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Biochemical biomarkers and metals in *Perna perna* mussels from mariculture zones of Santa Catarina, Brazil

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ABSTRACT

The activity of cholinesterase (ChE), glutathione-*S* transferase (GST), glutathione reductase (GR), glucose-6-phosphate dehydrogenase (G6PDH) and catalase (CAT) was evaluated in the gill and digestive glands of the *Perna perna* mussel transplanted to three non-contaminated mariculture zones under the influence of distinct physical–chemical characteristics. Differences among sites for ChE, GST and CAT activities in gill, as well as ChE, GST and G6PDH activity in digestive gland of mussels, were found and possibly related to differences in physicochemical characteristics of the sites and/or biological status of the mussels. Mussels that were transplanted to another, more urbanized site (Ponta do Lessa) with similar physicochemical characteristics to one of the farming sites (Sambaqui), was also chosen to evaluate biomarker responses to pollution. Activities of ChE, GST and GR in the digestive glands and CAT in the gills were higher in the polluted site. GR was the only biomarker to be unaltered in different farming sites, but induced in the pollution site. The trace metal concentrations in the mussels were low and unlikely to cause the changes observed in the biomarker levels. The present study strongly suggests that monitoring programs should compare sites with similar physicochemical characteristics when using a complementary biomarker approach. In addition, the baselines for the biomarkers and metal used in the present study can serve as a reference for the monitoring of these mariculture zones in future monitoring programs employing *P. perna*.

1. Introduction

Coastal zones of the Santa Catarina State in Southern Brazil have shown a significant increase in population density in the last decade, with a concomitant increase in sewage production affecting the environment quality (MMARH, 1996). An important economic activity in this region is mollusk farming, mainly of the brown mussel, *Perna perna*, and the pacific oyster, *Crassostrea gigas* (Poli and Littlepage, 1998). Santa Catarina produces more cultured mollusks than any other region in Brazil; therefore, the monitoring of the marine environmental quality is of primordial importance for establishing the most adequate areas for bivalve farming.

Mussels are filter-feeding bio-accumulators that have been used as sentinel organisms in numerous monitoring programs (NOAA, 1995). The analyses of biomarkers in these bivalves have been also incorporated into biomonitoring studies to evaluate the effects of pollutants (Viarengo et al., 2000) in areas contaminated with heavy metals (Najimi et al., 1997; Regoli and Principato, 1995), domestic sewage (Radenac et al., 1998), pesticide residues (Narbonne et al., 1991; Mora et al., 1999) and organic compounds (Akcha et al., 2000).

Cholinesterase (ChE) inhibition in sentinel organisms has been widely used as a marker of exposure to organophosphate and carbamate pesticides in biomonitoring programs (Mora et al., 1999; Walker et al., 1996; Monserrat and Bianchini, 1998) and, altered ChE activity have been also attributed to other classes of contaminants (Payne et al., 1996). Antioxidant enzymes, which help to protect cells against oxyradical damage resulting from exposure to certain pollutants, were also involved (Sies, 1991; Rodríguez-Ariza et al., 1992, 1993; Pedrajas et al., 1993;

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López-Barea, 1995; Bainy et al., 1996). Some transition metals and numerous aromatic compounds can generate reactive oxygen species (ROS) through redox cycling mechanisms (Garcia-Alfonso et al., 1995, 1996). Poorly coupled metabolism of polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) can also result in ROS (Bainy et al., 1996). Glutathione-*S* transferase (GST) conjugates different electrophilic compounds with tripeptide glutathione during phase II of biotransformation reactions, enhancing the polarity of these compounds in order to enable their excretion, and have frequently been used as an indicator of increased phase II reactions in contaminant-exposed animals (Dauterman, 1994; Martínez-Lara et al., 1996; Lenartova et al., 1996; García-Alfonso et al., 1998). The antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), along with ancillary enzymes, glucose-6-phosphate dehydrogenase (G6PDH) and glutathione reductase (GR) and conjugating enzymes, help to protect organisms from oxidative stress induced by exposure to contaminants in the environment (Akcha et al., 2000; Walker et al., 1996; Sies, 1991; Dauterman, 1994).

Despite the enormous array of data available on the levels of contaminants in mollusks around the world, there is little information about contaminants and/or biochemical responses in bivalves on the Brazilian coast. We previously found changes in GST activity in the digestive gland of *P. perna* transplanted to a site contaminated by domestic sewage and associated with a heavy rainfall period (Bainy et al., 2000). After one year of exposure, the levels of DNA damage increased as well (Almeida et al., 2003).

In this study, we evaluated the enzymatic biomarkers ChE, GST, GR, G6PDH and CAT in two important detoxification organs (the gill and the digestive gland) of *P. perna mussels* transplanted to three farming sites with different physicochemical characteristics. Mussels were also transplanted to a fourth site located in the same section of the bay as one of these mariculture sites, with similar physicochemical characteristics, but contaminated by urban sewage discharge. The first goal of this study was to evaluate which biomarker/organ combination is not erratic in mariculture sites, but instead altered by pollution. The second goal of this study was to show the levels of the metals As, Pb, Cr, Cd, Sn, V, Cu, Hg, Se, Hg, Ni, Zn and Ag in water and mussels kept in these four sites (Curtius et al., 2003). The results generated here can serve as reference for future monitoring studies in this mariculture/urban zone located along the central shore of Santa Catarina, as well as for biomarker studies in other regions of the world.

2. Materials and methods

2.1. Experimental design and sampling

The brown mussels, *P. perna* (30–40 mm), were collected at the Sambaqui Beach mariculture site (SAM), located in the North Bay of Florianópolis, SC, Brazil (27°28′30″S; 48°33′40″W; Fig. 1), close to the Mollusk Marine Laboratory (LMM, CCA, Federal University of Santa Catarina, UFSC). One group of mussels was kept at this farming site in a long line system, while other groups were transplanted to two other economically important farming zones of Santa Catarina State in the South Bay of Florianópolis (Ribeirão da Ilha Beach—RIB), approximately 15 miles away from SAM, and outside the Florianopolis Bays (Pinheira Beach—PIN), approximately 30 miles away from SAM.

A fourth group was transplanted to Ponta do Lessa (PL), a contaminated site which constantly receives untreated domestic sewage discharge from the city of Florianópolis (SC, Brazil). This area is frequently monitored by the *Fundação do Meio Ambiente* (FATMA: The Environmental Foundation), and the contamination is confirmed by much higher fecal coliform levels in the seawater compared to mariculture sites in Santa Catarina (Almeida et al., 2003). This contaminated site possesses physicochemical characteristics similar to SAM, since is located in the same region in the North Bay of Florianópolis, approximately 6 miles away from SAM. Based on this information, PL was considered a contaminated site, and SAM was considered a reference site for biomarker comparison using mussels.

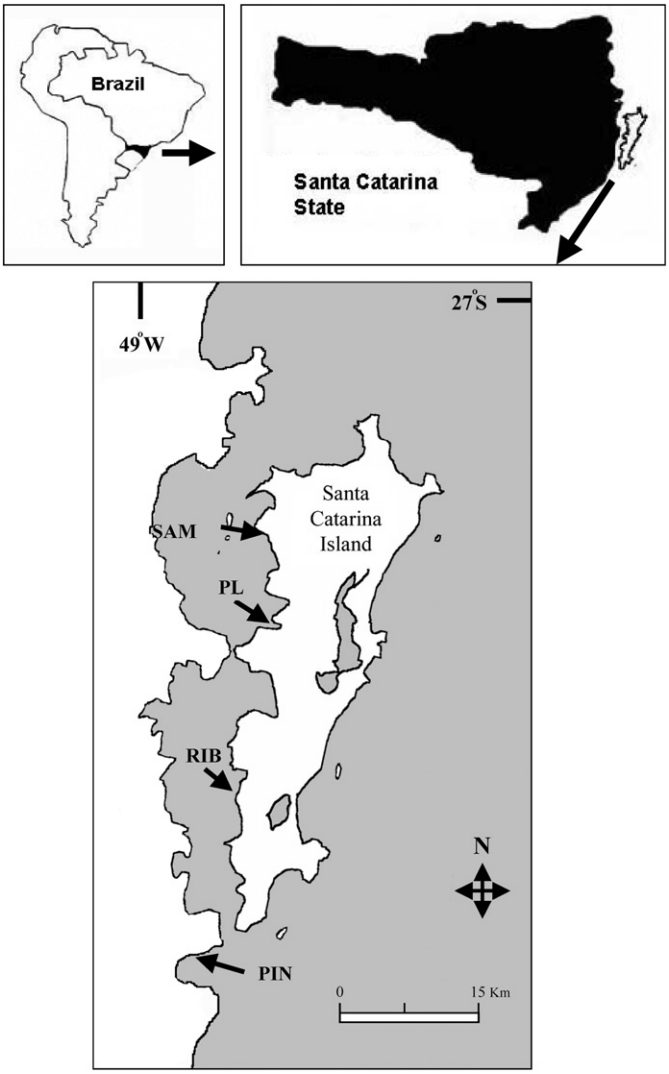

Fig. 1. Map of Santa Catarina Island showing the farming sites Pinheira Beach (PIN), Ribeirão da Ilha Beach (RIB) and Sambaqui Beach (SAM), and the contaminated site, Ponta do Lessa (PL).

