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Cellular Biomarkers in Native and Transplanted Populations of the Mussel *Perumytilus purpuratus* in the Intertidal Zones of San Jorge Bay, Antofagasta, Chile

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Abstract. Cellular biomarkers were measured in the mussel *Perumytilus purpuratus* from intertidal zones of San Jorge Bay, Antofagasta, Chile. They were also used to measure sublethal effects on individuals exposed to Cu under laboratory conditions. Lysosomal stability in hemocytes, and the degree of vacuolization and the content of lipofuscin granules in digestive cells were the cellular responses measured. Three study sites were established in San Jorge Bay: Coloso, E.R., and Reference. Both E.R. and Coloso receive effluent discharges. Reference does not receive any sewage discharges. Before sampling, mussels from Reference were transplanted into the intertidal zone of each site. Samplings were obtained at the beginning, after 45 days, and after 90 days after transplantation. Seawater samples for total dissolved Cu analysis and adult mussels (*P. purpuratus*) from native and transplanted populations were collected each time. Cellular biomarkers and Cu concentrations in gonads, gills, and remaining tissues (gut and muscle) were measured. Mussels from Reference were exposed to sublethal Cu concentrations (5, 10, 20, 30, 40, and 80 $\mu g\ L^{-1}$) during 45 days under laboratory conditions. Lysosomal stability was measured in mussel hemocytes by means of the neutral red retention assay. The degree of vacuolization and the extent of lipofuscin granules were determined in the digestive cells by image analysis of histological sections stained with the Schmorl's method. Seawater Cu concentrations and tissue Cu concentrations in *P. purpuratus* were higher in E.R. than in Reference and Coloso ($p < 0.02$). Native mussel populations from E.R. showed lower lysosomal stability ($p < 0.05$), higher vacuolization degree ($p < 0.001$), and lower amounts of lipofuscin granules ($p < 0.001$) than those from Coloso and Reference. Transplanted mussel to E.R. showed significant reduction in lysosomal stability ($p < 0.05$) and in extent of lipofuscin granules ($p < 0.05$) and significant increase in vacuolization degree ($p < 0.05$), whereas Reference and Coloso are not significantly dissimilar between them. Seawater Cu concentration was positively correlated with Cu content in gonads ($r^2 = 0.61$; $p < 0.02$), gills ($r^2 = 0.66$; $p < 0.01$), remaining tissues ($r^2 = 0.56$; $p < 0.05$), and the degree of vacuolization ($r^2 = 0.65$; $p < 0.01$) and negatively with lysosomal stability ($r^2 = 0.79$; $p < 0.001$) and lipofuscin granules extent ($r^2 = 0.53$; $p < 0.05$). Mussels exposed to Cu under laboratory conditions showed decreased lysosomal stability (over 30 $\mu g\ Cu\ L^{-1}$) ($p < 0.02$) and increased degree of vacuolization (at 80 $\mu g\ Cu\ L^{-1}$) ($p < 0.05$) and an increased lipofuscin granules extent (although differences among treatments were not statistically significant).

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Cellular biomarkers are used as measures of the sublethal effects of pollutants that can indicate a progressive cell damage with physiological consequences (Hebel *et al.* 1997). Thus measuring at the cellular level may provide a fast and sensitive indication of environmental pollution (Moore 1985; Viarengo and Canesi 1991; Lin and Steichen 1994).

Lysosomes constitute a group of subcellular structures involved in intracellular digestion that compartmentalize and accumulate a wide range of pollutants (Viarengo and Canesi 1991; Hole *et al.* 1993; Viarengo and Nott 1993). Thus pollution causes damage in these organelles, which can be measured by different means, including lysosomal stability tests (Lowe *et al.* 1992, 1995; Regoli 1992; Lin and Steichen 1994; Krishnakumar *et al.* 1994; Hole *et al.* 1995) and the measure of vacuolization and lipofuscin granules extent (Moore 1985; Viarengo *et al.* 1987; Cajaraville *et al.* 1989; Regoli 1992; Viarengo and Nott 1993; Etxeberria *et al.* 1995).

The measurement of lysosomal membrane stability is used as an integrative biomarker of cellular stress, as membrane integrity is affected by different pollutants (Moore 1985; Viarengo *et al.* 1987; Mayer *et al.* 1989; Lin and Steichen 1994). There is evidence of decreased lysosomal membrane stability in mussels exposed to pollutants under laboratory conditions and mussels collected from contaminated areas (Moore *et al.* 1978; Widdows *et al.* 1982; Ward 1990; Krishnakumar *et al.* 1994). Particularly, various investigations have been addressed to

study lysosomal membrane destabilization in hemocytes (Lowe *et al.* 1995; Svendsen and Weeks 1995).

In addition, digestive cells also posses a well-developed lysosomal system that is responsive to diverse pollutants, including heavy metals (Moore 1985, 1988). Exposure to pollutants causes changes in size and number of lysosomes, alterations in membrane permeability and osmotic disruption, which altogether lead to vacuolization of digestive cells (Moore 1985, 1988; Cajaraville *et al.* 1989; Lowe *et al.* 1992; Hole *et al.* 1995). Lipofuscin granules are insoluble structures formed by lipoproteins, debris of cellular membranes, and several organic and inorganic compounds (Viarengo and Canesi 1991; Viarengo and Nott 1993). Lipofuscin granules are the result of lipid peroxidation, and their accumulation within cells can reflect the organism metabolic age (Nicol 1987; Sheehy 1992; Donovan and Tully 1996). Lipofuscin granules can contribute to metal detoxification wherever they are retained as residual bodies or excreted by exocitosis (Moore 1985, 1988; Viarengo *et al.* 1987; Viarengo and Nott 1993).

The primary goal of this study was to measure cellular biomarkers in the mussel *Perumytilus purpuratus* from intertidal zones of San Jorge Bay, Antofagasta, Chile (23°28′–23°46′S; 70°24′–70°37′W). The degree of vacuolization and extent of lipofuscin granules in digestive cells and lysosomal stability in hemocytes were assessed in individuals collected from native and transplanted populations of *P. purpuratus*. Taking into account that the study area is characterized by intensive copper mining activity, seawater total dissolved Cu concentrations and tissue Cu concentrations in *P. purpuratus* were determined. On the other hand, cellular responses were measured in mussels exposed to sublethal Cu concentrations under laboratory conditions.

