.95	6.75	2.59	3.80	5.02	1.26	5.50	8.97	2.82	4.72	6.59

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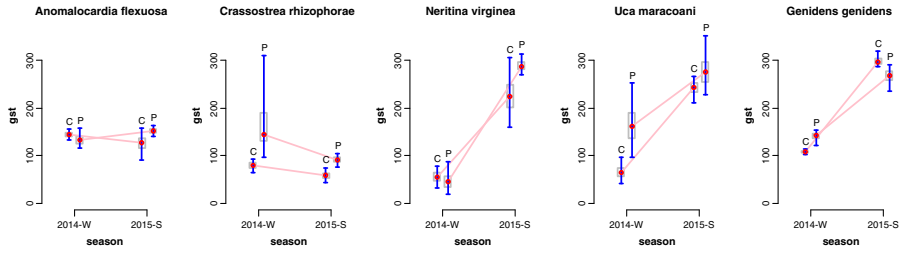
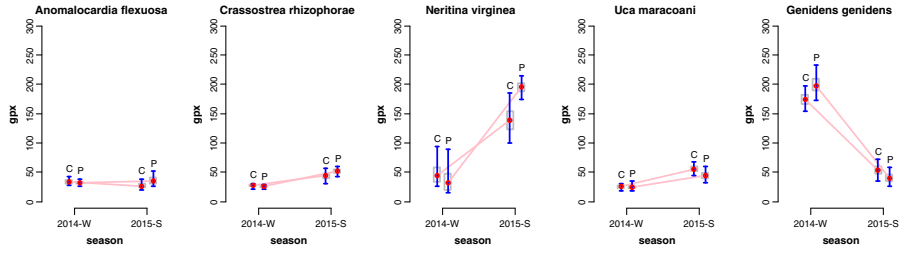
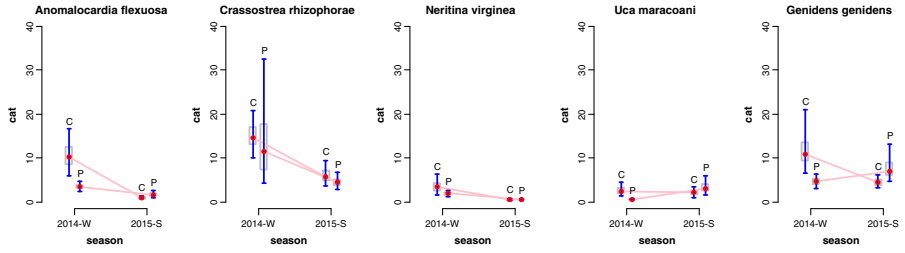


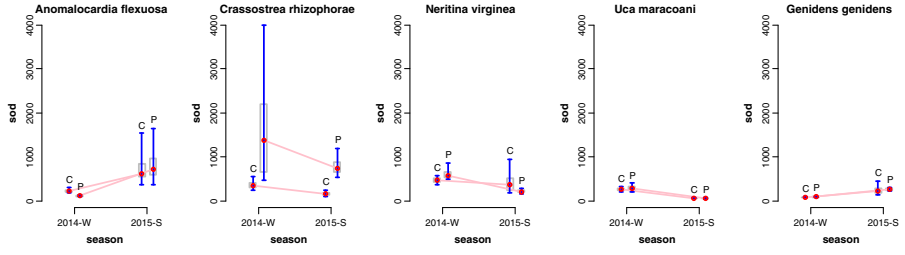
No significant effects | season (p=0.02) effect -28% condition(p=0.001) effect +61% | season (p<0.0001) effect +682% | season (p<0.0001) effect +287% | season (p<0.0001) polln.eff(W) +31% condition (p<0.0001) polln eff.(S) -10% interaction (p<0.0001)



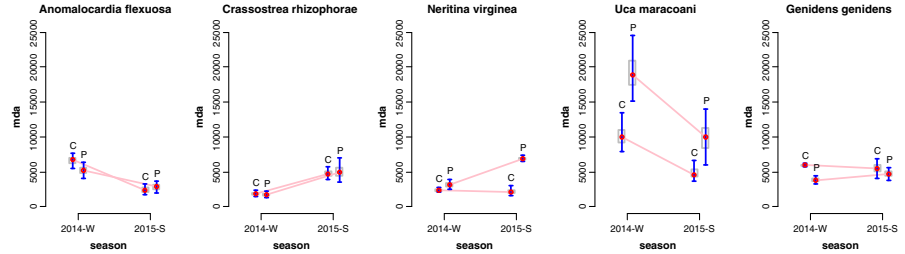
No significant effects | season (p=0.0001) seas.effect +73% | season (p<0.0001) poll neff.(W) -35% interaction(p=0.04) poll neff.(S) +59% | season(p=0.0003) effect +123% | season (p<0.0001) effect -78%



season (*p*<0.0001) poll neff.(W) -56% condition(*p*=0.04) polln.eff.(S)+50% interaction(*p*=0.03) | season (p=0.02) seas.effect -53% | season (p<0.0001) effect -73% | No significant effects | No significant effects



season(*p*=0.007) polln eff.(W) -49% condition(*p*=0.01) polln.eff.(S) +5% interaction(*p*=0.06) | condition (p<0.0001) eff.+242% | season (p<0.0001) effect -57% | season (p<0.0001) effect -78% | season (p<0.0001) effect +160% condition (p=0.03) effect +32%



season (*p*=0.001) season eff. -77% | season (p<0.0001) effect +166% | interaction(p<0.0001) polln eff. (W) +28% polln eff. (S) +263% | season (p<0.0001) effect -51% condition (p<0.0001) effect +97% | condition (p=0.04) effect -24%

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observed for GPx enzyme. MDA levels were lower in the polluted location than the reference one. The activity of SOD was significantly higher in summer than in winter and variation in the GST activity was mostly explained by the interaction between season and contamination condition (Fig. 2).

