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Trace metal and biomarker levels in tissues of *Argopecten purpuratus* in the north of Chile, and the potential use of this species as a bioindicator of metallic stress

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Abstract – The capacity to bioaccumulate trace metals present in San Jorge Bay, Antofagasta, Chile, was determined in northern scallop, *Argopecten purpuratus*, to examine the value of this important commercially species as a bioindicator of heavy metal pollution in areas where it is cultured. Scallops were sampled in summer 2009 in four sites: three natural populations (Coloso, Historic District and La Rinconada, marine reserve), and a commercial hatchery (Colorado). The concentrations of three heavy metals (copper, cadmium and lead) were then determined through stripping chronopotentiometric methods, and the levels of four biomarkers: three genes implicated in the stress and oxidative metabolism, i.e., glutathione peroxidase (GPx), glutathione s-transferase (GST) and heat shock protein 70 (HSP70), and a protein marker in the digestive gland and gill, a metallothionein (MT). The Historic District, located in the downtown area of the city, showed the highest metal concentration of all the sampled sites, as well as the highest levels of the four biomarkers. This can be largely attributed to the nearby international port area of the town and high sea traffic flow, exacerbated by the prevailing winds.

Keywords: Trace metals / Copper / Cadmium / Lead / Genetic marker / Metallothionein / Pollution / *Argopecten purpuratus* / Pacific Ocean

1 Introduction

Coastal marine ecosystems are affected by heavy anthropogenic pressure due to the proximity of human settlements. The main sources of pollution in the waters of bays and the coastal ocean are domestic, industrial and port wastes (Valdés et al. 2011). The quality of aquatic environments can be assessed through the analysis of organisms considered as indicators of pollution, such as bivalve molluscs (Salamanca et al. 2004; Yap et al. 2004; Tapia et al. 2010; Bustamante et al. 2012). Indeed, characteristics of their biology (e.g. capacity of bioaccumulation, resistance to physico-chemical stresses) and ecology (e.g. worldwide distribution, abundance of their populations), put bivalves among the best candidates to serve as bioindicator species (Phillips 1976; Farrington and Tripp 1993). These organisms can bioaccumulate a large variety of pollutants at levels higher than those present in the surrounding waters or sediments, and their behaviour can be recorded over short periods of time (Solé et al. 2000). These qualities suggest that bivalve molluscs could be used as a bioindicators for monitoring programs in coastal areas. It is also important to understand the complex mechanisms in mollusc populations that allow them to adapt to contaminated environments.

Among the pollutants discharged into the aquatic environment, heavy metals are of worldwide concern. Metal pollution in the aquatic environment can result both from natural and anthropogenic sources, such as mine washing or agricultural leaching (Ferreira-Cravo et al. 2009). Many studies have shown that exposure to metals, such as lead (Pb), cadmium (Cd), and copper (Cu), is associated with the induction of oxidative stress (see the review Valko et al. 2005). Antioxidant systems have great potential to provide insight on the consequences of metal exposure, since they constitute both coping mechanisms and potential targets. For example, many metals are known to generate oxidative stress either through direct generation of reactive oxygen species (ROS)

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or by scavenging thiols (glutathione and cysteine) that otherwise act as important non-enzymatic antioxidants (Zhang et al. 2010). Reactive oxygen species (ROS) are produced by basal cellular metabolism and also under certain environmental conditions. ROS include superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radicals (HO^-), and singlet oxygen ($^1O_2$). Oxidative stress caused by reactive oxygen species, or other non-oxygen containing free radicals, can increase lipid peroxidation, oxidation of proteins, and DNA damage. It can also affect cell viability by causing membrane damage and enzyme inactivity (Singh et al. 2006), and accelerate cell senescence and apoptosis (Kim and Phyllis 1998). To protect themselves against heavy metals and other toxic materials causing oxidative stress, aerobic organisms have evolved complex antioxidant defence systems.