After six months in the four sites (November/1999 to April/2000), twenty mussels from each site were collected (size 70–120 mm) in order to measure the biochemical parameters, and thirteen mussels were collected in order to analyze the levels of trace elements. Temperature, pH, turbidity and chlorophyll-*a* levels in the water were determined at the three farming sites (but not in PL) using standard methods (Strickland and Parsons, 1972; Lorenzen, 1967; Littlepage, 1998).

2.2. Biological parameters of mussels

After collection, shell length, soft tissue weight and gonad maturation stages (GMS) were recorded for each mussel. Mussels of both sexes were sampled in similar proportion for each site (50% male and 50% female). GMS was identified macroscopically following Lunetta (1969), and were found to be at IIIA for the full ripe or spawning gonads, IIIB for completely regressed gonads and IIIC for partially ripe gonads at the early gametogenic stage. The gill and digestive glands were excised from the mussels, immediately frozen in liquid nitrogen, and stored at −85 °C for subsequent biochemical analysis.

2.3. Homogenization and centrifugation of the samples

Mussel organs were homogenized (1:4 w/v) in a 20 mM Tris–HCl buffer containing 1 mM EDTA, 0.5 M sucrose, 1 mM DTT, 0.1 M PMSF, 0.15 M KCl pH 7.6, and using a Tissue Tearor (Biospec Prod. Inc.). The homogenate was divided in two parts: 1 ml was centrifuged at 9000*g* for 30 min at 4 °C before measuring the ChE activity in the supernatant (Najimi et al., 1997). The remaining homogenate was

centrifuged at 10,000g for 20 min and the supernatant centrifuged at 38,000g for 70 min at 4 °C, and GST, G6PDH, CAT and GR activities were measured in the cytosolic supernatant.

2.4. Enzyme activity and protein content

ChE activity in the gills and digestive glands were measured following Ellman et al. (1961), and using 0.3 mM acetylthiocholine in the assay. The increase in the rate of absorbance per minute was recorded at 412 nm, 25 °C, using an Ultrospec 3000 spectrophotometer (Pharmacia). The results are expressed in nmol of 5-thio-2-nitro-benzoic acid (NTB) produced per minute per milligram of protein. GST activity was measured following the method of Keen et al. (1976), modified for *P. perna* by Medeiros (2000), and using 2 mM 1-chloro,2,4 dinitrobenzene (CDNB) and 2 mM reduced glutathione (GSH), pH 7.0, which showed to be the best GST assay conditions for these tissues in this species. Absorbance change rate was recorded at 340 nm, 30 °C for 2 min. GST activity is expressed in μmol of 2.4 dinitrophenyl glutathione produced per minute per milligram of protein. CAT activity was quantified following Aebi's method (1984), which measures the H_2O_2 decomposition per minute at 240 nm. The results are expressed in units per milligram of protein. One unit of CAT is defined as the amount of enzyme able to decompose 1 μmol of H_2O_2 per minute. GR activity was evaluated using the protocol of Sies et al. (1979), and measured the NADPH oxidation per minute at 30 °C at 340 nm. The results are expressed in milliunits (mU) of GR per milligram of protein, where one GR unit is the amount of enzyme able to oxidize 1 μmol of NADPH per minute. The activity of G6PDH was calculated following Glock and Mclean (1953), by recording the $NADP^+$ reduction at 340 nm at 30 °C. Activity of G6PDH is expressed in mU of G6PDH per minute per milligram of protein, with one unit of G6PDH equaling the amount of enzyme that reduces 1 μmol of $NADP^+$ per minute. The protein content in the different fractions was evaluated following Peterson's method (1977), using BSA as standard.

2.5. Determination of trace elements in the seawater and in the mussels

Seawater samples were filtered at the time of collection through a 0.45 μm membrane and acidified for the determination of dissolved elements. The samples were analyzed in an inductively coupled plasma mass spectrometer (ICP-MS, Perkin-Elmer SCIEX) using electrothermal vaporization (ETV) for the direct determination of Cr, V, Ni, Mn, Ag and As (Pozebon et al., 1998; Chapple and Byrne, 1996). For the analysis of Pb, Cd, Hg, Se and Cu, the components were separated using the complexation-adsorption method (Dressler et al., 1998), with a flow injection (FI) system coupled to the ICP-MS. The accuracy of the methods was tested by the analysis of certified samples from the National Research Council of Canada.

For the determination of trace elements in mussel tissues, bi-distilled HNO_3 was added to 1.2 g of tissue (w/w) in a polytetrafluorethylene (PTFE) reactor and digested for 3 h at 110 °C. The analysis of total metal and semi-metal content in the mussel tissue solution was performed using ICP-MS (Wu et al., 1997; O'Connor, 1996). The accuracy of the methods was tested using the analysis of a certified oyster tissue sample from the National Institute of Standards and Technology (NIST).

2.6. Statistical analysis

The data distribution was tested with a normal probability plot (NPP) using Statistica for Windows 5.1 (Statsoft). Analysis of variance (ANOVA) was performed on the indices measured, followed by a post-hoc comparison of means using Tukey's honest significant difference (HSD) for equal and different sample sizes ($p<0.05$). A correlation matrix was produced using the biochemical data from each specimen (Pearson's coefficient, $p<0.05$).

3. Results

3.1. Physicochemical characterization of farming sites

During the six months of the experiment, only the site located outside the Florianópolis Bay systems and possessing high water circulation (PIN, Fig. 1) showed higher salinity, lower temperature and lower chlorophyll-*a* contents in the water when compared to RIB and SAM (Table 1). Higher levels of chlorophyll-*a* contents were found in SAM, which was the site with lower depth and the only one among the mariculture sites to possess predominantly muddy sediment and wind blowing in from the north (Table 1). Although there were some differences between the sites, the salinity, temperature and pH were within the appropriate range for *P. perna* cultivation.

Table 1
Physicochemical and other characteristics for the mariculture sites: Pinheira (PIN), Ribeirão (RIB) and Sambaqui (SAM). Chlorophyll *a*, salinity, temperature and pH from the surface water (20 cm depth) were measured along the six months ($n=6$) of experiment. Data are presented as mean ± standard deviation.

	PIN	RIB	SAM
Chlor. *a* (μg/L)	1.4 ± 0.8	3.9 ± 0.5	4.9 ± 2.7
Salinity	35.6 ± 0.6	32.6 ± 2.0	32.7 ± 1.7
T (°C)	21.9 ± 0.6	24.5 ± 2.1	24.6 ± 1.9
pH	8.3 ± 0.3	8.2 ± 0.1	8.2 ± 0.1
Depth (m)[a]	4.0–5.0	3.0–4.0	2.5–3.5
Circulation[a]	High	Intermediated	Intermediated
Bottom[a]	Sand	Sand	Muddy
Influent Wind[a]	South (S)	South (S)	North (N)

[a] Farming site characteristics reported by Ferreira et al. (2006).