Materials and Methods

Study Area

Three sampling sites were established in the distribution fringe of *P. purpuratus* of intertidal zones in San Jorge Bay (Figure 1): E.R. (Effluent Receptor) is a marine area that received an industrial-domestic effluent discharged without previous treatment during this study; Coloso is located near the port facilities of the copper mine Minera Escondida Ltda. and receives pretreated effluent discharges; and Reference is a place in which neither industrial discharges nor human settlement exist.

Sampling and Transplant of Mussels

Three cage transplant systems were installed in each site, consisting of aluminum quadrants (40 × 40 cm) fixed to rocks with 200 mussels from Reference (between 2.5 and 3.5 cm of length). Seawater Cu concentrations were determined in three samples taken around experimental the transplant cages at the beginning (March) and 45 (May) and 90 days (July) after transplantation. Simultaneously, 120 mussels were collected at each sampling site from both native and transplanted populations to analyze Cu concentrations in soft tissues and measure cellular biomarkers.

Exposure to Sublethal Copper Concentrations Under Laboratory Conditions

Mussels collected from Reference were exposed to sublethal Cu concentrations under laboratory conditions. Mussels were exposed during 45 days in semistatic systems with replacement of sea water every 48 h. The experimental design consisted of six Cu treatments (80, 40, 30, 20, 10, and 5 $\mu g\ L^{-1}$) plus a control, each one with 250 individuals maintained in 8-L (approximately 60 g wet weight L^{-1}) sea water with constant oxygenation. After exposure to Cu, cellular biomarkers were measured.

Copper Analysis in Sea Water and Soft Tissues of P. purpuratus

The seawater samples were immediately acidified with suprapure HNO_3 concentrate. Two hundred fifty milliliters of the samples were filtrated by 0.45 μm nitrocellulose membranes, previously washed with suprapure HCl 3 N. Then, Cu concentration was determined by anodic stripping voltametry (ASV) in a ISS-820 Radiometer (Roman and Rivera 1992). Cass-3 (National Research Council Canada, Division of Chemistry, Marine Analytical Standard Program) was used as standard. Tissue Cu concentrations were measured in three samples of composite soft parts (*i.e.*, gonad, gill, and remaining tissues), according to UNEP (1984). Tissues were dissected out with a titanium knife, mixed, and then homogenized with a T-25 Ultra-Turrax homogenizer, using a Teflon-coated tissue grinder. Each sample (0.4–0.6 g) was predigested overnight at ambient temperature with 10 ml concentrated HNO_3. Then the digestion was made with 10 ml trace metal–grade HNO_3 at 150°C for 4 h under pressure, using an acid-cleaned Teflon bomb. The cooled samples were redissolved in 25 ml HNO_3 3 N, and the resulting solution was filtrated (0.45 μm membrane). Cu 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.

Lysosomal Membrane Stability

Retention time of neutral red in hemocytes was measured in 10 mussels per sample of both field and laboratory Cu exposure, according to Lowe *et al.* (1992) and Svendsen and Weeks (1995). A 0.5-ml aliquot of blood sample was extracted from the adductor muscle of each individual and mixed with an equal volume of mussel physiological serum (Guilles 1975). This suspension was maintained in ice before use. A subsample of blood was used to test living cells with Eosin Y. The working solution of neutral red was prepared by diluting 10 μl stock solution (20 mg per 1 ml of dimethyl sulfoxide) with 5 ml physiological serum. In the assay, 50 μl blood suspension was dispensed in a glass slide, and 40 μl of working solution were added after 3 min. Each glass slide was then observed at the light microscope to determine the retention time, which corresponded to the time at which the dye leaches into the cytosol. The assay was terminated when the dye loss from lysosomes was evident in 80% of the cells.

Histological Analysis of Digestive Tubules

The digestive gland of 10 individual mussels per sample, in both field and laboratory experiments, was fixed with 4% formaldehyde in sea water, embedded in paraffin, and sectioned serially at 6 μm. Sections were stained for lipofuscin using the Schmorl's method (Pearse 1960;

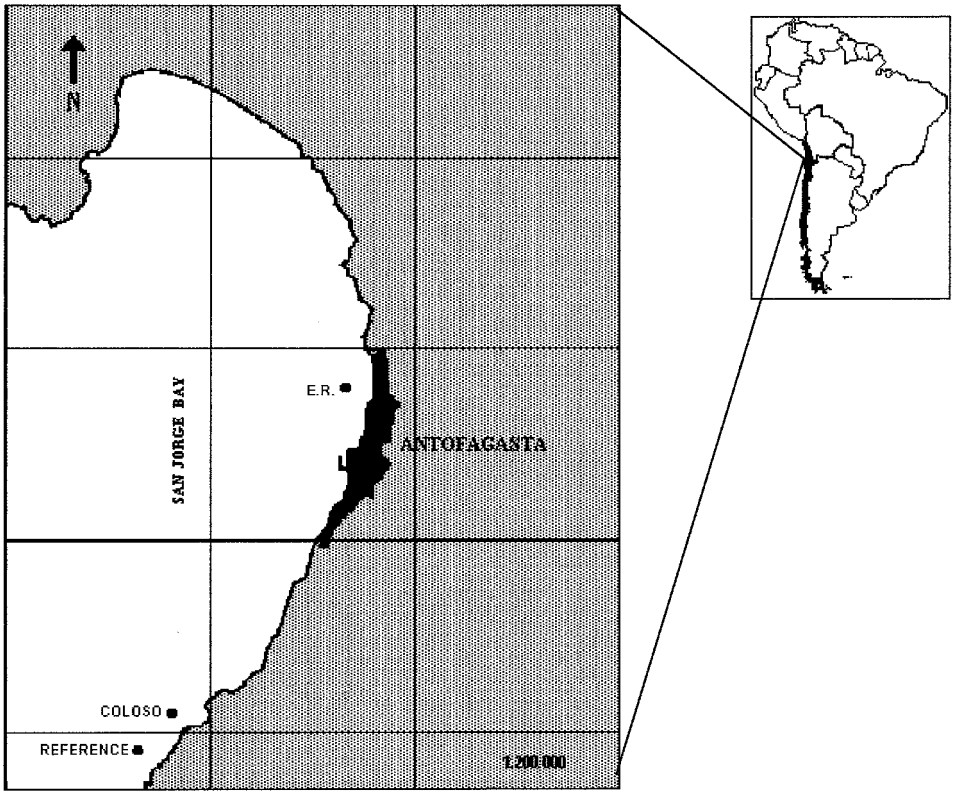

Fig. 1. Location of study intertidal sites in San Jorge Bay (Antofagasta-Chile)

Barka and Anderson 1967; Krishnakumar *et al.* 1994). Histological sections were observed through light microscope to determine the extent of vacuolization and lipofuscin granules in the digestive tubules. An area with tubules was chosen from each histological section in 100× micrographs. The presence of vacuoles and lipofuscin granules, was confirmed by transmission electron microscopy in digestive gland of mussels collected from the three sites.

