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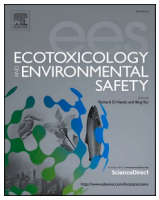

Research paper

Biochemical metal accumulation effects and metalloprotein metal detoxification in environmentally exposed tropical *Perna perna* mussels

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ABSTRACT

Marine bivalves have been widely applied as environmental contamination bioindicators, although studies concerning tropical species are less available compared to temperate climate species. Assessments regarding *Perna perna* mytilid mussels, in particular, are scarce, even though this is an extremely important species in economic terms in tropical countries, such as Brazil. To this end, *Perna perna* mytilids were sampled from two tropical bays in Southeastern Brazil, one anthropogenically impacted and one previously considered a reference site for metal contamination. Gill metallothionein (MT), reduced glutathione (GSH), carboxylesterase (CarbE) and lipid peroxidation (LPO) were determined by UV−vis spectrophotometry, and metal and metalloid contents were determined by inductively coupled plasma mass spectrometry (ICP−MS). Metalloprotein metal detoxification routes in heat-stable cellular gill fractions were assessed by size exclusion high performance chromatography (SEC−HPLC) coupled to an ICP−MS. Several associations between metals and oxidative stress endpoints were observed at all four sampling sites through a Principal Component Analysis. As, Cd, Ni and Se contents, in particular, seem to directly affect CarbE activity. MT is implicated in playing a dual role in both metal detoxification and radical oxygen species scavenging. Differential SEC−HPLC−ICP−MS metal-binding profiles, and, thus, detoxification mechanisms, were observed, with probable As-, Cu- and Ni-GSH complexation and binding to low molecular weight proteins. *Perna perna* mussels were proven adequate tropical bioindicators, and further monitoring efforts are recommended, due to lack of data regarding biochemical metal effects in tropical species. Integrated assessments, as performed herein demonstrate, are invaluable in evaluating contaminated aquatic environments, resulting in more accurate ecological risk assessments.

1. Introduction

Marine bivalves, such as mussels, have been widely used in field and laboratory studies in environmental monitoring programs worldwide (Yoloğlu et al., 2018). These animals display several characteristics that make them adequate bioindicators regarding local environmental pollution, such as their sessile nature, worldwide distribution, known biology and ecology and filter-feeding habits (Hauser-Davis and Lavradas, 2018).

Many metal contamination assessments have been carried out with temperate climate mussels, such as *Mytilus* sp. (Arienzo et al., 2019; Uluturhan et al., 2019; Rajaram et al., 2020), but studies on tropical

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species are less abundant, especially regarding metal contamination and its effects (El-Nemr et al., 2016; Denil et al., 2017; Vasanthi et al., 2017). This indicates a significant knowledge gap in this regard, as differences in sensitivity between species from different climatic zones are noted for diverse pollutants, resulting in differential biomarker responses (Kwok et al., 2007). Thus, more adequate biomonitoring efforts for a specific climate/region can be attained by assessing native species. In this scenario, the tropical mytilid mussel *Perna perna* is the most cultivated and most economically important throughout the Brazilian coast, as it reaches large sizes, grows relatively fast, has a high production rate and is both nutritious and easily collected (Baraj et al., 2003). Some studies have been previously carried out with *Perna perna* samples for environmental biomonitoring purposes by determining total metal loads (Lino et al., 2016; Lavradas et al., 2016a; Campolim et al., 2017), although biochemical metal effects are still relatively unknown for this species. In addition, although metal-rich granules and heat-stable proteins are known as biological detoxification fractions, aiming at reducing metal toxicity (Wallace et al., 2003), subcellular biological fractions have not been given much attention to in biomonitoring assessments in general, and even less so in mussel evaluations (Lavradas et al., 2016b).

One of the main metal biochemical effects consists in the disruption of cellular redox regulation or oxidative reaction cycles, producing, mainly, reactive oxygen species (ROS). If left unchecked, this may lead to oxidative stress (Buonocore and Groenendaal, 2007; Halliwell and Gutteridge, 2015), characterized by an imbalance between the formation rate of ROS and intracellular antioxidant defenses (Halliwell and Gutteridge, 2015). Oxidative stress may lead to several negative effects, including deleterious interactions with lipids, proteins or nucleic acids, leading to in biochemical and/or genetic damages (Costa et al., 2002; Ghezzi and Bonetto, 2003; Levine et al., 1990). Thus, assessing oxidative stress endpoints in bioindicator species is paramount in order to detect early negative contamination effects and allow for decision-making processes in metal contamination scenarios (Hauser-Davis and Lavradas, 2018; Monserrat et al., 2007).

Very limited biomarker investigations focusing on non-enzymatic biomarkers and oxidative stress endpoints, such as the tripeptide reduced glutathione (GSH), or lipid peroxidation, one of the mains oxidative stress cellular endpoints, for example, are available for *Perna perna* mussels, none very recent (De Almeida et al., 2004; El Jourmi et al., 2012; Trevisan et al., 2014) and, to the best of our knowledge, very few studies concerning enzymatic assessments are available, mainly concerning glutathione-S-transferase, glutathione reductase, catalase and acetylcholinesterase (Brahim Errahmani et al., 2014; De Almeida et al., 2004; Trevisan et al., 2014). Other enzymes, however, have not yet been assessed in a metal contamination scenario. For example, carboxylesterase (CarbE), a metabolic phase I enzyme, plays a role in interrupting the hydrolysis of many chemicals, thus being involved in xenobiotic detoxification (Solé et al., 2010). Its role in pesticide detoxification, considered specific of exposure to organophosphorous and carbamate compounds, has been well-described (Sogorb and Vilanova, 2002). However, this specific toxicological role of CarbE has received little attention in marine organisms in general, and no studies concerning inhibition by other pollutants, such as metals, are found in the literature for mussels. Therefore, its potential as a metal contamination biomarker is still unknown.

Mussel gills are always in direct and continuous contact with metals in the dissolved aqueous phase of the aquatic environment (Gagnon et al., 2006). This leads to contaminant accumulation at considerably faster rates in this tissue compared to other organs, resulting in gill metal storage (Dragun et al., 2004). Some authors have postulated that this may be due to gill mucus, which displays strong chelating properties, thus complexing with metals both dissolved in the aqueous phase and adsorbed to the particulate phase (Marigómez et al., 2002; Pagenkopf, 1983). Because of this, gills have been noted as an adequate organ for local metal contamination evaluations. In this context, the present study aimed, for the first time, to characterize and compare gill metal contamination, biochemical responses and metal detoxification routes in *Perna perna* mytilid mussels, from two tropical bays in southeastern Brazil, an impacted estuary and an area previously considered as a reference site in the literature, but which has been recently reported as highly contaminated by metals in recent years (Lavradas et al., 2016a), located in southeastern Brazil. This may, in turn, aid in decision-making processes concerning metal contamination and human consumption of these bivalves in the study areas.

