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Copper Metallothionein-like Proteins as Exposure Biomarker in Native and Transplanted Intertidal Populations of the Mussel *Perumytilus purpuratus* from San Jorge Bay, Antofagasta, Chile

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Metallothioneins (cytosolic and sulphydryl-rich proteins) have high affinity for divalent cations (Bonwick *et al.* 1990; Viarengo and Nott 1993; Viarengo *et al.* 1993). These proteins are involved in metal cell homeostasis, with possible biological functions in storage, transport or compartmentalization of essential metals (Di Giulio *et al.* 1995). Intracellular binding of metals to metallothionein has also been considered a detoxification mechanism, sequestering and preventing harmful interactions of heavy metal cations with biological molecules (Viarengo and Nott 1993). Accordingly, metallothioneins have been used as exposure biomarker for the detection of heavy metal pollution in the aquatic environment (Pavicic *et al.* 1993; Viarengo *et al.* 1997). Several papers have described the presence of metallothioneins in bivalves and the feasibility of using them to assess exposure to heavy metals in marine environments (Bebianno and Langston 1991; Pavicic *et al.* 1993; Viarengo *et al.* 1993, 1997; Bebianno and Machado 1997; Bordin *et al.* 1997; High *et al.* 1997; Baudrimont *et al.* 1999; Serafim and Bebianno 2001; Geret and Cosson 2002).

Northern Chile has been characterized by intensive copper mining activities, which have led to pollution by heavy metals in coastal ecosystems (Castilla and Correa 1997; Fariña and Castilla 2001). San Jorge Bay (Antofagasta), is a coastal area with sites subjected to anthropogenic activities. Some sites receive discharges of urban and industrial effluents and others remain without any anthropic intervention at all. Consequently, this area is particularly interesting for the study of copper dynamics in the marine environment in order to assess the potential effects of copper on the biota.

The main goals of this work were to measure copper metallothionein-like proteins in the mussel *Perumytilus purpuratus*, including native and transplanted populations in intertidal sites at San Jorge Bay, to assess its use as exposure biomarker for copper. Moreover, copper concentrations in seawater and in mussel soft tissues were related to copper metallothionein-like protein contents.

MATERIALS AND METHODS

Three field experimental sites were selected in the *P. purpuratus* intertidal fringe

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of San Jorge Bay (23°28'-23°46'S; 70°24'-70°37'W): Site 1 (Reference), south of Antofagasta, in which neither industrial discharges nor human settlements exist; Site 2 (Coloso), north of Reference site, near the port facilities of Minera Escondida Ltda. (a copper Mine); and Site 3 (Effluent Receptor), north of Antofagasta, which receives an urban-industrial effluent, discharged with no previous treatment In March 1998, three transplant cage systems were installed at each site. Cages consisted of aluminum quadrants of 40 x 40 cm, attached to the rocks. Approximately 200 mussels (ca. 3.0 cm length) collected from Site 1 (Reference) were meshed inside each cage. Seawater copper concentrations were measured in three samples taken around the cages at the beginning of the experiment (March) and after 45 (May) and 90 (July) days of exposure. At the same time, 120 individuals, both native and transplanted *P. purpuratus*, were collected and analyzed for copper contents and detection of copper metallothionein-like proteins in soft tissues.

One L seawater samples were acidified on site with concentrated suprapur HNO_3. 250 mL of each sample were filtered through a 0.45 µm nitrocellulose membrane, previously washed with suprapur HCl 3 N. Copper concentrations were determined by Anodic Stripping Voltametry (ASV) in a ISS-820 Radiometer (Román and Rivera 1992). Cass-3 (National Research Council Canada, Division of Chemistry, Marine Analytical Standard Program) was used as standard and 98.61% of recovery was obtained.

Three replicates of 10 mussels each were used to measure copper concentrations in gonads, gills and remaining tissues (included digestive and muscle tissues). The procedures used conform to UNEP (1984) recommendations. Tissues were dissected with a titanium knife, mixed and then homogenized with a T-25 Ultra-Turrax homogenizer, using a teflon-coated tissue grinder. About 0,5 g of each sample were pre-digested overnight at ambient temperature with 10 mL concentrate HNO_3. Digestion was performed with 10 mL trace-metal grade HNO_3 at 150 °C by 4 h, using an acid-cleaned teflon bomb. Samples were cooled and re-dissolved in 25 mL HNO_3 3 N, and the resulting solution was filtered through a 0.45 µm nitrocellulose membrane. Copper analysis was made using an Atomic Absorption Spectrophotometer GBC-905 PBT with flame atomization. DORM-1 (National Research Council Canada, Division of Chemistry, Marine Analytical Standard Program) was used as standard and 98.08% of recovery was obtained.