Image Analysis of Digestive Tubules

Light micrographs were scanned and analyzed using the program Idrisi for Windows (version 1.0). A segmentation procedure was applied to identify with different values the vacuoles, the lipofuscin granules and the rest of cell area to further quantify the number of pixels occupied by each of these structures. Thus both the relative section-areas of lipofuscin granules and vacuoles were estimated.

Data Analysis

Linear or power (X^r) transformations were used to standardize raw data. The symmetry and normality evaluation for each transformation was assessed using the Kolmogorov-Smirnov test. Significant differences were found using analysis of variance (ANOVA) from one to three ways, considering as dependent variable either seawater or tissue Cu concentration, neutral red retention time, and section-areas of vacuoles or lipofuscin granules. Analyzed factors were site (Reference, Coloso, and E.R.), date (March, May, and July), population condition (native or transplanted), and the interactions of these factors. For the laboratory experiments, one-way ANOVAs were applied considering Cu concentration as factor for the variables neutral red retention time and section-areas of vacuoles and lipofuscin granules. A Tukey HDS multiple comparison test was made when significant differences were found among treatments. Additionally, the relationships of seawater Cu concentration with the tissue Cu concentration, the neutral red retention time, and the section-areas of vacuolization and lipofuscin granules were estimated using minimal square linear regression. For these analyses, only native mussel populations were used because the results corresponding to the transplanted experiments in March could not be included.

All statistical tests were made using SYSTAT version 5.0 (Wilkinson 1992) and STATISTICA version 5.1 (1998). The significance level used was $\alpha = 0.05$.

Results

Table 1 shows mean and standard deviation values of total dissolved Cu concentration in sea water from the three study sites. Seawater Cu concentration in E.R. was significantly higher than in Coloso and Reference in all cases ($p < 0.02$). Accordingly, the three analyzed tissues (gonads, gills, and

Table 1. Seawater total dissolved Cu concentrations ($\mu g\ L^{-1}$) in the study sites from San Jorge Bay (mean ± SD, n = 3)

Sampling Date	Reference	Coloso	E.R.
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

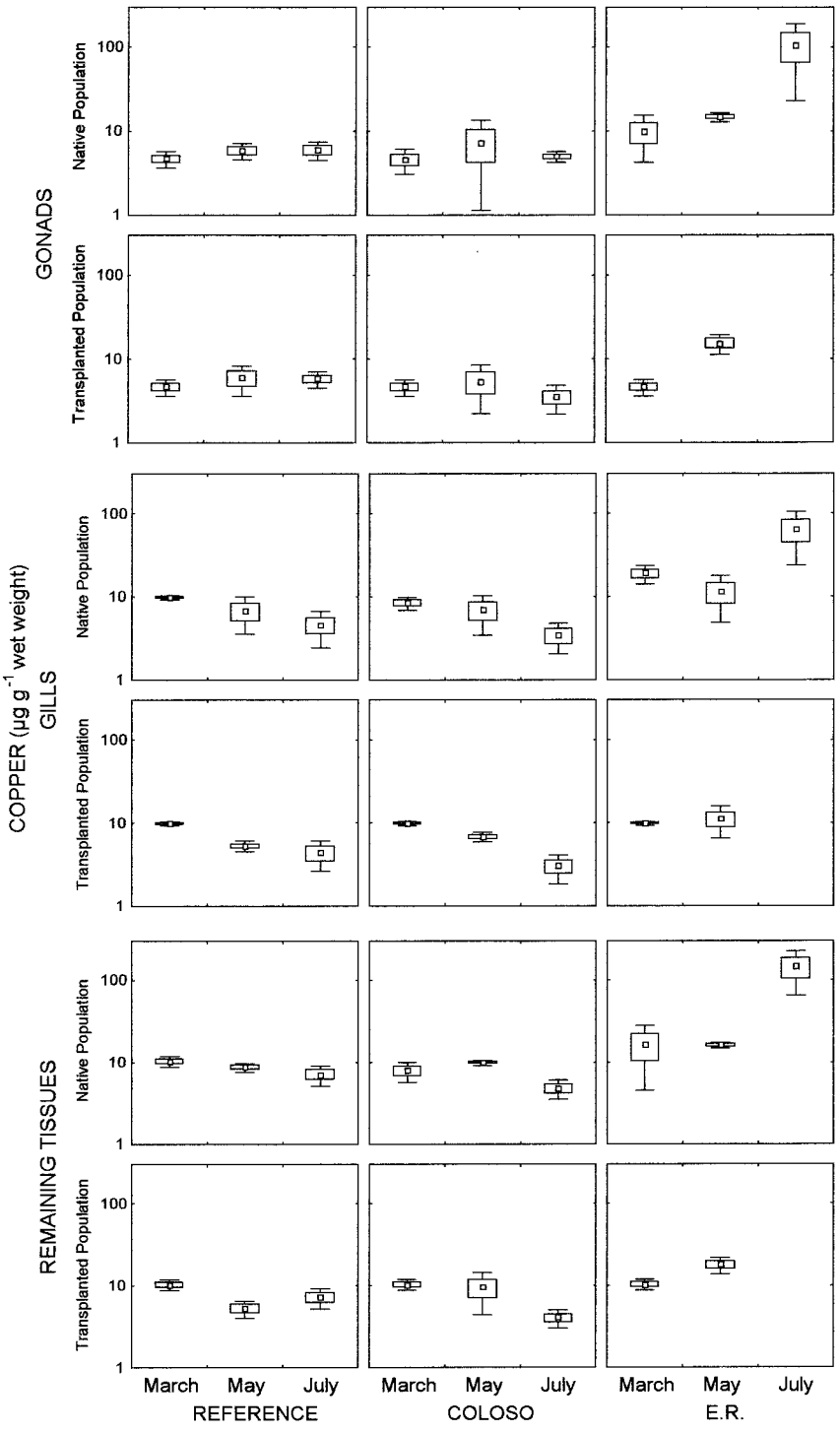

Fig. 2. *P. purpuratus.* Tissue Cu concentrations (gonads, gills, and remaining tissues) in native and transplanted mussel populations in the three study sites from San Jorge Bay (middle point: mean; box: mean ± SE; and lines: mean ± 1.96 * SE; n = 3)