2. Methodology

2.1. Study area

Guanabara Bay, located in the metropolitan area of the state of Rio de Janeiro, is one of the largest, albeit, most polluted, aquatic bodies in Brazil (Baptista Neto et al., 2005), exposed to many types of pollution sources due to its surrounding population of 11 million inhabitants, approximately 12,000 industries, oil refineries, two navy bases and several shipyards (Perin et al., 1997). Nevertheless, it is still one of the most important and productive Brazilian estuaries and a significant fishing site of both social and economic importance, displaying high local biota diversity, mainly due to its 90 km^2 area of fringing mangroves (Jablonski et al., 2006).

Ilha Grande Bay, located on the southern coast of the state of Rio de Janeiro, displays a very rugged character, with the presence of several smaller bays and over 350 islands, which decreases the local hydrodynamics. It is an important tourist destination in the state, and highly significant in terms of fishing productivity (CEPERJ, 2004; Kehrig et al., 1998). Although considered a reference location for biomonitoring assessments for many years, recent investigations have indicated increasing metal local levels (Lavradas et al., 2016a, 2016b). Pollution sources in the area include an oil terminal, a shipyard and two nuclear power plants, while atmospheric deposition and urban and industrial emissions from the states of São Paulo and Rio de Janeiro have also been noted as significant metal sources (Barros de Oliveira et al., 2012). A map of the mussel sampling sites located in both aforementioned bays is displayed in Fig. 1.

2.2. Mussel sampling

Perna perna mussels were manually sampled in the winter of 2015 at three different beaches within the anthropogenically impacted Guanabara Bay (GB), namely Diabo Beach, Urca Beach and Vermelha Beach, hereafter termed DB, UB and VB, respectively and from Ilha Grande Bay (IG). Several protein concentrations and enzymatic activities have been reported as increased during the reproductive period in mussels, due to increased metabolic activity and as a protection against oxidative stress (Petes et al., 2007, 2008). Therefore, recommendations have been made to avoid sampling during the reproductive period (Hauser-Davis and Lavradas, 2018). In Brazil, *Perna perna* mussels show reproductive peaks from April-June, in September and January, corresponding to Autumn, Spring and Summer (De, 1997). Thus, as our sampling was carried out in winter, reproductive effects are not a concern. Individuals ($n = 40$ from each beach) were immediately taken to the lab in Styrofoam boxes containing local seawater and measured (shell length, width and height). They were then dissected and the gill samples of 10 individuals were then pooled per site, totaling four composite gill samples per site (40 individuals). Subsequently, one aliquot of each composite sample was frozen at −20 °C in sterile polypropylene tubes for the enzymatic assessments, and another was freeze-dried for 48 h (Liotop 101, Liobrás, São Paulo, Brazil), for the protein and metal analyses, respectively.

2.3. Metallothionein (MT) extraction and determinations

MT were extracted according to the thermal procedure described by

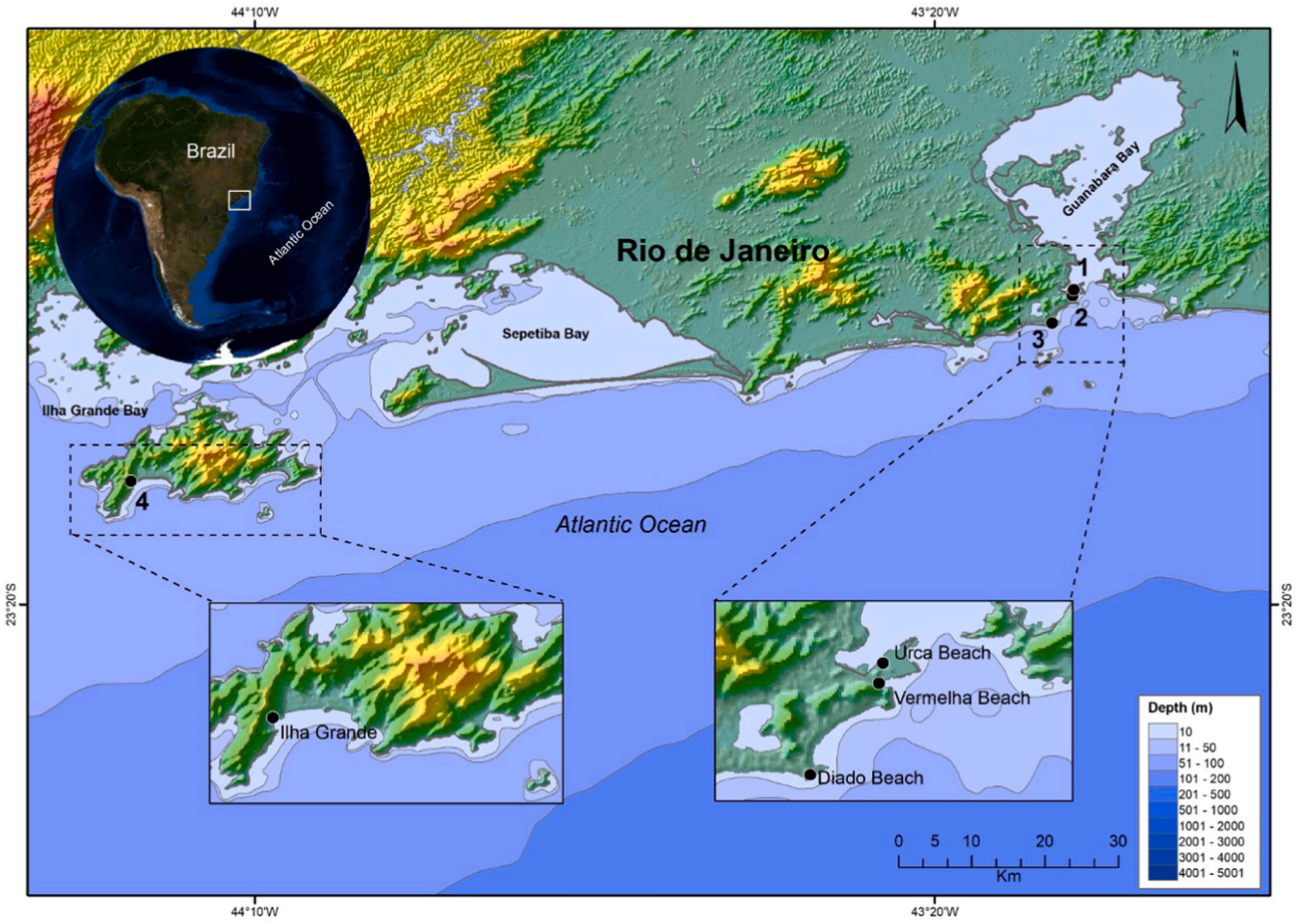

Fig. 1. Map of the mussel sampling sites, southeastern Rio de Janeiro, Brazil.

