

## Risk assessment of a coastal lacustrine environment using oxidative stress biomarkers present in the digestive gland of the Brazilian clam *Anomalocardia brasiliiana*.

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Research

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### CONFLICTS OF INTEREST

There are no conflicts of interest for any of the authors.

### CITATION

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### ABSTRACT

**Background:** Lacustrine systems are environments of great ecological and economical relevance, however, in the last decades they have been progressively threatened by several contaminants.

**Methods:** Total and fecal coliform concentrations in the environment, several biomarkers of oxidative stress as well as trace metal concentrations were examined in the digestive gland of the clam *Anomalocardia brasiliiana* sampled at four contaminated sites and compared to a reference site in a coastal lacustrine environment localized at the Santa Catarina Island, Southern Brazil. Comparisons of contaminated sites and the reference site were analyzed using one-way ANOVA with a minimal confidence interval of 5% ( $p < 0.05$ ).

**Results:** Compared to the reference site, lipoperoxidation (TBARS levels) in the digestive gland and coliform contents in the water were higher at all contaminated sites. Trace metal concentrations were high and

chromium (2.65-0.55 mg g<sup>-1</sup>) and lead (0.63-0.77 mg g<sup>-1</sup>) concentrations were found to be above the limit values allowed by international legislation (FDA). Antioxidant enzymes such as superoxide dismutase, catalase, glutathione reductase, glutathione peroxidase and glutathione S-transferase showed generally increased activity, while levels of reduced glutathione (GSH) showed decreased contents in clams sampled at the contaminated sites.

**Conclusions:** The results indicate that *A. brasiliiana* is facing a severe oxidative stress at the contaminated sites, which seems to be related to different contaminants but especially to fecal coliforms found in the water and to trace metals detected in the digestive gland. The clam *Anomalocardia brasiliiana* is suitable as a useful bioindicator species for aquatic risk assessments.

**Keywords:** Mollusk; *Anomalocardia brasiliiana*; Oxidative stress; Trace metals; Sewage.

## INTRODUCTION

The increased deterioration of the lacustrine system of the Lagoa da Conceição as well as other aquatic environments in the Santa Catarina Island is being demonstrated by several indicators of water quality in the last three decades [1-3], with some exceptions [4]. As a consequence, it shows increased nautic activity, diminished water quality, declining biodiversity and biomass production, and is contaminated, among others, by municipal sewage discharges and also trace metals mostly derived from intense nautic activity [4, 5]. Araújo [1] detected very high total and coliform contents ( $\geq 200$  -  $\geq 100,000/100\text{ml}$ ) in different aquatic systems of the island especially associated with local mangroves. Beside the intense harvesting of the clam to supply the numerous restaurants of the island, the mean length and weight of this species is being rapidly decreasing in the last years in this lagoon and other parts of the island as well as the coast of the Santa Catarina state [6].

Brazilian and worldwide coastal lagoon systems are environments of great ecological and economical relevance because they sustain the development of early stages of many invertebrate and vertebrate species [7]. Nevertheless, in the last decades they have been progressively threatened by several contaminants, including among others, sewage discharges, which may include trace metals, thereby posing severe environmental concerns, as in the particular case of the present study, the lacustrine environment of Lagoa da Conceição in south Brazil [5, 8]. Lacustrine sediments are important sinks for trace metals, which are released either from different natural or anthropogenic sources [9].

The biological effects of reactive oxygen species (ROS) are generally similar in aerobic organisms and these ROS, beside their important benefits involving cell signaling and development, immune system, among other functions, are capable to damage biologically important molecules [10]. An imbalance in redox reactions involving ROS and antioxidants can lead to a condition called oxidative stress (OS)[11], which typifies the toxicity induced by different xenobiotics present in the aquatic environment, including trace metals [12-15].

Bivalve mollusks are good bioindicators for aquatic pollution associated with augmented ROS generation and changes in their antioxidant defenses [12, 14-22]. Also, many studies on bivalve mollusks revealed positive correlations between different OS biomarkers and the presence of trace metals in the digestive gland [9, 12-17, 19-21, 23-34]. However, the related literature is very limited regarding the exposure of aquatic bioindicators to sewage discharges reflected on OS biomarkers either in mollusks [35] or even in fish [36, 37].

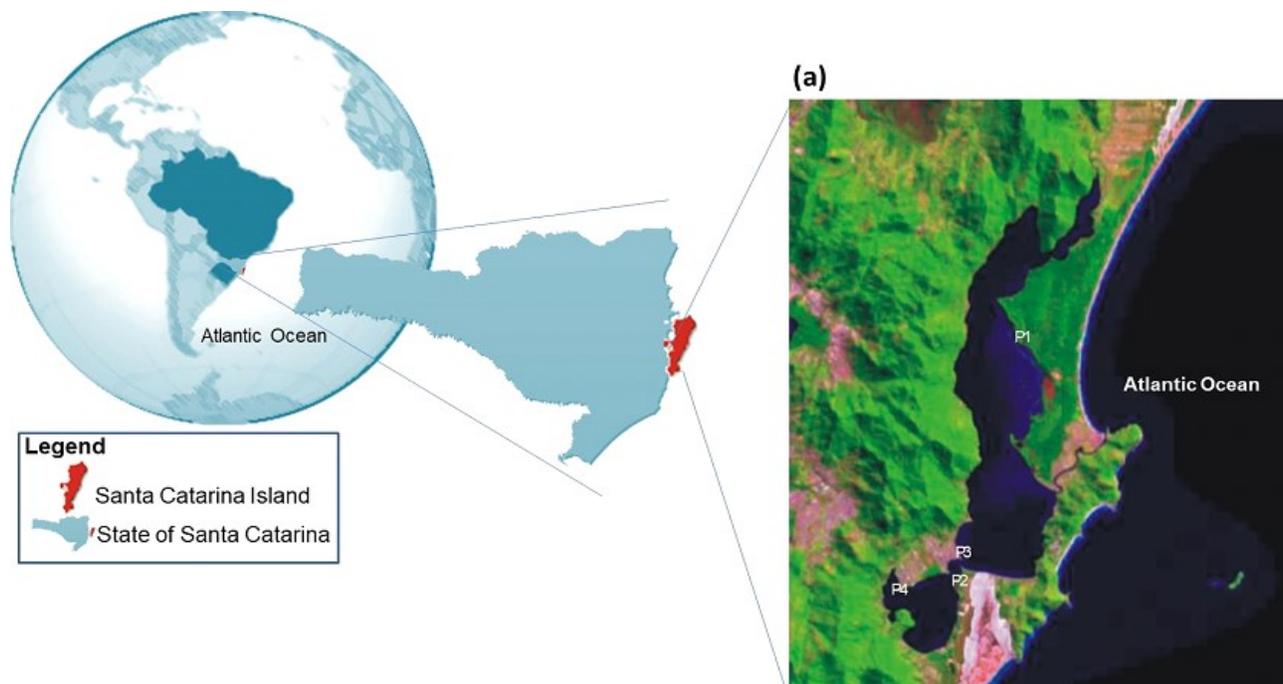
Therefore, the present study was conducted in order to evaluate the environmental risk assessment of contaminants of different anthropogenic sources such as coliform concentrations in the water and trace metals on OS biomarkers present in the digestive gland of the Brazilian clam *Anomalocardia brasiliiana*, sampled at four different contaminated sites, which were compared to a reference site in a coastal lacustrine environment localized at the Santa Catarina Island, Brazil.