remaining tissues) of E.R. mussels also showed significantly higher tissue Cu concentrations than Coloso and Reference mussels ($p < 0.001$) (Figure 2). In addition, Cu concentrations also varied among dates ($p < 0.03$). The highest Cu concentrations were recorded in native mussel populations from E.R. in July (34.3–222.9 µg L^{-1}).

Figure 3 shows the neutral red retention time in hemocytes (*i.e.*, lysosomal stability) of *P. purpuratus* individuals collected from the field at different dates. Hemocytes of native mussels from E.R. showed a significant lower neutral red retention time than those from Coloso and Reference ($p < 0.05$). Hemocytes of transplanted mussels to E.R. showed a gradual reduction in lysosomal stability during 90 days of exposure, with neutral red retention time significantly lower than those from Reference ($p < 0.05$).

Mussels exposed to Cu concentrations over 30 µg L^{-1} in the laboratory showed a significant reduction in neutral red retention time compared to controls ($p < 0.02$; Figure 4A).

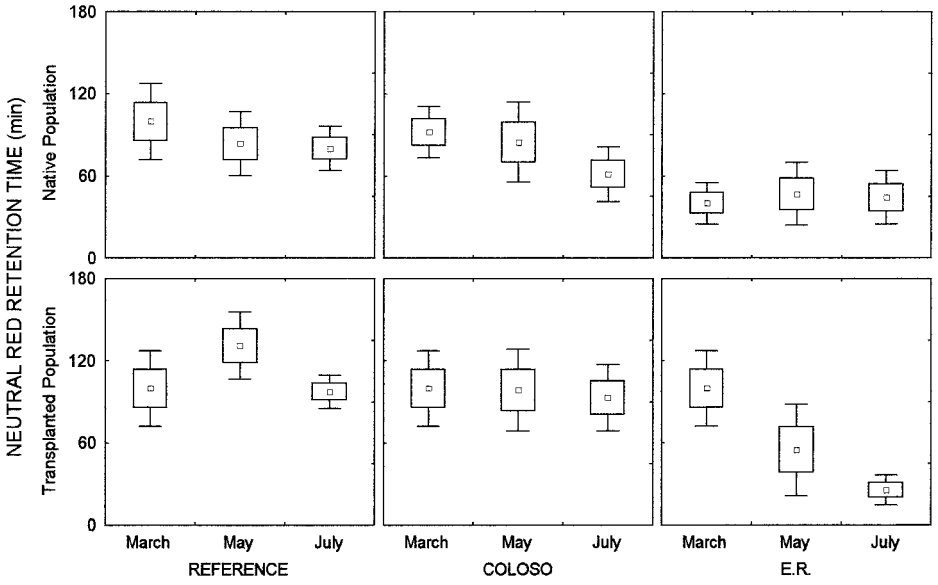

Fig. 3. *P. purpuratus.* Lysosomal stability in hemocytes (retention time of neutral red) in native and transplanted mussel populations in the three field sites from San Jorge Bay (middle point: mean; box: mean ± SE; and lines: mean ± 1.96 * SE; n = 10).

Digestive cells in mussels from Reference and Coloso showed lipofuscin granules of different sizes and a low degree of vacuolization in both native and transplanted populations (Figures 5A and 5B). In Coloso (Figure 5B), some individuals exhibited only small lipofuscin granules. Digestive cells of mussels from the native population in E.R. (Figure 5C) showed the cytoplasm occupied by vacuoles and reduction or absence of lipofuscin granules compared to those from Reference. Moreover, digestive cells of transplanted mussels showed a further increase in the extent of vacuolization in May and July (Figure 5D). These citoplasmatic structures were confirmed by transmission electron microscopy (Figure 6), where the vacuoles were observed as membrane-bound vesicles and lipofuscin granules as electron-dense membrane-bound structures of diverse sizes. This transmission electron micrograph from E.R. showed a great amount of vacuoles and the presence of small lipofuscin granules.

Native mussels from E.R. showed a significant higher vacuolization degree than those from the other sites ($p < 0.001$) and transplanted mussels from E.R. showed significant higher vacuolization than those from Reference in May ($p < 0.05$; Figure 7).

Mussels exposed to 80 $\mu g\ L^{-1}$ of Cu in laboratory experiments showed a significant increase in vacuolization section-area in the digestive cells in comparison with controls ($p < 0.05$; Figure 4B).

Figure 8 shows the ratio between lipofuscin granules and total section-area of digestive cells in native and transplanted populations of *P. purpuratus* in the three study sites. Native mussel populations from E.R. showed a significantly smaller amount of lipofuscin granules than mussels from the other sites ($p < 0.001$). Transplanted mussels also showed a reduction in section-area of lipofuscin granules in the three sites, with significant low extent occupied by these structures in mussels from E.R. in comparison with those from Reference in May ($p < 0.05$).

Exposure to sublethal Cu concentrations resulted in an increase in the amount of lipofuscin granules, although differences between control and Cu treatments were not statistically significant ($p > 0.388$; Figure 4C).

Regression coefficients between seawater Cu concentration and tissue Cu concentrations (*i.e.*, gonads, gills, and remaining tissues) and between seawater Cu concentration and cellular biomarkers were adjusted to linear models ($p < 0.05$; Table 2). Seawater Cu concentration showed a positive correlation with tissue Cu concentration and section-area of vacuolization, whereas seawater Cu concentration showed a negative correlation with lysosomal stability and section-area of lipofuscin granules.

