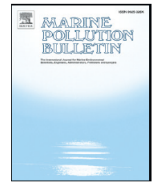


PAPER I

**BASELINE LEVELS OF OXIDATIVE STRESS BIOMARKERS IN
SPECIES FROM A SUBTROPICAL ESTUARINE SYSTEM
(PARANAGUÁ BAY, SOUTHERN BRAZIL)**

Adriana E. Sardi, Paul E. Renaud, Paulo da Cunha Lana, Lionel Camus

Published in *Marine Pollution Bulletin*



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

Adriana E. Sardi^{a, b, *}, Paul E. Renaud^a, Paulo da Cunha Lana^c and Lionel Camus^{a, b}

^a Fram Centre, Akvaplan-niva, 9296 Tromsø, Norway.

^b University of Tromsø, Faculty of Science, Faculty of Science and Technology, Department of Science & Safety, N-9037 Tromsø, Norway.

^c Centro de Estudos do Mar, Universidade Federal do Paraná (UFPR), PO Box 61, 83255-000 Pontal do Paraná, Paraná, Brazil.

*Address for correspondence:

Akvaplan-niva,

Fram Centre,

9296 Tromsø, Norway

Phone: +47 77 75 351

Fax: +47 77 75 03 01

e-mail: ads@akvaplan.niva.no

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.

Keywords: Antioxidant biomarkers, Oil contamination, Tropical species, Multivariate analysis, Monitoring design Brazil.

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 km² 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 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 brasiliiana*) is an abundant, in-faunal 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 $-80\text{ }^{\circ}\text{C}$ 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 $12,500\text{ g}$ for 30 min at $4\text{ }^{\circ}\text{C}$ in a Hermle z233 MK-2 microcentrifuge.

Aliquots were prepared on ice for enzymatic analyses and stored at $-80\text{ }^{\circ}\text{C}$ 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.

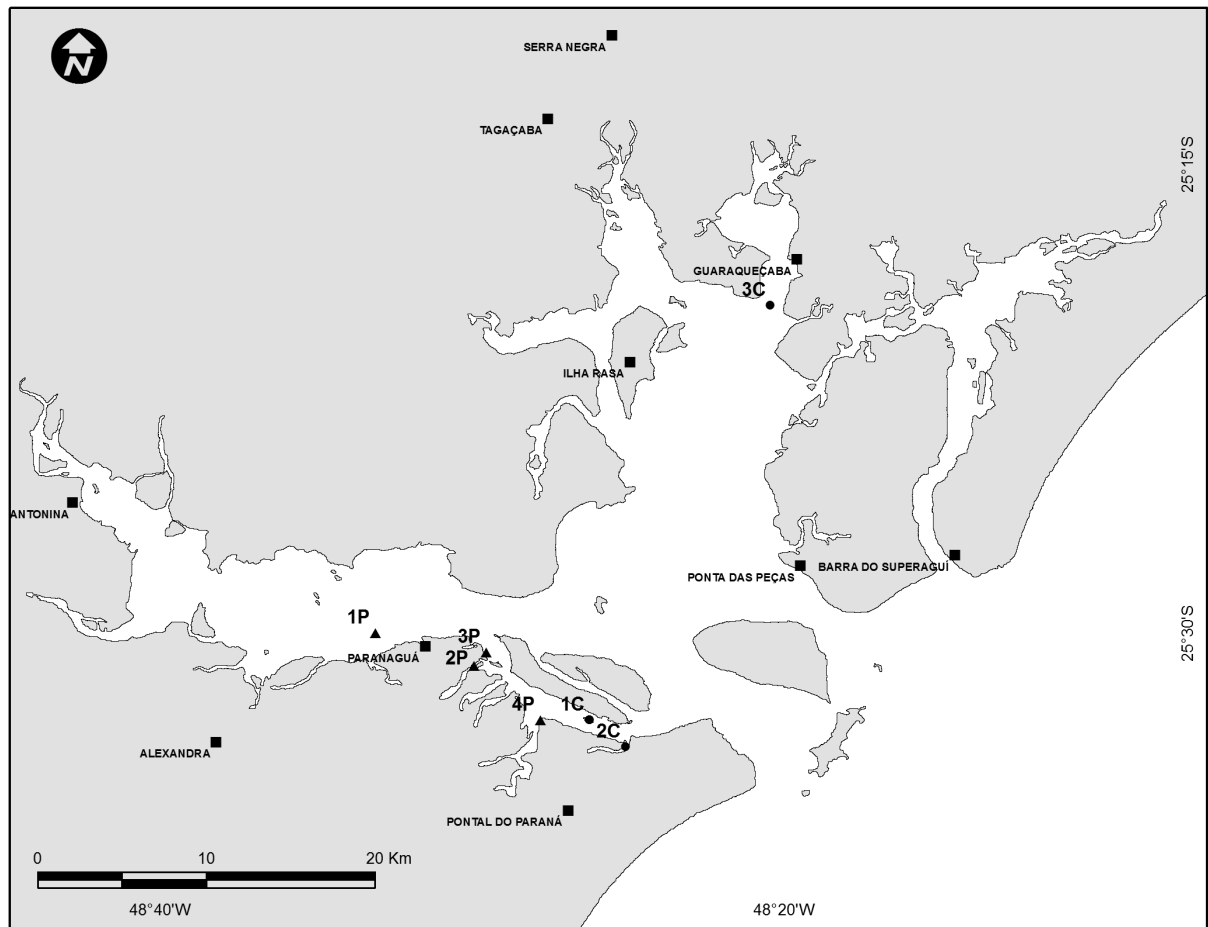


Figure 1. Sampling locations at the Paranaguá Estuarine System southern Brazil. Points labeled with C correspond to locations considered as control or reference, while points labeled with P refer to polluted locations. *Anomalocardia flexuosa* control 1C, polluted 3P; *Crassostrea rhizophorae* control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

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 pH 7.6, and 20 mmol L^{-1} H_2O_2 . Molar extinction coefficient employed was 40 mol L^{-1} cm^{-1} . Specific activity was 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 $\mu\text{mol min}^{-1} \text{mg}^{-1}$ protein and determined using the molar extinction coefficient of 0.622 $\text{mmol L}^{-1} \text{cm}^{-1}$.

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 $\text{mol L}^{-1} \text{cm}^{-1}$ and activity expressed as $\mu\text{mol min}^{-1} \text{mg}^{-1}$ 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).

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.mg⁻¹ of protein; GPx and GST: μMol.min⁻¹.mg⁻¹; of protein; SOD: U mg.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

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).