3.2. Biological characteristics of the mussels

After six months of being maintained in the four sites, mussels from PIN and SAM showed similar length (10.9 ± 0.5 and 10.8 ± 0.6 cm, respectively) and slightly (but significantly, $p<0.05$) higher values than those for RIB and PL (10.0 ± 0.54 and 7.7 ± 0.7 cm, respectively). In terms of the reproductive stage, mussels from PIN were predominantly IIIA and IIIB (45% and 40% of total, respectively) while RIB and SAM were mainly IIIB (35% and 45% of total, respectively) and IIIC (60% and 40% of total, respectively). All three stages were found in mussels in PL (30% IIIA, 35% IIIB and 35% IIIC of total).

3.3. Biomarkers in the three farming sites

Fig. 2 shows the activity of the different enzymes measured in the gills and digestive gland of mussels collected at the farming sites. The levels of ChE and GST activity for gill and digestive glands differed among sites. These differences were made clear by the ChE activity of the lower gills of the mussels in PIN, compared to RIB and SAM, and the differences in the ChE activity of the digestive glands of mussels among the three sites: RIB > PIN > SAM ($p<0.05$, Fig. 2A). The GST activity in the mussels in SAM was compared to that of the mussels in PIN and RIB. Higher levels were found in the gills and digestive glands of the SAM mussels than in those of PIN, and lower GST activity levels were found in SAM mussels when compared to the mussels from RIB ($p<0.005$, Fig. 2B). G6PDH digestive gland differences in biomarker levels among farming sites were also found through lower levels in SAM (Fig. 2D). Another difference found was that CAT levels in the gills were higher in PIN than they were in RIB (Fig. 2E). The activity of G6PDH in the gills, CAT in the digestive glands and GR in both organs were similar among the three sites ($p>0.05$).

Significant correlations with biomarkers in individual mussels (including mussel from the PL site) were found between the activity of G6PDH and GST in the gills ($r=0.39$, $n=34$) and the digestive glands ($r=0.34$, $n=40$), GST and GR in the gills ($r=0.48$, $n=34$) and the digestive glands ($r=0.79$, $n=38$) and GR and G6PDH in the gills ($r=0.47$, $n=35$) and the digestive glands ($r=0.40$, $n=38$) (data not shown).

3.4. Biomarkers at the contaminated site

Mussels maintained in the urban sewage contaminated site PL showed higher ChE (~150%), GST (~100%) and GR (~50%) activity in the digestive glands, and higher CAT (~50%) activity

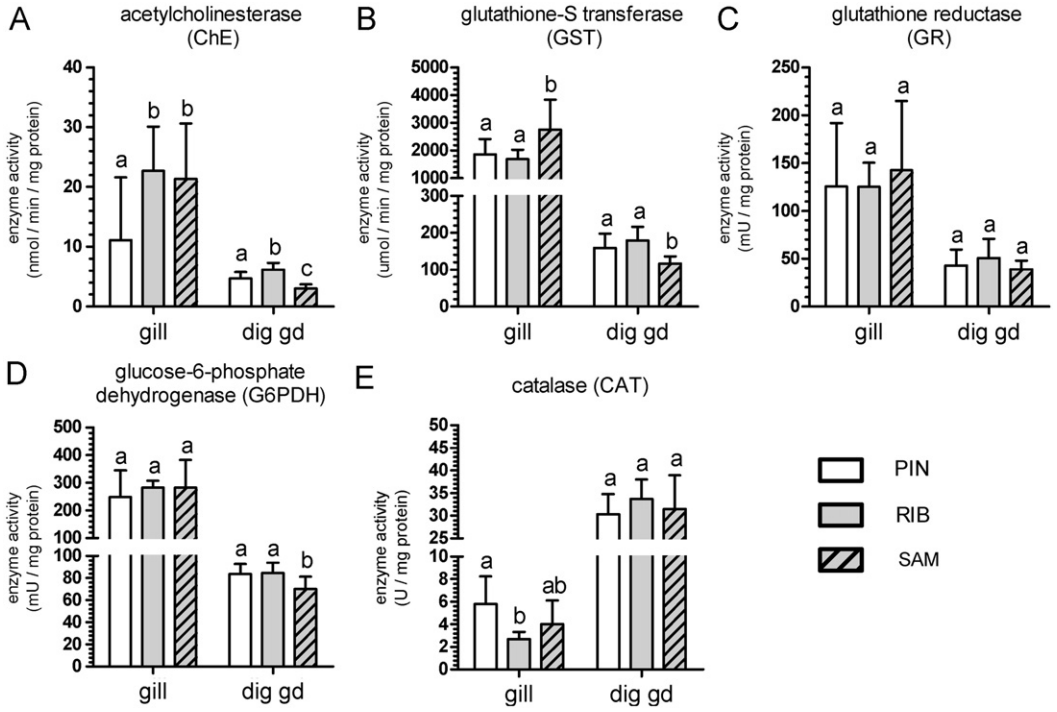

Fig. 2. Acethylcolinesterase (ChE) (A), glutathione-S transferase (GST) (B), glutathione reductase (GR) (C), glucose-6-phosphate dehydrogenase (G6PDH) (D) and catalase (CAT) (E) enzyme activity in the gills and digestive glands (dig gd) of *Perna perna* mussels kept at three farming sites with different physicochemical characteristics in Florianópolis, SC, Brazil for six months. Equal sign letters indicate that there was no difference among sites for a given organ (ANOVA, Tukey HSD, $p < 0.05$).

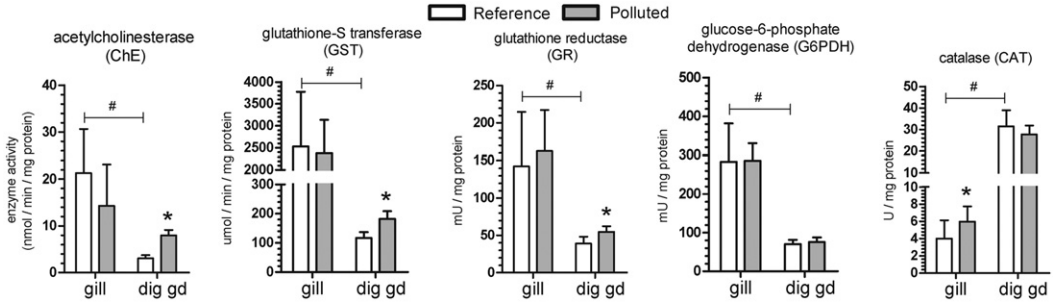

Fig. 3. Acethylcolinesterase (ChE), glutathione-S transferase (GST), glutathione reductase (GR), glucose-6-phosphate dehydrogenase (G6PDH) and catalase (CAT) enzyme activity in the gills and digestive glands (dig gd) of *Perna perna mussels* kept for six months in a reference site (Sambaqui Beach) and a urban sewage contaminated site (Ponta do Lessa), both located in the North Bay of Florianópolis, SC, Brazil. An asterisk (*) indicates a significant difference between sites for a given organ (ANOVA, *T*-student test, $p < 0.05$). A pound sign (#) indicates a significant difference between organs (gills and digestive glands) from the Sambaqui Beach reference site.

when compared to the reference site that was also located in Florianópolis, SC, North Bay SAM ($p < 0.05$, Fig. 3). The G6PDH activity in both organs was similar between polluted and reference sites ($p > 0.05$). When we compared polluted and reference sites, an absence of differences between the sites in terms of activity of ChE, GST and GR in the gills and CAT in the digestive glands coincided with high basal levels in these organs for the enzyme activities (differences between the levels in the gills and digestive glands in mussels from SAM were proven using the *T*-student test, $p < 0.05$, Fig. 3).

3.5. Trace element concentration in the seawater and in the mussels

The levels of dissolved trace elements in water from all sites were below the allowed limits, according to the Brazilian legislation for marine waters (CONAMA, 1986; Dressler et al., 1998) (Table 2). As, Pb, Se and Cd contents were similar at the

Table 2
Level of trace elements in water from three farming sites (Pinheira Beach—PIN; Ribeirão Beach—RIB and Sambaqui Beach—SAM) and one polluted site (Ponta do Lessa Beach—PL).