Discussion

In spite of the potential presence of other inorganic and organic pollutants, in this study we only measured Cu concentrations both in sea water and mussel tissues, because this metal is considered the most relevant contaminant in San Jorge Bay. E.R. shows higher seawater Cu concentrations (5.954 ± 0.797 $\mu g\ L^{-1}$) than Coloso (1.027 ± 0.351 $\mu g\ L^{-1}$) and Reference (0.782 ± 0.126 $\mu g\ L^{-1}$), with Cu levels consistently higher those indicated by U.S. EPA seawater quality criteria (2.9 $\mu g\ L^{-1}$; US EPA 1994). Similarly, a previous study in San Jorge Bay, detected higher seawater Cu concentrations in E.R. (2.363 ± 0.318 $\mu g\ L^{-1}$) than in Coloso (1.480 ± 0.523 $\mu g\ L^{-1}$) and Reference (1.119 ± 0.453 $\mu g\ L^{-1}$) (Rodriguez 1997).

Cu concentrations in tissues (gonads, gills, and remaining tissues) of E.R. mussels (31.25 ± 46.81 $\mu g\ g^{-1}$) were higher than those recorded in Coloso (6.27 ± 3.12 $\mu g\ g^{-1}$) and in Reference (6.05 ± 1.78 $\mu g\ g^{-1}$). The study by Rodriguez (1997) reported comparable total Cu concentrations (wet weight) in *P. purpuratus* soft tissues. In relation to mussel populations transplanted to E.R., in gonads and remaining tissues there was an increase in tissue Cu concentrations from March to May.

There was a positive correlation between Cu concentrations in tissues and seawater Cu concentrations. However, it is important to emphasize that, in spite of the fact that seawater

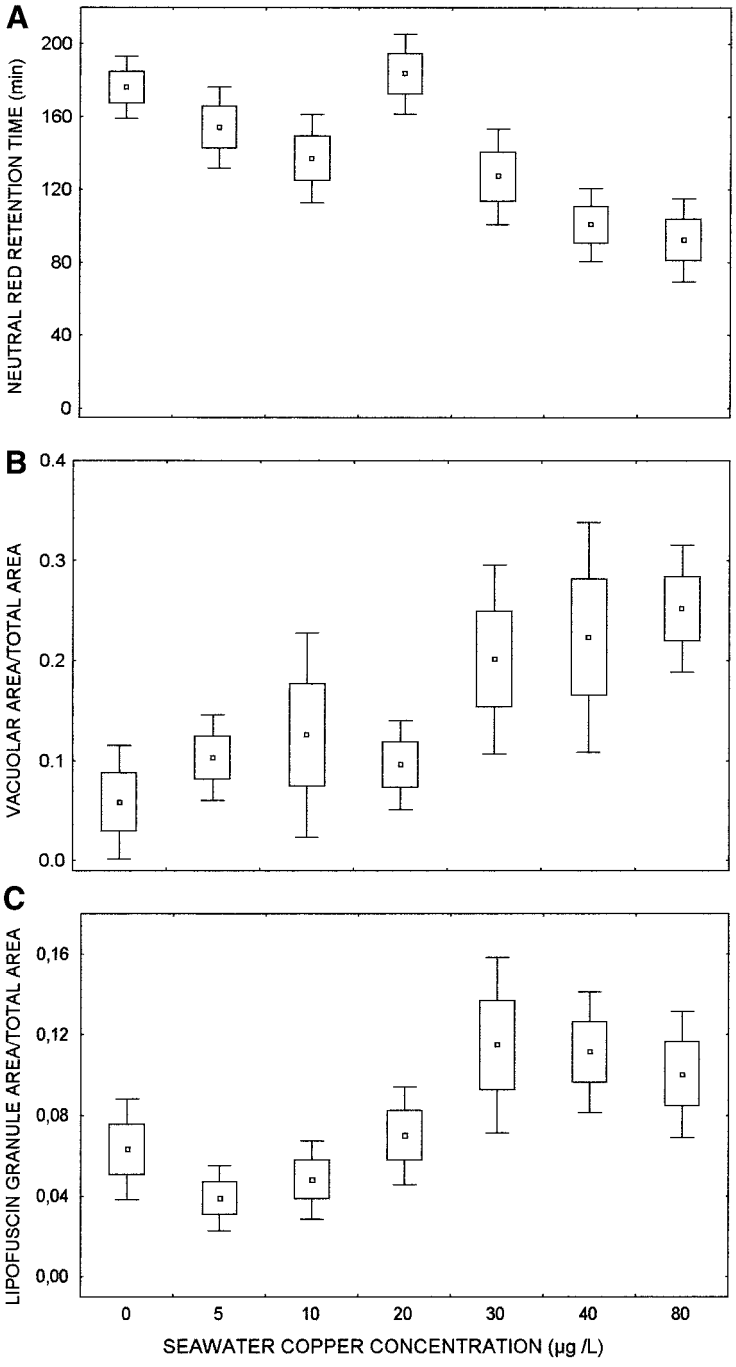

Fig. 4. Responses in cellular biomarkers of mussels exposed during 45 days to sublethal Cu concentrations under laboratory conditions. A: Lysosomal stability in hemocytes, determined by retention time of neutral red (n = 15). B: Vacuolization in digestive cells. Values correspond to the ratio between vacuolar section-area and digestive cell total section-area (n = 5). C: Lipofuscin granules in digestive cells. Values correspond to the ratio between lipofuscin granule section-area and digestive cell total section-area (n = 5) (middle point: mean; box: mean ± SE; and lines: mean ± 1.96 * SE)

Cu concentrations did not show significant differences among dates, native mussel populations from E.R. had higher Cu concentrations in July (105.522 ± 64.441 $\mu g\ g^{-1}$) than in March and May (14.538 ± 5.706 $\mu g\ g^{-1}$). It is known that mussels are good integrators of temporal and spatial variations of pollutants in aquatic environments, especially heavy metals (Phillips 1976; Cossa 1989; Rainbow and Phillips 1993; Soto *et al.* 1995; Rainbow 1995; San Francisco Estuary Institute 1997). Therefore differences in variability between seawater and mussel tissue Cu concentrations could be due to the bioaccumulation of Cu as a result of episodic events of pollution in E.R. between May and July.