Analyses of copper metallothionein-like proteins were performed in male and female specimens separately and an indirect method was used by measuring copper bound to partially purified metallothionein. Soft tissues from 20 mussels samples were dissected, pooled and homogenized in 3 volumes of 20 mM Tris-HCl (pH 8.6), 0.25 M sucrose, 0.5 M NaCl, 20 mM Hepes, 500 mM de NaCl, 1% β-mercaptoethanol (as reducing agent), 0.5 mM PMSF (protease inhibitor) and 0.02 % sodium azide (as an antibacterial agent). Then, the homogenate was centrifuged at 10.000 x g for 10 min. The supernatant was exposed to 100 °C during 5 min and then it was again centrifuged at 10.000 x g for 10 min (Stagg *et*

al. 1992; Bonwick *et al.* 1990). The final supernatant (*i.e.* cytosol) was stored at -20°C until required for chromatographic analysis. Finally, for each sample, 8 mL of supernatant (*i.e.* 7 g of soft tissue) were fractionated through gel chromatography using a Sephadex G-75 column (2.6 x 30 cm), eluted with 20mM Tris-HCl (pH 8.6). Fractions of 5 mL were collected in an automatic fraction collector. The proteins in eluted fractions were monitored at 280 nm using an UV spectrophotometer. Copper content was measured directly by flame atomic absorption spectrophotometry at intervals of 3 fractions app. Blue dextran, carbonic anhydrase, bovine albumin, cytochrome C and Zn-metallothionein (Metallothionein II, Zinc, Sigma Chemical Co., St. Louis, MO, USA) were used as protein standards to calibrate the column by molecular weight.

Statistical tests were applied using Systat version 5.0 (Wilkinson 1992) and Statistica version 5.1 (Statsoft, Inc 1998). The significance criteria used was α=0,05. Significant differences were found using ANOVA, considering either seawater, tissue copper concentration or copper concentration bound to metallothionein-like proteins as the dependent variable. Factors were site (Site 1, Site 2, Site 3), date (March, May, July) and population (Native, Transplanted). A Tukey HDS multiple comparison test was applied when significant differences were found between treatments.

RESULTS AND DISCUSSION

Table 1 shows total dissolved copper concentrations in seawater of the three experimental sites (mean ± standard deviation). ANOVA indicated that variance was mainly explained by differences between sites (96%). Multiple comparison tests showed that seawater copper concentrations in Site 3 were always significantly higher than those in Site 1 and Site 2 ($p<0.02$). Seawater copper contents in Site 3 were always above 4.5 $\mu g\ L^{-1}$ (5.9 ± 0.8 $\mu g\ L^{-1}$), exceeding USEPA seawater chronic quality criteria (3.1 $\mu g\ L^{-1}$, USEPA 1999). Similarly, a previous study in San Jorge Bay detected higher seawater copper concentrations in Site 3 (2.363 ± 0.318 $\mu g\ L^{-1}$) than in Site 1 (1.119 ± 0.453 $\mu g\ L^{-1}$) and Site 2 (1.480 ± 0.523 $\mu g\ L^{-1}$) (Rodríguez 1997).

Table 1. Total dissolved copper concentration in seawater ($\mu g\ L^{-1}$) from San Jorge Bay (mean ± standard deviation, n=3).

Sampling date	Site 1	Site 2	Site 3
March (initial)	0.833 ± 0.044	1.262 ± 0.423	6.241 ± 0.045
May (45 days)	0.831 ± 0.195	0.729 ± 0.156	5.112 ± 0.737
July (90 days)	0.683 ± 0.038	1.088 ± 0.260	6.331 ± 0.628

Table 2 shows copper concentration in soft tissues of native and transplanted populations of *P. purpuratus* (mean ± standard deviation). Transplants from Site 3 in July are not included, because transplant cages were destroyed. Copper concentrations in soft tissues of *P. purpuratus* of the three sites showed a similar tendency to that of seawater copper concentrations. Hence, ANOVA results for

Table 2. *Perumytilus purpuratus*. Tissue copper concentrations (gonads, gills and remaining tissues) in native and transplanted mussel populations in the three study sites (μg g^{-1}wet weight; mean ± standard deviation; n=3; R.T.: remaining tissues).

