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Chemical contamination in coastal areas alters shape, resistance and composition of carnivorous gastropod shells

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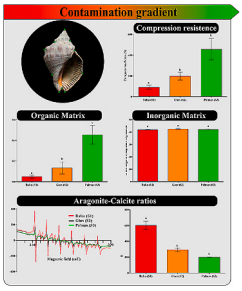
HIGHLIGHTS

- Contaminant levels were related to shell shape variations in *Stramonita brasiliensis*.
- Shells composition and resistance was influenced by contamination gradient.
- Calcite polymorphs was predominant in shells from polluted areas.
- Observed shell alterations were probably induced by chemical contamination.
- Shell alterations on carnivorous gastropods may be proposed as pollution biomarkers.

GRAPHICAL ABSTRACT



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ABSTRACT

Morphological, structural and compositional alterations in shells of molluscs have been proposed as putative biomarkers of chemical contamination in coastal zones. Despite this, few studies were carried out using top predator gastropods which tend to be more susceptible to contamination exposure. Thus, the present study assessed disturbances on shells of *Stramonita brasiliensis* considering compression resistance and organic and mineralogical matrix composition, related to morphometric alterations. Results showed reductions in compression resistance and organic matrix content associated with higher contaminated sites. In addition, a predominance of calcite polymorphs was seen in shells obtained in polluted areas. Such outputs were consistent with local contamination levels which may have induced the observed alterations. Thus, changes in mollusc shells showed good performance as potential biomarkers of coastal contamination, being probably observed in other species of carnivorous gastropods around the world.

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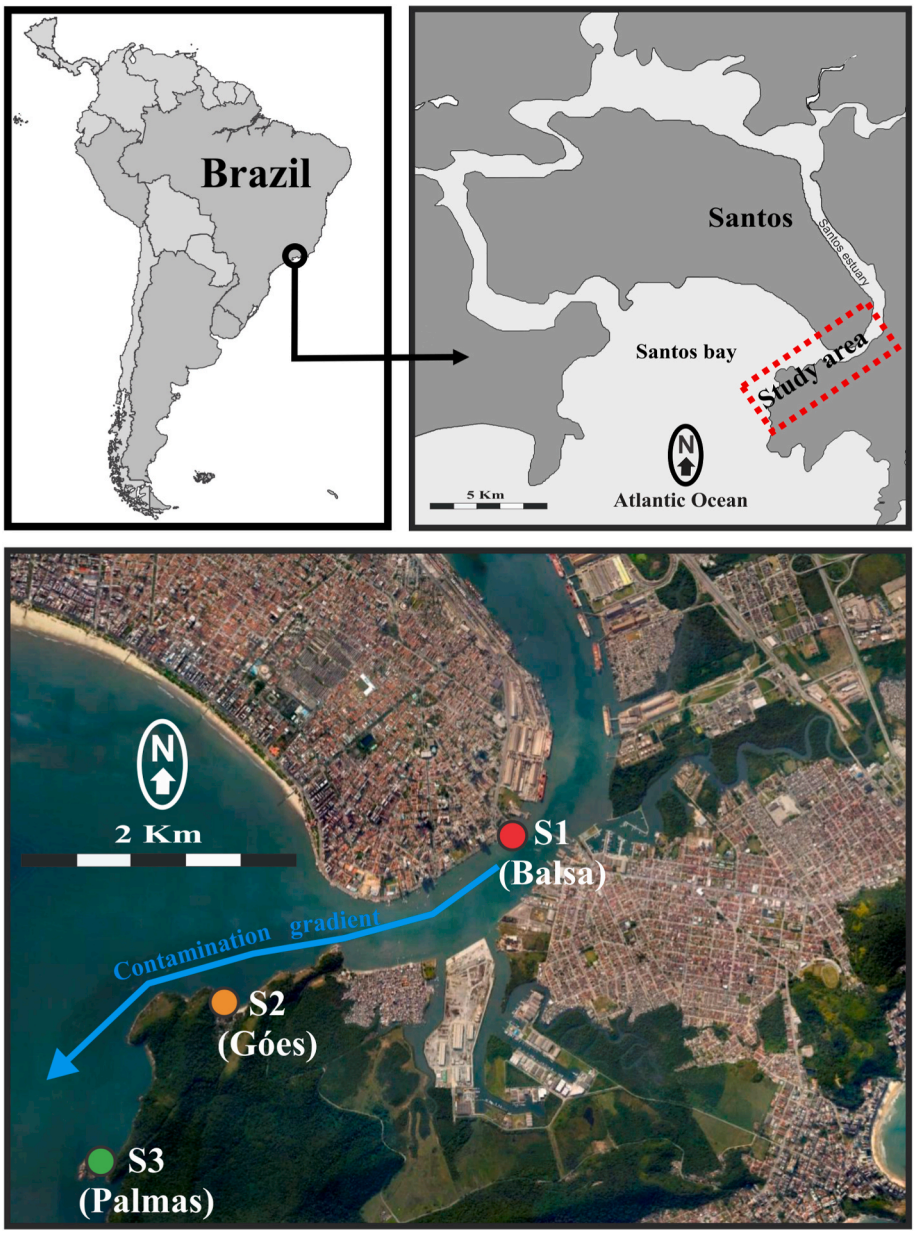

Fig. 1. Sampled sites in the Santos Estuarine System (SES; Brazil).

1. Introduction

Anthropogenic activities have increased over the years contributing to release of hazardous substances from different sources (Castro, 2019). Consequently, natural environments, especially coastal areas, has experienced a core set of impacts leading to disturbances on biological systems and threatening biodiversity (Abessa et al., 2018). In this regard, tools used to assess environmental quality play essential role to accurately estimate impacts and guide decision makers. In field conditions, assessments of contamination levels and their effects on biota have been carried out through different types of analytical determinations, often using highly complex, expensive, and time-consuming techniques (Castro, 2019). Alternatively, biological responses to contaminants exposure have enabled more effective tools with reduced costs. In addition, they provide ecological referenced data on environmental impacts in coastal zones (Lagadic, 2002; Márquez et al., 2011).