Antofagasta, the largest human settlement in northern Chile, is located on San Jorge Bay (Fig. 1). The main economic and industrial activities of this city are the loading of mineral concentrates – mainly copper and lead – and copper anodes and cathodes onto transporter ships, in addition to the exchange of merchandise produced in Chile and neighbouring countries. In the coastal area, wastes from old mining activities have persisted, subjected to the action of the tides, and between the years 1990 and 2006, a submarine emissary discharged a copper-contaminated effluent from a filtration plant (Valdés et al. 2011). For these reasons, and due to the high traffic flow in the city, we designed a field study to determine the relevance of using the scallop *A. purpuratus* as a bioindicator of metal contamination in San Jorge Bay. Scallops were sampled from four sites in summer 2009: three natural populations (Coloso, Historic District, and La Rinconada, marine reserve), and a commercial hatchery (Colorado). The concentration of three heavy metals (copper, cadmium and lead) was determined in the digestive gland and gills of these scallops, and four biomarkers were evaluated: three genes involved in stress and oxidative metabolism (GPx: glutathione peroxidase, GST: glutathione s-transferase and HSP70: heat shock protein 70), and a protein marker (metallothionein, MT). We used these two tissues because the gills are the first organ of the scallop to come into contact with the environment, and the digestive gland is the main site of xenobiotic uptake and oxyradical generating biotransformation enzymes (Livingstone et al. 1992). A set of biomarkers increases the potential to distinguish sites with different degrees of pollution (Dondero et al. 2006). Therefore, the identification of GPx, GST, HSP70 and MT fluctuations in *A. purpuratus* is important for the interpretation of field results on levels of heavy metals.

2 Materials and methods

2.1 Sample collection

Thirty scallops (70–60 mm shell length, close to commercial size) were collected from each of four sites on San Jorge bay, Antofagasta, Chile (Fig. 1): El Colorado (commercial hatchery, Lat 23° 29′ S 070° 25′ W), La Rinconada (marine reserve, Lat 23° 28′ S, 070° 30′ LW), Historic District (city centre, Lat 23° 38′ S 070° 24′ W), and Coloso (a copper shipping port, Lat 23° 45′ S, 070° 27′ W). Each scallop was

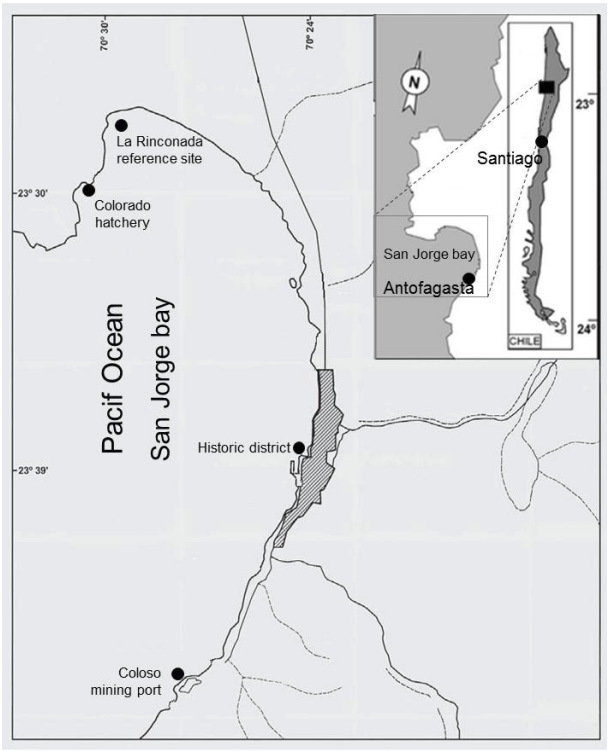

Fig. 1. Location of the study area in San Jorge bay, Antofagasta, Chile.

dissected and the gills and digestive gland separately homogenized and divided into 3 equal parts: one preserved and stabilized in RNALater® (Ambion, USA) for total RNA extraction, another put in extraction buffer (150 mM NaCl, 10 mM NaH_2PO_4, 1 mM phenylmethanesulfonyl fluoride, pH = 7.2) for protein extraction, and the remaining part immediately stored at −80 °C for later metal analysis. La Rinconada was used as the reference site because this area has the main natural population of the scallop *A. purpuratus*, an important national fishery resource, and has been a marine reserve since 1997. The site is protected from open fisheries, although extraction for research is permitted (Avendaño and Cantillánez 2008).