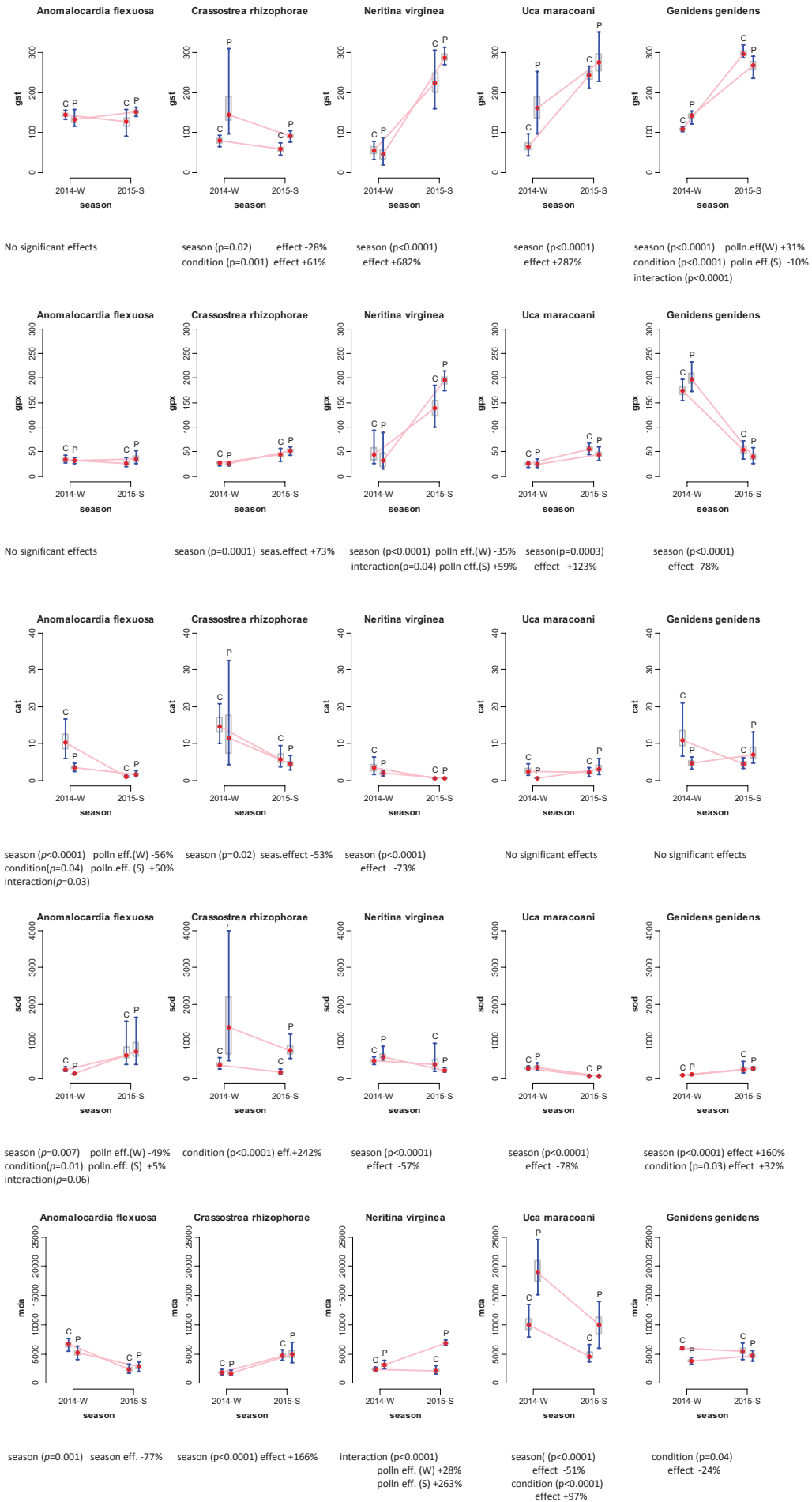


Figure 2. Confidence plots derived from log-transformed enzymatic activities in studied species. Plots represent the mean (red points), 50% confidence intervals (boxes) and 95% confidence intervals (dispersion lines). Effects of significant interaction are given as estimated changes between polluted (P) and control (C) samples for winter (W) and summer (S). When significant, the marginal effect of season and condition are also denoted. Enzyme activity units are, CAT: mMol.min⁻¹.mg⁻¹ of protein; GPx and GST: μMol.min⁻¹.mg⁻¹; of protein; SOD: U mg.ml⁻¹ of protein; MDA: nM g⁻¹ wet weight.

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 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.

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 (%)
<i>Anomalocardia flexuosa</i>	40.1	35.8	4.3
<i>Crassostrea rhizophorae</i>	35.7	21.8	13.9
<i>Neritina virginea</i>	55.9	51.5	4.4
<i>Uca maracoani</i>	34.9	28.5	6.4
<i>Genidens genidens</i>	45.9	41.6	4.3

For all the target species, season showed much lower P-values ($p < 0.0001$), 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.

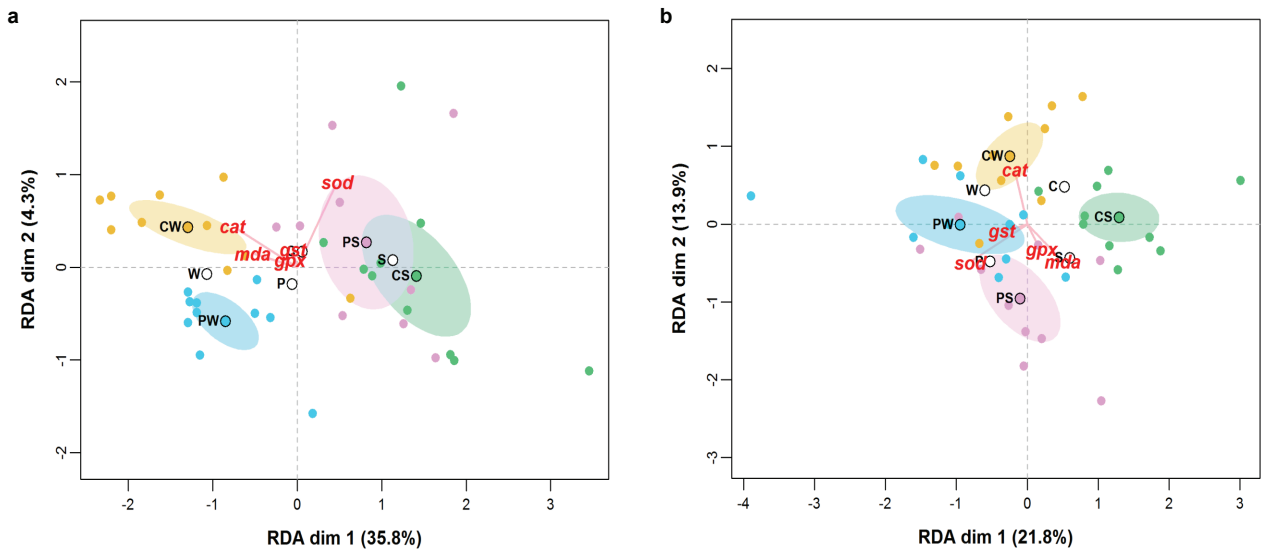


Figure 3. RDA biplots derived from log transformed enzymatic activities in a) *Anomalocardia flexuosa* and b) *Crassostrea rhizophorae* sampled during winter and summer seasons in locations with different levels of contamination. Ellipses represent 95% confidence intervals from centroids of the interaction 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).

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. S1a). 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. S1b). 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. S1c).

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).

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; *R*²: coefficient of determination, *P*: probability of *F*.

Species	Source of variation	<i>F</i>	<i>R</i> ²	<i>P</i>
<i>Anomalocardia flexuosa</i>	Season (Se)	20.14	0.34	<0.0001
	Condition (Cond)	0.84	0.01	0.47
	Se:Cond	3.14	0.05	0.024
<i>Crassostrea rhizophorae</i>	Season (Se)	10.26	0.18	<0.0001
	Condition (Cond)	9.55	0.17	<0.0001
	Se:Cond	1.29	0.02	0.26
<i>Neritina virginea</i>	Season (Se)	40.12	0.50	<0.0001
	Condition (Cond)	2.43	0.03	0.078
	Se:Cond	2.77	0.03	0.057
<i>Uca maracoani</i>	Season (Se)	16.55	0.28	<0.0001
	Condition (Cond)	2.93	0.05	0.026
	Se:Cond	1.68	0.02	0.15
<i>Genidens genidens</i>	Season (Se)	53.66	0.52	<0.0001
	Condition (Cond)	4.61	0.04	0.012
	Se:Cond	2.80	0.03	0.058

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).

