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Biomonitoring of antioxidant and oxidative stress responses in *Perinereis gualpensis* (Polychaeta: Nereididae) in Chilean estuarine regions under different anthropogenic pressure

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ABSTRACT

This study aimed to analyze oxidative stress parameters, including levels of the antioxidant glutathione (GSH), activity of glutamate-cysteine ligase (GCL) and glutathione-*S*-transferase (GST), total antioxidant capacity and protein oxidation, in the polychaete *Perinereis gualpensis* (Nereididae) collected from the Biobío, Itata, Valdivia and Lingue estuaries in Chile, which present different degrees of anthropogenic pressure. Sampling sites were characterized considering a geographic information system and the physicochemical characteristics of water and sediment. Significant differences ($p < 0.05$) were observed between the sampling sites for most of the responses (GSH, GCL, GST and antioxidant capacity), mainly related to human activities such as agriculture, industry, among others. Multivariate correlation analysis indicates a certain relationship of antioxidant responses with human activities, salinity, and worm weight, this last employed to standardize GST and antioxidant capacity. These results clearly indicate biomarker responses in *P. gualpensis* in Biobío and Valdivia estuaries, the more affected by human activities.

1. Introduction

Estuaries are very important in coastal aquatic ecosystems because they are regions of high productivity that are crucial for the life histories of many invertebrates, fish and birds (McLusky and Elliott, 2004). Consequently, the sustainability of these water bodies is vital for coastal regions both in ecological and economic terms. In general, estuaries are potentially exposed to chemical contaminants, transported by rivers from urban and industrial areas, because they are sediment deposition areas. Indeed, they have become the principal reservoir for a large quantity of chemical substances introduced into the aquatic ecosystem by human activity (Saiz-Salinas and Gonzalez-Oreja, 2000). As a result, to be able to determine the presence and/or potential risk of anthropogenic stressors, adequate marker organisms and responses at a sub-organism level need to be established for these ecosystems.

Central-southern Chile possesses a large number of estuaries with different environments: some are present in zones with high demographic density and important productive activities, while others receive little considerable anthropogenic pressure. The Biobío (36°48′S; 73°09′W), Itata (36°23′S; 72°52′W), Valdivia (39°50′S; 73°19′W) and Lingue (39°26′S; 73°11′W) estuaries, located within the most important estuarine systems in southern Chile, receive different levels of anthropogenic pressure depending on the intensity and type of nearby economic activity.

One of the most common and abundant species that is representative of the estuarine benthic macrofauna in these ecosystems are the polychaeta worms of the Nereididae family (Scaps, 2002), which are important ecologically because they are a food source for many birds and fishes (McLusky, 1989) as well as sedimentary processes such as bioturbation (Davey and Watson, 1995; Banta and Andersen, 2003). Furthermore, these organisms have been quite well used as contamination biomonitors (Scaps and Borot, 2000; Scaps, 2002; Geracitano et al., 2004a;

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Ferreira-Cravo et al., 2007). The nereidid polychaeta *Perinereis gualpesis* (Jeldes, 1963) is a very abundant species in estuaries in southern Chile, presenting densities higher than 2000 ind/m^2 (Jaramillo et al., 2001). Preliminary studies have shown that this worm is an efficient biomonitor for heavy metals in these environments (Bertrán et al., 2001a). Still, the sub-organism response level in these species has yet to be used as a biomarker, and it could be a potential tool to determine the state of environments experiencing numerous types of anthropogenic stressors.

Antioxidant responses can be used as potential biomarkers for aquatic contamination because different types of contaminants can directly or indirectly change the balance between the concentration of pro-oxidants and antioxidants (Monserrat et al., 2007), where these pro-oxidants are related to the generation of reactive oxygen species (ROS), which can trigger compensatory responses through the induction of antioxidant defense or eventually generate oxidative damage. As a result, these responses have been used as non-specific biomarkers for aquatic contamination (Bainy et al., 1996; Geracitano et al., 2004a) and have been successfully used to determine the general health state of estuarine ecosystems as well as to identify areas that have been impacted by a complex mixture of contaminants (Bainy et al., 1996; Geracitano et al., 2004a, b; Amado et al., 2006a, b). Within the antioxidant system, reduced glutathione (GSH) is considered to be one of the first lines of defense against the ROS (Cnubben et al., 2001; Dickinson and Forman, 2002). Some of the enzymes with antioxidant importance include glutamate cysteine ligase (GCL) and glutathione-*S*-transferase (GST), where the first is the rate-limiting enzyme for GSH synthesis and the last is a phase II enzyme related to the metabolism of certain organic lipophilic contaminants (Saint-Denis et al., 1999). Additionally, some studies consider the measure of total antioxidant capacity, related to both enzymatic and non-enzymatic antioxidants (Regoli and Winston, 1999; Regoli et al., 2002). In polychaete from Nereididae family, antioxidant and oxidative stress parameters has been employed in biomonitoring studies (Geracitano et al., 2004a, b; Perez et al., 2004; Ait Alla et al., 2006).

The validation of this type of sub-individual level response is related to a comparison between adequate reference sites, where a proper characterization of riverside use by photo-interpretation tools is required to precisely establish the level of anthropogenic pressure on the water bodies in the study area. Additionally, the potential relationship of these biochemical responses with abiotic factors the environment (such as the principal physicochemical variables of the water and sediment) and biotic factors (such as individual weight) of the ecosystem should be determined.

Thus, the objective of this study is to determine the levels of GSH, enzymatic activity of GCL and GST, total antioxidant capacity total and protein oxidation in *P. gualpensis* individuals collected in the Biobío, Itata, Valdivia and Lingue estuaries in order to determine the influence of anthropogenic pressure, the principal physicochemical variables present in these ecosystems and the individual weight variable. The levels of antioxidant enzymes and the oxidative damage observed in *P. gualpensis* are expected to present different response levels in relation to the occurrence of point and non-point sources of pollution in the drainage basin of each estuary, reflecting the different levels of anthropogenic pressures.

2. Material and methods

2.1. Study area

The Biobío, Itata, Valdivia and Lingue estuaries are located in central-southern Chile (39°49′S, 73°18′W, Fig. 1). Two sampling sites were identified in each estuary (Fig. 1), and sampling was performed during the warm season (January) of 2007 in order to obtain individuals in the same reproductive period. All the sites were established in areas with a considerable density of individuals. The different sampling sites were selected to establish a range of anthropogenic pressure and contamination types by choosing sites close to different potential contamination sources as well as sites in areas without considerable anthropogenic intervention. Other selection criteria included estuarine zones with urban settlements of more than 100,000 habitants (Biobío and Valdivia) and ones without nearby urban settlements (Itata, Lingue) (INE, 2002). Additionally, potential contamination areas were identified using historical data (Barra et al., 2009).

For a better characterization of the study areas, a Geographic Information System (GIS) layer was used to represent land use in the sampling sites and is based on the interpretation of aerial photographs (scale 1:70,000/1:115,000 for the period 1996–1998 CONAMA-CONAF-BIRF, 1999) combined with the Chilean Native Forest Census (*Catastro de Bosque Nativo de Chile*). The classification methodology was based in the work of Etienne and Prado (1982). Considering that the number of coverage types represented a great variety of land use, these were reclassified in four categories with GIS SIG ArcView 3.3 (ESRI, 2001): (1) agricultural and cattle-raising activities; (2) urban and/or industrial areas; (3) native vegetation with primary and/or secondary forests; and (4) wetlands (Table 1). Then, a 5-km circumference was defined for each previously geo-referenced sampling point and the land use present in the circumference areas was characterized (Fig. 1).