Erk and collaborators, using tris-2-carboxyethyl-phosphine (TCEP) as the reducing agent (Sigma-Aldrich, São Paulo, Brazil) (Erk et al., 2002; Getz et al., 1999; Tenório-Daussat et al., 2014). Briefly, the samples were homogenized in sterile polypropylene microtubes at a 1:20 ration in a buffer solution containing Tris-HCl, 20 mmol L^{-1} pH 8.6, phenyl methyl sulfonyl fluoride 0.5 mmol L^{-1} and 0.01% TCEP. The samples were then centrifuged in a refrigerated microtube centrifuge (Mikro 220 R, Hettich, Germany) at 20,000xg for 60 min at 4 °C and the supernatants were carefully removed and transferred to other sterile microtubes. The supernatants were subsequently heated at 70 °C for 10 min in a thermostatic water bath (Kacil, São Paulo, Brazil) and a new centrifugation at 20,000 x g was performed for 30 min at 4 °C. After this last centrifugation step, the supernatants containing the MT were again separated (hereby, termed the thermostable fraction, TSF) and the samples were then frozen at −20 °C until further analyses.

MT quantification was performed using a spectrophotometric method applying Ellman's (1959) reaction, in which the purified samples (hereby termed the thermostable fraction, TSF) were mixed with a HCl 1 mol L^{-1} and EDTA 4 mmolL^{-1} solution. Immediately afterwards, NaCl 2 mol L^{-1} containing 0.43 mmol L^{-1}, 5,5-dithiobis (2-nitrobenzoic acid) buffered with 0.2 mol L^{-1} sodium phosphate, pH 8.0, were added to the samples. These were then incubated in the dark for 30 min and their absorbances were measured in quartz microcuvettes at 412 nm on a UV–vis spectrophotometer (Lambda 35, Perkin Elmer).

MT concentrations were estimated using an analytical curve plotted using GSH as an external standard assuming a ratio of 1 mol MT = 20 mol GSH, as described previously (Kägi, 1991), and expressed as μmol g^{-1} dry weight (d.w.). All samples were analyzed in triplicate.

2.4. Reduced glutathione (GSH) extraction and determinations

GSH extraction was performed according to Beutler (1975), with modifications as applied by Wilhelm-Filho (2005). Briefly, approximately 25 mg of each sample was homogenized in 350 μL of 0.1 mol L^{-1} pH 7.0 sodium phosphate buffer containing sucrose 0.25 mol L^{-1} in inert atmosphere (nitrogen 99.9%). The samples were then centrifuged at 11.000xg in a Mikro 220R centrifuge (Hettich, Germany) for 30 min at 4 °C. The supernatants were removed and transferred to sterile microtubes and subsequently treated with 0.1 mol L^{-1} DTNB in a pH 8.0 sodium phosphate buffer at a 1:1 ratio. After 15 min incubation in the dark, the sample absorbances were determined at 412 nm in a UV−Vis spectrophotometer (Lambda 35, Perkin Elmer). GSH concentrations were estimated using an analytical curve plotted with GSH as the external standard, and expressed as μmol g^{-1} dry weight (d.w.).. All samples were analyzed in triplicate.

2.5. Carboxylesterase (CarbE) determinations

CarbE extraction and determination followed Morgan et al. (1994). Briefly, tissues were homogenized using a Potter−Elvehjem type homogenizer at a 1:4 ratio in 0.1 mol L^{-1} sodium phosphate buffer pH 7.7. p-nitrophenylacetate (p-NPA) (Sigma-Aldrich, São Paulo, Brazil) dissolved in acetonitrile, used as substrate at a final concentration of 5 mmol L^{-1}. The formed p-nitrophenol was determined during 3 min at 410 nm on a UV–Vis spectrophotometer (Shimadzu UV-160). The molar absorptivity coefficient of p-nitrophenol of 13,000 mol^{-1} L^{-1} cm^{-1} was used to calculate CarbE concentrations. One unit (U) of enzymatic

activity was defined as 1 μmol of the enzyme that catalyzes the conversion of one micromole of substrate per minute under the specified conditions of the assay, and activities were expressed as U mg^{-1} protein wet weight (w.w.). Total protein content was determined according to Peterson (1977).

2.6. Lipid peroxidation (LPO) determinations

Tissues were prepared according to the Cayman Chemical TBARS assay Kit manufacturer instructions. Briefly, 25 mg of each sample were homogenized using a Potter-Elvehjem type homogenizer in 250 μL of RIPA buffer (consisting of 0.5 mL Triton x-100, 2.5 mL of a 1 mol L^{-1} solution, Tris pH 7.5, 2.5 mL of 3 mol L^{-1} NaCl solution, 0.05 g SDS and 0.5 g sodium deoxycholate) (Sigma-Aldrich, São Paulo, Brazil) for 1 min in an ice bath. Subsequently, they were subjected to centrifugation for 10 min at 1600xg (at 4 °C) in an 5430R Eppendorf centrifuge (Hamburg, Germany). The resulting supernatants were transferred to other microtubes and stored at −20 °C for further analysis.

Lipid peroxidation levels were determined according to Esterbauer et al. (1991). through malondialdehyde (MDA) quantification by UV−Vis spectrophotometry following a trichloroacetic acid (TCA) and thiobarbituric acid (TBA) reaction and incubation for 60 min at 100 ºC in a water-bath, followed by centrifugation at 1600xg for 10 min. Absorbances were determined at 535 nm UV-Vis 1601 Shimadzu spectrophotometer (Japan) and expressed as μmol g^{-1} dry weight (d.w.). All samples were analyzed in triplicate.

2.7. Metal and metalloid determinations

Metal and metalloid sample preparations followed Lavradas et al. (2016a). The freeze-dried samples (150 mg) were decomposed in 1 mL of subdistilled nitric acid (67% v/v, Sigma-Aldrich, São Paulo) at 100 °C for 5 h. After cooling, the mixture volumes were adjusted to 10 mL and then adequately diluted with ultrapure water (resistivity> 18 MΩ cm) for subsequent analysis by inductively coupled plasma mass spectrometry (ICP−MS). The metals and metalloids were determined on an Elan DRC II (Perkin Elmer Sciex, Norwalk, CT, USA) spectrometer without the use of reaction cell. Sample introduction was performed using a Meinhard nebulizer with cyclonic chamber. Quality control was performed by a strict blank control, the analyses of replicates and certified reference materials. ICP−MS measurement conditions were optimized through the use of multi-elemental analytical curves using appropriate dilutions of a mixed standard solution (Merck IV) prepared in HNO_3 (v/v). Analytical curve coefficients were always higher than 0.995. Instrumental ICP-MS operating conditions were as follows: radiofrequency power of 1100 W, plasma gas flow rate of 17.0 L min^{-1}, auxiliary gas flow rate of 1.2 L min^{-1}, carrier gas flow rate of 0.98 L min^{-1} , Pt sampling and skimmer cones and dwell time of 30 ms per isotope.

Accuracy was assessed through the analysis of certified material DORM-4 (National Research Council, Canada). Certified DORM-4 recoveries (Table 1, Supplementary Material) ranged from 96.7% to 106.2% and were considered appropriate for the present study according to international standards (Eurachem, 1998; Ishak et al., 2015). All samples were analyzed in triplicate.

Table 1
Biometric data (displayed as mean ± standard deviation) of the mussel pools per sampling site.