Biomarker approaches are widely used to assess early-stage effects of chemicals on aquatic organisms (Abreu et al., 2020; Chiovatto et al., 2021; Fontes et al., 2021). However, the selection of good bioindicator models should consider preferably organism abundance, sedentarism (or low mobility), easily collection and, mainly mensurable changes when exposed to contaminants. For this reason, bivalve and gastropod species are often chosen to be used in monitoring studies of coastal contamination (Harayashiki et al., 2020b; Hong et al., 2020). Thus, common biomarker responses, such as imposex incidence, lysosomal membrane stability and DNA damage have been widely assessed in these organisms (Chiovatto et al., 2021; Gouveia et al., 2019; Grimón et al., 2016).

Molluscs have a rigid structure (shell) whose functions include support soft tissues and protect against predation and desiccation. These shells are often composed by an inorganic matrix based on $CaCO_3$ polymorphs (mainly aragonite and calcite) associated with small amounts (0.1–5% dry weight) of organic compounds such as proteins, glycoproteins, proteoglycans, polysaccharides, and chitin (Marin et al., 2012). It is known that the life-record of mollusks, including environmental changes information, may be stored in shell matrices, through structural, morphological, and chemical alterations (Harayashiki et al., 2020a). In this regard, studies using shells of gastropods exposed to

hazardous substances, evidenced alterations in shape and in the composition of both inorganic and organic matrices (Begliomini et al., 2017; Nuñez et al., 2012; Oliveira et al., 2020). Such changes, observed in field and laboratory conditions, have been attributed to coastal contamination and proposed as putative biomarkers of pollution (Begliomini et al., 2017; Gouveia et al., 2019).

Gouveia et al. (2022) assessed comparatively shell morphometry of three mollusc species with different feeding habits that were obtained along the same contamination gradient. This study pointed out the greater performance by the carnivore muricid *Stramonita brasiliensis*, that presented different shell shapes among sampled sites under different contamination levels which, in turn, were proposed as potential generalist proxies of coastal pollution. In fact, top predators, such as *S. brasiliensis*, are susceptible to biomagnification processes exhibiting measurable biological alterations (e.g., imposex) which have been historically used to monitor coastal contamination by tributyltin (Castro et al., 2012; Rossato et al., 2016). In addition, *S. brasiliensis* is widely distributed an abundant on rocky shore substrates, between Colombia and Uruguay (Claremont et al., 2011; Veiga et al., 2015), fulfilling relevant requirements for an ideal bioindicator.

Although the study by Gouveia et al. (2022) provides significant advances on the use of mollusc shells as potential contamination trackers, no endpoint related to structure and mineralogical matrix composition were so far investigated in shells of *S. brasiliensis*. In this regard, studies using patelliform gastropods (*Lottia subrugosa*) have already demonstrated disturbances on shell compression resistance and matrix compositions related to contamination exposure (Oliveira et al., 2020). Furthermore, no studies on aragonite-calcite ratios in mollusc shells obtained along environmental gradients of hazardous chemicals were so far performed. Therefore, we hypothesize that alterations in shell shape and inorganic matrix of carnivore gastropods are consistent with environmental contamination levels. Thus, the present study aimed to simultaneously assess alterations in shape, compression resistance and mineralogical matrix composition in shells of *S. brasiliensis* along a contamination gradient in Santos Estuarine System (SES; Brazil). Additionally, to confirm the contamination, chemical analyses (metals and organic compounds) were performed on *S. brasiliensis* soft tissues.

2. Materials and methods

2.1. Study area and sampling

Santos Estuarine System (SES) presents well documented contamination gradient considering different classes of residues, hazardous chemicals and toxic elements (Abreu et al., 2020; Begliomini et al., 2017; Kim et al., 2017; Pusceddu et al., 2019; Ribeiro et al., 2021; Torres et al., 2015). In fact, levels of polycyclic aromatic hydrocarbons, estrogens, antifouling biocides, polychlorinated biphenyls, organochlorine pesticides and metals were assessed in different environmental matrices showing higher concentrations at S1 (Balsa) which are progressively lower towards the Santos Bay (Palmas - S3). Further, intermediate contamination levels have been reported at S2 (Góes beach) (Fig. 1). Considering the study area, the variation of hydrodynamics and physicochemical parameters, such as salinity and pH, are negligible and the hard substrates available have similar lithological characters (Begliomini et al., 2017; Berbel et al., 2021; Gimiliani et al., 2016). Therefore, this set of characteristics make SES a natural laboratory that has already been used by our research team seeking to assess shell parameters as possible contamination biomarkers (Begliomini et al., 2017; Gouveia et al., 2022; Harayashiki et al., 2020a; Oliveira et al., 2020).

Adult specimens of *S. brasiliensis* (>20 mm, n = 40) were collected manually on consolidated substrates on each selected site (Fig. 1) in September 2019. Only living organisms were collected in order to avoid eroded shells. Specimens were transported inside plastic bags to laboratory and stored at −80 °C during at least 1 h. After, soft tissues were completely removed and shells were individually identified, immersed in sodium hypochlorite solution (5%), washed with distilled water, and air-dried.

3. Chemical analyses in soft tissues

Integral specimen soft tissues were pooled according to the sampling site and freeze-dried. These samples were analytically analyzed to determine concentrations of chemical elements (As, Cd, Cr, Cu, Fe, Mn, Pb and Zn), polycyclic aromatic hydrocarbons (PAH), polychlorinated biphenyls (PCB), total of DDT (DDT and its breakdown products DDE and DDD including p,p' and o,p' forms) and linear alkylbenzenes. For metal and metalloid determination, all samples (in triplicate) were weighted (~200 mg) in 50 mL conical tubes (Falcon Corning, Tamaulipas, Mexico), closed and predigested for 24 h with 2 mL of subboiled HNO_3. Then, 200 μL of H_2O_2 was added, and the mixture was heated at 100 °C during 1 h in a water-bath. After cooling, the volume made up to 30 mL with deionized water. The concentration of trace elements (As, Cd, Cr, Cu, Fe, Mn, Pb and Zn) was measured by inductively coupled plasma mass spectrometry (ICP-MS 7900, Agilent Technologies, Hachioji, Japan), according to Paniz et al. (2018). Calibration solutions for ICP-MS were prepared over the range of 1–200 μg L^{-1} from multi-element standard solutions containing 10 mg L^{-1} of each element (PerkinElmer, Norwalk, CT, USA).