The Biobio River estuary is located in an important urban-industrial area and the sampling sites were located in an area close to an oil refinery (BBI) and in an important sediment deposition site close to a densely populated and highly industrialized area (BBII) (Fig. 1, Table 1). In contrast, the Itata River estuary is located in a scarcely populated area and the sampling sites are located near to agricultural and livestock (iI) and forestry (iII) activities (Fig. 1, Table 1). The Valdivia River estuary presents an important anthropogenic gradient, and the first sampling site was located in an area with important demographic pressure and close to a water treatment plant discharge point (VI); the second sampling site was established in an area outside of the city (VII) with less demographic pressure but near important forestry activity (Fig. 1, Table 1). The Lingue River estuary corresponds to a small coastal-type basin with an important quantity of native forest and the land is not used for large-scale productive activity. The sampling sites were established in intertidal flat (LI) and preferably subtidal (L2) areas (Fig. 1, Table 1). To summarize, the available information points *a priori* to Biobío and Valdivia estuaries as the more impacted by human activities (Fig. 2).

2.2. Sampling

Similar sized, whole polychaetes were collected using a shovel and were manually extracted from the sediment (n=9–12) without damaging them. They were then sacrificed and transported on ice (−4 °C) for their subsequent conservation in an ultrafreezer at −80 °C. Prior to the respective biochemical analyses, wet worms were weighed on a digital scale. At each sampling site, salinity, temperature, oxygen and pH were measured. Sediment samples were also collected using a Corer with a 10-cm diameter. These samples were conserved at −20 °C until their analysis (Fig. 3).

2.3. Sediment analysis

To determine sediment size, a sub-sample was sized in standard grids of 4.0 and 1.0 phi in order to separate the principal textural fractions: mud, sand and gravel. To analyze the thick fraction (sand and gravel), a sub-sample of sized material was placed in a digital decantation tube (Emery type), and the size composition was estimated measuring the accumulated weight at different time intervals. Once each fraction's weight was obtained, the values were expressed in differential percentage with respect to total weight. Subsequently, the mean diameter (mean size) and dispersion parameters (selection) were estimated using the momentum method (McManus, 1988). The data were represented on a phi (ϕ) logarithmic scale. To analyze the fine sediment fraction (Mud and clay), a sub-sample was placed in a NaCl (35‰) electrolyte solution. This solution was placed in an ultra-sound for 5 min and then in a microparticle analyzer ELZONE® 282 PC. For mean size classification and selection, the data obtained were grouped according to Wentworth (1922) and Gray (1981). Total organic matter content was determined by the ash free dry weight (AFDW) method by ashing the samples in a furnace for 4 h at 550 °C.

2.4. Biochemical analysis

2.4.1. Tissue homogenization

For the biochemical measurements, organisms were homogenized (1:3 w/v) in ice-cold buffer with pH adjusted to 7.60 (20 mM Tris-base, 1 mM EDTA, 1 mM DL-dithiothreitol, 500 mM sucrose and 150 mM KCl) (Geracitano et al., 2002). Homogenates were centrifuged at 9000g for 45 min (4 °C) and the supernatants were collected and stored at −80 °C and employed later to determine total protein

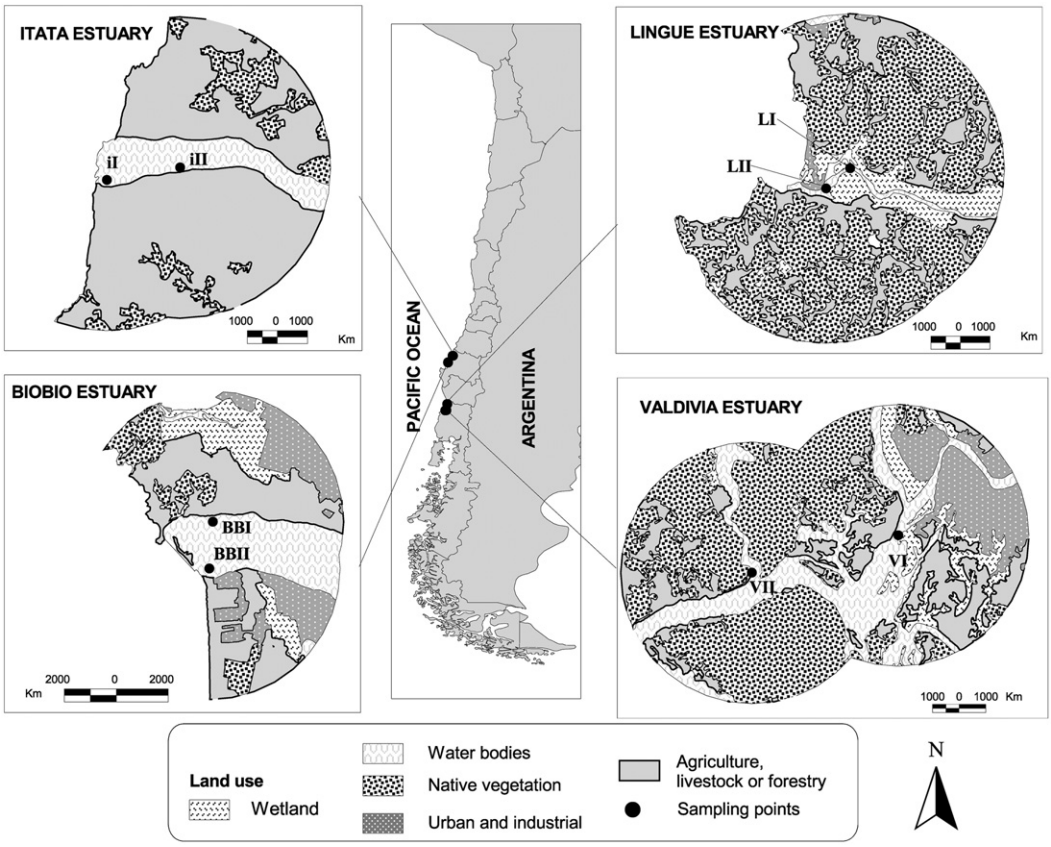

Fig. 1. Land-use map for a 5-km radius of the *Perinereis gualpensis* sampling sites in the four estuaries in Central-Southern Chile.

Table 1
Land uses (percentage) in a 5-km radius for the *Perinereis gualpensis* sampling sites in the four estuaries in Central-Southern Chile, January 2007.

Sites	General description	% of agriculture, livestock or forestry	% of urban construction and industries	% of native. vegetation	% of. wetlands[a]
Biobio I	Oil refinery and other industries	33.2	19.3	8.1	0.0
Biobio II	Highly urbanized and other industries	42.3	13.8	6.9	0.0
Itata I	Scarcely urbanized, livestock and agriculture	77.9	0.0	6.6	0.0
Itata II	Forestry and agriculture	75.6	0.0	10.7	0.0
Lingue I	Scarcely urbanized and native vegetation	28.8	0.5	58.1	10.3
Lingue II	Scarcely urbanized and native vegetation	28.7	0.9	58.7	9.9
Valdivia I	Highly urbanized and treatment plant	20.8	17.4	24.6	14.1
Valdivia II	Native vegetation and forestry	60.6	0.0	16.0	1.7

[a] Only wetlands connected with principal water body.

content, GSH content, GCL activity, total antioxidant capacity and oxidized proteins.