Sampling site	Length (mm)	Width (mm)	Height (mm)
Ilha Grande Bay	63.5 ± 2.5	24.1 ± 0.7	23.2 ± 0.8
Guanabara Bay (DB)	49.9 ± 3.0	23.3 ± 0.8	19.3 ± 1.4
Guanabara Bay (UB)	54.8 ± 2.9	29.0 ± 1.4	20.1 ± 1.4
Guanabara Bay (VB)	35.6 ± 1.8	16.4 ± 0.3	13.8 ± 0.7

2.8. SEC−HPLC−ICP−MS analyses

The TSF used for the MT determinations were analyzed by size exclusion (SEC) high-performance liquid chromatography (HPLC) coupled to an inductively coupled plasma mass spectrometer (ICP−MS) after total protein content determinations by Peterson's (1977) modified Lowry method, in order to inject the same amount of total protein (40 μg) for each sample (Lavradas et al., 2016b).

A NexIon 300X quadrupole inductively coupled plasma mass spectrometer (PerkinElmer, Norwalk, CT, USA), equipped with a collision and reaction cell, coupled by means of a 50 cm PEEK tubing to a 1100 Shimadzu HPLC pump with a UV detector (Shimadzu, São Paulo, Brazil), as the delivery system for the chromatographic separations. The mobile phase was prepared daily with ultrapure water (resistivity > 18 MΩ cm). No metal connectors or parts were used for this analysis, and potential element retentions were tested for at least 30 min after the last visible peak observed after each sample run, in order to overcome contamination problems or elemental losses, as described previously (González-Fernández et al., 2008). The instrumental operating conditions for the SEC−HPLC−ICP−MS analysis are those previously indicated in Table 1 (supplementary) coupled to a Superdex™ −75 (10 × 300 × 13 μm) (GE Healthcare, Uppsala, Sweden) column, using Tris–HCl 0.02 mol L^{-1} (pH 7.4) as the mobile phase, at a flow rate of 0.7 mL min^{-1} and injection volume of 20 μL.

The SEC column was calibrated using bovine serum albumin (BSA, 67 kDa, for Zn determination), metallothionein-I (MT, 1, 7 kDa, for Cd determination) and reduced glutathione (GSH, 0.3 kDa, for Cu determination), according to previous studies using this same technique (Gonzalez-Fernández et al., 2011; Hauser-Davis et al., 2012; Lavradas et al., 2016b).

2.9. Statistical analyses

Data normality was tested using the W Shapiro-Wilkes test. An ANOVA test was used to verify significant differences between the concentrations of the analytes in the mussel different tissues and between different sampling sites. Differences were considered significant at $p < 0.05$. A principal component analysis (PCA) was applied to detect interrelationships between all variables in the investigated gill samples from all sampling locations. The representative principal components (PCs) were chosen following the Kaiser's rules (Kaiser, 1960), where PCs with eigenvalues > 1.0 and individual variance > 10%. Statistical analyses were performed using the Prism® v.8.0 software and the PCA was constructed using the STATISTICA software (Statsoft).

3. Results and discussion

3.1. Biometric data

The biometric data (shell length, width and height, expressed as mean ± standard deviation) of the mussel samples assessed herein are displayed in Table 1. Statistically significant differences for length were observed, with IG mussel lengths significantly higher than those from all three GB beaches. Thus, data were normalized to the mean length of the mussels from each location, in order to remove size effects (Bjerkeng et al., 1998; Seixas et al., 2007; Nemr et al., 2012; Lavradas et al., 2016b).

3.2. Oxidative stress endpoints and metal concentrations

MT, GSH, CarbE and LPO values in *Perna perna* mussel gills analyzed in the present study are displayed in Fig. 2. MT, GSH and LPO values are expressed as μmol g^{-1} dry weight (d.w.) and CarbE values are expressed as U mg^{-1} protein.

The metal concentrations determined in the present study in *Perna perna* mussels from Guanabara and Ilha Grande Bay are displayed

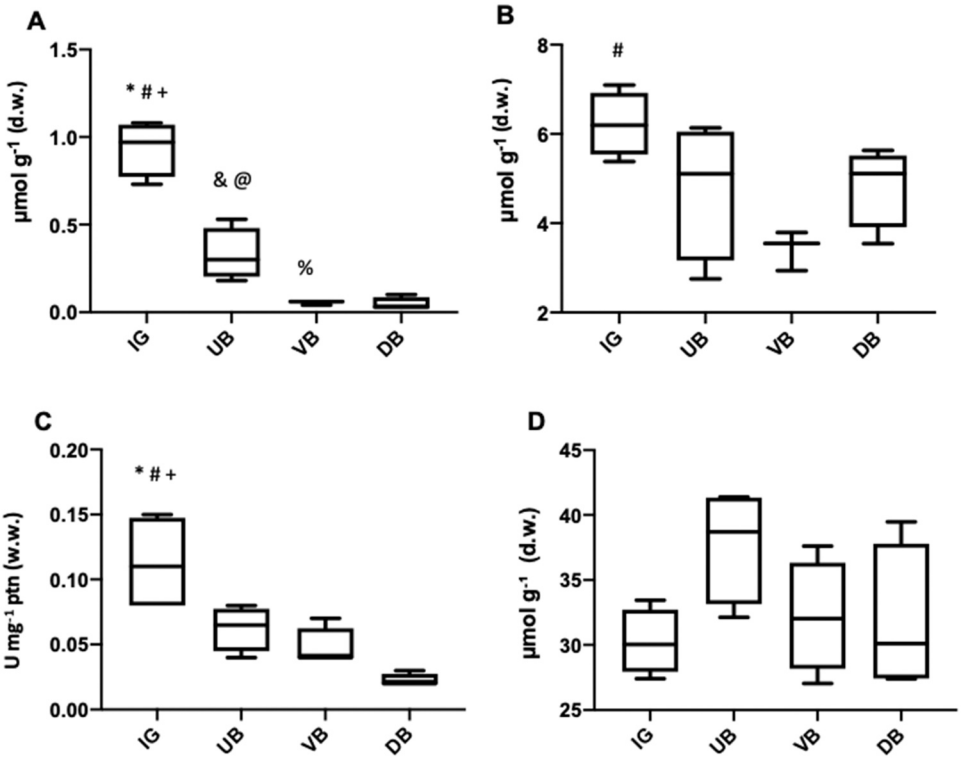

Fig. 2. (A) MT, (B) GSH, (C) CarbE and (D) LPO values in *Perna perna* mussel gills analyzed in the present study at Ilha Grande (IG), Diabo Beach (DB), Urca Beach (UB) and Vermelha Beach (VB). Same letters indicate significant differences ($p < 0.05$) between groups ($n = 4$, composite samples composed of 10 individuals from each sampling site). The boxplots display the upper and lower quartiles for each dataset and the median is represented by a short black line within the box. * Denotes significant differences between IG and DB; # denotes significant differences between IG and VB; + denotes significant differences between IG and D; & denotes significant differences between UB and VB; @ denotes significant differences between UB and DB, and % denotes significant differences between VB and DB (all $p < 0.05$).