PAHs, PCBs, DDTs (DDT and its breakdown products DDE and DDD including p,p' and o,p' forms) and LABs were analytically identified and quantified according to USEPA Methods (2021) (Extraction – EPA 3610C; Cleanup – EPA 3610, 3630, and 3640; Quantification – EPA 8270 and 8081B) and MacLeod et al. (1986) with some modifications (Jarcovis et al., 2021). In brief, 1 g of freeze-dried soft tissues from each site were pooled, homogenized, macerated and spiked using surrogate standards for PAHs (naphthalene-d_8, acenaphthene-d_{10}, phenanthrene-d_{10}, chrysene-d_{12}, and perylene-d_{12}), PCB 103 for PCBs and OCPs, and 1-C_{19}LAB (for LABs). The extracts, obtained in Soxhlet device with a mixture of n-hexane and dichloromethane, were concentrated and cleaned sequentially using silica-alumina column and gel permeation chromatography system. Finally, the extracts were concentrated again, and the internal standards ρ-terpheyl-d_{14} (PAHs), tetrachlorometaxylene (TCMX) for PCBs and OCPs, and 1-C_{12}LABs (LABs) were added. The separation was performed using an Agilent gas chromatography (GC) (Model 7890B) coupled to an Agilent triple quadrupole mass spectrometry system (MS/MS) (Model 7010B). Compounds were identified by matching the retention times with the results from standard compounds and by the ion mass fragments (m/z). Concentrations of individual compounds were obtained using the internal standard peaks area method and 10-point analytical curves for individual components.

The quality and reliability of the method, as well as its precision and accuracy, quality control was assessed using replicates, spiked samples, and standard reference materials (SRM 2974a, Organics in Freeze-Dried Mussel Tissue - *Mytilus edulis,* for the PAHs, purchased from the National Institute of Standards and Technology -NIST, USA- and SMR 1945 - organics in whale blubber, for the PCBs and OCPs) purchased from the National Institute of Standards and Technology (NIST, USA). The limit of quantification (LOQ), in dry weight (dw), for PAHs was 2.5 ng g^{-1}, for pesticides 0.1 ng g^{-1}, for PCBs 0.05 ng g^{-1}, and 10 ng g^{-1} for LABs.

4. Biometric and geometric morphometric analyses

Shell biometric parameters (length, width and height) were measured using a digital caliper (±0.01 mm). For geometric morphometric analyses the method described by Primost et al. (2015) was adopted. In brief, photos were obtained with the shell opening facing upwards using a digital camera (Canon T6i; 50 mm lens) and a portable stand. To reduce the effects of rotation (movement in the ventral-dorsal direction along the transverse axis of the shells), shells were fixed on a modeling clay. Subsequently, the images were treated in the tpsDig

Fig. 2. Ventral view of *Stramonita brasiliensis* shell with defined landmarks (green points). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

software to add 10 landmarks (digitalized by one observer – AJLAO) as shown in Fig. 2. Later, the reminiscent effects of rotation, translation and scale were eliminated using Procrustes an analysis in TPS Relw software, whose aligned coordinates of generated of Procrustes were used in the MorphoJ software for multivariate statistical analysis.

5. Compression analysis

Shells of 15 organisms from each sampling site were randomly selected and subjected to a compression test to quantify resistance to mechanical forces. The test was performed using a Universal EMIC Test Machine DL line. The compression function was selected with a defined feed rate of 1 mm min^{-1} and a measurement range between 22 and 1501 N. Shells were individually evaluated, and the maximum resistance strength was obtained by the analyses of the results generated in the software TESC SCRIPT, which consists of recording the force applied at the first significant break in the shells (O'Neill et al., 2018).

6. Thermogravimetric analysis (TGA)

Thermogravimetric analysis (TGA) is a destructive technique used to assess variations on sample mass as a function of temperature exposure (Lucas et al., 2001). This analysis was used to determine thermal degradation of organic and inorganic fractions (indirectly assessed by residue content) in triplicate from each sample. Before TGA analysis, a shell powder was obtained by macerating fragments from body whorl (last whorl and the most recently formed) of each samples using mortar and pestle according to Oliveira et al. (2020). The analysis used simultaneous measurements of thermogravimetric and differential thermal analyzes (Netzsch - 409 Cell) with samples heated until 1300 °C in a heating rate of 5 °C min^{-1}, using oxygen atmosphere at 50 mL min^{-1} of flow. The percentage of degraded shell mass was determined in thermal events occurred around 550 °C for organic fraction and as from 848 °C for inorganic residues (Silva et al., 2010). These analyzes were performed using three different polls individually obtained from five shells from each site.

7. Aragonite and calcite ratios in shells

These analyzes were performed with the same samples used in TGA, however, shell fragments were macerated using 2 mL of 95% ethanol and then ground to a slurry. These samples were placed on glass microscope slides and remained for 12 h to dry at room temperature resulting in a powder sample. The aragonite and calcite ratios were assessed using two approaches. Firstly, EPR measurements were carried out using a Miniscope EPR spectrometer (model 5500 - Freiberg Instruments) operating at X-band with 100 kHz modulation frequency, microwave power of 20 mW and field modulation amplitude of 0.1 T. The analysis was performed at room temperature using 150 mg of shell powder, in triplicate. The magnetic field range was between 300 and 370 mT seeking to detect small amounts of Mn^{2+} ions, which are indicative of the calcite phase in mollusc shells (Siriprom et al., 2011). Later, the peak-to-peak EPR intensity obtained were compared among studied sites. Subsequently aragonite-calcite ratios were also assessed according to Ries, 2011 using an X-ray diffractometer. Powdered shell was analyzed using a Rigaku model MiniFlex 600 X-ray diffraction system. X-ray diffraction data were collected at 40 kV and 15 mA with Cu Kα-radiation, and the 2θ scan range was from 20° to 60°, with a step size of 0.005° and scan speed of 0.6 s step to obtain precise measurements of calcite and aragonite peaks. The diffractograms were analyzed using the X'Pert HighScore Plus program for phase identification. The resulting X-ray diffraction pattern for each shell was used to determine the levels (percent) of calcite and/or aragonite using equations given in Ries (2011). The primary calcite peak (d(104)) corresponded to $2\theta = 29.5–30.2°$ on the X-ray diffraction pattern generated. The two primary aragonite peaks (d(111) and d(021)) corresponded to $2\theta = 26.3°$ and 27.2°, respectively (Milliman, 1974).