Metal	PIN	RIB	PL	SAM	CONAMA[a]
As	1.77 ± 0.09	1.63 ± 0.10	1.51 ± 0.06	1.75 ± 0.60	50
Pb	0.13 ± 0.04	0.15 ± 0.04	0.15 ± 0.01	0.10 ± 0.02	10
Cr	2.47 ± 0.4	2.09 ± 0.61	6.97 ± 0.8	2.55 ± 0.37	50
Cd	0.02 ± 0.004	0.03 ± 0.001	0.04 ± 0.008	0.03 ± 0.006	5
V	0.53 ± 0.03	0.69 ± 0.01	0.41 ± 0.03	0.59 ± 0.07	–
Cu	< LD	0.42 ± 0.6	< LD	< LD	50
Se	0.22 ± 0.01	0.23 ± 0.01	0.18 ± 0.01	0.21 ± 0.01	10
Ni	< LD	< LD	1.32 ± 0.06	< LD	100
Mn	0.98 ± 0.03	1.17 ± 0.11	1.16 ± 0.06	1.46 ± 0.21	100

Data expressed as $\mu g\ L^{-1}$ of seawater.

[a] Allowed limits for dissolved trace metals from saline waters Class V, according to the present Brazilian legislation ($\mu g\ L^{-1}$). Article 8, Resolution CONAMA number 20, from June 18, 1986.

Table 3
Level of trace elements in mussel *Perna perna* kept in three farming sites (Pinheira Beach—PIN; Ribeirão Beach—RIB and Sambaqui Beach—SAM) and one polluted site (Ponta do Lessa Beach—PL).

Metal	PIN	RIB	PL	SAM	BG*	ES*	HK*
As	13.75 ± 3.28^{a}	15.24 ± 3.13^{a}	13.19 ± 3.34^{a}	12.35 ± 4.24^{a}	–	–	–
Pb	0.24 ± 0.09^{a}	$0.44 \pm 0.21^{a,b}$	0.53 ± 0.14^{b}	$0.47 \pm 0.42^{a,b}$	4.21–5.09	0.06–0.85	1.0–17.8
Cr	0.88 ± 0.18^{a}	0.63 ± 0.17^{b}	0.86 ± 0.21^{a}	1.10 ± 0.15^{a}	–	–	0.93–20.93
Cd	0.48 ± 0.09^{a}	0.87 ± 0.20^{b}	0.66 ± 0.15^{a}	0.64 ± 0.23^{a}	–	0.05–0.90	0.41–4.10
Sn	0.02 ± 0.01^{a}	$0.03 \pm 0.01^{a,b}$	$0.03 \pm 0.02^{a,b}$	0.03 ± 0.02^{b}	–	–	–
V	7.75 ± 2.86^{a}	$10.93 \pm 5.10^{a,b}$	3.61 ± 0.77^{c}	1.86 ± 0.48^{c}	–	–	–
Cu	4.52 ± 1.22^{a}	5.27 ± 1.97^{a}	6.08 ± 1.18^{a}	5.29 ± 2.22^{a}	6.86–11.4	3.4–7.2	8.34–42.02
Se	1.69 ± 0.44^{a}	$1.88 \pm 0.48^{a,b}$	$2.19 \pm 0.70^{a,b}$	2.32 ± 0.72^{b}	–	–	–
Hg	nd	nd	nd	0.03 ± 0.01	–	–	–
Ni	2.87 ± 1.58^{a}	6.75 ± 2.70^{b}	8.49 ± 2.99^{b}	6.40 ± 2.92^{b}	5.14–8.27	–	2.82–25.32
Zn	51.9 ± 20.9^{a}	81.1 ± 29.6^{b}	90.5 ± 24.4^{b}	$68.3 \pm 19.70^{a,b}$	119–497	85.1–185.9	–
Ag	0.34 ± 0.22^{a}	2.01 ± 1.64^{b}	1.88 ± 0.61^{b}	1.63 ± 0.50^{b}	–	–	–

nd—under the method detection limit.

* represents levels of trace metals obtained in the literature for *Perna perna* from the Brazilian coastal areas Baía de Guanabara (BG; Rezende and Lacerda, 1986), Espírito Santo (ES; Furley and Oliveira Filho, 2000) and for *Perna viridis* from Hong Kong coast (HK; Chiu et al., 2000) Equal superscripted letters signs indicate that there was no difference among sites for a given trace element.

different sites. However, the levels of Cr and Ni were significantly higher at the PL, which suggests contamination at the site. Higher levels of V, Cu and Hg were detected at RIB, and higher levels of Mn were detected at SAM.

Different levels of trace elements were found in the mussels from the different sites (Table 3). Organisms from PIN showed lower levels of Ni, Zn and Ag than the organisms from the other sites located in the Florianópolis Bays. Mussels from PIN and RIB had higher levels of V than mussels kept in sites located at the Florianópolis North Bay PL and at SAM. When compared to other sites, Cr at RIB was lower, and Cd at RIB was higher. The contaminated site, PL, did not show any increased levels in metal contents when compared to SAM and RIB.

4. Discussion

Differences in some enzymatic biomarkers were found in gills and/or digestive glands of *P. perna* mussels transplanted to three farming zones. During the experiment, the farming zones were monitored for pollution using microbiological monitoring (e.g. fecal and total coliforms measurement, data not shown) and the analysis of trace elements in water, in which we recorded very low levels. Although the levels of trace elements in the water were below the limits set by Brazilian legislation, and although the levels measured in the mussels were in accordance with previous studies reported in the literature, some discrepancies were found between levels in the water and in mussels from different sites. However, no correlation was observed between the levels of trace elements found in water and the levels found in mussels. When we compared the levels of trace elements in the mussels and water between the different sites, some trace element levels were higher in the tissues of animals from sites at which the levels of the same element were not higher in the water (that is to say, the level was high in the specimen, but not in the water surrounding it), which suggests that the physicochemical parameters may also interfere with the rate of trace element uptake by mussels.

Low levels of microbiological markers and metals and the absence of important industrial or agricultural activities around the site led us to presume that the farming sites were not contaminated, or at least, that low contaminant levels were not high enough to cause enzymatic biomarker changes in the mussels during the six months of experiment.

We considered reasons other than pollution that could be involved in the enzymatic biomarker changes found in the farming sites, and identified the organ/biomarker combination that is unchanged in those sites. In addition, the enzymatic biomarker levels in mussels transplanted to an urban sewage contaminated site (Ponta do Lessa—PL) were also evaluated, and suggested important biomarker responses related to aquatic pollution.

4.1. Biomarker levels in farming sites

Both organs analyzed in the mussels showed differences in the activity of ChE and GST between the farming sites. ChE and GST enzymes have been extensively employed as biomarkers in coastal monitoring programs, but the prediction of baseline levels at reference sites is difficult, and the possible alteration by factors other than pollution (e.g. environmental and biological) has been a matter of concern in field studies (Najimi et al., 1997; Bend and James, 1978; Fisher et al., 2000).

ChE activity in the gills was lower in mussels kept at PIN, compared to RIB and SAM farming sites. Mussels from PIN showed slight biological differences, such as differences in sexual stage and shell size. Previous studies have also shown seasonal variation for ChE activity in aquatic organisms, which could be related to the reproductive cycle (Najimi et al., 1997). In mussels from Agadir Bay, the maximal and minimal ChE activities coincided with spawning or sexual rest, depending on the species of mussel in the study (Id-Halla et al., 1994). Najimi et al. (1997) suggests that these variations are related to different levels of cholinergic system activation during the reproductive cycle. In this study, mussels with empty gonad follicles (IIIB stage) exhibit the most ChE activity: higher than in specimens in the IIIA and IIIC stages (data not published). The low ChE activity observed in the mussels' gills at PIN may be associated with the higher frequency of animals in the IIIA and IIIC stages observed at this site (data not shown), and is not necessarily due to the presence of anticholinesterase compounds. The influence of environmental parameters that alter the ChE activity levels also have been pointed out by some authors (Radenac et al., 1998; Wu et al., 1997), and could not be ignored as a possible cause of the low levels of ChE activity observed at the PIN site.