Redundancy Analysis (RDA) revealed distinct patterns of enzymatic response, significantly influenced by season and contamination levels for the different species (Table 2, Fig. 3). Within this type of ordination, linear relationships between two sets of independent variables are found, and best-fit linear combinations are represented in a biplot. This analysis is the multivariate analog of regression, where an explanatory set of variables, here denoted as seasons, location condition, and their interaction, explain the observed variance in biomarker responses. For all the target species, season showed much lower *P*-values (< 0.0001), highlighting it as the most important factor structuring the antioxidant enzymatic activity (Table 3). In the case of the clam *A. flexuosa* the first (horizontal) axis showed lower MDA levels and lower CAT activity during austral summer. The vertical axis has a high (positive) weighting by SOD and lesser importance of CAT (Fig. 3a). The PERMANOVA analysis indicated a significant interaction between season and contamination condition (Table 3). This interaction effect can be easily distinguished by the overlapping of confidence ellipses from the summer and polluted group (PS) and reference summer group (CS). The effect of the interaction makes harder to identify unequivocally which enzymes were important in distinguishing differences between reference and contaminated areas.

The mangrove oyster *C. rhizophorae* showed a different pattern. RDA1 accounts for 21.8% of the total variability and corresponds to a seasonal shift from higher GPx and MDA in summer, and greater CAT activity in winter. Response to contamination is seen primarily on RDA2, which accounted for 13.9% of the total variability. Here we see higher activity of SOD and GST enzymes in polluted sites (Fig. 3b). The PERMANOVA test confirmed the statistical significance of the main factors (season and condition), but not their interaction (Table 3).

For *Neritina virginea*, the seasonal shift was mostly related with the first axis, which explained 51.5% of the total variance (Table 2) and was primarily caused by higher activities of GST and GPx during the summer season (Fig. S3a). For the rest of the species, both season and contamination conditions appeared as significant in structuring enzymatic responses (Table 3). However, season always had a stronger effect as observed by the percent of variance explained by the axis aligned with the seasonal shift (Table 2). Seasonal differences in the crab *U. maracoani* were mainly explained by higher activity of GST during summer and higher SOD during winter (Fig. S3b). The activity of GPx in *G. genidens* was higher in winter, whereas GST and SOD were higher in summer, and these enzymes contributed most to RDA1 (Fig. S3c).

Fig. 4 presents results from a redundancy analysis (RDA) where species and the interaction of season and contamination condition were included as explanatory variables. The model explained 23.4% of total variance. Levels of significance of the explanatory terms (species biomarkers activities) and the interaction between season and condition were always statistically significant ($p < 0.001$). From Fig. 4 it is evident that the enzymatic activity of the studied species is different (Fig. 4).

We carried out a quantitative comparison between the obtained baseline values with literature data from other Brazilian coastal habitats on the same or similar species (Table 4). The revision includes baseline and biomonitoring studies; also transplant, and field or laboratory exposure experiments. Baseline studies are here defined as investigations with a sampling conducted during different seasons, and that include a comparison between a reference and a polluted site. Biomonitoring studies included works that compared enzymatic activity along a gradient of pollution. In most papers data are presented as the range of mean activity in different treatments. Works for which the exact activity data were available are highlighted with a check mark on the "precise value" column; otherwise, table values were inferred from the original graphs. The revision includes studies conducted at 12 different locations from the Brazilian coast, (predominantly in the southern region). Only a few of the works followed the multi-species, and multi-biomarker assessment included in this study (Alves et al., 2002; Zanette et al., 2008; Pereira et al., 2014; Sandrini-Neto et al., 2016; Sardi et al., 2016). In total, antioxidant enzyme activity has been measured in 20 different species, 5 of which are included within this work. The activity of GST and CAT enzymes and levels of lipid peroxides were the endpoints more often employed (Table 4), and only 11 studies included multivariate analysis as a tool for data interpretation. In most cases, baseline values from Paranaguá Bay largely differ from literature values by orders of magnitude or are hard to compare given that different protocols were employed (Table 4).