In this study, we assessed cellular responses corresponding to three kinds of lysosomal alterations caused by pollutants in mussels: alterations in the extent of lipofuscin granules and vacuolization in digestive cells, and lysosomal stability in hemocytes. Significant differences were found in the three measured biomarkers in mussels collected from the studied sites as well as in mussels exposed to sublethal Cu concentrations in laboratory experiments.

Lysosomal stability has been defined as a very sensitive index of cellular condition (Moore 1978, 1985; Widdows *et al.* 1982; Viarengo *et al.* 1987). Concerning lysosomal stability in hemocytes, mussels collected in E.R. always showed shorter

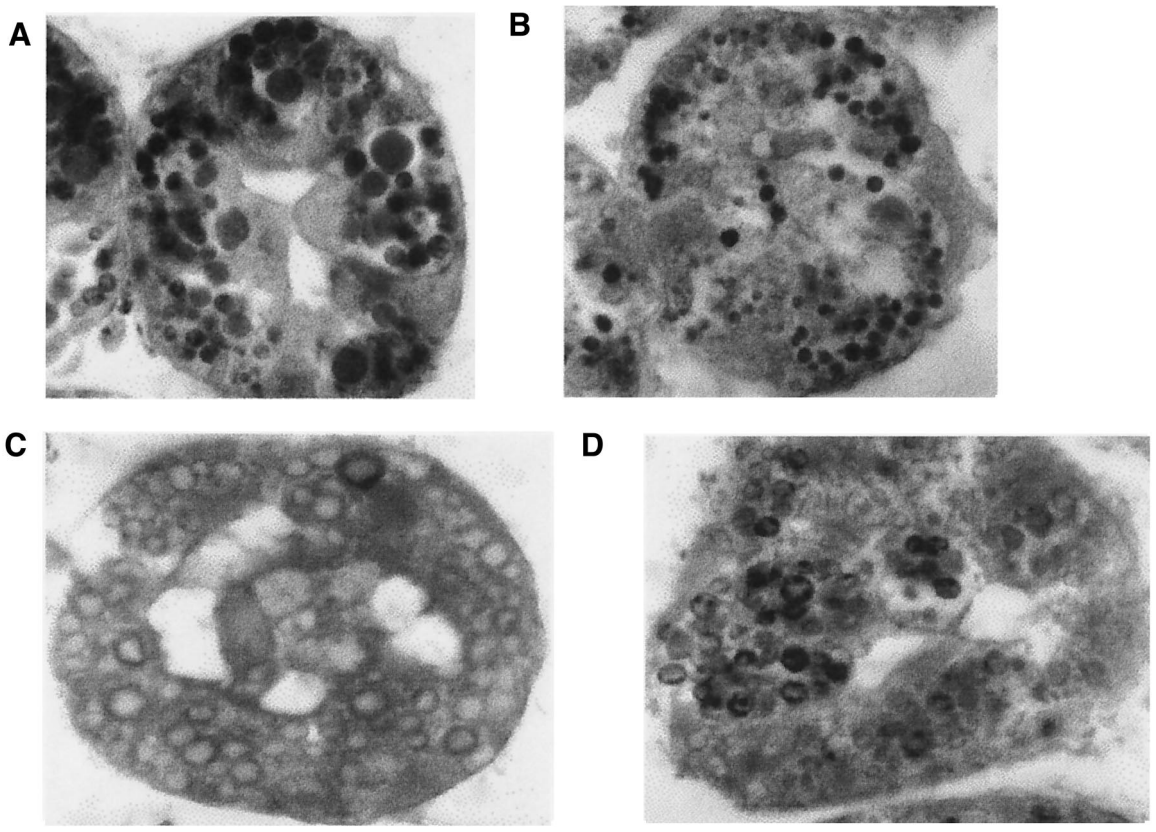

Fig. 5. *P. purpuratus.* Histological cuts of digestive cells (100×). Each photograph shows a transversal view of a digestive tubule, conformed by one layer of cells with a central lumen. A: Reference—native population—July. B: Coloso—native population—March. C: E.R.—Native population—May. D: Population transplanted to E.R.—May

retention times of neutral red (*i.e.*, lower lysosomal stability) than mussels from Coloso and Reference, in both native and transplanted populations.

It has been reported that alterations in mussel hemocytes have consequences in the immune defense mechanisms (Lowe *et al.* 1995). Lysosomal membrane destabilization provokes a release of hydrolitic enzymes, which can cause damage to cytosolic components due to proteolysis and lysis of organelles (Moore 1985; Cajaraville *et al.* 1989). Because lysosomal membranes may not recover integrity in the short term, this alteration could have negative effects on reproduction and growth in the long term (Moore *et al.* 1978; Lowe *et al.* 1995). It is worth to emphasize that the used technique to determine lysosomal stability (neutral red retention assay) possesses several advantages: low cost, short measuring time, and small sample size (only a tiny volume of blood is required) and, therefore, the same individual mussel can also be used to measure other biological responses.

With regard to the vacuolization in digestive cells, mussels collected in E.R. in all sampling dates showed section-areas of vacuolization larger than those from Coloso and Reference, especially in native populations.

Changes in size and number of lysosomes in the digestive cells in mussels have also been used as an indicator of environmental stress (Etxeberria *et al.* 1995). The enlargement of lysosomes could be due to alterations in fusion events in the lysosomal-vacuolar system of these cells and has benn related to the lysosomal membrane destabilization in the presence of pollutants (Moore 1985, 1988; Lowe *et al.* 1992; Hole *et al.* 1995).