With respect to bivalve size, higher ChE activity in the larger specimens of mussels *Mytilus edulis* in their first year of growth (1–4 cm) was found, and probably associated with nervous

system development during this period (Radenac et al., 1998). An inverse relationship between the ChE activity and length is observed in fish (Burgeot et al., 1996). However, in the present study, physicochemical characteristics were also observed at PIN (e.g. high circulation, depth and salinity but low food availability and temperature), which may represent important factors guiding the differences in the activity of ChE observed in this site. Regarding the digestive gland, ChE activity was different at the three sites, with lower levels in SAM (which is located in the North Bay of the city of Florianópolis), and higher levels in RIB (which is located in the South Bay). Environmental conditions and variability in organ responses have been reported as important factors involved in ChE responses (Radenac et al., 1998; Wu et al., 1997).

It should be noted that recent studied have reported that protease inhibitors, such as PMSF added to sample buffers in relative concentrations, may be able to inhibit ChEs in some organisms (Vioque-Fernández et al., 2007). Because this inhibitor was used to prepare the protein extracts of mussels used in this study, it is possible that this compound would be interfering with correct attribution of ChE activity. Further studies should be done avoiding the use of PMSF in sample extracts in order to better clarify this aspect. GST activity in the gills was higher in the mussels from SAM than in the mussels from the other farming sites, and GST activity in the digestive glands was lower. Higher levels of chlorophyll (Table 1) and turbidity (Ferreira et al., 2006) were found at this site, which suggests greater primary biomass productivity, and which could also indicate more food availability for the mussels at this site. It has been shown that food availability can account for changes in GST and CAT activities (Bocchetti and Regoli, 2006). Moreover, it is also possible that compounds present in water might influence this enzyme activity in an organ-specific manner. In fact, a negative correlation was observed between Se content in water and the activities of G6PDH and GR (data not shown). Decreases in these enzymes can cause a decrease in GSH, which leads to decreases in GST activity.

G6PDH activity in the mussels' digestive glands was also lower in SAM than in other sites. A statistically significant correlation was found between the GST and G6PDH activities in both organs. The results are consistent with the idea that G6PDH, together with GR, is involved in the maintenance of GSH levels, which, in turn, is used as substrate by GST (Sies, 1991; Reed, 1986; Keen et al., 1976).

Similar enzymatic activity (levels of GR in both organs, levels of G6PDH in the gills, and levels of CAT activity in the digestive glands) was found at the different sites, even when we considered the differences reported in the biological statuses of the mussels and the physicochemical characteristics of the farming sites. GR is involved in the maintenance of GSH levels, and since GSH have multiple roles in the cell, the absence of differences for this enzyme could be associated with the need to keep the GSH homeostasis in the cells. In addition, GR activity was positively correlated to GST and G6PDH activity in both organs, denoting the importance of the coordinated regulation of those enzymes in order to maintain the equilibrium between the cellular GSH/GSSG levels.

4.2. Biomarker changes at the contaminated site

The present study shows a markedly higher ChE activity in the digestive glands of mussels that were maintained at a sewage contaminated site (PL) for six months when compared to animals maintained at a reference site (SAM) for the same period. There is evidence in the literature supporting the existence of stimulatory effects in ChE activity by exposure to contaminants, such as lindane, Al, toluene, vinyl chloride and the organophosphate ethylparathion in rats (Bainy et al., 2006), as well as metals in fish (Jebali et al., 2006). However, this ChE increase is not like the well reported adverse effects caused by organophosphates and carbamate pesticides in vertebrates, which include the dose-dependent inhibition of ChE activity by such compounds (Carlock et al., 1999). ChE inhibition in fish has been also attributed to exposure to a wide range of contaminants, such as metals, pulp mill effluents, domestic sewage and PAHs (Payne et al., 1996).

ChE responses in invertebrates seem to vary according to the taxonomic group, contaminant class and exposure time, among other factors. The insect *Pterostichuscupreus cupreus* shows a classical vertebrate-like response to the organophosphorous insecticide dimethoate, denoted by ChE inhibition in a dose-dependent fashion and decrease in locomotor behavior (Jensen et al., 1997). However, the practical use of ChE as a biomarker in other invertebrates, such mollusks and crustaceans, has run into problems (Rickwood and Galloway, 2004). For example, no clear ChE inhibition *in vivo* has been observed in the *M. edulis* mussel or the *Penaeus duorarum* shrimp, although ChE activity is strongly inhibited *in vitro* in similar concentrations of the same anti-cholinesterase pesticides in both studies (Rickwood and Galloway, 2004). Similarly, no ChE effects were observed in the *C. gigas* oyster exposed to fenitrothion and phosalone (Bocquené et al., 1995). Vioque-Fernandes et al. (2009) observed an increase in ChE activity in the *Procambarus clarkii* (crustacean) exposed to a low dose of chlorpyrifos after a two-day exposure, followed by inhibition after a seven-day exposure. An increase in ChE activity in low doses of the pesticide chlorfenvinphos was also observed in the *M. edulis* mussel after a two-day exposure, but not after a four-day exposure (Rickwood and Galloway, 2004), and Matozzo et al. (2005) showed increases for the pesticide chlorpyrifos in the *Tapes philippinarum* clam. Bainy et al. (2006) and Najimi et al. (1997) both studied *P. Perna* mussels exposed to metal. Bainy et al. (2006) found substantial increases in ChE activity in the digestive glands, while Najimi et al. (1997) found increases throughout the mussel's entire body.

The higher ChE activity in *P. perna* from the PL polluted site may have been related to exposure to urban sewage discharge around this area. Possible ChE activator compounds that are commonly found in wastewater include EDCs such 4-nonylphenol (NP), which causes ChE inhibition in fish, planaria and animal cell culture (Li, 2008a, 2008b; Talorete et al., 2001) but which has been shown to cause an increase in ChE in the *T. philippinarum* clam after a range of tested doses (Matozzo et al., 2005). Nevertheless, the mechanisms involved in mussel ChE activation by contaminants in domestic sewage remain to be clarified. Possible mechanisms include modifications in the enzyme–substrate complex (Romani et al., 2003) or interaction with ChE/acetylcholine receptors, leading to acetylcholine accumulation in the synaptic cleft and activation of *de novo* ChE protein synthesis (Bainy et al., 2006).

Our findings were consistent with other studies, which also showed higher GST activity in organisms from polluted sites when compared to those from reference sites (Burgeot et al., 1996; Bainy et al., 2000; Lau and Wong, 2003; Manduzio et al., 2004). In addition, positive correlations between contaminant body burden and GST activity were also described (Cheung et al., 2001, 2002; Gowland et al., 2002). Elevated GST activity in *P. perna* mussels has previously been associated with the higher xenobiotic input produced by intense rainfall (Bainy et al., 2000).

In accordance with GST increase in the digestive glands of polluted mussels, GR activity increased as well. This enzyme catalyzes the reduction of oxidized glutathione (GSSG) to reduced glutathione (GSH) using electrons from NADPH (Sies, 1991; Keen et al., 1976; Reed, 1986). Increases in GR enzyme activity found at

the PL site could be explained by the need to cope with the GSH decrease caused by increased GST activity. GSHs function as non-enzymatic antioxidants that act as chain-breaking molecules that interrupt the autocatalytic spread of radical reactions, as well as co-substrates in some antioxidant enzymatic reactions catalyzed by GPx and GST (Storey, 1996). Therefore, increases in GR activity may be an important response for maintaining adequate GSH levels. In fact, decreases in GSH may contribute to higher levels of lipid peroxides (Doyotte et al., 1997; Cossu et al., 2000), which is one of the major contributors to the loss of cell function in oxidative stress situations. Moreover, GSH can participate in other important processes in cells, such as electron donation for glutathione peroxidases. Along this vein, Almeida et al. (2004) showed the possible important role of reduced glutathione (GSH) and phospholipid hydroperoxide glutathione peroxidase (PHGPx) activity in the maintenance of MDA levels when mussels were exposed to metals. Cossu et al. (2000) showed an increase in MDA levels, concomitant to decreases in reduced glutathione (GSH) levels and glutathione reductase (GR) activity in the bivalve *Unio tumidos* exposed to sites with high PAH levels.