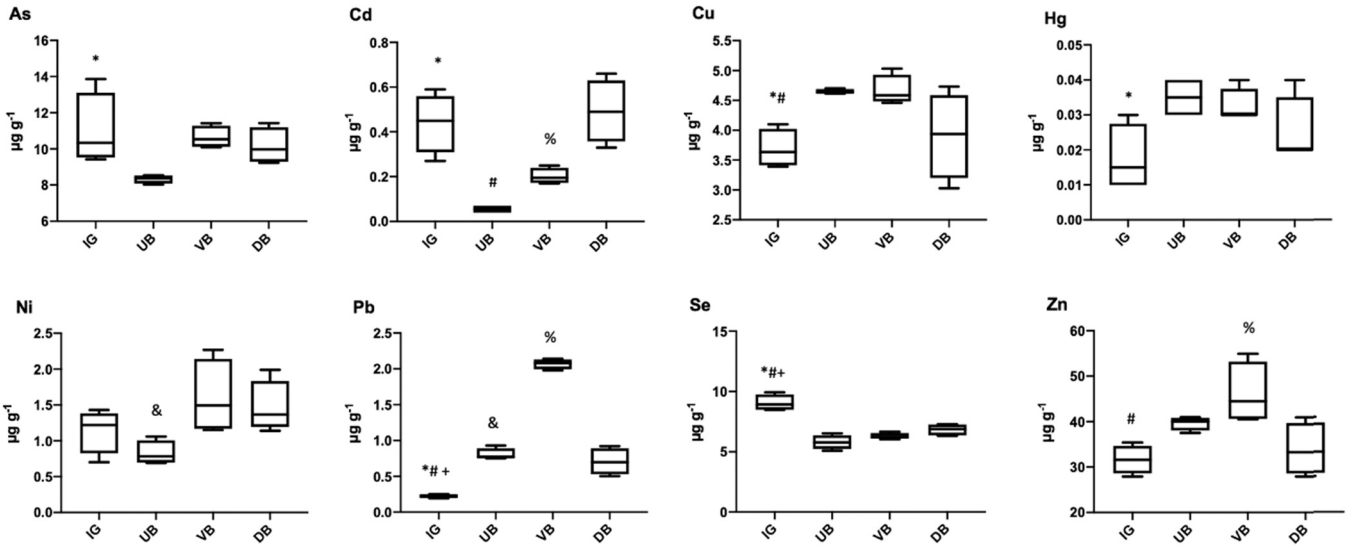

Fig. 3. Metal and metalloid concentrations in gills in *Perna perna* mussels from Ilha Grande (IG), Diabo Beach (DB), Urca Beach (UB) and Vermelha Beach (VB). Same letters indicate significant differences ($p < 0.05$) between sampling sites. The boxplots display the upper and lower quartiles for each dataset and the median is represented by a short black line within the box. The boxplots display the upper and lower quartiles for each dataset and the median is represented by a short black line within the box. * Denotes significant differences between IG and DB; # denotes significant differences between IG and VB; + denotes significant differences between IG and D; & denotes significant differences between UB and VB; @ denotes significant differences between UB and DB, and % denotes significant differences between VB and DB (all $p < 0.05$).

below in Fig. 3.

The PCA plots of the investigated oxidative stress endpoints and metals analyzed in the present study in *Perna perna* mussels are presented in Fig. 4. The PCA distinctly separated the mussel samples from each study area. Principal component 1 (PC1) was responsible for 45.19% of the total data variability. Each location was differentially correlated to certain variables. IG samples were primarily separated in PC1 from other locations and were associated to the following elements: Se (correlation coefficient, R, of 0.86), Cd (R 0.62), and the following biomarkers: MT (R 0.80), GSH (R 0.70) and CarbE (R 0.62). In PC1, both VB and UB samples were separated from IG and they were related to Cu ($R = -0.76$), Zn ($R -0.77$), Hg ($R -0.82$) and Pb ($R -0.83$). VB and UB could be discriminated in PC2, which was responsible for 23.46% of the total data distribution. In this component, VB was associated to Ni (R 0.80), As (R 0.82), and Cd (R 0.61). On the other hand, UB was correlated to LPO ($R = -0.72$). Finally, DB samples were correlated to PC3.