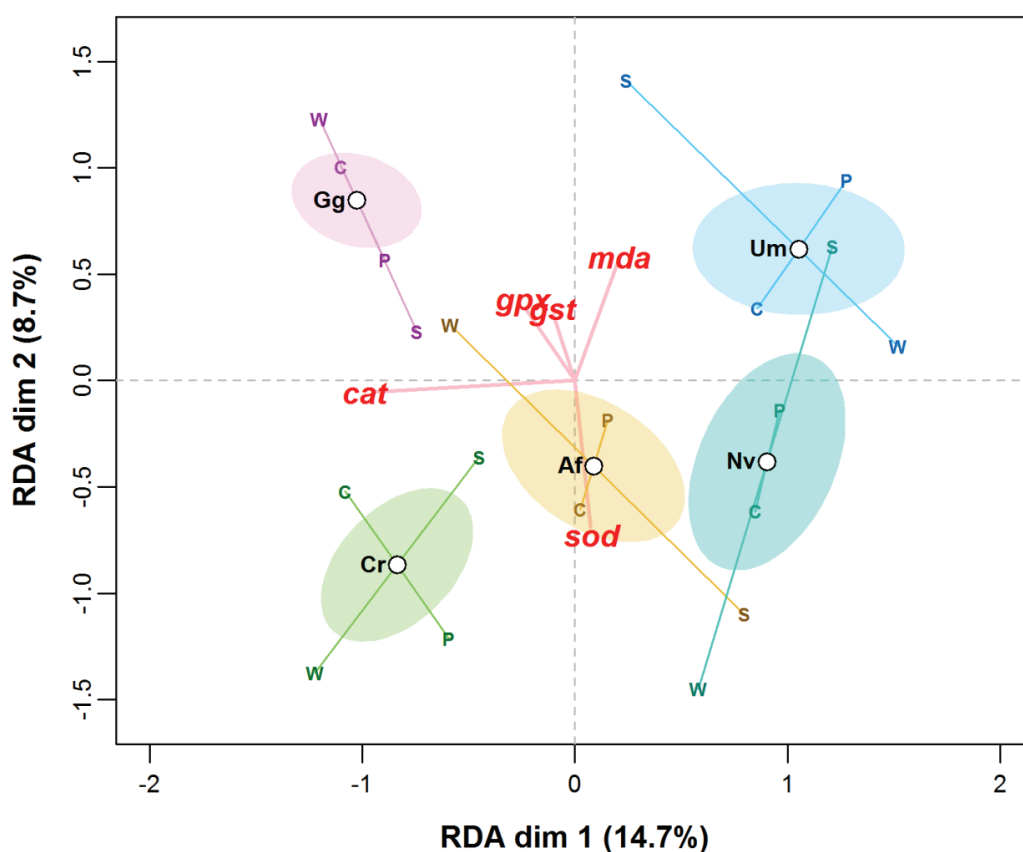


Figure 4. Redundancy analysis (RDA) for studied species. The analysis included species and the interaction between season and condition as predictor variables. Predictors accounted for 35.5% of the total variance. Ellipses represent 95% confidence centroids for each species and axis around it indicates 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: control; P: polluted; W: winter; S: summer.

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 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 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; Maranhão et al., 2012). Significant univariate variations of average values from reference vs. polluted sites were more frequent within end-points 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.

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
<i>Anomalocardia flexuosa</i>	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	Paranáguá Estuarine System, PR
<i>Anomalocardia flexuosa</i>	F	IM	F	DG	Range of the mean	x	150-210	18-28	100-240	60-158	0.5-3.2 e	Sandrini-Neto et al. 2016	Diesel oil exposure	Paranáguá Estuarine System, PR
<i>Anomalocardia flexuosa</i>	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. <i>In press</i>	Diesel oil exposure	Paranáguá Estuarine System, PR
<i>Cathorops spixii</i>	O	SB	Bm	L	Range of the mean	x				250-3500		Azevedo et al. 2009	Seasons and gradient of pollution	Santos estuarine system and Cananéia estuarine-lagoon complex, SP
<i>Cathorops spixii</i>	O	SB	Bm	L & M	Range of the mean	✓		0.51-1.18c		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
<i>Cathorops spixii</i>	O	SB	Bm	G, K & L	Range of the median	x			10-400 c	100-700 c	0.5-4 g	Gusso-Choueri et al. 2015	Seasons and gradient of pollution	MPA Cananéia-Iguape-Peruíbe, PR
<i>Genidens genidens</i>	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	Paranáguá Estuarine System, PR
<i>Hyphessobrycon reticulatus</i>	O	FW, B/P	Bm	L	Range of the mean	x		220-230		50-90 c	15-30 e	Katsumiti et al. 2013	Post-oil spill evaluation	Araucaria City, PR
<i>Microgogonias furnieri</i>	C	SB	B	L	Range of the mean	x		40-130		0.3-0.7	1000-3000 f	Amado et al. 2006a	Seasons and control vs. contaminated site	Patos Lagoon Estuary, RS
<i>Paralichthys orbignyanus</i>	C	SB	B	L	Range of the mean	x		1.5-2		0.75-2	50-180 f	Amado et al. 2006b	Seasons and control vs. contaminated site	Patos Lagoon Estuary, RS
<i>Poecilia vivipara</i>	O	P	L	DG, G & L	Range of the mean	x	5-90 a	1-110		12-800 c	-	Machado et al. 2014	Phenanthrene exposure	Casino Beach, RS
<i>Poecilia vivipara</i>	O	P	L	G, L & M	Range of the mean	x	3-30 a	1.5-1100		20-350	-	Machado et al. 2013	Cooper exposure	Casino Beach, RS
<i>Sciades herzbergi</i>	O	SB	Bm	L	Range of the mean	x		900-1000		2.0-2.1	-	Carvalho-Neto and Abreu-Silva 2010	Gradient of pollution	San Marcos Bay, MA
<i>Balanus improvisus</i>	F	RS	Bm	WB	Range of the mean	x		3.6-4.8		20-110	600-2200 f	Zanette et al. 2015	Seasons and gradient of pollution	Patos Lagoon Estuary, RS
<i>Crassostrea brasiliiana</i>	F	MR	L	DG & G	Range of the mean	x	30-120 a	60-450e	4-8 c	40-130 e	90-150 f	Lüchmann et al. 2011	Diesel oil exposure	Farmed animals Florianopolis, SC