Seasonality accounted for more of the observed variation in the antioxidant enzymes for all the target species than did contamination level. Seasonal changes in the biomarker response are known for several species (Nahrgang et al., 2010; Nahrgang et al., 2013; Gorbi et al., 2005; Orbea et al., 2002). These variations are often attributed to changes in temperature, salinity, food availability and reproductive cycle (Bocchetti and Regoli, 2006; Geracitano et al., 2004a; Manduzio et al., 2005). In our baseline assessment, seasonal effects on the antioxidant system were most evident for *N. virginea*, *U. maracoani* and *G. genidens*. The reproductive period for many subtropical catfish species occurs during warmer months (Schmidt et al., 2008). *G. genidens* has synchronous oocyte development and presents large oocytes and low fecundity (10–24 oocytes) rates. Male specimens incubate fertilized eggs in their mouth, reducing feeding (Chaves, 1994). Protection of relatively large eggs suggests that *G. genidens* is a K-strategist (Silva Junior et al., 2013), allocating significant amounts of energy for reproduction. As reproduction is an energetically demanding activity, basal metabolic rates tend to increase during gonad development, and so does the production of reactive oxygen species (ROS) (Alonso-Alvarez et al., 2004). This energy investment implies that organisms are potentially more sensitive to ROS produced following a hypothetical contamination event since their antioxidant defenses are already coping with oxidative imbalance caused by reproduction. *U. maracoani* behavior is modulated by tides; during flood tide individuals stay in their burrows and feed, whereas they copulate and fight during low tide when the mudflat is exposed. Hirose and Negreiros-Fransozo (2008) reported low reproduction rates during the summer and suggested that high temperatures and low salinities prevent individuals from exiting their burrows and copulating, thus regulating their reproductive behavior. *U. maracoani* reproduces throughout the year, as shown by the presence of females with eggs along the whole year with two intense reproductive peaks in

Table 2
RDA results for each species. Percentage of total variance explained by explanatory variables included in the model, seasons and contamination condition.

Species	Total variance explained (%)	RDA axis 1 (%)	RDA axis 2 (%)
Anomalocardia flexuosa	40.1	35.8	4.3
Crassostrea rhizophorae	35.7	21.8	13.9
Neritina virginea	55.9	51.5	4.4
Uca maracoani	34.9	28.5	6.4
Genidens genidens	45.9	41.6	4.3

Fig. 2. Confidence plots derived from log-transformed enzymatic activities in studied species. Plots represent the mean (red points), 50% confidence intervals (boxes) and 95% confidence intervals (dispersion lines). Effects of significant interaction are given as estimated changes between polluted (P) and reference (C) samples for winter (W) and summer (S). When significative, the marginal effect of season and condition are also denoted. Enzyme activity units are, CAT: $mMol.min^{-1} \cdot mg^{-1}$ of protein; GPx and GST: $\mu Mol \cdot min^{-1}.mg^{-1}$ of protein; SOD: U $mg \cdot ml^{-1}$ of protein; MDA: nM g^{-1} wet weight. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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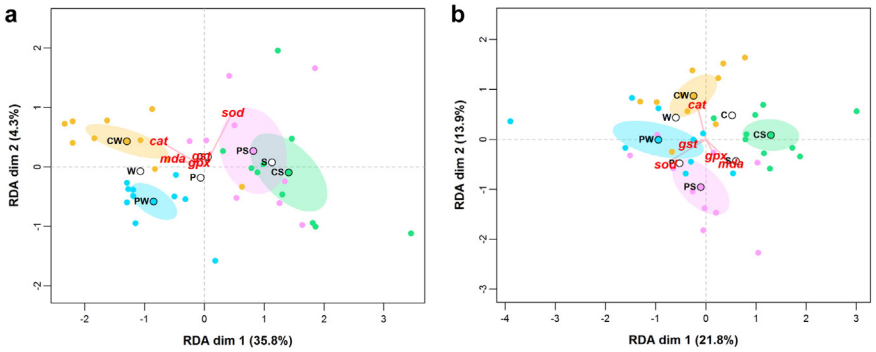

Fig. 3. RDA biplots derived from log transformed enzymatic activities in a) *Anomalocardia flexuosa* and b) *Crassostrea rhizophorae* sampled during winter and summer seasons in locations with different levels of contamination. Ellipses represent 95% confidence intervals from centroids of the interaction between season and condition. Abbreviations stand for: C: control; P: polluted; CW: control winter (yellow); PW: polluted winter (blue); CS: control summer (green); PS: polluted summer (purple).(For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

April (autumn) and November (spring) (Di Benedetto and Masunari, 2009). This result suggests that observed seasonal differences in the antioxidant capacity of *U. maracoani* from the PES are probably unrelated to reproduction. *N. virginea* populations are persistent throughout the year in PES, but show a seasonal pattern, with higher abundances during winter months when there is greater availability of detritus (Lana, 2003). In tropical Northeastern Brazil, the frequency of reproductive egg capsules is higher in the dry season, from July to December (Matthews-Cascon and Martins, 1999). As the PES has a wet subtropical climate with a dry season (winter) occurring between June–September and a rainy season (summer) between December to March, a direct extrapolation from Matthews-Cascon and Martins (1999) results to PES seems unreliable, making it difficult to correlate the observed seasonal variation with reproductive cues. Populations of *C. rhizophorae* and *A. flexuosa* from PES do not present a period of reproductive rest (Christo and Absher, 2004; Ferreira et al., 2015). The reproductive peak of *A. flexuosa* occurs in summer months from December to January (Ferreira et al., 2015) while a high percentage of *C. rhizophorae* with mature gonads were observed from January to March (Absher, 1989; Christo and Absher, 2004). Temperature rise and increases in food availability are suggested as the factors triggering gonad ripening (Christo and Absher, 2004).