Tissue	Population	Site 1		
		March	May	July
Gonads	Native	4.6 ± 0.9	5.8 ± 1.2	5.9 ± 1.3
	Transplanted	4.6 ± 0.9	5.9 ± 2.1	5.7 ± 1.1
Gills	Native	9.8 ± 0.5	6.8 ± 2.9	4.6 ± 1.9
	Transplanted	9.8 ± 0.5	5.3 ± 0.7	4.4 ± 1.5
R.T.	Native	10.3 ± 1.3	8.6 ± 0.9	7.1 ± 1.7
	Transplanted	10.3 ± 1.3	5.3 ± 1.1	7.2 ± 1.8
		Site 2		
Gonads	Native	4.5 ± 1.3	7.3 ± 5.5	4.9 ± 0.6
	Transplanted	4.6 ± 0.9	5.4 ± 2.8	3.5 ± 1.1
Gills	Native	8.4 ± 1.3	6.9 ± 3.0	3.4 ± 1.2
	Transplanted	9.8 ± 0.5	6.8 ± 0.8	3.0 ± 1.0
R.T.	Native	7.8 ± 1.9	9.8 ± 0.6	4.8 ± 1.1
	Transplanted	10.3 ± 1.3	9.4 ± 4.4	4.0 ± 0.9
		Site 3		
Gonads	Native	9.8 ± 5.0	14.5 ± 1.6	106.6 ± 74.1
	Transplanted	4.6 ± 0.9	15.3 ± 3.7	
Gills	Native	19.0 ± 4.3	11.4 ± 5.7	64.8 ± 36.2
	Transplanted	9.8 ± 0.5	11.1 ± 4.1	
R.T.	Native	16.4 ± 10.5	16.1 ± 1.2	145.1 ± 70.5
	Transplanted	10.3 ± 1.3	17.7 ± 3.5	

the three tissues analyzed showed the site factor as the main source of variance (46% of variance in gonads, 47% in gills and 49% in remaining tissues). Mussels from Site 3 showed significantly higher copper concentrations than mussels from Site 1 and Site 2 ($p<0.001$). Tissues showed significant differences in copper concentrations between dates and the interaction site-month Multiple comparisons showed that Site 3 had the highest tissue copper concentrations in July (between 34.3 and 222.9 μg g^{-1}). Otherwise, transplanted mussels from Site 3 showed increased copper concentrations from March to May. It is important to notice that, in spite of the fact that seawater copper concentrations did not show significant variations in time, native mussel populations from Site 3 had higher tissue copper concentrations in July (105.5 ± 64.4 μg g^{-1}) than individuals sampled in March and May (14.5 ± 5.7 μg g^{-1}). Differences in variability between seawater and tissue copper concentrations could be related to high bioavailability of copper between May and July, produced by episodic variability in the effluent pollutants load or temporal changes in seawater organic contents. This strongly suggests, what has been otherwise noted, that mussels are efficient integrators of temporal and spatial variations of pollutants (particularly heavy metals) in aquatic environments (Cossa 1989; Rainbow and Phillips 1993; Soto *et al.* 1995; Rainbow 1995). Results from native mussel populations were similar to those obtained by Rodríguez (1997), *i.e.* copper concentrations in Site 3 (16.5 ± 6.2 μg