Higher CAT activity was observed in the gills of mussels from the polluted site, suggesting a compensatory increase of this antioxidant to cope with higher H_2O_2 generation directly or indirectly induced by contaminants present at this polluted site. Also, it is important to consider that higher CAT activity can also indicate an increased peroxisomal proliferation induced by organic xenobiotics such as PAHs (Cajaraville et al., 2000). Therefore, modifications in the expression and activity of these enzymes could alter the level and effect of xenobiotics in the organism and thus protect the animal from damage due to oxidative metabolism of these compounds (Sheehan and Power, 1999; Winston and Di Giulio, 1991).

The increases in ChE, GST, GR (digestive gland) and CAT (gill) enzyme activity at the pollutant site PL could represent an effect from exposure to the sort of chemicals that are present in domestic sewage discharge. The composition of domestic sewage discharge in urban areas varies depending on the kind of anthropogenic activities. Metal, hydrocarbon and pesticide levels are typically low in domestic sewages from Brazilian urban areas, although some of those compounds may be present in high concentrations in some cases (Abessa et al., 2005). In this study, metal exposure did not seem to be the cause for the enzymatic changes observed in *P. perna*, as is shown here by the chemical analyses in the water and mussel tissues. Recently, a lot of attention has been given to the presence of various pharmaceuticals and personal care products (PPCPs) and surfactants in wastewater, many of them considered to be endocrine disruptor chemicals (EDCs) (Bolong et al., 2009). EDCs also have been shown to cause biological effects in mussels, including an increase in CAT and GST enzyme activities (Canesi et al., 2008).

The G6PDH activity in both organs was similar between polluted and reference sites. The CAT of the digestive glands and the activity of ChE, GST and GR in the gills were analyzed, and there was no difference found between the mussels from the polluted and the reference sites.

5. Conclusion

The use of biomarker responses in bivalves is sometimes controversial due to the variability in some enzyme activities related to gonad maturation stages, age, temperature and feeding stages of the specimens, making it difficult to clearly establish relationships between contaminants and biomarker changes (Najimi et al., 1997; Bend and James, 1978; Sheehan and Power, 1999; Fisher et al., 2000). In this study, some biomarkers were unchanged (GR in the gills and digestive glands, G6PDH in the gills, and CAT activity in the digestive glands) even though differences were observed in the biological status of the mussels and the physicochemical characteristic among farming sites. Among those biomarkers, GR was also induced in response to a polluted site, and can be described, in this particular case, as the most adequate biomarker of aquatic contamination. Because some enzymes (ChE, GST in the digestive glands and CAT in the gills) were present at both the polluted sites and the different farming sites, they could not be used to positively confirm that the differences observed at the polluted site were exclusively caused by pollution. These variations show that the different sites and enzymes require further study. Also, although higher enzyme activity was found in the gills than in the digestive gland (except for CAT activity), both tissues presented similar responses to the particular environmental characteristics of each site studied here. Trace metals in mussels were below the allowed limit levels for human consumption, and apparently were not involved in the biomarker responses. There is no previous data on the metal content in both mussels and water from the sites studied herein that would allow for comparisons over time. Therefore, the present characterization of biomarker levels in gills and digestive glands, as well as the trace metal levels in water and whole bodies of mussels, will be important in the development of future *Perna perna* monitoring strategies along the Brazilian coast. This study can also serve as a reference for similar studies employing Mitilidea bivalve mollusks worldwide in aquaculture and biomonitoring.

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References

Abessa, D.M.S., Carr, R.S., Rachid, B.R.F., Sousa, E.C.P.M., Hortelani, M.A., Sarkis, J.E., 2005. Influence of a Brazilian sewage outfall on the toxicity and contamination of adjacent sediments. Mar. Pollut. Bull. 50, 875–885.

Aebi, H., 1984. Catalase. In: Aebi, H. (Ed.), Methods of Enzymatic Analysis, 105. Academic Press, pp. 121–126.

Akcha, F., Izuel, C., Venier, P., Budzinski, H., Burgeot, T., Narbonne, J.-F., 2000. Enzymatic biomarker measurement and study of DNA adduct formation in benzo[a]pyrene-contaminated mussels, *Mytilus galloprovincialis*. Aquat. Toxicol. 49, 269–287.

Almeida, E.A., Bainy, A.C.D., Loureiro, A.P.M., Medeiros, M.H.G., Di Mascio, P., 2003. DNA and lipid damage in the brown mussel *Perna perna* from a contaminated site. Bull. Environ. Contam. Toxicol. 71, 270–275.

Almeida, E.A., Miyamoto, S., Bainy, A.C.D., De Medeiros, M.H.G., Di Mascio, P., 2004. Protective effect of phospholipid hydroperoxide glutathione peroxidase (PHGPx) against lipid peroxidation in mussels *Perna perna* exposed to different metals. Mar. Pollut. Bull. 49, 386–392.

Bainy, A.C.D., Almeida, E.A., Muller, I.C., Ventura, E.C., Medeiros, I.D., 2000. Biochemical responses in farmed mussel *Perna perna* transplanted to contaminated sites on Santa Catarina Island, SC, Brazil. Mar. Environ. Res. 50, 411–416.

Bainy, A.C.D., Saito, E., Carvalho, P.S.M., Junqueira, V.B.C., 1996. Oxidative stress in gill, erythrocytes, liver and kidney of Nile tilapia (*Oreochromis niloticus*) from a polluted site. Aquat. Toxicol. 34, 151–162.

Bainy, A.C.D., Medeiros, M.H.G., Di Mascio, P., Almeida, E.A., 2006. *In vivo* effects of metals on the acetylcholinesterase activity of the *Perna perna* mussel s digestive gland. Biotemas 19, 35–39.

Bend, J.R., James, M.O., 1978. Xenobiotic metabolism in marine freshwater species. In: Malins, D.C., Sargent, J.R. (Eds.), Biochemical and Biophysical Perspectives in Marine Biology, vol. 4. Academic Press, London, England, pp. 125–188.

Bocchetti, R., Regoli, F., 2006. Seasonal variability of oxidative biomarkers, lysosomal parameters, metallothioneins and peroxisomal enzymes in the