## MATERIALS AND METHODS

The study area is a mesohaline lacustrine environment connected to the Atlantic Ocean through a permanent channel of approximately 2 km of extension and around 20 km<sup>2</sup> of surface, called Lagoa da Conceição, a relatively large lacustrine system existent in the Santa Catarina Island, southern Brazil [4](Figure 1).

In the present study five sites were examined: a reference site ("Praia da Daniela", the reference site; 27°27'9.74"S - 48°32'17.73"W), here named Pc, localized near the Ratoles river, inside the "Estação Ecológica de Carijós", a local conservation unity created in 1987, at the north part of the Santa Catarina island. This area is characterized by a very low anthropogenic influence mainly because of its *status* of a protected area (Pereira, 2003). Four contaminated sites were also evaluated at the Lagoa da Conceição, which is localized in the city of Florianópolis, Santa Catarina Island, southern Brazil (27°34'14"- 27°35'31"S; 48°30'07" - 48°31'33"W). The four contaminated sites are the following (Figure 1): site "Rio Vermelho" (27°33'5.65"S - 48°26'51.57"W) here named P<sub>1</sub>, which faces, beside sewage discharges, a strong influence of

acid resins coming from a *Pinus* forest existent near the border of the lagoon. Site P<sub>2</sub> or “Osni Ortega” (27° 36’31.74”S - 48°27’47.32”W), which is localized near a local urban area with intense car traffic. Site P<sub>3</sub> or “Ponta das Almas” (27°36’17.13”S - 48°27’45.42”W), localized at the “Canto dos Araçás”, near an urban area and a recreational beach club. Finally, site P<sub>4</sub> or “LIC” (27°36’44.60”S - 48°28’51.29”W), which is localized near a dense urban area and next to a relatively large recreational beach club that has an intense nautic (mainly



**Figure 1.** Geographical localization of the study at Santa Catarina Island and space photograph from part of the Santa Catarina Island showing the Lagoa da Conceição and the four contaminated sites. P1: Rio Vermelho; P2: Osni Ortega; P3: Ponta das Almas; P4: LIC; Pc: Reference Site (Pontal Daniela) is not shown, which is localized in the north part of the island. The space photograph is available at [www.embrapa.gov.br](http://www.embrapa.gov.br)

### Analysis of physicochemical parameters and coliform contents

Analyses of physicochemical parameters such as water temperature, pH, conductivity, salinity, dissolved oxygen, as well as the analysis of total and fecal coliforms of the water were measured according to standard methods for water and wastewater examination [38]. Water was sampled at 30 cm of depth at the sites where the clams were collected.

### Study model

Fifteen adult individuals of *A. brasiliiana* (Gmelin, 1791) with length shell ranging from 20.5 cm to 31.8 cm, irrespective of sex evaluation were collected at the five sampling sites during the low tide period, in the same day, during early Fall (beginning of April). The 75 specimens were immediately transported to the la-

boratory in containers with water and sediment, which was also collected at the corresponding site. Immediately after arrival and before dissection, the clams were weighed (weight range: 4.51 - 8.13 g) and total length (length range: 22.8 - 34.2 mm) was measured. The digestive glands were excised and the corresponding portions were weighed for preparation of the homogenates and acid extracts. Aliquots of the supernatants were frozen and stored in liquid nitrogen (-170° C) regarding the enzymatic analysis, while those for GSH and TBARS quantifications were analyzed in same day in fresh homogenates to avoid interferences related to frozen samples [39].

### Preparation of tissue homogenates

The digestive glands were carefully excised, surface-dried with filter paper, weighed, washed in ice-cold saline solution and homogenized individually in a PBS

solution (pH 7.4) containing 0.1% Triton X-100, 0.12 M NaCl, 30mM Na-PO<sub>4</sub>, (1:9, wet tissue weight: buffer volume), containing freshly prepared protease inhibitors (0.3 mM PMSF and 0.05 mM trypsin inhibitor). Homogenizations were carried out in broken ice (5°C) using a tissue tearor for approximately 30 s, followed by centrifugation at 10,000 g for 5 min at 5°C. Aliquots of the supernatants were stored in liquid nitrogen and examined separately for each assay of enzymatic activity and TBARS quantification. All the biochemical measurements were carried out spectrophotometrically in a GBC UV/VIS 916 spectrophotometer (Sydney, NSW, Australia).

### Trace metal analysis

The soft parts of the clams were carefully excised, dried at 60°C and then pulverized to a homogeneous particle size. Aliquots of 200 mg from a pool of 15 individuals from each site were submitted to a digestion process with 3 ml of 0.5M HNO<sub>3</sub> at 120°C for 5 h, and 1 ml of 0.1M H<sub>2</sub>O<sub>2</sub> solution at 60°C for 1 h [40]. After digestion, each sample was diluted in 100 ml of water (Millipore) and analyzed by graphite furnace atomic absorption spectrophotometry (Varian Spectra-640Z, with Zeeman background corrector) regarding the concentrations of Pb, Cr, Zn, Ni and Cd. Values were reported as µg.g<sup>-1</sup>.