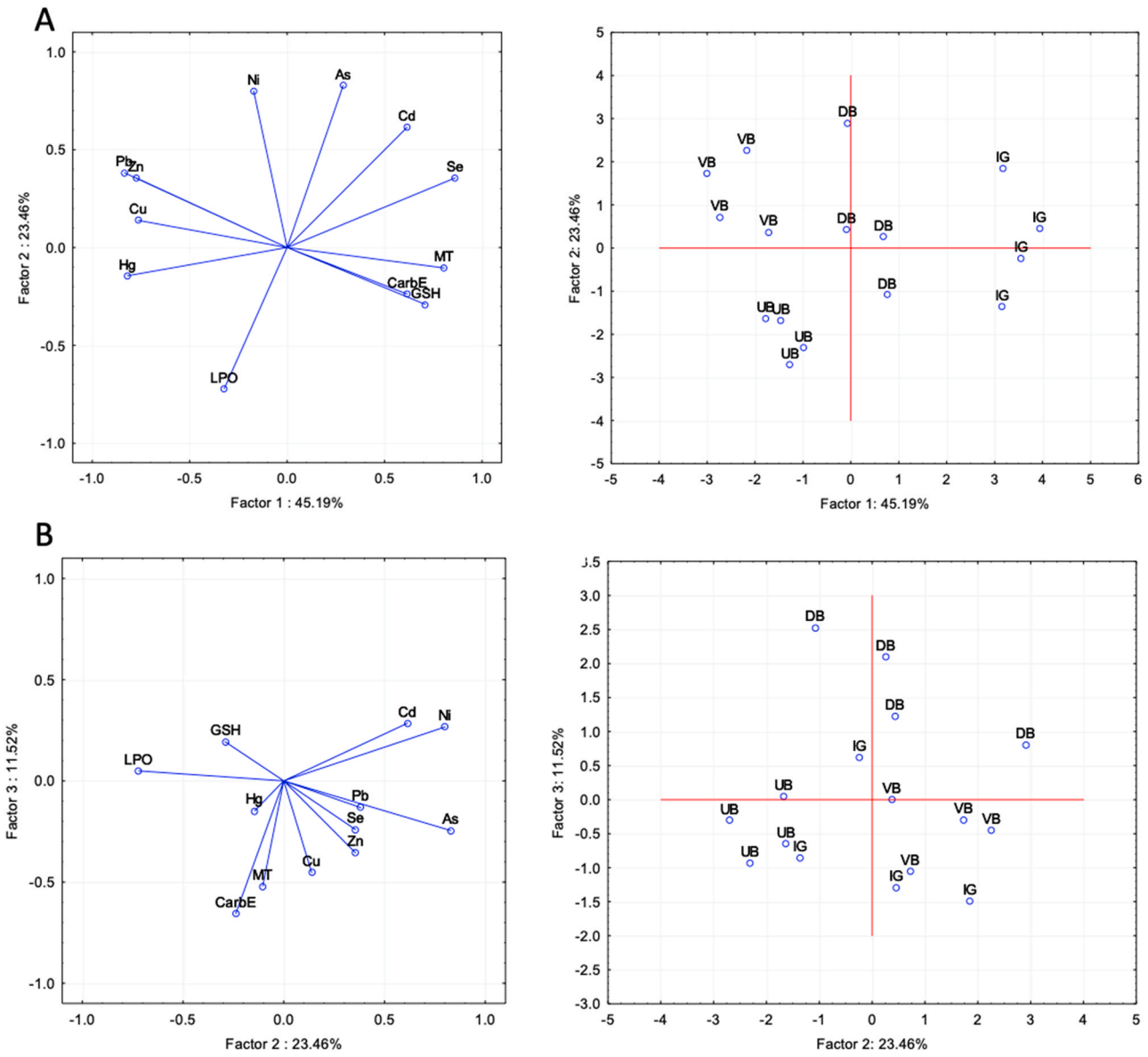

Fig. 4. PCAS of the investigated oxidative stress endpoints and elements determined in the present study in *Perna perna* mussels from Guanabara and Ilha Grande Bay. (A) PC1 *versus* PC2; (B) PC2 *versus* PC3.

The other locations were correlated to CarbE (R −0.65) and MT (R −0.52), separating them from DB.

Certain elements in *Perna perna* mussel gills contributed to higher oxidative stress biomarker induction than others, when comparing the four sampling locations, which can be observed by the protein content and enzymatic activity results displayed in Fig. 2 in association with the elemental concentrations displayed in Fig. 3. This was, in turn, corroborated by the PCA analysis concerning the correlations between the biochemical oxidative stress endpoints and the elements determined herein.

MT, GSH and CarbE activities followed almost the same trend, higher at IG, followed by UB, VB and DB, although GSH levels at VB were slightly lower than at DB, albeit non-significantly, probably due to differential contamination sources and effects. Higher IG MT and GSH content and CarbE activity seem to reflect higher metal contamination at this site, in accordance with the fact that this area has been recently reported as being metal-contaminated (Lavradas et al., 2016a, 2016b). This is corroborated by the high As, Se and Cd concentrations observed at this site, and by the PCA results, which indicate that IG samples displayed significant correlations between MT, GSH and CarbE and these elements. The observed lipid peroxidation levels and lack of LPO association observed in the PCA, however, indicate that organisms at IG are seemingly efficient in preventing at least certain deleterious cellular effects of these contaminants, since IG levels were the lowest from all areas. This, however, was not statistically significant. Interestingly, preliminary data from our research group has indicated, for the first time, higher CarbE activity in fish at higher dissolved oxygen concentrations (unpublished data). This may lead to further research associating this water quality parameter to modifications in chemical metal species and consequent CarbE activity alterations.

Regarding UB, protein content and enzymatic activities at this site were the second highest among the sampling locations, with Hg, Zn and Cu the highest at this location, indicating that these metals play an important role in inducing MT and GSH protein contents and CarbE activities. The high Zn and Cu concentrations observed at this sampling site may be due to the antifouling paints applied to the many leisure vessels constantly moored at UB. These metals have, indeed, been reported as potentially attaining toxic concentrations in high boat density

areas (Ytreber et al., 2010). This observation also corroborates the PCA analysis, where UB samples were more correlated to Hg, Cu, Zn. However, the PCA also indicates a significant correlation for Pb and LPO. As Pb is an extremely toxic metal, both carcinogenic and mutagenic, with severe neurotoxic effects and no safe threshold for exposure (ATSDR, 2017; Eisler, 1998), it is probable that even low doses of this metal lead to biochemical effects, in this case, LPO, corroborated by the higher LPO levels observed in Fig. 2 at this location.

VB samples were more correlated to the Hg, Cu, Zn, Pb and Ni and LPO as indicated by the PCA, also corroborating the detected metal levels and the oxidative stress biomarker trends observed herein displayed in Fig. 2. The PCA indicated that DB samples displayed significant correlation between GSH and CarbE, for As, Se, Ni and Cd. When observing metal concentrations, Hg levels were also high at this location, but not seemingly able to induce these oxidative stress biomarkers in gills, similarly to that reported in a previous study carried out by our research group for the muscle tissue and digestive gland of the sample mussel samples (Lavradas et al., 2016b). This may be due to the fact that only total Hg was determined, not methyl mercury (MeHg), the most bioavailable, and toxic Hg fraction, which may have yielded significant correlations to the assessed biomarkers. About 90% of total Hg content in aquatic food webs is present in this methylated form and has been reported as efficiently bioaccumulating and biomagnifying throughout food webs (Adams and Engel, 2014; Harding et al., 2018).

MT is one of the main biomarkers established by metal biomonitoring programs developed and implemented in the European community, such as MEDPOL (Programme for the Assessment and Control of Pollution in the Mediterranean region) and BEEP (Biological Effects of Environmental Pollution in marine coastal ecosystems) (Garrigues et al., 2002; Viarengo et al., 1999), as it is both involved in the homeostasis of metabolically important metals, such as Cu and Zn, and in the detoxification of toxic elements, such as As, Cd, Hg and Pb, among others (Viarengo et al., 1997). However, intracellular metal compartmentalization and deleterious effects are directly related to differences in metal metabolism and metal bioavailability (Decataldo et al., 2004; Marijić and Raspor, 2006; Monteiro et al., 2019). Therefore, metal correlation data for biological samples is not straightforward (M'Kandawire et al., 2017) and may vary according to location, metal speciation and species, among other aspects, through other, non-MT, detoxification pathways (Giguère et al., 2003). In the present study, MT was associated only to As, Se and Cd at IG, corroborating these affirmations, as all the other assessed elements are also known to bind to MT, inducing its synthesis in marine organisms (Kägi, 1991; Sarkar et al., 2006). Thus, metal speciation in the aquatic environment, and, therefore, bioavailability, as well as and other intracellular defenses, may also be responsible for this lack of correlation to MT.

In this regard, other cellular defense mechanisms may also be in force. For example, GSH, a non-enzymatic oxidative stress biomarker, is also known to bind to several metals, through its sulfhydryl groups, limiting their ability to counteract lipoperoxidation and DNA oxidation (Baer, 1996; Jozefczak et al., 2012), although its role as a primary antioxidant and first line of defense against reactive oxygen species is the most studied (Aguilar et al., 2016). One study, for example reported increased GSH in *Perna perna* mussels after exposure to Zn (Trevisan et al., 2014). Assessments for this tripeptide in this species are still, however, scarce.

The PCA also indicated an association between MT and GSH at IG and DB. Besides acting in essential metal homeostasis and toxic metal sequestration, MT has also been reported as a ROS scavenger when oxidant loads become too high for GSH to deal with on its own (Hauser-Davis et al., 2014; Kumari et al., 1998). Thus, it seems that MT in this case is playing a dual role regarding metal detoxification and ROS scavenging (Hauser-Davis et al., 2020), further corroborating that metal correlation data for biological samples is complex and requires further evaluation.