	F	RS	Bm	DG & G	Range of the mean	x	5-12 a	50-150	03-50	-	Souza et al. 2012	Gradient of pollution	Florianopolis, SC
<i>Crassostrea gigas</i>	F	MR	T	DG	Range of the mean	x	5-12 a	50-150	03-50	-	Souza et al. 2012	Gradient of pollution	Florianopolis, SC
<i>Crassostrea gigas</i>	F	MR	T	DG	Range of the mean	x	5-12 a	450-650	30-45		Zanette et al. 2008	Gradient of sewage pollution	São Jose, SC
<i>Crassostrea gigas</i>	F	MR	T	G	Range of the mean	x	50-70	50-70	95-110		Zanette et al. 2008	Gradient of sewage pollution	São Jose, SC
<i>Crassostrea rhizophorae</i>	F	MR	B	G	Mean (Min - Max)	✓	648.6 (21.4-8996.7)	9.16 (0.3-68.8) b	93.08 (26.5-525.1)	3296.7 (641.2-10561)	This study	Seasons and control vs. contaminated site	Paranaguá Estuarine System, PR
<i>Crassostrea rhizophorae</i>	F	MR	B	G	Range of the mean	x	100-130	100-120	20-55	100-250 f	Zanette et al. 2006	Seasons and gradient of pollution	Piraquê Estuarine Complex, ES
<i>Crassostrea rhizophorae</i>	F	MR	B	G	Range of the mean	x	50-120	50-120	10-50	100-125 f	Zanette et al. 2006	Seasons and gradient of pollution	Itamaracá Bay, PE
<i>Crassostrea rhizophorae</i>	F	MR	Bm	G	Range of the mean	✓	68.29-165.08 d	68.29-165.08 d	22.22-37.63 c	2.7-2.97 g	Marinho et al. 2012	Gradient of pollution	Paranaguá Estuarine Complex, PR
<i>Crassostrea rhizophorae</i>	F	MR	Bm	G	Range of the mean	✓	122.05-270.1 d	122.05-270.1 d	45.37-71.25 c	3.04-11.67 g	Marinho et al. 2012	Gradient of pollution	Santos estuarine system, SP
<i>Crassostrea rhizophorae</i>	F	MR	Bm	G	Range of the mean	x	60-112	60-112	10-50	100-1300 f	Zanette et al. 2006	Seasons and gradient of pollution	Paranaguá Estuarine Complex, PR
<i>Crassostrea rhizophorae</i>	F	MR	L	DG	Range of the mean	x	120-270	120-270	2-17		da Silva et al. 2005	Diesel oil exposure and salinity	Florianopolis, SC
<i>Crassostrea rhizophorae</i>	F	MR	L	G	Range of the mean	x	10-14	10-14	150-155		Alves et al. 2002	Furadan exposure	Florianopolis, SC
<i>Crassostrea rhizophorae</i>	F	MR	T	DG	Range of the mean	x	320-500	320-500	35-50		Zanette et al. 2008	Gradient of sewage pollution	São Jose, SC
<i>Crassostrea rhizophorae</i>	F	MR	T	G	Range of the mean	✓	107.64-232.45 c	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
<i>Crassostrea rhizophorae</i>	F	MR	T	G	Range of the mean	x	100-210	100-210	110-140		Zanette et al. 2008	Gradient of sewage pollution	São Jose, SC
<i>Laonereis acuta</i>	D	IM	B	BS	Range of the mean	✓	88.35-238.58 f	88.35-238.58 f			Ferreira-Cravo et al. 2007	Seasons, control vs. contaminated sites, and organisms body region	Patos Lagoon Estuary, RS
<i>Laonereis acuta</i>	D	IM	B	WB	Range of the mean	x	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
<i>Laonereis acuta</i>	D	IM	L	BS and Mucus	Range of the mean	x			2-8 c	0.2-4 g	Marques et al. 2013	Fullerene exposure	Patos Lagoon Estuary, RS
<i>Laonereis acuta</i>	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
<i>Laonereis acuta</i>	D	IM	L	WB	Range of the mean	x	15-67 a	1.5-4	0.025-0.045	43-60 f	Ventura-Lima et al. 2007	Arsenic exposure	Patos Lagoon Estuary, RS

<i>Laeoneis acuta</i>	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
<i>Laeoneis acuta</i>	D	IM	L	WB	Range of the mean	x	15-40 a	1.5-5.9	0.015-0.03		Geracitano et al. 2004a	Cooper exposure (acute)	Patos Lagoon Estuary, RS
<i>Laeoneis acuta</i>	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
<i>Laeoneis acuta</i>	D	IM	L	WB	Range of the mean	x	12-45 a	3-4.8	0.01-0.035		Geracitano et al. 2004a	Cooper exposure (chronic)	Patos Lagoon Estuary, RS
<i>Laeoneis acuta</i>	D	IM	L	WB	Range of the mean	x	1.5-2.5	0.005-0.050		125-750 f	da Rosa et al. 2008	Hydrogen peroxide exposure	Patos Lagoon Estuary, RS
<i>Laeoneis culveri</i>	D	IM	F	WB	Range of the mean	x	120-280	7-14	15-40	0.5-12 e	Sandri-Neto et al. 2016	Diesel oil exposure	Paranaguá Estuarine System, PR
<i>Laeoneis culveri</i>	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)	Sardi et al. in press	Diesel oil exposure	Paranaguá Estuarine System, PR
<i>Mytila guayanensis</i>	F	IM	Bm	DG	Range of the mean	x	290-300 a	3-6 a	1-5	110-300 i	Torres et al. 2002	Gradient of pollution	Florianópolis, SC
<i>Mytila guayanensis</i>	F	M	F	DG	Range of the mean	x			50-150 c	10-30 c	Marques et al. 2014	Exposure to diesel oil	Paranaguá Estuarine Complex, PR
<i>Mytilus edulis</i>	F	RS	Bm	G	Range of the mean	x	22-30 a	3-3.5	25-30		Rola et al. 2012	Gradient of pollution	Patos Lagoon Estuary, RS
<i>Perna perna</i>	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
<i>Perna perna</i>	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
<i>Perna perna</i>	F	RS	L	DG	Range of the mean	✓	138.47-178.09 a	9.74-11.44	6.85-8.19 d	80-100 i	Alves de Almeida et al. 2005	Air exposure and submersion	Farmed animals Florianópolis, SC
<i>Perna perna</i>	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	Noqueira et al. 2015	Diesel B5 exposure	Farmed animals Florianópolis, SC
<i>Perna perna</i>	F	RS	L	G	Range of the mean	✓	168.14-231.55 a	3.1-3.49	558.4-630.7 d	5-12 i	Alves de Almeida et al. 2005	Air exposure and submersion	Farmed animals Florianópolis, SC
<i>Perna perna</i>	F	RS	L	G	Range of the mean	x		14-16	370-380		Alves et al. 2002	Furadan exposure	Florianópolis, SC
<i>Perna perna</i>	F	RS	T	G	Range of the mean	✓	17.9-26.1	577.1-1762.7 c	669.8-1486.9 c		Pereira et al. 2014	Gradient of pollution	Santos estuarine system and São Sebastião Channel, SP
<i>Perna perna</i>	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

	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	Paranáguá Estuarine System, PR
<i>Neritina virginea</i>	G	SM	F	WB	Range of the mean	x	22-52	75-160	400-800	4-10 e	Sandrih-Neto et al. 2016	Diesel oil exposure	Paranáguá Estuarine System, PR
<i>Uca maracoani</i>	O	IM	B	H	Mean (Min -Max)	✓	1.8 (0.019-10) b	34.3 (2.1-84.7)	167.03 (9.6-441.7)	11629 (2840-34960)	This study	Seasons and control vs. contaminated site	Paranáguá Estuarine System, PR

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; Bn: bionitoring; 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 : $\mu\text{mol min}^{-1} \text{mg prot}^{-1}$; 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: 50% inhibition of xanthine oxidase reaction; i: nmol TBARS g-1 ww

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 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.

Acknowledgments

We thank Michael Greenacre for reviewing and advising the authors on the data analyses. We also thank Kristine Sperre, Bruno Costelini, Kalina Brauko, Thayanne Lima, Bianca Possamai, Angel Moreira and Carolina Capel, for helping with the animal sampling and dissection. This study was funded by the “Latin Amerika” research program of the Norwegian Research Council, attributed to Akvaplan-niva under the Project number 227180/H30.