Mediterranean mussel Mytilus galloprovincialis from Adriatic Sea. Chemosphere 65 (6), 913–921.
Bocquené, G., Bellanger, C., Cadiou, Y., Galgani, F., 1995. Joint action of combination of pollutants of the acetylcholinesterase activity of several marine species. Ecotoxicology 4, 266–279.
Burgeot, T., Bocquené, G., Porte, C., Dimeet, J., Santella, R.M., Garcia de la Parra, L.M., Pihol-Leszkowicz, A., Raoux, C., Galgani, F., 1996. Bioindicators of pollutant exposure in the northwestern Mediterranean Sea. Mar. Ecol. Prog. Ser. 131, 125–141.
Bolong, N., Ismail, A.F., Salim, M.R., Matsuura, T., 2009. A review of the effects of emerging contaminants in wastewater and options for their removal. Desalination 239, 229–246.
Cajaraville, M.P., Bebianno, M.J., Blasco, J., Porte, C., Sarasquete, C., Viarengo, A., 2000. The use of biomarkers to assess the impact of pollution in coastal environments of the Iberian Peninsula: a practical approach. Sci. Total Environ. 247, 295–311.
Canesi, L., Borghi, C., Ciacci, C., Fabbri, R., Lorusso, L.C., Vergani, L., Marcomini, A., Poiana, G., 2008. Short-term effects of environmentally relevant concentrations of EDC mixtures on *Mytilus galloprovincialis* digestive gland. Aquat. Toxicol. 87, 272–279.
Carlock, L.L., Chen, W.L., Cordon, E.B., Killeen, J.C., Manley, A., Meyer, L.S., Mullin, L.S., Pendino, K.J., Percy, A., Sargent, D.E., Seaman, L.R., Svanborg, N.K., Stanton, R.H., Van Goethem, I.L., 1999. Regulating and assessing risks of cholinesterase-inhibiting pesticides: divergent approaches and interpretations. J. Toxicol. Environ. Health, Part B 2, 105–160.
Chapple, G., Byrne, J.P., 1996. Direct determination of trace metals in seawater using electrothermal vaporization inductively coupled plasma mass spectrometry. J. Anal. Atom. Spectr. 11, 549.
Cheung, C.C.C., Zheng, G.J., Li, A.M.Y., Richardson, B.J., Lam, P.K.S., 2001. Relationships between tissue concentrations of polycyclic aromatic hydrocarbons and antioxidative responses of marine mussels, Perna viridis. Aquat. Toxicol. 52, 189–203.
Cheung, C.C.C., Zheng, G.J., Lam, P.K.S., Richardson, B.J., 2002. Relationships between tissue concentrations of chlorinated hydrocarbons (polychlorinated biphenyls and chlorinated pesticides) and antioxidative responses of marine mussels, Perna viridis. Mar. Pollut. Bull. 45, 181–191.
Chiu, S.T., Lam, F.S., Tze, W.L., Chau, C.W., Ye, D.Y., 2000. Trace metals in mussels from mariculture zones, Hong Kong. Chemosphere 41, 101–108.
CONAMA—Conselho Nacional do Meio Ambiente. 1986. Resolução N° 20, Ministerio do Desenvolvimento Urbano e Meio Ambiente, Brasilia, Brazil.
Cossu, C., Doyotte, A., Babut, M., Exinger, A., Vasseur, P., 2000. Antioxidant biomarkers in freshwater bivalves, *Unio tumidus,* in response to different contamination profiles of aquatic sediments. Ecotoxicol. Environ. Saf. 45, 106–121.
Curtius, A. J., Seibert E. L., Fiedler, H. D., Ferreira, J. F., Vieira, P.H.F., 2003. Avaliando a contaminação por elementos traço em atividades de maricultura. Resultados parciais de um estudo de caso realizado na Ilha de Santa Catarina. Quim. Nova 26, 44–52.
Dauterman, W.C., 1994. Metabolism of toxicants: phase II reactions. In: Hodgson, E., Levi, P.E. (Eds.), Introduction to Biochemical Toxicology. Appleton & Lange, Connecticut, USA, pp. 127–129.
Doyotte, A., Cossu, C., Jacquim, M.C., Babut, M., Exinger, A., Vasseur, P., 1997. Antioxidant enzymes, glutathione and lipid peroxidation of experimental or field exposure in the gills and the digestive gland of the freshwater bivalve *Unio tumidus*. Aquat. Toxicol. 39, 93–110.
Dressler, V.L., Pozebon, D., Curtius, A.J., 1998. Determination of heavy metals by inductively coupled plasma mass spectrometry after on-line separation and preconcentration. Spectroch. Acta Part B 53, 1527–1539.
Ellman, G.L., Courtney, K.D., Andres, V., Featherstone, R.M., 1961. A new and rapid colorimetric determination of acetylcholinesterase activities. Biochem. Pharmacol. 7, 88–95.
Ferreira, J.F., Besen, K., Wormsbecher, A.G., dos Santos, R.F., 2006. Physical–chemical parameters of seawater mollusc culture sites in Santa Catarina Brazil. J. Coastal Res. 2, 1122–1126.
Fisher, W.S., Lead, M.O., Winstead, J.T., Long, E.R., 2000. A survey of oyster *Crassostrea virginica* from Tampa Bay, Florida: associations of internal defense measurements with contaminant burdens. Aquat. Toxicol. 51, 115–138.
Furley, T.H., Oliveira Filho, A.C., 2000. Biomonitoring of heavy metals and organochlorinated compounds in a pulp mill effluent using introduced mussels. Aquat. Ecos. Health Manage. 3, 499–507.
Garcia-Alfonso, C., Lopez-Barea, J., Sanz, P., Repetto, G., Repetto, M., 1995. Stimulation of antioxidative enzymes by paraquat in cultured Vero cells. Vet. Human Toxicol. 37, 414–421.
García-Alfonso, C., López-Barea, J., Sanz, P., Repetto, G., Repetto, M., 1996. Changes in antioxidative activities induced by Fe(II) and Fe(III) in cultured Vero cells. Arch. Environ. Contam. Toxicol. 30, 431–436.
García-Alfonso, C., Repetto, G., Sanz, P., Repetto, M., López-Barea, J., 1998. Direct determination of glutathione-S-transferase and glucose-6-phosphate dehydrogenase activities in cells cultured in microtitre plates as biomarkers for oxidative stress. Alt. Lab. Animals 26, 321–330.
Glock, G.E., Mclean, P., 1953. Further studies on the properties and assay of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase of rat liver. Biochem. J. 55, 400–408.
Gowland, B.T.G., Mcintosh, A.D., Davies, I.M., Moffat, C.F., Webster, L., 2002. Implications from a field study regarding the relationship between polycyclic aromatic hydrocarbons and glutathione-S-transferase activity in mussels. Mar. Environ. Res. 54, 231–235.
Id-Halla, M., Bouhaimi, A., Moukrim, A., Kaaya, A., Zekhnini, A., 1994. Contribution a l'ètude de la biologie des moules dans la baie d'Agadir. Actes du colloque surla pêche maritime, Agadir, 9–11 juin 1994, pp. 30–37.
Jebali, J., Banni, M., Guerbej, H., Almeida, E.A., Bannaoui, A., Boussetta, H., 2006. Effects of malathion and cadmium on acetylcholinesterase activity and metallothionein levels in the fish *Seriola dumerilli*. Fish Physiol. Biochem. 32, 93–98.
Jensen, C.S., Garsdal, L., Baatrup, E., 1997. Acetylcholinesterase inhibition and altered locomotor behavior in the carabid beetle *Pterostichus cupreus*. A linkage between biomarkers at two levels of biological complexity. Environ. Toxicol. Chem. 16, 1727–1732.
Keen, J.H., Habig, W.H., Jakoby, W.B., 1976. Mechanism for several activities of the glutathione-S-transferases. J. Biol. Chem. 251, 6183–6188.
Lau, P.S., Wong, H.L., 2003. Effect of size, tissue parts and location on six biochemical markers in the green-lipped mussel, Perna viridis. Mar. Pollut. Bull. 46, 1563–1572.
Lenartova, V., Holovska, K., Martinez-Lara, E., Lopez-Barea, J., Barcena, J.A., Rosival, I., 1996. Changes in GST-isoenzyme pattern of some organs of sheep exposed to different levels of pollution. Comp. Biochem. Physiol. C 114, 153–158.
Li, M.H., 2008a. Effects of nonionic and ionic surfactants on survival, oxidative stress, and cholinesterase activity of planarian. Chemosphere 70, 1796–1803.
Li, M.H., 2008b. Effects of nonylphenol on cholinesterase and carboxylesterase activities in male guppies (*Poecilia reticulata*). Ecotoxicol. Environ. Saf. 71, 781–786.
Littlepage, J.L., 1998. *Oceanografia. Manual de técnicas oceanográficas para trabalhos em laboratório e a bordo*. EUFC, Fortaleza, Brazil.
López-Barea, J., 1995. Biomarkers in ecotoxicology: an overview. In: Degen, G.H., Seiler, J.P., Bentley, P. (Eds.), Toxicology in Transition. Springer, Berlin, pp. 57–79.
Lorenzen, C.S., 1967. Determination of chlorophyl and pheo-pigments: spectrophotometric equations. Limnol. Oceanog. 12, 343–346.
Lunetta, J.E., 1969. Fisiologia da reprodução de mexilhões (*Mytilus perna* L. Molusca Lamellibranchia). Bol. Zool. Biol. Mar. 26, 33–111.
Manduzio, H., Monsinjon, T., Galap, C., Leboulenger, F., Rocher, B., 2004. Seasonal variations in antioxidant defences in blue mussels Mytilus edulis collected from a polluted area: major contributions in gills of an inducible isoform of Cu/Zn-superoxide dismutase and of glutathione-S transferase. Aquat. Toxicol. 70, 83–93.
Martínez-Lara, E., Toribio, F., López-Barea, J., Bárcena, J.A., 1996. Glutathione-S-transferase isoenzyme patterns in the guilthead seabream (*Sparus aurata*) exposed to environmental contaminants. Comp. Biochem. Physiol. C 113, 215–220.
Matozzo, V., Tomei, A., Marin, M.G., 2005. Effects of 4-nonylphenol (xenoestrogen) and chlorpyrifos (organophosphorus pesticide) on acetylcholinesterase activity in the clam *Tapes philippinarum*. Fresenius Environ. Bull. 15, 710–714.
Medeiros, I.D., 2000. Padronização de Algumas Enzimas Detoxificantes Como Biomardores de Contaminação Aquática. Universidade Federal de Santa Catarina, Florianópolis, SC, Brazil.
MMARH, 1996. Macrodiagnóstico das zonas costeiras do Brasil na escala de união. Ministério do Meio Ambiente dos recursos hídricos e da Amazônia legal. Rio de Janeiro, RJ, Brasil.
Monserrat, J.M., Bianchini, A., 1998. Some kinetic and toxicology characteristics of thoracic ganglia cholinesterase of *Chasmagnatus granulata* (Decapoda, Grapsidae). Comp. Biochem. Physiol. C 120, 193–199.
Mora, P., Fournier, D., Narbonne, J., 1999. Cholinesterase from the marine mussels *Mytilus galloprovincialis* Lmk. and *M. edulis* L. and from the freshwater bivalve *Corbicula fluminea* Müller. Comp. Biochem. Physiol. C 122, 353–356.
Najimi, S., Bouhaimi, A., Daubeze, M., Zekhnini, A., Pellerin, J., Narbonne, J.F., Moukrim, A., 1997. Use of acetylcholinesterase in *Perna perna* and *Mytilus galloprovincialis* as a biomarker of pollution in Agadir Marine Bay (south of Morocco). Bull. Environ. Contam. Toxicol. 58, 901–908.
Narbonne, J.F., Garrigues, P., Ribera, D., Raoux, C., Mathieu, A., Lemaire, P., Salaun, J.P., Lafaurie, M., 1991. Mixed function oxygenase enzymes as tools for pollution monitoring. Field studies on the French coast of the Mediterranean Sea. Comp. Biochem. Physiol. C 100, 37–42.
NOAA, 1995. National Oceanic and Atmosferic Administration US Technical Memorandum. International Mussel Watch. Initial Implementation Phase Final Report, US Department of Commerce, USA.
O'Connor, T.P., 1996. Trends in chemicals concentrations in mussels and oysters collected along the US coast from 1986 to 1993. Mar. Environ. Res. 41, 183.
Payne, J.F., Mathieu, A., Melvin, W., Fancey, L., 1996. Acetylcholinesterase, an old biomarker with a new future? Field trials in association with two urban rivers and a paper mill in Newfoundland. Mar. Pollut. Bull. 32 (2), 225–231.
Pedrajas, J.R., Peinado, J., López-Barea, J., 1993. Purification of Cu,Zn-superoxide dismutase isoenzymes from fish liver: appearance of new isoforms as consequence of pollution. Free Rad. Res. Commun. 19, 29–41.
Peterson, G.L., 1977. A simplification of the protein assay method of Lowry et al. which is more generally applicable. Anal. Biochem. 83, 346–356.
Poli, C.R., Littlepage, J., 1998. Desenvolvimento do cultivo de moluscos no estado de Santa Catarina. Procedings of the I Congresso Sul-Americano de Aquicultura. 1, Recife, PE, Brazil, 45–49.
Pozebon, D., Dressler, V.L., Curtius, A.J., 1998. Study of interferences from Na and Cl using ETV-ICP-MS. Atom. Spectr. 19, 80–88.
Radenac, G., Bocquené, G., Fichet, D., Miramand, P., 1998. Contamination of a dredged-material disposal site (LaRochelle Bay, France). The use of the