Regarding CarbE, few studies in the literature regarding metal effects and mechanisms of action on this enzyme are available in marine organisms, most dealing only with its known role in pesticide detoxification (Escartín and Porte, 1997; Ozretić and Krajnović-Ozretić, 1992; Solé et al., 2018). Studies in this regard have assessed mostly in fish. For example, in a study carried out with zebrafish that verified the interference of copper, lead, iron and cadmium in the activities of CarbE, where this enzyme was inhibited *in vitro* with only a concentration of 20 mmol L^{-1}. *In vivo*, on the other hand, CarbE activity did not change at any time (two and seven days) and concentrations tested, except in the case of exposure to copper, which resulted in a decrease in the activity of this enzyme, indicating that metals may be esterase inhibitors in fish, and should be considered in environmental monitoring studies (De Lima et al., 2013). In another study, organotin compound (tributyltin, triphenyltin and dibutyl tin, a persistent metabolite) effects on this class of enzymes was investigated in the tropical marine fish species *Siganus canaliculatus*. The CarbE from the liver of this species was shown to be highly sensitive to these compounds, being significantly inhibited after exposure, indicating that this enzymatic class is effectively an adequate molecular biomarker of contamination by organotin compounds in fish (Al-Ghais et al., 2000). To the best of our knowledge, however, only one study evaluating CarbE activity specifically concerning metal exposure in mussels is available, albeit performed on the digestive gland, not in gills (Yoloğlu et al., 2018). The authors assessed correlations between metals in *Unio mancus* mussel digestive glands from a dam lake in Turkey, applying CarbE and several other biomarkers (glutathione-S-transferase (GST), acetylcolinesterase (AChE), glutathione reductase (GR) and metallothionein (MT)) following metal determinations. For CarbE in that study, statistical differences were observed between four sampling sites at the dam lake and between the winter and summer sampling campaigns. This enzyme was significantly correlated to Pb concentrations in winter, and Hg in summer and a PCA also indicated potential correlations to other elements (Cu, Fe, Ni, Pb, Zn, Hg and As) (Yoloğlu et al., 2018). Further studies, however, are required to validate CarbE as a biomarker of metal exposure.

3.3. SEC–HPLC–ICP–MS assessments

Differential chromatographic profiles for the *Perna perna* mussel gill TSF were observed. Not all analyzed elements exhibited a signal, but all that did, eluted between 5 and 30 min. No Cd or Hg peaks were observed in any of the samples. All the detected peaks were compared to the peaks from a protein mixture containing BSA (67 kDa), MT (7 kDa) and GSH (0.3 kDa), used as a calibration standard.

Table 2 displays the observed metalloprotein-bound metal and

Table 2
Metalloprotein-bound metals and metalloids in *Perna perna* gills from Ilha Grande Bay (IG), Diabo beach (DB), Urca beach (UB) and Vermelha beach (VB).

Sampling site	Metalloprotein-bound retention time (minutes)								
	6	11	14	16	19	21	24	25	28
Ilha Grande Bay	–	Cu, Ni, Zn	–	–	Cu, Ni, Zn	As, Cu, Ni , Zn	As	Cu, Ni, Se, Zn	–
Vermelha Beach	–	Cu, Ni, Zn	–	–	Cu, Ni, Pb, Zn	As, Cu, Ni, Zn	As, Sn	Cu, Ni, Se, Zn	–
Urca Beach	–	Cu, Ni, Zn	–	–	Cu, Ni, Pb, Zn	As, Cu, Ni , Zn	As, Ni	Cu, Se, Zn	–
Diabo Beach	Zn	Zn	Cu, Ni, Zn	Cu	Cu, Ni, Se, Zn	Cu, Ni	Ni	Cu, Se	Ni

metalloid retention times for *Perna perna* gills from Ilha Grande Bay, Urca Beach, Vermelha Beach and Diabo's beach.

Compared to the only other SEC–HPLC–ICP–MS assessment available for *Perna perna* mussels, where the muscle tissue and digestive glands of the same samples from the four study areas were analyzed (Lavradas et al., 2016b), a higher number of metal-protein complexation peaks was observed in gills compared to the other aforementioned tissues, probably representing a first detoxification mechanism defense concerning dissolved metal ions in the aquatic environment (Marigómez et al., 2002; Pagenkopf, 1983). On the other hand, no Cd peaks in gills were noted, whereas this element was eluted at 11 and 19 min in both muscle and digestive gland samples of the aforementioned study, although at very low intensities. No Hg peaks were detected in gills, nor in the previous assessment in muscle tissue and digestive gland (Lavradas et al., 2016b), perhaps indicating low environmental Hg levels, not enough to induce detoxification.

Fig. 5 display the chromatographic profiles for the determined analytes of each pooled sample from the four study areas.

The chromatograms for As showed an intense peak for Ilha Grande Bay, Urca Beach and Vermelha Beach samples, with a retention time of 24 min, an indication that this element is majorly bound to low molecular weight (LMW) metalloproteins (<0.3 kDa,). Since no calibration for these molecular weights was used, and no data was found in the literature, further studies regarding the possible binding between low molecular weight proteins and As are required. A smaller peak with a retention time of 21 min was also observed for these samples, a strong indicator that As is also bound to GSH in these samples. This assumption is supported by *in vitro* studies performed with GSH and As (Spuches et al., 2005). In addition, As-GSH complexes have also been observed in plants following As exposure, where they are stored in vacuoles, thus preventing transport throughout the organism and consequent cellular damage (Seth et al., 2012), and in both mice and rats (Kala et al., 2004; Suzuki et al., 2001). Although no studies in mussels are available, the data obtained herein may guide future research into As contamination and the GSH metabolism in these animals. No As peaks were observed for the Diabo Beach samples.

Several Cu peaks were detected eluting at 11 min for the Ilha Grande Bay, Urca Beach and Vermelha Beach samples, which may potentially be bound to BSA, as per certain *in vitro* studies (Singh, 2001). Regarding Diabo Beach, Cu-bound metalloprotein peaks were observed at 14 min and 16 min, indicating that some amount of gill Cu in *Perna perna* mussels is bound to proteins with molecular weight between 67 kDa and 7 kDa, in contrast to literature reports in mammals, indicating binding to proteins ranging from 4 to 20 kDa in mice brains (McCormick et al., 2013). Binding to CCS, a chaperone metalloprotein that plays a role in specific copper delivery to superoxide dismutase, is a possibility, as the molecular weight of CCS is of 54 kDa (Inesi, 2017). All samples displayed peaks at 21 min, suggesting a bond between Cu and GSH, as previously reported in *in vitro* assessments (Speisky et al., 2008) and generally taken to be a detoxifying mechanism used to eliminate the high electrophilicity and redox activity of free Cu ions (Aliaga et al., 2016). Another peak was noted at 24 min, again indicating that some Cu is bound to LMW proteins (<0.3 kDa), although no information regarding this was found in the literature.