2.2 Metal analysis

Homogenized tissue samples (100 mg) of digestive gland and gills were mineralized in 2 ml nitric acid (65%, Suprapur, Merck). After dilution in 0.5 M NaCl (SigmaUltra, Sigma), concentrations of copper, lead and cadmium were assessed by stripping chronopotentiometric methods. These methods are detailed in Riso et al. (1997a, 1997b) and were previously used for a number of different tissues (Tanguy et al. 2003; Marchand et al. 2004; David et al. 2012). In order to check the method accuracy, certified reference seawater samples were analyzed (NASS-5, CASS-3 and SLEW-2, National Research Council of Canada). Analyses were made by spiking each sample three times with standards. The reproducibility of the method was 4%.

Table 1. Combinations of primers used in the real-time PCR expression analysis.

Genes	GenBank accession n°	Primer sequences (sense and antisense)
Actin	FE895980	5′- AGGCTCCATCTTGGCATCTCT-3′
		5′- AGATTCGTCGTATTCCTGTTTGC-3′
Glutathione peroxidase (GPx)	FF147974	5′- CTGTCGCTGAGAAGTCGTAAATAGA-3′
		5′- ATAGGGTCCACTGTCTTTCTCTCTGT-3′
Glutathione S-transferase (GST)	ES469339	5′-CCTGGACAAAAGACCGAAGAC-3′
		5′-CACTCGGGTCAAGGGTTTACA-3′
Heat shock protein 70 (HSP 70)	FE896007	5′-TCCCCGCCTACTTCAATGAC-3′
		5′-CCAGCTATTATGCAGGCATCTTT-3′

2.3 Total RNA extraction and cDNA synthesis

Total RNA was extracted from the homogenized tissue of gills and digestive gland using TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's protocol. RNA was then re-suspended in nuclease-free water and checked for both quality and quantity using NanoDrop 1 000 (Thermo Scientific): ratios were 260/280 for quantity and 260/230 for quality. Reverse transcription was performed on 1 μg RNA from each sample, with an random hexamer primer (0.2 μg μl^{-1}) and RevertAid^{TM} H Minus M-MulVRT reverse transcriptase (Fermentas), according to the manufacturers' instructions.

2.4 Real-time PCR analysis

Real-time polymerase chain reaction (PCR) was performed with 4.86 μl cDNA (1/10 dilution) in a total volume of 10 μl, using a 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA). Reaction component concentrations were as follows: 1× Absolute QPCR SYBR Green ROX Mix (ABgene, Epsom, UK) and 70 nM of each specific primer (Boutet et al. 2008; Zapata et al. 2009, Table 1). Reactions were performed with activation by Thermo-Start DNA polymerase at 95 °C for 15 min followed by amplification of the target cDNA (45 cycles of denaturation at 95 °C for 30 s, annealing and extension at 60 °C for 1 min) and a melting curve program from 95 to 70 °C, decreasing by 0.5 °C every 10 s. Readings were taken at 60 °C. *A. purpuratus* actin was used as a housekeeping reference gene to normalize the expression levels between samples. Magnitude of change in gene expression relative to the population control was determined by the standard $2^{-\Delta\Delta CT}$ method of Livak and Schmittgen (2001).

2.5 Protein extraction and quantification of MT by ELISA

Localization of metallothionein proteins in scallop gill and digestive gland tissues was investigated using an antibody for a recombinant metallothionein protein (CgMT1) produced from the Pacific oyster *Crassostrea gigas* (Boutet et al. 2002), previously used in clams, (Moraga et al. 2002). Total protein was isolated from samples of gills and digestive glands homogenized in protein extraction buffer. Samples were then centrifuged at 11 000 g for 10 min at 4 °C, and the supernatant fractions containing proteins were collected in fresh tubes. Total proteins were quantified using the Dc protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA), with dilutions of bovine serum albumin (Sigma Chemical, St. Louis, MO, USA) as a standard. Optical density was measured at 620 nm using a microplate reader (Multiscan Transmit).