Variations in biomarker activities may also be related to the strong seasonal variations in local hydrological and hydrodynamic processes, as well as seasonal variation in the input of sewage and other contaminants. PES presents strong tidal regimes and significant seasonal differences in salinity, mainly driven by seasonal changes in precipitation (Lana et al., 2001). Characteristically, summer months have high temperatures (23–30 °C) and low salinities (12–29). In winter, salinity values are high (20–34), and temperature lower (18–25 °C) (Lana et al., 2001). Moreover, inputs of allochthonous dissolved organic matter (DOM) to PES are intensified during the summer season, which is characteristically a rainy season (Gusso-Choueri et al., 2011). Disposal of untreated sewage directly to the Cotinga sub-estuary is the first source of contamination in the studied area, and it depends on the number of inhabitants around the Paranaguá Bay, which fluctuates between seasons. The potential input of hydrocarbon contaminants from marine vessels also has a seasonal signal since activity in the Paranaguá Harbor has been 16% higher during the winter than during the summer for the past five years (APPA, 2016).

Although the seasonal signal was far more important for total variation, multivariate analysis revealed significant differences between reference and polluted locations in antioxidant biomarkers for *U. maracoani*, *C. rhizophorae*, and *G. genidens* and these results were not evident following the univariate approach. Similarly, Gagnon and Rawson (2016) observed deterioration on fish health only when integrating the biomarker responses with multivariate analysis; while individual biomarkers failed to detect exposure to xenobiotics.

The collection site for *U. maracoani* labeled as polluted is located at the mouth of the Guaraguaçu River, a 60 km long river that discharges

Table 3
Analysis of variance using permutation test (PERMANOVA) for enzymatic activities in tropical species collected at different seasons and locations with different levels of contamination. Statistically significant differences are highlighted in bold. Abbreviations stand for *F*: pseudo-F-ratio; R2: coefficient of determination, *P*: probability of F.

Species	Source of variation	*F*	R2	*P*
Anomalocardia flexuosa	Season (Se)	20.14	0.34	<**0.0001**
	Contamination condition (Cond)	0.84	0.01	0.47
	Se:Class	3.14	0.05	**0.024**
Crassostrea rhizophorae	Season (Se)	10.26	0.18	<**0.0001**
	Contamination condition (Cond)	9.55	0.17	<**0.0001**
	Se:Class	1.29	0.02	0.26
Neritina virginea	Season (Se)	40.12	0.50	<**0.0001**
	Contamination condition (Cond)	2.43	0.03	0.078
	Se:Class	2.77	0.03	0.057
Uca maracoani	Season (Se)	16.55	0.28	<**0.0001**
	Contamination condition (Cond)	2.93	0.05	**0.026**
	Se:Class	1.68	0.02	0.15
Genidens genidens	Season (Se)	53.66	0.52	<**0.0001**
	Contamination condition (Cond)	4.61	0.04	**0.012**
	Se:Class	2.80	0.03	0.058

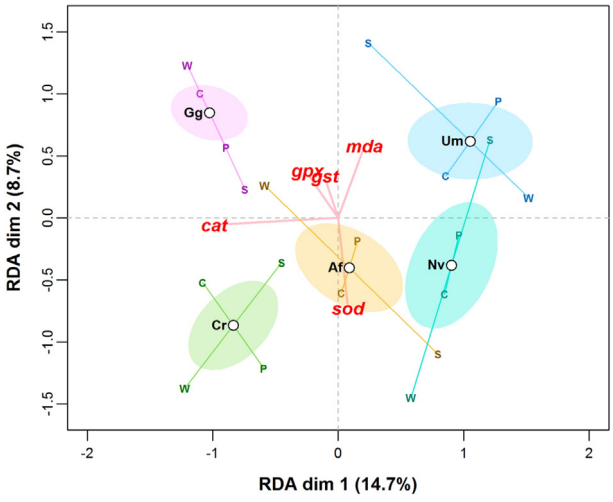

Fig. 4. Redundancy analysis (RDA) for studied species. The analysis included species and the interaction between season and condition as predictor variables. Predictors accounted for 35.5% of the total variance. Ellipses represent 95% confidence centroids for each species and axis around it indicates the distance from which season and condition centroids are placed. Abbreviations stand for: Af: *A. flexuosa*; Cr: *C. rhizophorae*; Gg: *G. genidens*; Nv: *N. virginea*; Um: *U. maracoani*; C: reference; P: polluted; W: winter; S: summer.

Table 4
Values of the studied biomarkers obtained in tropical and subtropical species from Brazilian estuaries. Results from this study are highlighted in bold.