2.4.2. Determination of total antioxidant capacity

Total antioxidant competence against peroxyl radicals was evaluated through ROS determination in tissue samples treated or not with a peroxyl radical generator, according to a new method described by Amado et al. (2009). Briefly, on a white 96-well microplate, 10 μL of the supernatant (prepared as described previously) of each tissue and protein concentration were pipetted into the wells, six wells per sample. The reaction buffer (127.5 μl), containing 30 mM HEPES (pH 7.2), 200 mM KCl and 1 mM $MgCl_2$, was added to the wells with samples. In three of the six wells of each sample, 7.5 μL of 2,2-azobis 2 methylpropionamidine dihydrochloride (ABAP; 4 mM; Aldrich) were added. In the other three wells, the same volume of ultrapure water was pipetted. After this, the microplate was put into a fluorescence microplate reader (Victor 2, Perkin Elmer), programmed to

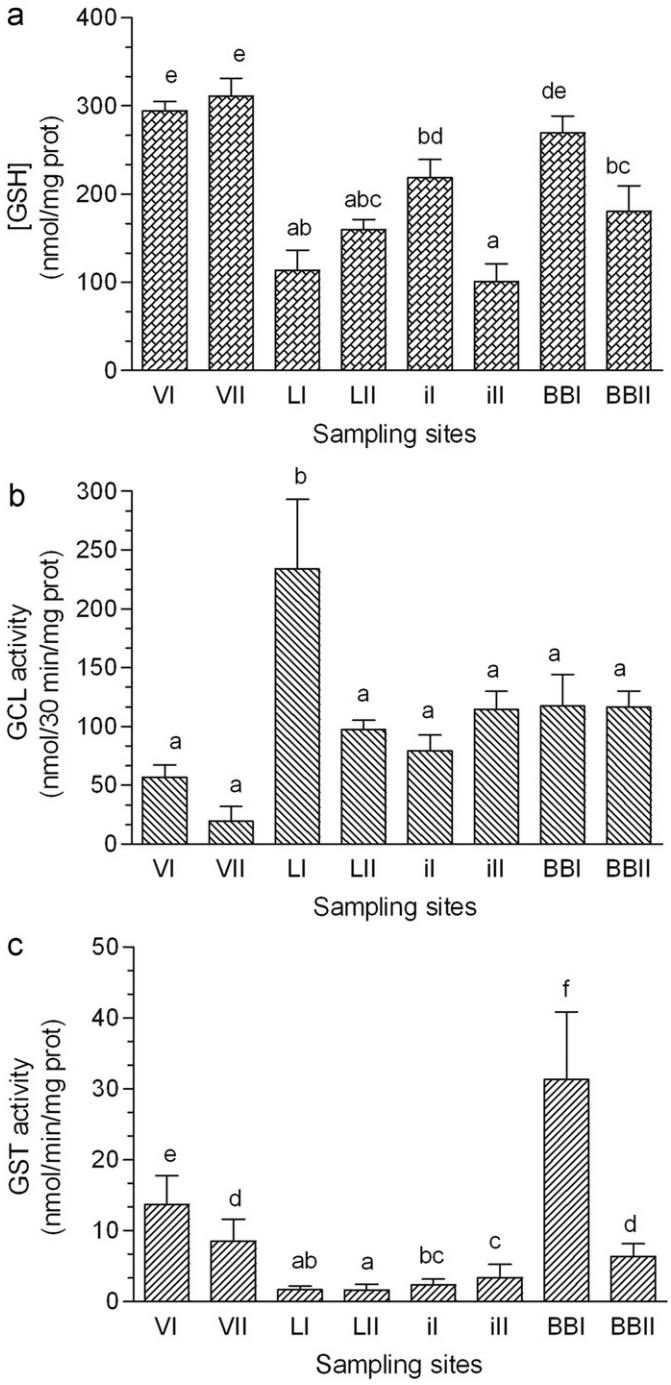

Fig. 2. Values for (a) reduced glutathion (GSH) activity, (b) glutamate cysteine ligase (GCL) activity and (c) glutathione-*S*-transferase (GST) activity in the tissue of *Perinereis gualpensis* collected in January 2007. The same letter indicates the absence of significant differences between sampling sites ($p > 0.05$).

maintain a temperature of 35 °C. At this temperature, peroxyl radicals were produced by thermal decomposition of ABAP (Winston et al., 1998). Immediately prior to microplate reading, 10 µl of the fluorescent probe 2,7-dichlorofluorescein diacetate (H_2DCF-DA) in a final concentration of 40 µM was added to all wells following the methodology employed by Ferreira-Cravo et al. (2007). H_2DCF-DA is cleaved by esterases that are present in the samples' supernatants. Thereafter, the non-fluorescent compound H_2DCF is oxidized by ROS to the fluorescent compound DCF, which is detected at wavelengths of 485 and 520 nm, for excitation and emission, respectively. The thermal decomposition of ABAP and ROS formation was monitored for 30 min, with readings every 5 min. Total fluorescence production was calculated by integrating the fluorescence units (FU) along the time of the measurement, after adjusting FU data to a second-order polynomial function. The relative difference between ROS area with and without ABAP was considered a measure of antioxidant capacity, with high area difference meaning

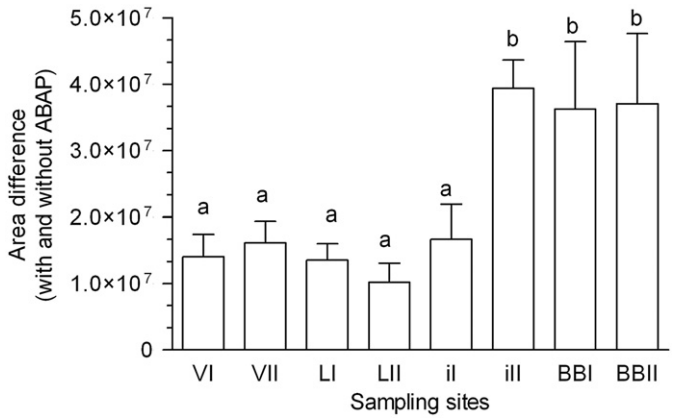

Fig. 3. Total antioxidant capacity activity in the tissue of *Perinereis gualpensis* collected in January 2007. The same letter indicate the absence of significant differences between sampling sites ($p > 0.05$).

low antioxidant capacity, since high fluorescence levels were obtaining after adding ABAP, meaning low competence to neutralize peroxyl radicals (Amado et al., 2009). The advantages of the employed methodology were related to the low homogenates volume needed to perform the analysis and its quickness, allowing the measurement of several samples by day.

2.4.3. Measurement of glutamate-cysteine ligase (GCL) activity and reduced glutathione (GSH) concentration

GCL and GSH activity levels were determined following White et al. (2003). This method is based in the reaction of naphthalene dicarboxialdehyde (NDA) with GSH or γ-glutamylcysteine (γ-GC) to form cyclic products that are highly fluorescent. In a 96-well round-bottom reaction plate, aliquots (25 µl) of GCL reaction cocktail (400 mM Tris, 40 mM ATP, 20 mM L-glutamic acid, 2.0 mM EDTA, 20 mM sodium borate, 2 mM serine and 40 mM $MgCl_2$) were added into each well. For assays, 25-µl aliquots of sample were pipetted into a pre-warmed (25 °C) reaction plate at 15-s time intervals. After 5 min of pre-incubation, the GCL reaction was initiated by adding 25 µl of 2 mM cysteine dissolved in buffer solution (100 mM Tris–HCl, 2 mM EDTA and 5 mM $MgCl_2 \cdot 6H_2O$, pH 7.75). In order to measure GCL activity, cysteine was not added to the GSH-baseline wells at this time. After 10 min, the GCL reaction was stopped by adding 25 µl of 200 mM sulfosalicilic acid to all wells, and then 25 µl of 2 mM cysteine was added to the GSH-baseline. An aliquot (20 µl) was mixed with 180 µl of NDA derivatization solution (50 mM Tris, pH 12.5; 0.5 N NaOH; and 10 mM NDA in dimethyl sulfoxide, 1.4/0.2/0.2 v/v/v) in a 96-well plate. The plate was covered to protect the wells from ambient light and was allowed to incubate at room temperature for 30 min. Following incubation, NDA-γ-GC or NDA–GSH fluorescence intensity was measured (472 nm excitation/528 nm emission) in a fluorescence microplate reader (Víctor 2, Perkin Elmer).