### Enzymatic activity

Superoxide dismutase activity was measured at 550 nm according to the method of cytochrome *c* reduction promoted by the superoxide anion [41]. In short, the activity was measured in aliquots of supernatants, which were added to a cuvette containing 2.0 ml of phosphate buffer 50 mM pH 7.8, EDTA 0.1 mM, cytochrome *c* 20 mM, xanthine 50 mM, 0.4 U of xanthine oxidase, obtaining a decrease of absorbance at A550, which was monitored during 2 minutes. Values were expressed in USOD g<sup>-1</sup> of tissue, considering that one arbitrary unit of SOD corresponds to the amount of sample able to inhibit 50% of the rate of cytochrome *c* reduction formation in the cuvette by superoxide anion. Catalase activity was evaluated by measuring the decrease in hydrogen peroxide concentration at 240nm [42] promoted by the enzyme present in the

sample. Decays in A<sub>240</sub> were registered during the first minute, in a cuvette containing 50 mM Na-phosphate, pH 7.0, and a freshly prepared 10 mM hydrogen peroxide solution. Hydrogen peroxide stock solution was previously titrated to ascertain the concentration. Values were expressed in mmol min<sup>-1</sup> g<sup>-1</sup> of tissue. Glutathione peroxidase (GPx) was measured at 340 nm through the glutathione/NADPH/glutathione reductase system, by the dismutation of *tert*-butylhydroperoxide [43], and expressed in µmol min<sup>-1</sup> g<sup>-1</sup>. The activity of glutathione reductase (GR) was measured at 340 nm through the oxidation rate of NADPH, which is proportional to the GSSG formation, in a reaction medium containing 0.1M NaPO<sub>4</sub> buffer, pH 7.0, 0.1% DPTA and 1mM GSSG [44], and expressed in µmol min<sup>-1</sup> g<sup>-1</sup>. The enzyme glutathione S-transferase (GST) was measured at 340 nm according to Habig et al. [45], using CDNB (1-chloro-2,4-dinitrobenzene) as substrate. In a cuvette containing 10 ml of CDNB 0.1 M, 10 ml of GR 0.1 M (GSH) and 970 ml of phosphate buffer 0.15 M, pH 7.0, while in the reference cuvette a medium containing 980 ml of the same phosphate buffer was used. The activity values were also expressed in µmol min<sup>-1</sup> g<sup>-1</sup>.

### Lipoperoxidation levels (TBARS)

Determination of thiobarbituric acid-reactive substances (TBARS) was used to assay endogenous lipid oxidation according to Ohkawa [46] and Bird and Draper [47], in fresh homogenates. Frozen samples were not used because even when reacted with BHT they showed further enhanced lipid autoxidation, and therefore, overestimation of TBARS levels [48]. Fresh homogenates were added to 0.2mM butylhydroxytoluene (BHT) to avoid further lipid oxidation. Tissue acid extracts were obtained by the addition of the homogenate to 12% trichloroacetic acid (1:4 v/v), followed by centrifugation. Supernatants were centrifuged at 5,000g for 5 min at 5°C, added to 0.67% (w/v) 2-thiobarbituric acid, maintained in boiling water for 60 min, cooled at 5°C for 30 min, and then measured spectrophotometrically at 535 nm. Absorbances were expressed as nmol TBARS g tissue<sup>-1</sup> ( $\epsilon_{535} = 153 \text{ mM}^{-1} \text{ cm}^{-1}$ ).

### Glutathione assay

Reduced glutathione (GSH) was measured according to Anderson [49] using the Elmann's reagent (DTNB). Tissue acid extracts were obtained by the addition of 12% trichloroacetic acid (1:4 v/v), followed by centrifugation at 5,000 g for 3 min at 5°C. Supernatants from the acid extracts were added to 0.25 mM DTNB in 0.1M Na-PO<sub>4</sub>, pH 8.0, and the formation of thiolate anion was determined at 412 nm during 3 minutes. Total glutathione (TG) was also measured at 412 nm according to the method of Tietze [50], and oxidized glutathione (GSSG) was calculated in equivalents of GSH (2 GSH = 1 GSSG). Values were expressed in mmol g<sup>-1</sup> of tissue using  $\epsilon = 14,1 \text{ mM}^{-1} \text{ cm}^{-1}$ .

### Chemicals

All reagents were purchased from Sigma Chemical Co. (Ohio, USA), with the exception of the standard working solutions for trace metal analysis, which were prepared from Merk Titrisol solution (Germany).

### Statistics

All experimental data are represented as mean  $\pm$  S.E.M. (n=15 per site). For testing the normality the following tests were carried out: D'Agostino & Pearson omnibus normality test and Shapiro-Wilk normality test. Statistically significant differences from the reference site were determined by one-way ANOVA and *post hoc* tests with multiple comparisons (Tukey-Kramer) posttest using GraphPad InStat version 3.01 and GraphPad Prism version 5.0, GraphPad software Inc, (La Jolla, California, USA). Differences were considered to be significant when  $p < 0.05$ .

## RESULTS

### Physicochemical parameters analyses

The physicochemical analyses revealed relatively small differences among the different sites examined (Table 1). Nevertheless, a relatively low value for dissolved oxygen was detected at site P<sub>4</sub>, which was approximately half (4.5 mg g<sup>-1</sup>) the values found in the other sites (6.9-10.7 mg g<sup>-1</sup>) (Table 1).

**Table 1.** Physicochemical parameters and total and fecal coliform concentrations in water collected at different sites of the Lagoa da Conceição. P<sub>1</sub>: Rio Vermelho; P<sub>2</sub>: Osni Ortiga; P<sub>3</sub>: Ponta das Almas; P<sub>4</sub>: LIC; P<sub>C</sub>: Reference Site (Pontal Daniela).

Parameter	P <sub>1</sub>	P <sub>2</sub>	P <sub>3</sub>	P <sub>4</sub>	P <sub>C</sub>	Unit
Conductivity	36.9	31.1	39.8	29.2	49.2	mS/Cm
Color	20.0	15.0	15.0	10.0	30.0	u.C.
BOD	12.0	9.0	10.2	9.6	5.4	mg/L
QOD	515.0	360.0	750.0	570.0	420.0	mg/L
Dissolved O <sub>2</sub>	10.7	9.3	7.2	4.5	6.9	mg/L
pH	8.5	8.3	8.27	7.87	8.30	Unity
Salinity	23.4	19.2	25.1	18.0	31.08	‰
STD	21.60	18.60	24.30	16.90	29.53	mg/L
Temperature	21.7	24.0	19.1	18.4	17.7	°C
Turbidity	6.0	2.4	6.0	1.2	14.6	NTU

Sites P<sub>1</sub>: Rio Vermelho; P<sub>2</sub>: Osni Ortiga; P<sub>3</sub>: Ponta das Almas; P<sub>4</sub>: Praia do LIC; P<sub>C</sub>: Pontal da Daniela (Reference Site).