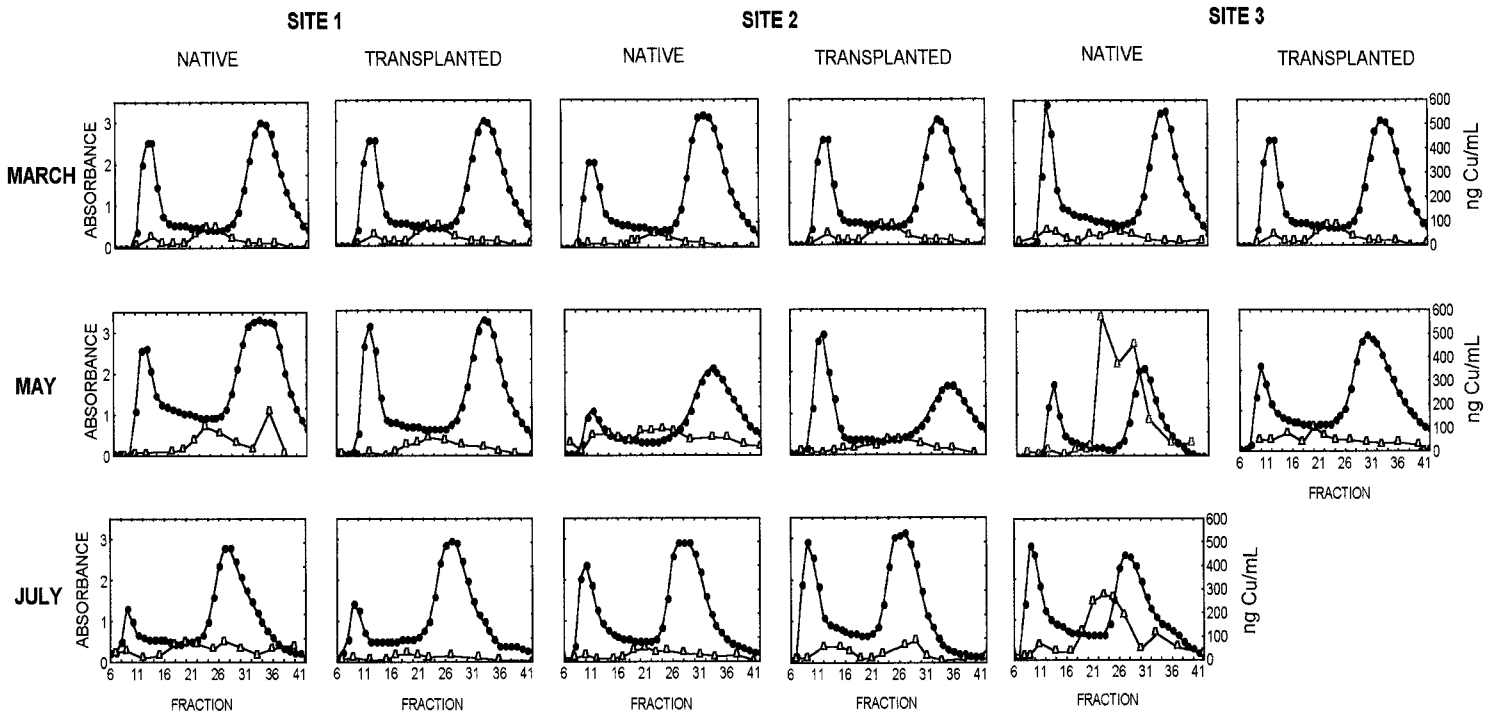

Figure 1. *Perumytilus purpuratus*. Chromatographic elution profiles of the cytosol on Sephadex G-75 column, in female specimens of native and transplanted populations from San Jorge Bay. (●) absorbance (280 nm); (△) copper concentration.