2.4.4. Measurement of glutathione-S-transferase (GST) activity

The activity of GST was measured by following the conjugation of 1 mM glutathione and 1 mM 1-chloro-2,4-dinitrobenzene (CDNB) at 340 nm as described by Habig et al. (1974) and Habig and Jakoby (1981).

2.4.5. Measurement of oxidized proteins

Protein carbonyl groups quantification was estimated in worm's tissues by one-dimensional electrophoresis and Western blot immunoassay. The detection of protein carbonyls involved the derivatization of carbonyl group with 2,4-dinitrophenyhydazine (DNPH, Sigma), which leads to the formation of a stable 2,4-dinitrophenyl (DNP) hydrazone product (Levine et al.,1990). The derivatization proteins were reacted with DNPH in a solution containing 12% SDS and DNPH/TFA stock solution: 20 mM DNPH in 20% (v/v) TFA (trifluoroacetic acid). Following incubation for 15 min at room temperature, the reaction mixture was neutralized with 2 M Tris-base containing 30% glyceraldehyde. Samples were then submitted to SDS-PAGE, electroblotted to PVDF membranes. Immune reactions for carbonyl content were performed with anti-DNP antibody after SDS-PAGE and Western transfer. Bands were visualized using a chromogenic immunodetection kit (Invitrogen) and analyzed after scanning the PVDF membranes (Fig. 4a).

2.5. Statistical analysis

The changes in enzymatic type responses were evaluated by one-tail analysis of variation (ANOVA, $p < 0.05$) followed by a Tuckey analysis to statistically determine the

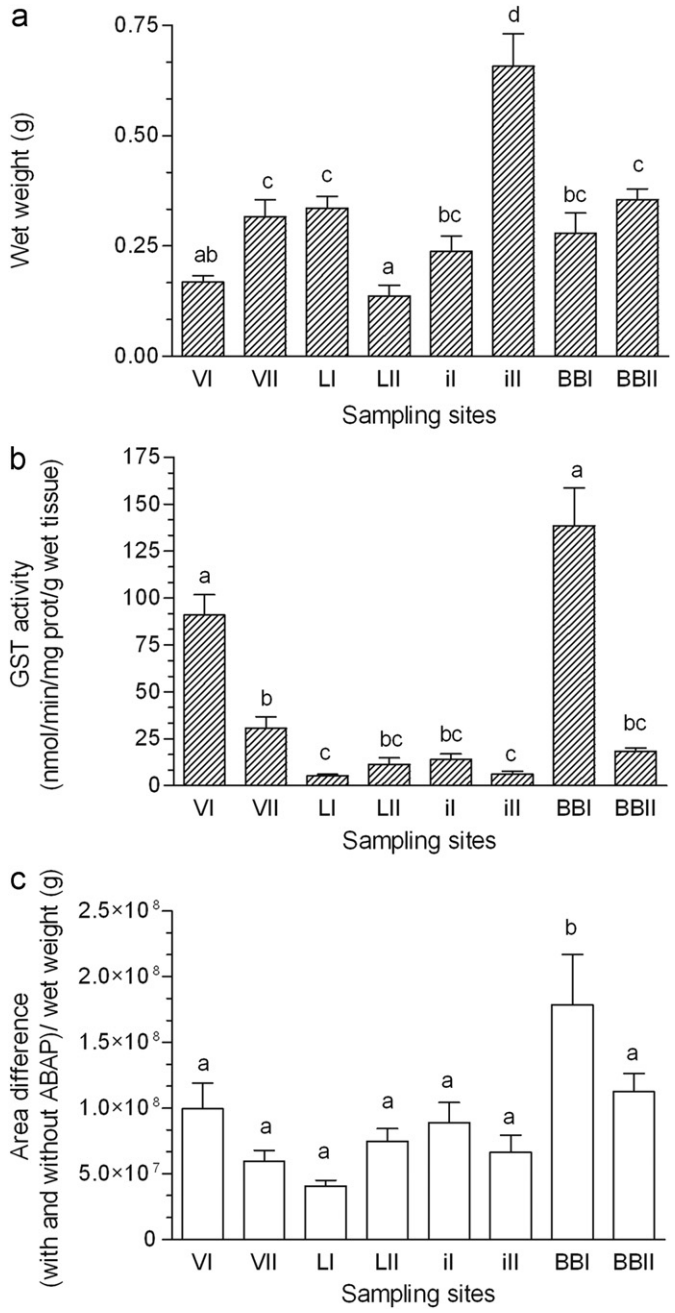

Fig. 4. Values of (a) whole individual wet weight, (b) glutathione-*S*-transferase (GST) normalized for individual wet weight, (c) total antioxidant capacity activity normalized to wet weight for *Perinereis gualpensis* collected in January 2007. The same letter indicate the absence of significant differences between sampling sites ($p > 0.05$).

differences between the sampling sites. Additionally, the set of environmental variables and the averaged oxidative stress values for each site were analyzed using the coefficient of Spearman, ρ_w (BIO-ENV analysis; Clarke and Warwick, 1994), where this coefficient acquires ρ_w values close to 1 when there is a good correlation and close to 0 when the correlation between variables is minimal or null. Thus, this method can be used to obtain the variable or combination of variables that best explain the set of oxidative stress parameters found in *P. gualpensis*. The multivariate analyses were performed using the software PRIMER V.6 developed in Plymouth Marine Laboratory (Clarke and Gorley, 2005). To characterize the sites and their relation with the set of analyzed responses, a principal component analysis (PCA) was performed using a multivariate matrix of the set of antioxidant responses and the weight variable prior to a standard normalization. To determine the importance of individual weight on antioxidant response related to a greater degree of anthropogenic intervention in these estuaries, these responses were normalized with individual wet weight, statistically determining differences between sampling sites ($p < 0.05$) with the non-parametric test Mann–Whitney for paired comparisons and with the Bonferroni correction.

3. Results

3.1. Environmental variables

Within the physicochemical parameters of the water column, the most evident differences between sampling sites were registered for the salinity values (Table 2). Slight latitudinal differences were observed with respect to the pH and temperature, where lower values were registered in the estuaries located in the southern part of the study area (Table 2). The sedimentologic variables in terms of organic material presented higher percentages in the sampling sites BBI, VI and VII (Table 2). The mean sediment particle size corresponded to medium-sized sand in the sampling sites BBI, BBII, iI, iII and VII (Table 2), while fine sand and very fine sand were found in the sites located farther south (LI and LII and VI, respectively) (Table 2). Poorly selected sediment, indicating a wide range of sizes, were found in the sampling sites BII, iII and VI, while the remaining sites presented medium selected sediments (BI, iI, LII and VII) and highly selected sediments (LI) (Table 2).