Enzyme-linked immunosorbent assays (ELISA) were performed with scallop proteins and the mollusc antibody as follows (Boutet et al. 2002). Microtiter plates were coated with 0.01, 0.05, 0.1, 0.25, and 0.5 μg of recombinant CgMT1 and 0.5 μg of MT from rabbit liver (Novus Biologicals) by overnight incubation at 4 °C. Active sites remaining on the plates were blocked with 200 μl per well of PBS (pH = 7.2) containing 0.1% Tween 20 (PBS-T) and 1% bovine serum albumin. After four washes with PBST, 40, 80, 120, and 180 ng of biotin-labelled IgG in 100 μl of PBS-T were added to the wells and incubated for 1 h 30 min at 37 °C. All combinations of CgMT1 and labelled IgG doses were tested to determine the appropriate reaction concentrations. After 4 washes with PBS-T, 100 μl ExtrAvidine peroxidase conjugate (Sigma) diluted 1:1000 in PBS-T was added per well and incubated for 1 h 30 min at 37 °C. After two washes with PBS-T and three with PBS, 100 μl o-phenylenediamine (Sigma), at a concentration of 0.06% in phosphatecitrate buffer (0.045 M citric acid, 0.11 M Na_2HPO_4, pH = 5.45) containing 0.001% H_2O_2, were added to the well. The reaction was stopped after 20 min with 2N H_2SO_4, and OD 492 was measured with a microplate reader (Multiscan Transmit). Samples of 20 μg per well of total protein extracted from the digestive gland and gills were then quantified in the same conditions, with the appropriate amount of IgG.

2.6 Western blots

Samples of digestive gland and gill proteins were electrophoresed on 15% SDS-polyacrylamide gel for 90 min at 110 V (constant current) and electrotransferred to nitrocellulose-membrane (Whatman, Germany) for 80 min at 105 V and 4 °C. The membrane was dried for 5 min at room temperature, washed three times for 5 min with 0.1 M phosphate buffered saline/0.1% tween-20 (PBST). The membrane was blocked for 1 h with blocking buffer (Bio-Rad labs, Hercules, CA), then washed three times for 5 min with PBST, then incubated overnight with 1% BSA in PBST containing biotin-labelled anti-CgMT antibody (1/500 dilution) with gentle agitation at 4 °C. The membrane was again washed three times for 5 min with PBST, then incubated with BAR solution (Bio-Rad labs, Hercules, CA) for 10 min with gentle agitation,

and washed three times for 5 min with 20% DMSO/PBST. The membrane was again washed three times for 5 min with PBST and then incubated with 1% BSA in PBST containing 1/1 000 Streptavidin-HRP (Bio-Rad labs, Hercules, CA) for 30 min with gentle agitation. Finally, the membrane was washed 3 times for 5 min with PBST a last time and the reactive band was visualized using Opti-4CN (Bio-Rad labs, Hercules, CA) according to the manufacturer's protocol.

2.7 Statistical analysis

All results are expressed as mean and standard deviation and were analysed by variance analysis (ANOVA). The threshold for significant differences was set at $p < 0.05$. When the effects of ANOVA were significant, the determination of the factors that could contribute significant differences was made by means of the Tukey multiple comparison test using the Statgraphics Plus version 5.1 statistical software (Statistical Graphics Corp, USA). The statistical analyses were made using the values of the real-time PCR tests, quantifications of MTs, and metal concentrations obtained for each biological sample collected at each sampling site. The graphs present mean values with their respective standard deviations.

3 Results

3.1 Heavy metal analysis

Metal concentrations in the digestive gland of scallops showed higher levels of Cu, Pb and Cd in the Historic District area than in the other three sites (Figs. 2A, B, C; $p < 0.05$). In the gills, a significant difference was observed in the Historic District for Cu and Pb concentrations (Figs. 2A, B) compared with the control site (La Rinconada), with Pb showing the highest concentration recorded in any of the four sampling sites (Fig. 2B). All sites showed high Cd concentrations in the digestive gland, with significant differences between the Historic District and the control (La Rinconada) (Fig. 2C; $p < 0.05$).

3.2 Relative gene expression

The study of the relative gene expression of the two genes associated with oxidative metabolism showed that only GST in the digestive gland (Fig. 3B; $p < 0.05$) in the Historic District sample is significantly different from the control. The coding gene of GPx registers a slight non-significant increase in the relative expression in both organs in the different sampling sites compared with the control site (Fig. 3A). The gene related to general stress (HSP70) shows the same trend as GST, with significant differences in the digestive gland compared with the control site (Fig. 3C; $p < 0.05$).