8. Statistical analyses

The data obtained from chemical analyzes, biometry, mechanical compression tests, TGA analysis (organic and inorganic degraded fractions), protein amounts and aragonite/calcite ratios were assessed with regard normality and homoscedasticity using Shapiro-Wilk and Levene tests, respectively. When these assumptions were attended, comparisons were performed by Analyses of Variance (ANOVA) followed Tukey *posteriori* tests. When ANOVA requirements were not meet, non-parametric Kruskal-Wallis analyses followed by Dunn's multi-comparative analyzes were performed. Such univariate analyses were performed using Statistica 13.0 software. The geometric morphometrics data were analyzed using MorphoJ software. The occurrence of allometric effects (relationship between shape and body size) were verified using multivariate regression (Klingenberg, 2011). Principal component analyses (PCA) were performed to determine alterations in the shape of the shells, while Canonical Variates Analysis (CVA), based on the Mahalanobis distance (Primost et al., 2015) was performed to verify the difference between the sampling points. All statistical tests used a significance level of 0.01.

9. Results and discussion

9.1. Contaminant levels in soft tissues

All analyzed contaminants (As, Cd, Cr, Cu, Fe, Mn, Pb, Zn, PAHs, PCBs, total of DDTs and LABs) were detected in soft tissues of *S. brasiliensis* (Fig. 3). In this regard, copper presented higher concentrations reaching 53.2 ± 3.3 μg g^{-1} in samples from S1. Similar levels were seen for As (36.8 ± 0.7 μg g^{-1}) Fe (36.3 ± 1.1 μg g-1) and Zn (37.1 ± 0.6 μg g^{-1}) in the same site. Concentrations below than 4 μg g^{-1} were measured for Mn, while Cd, Cr and Pb were detected in levels below than 1 μg g^{-1} (see Fig. 3a–h). The occurrence of such elements in environmental matrices from SES have been historically reported in sediments (Angeli et al., 2021; Buruaem et al., 2013; Kim et al., 2017) and biota (Bordon et al., 2012). Although *S. brasileinsis* is occasionally used as a food resource by local communities, there is no regulations on the levels of toxic elements accumulated in gastropod tissues. Based on the limits

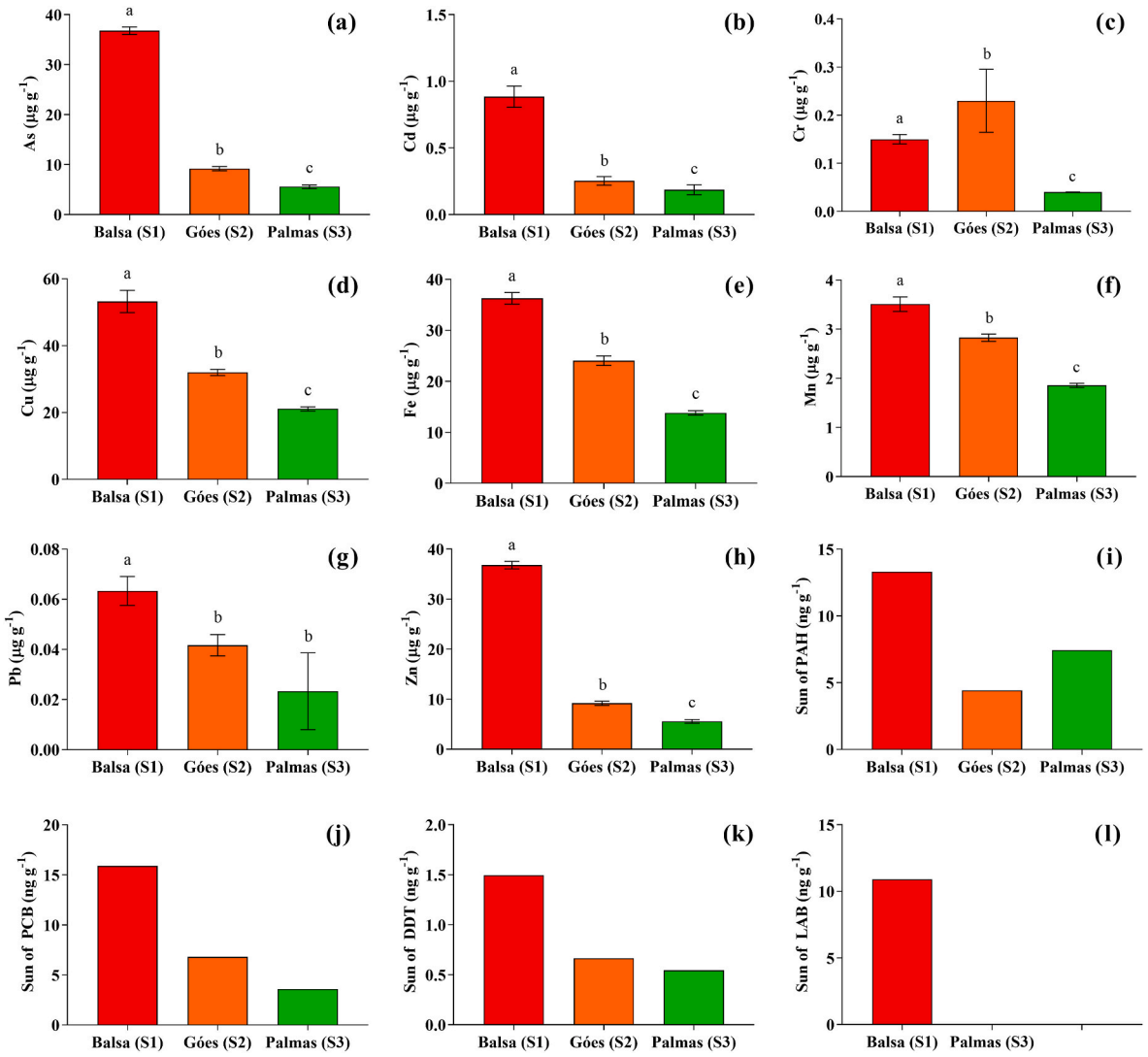

Fig. 3. Concentrations of elements (As, Cd, Cr, Cu, Fe, Mn, Pb and Zn), polycyclic aromatic hydrocarbons (∑PAH), polychlorinated biphenyls (∑PCB), total of DDT (DDT and its breakdown products DDE and DDD including p,p' and o,p' forms) and sun of linear alkylbenzenes in soft tissues of *Stramonita brasiliensis* collected along a contamination gradient in the Santos Estuarine System. Letters on the error bars denote statistical differences (Kruskal-Wallis, $p < 0.01$).