3.2. Antioxidant responses and weight

The significantly higher GSH values were found principally in those individuals collected in the Valdivia Estuary (VI and VII) and Biobío Estuary (BBI) in comparison with the majority of the other analyzed sites ($p < 0.05$). Indeed, these sites presented individuals whose values were greater than 250 nmoles of GSH/mg of protein, while the significantly lower GSH values were observed in the individuals collected in the Lingue estuary (LI and LII) and in the Itata estuary's more isolated site (iII) (Fig. 2a). The GCL values were significantly higher ($p < 0.05$) in individuals collected in LI in comparison with the individuals collected in the other estuaries, presenting values above 230 nmoles of GSH/h/mg of protein (Fig. 2b). Polychaetes from the BBI site presented significantly higher GST activity in comparison with the other sites ($p < 0.05$), while the individuals collected in the sites LI and LII presented significantly lower values for GST activity ($p < 0.05$; Fig. 2c). Total antioxidant activity indicated that the individuals located in iII together with the two sites located in the Biobío Estuary (BBI and BBII) presented significantly less total antioxidant capacity ($p < 0.05$) in comparison with the other sampling sites, while the site LII presented significantly higher antioxidant capacity with respect to the polychaetes of other estuarine regions ($p < 0.05$; Fig. 3). Protein oxidation did not find significant differences between the individuals collected in different estuaries ($p > 0.05$; data not shown). The polychaete's wet weight presented significantly higher values ($p < 0.05$) in iII, while the lower weight individuals were observed in LII (Fig. 4a). The GST values standardized for individual wet weight presented significantly higher values ($p < 0.05$) in the sites VI and BBI (Fig. 4b). Total antioxidant activity standardized for individual wet weight found significant differences ($p < 0.05$) in the BBI site with respect to the other studied sites (Fig. 4c).

3.3. Multivariate analysis

BIO-ENV analysis, which exhibits the maximum coefficient values for the range of the Spearman correlation (ρ_w) for the best combination of two or three variables included in the present study (Table 3), indicated that the following 13 variables had the highest correlation values: land use (% agricultural land use; % urban and industrial land use; % native vegetation; % wetlands); physicochemical variables (pH; temperature; salinity; dissolved oxygen; % organic matter; mean size; particle selection); and

Table 2
Mean values (± 1 standard deviation) for the physicochemical variables of the water column and sediments for the *Perinereis gualpensis* sampling sites in the four estuaries in Central-Southern Chile, January 2007.

Sites	pH	*T*(°C)	Salinity (psu)	O_2(mg/l)	Organic matter (%)	Mean grain size (Φ)	Sorting (Φ)
Biobio I	8.34 ± 0.01	25.38 ± 0.91	5.80 ± 6.03	4.43 ± 0.07	1.94 ± 0.15	1.53 ± 0.05	0.74 ± 0.05
Biobio II	8.15 ± 0.13	24.28 ± 5.54	6.83 ± 6.65	2.96 ± 0.29	9.18 ± 7.45	1.79 ± 0.71	1.37 ± 0.81
Itata I	8.17 ± 0.04	24.33 ± 1.12	12.91 ± 0.15	4.20 ± 0.36	2.22 ± 0.64	1.20 ± 0.03	0.77 ± 0.06
Itata II	8.08 ± 0.03	28.43 ± 0.50	0.10 ± 0.00	3.50 ± 0.52	1.59 ± 0.16	1.79 ± 0.84	1.26 ± 0.88
Lingue I	7.90 ± 0.17	23.43 ± 0.74	7.97 ± 0.12	4.48 ± 0.05	3.64 ± 0.37	2.12 ± 0.19	0.53 ± 0.07
Lingue II	7.71 ± 0.04	20.13 ± 0.21	13.40 ± 0.56	4.89 ± 0.36	1.85 ± 0.10	2.44 ± 0.06	0.61 ± 0.05
Valdivia I	6.73 ± 0.06	20.67 ± 0.06	2.30 ± 0.10	3.50 ± 0.03	7.11 ± 0.08	408 ± 0.89	2.41 ± 0.21
Valdivia II	8.00 ± 0.23	19.50 ± 0.00	11.23 ± 0.12	3.75 ± 0.10	6.83 ± 1.35	1.85 ± 0.03	0.83 ± 0.13

Table 3
Range of coefficient values of the Spearman correlation (ρ_w) for the environmental variables measured in the different sampling sites and antioxidant responses found for the *Perinereis gualpensis* (BIO-ENV), January 2007.

Number of variables	Best variable combinations (ρ_w)
3	Weight, salinity, % native vegetation (0.547)
3	Weight, salinity, *T* (0.525)
2	Weight, % native vegetation (0.494)
1	Sorting (Φ) (0.391)

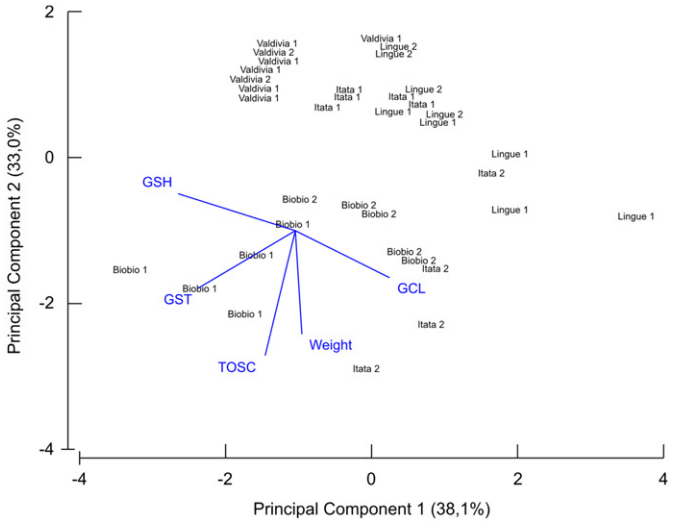

Fig. 5. Principal component analysis (PCA) for the different antioxidant responses and weight for the different sampling sites. The variability percentage explained by each principal axis is provided.

weight, where the combination of weight, salinity, and % native vegetation presented the highest correlation values (ρ_w=0.547) (Table 3). On the other hand, the best combination of two variables to explain the antioxidant response values was given by the variables weight and % native vegetation (Table 3). The variable particle selection was the one that best explained the antioxidant response values (Table 3). The PCA indicated that the first two components explain 71.1% of the variation (Fig. 5). It also indicated that the sites BBI, BBII and iII presented the greatest degree of difference with the other sites. Furthermore, it could be observed in the ordering that in comparison with the other sites, the individuals from BBI were more related with the variables GST, antioxidant capacity and individual weight, where weight was equally related with the individuals of the site iII (Fig. 5). Furthermore, the sites BBI, VI and VII were found to be related for the GSH concentration (Fig. 5).

4. Discussion

It can be observed that the sampling sites present different degrees and types of anthropogenic intervention (principally due to urban and industrial or agricultural activities), where the BiobíoBiobío, Valdivia and Itata estuaries present the greatest degree of anthropogenic intervention.

The organic matter content in the sediment presents percentages that are similar to those found for other estuaries in central-southern Chile (Jaramillo et al., 2001) and in South America (Ieno and Bastida, 1998). Still, the higher percentages found in the sites BBII, VI and VII suggest an important organic enrichment due to human activities and that is associated to low hydrodynamics (Valdovinos, 2001). The sediment's texture coincides with the results previously described for the estuaries in southern Chile, where sand is the principal component followed by the muddy fraction (Jaramillo et al., 1985; Bertrán, 1989). The predominance of the sandy fraction in these environments is mainly related to estuarine dynamics, which impede the deposition of a considerable quantity of fine particles (Bertrán et al., 2001b). Indeed, and in agreement with the particle selection, the results indicate that the sites that presented poor particle selection would be related to environments where there is great variability in hydrodynamic processes. For example, the estuaries located in the northern part of the study area (e.g. the BiobíoBiobío estuary) present water flow variation dynamics associated to snowmelt and rain regimes (Bertrán et al., 2001b).