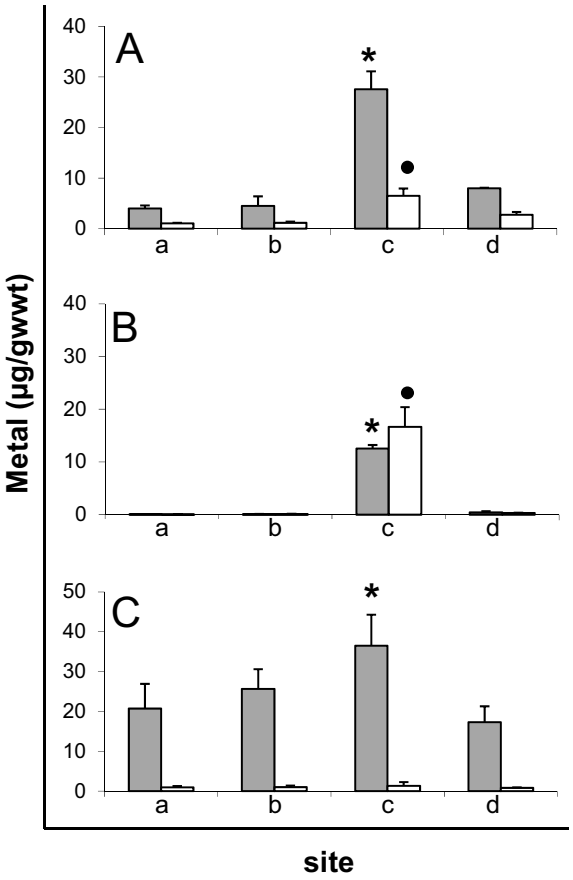

Fig. 2. Concentrations of the metals copper (A), lead (B) and cadmium (C) in *Argopecten purpuratus* from different sites in San Jorge bay, Antofagasta, Chile. (a) El Colorado, (b) La Rinconada, (c) Historic district, (d) Coloso. (■) Digestive gland, (□) gills. Bars represent the mean of 100 mg of pooled sample from each population (30 individuals per site); error bars correspond to standard deviations. (*) Indicates a significant difference at $p < 0.05$ between the digestive gland of each site compared with La Rinconada. (•) Indicates a significant difference between the gills of each site compared with La Rinconada.

3.3 Analysis of the expression of metallothionein

Figure 4 is the result of a Western blot analysis of total proteins of the digestive gland and gills in the presence of a polyclonal antibody designed for *Crassostrea gigas*. Expression of the metallothionein (MT) protein with a molecular weight of ca. 16 kDa is seen in the digestive gland (Fig. 4B) as well as in the gills (Fig. 4C).

Quantification of MT using the ELISA test (Fig. 5) showed significant differences ($p < 0.05$) in the digestive gland of scallops in the different sampling sites compared with the reference site. The highest MT concentration was recorded in the Historic District (0.075 mg g^{-1} wwt), followed by Coloso (0.055 mg g^{-1} wwt) and Colorado (0.054 mg g^{-1} wwt). For the gills, no significant differences were found between the sampled sites and the control site.

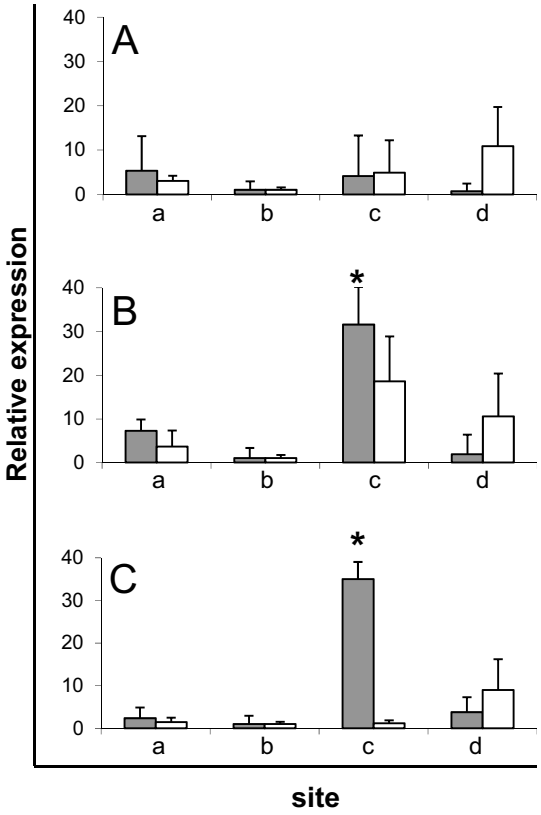

Fig. 3. Relative expression of GPX (A), GST (B) and HSP70 (C) genes in *A. purpuratus* from different sites in San Jorge bay, Antofagasta, Chile. (a) El Colorado, (b) La Rinconada, (c) Historic district, (d) Coloso. (■) Digestive gland, (□) gills. Bars represent the mean of 30 individuals per site; error bars correspond to standard deviations. (*) Indicates a significant difference at $p < 0.05$ between the digestive gland of each site compared with La Rinconada.