stablished by national and international regulations, for bivalve tissues, the values found were below the safe limits (European Commission, 2006). Similar pattern was also seen considering concentrations of legacy microcontaminants such as PAHs (4.4–13 ng g^{-1}), PCBs (3.6–15 ng g^{-1}) and total of DDTs (0.5–1.5 ng g^{-1}), which were below the threshold levels proposed by OSPAR - Oslo Paris Commission (2009) even in the most contaminated zones. In addition, linear alkylbenzenes (LABs) were seen only in S1 in concentrations around 11 ng g^{-1} (see Fig. 3i-l). These classes of organic contaminants have also been historically found in sediments, water, suspended particulate material, and bivalves, from SES (Bícego et al., 2006; Martins et al., 2010; Torres et al., 2015; Fontenelle et al., 2019), further, some PAHs have been found in concentrations higher than the threshold-effects level (TEL) in the SES sediments (Fontenelle et al., 2019). The presence of dibenz[a,h] anthracene in concentrations higher than the TEL in the sediments of SES prompts concern for the environmental health of the bay because it is considered a carcinogenic (Wood et al., 1981; de Albergaria-Barbosa et al., 2017).

According to Gouveia et al. (2022), the concentrations measured in *S. brasiliensis* were in the same order of magnitude, but lower than those found in oysters (*Crassostrea braziliana*) simultaneously collected in the same sites. Although the use of filter-feeding bivalves is more frequent in assessments of the aquatic contamination, gastropods have also been used as sentinels of chemicals released in these areas (Abdel Gawad, 2018; Amin et al., 2009; Uc-Peraza et al., 2022). Indeed, bivalve and gastropod species can internalize trace elements and organic microcontaminants through bioaccumulation and biomagnification mechanisms (Wang and Ke, 2002). Further, the concentrations of organic and inorganic molecules in soft tissues of molluscs depend on the accumulation strategy adopted by each species, besides being highly influenced by biochemical processes, which mediate the metabolic pathways (Langston et al., 1998; Rossato et al., 2018). Moreover, other factors such as local hydrodynamics, pH, temperature, sources proximity and physicochemical properties of assessed molecules may influence the balance between excretion and accumulation rates in soft tissues (Castro, 2019). Overall, the measured concentrations of toxic elements and organic microcontaminants were higher in S1, progressively decreasing towards S2 and S3 (Fig. 3). Such findings are in accordance with several studies, based on sediments levels, reporting contamination gradients along the lower estuary of SES (Abreu et al., 2020; Begliomini et al., 2017; Kim et al., 2017; Pusceddu et al., 2019). At SES, port terminals, industrial facilities and sewage effluents are mostly located along the middle and upper estuary (Abreu et al., 2020; Pusceddu et al., 2019). Therefore, the contaminant load released in these areas is transported

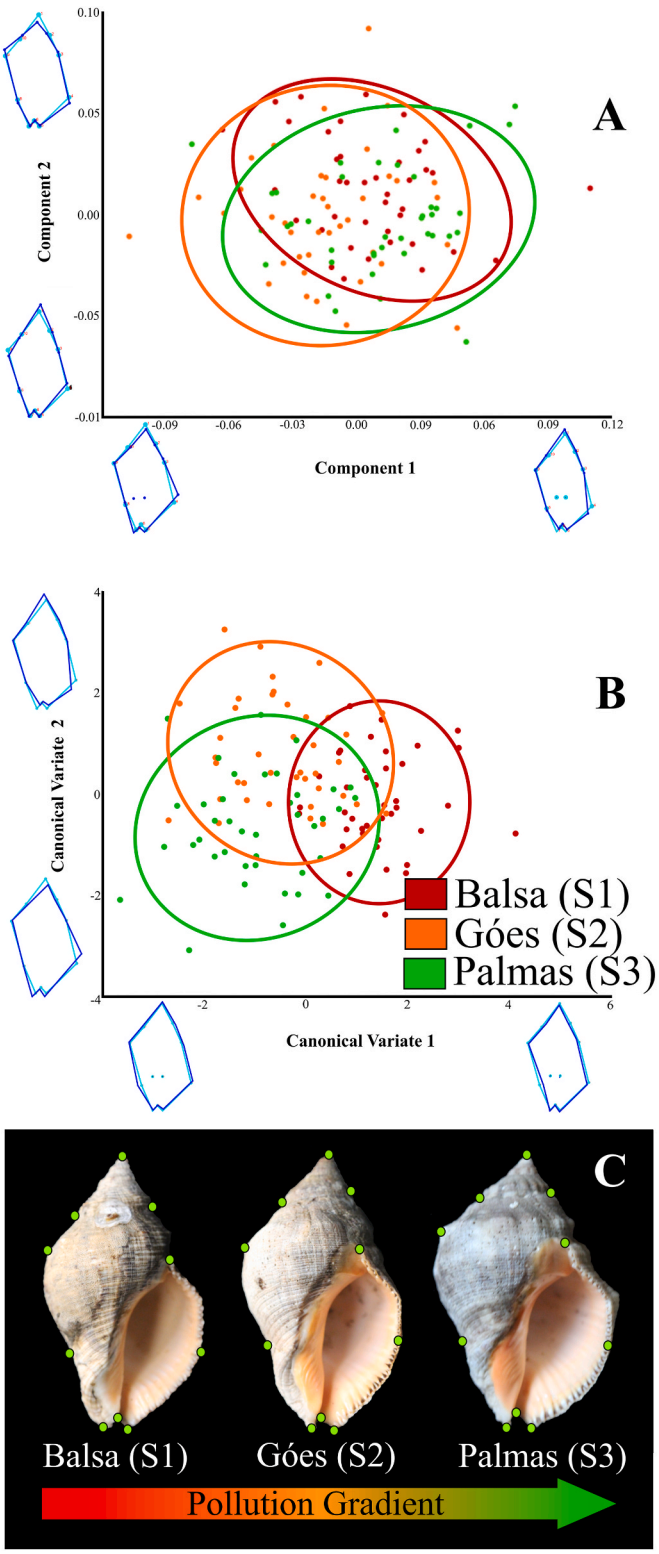

Fig. 4. Principal components analyzes (A), Canonical variables analyzes (B) and ventral view of *Stramonita brasiliensis* shells (C) with defined landmarks (green points) collected along a contamination gradient in the Santos Estuarine System. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