References

- Absher, T.M., 1989. Populações naturais de ostras do gênero *Crassostrea* do litoral do Paraná - Desenvolvimento larval recrutamento e crescimento. Dissertação de doutorado Universidade de São Paulo Instituto Oceanográfico (185p).
- Administração dos Portos de Paranaguá e Antonina – APPA, 2016. (Retrieved from) <http://www.portosdoparanapgovbr/modules/conteudo/conteudophp?conteudo=369>.
- Aebi, H., 1984. Catalase in vitro. *Methods Enzymol.* 105, 121–126.
- Alonso-Alvarez, C., Bertrand, S., Devevey, G., Prost, J., Faivre, B., Sorci, G., 2004. Increased susceptibility to oxidative stress as a proximate cost of reproduction. *Ecol. Lett.* 7, 363–368.
- Alves de Almeida, E., Celso Dias Bainy, A., Paula de Melo Loureiro, A., Regina Martinez, G., Miyamoto, S., Onuki, J., Barbosa, L.F., Garcia, Prado, F.M., Ronsen, G.E., Sigolo, C.A., Brochini, C.B., Martins, A.M.G., Gennari de Medeiros, M.H., Di Mascio, P., 2007. Oxidative stress in *Perna perna* and other bivalves as indicators of environmental stress in the Brazilian marine environment: antioxidants, lipid peroxidation and DNA damage. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* 146 (4), 588–600.
- Alves, S.R.C., Severino, P.C., Ibbotson, D.P., da Silva, A.Z., Lopes, F.R.A.S., Sáenz, L.A., Bainy, A.C.D., 2002. Effects of furadan in the brown mussel *Perna perna* and in the mangrove oyster *Crassostrea rhizophorae*. *Mar. Environ. Res.* 54 (3–5), 241–245.
- Amado, L.L., da Rosa, C.E., Leite, A.M., Moraes, L., Pires, W.V., Pinho, G.L.L., Martins, C.M.G., Robaldo, R.B., Nery, L.E.M., Monserrat, J.M., Bianchini, A., Martinez, P.E., Geracitano, L.A., 2006a. Biomarkers in croakers *Micropogonias furnieri* (Teleostei: Sciaenidae) from polluted and non-polluted areas from the Patos lagoon estuary (southern Brazil): evidences of genotoxic and immunological effects. *Mar. Pollut. Bull.* 52, 199–206.
- Amado, L.L., Robaldo, R.B., Geracitano, L., Monserrat, J.M., Bianchini, A., 2006b. Biomarkers of exposure and effect in the Brazilian flounder *Paralichthys orbignyanus* (Teleostei: Paralichthyidae) from the Patos lagoon estuary (southern Brazil). *Mar. Pollut. Bull.* 52 (2), 207–213.
- Azevedo, J.S., Fernandez, W.S., Farias, L.A., Fávoro, D.T.I., Braga, E.S., 2009. Use of *Cathorops spixii* as bioindicator of pollution of trace metals in the Santos Bay, Brazil. *Ecotoxicology* 18 (5), 577–586.

- Azevedo, J.S., Braga, E.S., de Assis, H.C.S., Oliveira Ribeiro, C.A., 2013. Biochemical changes in the liver and gill of *Cathorops spixii* collected seasonally in two Brazilian estuaries under varying influences of anthropogenic activities. *Ecotoxicol. Environ. Saf.* 96, 220–230.
- Bocchetti, R., Regoli, F., 2006. Seasonal variability of oxidative biomarkers, lysosomal parameters, metallothioneins and peroxisomal enzymes in the Mediterranean mussel *Mytilus galloprovincialis* from Adriatic Sea. *Chemosphere* 65, 913–921.
- Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72, 248–254.
- Brauko, K.M., Muniz, P., Martins, C.D.C., Lana, P.C., 2016. Assessing the suitability of five benthic indices for environmental health assessment in a large subtropical south American estuary. *Ecol. Indic.* 64 (May), 258–265.
- Brown, R.J., Galloway, T.S., Lowe, D., Browne, M.A., Dissanayake, A., Jones, M.B., Depledge, M.H., 2004. Differential sensitivity of three marine invertebrates to copper assessed using multiple biomarkers. *Aquat. Toxicol.* 66, 267–278.
- Cardoso, F.D., Dauner, A.L.L., Martins, C.C., 2016. A critical and comparative appraisal of polycyclic aromatic hydrocarbons in sediments and suspended particulate material from a large south American subtropical estuary. *Environ. Pollut.* 214, 219–229.
- Carvalho-Neta, R.N.F., Abreu-Silva, A.L., 2010. *Sciades herzbergii* Oxidative stress biomarkers: an *in situ* study of an estuarine ecosystem (São Marcos' bay, Maranhão, Brazil). *Braz. J. Oceanogr.* 58, 11–17 SPEC. ISSUE.
- Chaves, P.T.C., 1994. A incubação de ovos e larvas em *Genidens genidens* (Valenciennes) (Siluriformes, Ariidae) da baía de Guaratuba, Paraná, Brasil. *Rev. Bras. Zootec.* 11 (4), 641–648.
- Christo, S.W., Absher, T., 2004. Reproductive period of *Crassostrea rhizophorae* (Guilding, 1828) and *Crassostrea brasiliiana* (Lamarck, 1819) (Bivalvia:Ostreidae) in Guaratuba Bay: Paraná, Brazil. *J. Coast. Res.* 39.
- Combi, T., Taniguchi, S., de Lima Ferreira, P.A., Mansur, A.V., Figueira, R.C.L., de Mahiques, M.M., Martins, C.C., 2013. Sources and temporal patterns of polychlorinated biphenyls around a large south American grain-shipping port (Paranaguá estuarine system, Brazil). *Arch. Environ. Contam. Toxicol.* 64, 573–582.
- da Rosa, C.E., Bianchini, A., Monserrat, J.M., 2008. Antioxidant responses of *Laonereis acuta* (Polychaeta) after exposure to hydrogen peroxide. *Braz. J. Med. Biol. Res.* 41 (2), 117–121.
- da Silva, A.Z., Zanette, J., Ferreira, J.F., Guzinski, J., Marquesa, M.R.F., Bairy, A.C.D., 2005. Effects of salinity on biomarker responses in *Crassostrea rhizophorae* (Mollusca Bivalvia) exposed to diesel oil. *Ecotoxicol. Environ. Saf.* 62, 376–382.
- de Abreu-Mota, M.A., de Moura Barboza, C.A., Bicego, M.C., Martins, C.C., 2014. Sedimentary biomarkers along a contamination gradient in a human-impacted sub-estuary in southern Brazil: a multi-parameter approach based on spatial and seasonal variability. *Chemosphere* 103, 156–163.
- De Luca-Abbott, S.B., Richardson, B.J., McClellan, K.E., Zheng, G.J., Martin, M., Lam, P.K.S., 2005. Field validation of antioxidant enzyme biomarkers in mussels (*Perna viridis*) and clams (*Ruditapes philippinarum*) transplanted in Hong Kong coastal waters. *Mar. Pollut. Bull.* 51 (8–12), 694–707.
- Depledge, M., Galloway, T.S., 2015. Healthy animals healthy ecosystems. *Front. Ecol. Environ.* 3, 251–258.