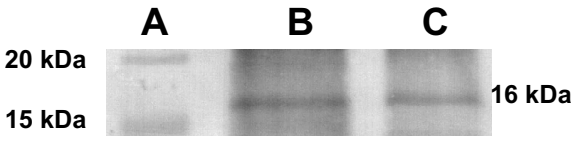

Fig. 4. Western blot of *A. purpuratus* digestive gland and gills proteins probed with mollusc biotin-labelled anti-CgMT antibody at a 1/500 dilution. (A) Protein standards (Bio-Rad labs, Hercules, CA); (B) digestive gland; (C) gills.

4 Discussion

4.1 Heavy metal analysis

The heavy metal concentrations recorded in the scallops varied between the four sampling sites in San Jorge bay, with those from the Historic District showing the highest metal concentrations in the two sampled organs (digestive gland and gills). High Cu concentrations in the Historic District were also found by Salamanca et al. (2004) in a study that determined copper in the tissues of the mussel *Perumytilus purpuratus*.

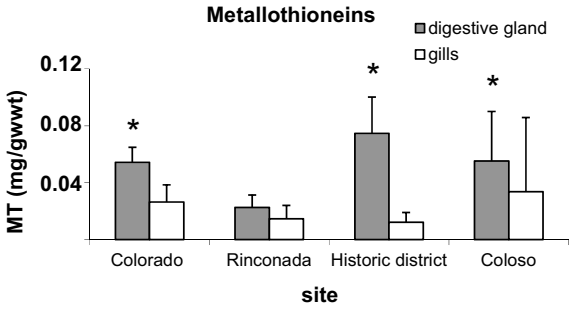

Fig. 5. Quantification of metallothioneins (MTs) in *A. purpuratus* from different sites on San Jorge bay, Antofagasta, Chile. Bars represent the mean of 30 individuals per site; error bars correspond to standard deviations (*) indicates a significant difference at $p < 0.05$ between the digestive gland of each site compared with La Rinconada.

Valdés et al. (2010) also found the same trend for this metal in their analyses of deep water and sediments, as well as in surface waters. One of the main causes of the high metal concentrations found is the closeness of this site to the international port of the city of Antofagasta. Since 1943, this port has been a centre for the loading and unloading of copper, lead, and other metallic and non-metallic products. Storage facilities have long been rudimentary and precarious, which, together with the prevailing weather conditions and wind in this area, has led to the dispersion of the stacked metals (Valdés et al. 2010).

The high Pb concentrations recorded at the Historic District site are probably due to the combined action of the wind, port activities (Salamanca et al. 2000), the presence of fuel terminals, and the effluent from the discharge of secondary water from the household sewage of the city of Antofagasta into San Jorge bay (Valdés et al. 2011). Like Antofagasta, Concepción (in the south of Chile) has a very active port, and studies by Muñoz (2002) recorded a large amount of atmospheric lead present in the bay's marine sediments due to the high anthropic activity.

Cadmium was another metal found in the scallop tissues, and, as in the case of Cu and Pb, the highest concentration was recorded at the Historic District site in the digestive gland. Cd is one of the most serious pollutants affecting the environment and human beings because it is teratogenic and carcinogenic (Nawrot et al. 2006). In all the sampling sites, considerable Cd concentrations were recorded in the digestive gland: up to nearly 132 μg g^{-1} dry wt. Such high levels can be explained because scallop species typically have higher Cd concentrations than other bivalves, due to their high assimilation efficiency and low rate of elimination (Pan and Wang 2008a). Other studies typically found much lower levels of heavy metals than those we recorded. A study made on the scallop *Chlamys nobilis* in Hong Kong recorded up to 28 μg g^{-1} dry wt Cd (Pand and Wang 2008b). Also, in the Maule region of the south of Chile, maxima of 4.3, 3.2 and 3.4 μg g^{-1} dry wt were recorded on the bivalves *Ameghinomya antiqua*, *Aulacomya atra* and *Mytilus chilensis*, respectively (Tapia et al. 2010). To date, the study by Brook and Rumsby (1965) on *Pecten novaezalandiae* has recorded higher values (299 μg g^{-1}) than those obtained in our research.