With respect to antioxidant responses, GSH levels responded principally in sites related to estuaries receiving greater anthropogenic pressure Indeed, the induction of GSH reflects an adaptation to pollutants and has been shown to play a critical role in maintaining cellular homeostasis (Doyotte et al., 1997). In this context, the transcription Nrf2 factor plays a central role in the constitutive and inducible expression of several phase II proteins (Nguyen et al., 2000). The migration of this factor from the cytoplasm to the nucleus under pro-oxidant conditions signals the expression of important genes to antioxidant defense, including those coding for the catalytic subunit of GCL and several GST isoenzymes (Lee and Surh, 2005). The significantly higher GCL values found in site LI suggest that the individuals sampled would be exposed to a pro-oxidant environment that favors an increase in the expression of catalytic and regulatory sub-units of GCL. Authors, like Maher (2005), comment that moderate stress can in fact increase antioxidant defenses as a mechanism to provide protection for the ongoing stress, enabling the cells to

more competently face more severe stress. Additionally, even when the Lingue estuary was not in an area with great anthropogenic pressure, a pro-oxidant effect would be due principally to the condition as a intertidal flat, in which both the sediment and the individuals would be exposed to oxygen saturation conditions resulting principally from the penetration of atmospheric oxygen in the first sediment layers. Similarly, the sediment conditions of this estuary show a greater particle selection, a variable that together with the shallow waters that are present in this estuary, would have an influence on sediment permeability and consequently oxygen penetration (Huettel and Rusch, 2000).

The activity of GST in the BBI site is relatively similar to those found in other polychaetes of this family, such as *Hediste diversicolor*, in contaminated sites (Perez et al., 2004), and considerably lower that found for *Nereis diversicolor* in areas with important industrial activity and domestic effluent discharge (Ait Alla et al., 2006). This result indicates that even if these individuals would be exposed to contaminated conditions – where detoxification activity should be greater – their values would vary between species of this family and would also be influenced by the degree and type of contamination. Indeed, the significant differences in GST with respect to other sites would present an important relation with respect to the results reported by Gavilán et al. (2001) for EROD activity in fish (*Mugil cephalus)* for this estuary, where there would be a relationship with the metabolism of lipophilic organic pollutants (Van der Oost et al., 2003) an effect linked to the presence of PAH in sediments due to the petrochemical industry located in the lower part of the Biobío River and close to this site (Sanchez-Hernandez et al.,1998; Inzunza et al., 2006; Barra et al., 2009). Also, studies performed on *P. gualpensis* from this estuary indicate an important accumulation of heavy metals from the sediment (Bertrán et al., 2001a), which when combined with the fact that this water body suffers a large variety of anthropogenic-originated stressors (cellulose industry, urban effluents, agricultural use, etc.) (Barra et al., 2009), suggests the existence of a multiple contamination sources. Similarly, the increase in GST activity in the Valdivia estuary near discharge points of a water treatment plant would indicate the presence of certain compounds capable of inducing detoxifying activity in *P. gualpensis*.

The antioxidant capacity was lower in the sites located in the Biobío estuary and in site iII, and this variation indicate a potential higher susceptibility to oxidative damage by specific oxyradicals, a situation that has already been demonstrated in other aquatic organisms (Regoli, 2000; Frenzilli et al., 2001; Regoli et al., 2002). Still, the lack of increased oxidative damage at the protein level indicates that the use of antioxidants was sufficient to prevent this type of effect. This response type has already been observed in other polychaeta species, for example for *Laeonereis acuta*, even when there were differences in antioxidant responses in populations facing different degrees of contamination, these responses were sufficient to prevent oxidative damaged at the lipid level (Geracitano et al., 2004a).

The relation between weight and the set of antioxidant responses, observed in the set of variables that explained these responses with the correlation coefficient as well as the changes observed in the GST and antioxidant capacity responses in the sampling sites, indicate in the majority of the cases that these variables should be considered in the sites that present more intervention and that future studies should compare or standardize individuals at a similar weight. Additionally, assuming that the heavier individuals represent older individuals, future studies should consider that antioxidant concentration diminishes in older individuals as demonstrated in numerous studies (Golden et al., 2002; Lithgow and Andersen, 2005). In the case of *P. gualpensis*, the differences in weight between sites in a similar time period could be because these populations correspond to individuals that have not found adequate reproduction conditions and are capable of delaying their reproductive cycle when the conditions are inadequate, a situation observed in this polychaeta family, that present semelpary as a principal reproductive characteristic (Finch, 1994), or it could be because their exposure to certain stressors have generated disturbances in their reproductive processes. On the other hand, the relation of the variable salinity with the set of antioxidant responses is not conclusive principally due to the high temporal variability of this parameter in estuarine environments. Still, in laboratory experiments, authors such as Scaps and Borot (2000) have found variations in sub-individual level responses, such as the acetylcolinesterase with respect to different salinities, which would suggest the need to evaluate with greater precision the effect of these variables on this response type. The relation of percentage of native forest coverage with the set of responses analyzed would respond to the differentiation of responses between sites with lower anthropogenic impact, principally related with the conservation and/or preservation of riverside and forest vegetation near the estuaries. This result indicates that the presence of native vegetation near these water bodies is an indicator of lower anthropogenic intervention and consequently would allow inference of an ecosystem with better water quality, as widely suggested for continental water ecosystems as possessing a high buffering capacity with respect to non-punctual contamination sources (Lowrance et al., 1997). However, the presence of native vegetation does not represent an indicator for all the areas without anthropogenic intervention since point pollution sources, found in the lower part of the Biobío River, do produce an alteration in water quality even when there are important areas of native vegetation.

Indeed, since the correlations found cannot completely explain these responses (ρ_w=0.547), and the relation of the set of physicochemical variables and land use of these environments with the set of antioxidant responses observed in the present study are not conclusive and require further studies, principally related with including other types of variables and experiences in order to determine potential cause–effect relations.

According to the PCA based in the multivariate matrix of the principal responses to oxidative stress and in relation to the different sampling sites, it can be observed that one of the sites located on the Biobío River differs from the other sites in its GST and total antioxidant capacity activity. Thus, according to the site characteristics, it can be established that these responses found in *P. gualpensis* are adequate biomarkers to establish areas with an elevated condition of contamination, related principally with lipophilic organic compounds or a multi-contaminated condition, a situation that was also established by other authors for invertebrate species (Regoli, 2000; Perez et al., 2004; Ait Alla et al., 2006). Additionally, the GSH concentration, due to its relation to areas with greater anthropogenic intervention, could also be used as a potential biomarker to establish impact areas as found for other invertebrate species with respect to short- and medium-term exposure of lipophilic compounds (Richardson et al., 2008).

5. Conclusions

This study provides the first baseline for the characterization of antioxidant responses in *P. gualpensis*, indicating that this species is adequate for monitoring environmental disturbances in different Chilean estuary areas of the South-East Pacific where this species is present. Antioxidant responses, such as GST, GSH

and total antioxidant capacity, are presented as potential biomarkers for exposure to different contamination conditions, although the variable of individual weight will be fundamental for future evaluation of this type and other types of responses at a sub-individual level in these organisms. Finally, the use of GIS is presented as a complementary tool for a better characterization of these important ecosystems. The obtained results clearly indicate biomarker responses in *P. gualpensis* in Biobío and Valdivia estuaries, the more affected by human activities.