### Coliforms, trace metals and *Pinus* resins

High total ( $\geq 1732.9/100\text{ml}$ ) and fecal coliform contents (1732.9 – 111.9/100ml) were found in all contaminated sites examined compared to the reference site (81.6 and 18.3 total and fecal coliforms/100ml, respectively; Table 1). Site P<sub>2</sub> also showed high TBARS contents together with high coliform contents and low trace metal contents in the digestive gland of *A. brasiliiana* (Table 1; Figure 1). Similarly, site P<sub>4</sub> showed the lowest coliform and TBARS contents together with a relatively low trace metal content (Tables 1,2).

Trace metal concentrations in the digestive gland of *A. brasiliiana* showed values according to the following decreasing sequence: P<sub>3</sub>>P<sub>1</sub>>P<sub>2</sub>>P<sub>C</sub>>P<sub>4</sub> (Table 2). Ni showed the highest and Cd the lowest concentrations in the digestive gland of *A. brasiliiana* (Table 2). However, these concentrations were relatively high when calculated in a wet weight basis. Pb showed 0.02; 0.09; 0.63; 0.77; 0.16 mg g<sup>-1</sup> and Cd showed 0.04; 0.09; 0.10; 0.12; 0.10 mg g<sup>-1</sup> for sites P<sub>C</sub>, P<sub>1</sub>, P<sub>2</sub>, P<sub>3</sub>, and P<sub>4</sub> (reference site), respectively (Table 2).

**Table 2 .** Concentrations of trace elements (Pb, Cr, Zn, Ni and Cd) in the digestive gland of *Anomalocardia brasiliiana* (n=15) sampled at different sites of the Lagoa da Conceição. Site P<sub>1</sub>: Rio Vermelho; Site P<sub>2</sub>: Osni Ortiga; P<sub>3</sub>: Site Ponta das Almas; Site P<sub>4</sub>: LIC; Site P<sub>C</sub>: Reference site (Pontal Daniela).

	Trace metal (mg.g <sup>-1</sup> )					Coliforms (N 100 ml <sup>-1</sup> )
	Pb	Cr	Zn	Ni	Cd	
P <sub>1</sub> RIO VERMELHO	0.02	1.28	0.20	0.77	0.04	$\geq 2.419$
P <sub>2</sub> OSNI ORTIGA	0.09	0.68	0.38	0.44	0.09	1.733
P <sub>3</sub> PONTA ALMAS	0.62	2.65	8.21	0.80	0.10	961
P <sub>4</sub> PRAIA DO LIC	0.77	0.55	9.36	0.41	0.10	112
P <sub>C</sub> PONTAL DANIELA	0.16	0.67	10.09	0.50	0.10	18.3

Site P<sub>1</sub>: Rio Vermelho; Site P<sub>2</sub>: Osni Ortiga; P<sub>3</sub>: Site Ponta das Almas; Site P<sub>4</sub>: LIC; Site P<sub>C</sub>: Reference site (Pontal Daniela).

### Biomarkers of oxidative stress

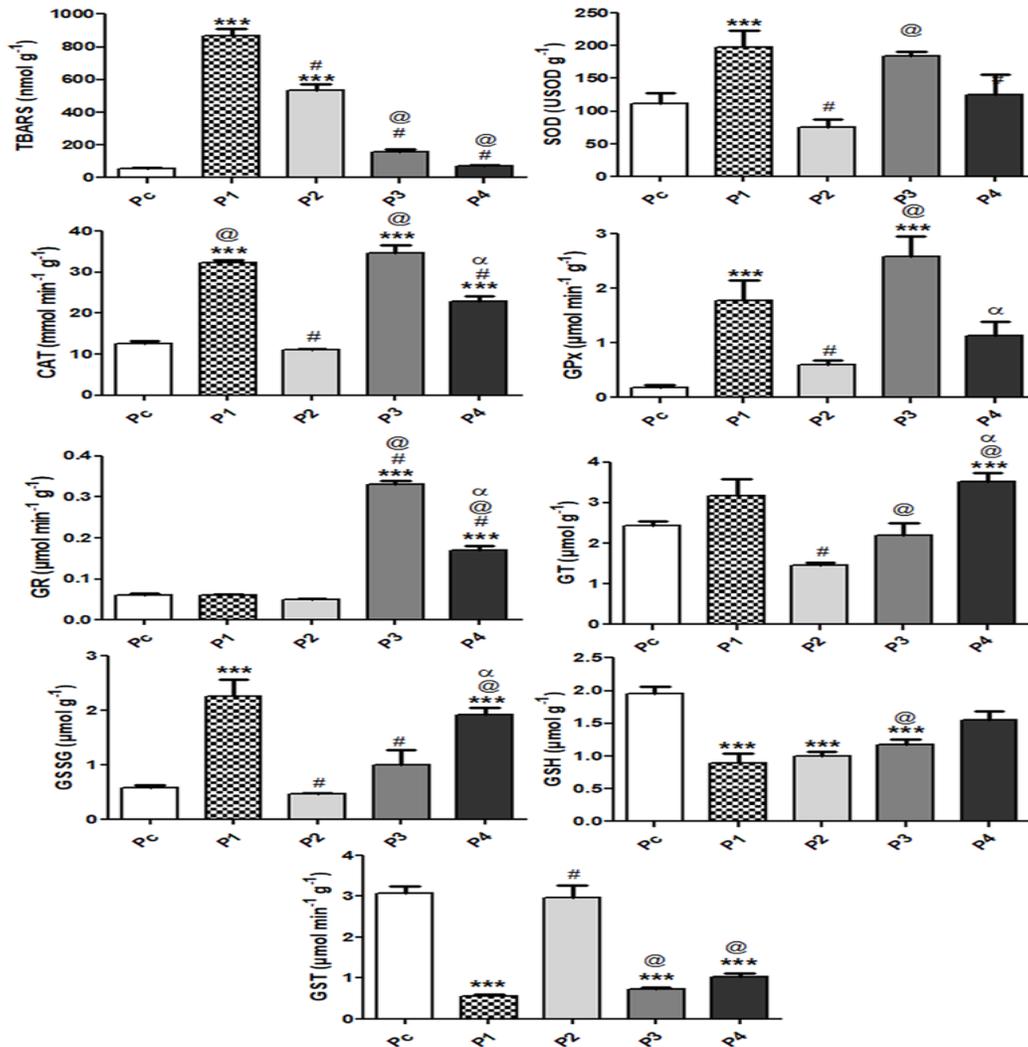
Figure 2 shows the OS biomarkers in the digestive gland of *A. brasiliiana* samples from different sites of the Lagoa da Conceição (content of TBARS, enzymatic activity of SOD, CAT, GPx, GR and GST; as well as TG, GSH and GSSG levels). The levels of TBARS found in the digestive organ of *A. brasiliiana* collected in the reference site (P<sub>C</sub>) and in the contaminated sites P<sub>1</sub>, P<sub>2</sub>, P<sub>3</sub> and P<sub>4</sub> were: 57.65±5.98; 870.6±39.1; 534.5±40.3; 160.8±12.7 and 72.16±7.31 nmol TBARS g<sup>-1</sup>, respectively. Excepting for sites P<sub>3</sub> and P<sub>4</sub>, high TBARS contents were found in the digestive organ of *A. brasiliiana* collected in the other contaminated sites P<sub>2</sub> and P<sub>3</sub> compared to the reference site P<sub>C</sub> (p<0.0001 when compared to P<sub>C</sub>; Figure