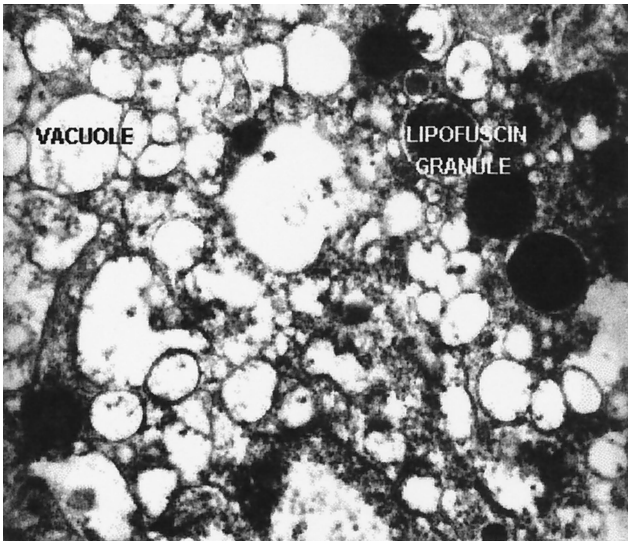

Fig. 6. *P. purpuratus.* Transmission electron micrograph of digestive cells from E.R. (15,000×) showing lipofuscin granules as electron-dense membrane-bound structures, lysosomes, and vacuoles. Note characteristic small granules and abundant large vacuoles

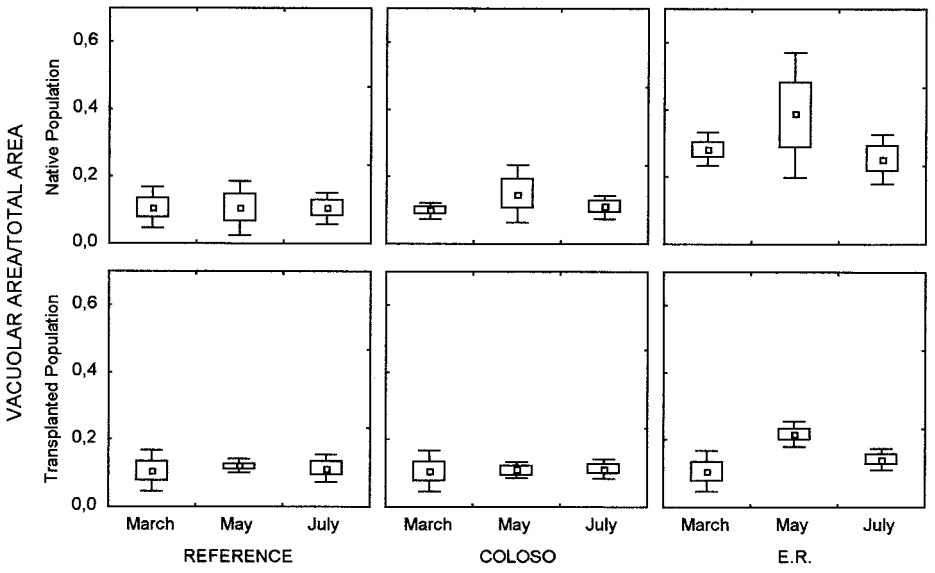

Fig. 7. *P. purpuratus.* Vacuolization degree in digestive cells of native and transplanted mussels in the three sites from San Jorge Bay. Values correspond to the ratio between vacuolar section-area and digestive cell total section-area. Data obtained using IDRISI image analysis (middle point: mean; box: mean ± SE; and lines: mean ± 1.96 * SE; n = 10)

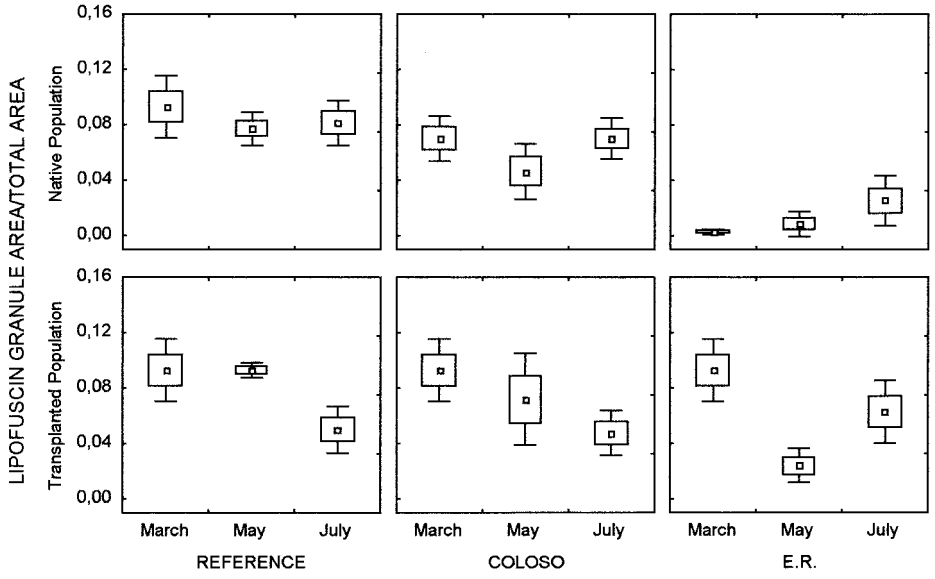

Fig. 8. *P. purpuratus.* Lipofuscin granules extent in digestive cells of native and transplanted mussels in the three sites from the San Jorge Bay. Values correspond to the ratio between lipofuscin granule section-area and digestive cell total section-area. Data obtained using IDRISI image analysis. (middle point: mean; box: mean ± SE; and lines: mean ± 1.96 * SE; n = 10)

In digestive cells of native and transplanted mussel populations from Coloso and Reference, the section-area occupied by lipofuscin granules did not show significant differences, being always detected in both sites. On the contrary, in native mussel populations from E.R., lipofuscin granules were absent or occupied significantly smaller section-areas than those in Coloso and Reference. A reduction in the lipofuscin section-area was observed in mussels transplanted to E.R. compared to Reference mussels, in May and July.

The content of lipofuscin granules in digestive cells in marine invertebrates, increases with exposure to pollutants and with organism age (Viarengo and Nott 1993). It has been suggested that lipofuscin granules are involved in the compartmentalization of metals into the lysosomes, acting as a mechanisms of metal detoxification. Moreover, it has been reported that the presence of a great amount of lipofuscin granules provides evidence of lysosomal pathology (Moore 1988).

With regard to relations between seawater Cu concentrations and cellular biomarkers, the Cu concentration was negatively correlated with lysosomal stability and positively correlated with vacuolization.