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References

Ait Alla, A., Mouneyrac, C., Durou, C., Moukrim, A., Pellerin, J., 2006. Tolerance and biomarkers as useful tools for assessing environmental quality in the Oued Souss estuary (Bay of Agadir, Morocco). Comp. Biochem. Physiol. C 143, 23–29.

Amado, L.L., da Rosa, C.E., Meirelles Leite, A., Moraes, L., Vaz Pires, W., Pinho, G.L.L., Martins, C.M.G., Robaldo, R.B., Nery, L.E.M., Monserrat, J.M., Bianchini, A., Martínez, P.E., Geracitano, L.A., 2006a. Biomarkers in croakers *Micropogonias furnieri* (Teleostei, Scianidae) from polluted and non-polluted areas from the Patos Lagoon estuary (Southern Brazil): evidences of genotoxic and immunological effects. Mar. Pollut. Bull. 52, 199–206.

Amado, L.L., Robaldo, R.B., Geracitano, L., Monserrat, J.M., Bianchini, A., 2006b. Biomarkers of exposure and effect in the Brazilian flounder *Paralichthys orbignyanus* (Teleostei: Paralichthyidae) from the Patos Lagoon estuary (Southern Brazil). Mar. Pollut. Bull. 52, 207–213.

Amado, L.L., Garcia, M.L., Ramos, P.B., Freitas, R.F., Zafalon, B., Ferreira, J.L.R., Yunes, J.S., Monserrat, J.M., 2009. A method to measure total antioxidant capacity against peroxyl radicals in aquatic organisms: application to evaluate microcystins toxicity. Sci. Tot. Environ. 407, 2115–2123.

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 (Oreochromisniloticus) from a polluted site. Aquat. Toxicol. 34, 151–162.

Banta, G.T, Andersen, O., 2003. Bioturbation and the fate of sediment pollutants. Experimental case studies of selected infauna species. Vie Mileu 53 (4), 233–248.

Barra, R., Quiroz, R., Saez, K., Araneda, A., Urrutia, R., Popp, P., 2009. Sources of polycyclic aromatic hydrocarbons (PAHs) in sediments of the Biobío River in south central Chile. Environ. Chem. Lett. 7, 133–139.

Bertrán, C., 1989. Zonación y dinámica temporal de la macroinfauna intermareal en el estuario del Río Lingue (Valdivia, Chile). Rev. Chil. Hist. Nat. 62, 19–32.

Bertrán, C., Tapia, J., Parra, O., Basualto, S., 2001a. *Perinereis gualpensis* Jeldes (Annelida, Polychaeta) como biomonitor de metales pesados en la desembocadura del Río Biobío (Chile). Inf. Tecnol. 12 (4), 59–64.

Bertrán, C., Arenas, J., Parra, O., 2001b. Macrofauna del curso inferior y estuario del río Biobío (Chile): cambios asociados a variabilidad estacional del caudal hídrico. Rev. Chil. Hist. Nat. 74, 331–340.

Clarke, K.R., Warwick, R.M., 1994. In: Change in Marine Communities. An Approach to Analysis and Interpretation. Natural Environment Research Council, United Kingdom.

Clarke, K.R., Gorley, R.N., 2005. In: PRIMER V.6: User Manual/Tutorial. PRIMER-E Ltd., Playmouth, United Kingdom.

Cnubben, N.H.P., Rietjens, I.M.C.M., Wortelboer, H., van Zanden, J., van Bladeren, P.J., 2001. The interplay of glutathione-related processes in antioxidant defense. Environ. Toxicol. Pharmacol. 10, 141–152.

CONAMA-CONAF-BIRF, 1999. Catastro y evaluación de los recursos vegetacionales nativos de Chile. Corporación Nacional Forestal, Comisión Nacional del Medio Ambiente, Banco Interamericano de Reconstrucción y Fomento. Informe Regional: Octava, Novena y Décima Región. CONAF-CONAMA, Santiago, Chile.

Davey, J.T., Watson, P.G., 1995. The activity of *Nereis diversicolor* (Polychaeta) and its impact on nutrient fluxes in estuarine waters. Ophelia 41, 55–70.

Dickinson, D.A., Forman, H.J., 2002. Glutathione in defense and signaling: lessons from a small thiol. Ann. N.Y. Acad. Sci. 973, 488–504.

Doyotte, A., Cossu, C., Jacquin, M.C., Babut, M., 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.

ESRI, 2001. In: ArcGIS 8. Using ArcGIS Geostatistical Analyst. ESRI, Redlands, CA.

Etienne, M., Prado, C., 1982. Descripción de la Vegetación mediante cartografía de ocupación de tierras. Ciencias Agrícolas No. 10, Universidad de Chile, Facultad de Cs. Agrarias y Forestales, UNESCO-MAB.

Ferreira-Cravo, M., Reinhardt-Piedras, F., Barros-Moraes, T., Ribas-Ferreira, J.L., de Freitas, D.P.S., Dornelles-Machado, M., Geracitano, L.A., Monserrat, J.M., 2007. Antioxidant responses and reactive oxygen species generation in different body regions of the estuarine polychaeta *Laeonereis acuta* (Nereididae). Chemosphere 66 (7), 1367–1374.

Finch, C., 1994. Latent capacities for gametogenic cycling in the semelparous invertebrate *Nereis*. Proc. Natl. Acad. Sci. 91, 11769–11770.

Frenzilli, G., Nigro, M., Scarcelli, V., Gorbi, S., Regoli, F., 2001. DNA integrity and total oxyradical scavenging capacity in the Mediterranean mussel, *Mytilus galloprovincialis*: a field study in a highly eutrophicated coastal lagoon. Aquat. Toxicol. 53 (1), 19–32.

Gavilán, J.F., Barra, R., Fossi, M.C., Casini, S., Salinas, G., Parra, O., Focardi, S., 2001. Biochemical biomarkers in fish from different river systems reflect exposure to a variety of anthropogenic stressors. Bull. Environ. Contam. Toxicol. 66, 476–483.

Geracitano, L.A., Monserrat, J.M., Bianchini, A., 2002. Physiological and antioxidant enzyme responses to acute and chronic exposure of *Laeonereis acuta* (Polychaeta, Nereididae) to copper. J. Exp. Mar. Biol. Ecol. 227, 145–156.

Geracitano, L.A., Bocchetti, R., Monserrat, J.M., Regoli, F., Bianchini, A., 2004a. Oxidative stress responses in two populations of *Laeonereis acuta* (Polychaeta, Nereididae) after acute and chronic exposure to copper. Mar. Environ. Res. 58, 1–17.

Geracitano, L.A., Monserrat, J.M., Bianchini, A., 2004b. Oxidative stress in *Laeonereis acuta* (Polychaeta, Nereididae): environmental and seasonal effects. Mar. Environ. Res. 58, 625–630.

Golden, T.R., Hinerfeld, D.A., Melov, S., 2002. Oxidative stress and aging: beyond correlation. Aging Cell. 1, 117–123.

Gray, J.S., 1981. In: The Ecology of Marine Sediments. Cambridge University Press, Cambridge.

Habig, W.H., Pabst, M.J., Jakoby, W.B., 1974. Glutathione S-transferases: the first enzymatic step in mercapturic acid formation. J. Biol. Chem. 249, 7130–7139.