2). In addition, the digestive organ of *A. brasiliiana* collected at P<sub>1</sub> presented the higher levels of TBARS when compared to all other contaminated sites (p<0.001), while their levels at P<sub>2</sub> were higher than the TBARS content from samples collected at P<sub>3</sub> and P<sub>4</sub> sites (p<0.001).

The mean GSH concentration found in the digestive gland of *A. brasiliiana* from the reference site P<sub>C</sub> (1.96±0.10 mmol g<sup>-1</sup>) was higher (p<0.0001) than those from the polluted sites (P<sub>1</sub>: 0.90±0.14; P<sub>2</sub>: 1.00±0.07; P<sub>3</sub>: 1.18±0.08; P<sub>4</sub>: 1.55±0.14 mmol g<sup>-1</sup>). Moreover, GSH concentration in samples collected at P<sub>3</sub> was higher and at P<sub>4</sub> was lower than those obtained in samples from P<sub>2</sub> (p<0.0001). As expected, an inverse correlation between GSH and GSSG con-

centrations was evident in the digestive gland sampled in the contaminated sites of the Lagoa da Conceição (Pc:  $0.58 \pm 0.05$ ; P<sub>1</sub>:  $2.26 \pm 0.31$ ; P<sub>2</sub>:  $0.46 \pm 0.02$ ; P<sub>3</sub>:  $1.01 \pm 0.26$ ; P<sub>4</sub>:  $1.96 \pm 0.13$  mmol g<sup>-1</sup>)(Figure 2). The digestive glands of animals sampled at the reference site showed the lowest contents of oxidized glutathione compared to the contaminated sites P<sub>1</sub> and P<sub>4</sub> ( $p < 0.0001$ ), while sites P<sub>2</sub> and P<sub>3</sub> GSSG concentrations similar to those obtained at the reference site. Total glutathione (TG) contents found in clams col-

lected at the reference site ( $2.44 \pm 0.11$  mmol g<sup>-1</sup>) were similar to those found in site P<sub>1</sub> ( $3.16 \pm 0.43$  mmol g<sup>-1</sup>), site P<sub>2</sub> ( $1.46 \pm 0.06$  mmol g<sup>-1</sup>) and site P<sub>3</sub> ( $2.19 \pm 0.32$  mmol g<sup>-1</sup>), while higher contents were found in site P<sub>4</sub> ( $3.51 \pm 0.22$  mmol g<sup>-1</sup>;  $p < 0.0001$ ). On the other hand, GSSG content from digestive glands of animals sampled at P<sub>2</sub> were lower than that observed at P<sub>1</sub> ( $p < 0.001$ ). Significant difference in GSSG content was also observed after comparing data from P<sub>3</sub> and P<sub>4</sub> samples versus P<sub>2</sub> ( $P < 0.001$ )(Figure 2).



**Figure 2.** OS biomarkers in the digestive gland of *Anomalocardia brasiliiana* (n=15) sampled at different sites of the Lagoa da Conceição: contents of TBARS; reduced glutathione (GSH); total glutathione (TG); oxidized glutathione (GSSG); activity of superoxide dismutase (SOD); catalase (CAT); glutathione peroxidase (GPx); glutathione reductase (GR) and glutathione S-transferase (GST). Data are reported as means  $\pm$  SEM. Statistically significant differences, as determined by one-way ANOVA followed by Tukey-Kramer multiple comparison test are indicated: \*\*\* $P < 0.0001$  compared with Pc (reference site); # $P < 0.001$  compared with P<sub>1</sub>;  $\alpha P < 0.001$  compared with P<sub>2</sub>; a $P < 0.001$  compared with P<sub>3</sub>. Sites P<sub>1</sub>: Rio Vermelho; P<sub>2</sub>: Osni Ortiga; P<sub>3</sub>: Ponta das Almas; P<sub>4</sub>: Praia do LIC; P<sub>C</sub>: Pontal da Daniela (Reference Site).

SOD activity showed higher values at the contaminated site P<sub>1</sub> (198.2±25.5 U SOD g<sup>-1</sup>) when compared to the reference site (111.6±6.2 U SOD g<sup>-1</sup>). The activity of the enzyme in the samples collected at site P<sub>2</sub> (74.9±13.0 U SOD g<sup>-1</sup>) was similar to that observed in the digestive organ of *A. brasiliensis* collected at P<sub>c</sub>, but lower than the SOD activity from P<sub>1</sub> samples (p<0.001). Moreover, significant difference in SOD activity was obtained between P<sub>2</sub> and P<sub>3</sub> samples (75.3±13.0 and 184.9±13.0 U SOD g<sup>-1</sup>, respectively; p<0.001)(Figure 2).