Species	Feeding	Habitat	Type of work	Tissue	Data reported	Precise values	SOD	CAT	GPx	GST	LPO	Reference	Treatment	Location and State
Anomalocardia flexuosa	**F**	**IM**	**B**	**DG**	**Mean (Min - Max)**	✓	**401.4 (68.3–2891.2)**	**4.1 (0.2–26.5)[b]**	**31.54 (10.67–85.23)**	**137.86 (19.9–207.9)**	**4331.7 (11.9–9074.07)**	**This study**	**Seasons and control vs. contaminated site**	**Paranaguá Estuarine System, PR**
Anomalocardia flexuosa	F	IM	F	DG	Range of the mean	✗	150–210	18–28	100–240	60–158	0.5–3.2[e]	Sandrini-Neto et al. (2016)	Diesel oil exposure	Paranaguá Estuarine System, PR
Anomalocardia flexuosa	F	IM	L	DG	Mean (Min - Max)	✓	742.4 (607.9–955.7)	67.4 (51.6–92.6)	238.2 (210–278.1)	174.9 (128.2–233.2)	17.7 (13.2–22.9)[e]	Sardi et al. (in press)	Diesel oil exposure	Paranaguá Estuarine System, PR
Cathorops spixii	O	SB	Bm	L	Range of the mean	✗					250–3500	Azevedo et al. (2009)	Seasons and gradient of pollution	Santos estuarine system and Cananéia estuarine-lagoon complex, SP
Cathorops spixii	O	SB	Bm	L & M	Range of the mean	✓		0.51–1.18[c]		115–182[c]	0.004–0.01[e]	Azevedo et al. (2013)	Seasons and gradient of pollution	Santos estuarine system and Cananéia estuarine-lagoon complex, SP
Cathorops spixii	O	SB	Bm	G, K & L	Range of the median	✗			10–400[c]	100–700[c]	0.5–4[g]	Gusso-Choueri et al. (2011)	Seasons and gradient of pollution	MPA Cananéia-Iguape-Peruíbe, PR
Genidens genidens	**O**	**SB**	**B**	**L**	**Mean (Min - Max)**	✓	**137.2 (44.5–788)**	**7.5 (0.2–58.9)[b]**	**129.7 (7–297)**	**182.3 (79.9–365.2)**	**5234 (519.1–9060.3)**	**This study**	**Seasons and control vs. contaminated site**	**Paranaguá Estuarine System, PR**
Hyphessobrycon reticulates	O	FW, B/P	Bm	L	Range of the mean	✗		220–230		50–90[c]	15–30[e]	Katsumiti et al. (2013)	Post-oil spill evaluation	Araucaria City, PR
Micropogonias furnieri	C	SB	B	L	Range of the mean	✗		40–130		0.3–0.7	1000–3000[f]	Amado et al. (2006a)	Seasons and control vs. contaminated site	Patos Lagoon Estuary, RS
Paralichthys orbignyanus	C	SB	B	L	Range of the mean	✗		1.5–2		0.75–2	50–180[f]	Amado et al. (2006b)	Seasons and control vs. contaminated site	Patos Lagoon Estuary, RS
Poecilia vivipara	O	P	L	DG, G & L	Range of the mean	✗	5–90[a]	1–110		12–800[c]	–	Machado et al. (2014)	Phenanthrene exposure	Casino Beach, RS
Poecilia vivipara	O	P	L	G, L & M	Range of the mean	✗	3–30[a]	1.5–1100		20–350	–	Machado et al. (2013)	Cooper exposure	Casino Beach, RS
Sciades herzbergi	O	SB	Bm	L	Range of the mean	✗		900–1000		2.0–2.1	–	Carvalho-Neta and Abreu-Silva (2010)	Gradient of pollution	San Marcos Bay, MA
Balanus improvisus	F	RS	Bm	WB	Range of the mean	✗		3.6–4.8		20–110	600–2200[f]	Zanette et al. (2015)	Seasons and gradient of pollution	Patos Lagoon Estuary, RS
Crassostrea brasiliana	F	MR	L	DG & G	Range of the mean	✗	30–120[a]	60-450[e]	4–8[c]	40–130[e]	90–150[f]	Lüchmann et al. (2011)	Diesel oil exposure	Farmed animals Florianopolis, SC
Crassostrea gigas	F	RS	Bm	DG & G	Range of the mean	✗	5–12[a]	50–150		03–50	–	Souza et al. (2012)	Gradient of pollution	Florianopolis, SC
Crassostrea	F	MR	T	DG	Range of	✗		450–650		30–45		Zanette et al.	Gradient of sewage	São Jose, SC

(continued on next page)

Table 4 (*continued*)