Habig, W.H., Jakoby, W.B., 1981. Assays for differentiation of glutathione S-transferases. Meth. Enzymol. 77, 398–405.

Huettel, M., Rusch, A., 2000. Transport and degradation of phytoplankton in permeable sediment. Limnol. Oceanogr. 45, 534–549.

Ieno, E.N., Bastida, R.O., 1998. Spatial and temporal patterns in coastal macrobenthos of Samborom Bay, Argentina: a case of study of very low diversity. Estuaries 21, 690–699.

INE, 2002. In: Censo nacional de población y vivienda: Chile. Instituto Nacional de Estadística, Santiago, ChileCenso nacional de población y vivienda: Chile. Instituto Nacional de Estadística, Santiago, Chile.

Inzunza, B., Orrego, R., Peñalosa, M., Gavilán, J.F., Barra, R., 2006. Analysis of CYP4501A1, PAHs metabolites in bile, and genotoxic damage in *Oncorhynchus mykiss* exposed to Biobío River sediments. Central Chile. Ecotoxicol. Environ. Saf. 65 (2), 242–251.

Jaramillo, E., Mulsow, S., Navarro, R., 1985. Intertidal and subtidal macroinfauna in the Queule river Estuary, South of Chile. Rev. Chil. Hist. Nat. 74, 331–340.

Jaramillo, E., Contreras, H., Quijón, P., 2001. Variabilidad estacional e interanual en las abundancias poblacionales de la macroinfauna intermareal del estuario del río Queule, centro-sur de Chile. Rev. Chil. Hist. Nat. 74 (2), 455–468.

Lee, J., Surh, Y., 2005. Nrf2 as a novel molecular target for chemoprevention. Cancer Lett. 224, 171–184.

Levine, R.L., Garland, D., Oliver, C.N., Amici, A., Climent, I., Lenz, A., Ahn, B., Shaltiel, S., Stadtman, E.R., 1990. Determination of carbonyl content in oxidatively modified proteins. Methods Enzymol. 186, 464–478.

Lithgow, G.J., Andersen, J.K., 2005. Models of oxidative stress in the biology of aging. Drug Discov. Today 2 (4), 273–277.

Lowrance, R., Altier, L.S., Newbold, J.D., Schnabel, R.R., Groffman, P.M., Denver, J.M., Correll, D.L., Gilliam, J.W., Robinson, J.L., Brinsfield, R.B., 1997. Water quality functions of riparian forest buffers in Chesapeake Bay watersheds. Environ. Manage. 21, 687–712.

McLusky, D., 1989. The Estuarine Ecosystem, 2nd ed. Blackieand Sons, London, UK.

McLusky, D.S., Elliott, M., 2004. The Estuarine Ecosystem—Ecology, Threats, and Management third ed. Oxford University Press.

McManus, J., 1988. Grain size determination and interpretation. In: Tucker, M. (Ed.), Techniques in Sedimentology. Blackwell Science, Oxford, pp. 63–85.

Maher, P., 2005. The effects of stress and aging on glutathione metabolism. Ageing Res. Rev. 4 (2), 288–314.

Monserrat, J.M., Martínez, P.E., Geracitano, L.A., Amado, L.L., Martins, C.M., Pinho, G.L., Chaves, I.S., Ferreira-Cravo, M., Ventura-Lima, J., Bianchini, A., 2007. Pollution biomarkers in estuarine animals: critical review and new perspectives. Comp. Biochem. Physiol. C 146 (1–2), 221–234.

Nguyen, T., Huang, H.C., Pickett, C.B., 2000. Transcriptional regulation of the antioxidant response element. Activation by Nrf2 and repression by MafK. J. Biol. Chem. 275, 15466–15473.

Perez, E., Blasco, J., Solé, M., 2004. Biomarker responses to pollution in two invertebrate species: *Scrobicularia plana* and *Nereis diversicolor* from the Cadiz bay (SW Spain). Mar. Environ. Res. 58, 275–279.

Regoli, F., Winston, G.W., 1999. Quantification of total oxidant scavenging capacity of antioxidants for peroxynitrite, peroxyl radicals, and hydroxyl radicals. Toxicol. Appl. Pharm. 156, 96–105.

Regoli, F., 2000. Total Oxyradical Scavenging Capacity (TOSC) in polluted and translocated mussels: a predictive biomarker of oxidative stress. Aquat. Toxicol. 50, 351–361.

Regoli, F., Gorbi, T., Frenzilli, G., Nigro, M., Corsi, I., Focardi, S., Winston, G.W., 2002. Oxidative stress in ecotoxicology: from the analysis of individual antioxidants to a more integrated approach. Mar. Environ. Res. 54, 419–423.

Richardson, B.J., Mak, E., De Luca-Abbott, S.B., Martin, M., McClellan, K., Lam, P.K.S., 2008. Antioxidant responses to polycyclic aromatic hydrocarbons and organochlorine pesticides in green-lipped mussels (*Perna viridis*): do mussels "integrate" biomarker responses? Mar. Pollut. Bull. 57, 503–514.

Saint-Denis, M., Narbonne, J.F., Arnaud, C., Thybaud, E., Ribera, D., 1999. Biochemical responses of the earthworm *Eisenia fetida* andrei exposed to contaminated artificial soil: effects of benzo(a)pyrene. Soil Biol. Biochem. 31, 1837–1846.

Saiz-Salinas, J.I., Gonzalez-Oreja, J.A., 2000. Stress in estuarine communities: lessons from the highly-impacted Bilbao estuary (Spain). J. Aquat. Ecosyst. Stress Recov. 7, 43–55.

Sanchez-Hernandez, J.C., Fossi, M.C., Leonzio, C., Focardi, S., Barra, R., Gavilán, J.F., Parra, O., 1998. Use of biochemical biomarkers as a screening tool to focus the chemical monitoring of organic pollutants in the Biobío river basin (Chile). Chemosphere 37, 699–710.

Scaps, P., Borot, O., 2000. Acetycholinesterase activity of the polyhaete *Nereis diversicolor*: effects of temperature and salinity. Comp. Biochem. Physiol. C 125, 377–383.

Scaps, P., 2002. A review of the biology, ecology and potential use of the common ragworm *Hediste diversicolor* (O.F. Müller) (Annelida: Polychaeta). Hydrobiologia 470, 203–218.

Valdovinos, C., 2001. Biodiversidad de los fondos blandos sublitorales: Macroinvertebrados del Golfo de Arauco (Chile). In: Alveal, K., Antezana, T. (Eds.), Sustentabilidad de la biodiversidad. Universidad de Concepción, Concepción, pp. 635–662.

Van der Oost, R., Beber, J., Vermeulen, N.P.E., 2003. Fish bioaccumulation and biomarkers in environmental risk assessment: a review. Environ. Toxicol. Pharmacol. 13, 57–149.

Wentworth, C.K., 1922. A scale of grade and class terms for clastic sediments. J. Geol. 30, 377–392.

White, C.C., Viernes, H., Krejsa, C.M., Botta, D., Kavanagh, T.J., 2003. Fluorescence-based microtiter plate assay for glutamate–cysteine ligase activity. Anal. Biochem. 318 (2), 175–180.

Winston, G.W., Regoli, F., Dugas Jr., A.J., Fong, J.H., Blanchard, K.A., 1998. A rapid gas chromatographic assay for determining oxyradical scavenging capacity of antioxidants and biological fluids. Free Radic. Biol. Med. 24, 480–493.