Catalase activity showed variable responses at the different contaminated sites when compared to the reference site (12.6±0.7 μmol min<sup>-1</sup> g<sup>-1</sup>). Sites P<sub>1</sub>, P<sub>3</sub> and P<sub>4</sub> showed higher catalase activity than P<sub>c</sub> (32.3±3.6; 64.8±1.8 and 22.9±1.2 μmol min<sup>-1</sup> g<sup>-1</sup>, respectively; p<0.001), while site P<sub>2</sub> showed the lowest activity (11.2±0.2 μmol min<sup>-1</sup> g<sup>-1</sup>; p<0.001) among the contaminated sites, which was not significantly different from the reference site. Although the SOD activity in the digestive organ of *A. brasiliensis* collected at P<sub>4</sub> was higher than that obtained in samples collected at reference site (P<0.0001), it was lower than that observed at P<sub>1</sub> and P<sub>3</sub> (p<0.001)(Figure 2).

Glutathione peroxidase activity at the reference site was much lower (0.19±0.04 μmol min<sup>-1</sup> g<sup>-1</sup>) than that found in the contaminated sites P<sub>1</sub> and P<sub>3</sub> (1.78±0.37; 2.59±0.37 μmol min<sup>-1</sup> g<sup>-1</sup>, p<0.001). Site P<sub>3</sub> showed the highest value (2.59±0.37 μmol min<sup>-1</sup> g<sup>-1</sup>; p<0.0001), almost 14 times higher compared to the reference site, while sites P<sub>1</sub> and P<sub>4</sub> showed roughly ten and six fold higher GPx activity compared to the reference site (1.78±0.04 and 1.13±0.26 μmol min<sup>-1</sup> g<sup>-1</sup>, respectively) (Figure 2). Site P<sub>2</sub> showed the lowest value (0.60±0.08 μmol min<sup>-1</sup> g<sup>-1</sup>) among the contaminated sites, even so the value was *ca.* 3 times higher compared to the reference site. Glutathione reductase activity found at sites P<sub>1</sub> and P<sub>2</sub> (0.06±0.01 μmol min<sup>-1</sup> g<sup>-1</sup> and 0.04±0.01 μmol min<sup>-1</sup> g<sup>-1</sup>, respectively) were similar to that of the reference site (0.06±0.01 μmol min<sup>-1</sup> g<sup>-1</sup>; Figure 2). On the other hand, sites P<sub>3</sub> and P<sub>4</sub> showed higher GR activity (0.32±0.01 μmol min<sup>-1</sup> g<sup>-1</sup> and 0.16±0.01 μmol min<sup>-1</sup> g<sup>-1</sup>) compared to the reference site (p<0.0001) and to P<sub>1</sub> and P<sub>2</sub> sites (p<0.001) (Figure 2).

The enzymatic activity of GST found in the digestive organ of *A. brasiliensis* collected in the reference site and in the contaminated sites P<sub>1</sub>, P<sub>2</sub>, P<sub>3</sub> and P<sub>4</sub> were respectively: 3.09±0.17; 0.56±0.04; 2.97±0.30; 0.73±0.050 and 1.03±0.08 μmol min<sup>-1</sup> g<sup>-1</sup>. The GST activity observed in samples collected at reference site and at site P<sub>2</sub> presented very similar values. The activity of this enzyme in the digestive organ of *A. brasiliensis* collected at P<sub>1</sub>, P<sub>3</sub> and P<sub>4</sub> contaminated sites was lower than those showed at P<sub>c</sub> or P<sub>2</sub> sites (p<0.0001; p<0.001, respectively)(Figure 2).

## DISCUSSION

### Physicochemical parameters

Very high contents of total and fecal coliforms were detected in three contaminated sites, especially in P<sub>1</sub> and P<sub>2</sub>, and also in P<sub>3</sub>, when compared to the reference site P<sub>c</sub>. Sites P<sub>1</sub> and P<sub>2</sub> revealed values that were well above the limit allowed by the local environmental agency (FATMA) and Brazilian legislation [51]. These results clearly indicate that there is a strong anthropogenic environmental stress, considering that all the contaminated sites are under an accelerated and often illegal urban occupation, including the use of frequent clandestine (*in natura*) sewage discharges directly to this lacustrine system [2, 52, 53], as well as in other aquatic environments existent in the Santa Catarina island [1].

The clams were collected by purpose after the summer season (middle April of 2002, beginning of Fall) to take into account the seasonal anthropogenic impact on this lacustrine system. Corroborating the results obtained in present study, the time of sampling occurred just after the high tourist influx during high summer (January-February), when such transient population often promotes temporary increases of 3-5 fold of the normal population of the Santa Catarina Island. Accordingly, near the site P<sub>4</sub> is very common to visualize algae bloom associated with a local and predominant bad smell during spring and summer [53]. Such conditions are probably consequence of augmented sewage discharges together with the particularly local low water circulation near site P<sub>4</sub>, with the consequent oxygen deficit, which coincided with the lowest dissolved oxygen concentrations found at this site in the

present study, and also with the high local H<sub>2</sub>S production [53]. In this regard, the evaluation of trace metal contamination in surface sediments of the Guanabara Bay (Brazil) showed the importance of hydrogen sulfide, which are trapped in the bottom thereby maintaining these metals less oxygenated and less soluble for contamination of the benthic fauna [54].

In contrast, P<sub>2</sub> showed the second highest coliform content in the water, thus indicating a contaminated site, but also showed the lowest value for the chemical oxygen demand, thus conversely suggesting a relatively low contaminated site. On the other hand, total and coliform values obtained for the reference site as well as considering the physicochemical parameters obtained, ensure its use as a control site in the present study. Accordingly, the measured biochemical oxygen demand (BOD) was approximately half at the reference site compared to the contaminated sites.