A fundamental factor affecting the spatial distribution of the metals is the system of currents in the bay (not determined in the present study). The area is subject to great wind variation, which is predominantly south and southwest (Rutllant et al. 1998), with a current field that suggests that the bay is filled through the north and empties at the south end (Escribano and Hidalgo 2001). These characteristics of the bay would influence the distribution of the metals, which decrease to the north and increase in the Historic District (downtown), due to anthropic action. Studies made of the sediments and the water column in the bay reported the same trend found in the present work (Valdés et al. 2010, 2011), but opposite to that of Salamanca et al. (2004), where the Cu concentration increased toward the north of the bay, and in the case of Pb the distribution was quite low.

4.2 Relative gene expression

The levels of mRNA expression are similar for two of the three genes selected in this study, presenting the same pattern (GST and HSP70), with overexpression in the digestive gland in the Historic District, the site with the highest recorded metal concentration.

One of the possible causes of the overexpression of mRNA of HSP70 is the high metal concentration recorded in the historic district. It is known that heat shock genes (HSPs) are induced by a number of factors other than heat, including ischemia, inflammation, osmotic and oxidative stress, and heavy metals, which affect cell protein structure and function (Feder and Hofmann 1999). All of these factors are related to cellular aging in bivalves (Ivanina et al. 2008). Similar results were obtained for the expression of the HSP70 gene in the mussel *Modiolus modiolus* obtained from a bay with municipal marine outfalls, where an overexpression of mRNA was seen in the hepatopancreas (Veldhoen et al. 2009) and adductor muscle (Veldhoen et al. 2011). Experimental exposure to heavy metals such as Cu, Pb and Hg also increased the expression of HSP70 in the digestive gland and gills of the mussel *Dreissena polymorpha* (Navarro et al. 2011). Likewise, in the oyster *Crassostrea virginica*, a strong overexpression of the gene was seen in the hepatopancreas and gills when they were exposed to high Cd concentrations and temperature (Ivanina et al. 2009). The increased expression of the mRNA of HSP70 in bivalves is also associated with the response to stress after exposure to metals and xenobiotics (Franzellitti and Fabri 2005; Dondero et al. 2006; Song et al. 2006).

The two genes related to oxidative metabolism (GPx and GST) showed the same pattern in the expression levels of their mRNA in the sites with lower metal concentrations, in contrast to the Historic District, where the expression of the mRNA of GST showed a significant overexpression in the digestive gland. Although a slight increase in the expression of the mRNA of GPx in the sampling sites was recorded, this increase was not statistically significant compared with the control (La Rinconada). One of the causes for the reduction of the activity of GPx is probably the decrease in the amount of glutathione (Jadhav et al. 2007), due to the affinity of heavy metals for thiol groups, leading to the intracellular exhaustion of these groups, thereby altering the nature and activity of the proteins within the cells and possibly contributing to oxidative stress (Becker and Soliman 2009). This is consistent with previous studies that showed a decrease of the expression levels of the mRNA of GPx in the Asian scallop *Corbinucula fluminea* and the gastropod *Achatina fulica*, with an increased level of $CdCl_2$ (Chandran et al. 2005; Legeay et al. 2005), and in the bivalve *Chamys farreri* exposed to Pb (Zhang et al. 2010). However, GST codes one of the many enzymes of phase II of the respiratory chain that catalyze the conjugation of glutathione with various electrophilic substances, and has a preventive role against oxidative damage through conjugation of the decomposition products of lipid peroxides with glutathione (Ketterer et al. 1983). The observed increase in GST activity in the present study could be due to the natural activation of the antioxidant defence system for the presence of a high concentration of metals and other xenobiotics (not determined for this study), and suggests that detoxification processes against pro-oxidation forces have been induced. This increase has also been reported in other bays that show some degree of pollution caused by municipal outfall, with an increase in GST expression observed in the digestive gland and gills of the scallop *Argopecten gibbus* (Quinn et al. 2005). Additionally, an effect was observed in the gills of *Mytilus galloprovincialis* in lakes with high tourist activity, caused by activation of detoxification processes (Maria et al. 2009). In another study on the same organism, Cu treatments led to an increase of GST activity in the digestive gland as well as in the gills; this decreased the total amount of GSH possibly reflecting an increased utilization of GSH in conjugation reactions involved in the metabolism of lipid hydroperoxide and carbonyl compounds formed by the Cu-induced peroxidation of cellular membranes (Canesi et al. 1999).