Species	Feeding	Habitat	Type of work	Tissue	Data reported	Precise values	SOD	CAT	GPx	GST	LPO	Reference	Treatment	Location and State
gigas					the mean							(2008)	pollution	
Crassostrea gigas	F	MR	T	G	Range of the mean	✗		50–70		95–110		Zanette et al. (2008)	Gradient of sewage pollution	São Jose, SC
Crassostrea rhizophorae	**F**	**MR**	**B**	**G**	**Mean (Min - Max)**	✓	**648.6 (21.4–8896.7)**	**9.16 (0.3–68.8)[b]**	**36.9 (12.6–76.5)**	**93.08 (26.5–525.1)**	**3296.7 (641.2–10,561)**	**This study**	**Seasons and control vs. contaminated site**	**Paranaguá Estuarine System, PR**
Crassostrea rhizophorae	F	MR	B	G	Range of the mean	✗		100–130		20–55	100–250[f]	Zanette et al. (2006)	Seasons and gradient of pollution	Piraquê Estuarine Complex, ES
Crassostrea rhizophorae	F	MR	B	G	Range of the mean	✗		50–120		10–50	100–125[f]	Zanette et al. (2006)	Seasons and gradient of pollution	Itamaracá Bay, PE
Crassostrea rhizophorae	F	MR	Bm	G	Range of the mean	✓			68.29–165.08[d]	22.22–37.63[c]	2.7–2.97[g]	Maranho et al. (2012)	Gradient of pollution	Paranaguá Estuarine Complex, PR
Crassostrea rhizophorae	F	MR	Bm	G	Range of the mean	✓			122.05–270.1[d]	45.37–71.25[c]	3.04–11.67[g]	Maranho et al. (2012)	Gradient of pollution	Santos estuarine system, SP
Crassostrea rhizophorae	F	MR	Bm	G	Range of the mean	✗		60–112		10–50	100–1300[f]	Zanette et al. (2006)	Seasons and gradient of pollution	Paranaguá Estuarine Complex, PR
Crassostrea rhizophorae	F	MR	L	DG	Range of the mean	✗		120–270		2–17		da Silva et al. (2005)	Diesel oil exposure and salinity	Florianopolis, SC
Crassostrea rhizophorae	F	MR	L	G	Range of the mean	✗		10–14		150–155		Alves et al. (2002)	Furadan exposure	Florianopolis, SC
Crassostrea rhizophorae	F	MR	T	DG	Range of the mean	✗		320–500		35–50		Zanette et al. (2008)	Gradient of sewage pollution	São Jose, SC
Crassostrea rhizophorae	F	MR	T	G	Range of the mean	✓			107.64–232.45[c]	39.69–139.22[c]	6.63–11.96[g]	Pereira et al. (2014)	Gradient of pollution	Santos estuarine system and São Sebastião Channel, SP
Crassostrea rhizophorae	F	MR	T	G	Range of the mean	✗		100–210		110–140		Zanette et al. (2008)	Gradient of sewage pollution	São Jose, SC
Laeonereis acuta	D	IM	B	BS	Range of the mean	✓					88.35–238.58[f]	Ferreira-Cravo et al. (2007)	Seasons, control vs. contaminated sites, and organisms body region	Patos Lagoon Estuary, RS
Laeonereis acuta	D	IM	B	WB	Range of the mean	✗	35–52[a]	2.5–7.5		0.0125–0.06	125–600[f]	Geracitano et al. (2004b)	Seasons and control vs. contaminated site	Patos Lagoon Estuary, RS
Laeonereis acuta	D	IM	L	BS and Mucus	Range of the mean	✗				2–8[c]	0.2–4[g]	Marques et al. (2013)	Fullerene exposure	Patos Lagoon Estuary, RS
Laeonereis acuta	D	IM	L	Mucus	Mean	✓	15.07[a]	16.6	0.052	not detected		Moraes et al. (2006)	Antioxidant enzymes in mucus secretion	Patos Lagoon Estuary, RS
Laeonereis acuta	D	IM	L	WB	Range of the mean	✗	15–67[a]	1.5–4		0.025–0.045	43–60[f]	Ventura-Lima et al. (2007)	Arsenic exposure	Patos Lagoon Estuary, RS
Laeonereis acuta	D	IM	L	WB	Range of the mean	✓	18.21–20.49[a]	2.41–3.24		0.016–0.027	337.88–481.87[f]	Geracitano et al. (2002)	Cooper exposure (acute)	Patos Lagoon Estuary, RS

Laeonereis acuta	D	IM	L	WB	Range of the mean	✗	15–40[a]	1.5–5.9	15-30[c]	0.015–0.03		Geracitano et al. (2004a)	Cooper exposure (acute)	Patos Lagoon Estuary, RS
Laeonereis acuta	D	IM	L	WB	Range of the mean	✓	3.54–38.19[a]	2.16–4.06		0.012–0.022	833.4–871.6[f]	Geracitano et al. (2002)	Cooper exposure (chronic)	Patos Lagoon Estuary, RS
Laeonereis acuta	D	IM	L	WB	Range of the mean	✗	12–45[a]	3–4.8		0.01–0.035		Geracitano et al. (2004a)	Cooper exposure (chronic)	Patos Lagoon Estuary, RS
Laeonereis acuta	D	IM	L	WB	Range of the mean	✗		1.5–2.5	0.005–0.050		125–750[f]	da Rosa et al. (2008)	Hydrogen peroxide exposure	Patos Lagoon Estuary, RS
Laeonereis culveri	D	IM	F	WB	Range of the mean	✗	120–280	7–14	100–220	15–40	0.5–12[e]	Sandrini-Neto et al. (2016)	Diesel oil exposure	Paranaguá Estuarine System, PR
Laeonereis culveri	D	IM	L	WB	Mean (Min - Max)	✓	923.8 (703.9–1228.1)	24.4 (13.6–44.8)	282.6 (260.4–320.8)	36.4 (28.05–48)	20.4 (7.9–32.2)[e]	Sardi et al. (in press)	Diesel oil exposure	Paranaguá Estuarine System, PR
Mytella guayanensis	F	IM	Bm	DG	Range of the mean	✗	290–300[a]	3–6[a]	1–5	10–25	110–300[h]	Torres et al. (2002)	Gradient of pollution	Florianopolis, SC
Mytella guayanensis	F	M	F	DG	Range of the mean	✗			50–150[c]	10–30[c]		Marques et al. (2014)	Exposure to diesel oil	Paranaguá Estuarine Complex, PR
Mytilus edulis	F	RS	Bm	G	Range of the mean	✗	22–30[a]	3–3.5		25–30		Rola et al. (2012)	Gradient of pollution	Patos Lagoon Estuary, RS
Perna perna	F	RS	Bm	G	Range of the mean	✓		7.59–11.81		220.63–531.71		Pereira et al. (2007)	Seasons and gradient of pollution	São Sebastião Channel, SP
Perna perna	F	RS	Bm	G	Range of the mean	✓		7.59–11.81		220.63–358.88[d]		Pereira et al. (2007)	Seasons and gradient of pollution	Santos estuarine system and São Sebastião Channel, SP
Perna perna	F	RS	L	DG	Range of the mean	✓	138.47–178.09[a]	9.74–11.44	6.85–8.19[d]	129.55–197.4	80–100[h]	Alves de Almeida et al. (2007)	Air exposure and submersion	Farmed animals Florianopolis, SC
Perna perna	F	RS	L	DG & G	Range of the mean	✓	14.24–105.2[a]	4.72–24.49	0.0017–0.0133	0.14–1.34		Nogueira et al. (2015)	Diesel B5 exposure	Farmed animals Florianopolis, SC
Perna perna	F	RS	L	G	Range of the mean	✓	168.14–231.55[a]	3.1–3.49	8.2–9.3[d]	558.4–630.7[d]	5–12[h]	Alves de Almeida et al. (2007)	Air exposure and submersion	Farmed animals Florianopolis, SC
Perna perna	F	RS	L	G	Range of the mean	✗		14–16		370–380		Alves et al. (2002)	Furadan exposure	Florianopolis, SC
Perna perna	F	RS	T	G	Range of the mean	✓		17.9–26.1	577.1–1762.7[c]	669.8–1458.9[c]		Pereira et al. (2014)	Gradient of pollution	Santos estuarine system and São Sebastião Channel, SP
Perna perna	F	RS	T	G	Range of the mean	✓		14.7–26.9	32.3–1762.7[c]	182.5–1458.9[c]		Pereira et al. (2010)	Seasons and gradient of pollution	Santos estuarine system and São Sebastião Channel, SP
Neritina virginea	**G**	**SM**	**B**	**WB**	**Mean (Min - Max)**	✓	**394.5 (63.9–1787.8)**	**1.5 (0.1–11.1)[b]**	**104.5 (6.2–270.5)**	**155.5 (3.7–399.8)**	**3643.3 (882.3–8452)**	**This study**	**Seasons and control vs. contaminated site**	**Paranaguá Estuarine System, PR**
Neritina virginea	G	SM	F	WB	Range of the mean	✗	140–350	22–52	75–160	400–800	4–10[e]	Sandrini-Neto et al. (2016)	Diesel oil exposure	Paranaguá Estuarine System, PR
Uca maracoani	**O**	**IM**	**B**	**H**	**Mean (Min - Max)**	✓	**188.9 (11.6–688.6)**	**1.8 (0.019–10)[b]**	**34.3 (2.1–84.7)**	**167.03 (9.6–441.7)**	**11,629 (2840–34,960)**	**This study**	**Seasons and control vs. contaminated site**	**Paranaguá Estuarine System, PR**