#### **Coliforms, trace metals, *Pinus* resins and lipoperoxidation levels**

High total and fecal coliform contents were detected in all contaminated sites compared to the reference site, indicating a strong anthropogenic influence on these areas, most of them probably related to urban sewage discharges. Site P<sub>2</sub> showed high TBARS contents together with high coliform contents and low QOD in the water, together with low trace metal contents in the digestive organ of *A. brasiliensis*. Coherently, site P<sub>4</sub> showed the lowest coliform and TBARS contents combined with a relatively low trace metal contents. These results suggest that TBARS concentrations in the digestive gland of *A. brasiliensis* could be more associated with the coliform contents in the surrounding water rather than with the trace metal contents *per se*. Accordingly, not all heavy metals induce OS in different organisms, as was found in the digestive gland of *Mytilus galloprovincialis*, showing that only copper was able to enhance lipoperoxidation while cadmium and zinc did not [26].

Likewise, site P<sub>1</sub> was the solely site here examined that was heavily contaminated by several acid resin components such as primaric, adiabatic and dehydroabiatic acids coming from a vicinal forest of *Pinus elliotti*, which drains into the lagoon and are cu-

mulative and highly toxic to aquatic organisms [52]. On the other hand, elevated lipoperoxidation processes were directly associated with trace metal contents in other mussels sampled in polluted areas [26, 31]. Similarly, a high correlation between trace metal and TBARS concentrations was also detected by our research group in the digestive gland of the local mangrove mussel *Mytella guyanensis* [19]. Also similar to the present findings, enhanced OS biomarkers were detected after 1-3 weeks in the freshwater clam *Unio tumidus* transplanted to contaminated sites was well correlated with the worsening of water quality [55],

However, low TBARS contents were found in the mollusks *Chamaelea gallina* and *Cassostrea gigas* sampled in polluted areas of the Spanish coast [56], and the authors suggested that the lowering of such lipoperoxidation could be a compensation of the elevation of the antioxidant defenses. This interpretation coincides with ours, excepting for the relatively low GST activity detected in site P<sub>1</sub>, probably as a consequence of the inhibitory effect produced by the resin contamination derived from *Pinus elliotti*. Accordingly, Adler-Ivanbrook and Breslin [30] also found elevated levels of Cu, Cr and As in the blue mussel *Mytilus edulis* exposed to wood treated with these heavy metals.

Thus, the apparent association between the water contents of fecal coliforms and the different biomarkers of OS found in the digestive gland of the clam including the TBARS contents, seems not to be sufficient to explain the antioxidant responses here obtained. Such antioxidant responses may be attributable to the trace metals found in the digestive gland, probably reflecting the concentrations found in the sediment and water of this lacustrine system [5].

Very similar profiles were found between the trace metal concentrations of the digestive gland of *A. brasiliensis* and those found in the sediments in the same lacustrine system [3, 5], probably due to the bioaccumulation process associated with bivalve filter-feeding [7]. In this regard, many bivalve species can accumulate trace metals in concentrations directly proportional to that of the environment, and the main site of trace metal metabolism and accumulation is the digestive gland [28, 57-59]. In the present study the

digestive gland of *A. brasiliiana* also showed a similar profile, exhibiting roughly 20 to 100 times lower trace metal concentrations than those found in the sediments [3, 5], although still higher when compared to the concentrations found in the surrounding water. As mentioned in the related literature, such heavy metal concentrations vary according to seasonal and other environmental changes such as the intensity of rain-falls [28]. In addition, antioxidants in mollusks also respond to seasonal and ontogenetic variations, irrespective to the presence of pollutants present in the environment [14, 39, 60-62]. Therefore, such natural contributions must be taken into consideration when focusing environmental contamination in monitoring programs.

When the concentrations of Pb, Ni and Cd found in the digestive gland of *A. brasiliiana* were compared to those found in the digestive gland of the mangrove mussel *Mytella guyanensis*, they showed very similar values [4, 19]. Accordingly, other related study on the bivalves *Chione* sp., *C. palmula* and *C. corteziensis* from Mexico [63] revealed concentrations of Cd, Ni, Cr, Zn and Pb close to those found in the digestive gland of *A. brasiliiana* (this study). Most of these concentrations were within the values recommended by FDA for marine animals (0.6 – 0.9 mg.g<sup>-1</sup> for Cd and 0.4-0.6 mg.g<sup>-1</sup> for Pb). However, for Cr concentrations the values found in *A. brasiliiana* sampled at all sites examined were above (range 0.55 – 2.65 mg.g<sup>-1</sup>) those allowed by the FDA (0.3 – 0.4 mg.g<sup>-1</sup>), as well as the Pb concentrations found in the sites P<sub>3</sub> and P<sub>4</sub> (0.62 and 0.77 mg.g<sup>-1</sup>, respectively), which were also above the limit recommended by the FDA.

The concentrations of Cd and Pb found in the digestive gland of *Mytilus galloprovincialis* caged for one month in contaminated sites of Gulf of Patras (Greece) were even higher [64]. Not surprisingly, they found similar trace metal concentration ratios between sediments and digestive gland for Zn and Cu, higher values for Hg (1-3 times), Pb (2-4 times), Cr (15-40 times), while in the present study these ratios for Zn and Pb were much higher (~40 and ~60 times, respectively). Surprisingly, Cd concentrations in the digestive organ of *M. galloprovincialis* were even higher than in the contaminated sediment [64], indicating a

high trace metal bioaccumulation in this mussel species. The highest trace metal accumulated by the digestive organ of *A. brasiliiana* was also Cd, but the values were roughly five fold lower than those found in the surrounding sediment [3, 5]. All these results reinforce that bivalve mollusks have a bioaccumulation capability related to their filter-feeding nutrition [21], which are directly proportional to levels existent in the environment [14, 28, 57-59].

### Biomarkers of oxidative stress

Excepting in site P<sub>2</sub>, the SOD activity showed higher values in clams sampled at the contaminated sites. Similar results were obtained in *Mytilus* sp [65], in the oyster *Saccostrea cucullata* [66], and in the clam *Tapes philippinarum* exposed to aquatic contaminants [67]. Conversely, *Mytella guyanensis* did not show differences in the SOD activity for contaminated sites compared to a reference site [19], and similar finding was also obtained for *Mytilus edulis* [68] and *Perna viridis* [31]. At site P<sub>2</sub> SOD activity, as well as the other antioxidant enzymes examined (see below) was not different from the reference site. However, this site revealed the second highest coliform content in the water and the lowest value for the chemical oxygen demand (360 mg/L). While the former value corroborates the category of a contaminated site, the latter value suggests a relatively low contamination. Thus, considering the site P<sub>2</sub> and excepting for the relatively high fecal coliform contents found in the water, all the antioxidant enzymes here evaluated were downregulated, in accordance to the apparent low levels of environmental contamination of this site compared to the other contaminated sites.