In many cases, exposure to environmental contamination is correlated with changes in the conditions of biomolecules, usually related to oxyradical metabolism and the detoxification of metals and drugs (Gorbi et al. 2008), and these can modify the abundance of mRNA within the transcriptome in the affected tissues (Tanguy et al. 2005; Venier et al. 2006).

4.3 Quantification of metallothionein (MT)

The multifaceted role of MTs includes involvement in homeostasis, protection against heavy metals and oxidant damage, metabolic regulation, sequestration and/or redox control, and could be induced by heavy metals, which are otherwise able to hinder gametogenesis, suppress embryogenesis, and hamper development (Mao et al. 2012). These properties of MT indicate that it is an important factor affecting metal toxicity in aquatic invertebrates (Xie and Klerks 2004). Before quantifying MT in the tissues of the scallop *Argopecten purpuratus*, the effectiveness of the CgMT antibody obtained from the proteins of the oyster *Crassostrea gigas* (Boutet et al. 2002) was tested by a cross-reaction of the proteins isolated from the digestive gland and the gills of the scallop. The results revealed a band of approximately 16 kDa for each tissue. The MT isoforms that were recorded from *A. purpuratus* are within the range found in the studies performed on *Mytilus edulis*, showing two MT isoforms, MT10 of 10 kDa and MT20 of 20 kDa (Mackay et al. 1993). These results suggest the presence of at

least one type of MT in the digestive gland and gill, validating this polyclonal antibody as a suitable method to quantify the presence of these proteins in the tissues of scallops and possibly in other molluscs. The increase in the expression of MT protein is partly due to the metal concentrations, which had accumulated at significantly higher levels in the digestive gland at the tested sites compared to scallops obtained from the control site La Rinconada. It is important to note that the expression of MT is induced by a wide range of substances and stimuli, including heavy metals, oxidative stress, hypoxia, and endocrine signals (Kägi and Schäffer 1988). Our results confirm that MT acts as a specific indicator of the biological effects of heavy metals in marine organisms, in agreement with previous studies on the mussel *Mytilus galloprovincialis* exposed to Cd^{2+}, which induced a significant increase of MT levels in the digestive gland (Pytharopoulou et al. 2011), and in *Dreissena polymorpha*, where Cd was the main inducer of MT levels compared with other metals tested (Navarro et al. 2011).

The ability to synthesize and accumulate MT in response to metal exposure to metals varies among marine molluscs. For example, in *Crassostrea gigas*, protein levels depend on the metal concentration in the sampling sites (Boutet et al. 2002); in contrast, in the digestive gland of *M. galloprovincialis*, natural factors contribute more to MT content than sub-lethal Cd concentrations (Raspor et al. 2004). In other bivalves, such as the scallop *Argopecten gibbus*, it was found that MT levels increase significantly in the gills in sites contaminated with municipal sewage (Quinn et al. 2005). Other factors must be considered such as physiological changes caused by gonad development and the abundance of food, which can contribute to high variability in MT levels, as in the case of the bivalve *Cerastoderma glaucum*, which can interfere in the estimation of the changes induced by anthropogenic sources (Machreki-Ajmia et al. 2011).

5 Conclusion

The bay of San Jorge in Antofagasta has a well-defined sector around the city's international port, which has a high heavy metal content. The port area has historically been used for the loading and unloading of minerals for more than 60 years, and has seen an massive increase in traffic flow. The abundance of heavy metals is reflected in the data presented in our present study, showing that the battery of biomarkers we measured in *Argopecten purpuratus* is sensitive to the levels of heavy metals recorded in the environment, and illustrates the feasibility of using them as molecular biomarkers of heavy metal contamination in ecosystems. Furthermore, these results demonstrate that, in the future, *A. purpuratus* may be utilized as a promising tool for the evaluation of environment risk.

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