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Functional group: C: carnivorous; D: detritivorous; F: filter feeder; G: grazer; O: omnivorous.
Habitat: B/P: benthopelagic; FW: fresh water; IM: intertidal mudflat; MR: mangrove roots; P: pelagic; RS: rocky shores; SB: subtidal benthos; SM: salt marshes.
Type of work B: Baseline; Bm: biomonitoring; F: Field study; L: Laboratory study; T: Transplant experiment.
Tissue BS: body sections; DG: digestive gland; G: gills; H: hepatopancreas; K: Kidney; L: liver; M: muscle; WB: whole body.
SOD: Pyrogallol oxidation U mg prot-1; CAT, GPx and GST: μmol min-1 mg prot-1; LPO: MDA nmol g-1 wet weight.
[a] Cytochrome c reduction U mg prot-1.
[b] mmol·min-1 mg prot-1.
[c] nmol·min-1 mg prot-1.
[d] pmol·min-1 mg prot-1.
[e] nmol·mg prot-1.
[f] nmol CHP g wet weight-1.
[g] TBARS mg of TBARS mg-1 prot.
[h] nmol TBARS g^{-1} ww.

freshwater and large amounts of terrigenous organic matter into the Continga sub-estuary (de Abreu-Mota et al., 2014). Although no chemical data on PAH contamination is available for this sampling site, it is known that sediments with high organic carbon contents tend to adsorb hydrophobic compounds, as shown by Froehner et al. (2011). Regarding *G. genidens*, contamination levels of PAH in the polluted site doubled those found in the reference site (see Table S1), which is located in Guaraqueçaba Bay, a preserved area (Lana et al., 2001). Limiting the contamination occurring in the area to a comparison of PAH contamination solely is unrealistic, yet our results allowed separating these two locations. However, the seasonal effect in antioxidant biomarkers of *G. genidens* and *U. maracoani* was stronger (as measured by the pseudo-F ratio and the percentage explained by RDA2) than that observed for the contamination condition.

C. rhizophorae biomarker activity also allowed to discriminate between reference (PAH in sediments 13.09 ng g^{-1} Sandrini-Neto et al., 2016) and polluted locations (89.14 ng g^{-1} Rizzi et al., 2016). Previous studies have also highlighted the response of biotransformation and antioxidant enzymes from the mangrove oyster as suitable biomarkers for contamination (Alves et al., 2002; Zanette et al., 2006; Zanette et al., 2008; Maranho et al., 2012). Significant univariate variations of average values from reference vs. polluted sites were more frequent within endpoints measured for the mangrove oyster, the clam and catfish species. This result was not consistent with multivariate results for *A. flexuosa*, in which the interaction between season and condition proved significant for *A. flexuosa*, with the variation in the biomarker response between locations more evident in winter.