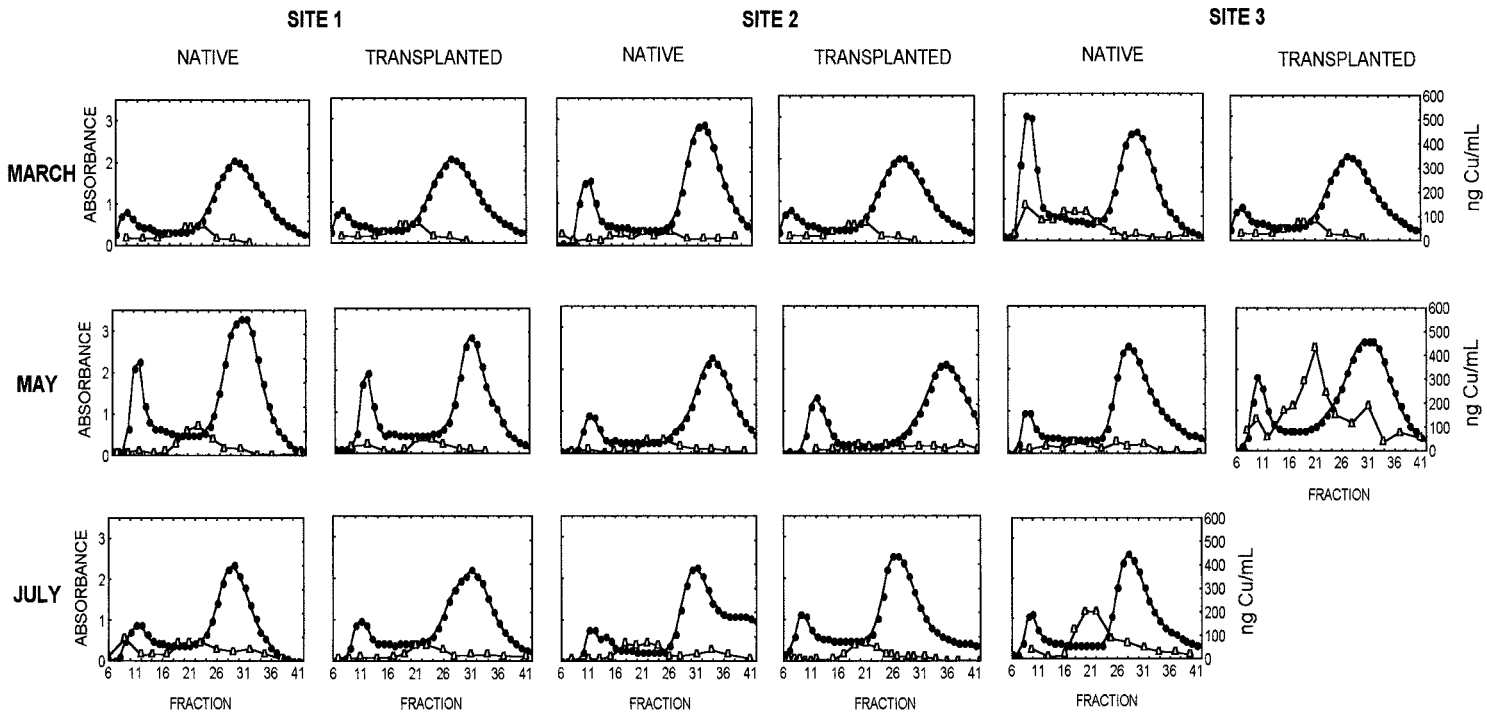

Figure 2. *Perumytilus purpuratus.* Chromatographic elution profiles of the cytosol on Sephadex G-75 column, in male specimens of native and transplanted populations from San Jorge Bay. (●) absorbance (280 nm); (△) copper concentration.

g^{-1}) were higher than those measured in Site 1 (3.6 ± 0.4 μg g^{-1}) and Site 2 (5.6 ± 1.3 μg g^{-1}).

Figures 1 and 2 show absorbance at 280 nm and copper concentration in chromatographic fractions of homogenates of *P. purpuratus* populations from the three sampling sites. All samples showed a similar profile of absorbance, that coincides with the typical chromatographic elution profiles described for cytosol from mollusks tissues. The earlier peak corresponds to the high molecular weight protein pool (HMW), *e.g.* enzimes and hemocianine, the second peak corresponds to very low molecular weight compounds (VLMW), that include amino acids. Between these peaks, medium and low weight molecular proteins are eluted, including metal-binding proteins (Bebianno and Langston 1991; Bebianno *et al.* 1992; Viarengo *et al.* 1993; Pavicic *et al.* 1993). Copper detected in the last fractions of this range was expected to correspond to the concentration of this metal-bound metallothionein-like proteins, since Zn-metallothionein standard (*ca.* 10,000 daltons) eluted in fraction 21. In spite of the variability, copper concentration in this fraction in samples from Site 3 were significantly higher than those from the other sites (Site 1 and 2; $p<0.05$). The highest peaks of copper were present in male and female samples from Site 3.

There was a coincidence among copper concentrations in seawater, tissues and copper bound to metallothionein-like proteins in *P. purpuratus* populations from San Jorge Bay. This implies that mussels from Site 3 (Effluent Receptor) are exposed to higher copper concentrations than those from Site 1 (Reference) and Site 2 (Coloso) and suggests that the exposure to this metal produced an increase of copper bound to metallothionein-like proteins. In another paper (Riveros *et al.* 2002) we reported that cellular biomarkers (lysosomal stability in hemocytes and amount of lipofuscine granules and vacuoles in digestive cells) in *P. purpuratus* detected adverse biological changes produced by environmental conditions in Site 3. Accordingly, these results further support that metallothionein-like proteins in *P. purpuratus* offer a useful approach to assess exposure to heavy metals in aquatic environments in the Chilean coast, although more studies and data are desirable to validate this specific exposure biomarker as reliable tool for pollution survey.

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