- Development Core Team, R, 2009. R: a language and environment for statistical computing R foundation for Statistical Computing Vienna Austria (<http://www.R-project.org>).
- Di Benedetto, M., Masunari, S., 2009. Estrutura populacional de *Uca maracoani* (Decapoda Brachyura Ocypodidae) no Baixo Mirim Baía de Guaratuba Paraná. *Iheringia Série Zoologia* 99, 381–389.
- Di Toro, D.M., Zarba, C.S., Hansen, D.J., Berry, W.J., Swartz, R.C., Cowan, C.E., Pavlou, S.P., Allen, H.E., Thomas, N.A., Paquin, P.R., 1991. Technical basis for establishing sediment quality criteria for nonionic organic chemicals using equilibrium partitioning. *Environ. Toxicol. Chem.* 10, 1541–1583.
- Egres, A.G., Martins, C.C., de Oliveira, V.M., Lana, P.C., 2012. Effects of an experimental *in situ* diesel oil spill on the benthic community of unvegetated tidal flats in a subtropical estuary (Paranaguá Bay, Brazil). *Mar. Pollut. Bull.* 64, 2681–2691.
- Ferreira, A.L., Neto, R.L.B., Kolm, H.E., Absher, T.M., 2015. Relationship between reproductive cycle of *Anomalocardia brasiliiana* (Mollusca: Veneridae) and the suspended particulate matter in the Paranaguá estuarine complex, Brazil. *Pan Am. J. Aquat. Sci.* 10 (1), 44–54.
- Ferreira-Cravo, M., Piedras, F.R., Moraes, T.B., Ferreira, J.L.R., de Freitas, D.P.S., Machado, M.D., Geracitano, L.A., Monserrat, J.M., 2007. Antioxidant responses and reactive oxygen species generation in different body regions of the estuarine polychaeta *Laeonereis acuta* (Nereididae). *Chemosphere* 66 (7), 1367–1374.
- Froehner, S., Maceno, M., Machado, K.S., 2011. Predicting bioaccumulation of PAHs in the trophic chain in the estuary region of Paranaguá, Brazil. *Environ. Monit. Assess.* 174 (1–4), 135–145.
- Gagnon, M.M., Rawson, C.A., 2016. Integrating multiple biomarkers of fish health: a case study of fish health in ports. *Arch. Environ. Contam. Toxicol.* 70 (2), 192–203.
- Galloway, T.S., Brown, R.J., Browne, M.A., Dissanayake, A., Lowe, D., Jones, M.B., Depledge, M.H., 2004. A multibiomarker approach to environmental assessment. *Environ. Sci. Technol.* 38, 1723–1731.
- Gao, R., Yuan, Z., Zhao, Z., X., G., 1998. Mechanism of pyrogallol autoxidation and determination of superoxide dismutase enzyme activity. *Bioelectrochem. Bioenerg.* 45, 41–45.
- Geracitano, L., Monserrat, J.M., Bianchini, A., 2002. Physiological and antioxidant enzyme responses to acute and chronic exposure of *Laeonereis acuta* (Polychaeta, Nereididae) to copper. *J. Exp. Mar. Biol. Ecol.* 277 (2), 145–156.
- Geracitano, L., Monserrat, J.M., Bianchini, A., 2004a. Oxidative stress in *Laeonereis acuta* (Polychaeta, Nereididae): environmental and seasonal effects. *Mar. Environ. Res.* 58 (2–5), 625–630.
- Geracitano, L., Bocchetti, R., Monserrat, J.M., Regoli, F., Bianchini, A., 2004b. Oxidative stress responses in two populations of *Laeonereis acuta* (Polychaeta, Nereididae) after acute and chronic exposure to copper. *Mar. Environ. Res.* 58 (1).
- Gong, Y., Zhao, X., Cai, Z., O'Reilly, S.E., Hao, X., Zhao, D., 2014. A review of oil, dispersed oil and sediment interactions in the aquatic environment: influence on the fate, transport and remediation of oil spills. *Mar. Pollut. Bull.* 79 (1–2), 16–33.
- Gorbi, S., Baldini, C., Regoli, F., 2005. Seasonal variability of metallothioneins, cytochrome P450, bile metabolites and oxyradical metabolism in the European eel *Anguilla anguilla* (Anguillidae) and striped mullet *Mugil cephalus* (Mugilidae). *Arch. Environ. Contam. Toxicol.* 49, 62–70.
- Greenacre, M., 2016. Data reporting and visualization in ecology. Submitted to *Polar Biology*, pp. 1–33.

- Gusso-Choueri, P.K., Choueri, R.B., Lombardi, A.T., Machado, E.C., 2011. The dynamics of fluorescent dissolved organic matter in the Paranaguá estuarine system, southern Brazil. *Braz. J. Oceanogr.* 59 (4), 311–326.
- Hafeman, D.G., Sunde, R.A., Hoekstra, W.C., 1974. Effect of dietary selenium on erythrocyte and liver glutathione peroxidase in the rat. *J. Nutr.* 104, 580–587.
- Hirose, G.L., Negreiros-Fransozo, M.L., 2008. Population biology of *Uca maracoani* Latreille 1802-1803 (Crustacea, Brachyura, Ocypodidae) on the south-eastern coast of Brasil. *Pan.-Ami. J. Aquat. Sci.* 3, 373–383.
- Katsumiti, A., Domingos, F.X.V., Azevedo, M., da Silva, M.D., Damian, R.C., Almeida, M.I.M., Silva de Assis, H.C., Cestari, M.M., Randi, M.A.F., Oliveira Ribeiro, C.A., Freire, C.A., 2009. An assessment of acute biomarker responses in the demersal catfish *Cathorops spixii* after the Vicuña oil spill in a harbour estuarine area in southern Brazil. *Environ. Monit. Assess.* 152, 209–222.
- Katsumiti, A., França, P.P., Silva Costa, G.P., Zandoná, E.M., Benincá, C., de Assis, H.C.S., Roche, H., Oliveira-Ribeiro, C.A., 2013. Evaluation five years after a Refinery oil spill in freshwater wetland - Paraná state, southern of Brazil. *Ecotoxicol. Environ. Contami.* 8 (2), 77–87.
- Keen, J.H., Habig, W.H., Jakoby, W.B., 1976. Mechanism for several activities of the glutathione-S-transferase. *J. Biol. Chem.* 251, 6183–6188.
- Lana, P.C., 2003. As marismas da Baía de Paranaguá: características gerais, modos de apropriação e implicações para a legislação ambiental. *Desenvolvimento E Meio Ambiente* 8, 11–23.
- Lana, P.C., Marone, E., Lopes, R.M., Machado, E.C., 2001. The subtropical estuarine complex of Paranaguá Bay, Brazil. *Coastal Marine Ecosystems of Latin America. Ecological Studies* 144, pp. 131–145.
- Lüchmann, K.H., Mattos, J.J., Siebert, M.N., Granucci, N., Dorrington, T.S., Bicego, M.C., Taniguchi, S., Sasaki, S.T., Daura-Jorge, F.G., Bairy, A.C.D., 2011. Biochemical biomarkers and hydrocarbons concentrations in the mangrove oyster *Crassostrea brasiliiana* following exposure to diesel fuel water-accommodated fraction. *Aquat. Toxicol.* 105 (3–4), 652–660.
- Luoma, S.N., 1996. The developing framework of marine ecotoxicology: pollutants as a variable in marine ecosystems? *J. Exp. Mar. Biol. Ecol.* 200, 29–55.
- Machado, A.A., Hoff, M.L.M., Klein, R.D., Cardozo, J.G., Giacomini, M.M., Pinho, G.L.L., Bianchini, A., 2013. Biomarkers of waterborne copper exposure in the guppy *Poecilia vivipara* acclimated to salt water. *Aquat. Toxicol.* 138-139, 60–69.
- Machado, A.A., Hoff, M.L.M., Klein, R.D., Cordeiro, G.J., Lencina Avila, J.M., Costa, P.G., Bianchini, A., 2014. Oxidative stress and DNA damage responses to phenanthrene exposure in the estuarine guppy *Poecilia vivipara*. *Mar. Environ. Res.* 98, 96–105.
- Manduzio, H., Rocher, B., Durand, F., 2005. The point about oxidative stress in molluscs. *Inf. Syst. J.* 91–104.
- Maranho, A.L., Pereira, C.D.S., Choueri, R.B., Cesar, A., Gusso-Choueri, P.K., Torres, R.J., de Souza Abessa, D.M., Morais, R.D., Mozeto, A.A., Del Valls, A.T., Martín-Díaz, M.L., 2012. The application of biochemical responses to assess environmental quality of tropical estuaries: field surveys. *J. Environ. Monit.* 14 (10), 2608.
- Marques, B.F., Cordeiro, L.F., Kist, L.W., Bogo, M.R., López, G., Pagano, G., Tomazi, M.D., Machado de Carvalho, L., Kulkamp-Guerreiro, I.C., Monserrat, J.M., 2013. Toxicological effects induced by the nanomaterials fullerene and nanosilver in the polychaeta *Laeonereis acuta*