By studying different species, we incorporated the habitat diversity of PES in our survey. The clam *A. flexuosa* and the fiddler crab *U. maracoani* occur in unvegetated tidal mudflats; the oyster *C. rhizophorae* occur in mangroves, *N. virginea* in salt marshes and the catfish *G. genidens* in shallow subtidal habitats. Besides habitat preferences, the target species belong to diverse feeding guilds. Exposure pathways to contaminants are unique for each species, potentially explained by changes in contaminant bioavailability given contaminant partitioning properties between sediment, pore water and overlying water (Di Toro et al., 1991; Gong et al., 2014). However, we expected to find a common or shared biomarker response among very diverse organisms. Our results demonstrate that the integrated responses of biomarkers are highly species-specific, and significantly affected by seasonality and contamination levels. As natural biochemical signals required for normal homeostasis, biomarkers are indeed presumed to vary among species that widely differ in their phylogenetic relationships, feeding guilds, and habitats. Similar comparisons of multi-biomarker responses in a set of diverse organisms are still scarce in the literature, and consistent biomarker validation has been done for only a few species, mainly bivalves. As a result, biomarker responses in selected indicator species may not reflect the range in sensitivity of other species or functional groups within a community. This obviously may hinder the development of consistent strategies for species selection in monitoring programs.

For practical purposes, the interpretation of biomarker responses to seasonal variation and varying contamination conditions should naturally lead to the selection of indicator species. Based on the responsiveness of their measured endpoints, both in univariate and multivariate approaches, the bivalves *A. flexuosa*, *C. rhizophorae* and the catfish *G. genidens* are herein proposed as relevant contamination sentinels, since their biochemical responses were more easily discriminated between reference and polluted locations. Filter feeders such as *A. flexuosa* and *C. rhizophorae* are more exposed to the water-soluble fraction of contaminants than detritivores, grazers or carnivores. Epifaunal bivalves are frequent targets in pollution monitoring studies because of their sessile lifestyle, high filtration capacity, and ability to accumulate contaminants (De Luca-Abbott et al., 2005; Nahrgang et al., 2013). A sessile lifestyle is usually associated with constant exposure pathways to contaminants. However, contaminants often show complex distributions among suspended particles, sediments, solution, pore water and

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food. Exposure to contaminants thus depends on the way each species "samples" their complex milieu (Luoma, 1996). In this sense, bivalves are mostly exposed to contaminants suspended or dissolved in the seawater and, therefore, their antioxidant response mainly responds to a water column-influenced exposure pathway (De Luca-Abbott et al., 2005). Infaunal suspension-feeding species are also susceptible to contaminants present in the sediment. A recent study by Cardoso et al. (2016) demonstrated that most of the PAH contamination at PES is associated with suspended particulate matter. Omnivorous species such as *G. genidens* are exposed to water and sediment contamination and also to contaminants bioaccumulated in their food.

Baseline enzymatic levels in PES were levels of magnitude higher than literature data (see for example CAT activity). Although much effort has been recently put in standardizing individual biomarkers and characterizing their "normal" response range (Wells and Balls, 1994; Viarengo et al., 2000), different protocols and laboratory conditions may explain some of the observed variation between our and literature data. To consolidate the use of biomarkers into routine environmental monitoring, standardizations and quality control routines are much needed. Besides, biomarker responses are known to vary considerably at different spatial scales and at various temporal scales (Brown et al., 2004; Depledge and Galloway, 2015). Quality control routines and comparisons with results available in the literature may become a downside for interpretation and implementation of biomarkers within environmental monitoring. To deal with this, we propose the implementation of multivariate tools to at least provide qualitative comparisons between widely varying data. Biomarker-based biomonitoring studies have traditionally made little use of such multivariate approaches, which are routine in ecological research. Only 10 of the 35 reviewed studies employed multivariate analysis, and its use was mostly restricted to baseline and biomonitoring routines. However, in all cases, multivariate analysis was restricted to principal component analysis (PCA). None of these studies used contamination as a factor or as a structuring variable that would influence organisms' antioxidant machinery as we have done. Within this framework, understanding the ecological and biological circumstances for which pollutants effects are significant becomes the main objective, pushing to a second plane the identification of the best tool (or biomarker) to demonstrate 'damage' from pollutants.

Determining biomarker baseline levels is mandatory for the proper implementation of biomonitoring programs. This study explores the spatial and temporal variation in biomarker levels in a subtropical estuary, and can thus be used as a starting point for future biomonitoring programs. Our results are a necessary step towards the consistent choice of sentinel species for biomarker-based monitoring in tropical and subtropical estuaries. We also propose multivariate approaches, such as RDA, as a better strategy to visually present the results and to quantitatively assess variability in multi-species and multi-biomarker studies. Antioxidant biomarkers were highly species-specific and strongly affected by seasonality. All target species, excepting *N. virginea,* responded secondarily to varying levels of contamination by presenting varying overall antioxidant responses. The bivalve species *A. flexuosa*, *C. rhizophorae,* and the catfish *G. genidens* are proposed as sentinels of contamination since the integrated response of their antioxidant enzymes allowed discrimination of locations with different levels of contamination. Moreover, these species are abundant, economically important, and widely distributed. However, further experimental work is needed to establish better causality relationships between contamination levels and biological responses. Such approaches will be crucial to better understand antioxidant biomarkers responses under background natural conditions and for developing cost-effective and ecologically sound monitoring programs in tropical regions.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.marpolbul.2016.08.014.

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