acetylcholinesterase activity of *Mytilus edulis* (L.) as a biomarker of pesticides: the need for a critical approach. Biomarkers 3, 305–315.
Reed, D.J., 1986. Regulation of reductive processes by glutathione. Biochem. Pharmacol. 35, 7–13.
Regoli, F., Principato, G., 1995. Glutathione-dependent and antioxidant enzymes in mussel, *Mytilus galloprovincialis*, exposed to metals under field and laboratory conditions: implications for the use of biochemical biomarkers. Aquat. Toxicol. 31, 143–164.
Rezende, C.E., Lacerda, L.D., 1986. Metais pesados em mexilhões (*Perna perna*) no litoral do estado do Rio de Janeiro. Ver. Bras. Biol. 46, 239–247.
Rickwood, C.J., Galloway, T.S., 2004. Acetylcholinesterase inhibition as a biomarker of adverse effect - A study of Mytilus edulis exposed to the priority pollutant chlorfenvinphos. Aquat. Toxicol. 67, 45–56.
Rodríguez-Ariza, A., Abril, N., Navas, J.I., Dorado, G., López-Barea, J., Pueyo, C., 1992. Metal, mutagenicity and biochemical studies on bivalve molluscs from Spanish coasts. Environ. Mol. Mutagenesis 19, 112–124.
Rodríguez-Ariza, A., Martínez-Lara, E., Pascual, P., Pedradas, J.R., Abril, N., Dorado, G., Toribio, F., Bárcena, J.A., Peinado, J., Pueyo, C., López-Barea, J., 1993. Biochemical and genetic indices of marine pollution in Spanish littoral. Sci. Total Environ. 134 (1), 109–116.
Romani, R., Antognelli, C., Baldracchini, F., De Santis, A., Isani, G., Giovannini, E., Rosi, G., 2003. Increased acetylcholinesterase activities in specimens of Sparus auratus exposed to sublethal copper concentrations. Chem-Biol. Interact. 145, 321–329.
Sheehan, D., Power, A., 1999. Effects of seasonality on xenobiotic and antioxidant defence mechanisms of bivalve molluscs. Comp. Biochem. Physiol. C 123, 193–199.
Sies, H., 1991. Oxidative stress: from basic research to clinical application. Am. J. Med. 91–3C, 32–38.
Sies, H., Koch, O.R., Martino, E., Boveris, A., 1979. Increased biliary glutathione disulfide release in chronically ethanol treated rats. FEBS Lett. 103, 287–290.
Storey, K.B., 1996. Oxidative stress: animal adaptations in nature. Braz. J. Med. Biol. Res. 29, 1715–1733.
Strickland, J.D., Parsons, T.R., 1972. A Practical Handbook of Seawater Analysis 2nd ed. Ottawa Queen's Printer 310 pp.
Talorete, T.P.N., Isoda, H., Maekawa, T., 2001. Alkylphenolic compounds and their effect on the injury rate, survival and acetylcholinesterase activity of the rat neuronal cell line PC12. Cytotechnol. 36, 163–169.
Viarengo, A., Lafaurie, M., Gabrielides, G.P., Fabbri, R., Marro, A., Romeo, M., 2000. Critical evaluation of an intercalibration exercise undertaken in the framework of the MED POL biomonitoring program. Mar. Environ. Res. 49, 1–8.
Vioque-Fernández, A., Almeida, E.A., López-Barea, J., 2007. Esterases as pesticide biomarkers in crayfish (*Procambarus clarkii*, Crustacea): tissue distribution, sensitivity to model compounds and recovery from inactivation. Comp. Biochem. Physiol. C 145 (3), 404–412.
Vioque-Fernandez, A., Almeida, E.A., Lopez-Barea, J., 2009. Biochemical and proteomic effects in *Procambarus clarkii* after chlorpyrifos or carbaryl exposure under sublethal conditions. Biomarkers 14, 299–310.
Walker, C.H., Hopkin, S.P., Sibly, R.M., Peakall, D.B., 1996. Principles of Ecotoxicology. Taylor and Francis Ltd., London, England 321 pp.
Winston, G.W., Di Giulio, R.T., 1991. Prooxidant and antioxidant mechanism in aquatic organisms. Aquat. Toxicol. 19, 137–161.
Wu, S.L., Feng, X.B., Wittmeier, A., 1997. Microwave digestion of plant and grain reference materials in nitric acid or a mixture of nitric acid and hydrogen peroxide for determination of multi-elements by inductively coupled plasma mass spectrometry. J. Anal. Atom. Spectrom. 12, 797.