and diluted towards the lower estuary and Santos Bay (Begliomini et al., 2017; Oliveira et al., 2020). This gradient pattern was already descripted by an integrated quality assessment of sediments from SES, pointing out associated biological effects to aquatic organisms (Buruaem et al., 2013).

9.2. Biometric and geometric morphometrics alterations in shells

Considering all sampled sites, the shell lengths ranged from 33.4 ± 6.8 to 50.7 ± 6.3 mm, while shell height were between 20.7 ± 5.0 and 31.3 ± 8.8 mm. The shell height varied from 18.7 ± 2.8 and 27.7 ± 4.1 mm. Thus, the biometric parameters of the shells showed significant differences ($p < 0.01$) among the sampling sites (see Fig. S1 in supplementary material). In general, specimens sampled at Goes (S2) presented larger sizes than the ones sampled at Balsa (S1) and Palmas (S3). Considering all sites, shell length was positively correlated with width (Spearman, $p < 0.0001$ and $r = 0.96$) and height (Spearman $p < 0.0001$ and $r = 0.97$) (see Fig. S2 in supplementary material). These findings were also confirmed by multivariate regression analyses ($p > 0.01$) considering shell shapes. Such correlations indicate similar grow rates in different parts of organism bodies. Therefore, allometric effects among the analyzed samples were seen, as already reported to *S. brasiliensis* by Gouveia et al. (2022). According to several studies using geometric morphometrics analyzes of mollusc shells, bias may arise when allometric effects are present. However, these effects are removed mathematically by subsequent analyzes using the regression residuals of the allometric-free shell shape variables (Márquez et al., 2011; Primost et al., 2015). This approach has been used successfully in studies evaluating variations in the shape and structure of mollusc shells along the contamination gradients (Harayashiki et al., 2021).

Considering new shape variables, based on regression residuals to remove allometric effects, the PCA showed that 8 principal components explained 91.2% of the variation in shell shapes. The first principal component (PC1 = 28.5%) was related to shell opening (Fig. 4A), with positive values indicating larger shell opening and negative values indicating an opposite pattern. On the other hand, the second component (PC2 – 17.7%) pointed out differences in the region of the spires of the shells (Fig. 4A) with positive values indicating better defined spires. According to canonical variables analyses (Fig. 4B), the shells obtained in the three sampled sites differ from each other ($p < 0.001$). Considering the grouping between organism collected in S1, a smaller form variety was verified (Fig. 4B). Furthermore, the separation between the organisms from S3 (Palmas) and S1 (Balsa) occurred according to variations observed in CV1, while the organisms from S2 (Góes) were separated from those catch in S3 by alterations in CV2 (Fig. 4B). Thus, shells of *S. brasiliensis* from S3 (less contaminated site) were grouped in the negative values of CV1 and CV2, presenting more rounded shapes and larger shell openings (Fig. 4C). On the other hand, organisms from S1 had positive CV1 values, i.e., shells with a thinner shape. While specimens from S2 presented smaller openings (positive CV2 values). Therefore, the shells of *S. brasiliensis* showed shape variations consistent with contamination levels measured in soft tissues. Similar pattern, with thinner shells founded in high contaminated zones, were previously reported for the carnivore gastropods *Odontocymbiola magellanica* (Márquez et al., 2011). Indeed, different kinds of malformations in mollusc shells have been attributed to contaminant exposure by several studies performed worldwide (Alzieu et al., 1986; Begliomini et al., 2017; Faubel et al., 2008; Primost et al., 2015a; Slama et al., 2021). However, the specific molecule or group of molecules, inducing these changes remains unknown being considered generalist biomarkers (Harayashiki et al., 2020a).

10. Compression resistance in shells

The mechanical compression analyses showed that it was necessary to apply an average of 91 ± 44, 197 ± 100 and 458 ± 263 N of

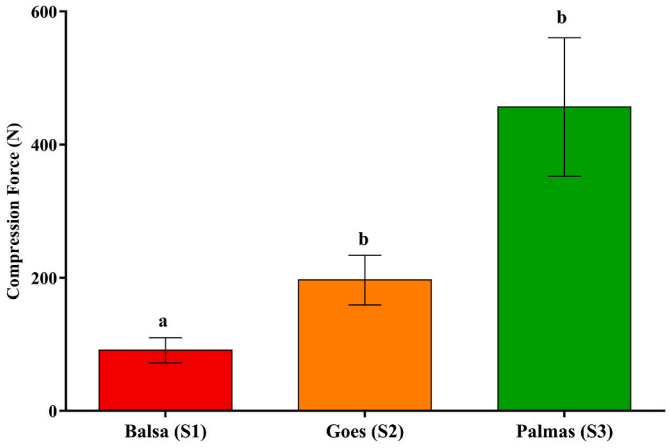

Fig. 5. Means and standard deviations of the compression force necessary to produce a first rupture in shells of *Stramonita brasiliensis* collected along a contamination gradient in the Santos Estuarine System. Letters on the error bars denote statistical differences (Kruskal-Wallis, $p < 0.01$).

compression force to produce the first rupture in shells obtained in Balsa (S1), Góes (S2) and Palmas (S3), respectively (Fig. 5). These results showed significant variations ($p < 0.0001$), demonstrating that shells collected at Palmas (less contaminated site) required significantly more compression force to induce the first rupture. In addition, the shells of organisms obtained in contaminated areas (Góes and Balsa), required progressively less energy to break. Thus, as seen in shell shapes, the compression resistance presented significant correlations (Spearman, $p < 0.01$ and $r > 0.70$) with the most of contaminants measured in soft tissues of *S. brasiliensis*. Likewise, Oliveira et al., (2020) assessing shell alterations in patelliform gastropods of the specie *Lottia subrugosa* founded more resistant shells at the same sites (S2 and S3) from SES. This study also highlighted, that such structural changes can lead to greater vulnerability to predators as crabs, which feed of *L. subrugosa* and *S. brasiliensis* (Matthews, 1968). Moreover, the fragility observed in shells from more contaminated areas, suggest damages on the organic matrix or in mineralogical balance of inorganic matrix, which usually gives resistance and elasticity to mollusc shells (Marin et al., 2007).