Similarly, catalase activity showed generally significant higher activity in clams sampled at all the contaminated sites compared to the reference site, again with the exception of site P<sub>2</sub>, which showed a similar value of that found at the reference site. High CAT activity was also found by our research group in the digestive gland of the mangrove mussel *Mytella guyanensis*, also comparing contaminated sites with a reference site [19]. Similar responses were also obtained in *Saccostrea cucullata* [66], in *Mytilus* sp [65] and in *Crassostrea rhizophora* and *Perna perna* ex-

posed to pollutants [69], while Cheung and collaborators [31] in *Perna viridis* and Matozzo and collaborators [67] in *Tapes philippinarum* did not detect such correlation.

In parallel to SOD and CAT activity, glutathione peroxidase activity in clams sampled at the reference site were much lower than those found at the contaminated sites, sites P<sub>1</sub> and P<sub>4</sub> showing GPx activity roughly ten fold higher than that of the reference site. This similar enzymatic profile is not surprisingly, because both antioxidant enzymes possess a very close functional convergence in vertebrates and invertebrates [70]. Accordingly, enhanced GPx activity was also found in other mollusk species exposed to aquatic contaminants [19, 31, 68]. However, the present results are not in agreement with authors that do not consider GPx as a good OS biomarker in mussels [71], contrasting with others who also have recommended GPx as an excellent biomarker of environmental contamination in the pearl oyster *Pinctada martensii* [72].

As occurred in the other antioxidant enzymes, excepting for the clams collected at site P<sub>2</sub>, glutathione reductase activity showed higher activity compared to those collected at the reference site. The main role of GR is to maintain constitutive high cellular GSH levels, which are important to cell homeostasis and to avoid OS derived from xenobiotic exposure [73]. Therefore, elevated GR activity suggests a compensatory response to recover the depletion of GSH [10]. *Mytella guyanensis* sampled in contaminated sites also revealed elevated GR activity [19], while Cheung and collaborators [31] did not find differences in GR activity in *Perna viridis* collected in polluted sites from Hong Kong.

GST activity showed the highest value at the reference site, while the digestive gland from clams collected at the different contaminated sites showed *a priori* an unexpected lower activity, again with the exception of clams sampled at site P<sub>2</sub>, which showed a mean value similar to that of the reference site. Mussels acutely exposed to contaminated areas usually show enhanced GST activity and/or expression [19, 31, 74]. However, unchanged GST activity was found in *Unio tumidus* [75], and in *Perna perna* and

*Crassostrea gigas* [69] exposed to different pollutants. The apparent inhibition of GST activity detected in all the contaminated sites combined with the upregulation of all other antioxidant enzymes probably indicates that this clam is facing a severe and chronic oxidative insult at all the contaminated sites, excepting at P<sub>2</sub> and at the reference site. Similar results on GST inhibition were already described in fish [48, 61, 76].

An inverse correlation between GSH and GSSG concentrations was evident in the digestive gland of *A. brasiliiana* sampled at the contaminated sites of the Lagoa da Conceição. The mean GSH concentrations found in the digestive gland of *A. brasiliiana* sampled at all the four contaminated sites were lower compared to the reference site, indicating that GSH is being depleted by such contaminants. Low GSH contents were also found in other mussel species exposed to contaminated areas [31], and in a mussel exposed to metal contaminants, which was inversely correlated with the lipid peroxidation values [24]. Likewise, the digestive gland of clams sampled at the reference site showed the lowest contents of oxidized glutathione (GSSG) compared to the contaminated sites, again excepting site P<sub>2</sub>, which exhibited a value very similar to that of the reference site. High GSSG contents were also found by our research group in the digestive organ of *M. guyanensis* sampled in two polluted mangroves compared to a reference site [19]. Despite the relatively high TBARS and coliform contents found respectively in the digestive gland and in the surrounding water, and according to the responses obtained in all antioxidant enzymes, site P<sub>2</sub> coherently revealed lower GSSG contents, suggesting that these clams were less affected by local contaminants compared to the other sites.

Total glutathione contents found in the digestive gland of *A. brasiliiana* collected at the reference site were similar to those found in site P<sub>3</sub>, while higher contents were found in sites P<sub>1</sub> and P<sub>4</sub>, and again a different profile was obtained for site P<sub>2</sub>, exhibiting lower TG values. The relatively high total glutathione contents found in the two contaminated sites may reflect the necessity of *A. brasiliiana* to further synthesize this important antioxidant tripeptide to compensate the OS associated with local contamination. In accordance with such inference, the lower apparent investment in glutathione in clams collected at site P<sub>2</sub>

was well in line with the results obtained for the other OS biomarkers, which also showed minor changes related to this site when compared to the other contaminated sites.

In summary, the trace metal concentrations found in the digestive gland of *A. brasiliiana* collected in contaminated sites showed a very similar profile with the concentrations formerly found in the sediments [3, 5], probably reflecting the bioaccumulation process associated with bivalve filter-feeding foraging process. Some of these metal concentrations (Pb and Cr) were higher than those allowed by the related legislation of different countries (Brazil, USA and Spain). Accordingly, the concentrations of total and fecal coliforms were elevated in the water collected at all contaminated sites compared to the reference site, while one site (P<sub>1</sub>) was particularly contaminated by acid resins drained from a *Pinus* forest. Similarly, the biomarkers of oxidative damage such as TBARS and GSSG concentrations were generally higher in the digestive gland of clams collected in contaminated sites, results that are in parallel to the response obtained for most of the antioxidant enzymes, which showed generally higher activity in the contaminated sites compared to the reference site. Also, excepting in one contaminated site (P<sub>2</sub>), which seems to be less affected by the different contaminants here examined, GST activity was lower in clams collected in all contaminated sites. This inhibition of GST together with the concomitant depletion of GSH suggests that *A. brasiliiana* is gradually losing a full compensatory antioxidant compensation in the contaminated sites. However, as mentioned by Regoli and Giuliani [77] caution must be taken when making such inferences with OS biomarkers, because “in monitoring studies (...) antioxidants could represent a snapshot of cell activity at a given time, not an effective endpoint of environmental pollutants”.

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