The chromatographic Ni profiles for Vermelha Beach, Ilha Grande Bay and Urca Beach were remarkably similar, displaying only a slight variation in peak intensity. As supported by *in vitro* studies, the retention time of 11 min indicates a probable bond to BSA (Topală et al., 2014). The Ni peaks observed at 19 and 21 min point towards a Ni-GSH complex, which has also been previously reported in *in vitro* assessments (Krężel and Bal, 2004), including in mammalian cells (Salnikow et al., 1994), as Ni exhibits very high affinity for cysteine (Costa et al., 1994). The retention time of 25 min indicates that some Ni is also bound to LMW proteins (<0.3 kDa), contrasting with literature reports for other species, which report that only a very small amount of Ni is bound to LMW in human blood (Lucassen and Sarkar, 1979). The Diabo Beach samples exhibited a slightly different chromatographic profile than Vermelha Beach, Ilha Grande Bay and Urca Beach, with many low intensity peaks and retention times of 14 min, indicating binding metalloproteins with molecular weight between 67 kDa and 7 kDa, while the peak at 19 min indicates that a part of the total Ni is bound to metalloproteins with a molecular weight between 7 kDa and 0.3 kDa, and the peak observed at 21 min points again towards a Ni-GSH complex. Ni was also noted as bound to LMW proteins (<0.3 kDa) at 24 and 28 min.

The chromatographic profile for Pb exhibited binding to LMW ranging from 7 kDa to 0.3 kDa due to a peak at 19 min for the Vermelha Beach and Urca Beach samples. This may be indicative of lead-binding proteins, a series of low molecular weight proteins, analogous to MT, which complex to lead, resulting in a nontoxic form, reported in several species such as chickens, mice, rats, dogs and humans (Gonick, 2011). No studies, however, are available in this regard for mussels. No peaks were observed for this metal for the Diabo Beach and Ilha Grande Bay samples.

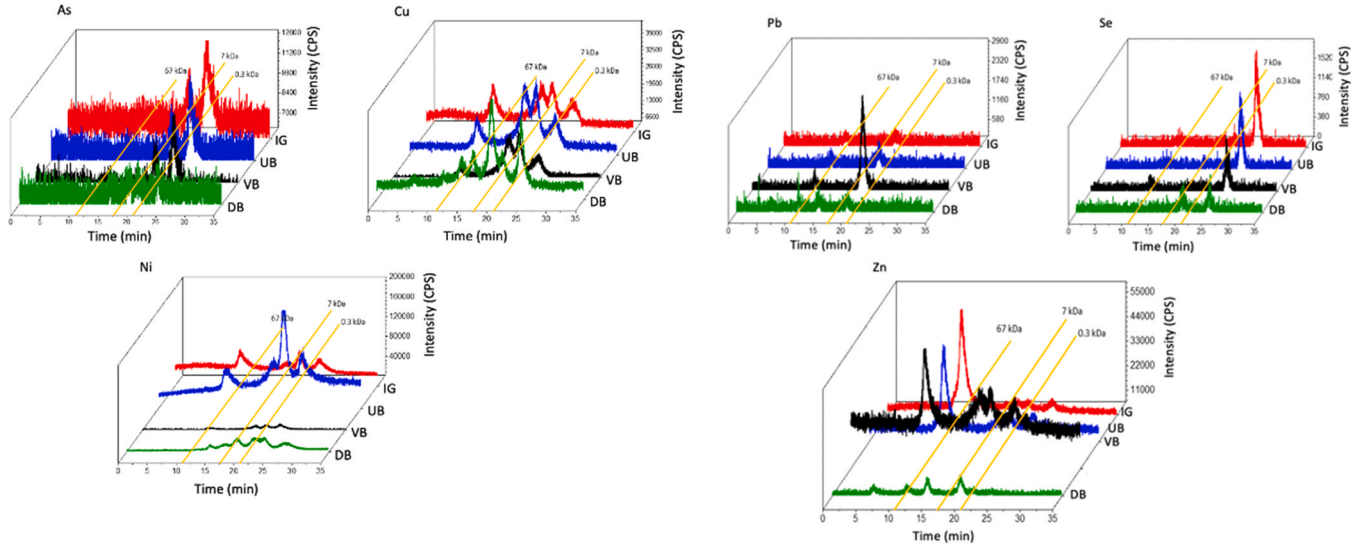

Fig. 5. Protein-bound As, Cu, Ni, Pb, Se and Zn detected by SEC–HPLC–ICP–MS in *Perna perna* mussel gills at Ilha Grande Bay (IG – red line, first line from back to front), Urca beach (UB – blue line, second line from back to front), Vermelha beach (VB – black line, third line from back to front) and Diabo Beach (DB – green line, fourth line from back to front). BSA (67 kDa), MT (7 kDa) and GSH (0.3 kDa) were used as the standard protein sizes.

Regarding Se, all samples peaked at 25 min, indicating that this element is bound to LMW metalloproteins (<0.3 kDa), which has been reported previously for fish (Åkesson and Srikumar, 1994), turtles (Anan et al., 2011), rats (García-Sevillano et al., 2014) and whales (Bryan et al., 2017). Further selenium-protein binding studies are paramount to assess seafood selenium health benefit values, as selenium bioavailability varies according to its biochemical form (Daniels, 1996). In addition, the Diabo Beach sample also exhibited a peak at 19 min, indicating proteins bound to Se ranging from 7 kDa to 0.3 kDa.

Regarding Zn, peaks were observed at 11 min for all samples, indicating probable binding between Zn and albumin, as albumin is a major Zn transporter protein previously pointed out by *in vitro* studies (Krężel and Bal, 2004), and concerning intestinal cells from perfused rats (Smith et al., 1979). Another peak was noted at 19 min, indicating binding at molecular weights between 7 kDa and 0.3 kDa, which has been previously reported for terrestrial slugs (Dvorak et al., 2019) and biological human fluids (Arver, 1982). In addition, all samples shared a 25 min peak, indicating binding to LWM metalloproteins (<0.3 kDa). Other peaks were also observed for the Diabo Beach samples, with retention times of 6 (higher than 67 kDa) and 15 min (between 67 and 7 kDa).

4. Conclusions

Differential associations between the assessed metals and oxidative stress endpoints were observed in *Perna perna* gill tissues from the four sampling locations in southeastern Brazil. In particular, As, Cd, Ni and Se contents seem to directly affect CarbE activity, although studies are notoriously lacking in the literature. In addition, MT seems to play a dual role in these contamination scenarios, both in metal detoxification and ROS scavenging, due to statistically significant associations with GSH. Further monitoring efforts are recommended at all sampling sites, due to lack of data on tropical mussel species concerning biochemical metal effects, especially at IG, recently described as metal contaminated. The SEC−HPLC−ICP−MS proved useful in characterizing metalloprotein binding to the assessed metals in *Perna perna* gills in response to environmental metal contamination. In this regard, probable complexation to GSH was observed for As, Cu and Ni, while binding to LMW proteins was noted for all analytes. Mass spectrometry identification of the extracted proteins in the mussel gill TSF is a logical next step for the specific identification of potential metal-detoxification biomarkers and biochemical detoxification pathways in *Perna perna* mussel gill TSF. In sum, the integrated assessments carried out herein demonstrate the importance of *Perna perna* mussels as tropical bioindicators, and are invaluable in evaluating contaminated aquatic environments, resulting in more accurate ecological risk assessments.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2020.111589.

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