11. Thermogravimetric analysis (TGA) in shells

The samples from Balsa (S1), Goes (S2) and Palmas (S3) presented two thermal events well defined by the thermogravimetric and differential thermal curves. During the first thermal event, observed around 500 °C, degradation of the organic matter fraction take place as point out by Silva et al. (2010). The mass loss was 0.45 ± 0.2% for samples from S3, while in shells obtained at S2 and S1 the percentages of organic losses were 0.13 ± 0.1 and 0.05 ± 0.02, respectively. Such values corroborate with previous studies, which have shown that organic matrix of mollusc shells represents <5% of total shell weight (Arivalagan et al., 2016; Marin et al., 2007; Oliveira et al., 2020). Therefore, the organic content in shells from the more contaminated site (S1) were significantly lower ($p < 0.001$) than S2 and S3 (Fig. 6A) and positively correlated (Spearman, $p < 0.01$ and $r = 0.97$) with compression resistance. These findings indicates that shell organic matrix play essential role in shell structure and compressive strength of *S. brasiliensis*, as pointed out by Oliveira et al. (2020), assessing similar endpoints in *L. subrugosa* from SES. In fact, organic matrix is known to control important biomineralization steps in mollusc shells, mediating ion transport and growth through specific metabolic pathways (Harayashiki et al., 2020a). Thus, additional studies should investigate the influence of contaminant exposure on organic matrix functioning, including proteomic analysis, seeking to better address shell shape alterations.

The second TGA event was associated with the $CaCO_3$ decarbonation reaction, due to the formation of CaO and CO_2 (Shaikh and Supit, 2014), which is main constituent of the shells (Marin et al., 2007). During this even, occurred by an endothermic process as from 848 °C, the percentages of mass losses were of 42.0 ± 0.8, 42.5 ± 0.7 and 42.1 ± 1.0 for S1, S2 and S3, respectively (Fig. 6B). Therefore, no significant differences were seen among the studied sites ($p > 0.05$) suggesting similar amounts of inorganic phase in shell matrices regardless different contamination levels in studied sites. Despite the absence of significant differences, it is important to highlight that shell inorganic matrix is constituted by different polymorphs (for instance, aragonite and calcite), which are subject to imbalances as a result of environmental stress (Hubbard et al., 1981).

12. Aragonite and calcite ratios in shells

Molluscs shells are composed of different calcium carbonate polymorphs including mostly aragonite and calcite (Marin et al., 2012). In general, both minerals are present in shells, distributed in separate layers playing essential role on physical properties and morphology. Thus, the determination of aragonite-calcite ratios in shell of organisms exposed to environmental stressors such as chemical pollution and ocean acidification may enable tools for environmental assessments (Piwoni-Piórewicz et al., 2017). In this regard, Electron Paramagnetic Resonance (EPR) spectroscopy has been used to detect traces of Mn^{2+} ions in carbonate shells, which are related to calcite composite structures (Siriprom et al., 2012). According to obtained EPR spectra (Fig. 7A), the intensity of hyperfine signals due to the Mn^{2+} ions were higher in the samples from Balsa (S1) and progressively decreasing in sites under lower contamination levels (S2 and S3). In addition, significant differences were observed comparing peak-to-peak EPR intensities

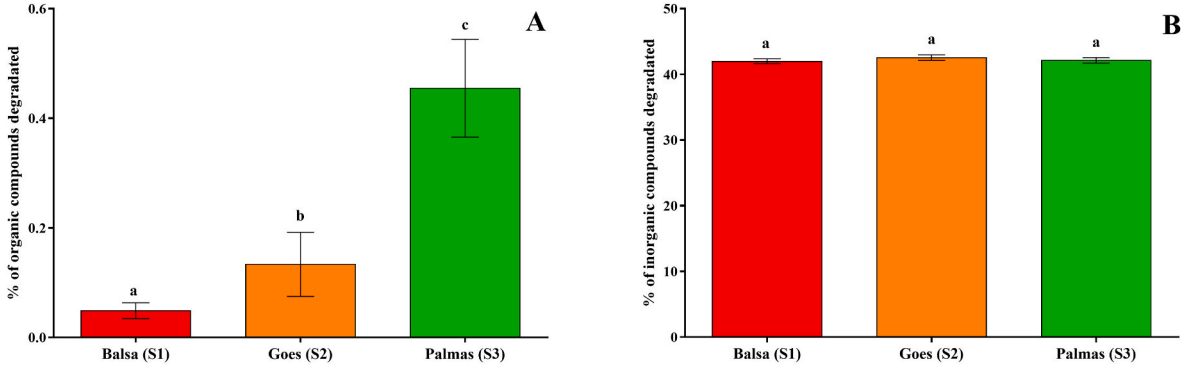

Fig. 6. Mass degradation (mean ± SD, %) of organic (A) and inorganic (B) compounds during thermogravimetric analysis in shells of *Stramonita brasiliensis* collected along a contamination gradient in the Santos Estuarine System. Letters on the error bars denote statistical differences Letters on the error bars denote statistical differences (Kruskal-Wallis, $p < 0.01$).