- (Nereididae) and in the bacteria communities living at their surface. *Mar. Environ. Res.* 89, 53–62.
- Marques, J.A., Silva de Assis, H.C., Guiloski, I.C., Sandrini-Neto, L., Carreira, R.S., Lana, P.C., 2014. Antioxidant defense responses in *Mytella guyanensis* (Lamarck, 1819) exposed to an experimental diesel oil spill in Paranaguá Bay (Paraná, Brazil). *Ecotoxicol. Environ. Saf.* 107C, 269–275.
- Martins, C.C., Braun, J.A.F., Seyffert, B.H., Machado, E.C., Fillmann, G., 2010. Anthropogenic organic matter inputs indicated by sedimentary fecal steroids in a large South American tropical estuary (Paranaguá estuarine system, Brazil). *Mar. Pollut. Bull.* 60 (11), 2137–2143.
- Matthews-Cascon, H., Martins, I., 1999. Notes on the reproduction of *Neritina virginea* (Linnaeus 1758) in Northeast Brazil (Mollusca: Gastropoda: Neritidae). *Arquivos de Ciências Do Mar* 32, 129–132.
- Moraes, T.B., Ferreira, J.L.R., da Rosa, C.E., Sandrini, J.Z., Votto, A.P., Trindade, G.S., Geracitano, L.A., Abreu, P.C., Monserrat, J.M., 2006. Antioxidant properties of the mucus secreted by *Laeonereis acuta* (Polychaeta, Nereididae): a defense against environmental pro-oxidants? *Comp. Biochem. Physiol. Toxicol. Pharmacol.* 142 (3–4), 293–300.
- Nahrgang, J., Camus, L., Broms, F., Christiansen, J.S., Hop, H., 2010. Seasonal baseline levels of physiological and biochemical parameters in polar cod (*Boreogadus saida*): implications for environmental monitoring. *Mar. Pollut. Bull.* 60, 1336–1345.
- Nahrgang, J., Brooks, S.J., Evenset, A., Camus, L., Jonsson, M., Smith, T.J., Lukina, J., Frantzen, M., Giarratano, E., Renaud, P.E., 2013. Seasonal variation in biomarkers in blue mussel (*Mytilus edulis*), Icelandic scallop (*Chlamys islandica*) and Atlantic cod (*Gadus morhua*): implications for environmental monitoring in the Barents Sea. *Aquat. Toxicol.* 127, 21–35.
- Nogueira, L., Garcia, D., Trevisan, R., Sanches, A.L.M., da Silva Acosta, D., Dafre, A.L., Oliveira, T.Y.K., de Almeida, E.A., 2015. Biochemical responses in mussels *Perna perna* exposed to diesel B5. *Chemosphere* 134, 210–216.
- Oksanen, J., Blanchet, F.G., Kindt, R., Legendre, P., Minchin, P.R., O'Hara, R.B., Simpson, G.L., Solymos, P., Henry, M., Stevens, H., Wagner, H., 2013. *Vegan: Community Ecology Package*. R package version 2.0-10. <http://CRAN.R-project.org/package=vegan>.
- Orbea, A., Ortiz-Zarragoitia, M., Solé, M., Porte, C., Cajaraville, M.P., 2002. Antioxidant enzymes and peroxisome proliferation in relation to contaminant body burdens of PAHs and PCBs in bivalve molluscs, crabs and fish from the Urdaibai and Plentzia estuaries (Bay of Biscay). *Aquat. Toxicol.* 58, 75–98.
- Pereira, C.D.S., Abessa, D.M.S., Bainy, A.C.D., Zaroni, L.P., Gasparro, M.R., Bicego, M.C., Taniguchi, S., Furley, T.H., de Sousa, E.C.P.M., 2007. Integrated assessment of multilevel biomarker responses and chemical analysis in mussels from São Sebastião, São Paulo, Brazil. *Environ. Toxicol. Chem.* 26 (3), 462–469 (SETAC).
- Pereira, C.D.S., Martin-Diaz, M.L., Catharino, M.G.M., Cesar, A., Choueri, R.B., Tanigushi, S., Abessa, D.M.S., Bicego, M.C., Vasconcellos, M.B.A., Bainy, A.C.D., Sousa, E.C.P.M., DeValls, T.A., 2010. Chronic contamination assessment integrating biomarkers' responses in transplanted mussels—a seasonal monitoring. *Environ. Toxicol.* 24 (3).
- Pereira, C.D.S., Abessa, D.M.S., Choueri, R.B., Almagro-Pastor, V., Cesar, A., Maranhão, L.A., Martín-Díaz, Torres, R.J., Gusso-Choueri, P.K., Almeida, J.E., Cortez, F.S., Mozeto, A.A., Silbiger, H.L.N., Sousa, E.C.P.M., del Valls, T.A., Bainy, A.C.D., 2014. Ecological relevance of Sentinels' biomarker responses: a multi-level approach. *Mar. Environ. Res.* 96, 118–126.
- Rapoza, 2015. <http://www.forbes.com/sites/kenrapoza/2015/06/15/how-important-is-brazil-to-the-worlds-oil-market/#42564fd91908>.