Contrary to what was expected, the extent of lipofuscin granules showed a negative correlation with seawater Cu concentration. The absence or reduced occurrence of lipofuscin granules could be explained by the high degree of vacuolization of digestive cells in E.R. individuals, together with a significant reduction in cytoplasmatic volume. The results are similar those reported by Moore *et al.* (1978), who found that mussels *Mytilus edulis* showed an increase in vacuolization and a reduction in the number of lipofuscin granules in digestive cells when exposed to anthracene. Because E.R. is a receptor site of industrial and domestic effluent discharges with organic and inorganic pollutants, it is possible that mussels from this site have been in so poor a physiological condition that they showed a reduction in cytoplasm space and did not have a normal cellular function to develop mechanism of detoxification, as the formation of lipofuscin granules. If we compare the sum of vacuolar and lipofuscin section-areas in digestive cells

Table 2. Linear regression analysis of seawater Cu concentration versus tissues Cu concentration and versus cellular biomarkers in *Perumytilus purpuratus*

Variable	Intercept	Slope	r^2	F	p
Ln (Cu in gonads)	1.393	0.288	0.605	10.718	0.014
Ln (Cu in gills)	1.531	0.272	0.662	13.729	0.008
Ln (Cu in remaining tissues)	1.784	0.282	0.557	8.803	0.021
Neutral red retention time	90.594	−7.789	0.788	25.938	0.001
Vacuolization degree	0.097	0.021	0.652	13.143	0.009
Lipofuscin granules extent	0.079	−0.007	0.534	8.019	0.025

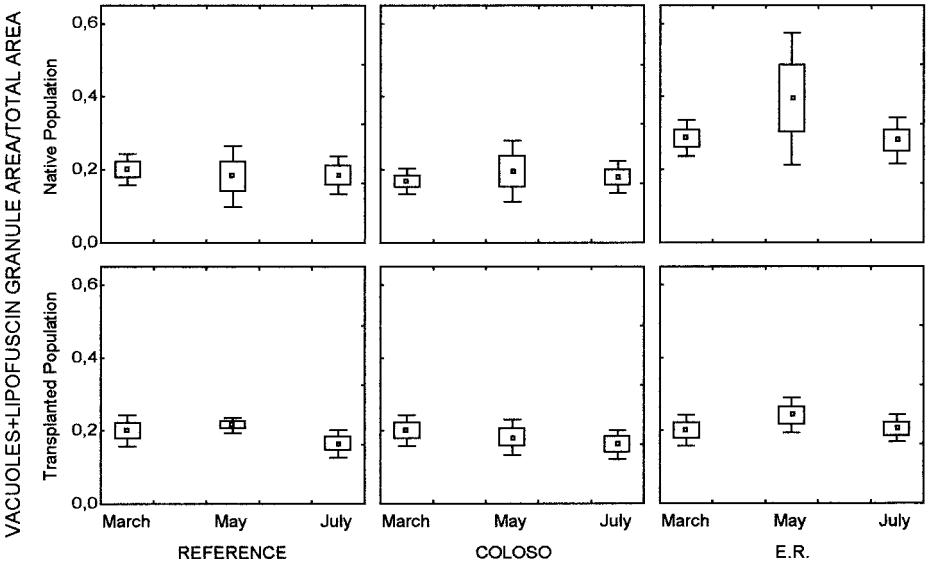

Fig. 9. *P. purpuratus.* Sum of vacuolar and lipofuscin granule section-areas in digestive cells of native and transplanted mussels in the three sites from the San Jorge Bay as integrated index of cellular damage. Values correspond to the ratio between vacuoles plus lipofuscin granule section-area and digestive cell total section-area. Data obtained using IDRISI image analysis (middle point: mean; box: mean ± SE; and lines: mean ± 1.96 * SE; n = 10)

of *P. purpuratus* to obtain an integrated index of cellular damage (Figure 9), the highest section-areas were detected in native mussel populations of E.R. in all dates ($p < 0.05$).

After 90 days of transplantation from Reference to the three study sites, no significant differences were detected in the biomarker measured in mussels from Coloso and Reference, whereas mussels transplanted to E.R. showed changes. These results are in agreement with those obtained in mussels from native populations, although the biomarker changes in transplant experiments were of less magnitude. Accordingly, it can be concluded that the use of transplanted mussels in biomarker assessment is a suitable tool to obtain information about biological effects in field experiments to known time of exposure.

Several studies have reported pollutants effects on the basis of the cellular biomarkers used in this investigation. Destabilization of lysosomal membranes in mussel cells has been observed in areas near urban and industrial discharges, with presence of either organic chemicals (aromatic hydrocarbons, polychlorinated biphenyls, DDTs, HCH, and Aroclor 1254; Moore *et al.* 1978; Widdows *et al.* 1982; Krishnakumar *et al.* 1994; Lowe *et al.* 1995) or metals (mercury, cobalt, zinc, and copper; Ward 1990; Krishnakumar *et al.* 1994; Lowe *et al.* 1995). There is evidence of lysosomal enlargement in digestive cells of mussels obtained from native populations living in zones polluted by heavy metals such as manganese, iron, and lead (Regoli 1992) and from areas near sewage discharges of urban and industrial origin with high presence of organic and inorganic pollutants (Etxeberria *et al.* 1995). There is also evidence of increasing levels of lipofuscin granules in digestive cells of mussels exposed to organic and inorganic pollutants (Regoli 1992; Krishnakumar *et al.* 1994).

Exposure of *P. purpuratus* individuals to sublethal Cu concentrations in laboratory conditions produced changes in the three cellular biomarkers studied. Exposure to Cu produces a decrease in lysosomal membrane stability (with significant effects over 30 $\mu g\ L^{-1}$), an increase in the degree of vacuolization (with significant effects only at 80 $\mu g\ L^{-1}$) and an increase in the extent of lipofuscin granules (although there were no statistically significant effect at 80 $\mu g\ L^{-1}$).

The biomarker responses under laboratory conditions, were significant over 30 $\mu g\ L^{-1}$ of Cu that are about sixfold higher than levels of Cu in sea water of E.R. ($5.954 \pm 0.797\ \mu g\ L^{-1}$). In spite of that these seawater Cu concentrations never were found in the field, *P. purpuratus* populations from E.R. showed higher cellular responses than mussels exposed to Cu under laboratory conditions. These results in E.R. could be explained by the presence of Cu and other pollutants with synergic effects. It is important to mentioned that there is no enough information about multiple pollutants effects in cellular biomarkers, therefore, new studies are necessary in that direction to validate these biomarkers as suitable tools for pollution survey.

Finally, we can conclude that the measurement of these cellular biomarkers in both native and transplanted populations of *P. purpuratus* offers a reliable approach to pollution assessment in Chilean coastal environments, similar to that applied in other mussel species for other geographical areas.

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