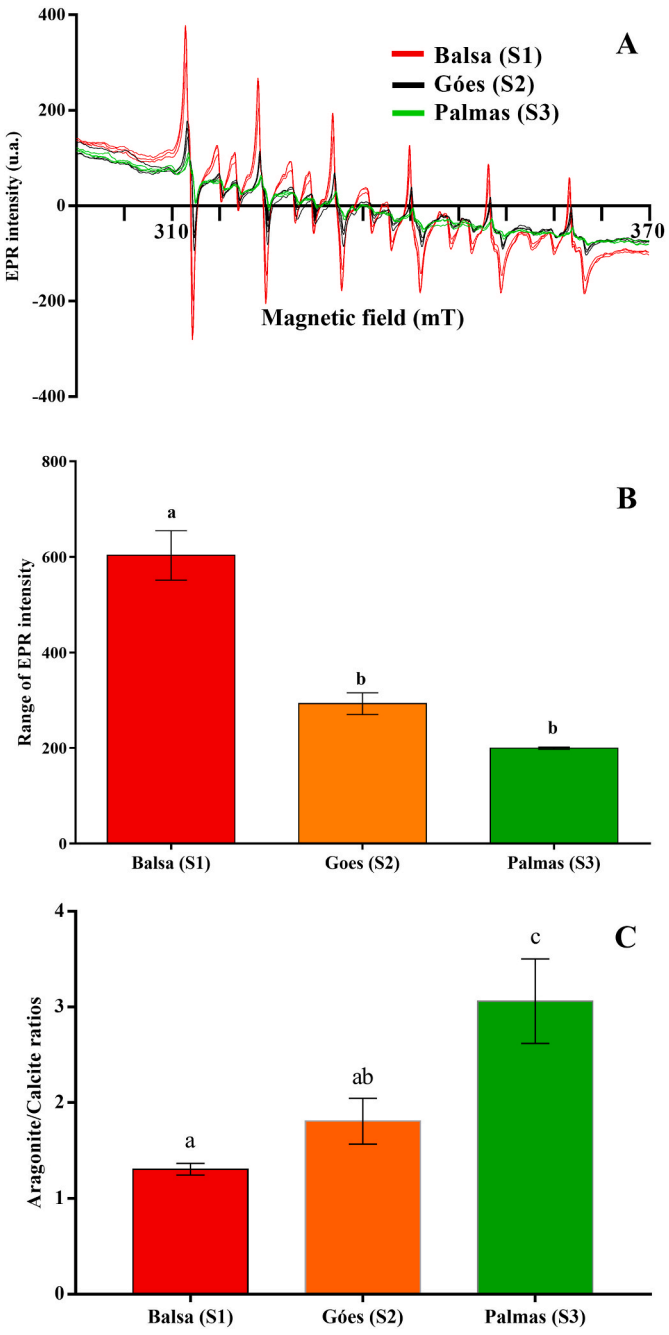

Fig. 7. Electron paramagnetic resonance (EPR) spectra (A), EPR intensities (B) and Aragonite/Calcite ratios (C) obtained for shells of *Stramonita brasiliensis* collected along a contamination gradient in the Santos Estuarine System. Letters on the error bars denote statistical differences (Kruskal-Wallis test, $p < 0.01$).

from each sampled site (Fig. 7B). Such results suggested a predominance of calcite polymorphs in shells from polluted areas (S1), which was confirmed by Rietveld refinement over XRD analyzes assessing the ratios between two crystalline phases (Fig. 7C). In fact, aragonite-calcite ratios significatively ($p < 0.01$) increased from S1 (1.3 ± 0.1) to S3 (3.1 ± 0.4) confirming similar pattern reported by Siriprom et al. (2011). In addition, aragonite-calcite ratios were significantly correlated to % of organic matter fraction (Spearman, $p < 0.01$ and $r = 0.99$) compression resistance (Spearman, $p < 0.01$ and $r = 0.99$) observed in shell of *S. brasiliensis*.

Calcite is mechanically weaker and has lower density and elasticity than aragonite composites (Barclay et al., 2020; Zuschin et al., 2003). Therefore, the organisms inhabiting contaminated zones and presenting lower aragonite-calcite ratios are probably more vulnerable to crushing due to predators and local hydrodynamics. Such findings corroborate results obtained in compression resistance analysis which also pointed to higher shell vulnerability of organisms from S1 (see Fig. 4). Similar pattern was reported to limpets (*Lottia subrugosa*) collected in the same studied area by Oliveira et al., (2020) indicating that environmental conditions influence mollusc shell structure. Moreover, it has been postulated that shell morphology is also dependent of crystalline structure (Siriprom et al., 2011) suggesting that unbalances on aragonite-calcite ratios may be related to morphometric variations observed in *S. brasiliensis* by the present study.

Most of studies assessing aragonite-calcite ratios in mollusc shells have related the observed results to ocean and/or coastal acidification, besides temperature and salinity variations. It is predicted that undersaturation of aragonite in shallow waters may affect organisms with aragonitic shells such as benthic molluscs and pteropods (Figuerola et al., 2021). However, in the present study the organisms were collected within zones with no spatial variations of pH and temperature (Berbel et al., 2021). Therefore, aragonite-calcite ratios observed in shells of *S. braziliensis,* along the contamination gradient, cannot be attributed to coastal acidification. In this regard, sewage pollution, confirmed by measured levels of LABs and other contaminants in SES (see Fig. 3), was suggested to influence the shells mineralogy of *Mytilus edulis* living in the Tay estuary (Hubbard et al., 1981). Therefore, our results support the hypothesis that aragonite/calcite unbalances in mollusc shells can also be induced by exposure to hazardous substances. Although further, studies on this topic are needed, our findings suggested these core set of shell alterations as putative biomarkers of chemical contamination in coastal zones.

13. Conclusion

Variations in morphometry, resistance and in composition of both, organic and inorganic matrices, were verified in shells of *S. brasiliensis* along a contamination gradient in SES. This set of findings were consistent with local contamination levels which may have induced the detected changes in the studied populations. In addition, alterations in calcium carbonate polymorphs, mostly attributed to coastal and oceanic acidification, seem to be also affected by chemical pollution. Thus, changes in mollusc shells showed good performance as potential biomarkers of coastal contamination, being probably observed in other species of carnivorous gastropods around the world. Additional proteomic analysis and experimental studies under controlled conditions should be performed seeking to better address shell alterations as biomarkers of costal contamination.

Author statement

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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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2022.135926.

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