- Rizzi, J., Pérez-Albaladejo, E., Fernandes, D., Contreras, J., Froehner, S., Porte, C., 2016. Characterization of Quality of Sediments from Paranagua Bay (Brazil) Combined in Vitro Bioassays and Chemical Analyses. *Environ. Toxicol. Chem.* <http://dx.doi.org/10.1002/etc.3553>.
- Rola, R.C., Monteiro, M.d.C., da S., Reis S.R., Sandrini, J.Z., 2012. Molecular and biochemical biomarkers responses in the mussel *Mytilus edulis* collected from southern Brazil coast. *Mar. Pollut. Bull.* 64 (4), 766–771.
- Sandrini-Neto, L., Martins, C.C., Lana, P.C., 2016. Are intertidal soft sediment assemblages affected by repeated oil spill events? A field-based experimental approach. *Aquat. Toxicol.* 177, 237–249.
- Sardi, A.E., Sandrini-Neto, L., Pereira, L.S., de Assis, H.C.S., Martins, C.C., Lana, P.C., Camus, L., 2016. Oxidative stress in two tropical species after acute exposure to diesel oil. *Environ. Sci. Pollut. Res.* <http://dx.doi.org/10.1007/s11356-016-7280-2>.
- Schmidt, T.C.S., Martins, I.A., Reigada, A.L.D., Dias, J.F., 2008. Taxocenose de bagres marinhos (Siluriformes Ariidae) da região estuarina de São Vicente SP Brasil. *Biota Neotropica* 8, 73–81.
- Shaw, J.P., Large, A.T., Donkin, P., Evans, S.V., Staff, F.J., Livingstone, D.R., Chipman, J.K., Peters, L.D., 2004. Seasonal variation in cytochrome P450 immunopositive protein levels, lipid peroxidation and genetic toxicity in digestive gland of the mussel *Mytilus edulis*. *Aquat. Toxicol.* 67 (4), 325–336.
- Silva Junior, D.R., Carvalho, D.M.T., Vianna, M., 2013. The catfish *Genidens genidens* (Cuvier, 1829) as a potential sentinel species in Brazilian estuarine waters. *J. Appl. Ichthyol.* 29 (6), 1297–1303.
- Souza, D.S.M., Ramos, A.P.D., Nunes, F.F., Moresco, V., Taniguchi, S., Guiguet Leal, D.A., Sasaki, S.R., Bicego, M.C., Montone, R.C., Durigan, M., Teixeira, A.L., Pilotto, M.R., Delfino, N., Franco, R.M.B., de Melo, C.M.R., Bairy, A.C.D., Barardi, C.R.M., 2012. Evaluation of tropical water sources and mollusks in southern Brazil using microbiological, biochemical, and chemical parameters. *Ecotoxicol. Environ. Saf.* 76 (1), 153–161.
- Souza, F.M., Brauko, K.M., Lana, P.C., Muniz, P., Camargo, M.G., 2013. The effect of urban sewage on benthic macrofauna: a multiple spatial scale approach. *Mar. Pollut. Bull.* 67, 234–240.
- Torres, M.A., Testa, C.P., Gáspari, C., Masutti, M.B., Panitz, C.M.N., Curi-Pedrosa, R., de Almeida, E.A., Di Mascio, P., Wilhelm Filho, D., 2002. Oxidative stress in the mussel *Mytella guyanensis* from polluted mangroves on Santa Catarina Island, Brazil. *Mar. Pollut. Bull.* 44 (9), 923–932.
- Ventura-Lima, J., Sandrini, J.Z., Cravo, M.F., Piedras, F.R., Moraes, T.B., Fattorini, D., Notti, A., Regoli, F., Geracitano, L.A., Marins, L.F.F., Monserrat, J.M., 2007. Toxicological responses in *Laeonereis acuta* (annelida, polychaeta) after arsenic exposure. *Environ. Int.* 33 (4), 559–564.
- Viarengo, A., Lafaurie, M., Gabrielides, G.P., Fabbri, R., Marro, A., Roméo, M., 2000. Critical evaluation of an intercalibration exercise undertaken in the framework of the MED POL biomonitoring program. *Mar. Environ. Res.* 49 (1), 1–18.
- Viarengo, A., Lowe, D., Bolognesi, C., Fabbri, E., Koehler, A., 2007. The use of biomarkers in biomonitoring: a 2-tier approach assessing the level of pollutant-induced stress syndrome in sentinel organisms. *Comp. Biochem. Physiol., Part C: Toxicol. Pharmacol.* 146 (3), 281–300.
- Wells, D.E., Balls, H.R., 1994. QUASIMEME: quality assurance of information for marine environmental monitoring in Europe. *Mar. Pollut. Bull.* 29, 143–145.

- Zanette, J., Monserrat, J.M., Bianchini, A., 2006. Biochemical biomarkers in gills of mangrove oyster *Crassostrea rhizophorae* from three Brazilian estuaries. *Comp. Biochem. Physiol. Toxicol. Pharmacol.* : CBP 143 (2), 187–195.
- Zanette, J., Nunes, F.F., Medeiros, I.D., Siebert, M.N., Mattos, J.J., Lüchmann, K.H., Rodrigues de Melo, C.M., Bainy, A.C.D., 2008. Comparison of the antioxidant defense system in *Crassostrea rhizophorae* and *Crassostrea gigas* exposed to domestic sewage discharges. *Mar. Environ. Res.* 66 (1).
- Zanette, J., Monserrat, J.M., Bianchini, A., 2015. Biochemical biomarkers in barnacles *Balanus improvisus*: pollution and seasonal effects. *Mar. Environ. Res.* 103, 74–79.

Supplementary material

Table S1. Sampled species, location name, coordinates, location code as represented in figure 1, total PAHs in sediment and reference, animal size and target tissue employed for biomarker analysis. The contamination condition factor indicates the level of contamination of the chosen locations.

Species	Contamination condition	Latitude (S)	Longitude (W)	Code	Σ PAHs in sediments (ng g ⁻¹)	Reference	Animal size (mm)	Target tissue
<i>Anomalocardia flexuosa</i>	Reference	-25.545528	-48.437169	1C	13.09	Sandrini-Neto et al. 2016	20 - 24	Digestive gland
	Polluted	-25.509933	-48.492133	3P	89.14	Rizzi et al. 2016		
<i>Crassostrea rhizophorae</i>	Reference	-25.545528	-48.437169	1C	13.09	Sandrini-Neto et al. 2016	40 - 60	Digestive gland
	Polluted	-25.516986	-48.498717	2P	89.14	Rizzi et al. 2016		
<i>Neritina virginea</i>	Reference	-25.545528	-48.437169	1C	13.09	Sandrini-Neto et al. 2016	5 - 10	Whole body
	Polluted	-25.509933	-48.492133	3P	89.14	Rizzi et al. 2016		
<i>Uca maracoani</i>	Reference	-25.559943	-48.417816	2C			50 - 80	Hepato-pancreas
	Polluted	-25.546056	-48.463167	4P	40.83	Froehner et al. 2011		
<i>Genidens genidens</i>	Reference	-25.324639	-48.340361	3C	37.27	Rizzi et al. 2016	280 - 350	Liver
	Polluted	-25.499639	-48.551278	1P	69.71	Rizzi et al. 2016		

Table S2. Significance test for RDA models employed. Models included enzyme measurements for all studied species. Significance was tested using PERMANOVA. Abbreviations stand for *F*: F-ratio; *P*: probability of *F* for test ran within terms of the model or with margins. An RDA including species, season, contamination condition and the interaction between season and condition was employed. Levels of significance were assessed with the ANOVA function in the *vegan* R package. This function determines the importance of the RDA model constraints (experimental factors) by performing an ANOVA-like permutation test (Oksanen et al. 2013). Since the interaction of factors was significant ($p = 0.01$; Table S2), the model was reduced to species and the interaction between season and contamination condition factors.

Model	Factor	<i>F</i>		<i>P</i>	
		Terms	Margins	Terms	Margins
All biomarkers	Species	21.67	21.49	0.001	0.001
Explanatory variables: Species + Season + Contamination condition + Season:Condition	Season	14.94	n.s.	0.001	n.s.
	Condition	3.62	n.s.	0.009	n.s.
	Se:Co	3.69	3.69	0.011	0.01
All biomarkers	Species	21.67	-	0.001	-
Explanatory variables: Season + Season:Condition	Se:Co	7.42	-	0.001	-

Figure S1. RDA biplots derived from log transformed enzymatic activities in a) *Neritina virginea*, b) *Uca maracoani* and c) *Genidens genidens* 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).

